Accepted Manuscript

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DOI: http://dx.doi.org/doi:10.1016/j.aquatox.2015.05.012
Reference: AQTOX 4125
PII: S0166-445X(15)00150-2

To appear in: *Aquatic Toxicology*

Received date: 31-3-2015
Revised date: 11-5-2015
Accepted date: 15-5-2015

Please cite this article as:
Esperanza, Marta, Seoane, Marta, Rioboo, Carmen, Herrero, Concepción, Cid, Ángeles, Chlamydomonas reinhardtii cells adjust the metabolism to maintain viability in response to atrazine stress. Aquatic Toxicology
http://dx.doi.org/10.1016/j.aquatox.2015.05.012

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Chlamydomonas reinhardtii cells adjust the metabolism to maintain viability in response to atrazine stress

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Highlights

Results indicate energy alterations in *C.reinhardtii* cells exposed to atrazine
Cellular metabolic activity decreased in atrazine treated cells
Cytoplasmic and mitochondrial membrane potentials were altered in exposed cells
Atrazine exposed cells were able to maintain cellular viability
RNA-Seq revealed upregulated transcripts related to heterotrophic energy generation

Abstract

Chlamydomonas reinhardtii cells were exposed to a sublethal concentration of the widespread herbicide atrazine for 3 and 24 h. Physiological parameters related to cellular energy status, such as cellular activity and mitochondrial and cytoplasmic membrane potentials, monitored by flow cytometry, were altered in microalgal cells exposed to 0.25 μM of atrazine. Transcriptomic analyses, carried out by RNA-Seq technique, displayed 12 differentially expressed genes between control cultures and atrazine-exposed cultures at both tested times. Many cellular processes were affected, but the most significant changes were observed in genes implicated in amino acid catabolism and respiratory cellular process. Obtained results suggest that photosynthesis inhibition by atrazine leads cells to get energy through a heterotrophic metabolism to maintain their viability.

Keywords: microalga, atrazine, cytotoxicity, RNA-Seq, flow cytometry

Abbreviations

a.u.: arbitrary units, $DiBAC_4(3)$: lipophilic anionic oxonol dye bis-(1,3-dibu- tylbarbituric acid) trimethine oxonol, FCM: flow cytometry, FDA: fluorescein diacetate, FDR: false discovery rate, FS: forward scatter light, JC-1: 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide, LFC: Log2 fold change, PI: propidium iodide, PS: photosystem, ROS: reactive oxygen species, RPM: reads per million mapped reads, SS: side scatter light, TCA: tricarboxylic acid cycle.

1. Introduction

Anthropogenic activity is a constant menace to the stability of ecosystems and the ecological risk assessment of organic pollutants, including herbicides, has become a priority field in current ecotoxicological research. In this sense, aquatic systems are recipients of many chemicals with potential deleterious effects on various physiological and biological processes of their biota. A considerable amount of the herbicides applied in crop fields enters into freshwater aquatic ecosystems through surface runoff or leaching, leading to environmental contamination (Törnqvist et al., 2011). These pollutants exert their toxicity on different types of organisms that were not the original target, such as unicellular algae, the primary producers in the aquatic food web. Adverse herbicide effects on microalgae could also alter higher trophic levels, potentially compromising biodiversity, structure and function of freshwater ecosystems (Campanella et al., 2001; Rioboo et al., 2007).

One of the herbicides most extensively applied in agriculture all over the world is atrazine. It was found in many surface and ground waters and listed as priority substance under the European Water Framework Directive as described in Directive 2013/39/EU. Atrazine inhibits photosynthesis blocking the photosynthetic electron transport at photosystem II (Rutherford and Krieger-Liszkay, 2001) and thereby energy production, preventing CO_2 fixation in target and non-target organisms. Its widespread application, persistence, and mobility have led to its frequent detection in ground and surface water sources (Hayes et al., 2010) at concentrations exceeding 10 μg L^{-1} (USEPA 2012). Atrazine-induced detrimental effects on the aquatic ecosystem and alterations in aquatic community structure have been reported previously (Choi et al., 2012, Didur et al., 2012, Sjollema et al., 2014; Weiner et al., 2004). This herbicide has also been reported to affect the human endocrine, central nervous, immune, and reproductive systems (Liu et al., 2014).

Microalgae have been proposed as an alternative to traditional analyses for ecotoxicological laboratory studies due to their short generation times and rapid responses to environmental changes. These organisms are extremely useful as tools to assess the toxicity of contaminants in water (Ma et al., 2006), using standardized tests (e.g. according to ISO (2012) and OECD (2011) guidelines) which focus on growth inhibition of these unicellular algae. Currently, with the development of the omics, new alternatives arise to study the effects of pollutants on microalgae (Jamers et al., 2009). In particular, transcriptomic analysis has a great potential to investigate subcellular mechanisms of stress and responses affecting growth and cellular physiology and biochemistry. Among aquatic microalgae, the freshwater species *Chlamydomonas reinhardtii* is currently used extensively in biological research and in molecular genetic studies as a biological model because of its ease of cultivation, rapid growth, possibility of inducing sexual reproduction and because it is haploid (Harris, 1989, 2001), and its genome has been sequenced (Merchant et al., 2007).

The aim of the present work has been the study of the response of the freshwater microalga *Chlamydomonas reinhardtii* exposed to a sublethal concentration of the herbicide atrazine for 24 hours, focused on the possible alteration of the cellular activity, as well as the mitochondrial and cytoplasmic membrane potentials. These parameters, related to cellular energy status, were monitored by flow cytometry (FCM) as potential good markers of cytotoxicity, since this technique allows the rapid analysis of a high number of cell functions under near *in vivo* conditions. Changes in the cellular transcriptome between control cells and cells exposed to atrazine for 3 and 24 h, were also determined using the RNA-Seq technique.

2. Materials and methods

2.1. Microalgal cultures

The unicellular green alga *Chlamydomonas reinhardtii* Dangeard (strain CCAP 11/32A mt+) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK). *C. reinhardtii* cells were cultured in Tris-minimal phosphate medium (Harris, 1989) on a rotary shaker set at 150 rpm, under controlled conditions: 22 ± 1°C and iiluminated with 100 µmol photon m^{-2} s^{-1} under a 12:12 h light:dark cycle. Cells in mid-logarithmic growth phase were used as inoculum for the different assays. Initial cell density for each experiment was 2 x 10^5 cells mL^{-1}.

Atrazine concentration used (0.25 µM) in the present study was based on 96 hours EC_{50} determined by previous toxicity test (data not shown). Before each experiment, fresh stock solutions of atrazine were prepared by dissolving the pure compound (Sigma-Aldrich, MW: 215.68) in methanol and filtering through 0.2 µm membrane filters. No significant differences between nominal and effective concentration of atrazine were found using a gas chromatography/mass spectrometry analysis.

All cultures were carried out in duplicate and the different analyses were done after 3 and 24 hours of culture. These time points were selected based on previous cytometry studies where the effects of atrazine were analysed every hour during 24 hours; after 3 h of atrazine exposure changes in the cellular metabolic activity were detected, whereas after 24 h a light:dark cycle was completed.

2.2. Flow cytometric analyses

FCM analyses of *C. reinhardtii* cells were performed on a Beckman-Coulter Gallios flow cytometer fitted with 488 nm and 633 nm excitation lasers, detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to different wavelength intervals: 505-550 nm (FL1), 550-600 nm (FL2), 600-645 nm (FL3) and >645 nm (FL4). The 488-nm argon-ion laser was used as excitation source for all the probes assayed. Forward scatter (FS, an estimation of cell size) and red autofluorescence (FL4 channel, an estimation of cell chlorophyll *a* content) dot-plots were used to characterise the microalgal population, setting gating levels in order to exclude non-microalgal particles. At least 10.000 gated cells per sample were collected and analysed using Kaluza software version 1.1 (Beckman Coulter).

Cell suspensions (2 × 10^5 cells mL^{-1}) were incubated with the appropriate fluorochrome at room temperature and in darkness. The lowest fluorochrome concentration and the shortest incubation time were chosen in order to obtain significant and stable staining of cells without toxicity being developed. All FCM determinations were performed at least twice and duplicate samples were run on the flow cytometer.

2.2.1. Growth measurement

Growth of microalgal cultures was measured by counting culture aliquots in the flow cytometer using a suspension of fluorochrome-containing micro-spheres for its calibration (Flow Count Fluorospheres, Beckman Coulter Inc.).

Growth rates (μ) expressed as day^{-1} were calculated via the formula $\mu=[\ln(Nt)-\ln(N_0)]/\ln 2(t-t_0)$ where N_t is the cell density at time t and N_0 is the cell density at time 0.

2.2.2. Cell viability determination

Propidium iodide (PI) was used to discriminate between viable non-fluorescent cells and non-viable fluorescent cells with cell membrane damage. *C. reinhardtii* cells were incubated with 4 µM of PI for 15 min prior samples were analyzed by FCM (Prado et al., 2009a). The orange fluorescent emission of PI was

collected in the FL3 channel indicated above. Results were expressed as the percentage of viable cells *vs*. the total amount of cells analysed by FCM.

2.2.3. Cellular metabolic activity determination: kinetic assay

Cellular activity level was evaluated using a kinetic approach to the fluorescein diacetate (FDA)-based cell esterase activity assay (Prado et al., 2012a). Cell suspensions were stained with 0.24 µM FDA. FDA-dependent fluorescence generation rates (indicative of the metabolic activity level, and expressed as arbitrary relative fluorescence units per min, a.u.), were calculated by regression analysis of mean values of green-fluorescence intensity (normalized to cell size values estimated using FS) over time.

2.2.4. Cytoplasmic membrane potential assessment

This parameter was monitored using a slow-response potentiometric probe with the lipophilic anionic oxonol dye bis-(1,3-dibu- tylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) as previously described in Prado et al. (2012a). *C. reinhardtii* cell suspensions were stained with 0.97 µM $DiBAC_4(3)$ for 10 min. $DiBAC_4(3)$ green fluorescent emission was collected in the FL1 channel indicated above. Results were expressed as the percentage of depolarised cells *vs.* the total amount of cells analysed per culture.

2.2.5. Mitochondrial membrane potential measurement

Changes in mitochondrial membrane potential of *C. reinhardtii* cells after treatment with atrazine were evaluated by staining cells with the lipophilic cationic probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) (Prado et al., 2012b). *C. reinhardtii* cells were incubated with 3 µM of JC-1 for 20 min. Mitochondrial membrane potential results were expressed as the mean orange (JC-1 oligomers) / green (JC-1 monomers) fluorescence intensity ratio which only depends on the mitochondrial membrane potential measurement (Cassart et al., 2007).

2.3. Total RNA extraction and RNA-Seq

RNA was isolated from control and atrazine exposed (0.25 µM) cells after 3 and 24 h of culture, using the reagent NZYol (NZYTech). Frozen samples were homogenised using a mortar and pestle under liquid nitrogen. 1 mL of NZYol was added directly to the homogenate, and transferred to a nuclease-free 1.5 mL tube. Then, 0.2 volumes of chloroform-isoamil alcohol (24:1) were added, the mixture was centrifuged, and the supernatant was recovered into a new tube. One volume of ice-cold isopropanol was added, and the mixture was kept at -20 ºC overnight in order to precipitate the RNA. The samples were centrifuged, and the supernatant discarded. The pellet was washed with 75 % ethanol. The ethanol was discarded, and the pellet resuspended in a final volume of 30 µL (Chomczynski and Sacchi, 2006).

After that, a DNase treatment was carried out with 2.5 µL of RQ1 RNase-Free DNase 10X Reaction Buffer (Promega), 2 units of RQ1 RNase-Free DNase (Promega), and 21.5 µL of the RNA solution. RNA concentration and integrity were measured in an Agilent 2100 Bioanalyzer.

Then a cDNA library construction and Illumina HiSeq 2000 sequencing were done with the collaboration of AllGenetics & Biology, SL. A total number of 8 cDNA libraries were constructed, one per sample (2 control and 2 treated samples at 3 h and 2 control and 2 treated samples at 24 h), using the TruSeq RNA Sample Preparation Kit v2 (Illumina), strictly following the manufacturer's instructions and the pool was sequenced in a HiSeq 2000 PE100 lane.

2.4. Data analysis

For the FCM data analysis mean values ± standard deviation (SD) of at least two experiments were

calculated and statistically analysed by an overall one-way analysis of variance (ANOVA) using SPSS Statistic software (version 21.0, SPSS, IBM). A p-value < 0.05 was considered statistically significant. When significant differences were observed, controls *vs.* treated means were compared using the Dunnett test.

For the RNA-Seq data the bioinformatic analysis was carried out by ECSEQ Bioinformatics, using the "edgeR 3.8.5" program for the analysis of differential expression of the genes. Only genes with a "False Discovery Rate" (FDR) ≤ 0.01 and a "Log2 Fold Change" (LFC) ≥ 1 or ≤ -1 were selected. Then, differentially expressed genes were classified manually by categories of functions previously described in Hemschemeier et al., (2013), using information from Ensembl Plants 22 *Chlamydomonas reinhardtii* v3.1. Furthermore, GO enrichment analyses were performed using the Algal Functional Annotation Tool at http://pathways.mcdb.ucla.edu/algal/index.html (Lopez et al., 2011).

3. Results and discussion

3.1. Growth and cell viability

Growth inhibition and cell viability were analysed to assess potential toxic effects of atrazine at a population level. Growth rates were calculated after 24 h of culture. Atrazine exposure induced a significant inhibitory effect on *C. reinhardtii* growth in comparison with control cultures ($p < 0.05$). Growth rates (μ) at 24 h were 1.73 ± 0.20 day^{-1} for control cultures and 0.94 ± 0.03 day^{-1} for cultures exposed to atrazine; therefore, this herbicide caused an inhibition of growth of 45.6 %.

To determine whether the significant decrease recorded in the *C. reinhardtii* proliferation was associated with the loss of cell viability, a FCM assay based on dye exclusion of the probe PI was used to identify cells with intact plasma membrane. The percentage of *C. reinhardtii* viable cells, with intact plasma membrane (PI-cells), after 3 and 24 h of atrazine exposure remained close to 95% for both control cultures and atrazine exposed cultures ($p > 0.05$) (Figure 1).

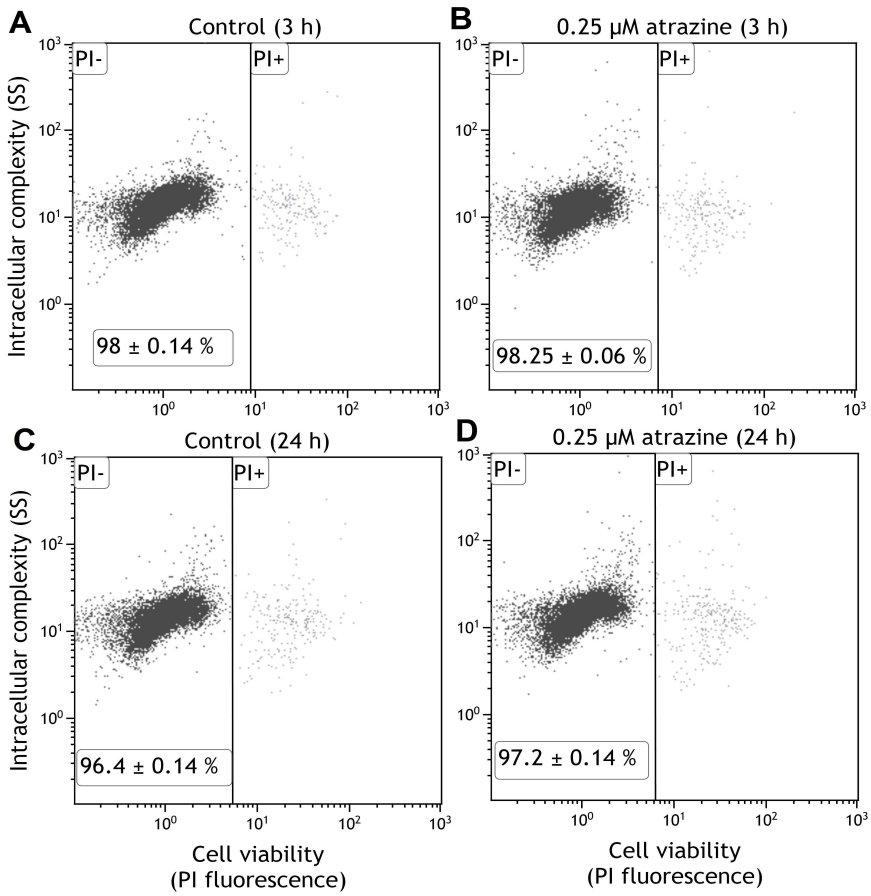

Figure 1. Cell viability of *C. reinhardtii* in control and atrazine exposed (0.25 µM) cultures. **(A)** and **(C)** control cultures at 3 and 24 h, respectively; **(B)** and **(D)** atrazine exposed cultures at 3 and 24 h, respectively. Results are expressed as percentage of viable cells (PI negative) *vs.* the total amount of cells analysed by FCM. Values are shown as mean ± SD of experimental duplicates.

The toxicity of atrazine on *C. reinhardtii* growth has been reported previously (Fischer et al., 2010; Reboud et al., 2007). In the present study, although a significant decrease in the growth rate was observed, cell viability was not affected at the tested concentration; this means that 0.25 µM of atrazine is a sublethal concentration (Figure 1). It has been also reported that microalgal cells exposed to atrazine were not able to complete their cell division but maintained their metabolic activity and cell viability (Gónzalez-Barreiro et al., 2004, 2006). The potential stimulation of the antioxidant defense mechanisms (Chankova et al., 2014; Dewez et al., 2005) could be involved in this maintenance. Obtained results indicate that cytoplasmic membrane integrity was affected only after other cellular parameters had already been damaged in a strong way by the herbicide.

3.2. Cellular metabolic activity

To further characterize the potential cytotoxic effect of atrazine on metabolic activity of *C. reinhardtii* cells, a kinetic approach to the FDA-based cell esterase activity assay was used taking into account only metabolically active cells. *In fluxo* analysis of cellular non-specific esterase activity revealed that atrazine exposure induced a significant ($p < 0.05$) reduction in metabolic activity of *C. reinhardtii* cells with respect to control cultures. Cell activity decreased 31 % and 29 % at 3 and 24 h, respectively, in cultures exposed to 0.25 µM of the herbicide (Figure 2).

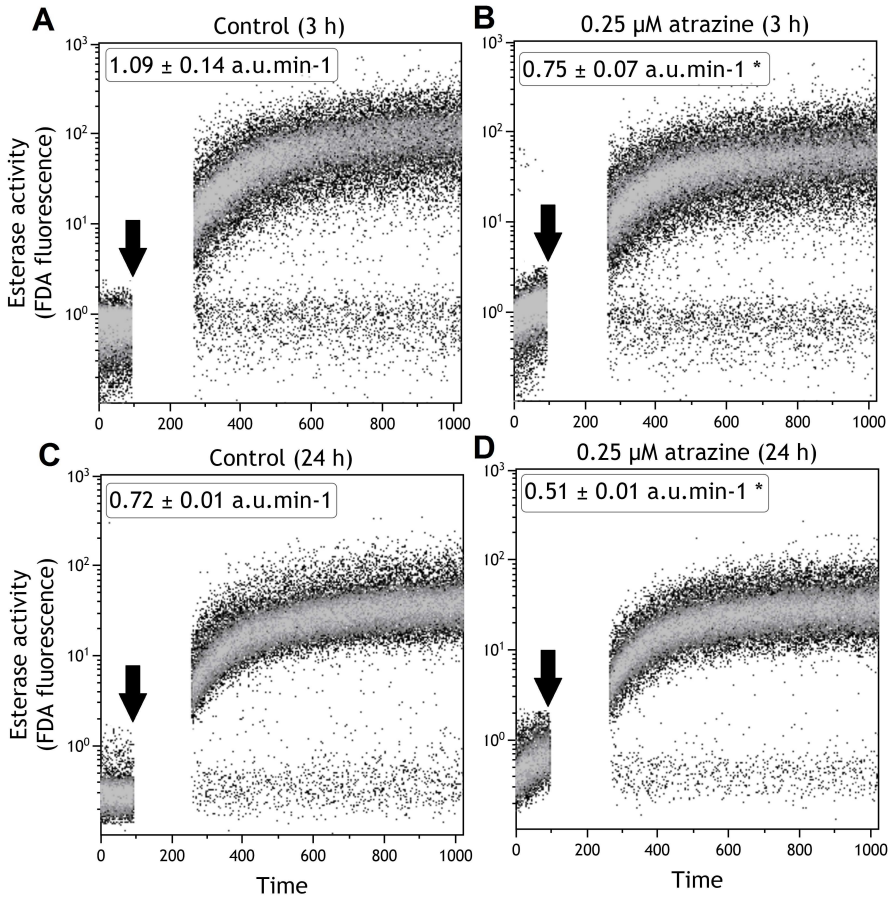

Figure 2. Metabolic activity of *C. reinhardtii* in control and atrazine exposed (0.25 µM) cultures. **(A)** and **(C)** control cultures at 3 and 24 h, respectively; **(B)** and **(D)** atrazine exposed cultures at 3 and 24 h, respectively. Arrows indicate the time of addition of FDA to unstained suspensions. Fluorescence generation rates are expressed in fluorescence arbitrary units min^{-1}. Kinetic plots represent one of two experiments. Values are shown as mean ± SD of experimental duplicates. Asterisks (*) indicate significant differences ($P < 0.05$) *vs.* non-treated control.

Non-significant changes in the percentage of viable cells were observed (Figure 1), whereas cell activity in viable cells was affected at tested times (Figure 2). These results could pontetially be explained by growth data at 24 h, since a reduction about 50% of the growth was observed in the cultures exposed to atrazine with respect to control cultures. Our results confirm previous findings regarding a decline in esterase activity in *Chlamydomonas* cells exposed to different herbicides (Jamers and De Coen, 2010, Nestler et al, 2012; Prado et al, 2009a) and other aquatic pollutants (Prado et al., 2015). Obtained results could indicate that the mechanism of action of atrazine generates oxidative stress that causes redox misbalances and alterations in protein synthesis and, therefore, a general decrease in cellular activity. A good correlation between the rate

of conversion of FDA to fluorescein (esterase activity) and photosynthetic activity has also been described in unicellular algae (Brookes et al., 2000). Taking into account this correlation, the inhibition of the esterase activity could be due to the oxidative damage provoked by atrazine at photosystem II level in microalgal cells.

3.3. Cytoplasmic and mitochondrial membrane potentials

FCM analysis of $DiBAC_4(3)$-stained cells showed that atrazine affected cytoplasmic membrane potential of *C. reinhardtii* cells, leading to the depolarization of the plasma membrane, i.e. to a huge fluorochrome influx to the cells. A significant increase in the percentage of cells with a depolarised cytoplasmic membrane was observed in cultures exposed to the herbicide with respect to control cultures at both tested times. Cultures exposed to the sublethal atrazine concentration showed an increase in depolarised cells of 33 % and 31 % at 3 and 24 h, respectively (Figure 3, A).

C. reinhardtii cells were also stained with the mitochondria-specific dye, JC-1, widely used to monitor mitochondrial membrane potential. Atrazine exposure caused a change of mitochondrial membrane potential at tested times, as evidenced by the significantly higher orange (JC-1 oligomers)/green (JC-1 monomers) fluorescence ratio values recorded in atrazine-treated cells relative to those in non-treated controls ($p < 0.05$). After 3 h and 24 h of exposure, values of mitochondrial membrane potential in treated cells increased 2.18- and 1.36-fold, respectively (Figure 3, B).

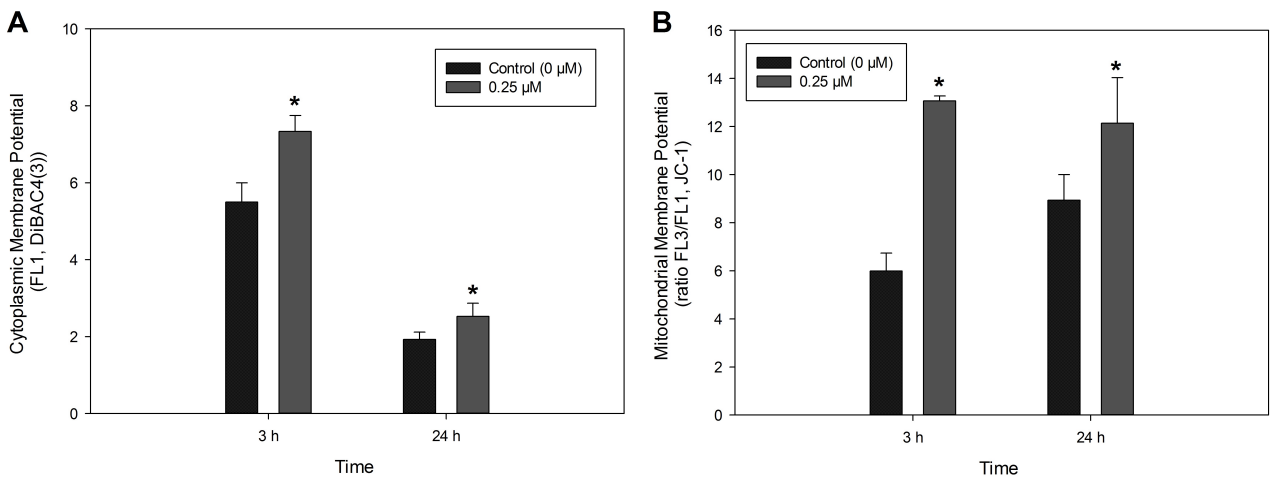

Figure 3. Cytoplasmic **(A)** and mitochondrial **(B)** membrane potentials of *C. reinhardtii* in control cultures and atrazine exposed (0.25 µM) cultures at 3 and 24 h. Cytoplasmic membrane potential data were expressed as the percentage of depolarised cells *vs*. the total amount of cells analysed per culture. Mitochondrial membrane potential changes were expressed as the mean orange (JC-1 oligomers)/green (JC-1 monomers) fluorescence intensity ratio. Values are shown as mean ± SD of experimental duplicates. Asterisks (*) indicate significant differences ($P < 0.05$) *vs.* non-treated control.

These results together viability data indicate that the induced damage in the plasma membrane begins with changes in the permeability properties (depolarization) before the disruption of the membrane integrity (Prado et al., 2012b, 2015). Previous studies in different microorganisms have also reported this change in the permeability of the cytoplasmic membrane (Bouix et al., 2015, Lloyd et al., 2004; Wickens et al., 2000). Any alteration in the cytoplasmic membrane potential can be considered a fast and sensitive indicator of the presence of stress factors that can lead to important physiological changes for the proper functioning of *C.*

reinhardtii cells. The depolarization of the membrane was also related to the inhibition of H^+-ATPases and NA^+/K^+-ATPases pumps in membrane, that are considered the main generators of electrochemical gradients in plants (Krol et al., 2003). Due to these changes it is normal that the cellular activity mentioned earlier was affected (Figure 2).

A significant hyperpolarization of the mitochondrial membrane potential was observed in *C. reinhardtii* cells after 3 and 24 h of atrazine exposure at assayed concentration (Figure 3, B). The mechanism of action of atrazine affects the photosynthesis, but could also provoke perturbations on mitochondrial respiratory chain. Several authors reported that physiologically, mitochondrial hyperpolarization could be due to an increase in respiration, with a higher electron flux, more active oxidative phosphorylation and consequently, possible leaks of electrons that favored the generation of ROS, as a by-product of the ATP-generating process (Huc et al., 2012, Loseva et al., 2004; Wallace, 2005). Atrazine toxicity on mitochondrial respiratory chain has been studied in murine and human cells (Chen et al., 2015; Koo et al., 2012). Furthermore, other studies in animal and vegetal cells have observed a protonphobic effect exerted by herbicides, such as paraquat, on mitochondria, *i. e.*, this herbicide causes a depolarization of the inner mitochondrial membrane and an uncoupling of the oxidative phosphorylation (Costantini et al., 1995, Palmeira et al., 1995, Prado et al., 2012b; Vicente et al., 2001).

3.4. Transcriptomic analyses

The RNA-Seq data showed 12 differentially expressed genes (FDR ≤ 0.01 and LFC ≥ 1 or ≤ - 1) between control cultures and cultures exposed to atrazine at both tested times (3 and 24 h). Amount these 12 genes, 11 were upregulated and only 1 was downregulated in cultures exposed to the herbicide with respect to non-treated cultures. All these differentially expressed genes were classified according to the biological process wherein they are involved and to their molecular functions in the following categories: amino acids, energy, metabolism, ROS and stress, gene expression and redox (Table 1).

Table 1. Differentially expressed genes of *C. reinhardtii*. Comparison of transcript abundances and fold-changes in cultures exposed to 0.25 µM of atrazine for 3 h and 24 h *versus* control cultures. For each comparison, a gene was deemed differentially expressed if it fitted the following criteria: significant Log Fold Change (LFC ≤ -1 or ≥ 1) and significant False Discovery Rate (FDR ≤ 0.01). RPM means Reads Per Million Mapped Reads.

Regarding to the amino acid category, 4 transcripts are related to amino acid catabolism (*MCCA, AAH1*, *GDH2* and *CLR21*) and all these genes were significantly (FDR ≤ 0.01 and LFC ≥ 1 or ≤ -1) upregulated at both tested times (Table 1). The early accumulation of transcripts coding for amino acid catabolic enzymes or proteins related to amino acid catabolism pathways suggests that *C. reinhardtii* resorts first to amino acid degrading pathways to obtain energy, when the photosynthetic process is affected (Hemschemeier, 2013). Amino acids can be sorted as glucogenic or ketogenic according to the metabolic fate of their carbon skeletons. Several studies have shown that in microalgal species such as *C. reinhardtii* and *Chlorella vulgaris*, glucogenic amino acids can be converted to glucose through gluconeogenesis and ketogenic amino acids can be transformed into ketone bodies through ketogenesis and their degradation yield acetyl-CoA (Ahii et al., 2015; Johnson and Alric, 2013). These ketone bodies are produced to be used as energy. In *Arabidopsis*, the catabolism of amino acids was also proposed to fuel the tricarboxylic acid cycle (TCA)

(Caldana et al., 2011).

The upregulated transcript of *MCCA* encodes the methylcrotonoyl-CoA carboxylase alpha subunit that is involved in the degradation of the branched-chain amino acid leucine. Specifically, the protein product is responsible for the fourth step in the breakdown of leucine, a ketogenic amino acid that is a building block of many proteins. So, Leu catabolism finally yields acetyl-CoA, for the formation of ketones or to be used as substrate for the TCA cycle (Johnson and Alric, 2013). The *AAH1* transcript was the most upregulated at 3 h (Table 1); it encodes a phenylalanine hydroxylase responsible for the conversion of phenylalanine to another amino acid, tyrosine (Vallon and Spalding, 2009). Tyrosine can also be broken down into smaller molecules that are used to produce energy. These two aromatic amino acids are both glucogenic and ketogenic. *GDH2* encodes a glutamate dehydrogenase that is involved in the glutamate catabolism (Vallon and Spalding, 2009). *CLR21* was the most upregulated transcript at 24 h (Table 1); it encodes a CLR family protein that is known to be involved in methionine catabolism. Both amino acids (Glu and Met) are glucogenic amino acids and the carbon skeletons originating from their oxidation can feed into the TCA cycle, leading to an accumulation of intermediates that can be further converted into longer-carbon-chain compounds via gluconeogenesis (Johnson and Alric, 2013).

Regarding to the energy category, 4 transcripts (*ICL1*, *PCK1B*, *ETFA* and *ETFB*) were significantly (FDR ≤ 0.01 and LFC ≥ 1 or ≤ - 1) upregulated at both tested times (Table 1). The fact that most of the genes were related with energy generation pathways suggest that *C. reinhardtii* resorts to all available energy sources to properly acclimate to the stress conditions to maintain a healthy energy status. The *ICL1* transcript encodes the isocitrate lyase, a key enzyme of the glyoxylate cycle. The glyoxylate cycle generates intermediates that can be used to synthesize glucose (via gluconeogenesis). ICL1 appears to function primarily in channeling acetyl-CoA through the glyoxylate shunt pathway for synthesis of amino acid precursors. This pathway bypasses a part of the TCA-cycle and produces glyoxylate and succinate. Glyoxylate in turn combines with another molecule of acetyl-CoA and generates malate, which then is converted to oxaloacetate by the enzyme malate dehydrogenase. Oxaloacetate could then be used (i) for the generation of aspartate and the pool of amino acids derived from it, (ii) to feed into the reverse glycolysis pathway via the generation of phosphoenolpyruvate by the reversible enzyme phosphoenolpyruvate carboxykinase (PCK1), also upregulated (Table 1) and (iii) to enter in another round of TCA-cycle/glyoxylate cycle to replenish TCA-cycle intermediates (Subramanian et al., 2014). In microorganisms, glucose is frequently not available, and other simple carbon compounds provide the only accessible carbon. The TCA cycle, with its two decarboxylation steps, does not permit assimilation of carbon and thus does not provide a route for the synthesis of macromolecules from C2 compounds. However, the glyoxylate pathway bypasses these decarboxylations, allowing C2 compounds to serve as carbon sources in gluconeogenesis and to be incorporated into glucose and, from there, into amino acids, DNA, and RNA (Lorenz and Fink, 2002). The study of Plancke et al. (2014) on cells of *C. reinhardtii* showed that ICL1 has a central role in cellular metabolism and the lack of isocitrate lyase is responsible for strong decrease in respiration rates and in the amounts of enzymes of the glyoxylate cycle and gluconeogenesis and, as a consequence, stimulation of amino acid synthesis from organic acids was observed. Furthermore, the higher ICL activity found in the present study may be an adaptation to promote mixotrophic carbon assimilation when photosynthetic carbon dioxide fixation is impaired (Sztrum et al., 2012) so this is related to the mechanism of action of atrazine. In other studies with *C. reinhardtii*, the

abundances of transcripts associated with amino acid catabolism (e.g. *MCCA*) was shown to increase significantly when photosynthetic O_2 evolution was blocked by DCMU (Hemschemeier et al., 2013).

Other relevant transcripts related to energy metabolism were upregulated in this study. The *PCK1B* transcript encodes a phosphoenolpyruvate carboxykinase and the increased amounts of this gene suggested the induction of gluconeogenesis mentioned above. Also *ETFA* and *ETFB* were upregulated and these two transcripts encode the *α*- and β-subunit of electron-transfer flavoprotein. The increased abundances of transcripts related to amino acid catabolism together with the upregulation of *ETFA* and *ETFB* genes was also found by Hemschemeier et al. (2013) in dark-anoxic *C. reinhardtii* cells. Genetic and metabolic data indicate that plants subjected to darkness oxidatively degrade mainly Leu, Ile, Val, Phe, Trp, and Tyr and transfer the electrons to the mitochondrial electron transport chain via electron-transfer flavoprotein and electron-transfer flavoprotein:ubiquinone oxidoreductase (ETF/ETFQO). Thereby, the ETF/ETFQO system allows cells to use electrons derived from diverse catabolic pathways for respiratory energy generation (Ishizaki et al., 2005, 2006).

Trancriptomic data correlated well with data obtained by flow cytometry. In general, the RNA-Seq data showed increased abundances of transcripts related to heterotrophic energy generation and electron disposal. These results are related with those obtained in the cytoplasmic membrane potential assay since this parameter provides information about the current transport and energy status of the cell (Konrad and Hedrich, 2008). Perturbations of the cytoplasmic membrane potential provide a sensitive and rapid indication of physicochemical changes in the extracellular environment and therefore they have been suggested to be mediators of subsequent physiological cellular responses to environmental stress factors (Rabinovitch and June, 1990). In the present work, a significant decline in cellular activity (Figure 2) and a depolarization of the plasma membrane (Figure 3, A) were observed in the *C. reinhardtii* cells exposed to a sublethal atrazine concentration. The plasma membrane is the chemiosmotic barrier that provides the interface between the organism and its external environment. Across this phospholipid bilayer, a transmembrane electrochemical potential (negative inside) plays a pivotal role in the control of solute exchange, with the implication of large gradients between intracellular and extracellular concentrations of a variety of ions. It is an energy-requiring disequilibrium whose maintenance depends on the action of different channels and pumps (Lloyd et al. 2004). For this reason, *C. reinhardtii* cells under atrazine stress conditions required all energy storages for the preservation of their cytoplasmic membrane potential, viability and cellular homeostasis.

Flow cytometry data also showed an increase in the mitochondrial membrane potential (Figure 3, B), which can be interpreted as a stimulation of the mitochondrial activity and the data could support the hypothesis that another important source of energy for the microalgal cells might proceed from respiration (Loseva et al., 2004). As mentioned before, *ETFA* and *ETFB* genes, involved in the respiratory electron transport of the mitochondria, were upregulated. Cells might use electrons derived from diverse catabolic pathways, such as amino acid degradation, for respiratory energy generation. *C. reinhardtii* cells under atrazine stress conditions change their photosynthetic metabolism to a heterotrophic one, because the herbicide exerts its mechanism of action on the photosystem II (Marchetti et al., 2013; Rutherford and Krieger-Liszkay, 2001), thus reducing the photosynthetic activity and due to this reason the microalgal cells try to get energy through heterotrophic metabolism for being able to maintain its viability (Figure 1).

In the gene expression category, one transcript (*FKB16-1*) was upregulated in cultures exposed to the

herbicide (Table 1). This gene encodes a peptidyl-prolyl cis-trans isomerase involved in post-transcriptional modification processes such as protein folding. In the ROS and stress category, the *GOX9* transcript was upregulated. This gene encodes a glyoxal or galactose oxidase related with antioxidant defense mechanisms. These results suggest that atrazine increased the intracellular ROS levels and provoked an oxidative stress in *C. reinhardtii* cells (Mofeed and Mosleh, 2013). We found similar results with the herbicide paraquat in cells of *C. moewusii (*Prado et al., 2012a) since this compound induced oxidative stress and a decrease in metabolic activity.

Also, as a result of amino acid catabolism, large amount of ammonium is produced and this ammonium should be excreted for the maintenance of the osmotic equilibrium of the cell. The induction of ammonium transport was indicated by increased amount of the *RHP-2* trancript since it encodes a Rh protein implicated in ammonium/CO_2 transmembrane transport (Soupene et al., 2004). Moreover, regarding to nitrogen metabolism, there was one downregulated transcript (*NIT1*) in cultures exposed to atrazine (Table 1). *NIT1* encodes a nitrate reductase, which catalyses the first step of nitrate assimilation. Nitrate is reduced to nitrite by nitrate reductase and then, nitrite reductase catalyses its reduction to ammonium. The expression of *NIT1* is repressed by ammonium and induced by ammonium starvation (de Montaigu et al., 2010). In this case, this gene was downregulated due to the amounts of ammonium produced as we discussed above. Nitrate reductase activity decreased in cells of *C. moewusii* exposed to paraquat (Prado et al., 2009b) in a concentration-dependent manner and similar results were also obtained in *C. reinhardtii* exposed to atrazine (unpublished data). Decreased expression of genes controlling nitrogen assimilation has also been observed upon exposure to heavy metals and atrazine in the cyanobacteria *Microcystis aeruginosa* (Quian et al., 2012). Nitrogen assimilation and carbon fixation are highly coordinated in unicellular algae, so that an herbicide that finally should interfere in the photosynthetic carbon fixation goes on to also interferes in enzymatic activities in nitrogen assimilation (Prado et al., 2009b).

4. Conclusions

A sublethal concentration of atrazine alters the energy status of *Chlamydomonas reinhardtii* cells. Using flow cytometric protocols a decrease of cellular activity, a plasma membrane depolarization and a hyperpolarization of the mitochondrial membrane were detected in atrazine exposed cells respect to control cells. RNA-Seq analysis also indicated this energy alteration since the transcriptomic data revealed higher abundances of transcripts related to heterotrophic energy generation and electron disposal in the stressed cells. Despite these alterations, the cells were able to overcome stress and maintain cellular viability, thus *C. reinhardtii* showed an important adaptability to the adverse conditions.

Acknowledgements

This work was carried out with the financial support of the Spanish Ministerio de Economía y Competitividad (CGL2010-15993/BOS). M. E. acknowledges a pre-doctoral fellowship from the Xunta de Galicia (Spain).

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Figure 1. Cell viability of *C. reinhardtii* in control and atrazine exposed (0.25 µM) cultures. **(A)** and **(C)** control cultures at 3 and 24 h, respectively; **(B)** and **(D)** atrazine exposed cultures at 3 and 24 h, respectively. Results are expressed as percentage of viable cells (PI negative) *vs.* the total amount of cells analysed by FCM. Values are shown as mean ± SD of experimental duplicates.

Figure 2. Metabolic activity of *C. reinhardtii* in control and atrazine exposed (0.25 µM) cultures. **(A)** and **(C)** control cultures at 3 and 24 h, respectively; **(B)** and **(D)** atrazine exposed cultures at 3 and 24 h, respectively. Arrows indicate the time of addition of FDA to unstained suspensions. Fluorescence generation rates are expressed in fluorescence arbitrary units min^{-1}. Kinetic plots represent one of two experiments. Values are shown as mean ± SD of experimental duplicates. Asterisks (*) indicate significant differences ($P < 0.05$) *vs.* non-treated control.

Figure 3. Cytoplasmic **(A)** and mitochondrial **(B)** membrane potentials of *C. reinhardtii* in control cultures and atrazine exposed (0.25 µM) cultures at 3 and 24 h. Cytoplasmic membrane potential data were expressed as the percentage of depolarised cells *vs*. the total amount of cells analysed per culture. Mitochondrial membrane potential changes were expressed as the mean orange (JC-1 oligomers)/green (JC-1 monomers) fluorescence intensity ratio. Values are shown as mean ± SD of experimental duplicates. Asterisks (*) indicate significant differences ($P < 0.05$) *vs.* non-treated control.

Table 1. Differentially expressed genes of *C. reinhardtii*. Comparison of transcript abundances and fold-changes in cultures exposed to 0.25 µM of atrazine for 3 h and 24 h *versus* control cultures. For each comparison, a gene was deemed differentially expressed if it fitted the following criteria: significant Log Fold Change (LFC ≤ -1 or ≥ 1) and significant False Discovery Rate (FDR ≤ 0.01). RPM means Reads Per Million Mapped Reads.

Categories	Gene ID	Gene name	Average RPM (treatment) 3H	Average RPM (control) 3H	Log Fold Change 3H	FDR 3H	Average RPM (treatment) 24H	Average RPM (control) 24H	Log Fold Change 24H	FDR 24H	Description
Upregulated											
Amino acids	205945	AAH1	41.44	9.58	2.23	9.51e-25	71.28	22.23	1.76	9.44e-13	Aromatic amino acid hydroxylase-related protein
Amino acids	193008	MCCA	22.68	6.17	1.98	3.21e-13	49.49	20.91	1.31	2.51e-05	Methylcrotonoyl-CoA carboxylase alpha subunit
Amino acids	82916	GDH2	260.37	77.54	1.86	1.12e-11	484.00	208.26	1.30	3.58e-07	Glutamate dehydrogenase
Amino acids	186295	CLR21	5.90	3.00	1.09	0.00149	2.77	0.36	2.96	2.18e-06	Predicted protein of CLR family
Energy	191668	ICL1	3597.57	1628.07	1.26	9.73e-07	1735.18	730.41	1.31	0.00286	Isocitrate lyase
Energy	196612	PCK1B	2076.78	1022.10	1.13	0.000124	1190.77	588.79	1.08	0.000768	Phosphoenolpyruvate carboxykinase, splice variant
Energy	189344	ETFA	10.27	5.17	1.11	0.000147	6.63	2.19	1.66	2.51e-05	Electron transfer flavoprotein alpha subunit
Energy	180281	ETFB	4.44	1.98	1.27	0.000752	3.72	1.28	1.61	0.000574	Electron transfer flavoprotein beta subunit

Metabolism	24240	RHP-2	86.95	31.51	1.58	1.06e-10	71.79	28.32	1.42	5.72e-09	Rh protein
ROS and stress	196820	GOX9	11.42	4.95	1.33	9.28e-06	10.71	4.25	1.41	0.000724	Glyoxal or galactose oxidase
Gene expression	142698	FKB16-1	28.18	14.02	1.12	2.25e-06	50.88	25.99	1.05	0.000409	Peptidyl-prolyl cis-trans isomerase
Downregulated											
Redox	184661	NIT1	23.79	68.81	-1.43	2.59e-07	22.15	62.42	-1.43	3.53e-07	Nitrate reductase