# Early alterations on photosynthesis-related parameters in *Chlamydomonas reinhardtii* cells exposed to atrazine: A multiple approach study

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## Highlights

- Pigment content and chlorophyll a fluorescence decreased in atrazine exposed cells.
- Atrazine induced an increase of reactive oxygen species level in treated cells.
- RNA-Seq analysis showed 9 differentially expressed photosynthesis-related genes.
- Proteomic analysis revealed changes in 5 proteins related to photosynthesis.

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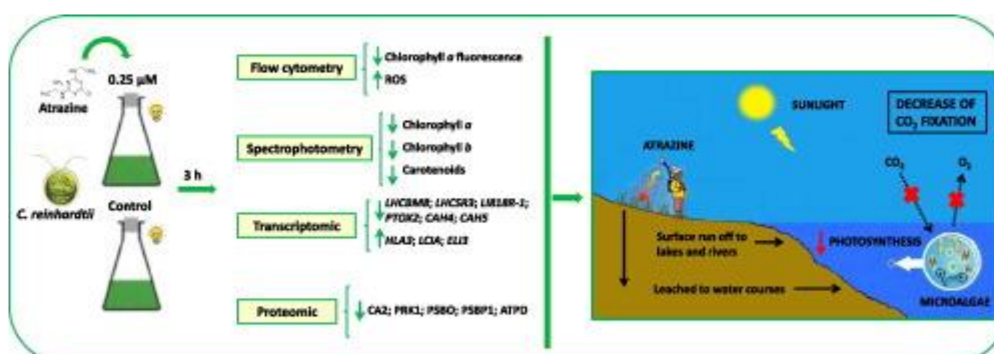
## Abstract

*Chlamydomonas reinhardtii* cells were exposed to a sublethal concentration of the widespread herbicide atrazine for 3 h. Physiological cellular parameters, such as chlorophyll *a* fluorescence and oxidative stress monitored by flow cytometry and pigments levels were altered in microalgal cells exposed to 0.25 μM of atrazine. Furthermore, the effects of this herbicide on *C. reinhardtii* were explored using "omics" techniques. Transcriptomic analyses, carried out by RNA-Seq technique, displayed 9 differentially expressed genes, related to photosynthesis, between control cultures and atrazine exposed cultures. Proteomic profiles were obtained using iTRAQ tags and MALDI-MS/MS analysis, identifying important changes in the proteome during atrazine stress; 5 proteins related to photosynthesis were downexpressed. The results of these experiments advance the understanding of photosynthetic adjustments that occur during an early herbicide exposure. Inhibition of photosynthesis induced by atrazine toxicity will affect the entire physiological and biochemical states of microalgal cells.


## Graphical abstract





## Keywords

- Microalga;
- Atrazine;
- Flow cytometry;
- iTRAQ;
- MALDI;
- RNA-Seq


## Abbreviations

- a.u., arbitrary units;

- CCM, carbon concentrating mechanism;
- Ci, inorganic carbon dioxide;
- CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate;
- EB, etidium bromide;
- FCM, flow cytometry;
- FDR, false discovery rate;
- FS,forward scatter light;
- HE, hydroethidine;
- iTRAQ, isobaric tags for relative and absolute quantitation;
- LFC, log2 fold change;
- MALDI-TOF-MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry;
- PSII, photosystem II;
- ROS, reactive oxygen species;
- RPM, reads per million mapped reads;
- SS, side scatter light;
- TCA,tricarboxylic acid cycle

## 1. Introduction

The occurrence of anthropogenic stress-induced chemicals, such as herbicides, in the aquatic environment presents a serious problem. These man-made organic toxicants are released into the aquatic environment and can affect non-target species. One of the herbicides most extensively applied in agriculture all over the world is atrazine. Its widespread application, persistence, and mobility have led to its frequent detection in ground and surface water sources (Hayes et al., 2010) and to list it as priority substance under the European Water Framework Directive as described in Directive 2013/39/EC (European Council, 2013). Atrazine inhibits photosynthesis blocking the photosynthetic electron transport at photosystem II (Rutherford and Krieger-Liszkay, 2001) and thereby energy production drops, preventing $CO_2$ fixation in target and non-target organisms. 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 and Weiner et al., 2004).

Microalgae have been recommended as test organisms in ecotoxicological studies because of their ecological relevance and sensitivity (Ma et al., 2006). All

primary producers are the basis of the aquatic food web; therefore it is an interesting study of their photosynthetic state since any disturbance in productivity of the microalgae community can induce direct structural changes in the rest of the ecosystem (Campanella et al., 2001, Martinez et al., 2014 and Rioboo et al., 2007). Regarding toxicity investigations and risk assessment with microalgae, integral endpoints, such as growth and reproduction, are traditionally monitored. However, before these endpoints show a significant change, the exposure may affect other cellular physiological parameters at much lower toxicant concentrations (Nestler et al., 2012).

The photosynthetic state of microalgae can be measured by quantifying the pigment content, which has been used as biomarker of exposure to herbicides in algae (Couderchet and Vernet, 2003). Fluorescence measurements have also been proposed as simple, rapid and sensitive methods to detect the photoinhibitory effects of environmental stressors on phytoplankton (Geoffroy et al., 2007 and Juneau et al., 2002). Flow cytometry (FCM) is an alternative to the standard algal population based endpoints, since it allows the characterization of the microalgal response at a single-cell level close to *in vivo* conditions. The *in vivo* chlorophyll *a* fluorescence of green algae can be used as a tool to detect negative alterations on photosynthesis ( Bi Fai et al., 2007,Chalifour et al., 2009, Cid et al., 1995, Ekelund and Aronson, 2007, González-Barreiro et al., 2004 and Prado et al., 2011).

Currently, with the development of the omics, new alternatives arise to study the effects of pollutants on microalgae (Dowling and Sheehan, 2006, Esperanza et al., 2015,Jamers et al., 2009 and Monsinjon and Knigge, 2007). Transcriptomics and proteomics can be used to detect and characterize responses to external stimulus, and have a great potential for investigating stress mechanisms and responses affecting growth and other physiological and biochemical endpoints (Jamers and De Coen, 2010, Nestler et al., 2012 and Subramanian et al., 2014), being increasingly applied in ecotoxicology and other fields of biological science. The availability of the sequenced genome of the microalgal species *Chlamydomonas reinhardtii*, with adequate annotation and metabolic pathway information, facilitates high-throughput analyses of transcriptional and proteomic profiling ( Merchant et al., 2007).

Previous research has demonstrated that *C. reinhardtii* cells exposed to atrazine change their metabolism and get energy mainly by heterotrophic pathways ( Esperanza et al., 2015). In a global climate change scenario with a dramatic increase in $CO_2$ levels, it is very important to know the behavior of carbon fixing organisms. Thus, the aim of the present study was to determine alterations in parameters directly or indirectly related to photosynthesis after only 3 h of exposure to the herbicide. For this

purpose, different methodological approaches were applied. First, a traditional spectrophotometric method for the quantification of pigments content was carried out. Additionally, flow cytometry was used to determine chlorophyll *a* fluorescence and reactive oxygen species level (ROS). Moreover, unlike standard ecotoxicity studies, detailed quantitative transcriptomic profiling of algal cells exposed to atrazine compared with those cultured without herbicide was assessed, looking for changes in the regulation of transcription. Furthermore, a proteomic analysis was carried out to observe early protein alterations due to the stress directly caused by the herbicide.

## 2. Materials and methods

### *2.1. Microalgal cultures*

The unicellular green alga *C.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 illuminated 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 $2 \times 10^5$ cells $mL^{-1}$.

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. Also control cultures were included, to which only methanol was added. No significant differences between nominal and effective concentration of atrazine were found using a gas chromatography/mass spectrometry analysis.

All cultures were set up in triplicate for 3 h and at least two independent experiments were carried out for each parameter analyzed. This time point was selected considering previous cytometry studies where the effects of atrazine were analyzed every hour during 24 h and changes in the cellular metabolic activity and ROS formation were detected after 3 h of atrazine exposure (unpublished data).

### *2.2. Growth measurement*

A growth inhibition test for *C. reinhardtii* using atrazine concentrations ranged from 0.1 to 2 μM was carried out to determine the herbicide concentration used for the following determinations of the present study. Cell density was daily determined for 96 h by counting culture aliquots in the flow cytometer. For absolute cell counting, a suspension of fluorescent polystyrene microspheres (Flow-Count Fluorospheres;

Beckman Coulter) with known concentration was added as an internal reference to all cell samples. Growth rates (μ) expressed as $day^{-1}$ were calculated *via* the formula $\mu = [\ln(N_t) - \ln(N_0)] / \ln 2(t - t_0)$ where $N_t$ is the cell density at time t and $N_0$ is the cell density at time 0. The 96 h $EC_{50}$ value for growth was calculated, based on growth rate data, using the computer program CompuSyn (Chou and Martin, 2005).

### *2.3. Photosynthetic pigment content*

Pigments were extracted from a concentrated algal sample in a 90% acetone aqueous solution and determined by measuring the absorbance of the extract using a Shimadzu UV-1700 spectrophotometer at appropriate wavelengths (664, 647 and 480 nm). The resulting absorbance measurements were translated to chlorophylls and carotenoids according to Jeffrey and Humphrey (1975) and Strickland and Parsons (1972), respectively. The equations used to calculate the pigment concentrations in the extract are:

Chlorophyll *a* = 11.93 A664 − 1.93 A647

Chlorophyll *b* = 20.36 A647 − 5.50 A664

Carotenoids = 4.0 A480

where total chlorophylls *a* and *b* and carotenoids represent the pigment concentrations of extract in μg $ml^{-1}$, and A664, A647 and A480 represent the absorbances measured at 664, 647 and 480 nm, respectively.

### *2.4. Flow cytometry 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 characterize the microalgal population, setting gating levels in order to exclude non-microalgal particles. At least 10 000 gated cells per sample were collected and analyzed using Kaluza software version 1.1 (Beckman Coulter). All FCM determinations were performed at least twice and duplicate samples were run on the flow cytometer.

### 2.4.1. Inherent cell properties: chlorophyll *a* fluorescence

The red autofluorescence of *C. reinhardtii* cells, related to chlorophyll *a* fluorescent emission, was analyzed to study the potential changes in this pigment content or changes in the functioning of the PSIII. Results were expressed as the mean red fluorescence in the FL4 channel fluorescence intensity in a.u. ( Prado et al., 2011).

### 2.4.2. Oxidative stress determination

Oxidative stress in *C. reinhardtii* was evaluated by FCM using determinations of intracellular levels of superoxide anion radical ($O_2^-$) with an oxidation-sensitive fluorescent dye, hydroethidine (HE; Molecular Probes, Inc.). HE was added at a final concentration of 16 µM for 30 min to the cell suspensions ($2 \times 10^5$ cells $mL^{-1}$). Results were expressed as the mean orange-ethidium bromide (EB) fluorescence in the FL3 channel fluorescence intensity in a.u. (Prado et al., 2012a).

## *2.5. Total RNA extraction and RNA-Seq*

RNA was isolated from control and atrazine exposed (0.25 µM) cells after 3 h of atrazine exposure, using the reagent NZYol (NZYTech), following the procedure described previously in Esperanza et al. (2015).

Then a cDNA library construction and Illumina HiSeq 2000 sequencing were done with the collaboration of AllGenetics & Biology, SL. A total number of 4 cDNA libraries were constructed, one per sample (2 control and 2 treated samples), 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.

## *2.6. Protein extraction, identification and quantifying*

The protein extraction was carried out following the protocol of Damerval et al. (1986)with some modifications. Cells were harvested by centrifugation to get a pellet of $10 \times 10^7$cells, pellet was resuspended in an extraction buffer (50 mM Tris HCl, pH 8.8 with 150 mM NaCl), suspended cells were lysed by sonication. For TCA/acetone method, samples were centrifuged and supernatants were taken in fresh tubes and mixed with 10% of trichloroacetic acid (TCA) in cold acetone with 0.07% of β-mercaptoethanol to 0.07%. This mixture was incubated at 20 °C overnight. After this time, the mixture was centrifuged and the precipitate was washed 3 times with a cold mixture of acetone and 0.07% of β-mercaptoethanol, with their respective

centrifugations. Then the pellet was allowed to dry. Finally, the pellet was resuspended in a solubilization buffer (30 mM Tris, 8 M Urea, 2 M thiourea and 4% of CHAPS).

Protein concentration of samples was determined by the Bradford method modified byRamagli and Rodriguez (1985). The absorbance was measured in a spectrophotometer at 595 nm against a blank of solubilization buffer, using Bovine serum albumin (BSA) as a standard.

Trypsin digestion, iTRAQ labeling, liquid chromatography, mass spectrometry, protein identification and quantifying were performed as previously described in Fernández-Puente et al. (2011) and Gayoso et al. (2014). These proteomic analyses were carried out with the collaboration of the Osteoarticular and Aging Research Lab, Proteomics-ProteoRed/ISCIII, INIBIC-Complejo Hospitalario Universitario de A Coruña.

### 2.7. Statistical analyses

For the photosynthetic pigment content and FCM data, mean values ± standard deviation (SD) of at least two experiments were statistically analyzed by an overall one-way analyses of variance (ANOVA) using SPSS Statistic software (version 21.0, SPSS, IBM). A $p$-value of $< 0.05$ was considered statistically significant. When significant differences were observed, means of treated cultures were compared with control means 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 “$\log_2$ fold change” (LFC) ≥ 1 or ≤− 1 were selected.

For proteomic analysis, the normalization tools and statistical package from ProteinPilot 4.0 software (AB Sciex) were employed. A stringent > 95% confidence interval was applied (equivalent to ProteinPilot Unused score > 1.3). Spectra were searched against the *Chlamydomonas* protein database v5.3 (19,529 Protein Sequences downloaded from Phytozome). After searching, protein IDs that had a ProteinPilot Unused Score of at least 1.3 (corresponding to a 95% Confidence Interval) were accepted. The ProteinPilot software also calculated a confidence percentage, the unused score, which reflects the probability of a hit being a “false positive,” at a 95% confidence. Although this software automatically accepts all peptides with an identification level of > 1%, only proteins having at least one peptide above the 95% confidence level were initially recorded. Searches against a concatenated database containing both forward and reversed sequences allowed the false discovery rate to be kept at 1%. Data were normalized for loading error by bias and the background

correction was calculated using the Pro Group algorithm (ABSciex). A $p \leq 0.05$ or a ratio of ≥ 1.2 or ≤ 0.8 was considered significant (over- or underexpressed).

Then, differentially expressed genes and proteins were classified manually by categories of functions previously described in Hemschemeier et al. (2013), using information from Ensembl Plants 22 *C.reinhardtii* v3.1. Furthermore, GO (Gen Ontology) 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*

Growth data indicated that atrazine treatment induced a significant ($p < 0.05$) inhibitory effect on the proliferation of *C. reinhardtii* in a concentration-dependent manner ( Table 1). Control cultures showed significantly ($p < 0.05$) higher cell numbers per mL medium and higher average growth rates than the atrazine exposed population after 96 h (Table 1). The $EC_{50}$ for growth after 96 h value, based on growth rate data was 0.231 μM. Then, the sublethal atrazine concentration used in the following analyses of the present study was 0.25 μM, a concentration close to this 96 h $EC_{50}$ value.

Table 1. Effect of atrazine on the growth of *C. reinhardtii* cells after 96 h of exposure. Values are shown as mean ± SD of experimental triplicates. Asterisks (*) indicate significant differences ($p < 0.05$) *versus* non-treated control.

| Atrazine (μM) | Cell density (cells × $10^4$ $mL^{-1}$) | Growth rates (μ) ($day^{-1}$) |
|---|---|---|
| Control | 457.50 ± 20.30 | 1.13 ± 0.02 |
| 0.1 | 256.00 ± 11.46* | 0.92 ± 0.02* |
| 0.25 | 74.90 ± 1.30* | 0.48 ± 0.01* |
| 0.5 | 56.20 ± 0.85* | 0.37 ± 0.01* |
| 1 | 22.50 ± 0.40* | 0.04 ± 0.00* |
| 2 | 20.30 ± 0.30* | 0.01 ± 0.00* |

## *3.2. Photosynthetic pigment content and chlorophyll a fluorescence*

After 3 h of exposure to a sublethal atrazine concentration (0.25 μM), chlorophyll *a* and total carotenoid contents of *C. reinhardtii* biomass were significantly reduced ($p < 0.05$) with respect to control cultures, 26.7% and 29.4%, respectively (Fig.

1). However a non-significant reduction (19.1%, $p > 0.05$) in the cellular amount of chlorophyll *b* was observed in cultures exposed to atrazine ( Fig. 1).

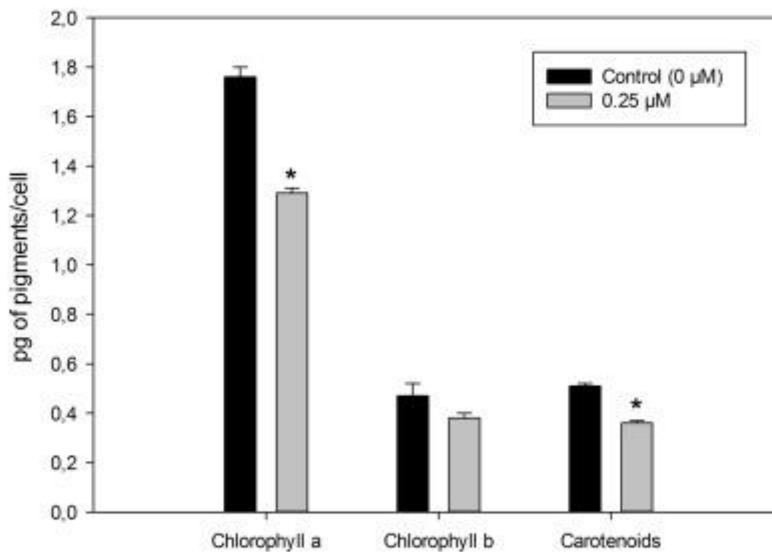



Fig. 1. Photosynthetic pigment content of *C. reinhardtii* cells in control cultures and atrazine exposed (0.25 μM) cultures at 3 h. Photosynthetic pigment content values are shown as mean ± SD of experimental duplicates, expressed as pg of pigments per cell. Asterisks (*) indicate significant differences ($p < 0.05$) *versus* non-treated control.

FCM data showed a significant reduction ($p < 0.05$) of 16.6% of the chlorophyll *a* fluorescence (2065 ± 35.36 a.u.) with respect to control cultures (2475 ± 77.78 a.u.) after 3 h of exposure to the herbicide (Fig. 2A).

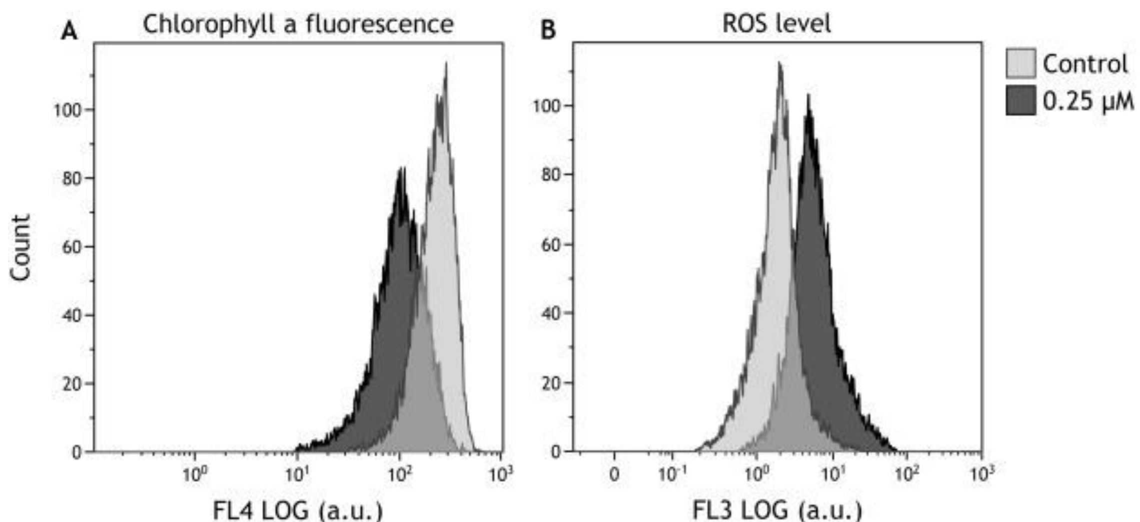



Fig. 2. Overlay histograms of flow cytometric analysis of chlorophyll *a* autofluorescence (A) and intracellular levels of superoxide anion radical (B) of *C. reinhardtii* cells in control cultures and atrazine exposed (0.25 μM) cultures at 3 h. (*Y*-axis: count; *X*-axis: fluorescence intensity in arbitrary units, a.u.).

Chlorophyll *a* fluorescence is a function of the cell pigment content and the photochemical activity of PSII in the photosynthetic electron transport chain ( Franklin

et al., 2001). Thus, in this study, the observed reduction of the autofluorescence can be explained by the decrease in cellular pigment content.

Furthermore, chlorophyll *a* fluorescence emission analysis of PSII from the photosynthetic apparatus of algae makes possible the characterization of the effects and modes of action of different kinds of environmental stressors and water pollutants such as herbicides ( Brack and Frank, 1998). Obtained results indicated a reduction of photosynthetic activity and this is related to the atrazine mechanism of action on the D1 protein of PSII ( Chalifour et al., 2009 and Didur et al., 2012). Photosynthesis inhibition induced by atrazine toxicity will affect the entire physiological state and cell growth process ( Bi Fai et al., 2007 and Esperanza et al., 2015).

These results, decrease in the cellular pigment content and the reduction of the chlorophyll *a* fluorescence caused by atrazine ( Fig. 1 and Fig. 2A), are in accordance with results obtained in previous studies in which it is reported that microalgal cells, under the stress induced by this herbicide, change their photoautotrophic metabolism, inefficient under these conditions, to an heterotrophic metabolism ( Esperanza et al., 2015 and González-Barreiro et al., 2004).

### *3.3. Oxidative stress*

In order to test the relationship between atrazine treatment and oxidative stress induction in *C. reinhardtii*, FCM analysis of reactive oxygen species (ROS) generation level was evaluated. The exposure to atrazine caused a remarkable accumulation of ROS in *C. reinhardtii* cells, resulting in a significant increase ($p < 0.05$) in the intracellular level of superoxide anion radical ($O_2 \bullet^-$) in treated cultures (46.95 ± 0.83 a.u.) with respect to control cultures (34.70 ± 0.99 a.u.) (Fig. 2B). This increase in cellular ROS level is related to the reduction of pigments and autofluorescence, since it has been well documented that a photosynthetic efficiency decrease can enhance ROS production in microalgal cells, followed by oxidative damages and function abnormalities (Liu et al., 2012).

Although ROS were classically considered toxic metabolic byproducts that ultimately lead to cell death, it is now recognized that ROS act as central secondary messengers involved in compartmentalized signaling networks (Rosenwasser et al., 2014). This function suggests that cells have evolved strategies to utilize ROS as biological signals that control various genetic stress programs. This interpretation is based on the unstated assumption that a given ROS can interact selectively with a target molecule that perceives the increase in ROS concentration, and then translates this information into a change of gene expression, such as changes in transcriptional activity (Laloi et al., 2004).

Results obtained showed a significant increase in intracellular ROS levels in the cultures exposed to atrazine (Fig. 2B). Toxic action involving ROS formation and oxidative stress have been reported in ecotoxicological studies with unicellular algae exposed to atrazine (Qian et al., 2009 and Mofeed and Mosleh, 2013), paraquat (Jamers and De Coen, 2010,Nestler et al., 2012, Prado et al., 2012a and Prado et al., 2012b), and metals (Melegari et al., 2013 and Perreault et al., 2012). *C. reinhardtii* is sensitive to the oxidative action of atrazine and this can be related to the decrease observed in the cellular content of chlorophyll, as has been reported in plants ( Ekmekci and Terzioglu, 2005).

In a previous study with atrazine the cellular activity of microalgal cells was affected significantly at tested concentration, however non-significant changes in cell viability were observed (Esperanza et al., 2015). ROS generated by the mechanism of action of atrazine, as described above, provoked redox misbalances and alterations in gene regulation and protein synthesis, and therefore a general decrease in the cellular activity.

### 3.4. Transcriptomic analyses

Obtained RNA-Seq data showed 185 differentially expressed genes (FDR ≤ 0.01 and LFC ≥ 1 or ≤− 1) between control cultures and cultures exposed to atrazine (0.25 μM) at tested time (3 h). These genes were classified manually in 13 categories of functions: photosynthesis, metabolism, gene expression, energy, amino acids, cell cycle, redox, lipids, regulation, ROS and stress, proteases, other and unknown (Hemschemeier et al., 2013). Of this amount of genes, 124 showed a reduction in their expression, while 61 genes showed an increase (Fig. 3).

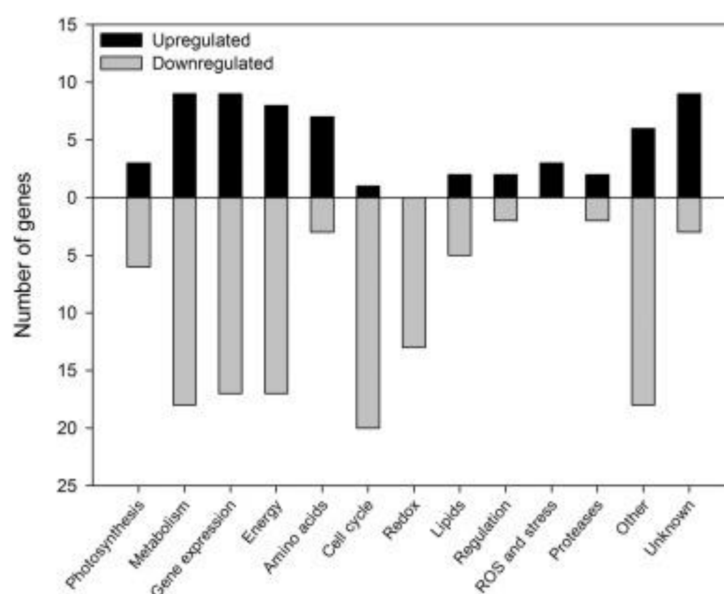


Fig. 3. Differentially expressed genes (FDR ≤ 0.01 and LFC ≥ 1 or ≤− 1) after 3 h of exposure to 0.25 μM of atrazine with respect to the control divided into 13 categories. Separated by a horizontal axis the genes upregulated are in the top and the downregulated are in the bottom.

Besides the large number of altered genes, this study was only focused on those related directly or indirectly to photosynthesis processes (9). Within this category, 6 of the 9 genes (*LHCBM8*, *LHCSR3*, *LI818R-1*, *PTOX2*, *CAH4* and *CAH5*) were significantly downregulated and 3 were significantly upregulated (*HLA3*, *LCIA* and *ELI3*) ( Table 2).

Table 2. Differentially expressed genes related to photosynthesis of *C. reinhardtii*. Comparison of transcript abundances and fold-changes in cultures exposed to 0.25 μM of atrazine for 3 h *vs.* control cultures. For each comparison, a gene was deemed differentially expressed if it fitted the following criteria: significant "$\log_2$ fold change" (LFC ≤ − 1 or ≥ 1) and significant "false discovery rate" (FDR ≤ 0.01). RPM means "Reads per million mapped reads".

| Gene ID | Gene name | Average RPM (treatment) 3H | Average RPM (control) 3H | $\log_2$fold change 3H | FDR 3H | Description |
|---|---|---|---|---|---|---|
| Downregulated | | | | | | |
| 205752 | *LHCBM8* | 266.80 | 595.64 | − 1.05 | 0.000119 | Chlorophyll *a*–*b* binding protein of LHCII |
| 184730 | *LHCSR3* | 24.00 | 168.52 | − 2.70 | 4.36e − 43 | Stress-related chlorophyll *a*/*b* binding protein 3 |
| 184724 | *LI818R-1* | 8.78 | 29.62 | − 1.64 | 5.53e − 12 | Stress-related chlorophyll *a*/b binding protein 1 |
| 205757 | *PTOX2* | 24.18 | 67.80 | − 1.37 | 1.92e − 07 | Alternative oxidase, plastid terminal oxidase 2 |
| 24552 | *CAH4* | 272.75 | 601.41 | − 1.03 | 2.23e − 05 | Mitochondrial carbonic anhydrase, beta type |
| 196876 | *CAH5* | 147.79 | 317.96 | − 1.00 | 0.000493 | Mitochondrial carbonic anhydrase, beta type |
| Upregulated | | | | | | |
| 134058 | *HLA3* | 348.89 | 161.78 | 1.21 | 0.00172 | ABC transporter, multidrug resistance-related proteins (MRP) subfamily |

| 135648 | *LCIA* | 94.31 | 29.03 | 1.82 | 1.48e − 10 | Low-$CO_2$-inducible membrane protein |
|---|---|---|---|---|---|---|
| 148916 | *ELI3* | 5.48 | 2.22 | 1.40 | 0.000105 | Early light inducible chlorophyll *a*/b binding protein |

The *LHCBM8*, *LHCSR3* and *LI818R-1* transcripts encode early light inducible (*LHCBM8*) and stress-related (*LHCSR3* and *LI818R-1*) chlorophyll *a*/*b* binding proteins that play a key role in the light-harvesting complex. In this study all of these transcripts were significantly downregulated ( Table 2). In eukaryotic oxygenic photosynthetic organisms, light-harvesting chlorophyll *a*/*b* binding proteins function in the collection and transfer of light energy to the reaction centers of the photosystems II and I. Additionally these proteins are also involved in light dissipation and energy quenching. Therefore, light-harvesting proteins are important components of the photosynthetic machinery that optimize photosynthetic function and minimize photooxidative damage in response to light quantity and quality ( Stauber et al., 2003). Maruyama et al. (2014) showed that the transcription of the *LHCSR3* gene, the most downregulated in the current study ( Table 2), was suppressed by treatment with a photosystem II inhibitor (DCMU). Bonente et al. (2011) have also shown that *LHCSR3*, essential for energy quenching in *C. reinhardtii*, is a pigment-binding protein with the properties of a constitutive quencher. *LI818R-1* protein has been suggested to play a role in protecting chlorophyll proteins from excitation pressure under low $CO_2$ as well as high light conditions ( Iwai et al., 2007 and Richard et al., 2000). The decrease in the level of transcription of these genes in treated cells is in accordance with the mechanism of action of atrazine that inhibits PSII and the decrease in chlorophyll *a* content and its fluorescence ( Figs. 1 and 2A).

The *PTOX2* transcript encodes a plastid terminal oxidase 2, the oxidase most predominantly involved in chlororespiration in *Chlamydomonas* species ( Houille-Vernes et al., 2011). Transcriptomic data revealed that also the amounts of *PTOX2* decrease significantly in this study ( Table 2). Chlororespiration has been defined as an alternative respiratory electron transport chain in interaction with the photosynthetic electron transport chain in thylakoid membranes of chloroplasts (Peltier and Cournac, 2002). It works as a valve to dissipate excess NADPH and maintain redox homeostasis. Chlororespiration involves two proteins that have been recently identified in*Chlamydomonas*: a NADPH dehydrogenase (NDA2), and a plastid terminal oxidase (PTOX2). NDA2 reduces plastoquinones from NADPH, and PTOX2 regenerates oxidized plastoquinones at the expense of $O_2$. Activity of these two

chloroplast enzymes, which act in tandem, results in the oxidation of NADPH and the consumption of $O_2$(Johnson and Alric, 2013). Downregulation of *PTOX2* and the observed ROS production can be related to the mechanism of action of atrazine because, as mentioned before, this herbicide blocks the photosynthetic electron transfer to the plastoquinone pool, leading to production of triplet chlorophyll and $O_2^-$. PTOX2 is also important for carotenoid biosynthesis during chloroplast biogenesis because its activity prevents the over-reduction of the plastoquinone pool. Plants without this enzyme exhibit phenotypes of variegated leaves with white patches because the carotenoid synthesis pathway slows down due to the lack of oxidized plastoquinone which oxidizes phytoene, a carotenoid intermediate (Carol and Kuntz, 2001). Therefore, the observed downregulation of the*PTOX2* transcript could be related to the decrease in the carotenoid contents ( Fig. 1). A lack of *PTOX2* indirectly causes photodamage during plastid development because protective carotenoids are not synthesized ( Aluru and Rodermel, 2004). Excess light captured by the light-harvesting complex of PSII is dissipated by carotenoids. The lack of sufficient amount of carotenoids and chlorophyll (Fig. 1) generates reactive oxygen radicals which in turn lead to the photooxidation of plastid components (Aluru and Rodermel, 2004). Hence, the decreased amounts of this transcript could also be related to the accumulation of ROS observed in this study in the cultures treated with atrazine (Fig. 2B).

The transcripts *CAH4* and *CAH5* encode beta carbonic anhydrases (CAs) which are zinc-containing metalloenzymes that catalyse the reversible interconversion of $CO_2$ and $HCO_3^-$, and these two transcripts were also significantly downregulated in cultures exposed to atrazine (0.25 μM) for 3 h with respect to control cultures (Table 2). Although these beta carbonic anhydrases are localized in the mitochondria ( Eriksson et al., 1995 and Eriksson et al., 1996), they could affect chloroplast related processes, being linked to the carbon concentrating mechanism (CCM) (Moroney and Ynalvez, 2007). The role of these mitochondrial CAs has been suggested as buffering matrix $H^+$ upon the initiation of photorespiration when the cells are transferred from high to low $CO_2$conditions (Eriksson et al., 1996). When subjected to low-$CO_2$ environments, mitochondria are found to migrate around the periphery of the chloroplast, and CAH4 and CAH5 may function in the capture of $CO_2$ escaping the chloroplast, rapidly converting these molecules to $HCO_3^-$, which can be better retained by the cell ( Giordano et al., 2003, Moroney et al., 2011 and Raven, 2001). Atrazine exposed cells acts like cells grown in dark conditions changing their autotrophic metabolism (Esperanza et al., 2015), according to results obtained by Hemschemeier et al. (2013) when *C. reinhardtii* cells were studied under anoxic and dark conditions. Since

the CCM is only activated in the presence of light, enzymes related to this process, such as CAH4 and CAH5, were expected to be downregulated ( Tirumani et al., 2014).

RNA-Seq data also revealed the upregulation of three transcripts (*HLA3*, *LCIA* and *ELI3*) ( Table 2). HLA3 and LCIA were identified as Ci transporters candidates. *HLA3* (high light-induced gene 3, also known as Mrp1 for multidrug resistance-related protein 1) has been annotated as a putative ATP-binding cassette-type transporter, containing a single ABC-MRP domain and it was predicted to be localized to the plastid membrane ( Winck et al., 2013). *LCIA*, also known as Nar1.2, was identified as a limiting-$CO_2$-inducible gene in a large-scale analysis of gene expression profiles in *C. reinhardtii* ( Miura et al., 2004). LCIA has six predicted transmembrane domains, and possesses a domain of the formate/nitrite transporter protein family. Members of this protein family have been identified in the genome of prokaryotes, yeast and green algae *Chlamydomonas*,*Chlorella* and *Volvox*. It has been reported *HLA3* increases in *C. reinhardtii*, under low $CO_2$ conditions (Jungnick et al., 2014). LCIA was proposed as a candidate Ci transporter localized in the chloroplast membrane of *Chlamydomonas* cells ( Winck et al., 2013) rather than a nitrite transporter, as are the remaining *NAR1* gene family members of *C. reinhardtii*, because the expression of *LCIA* is regulated by $CO_2$ irrespective of the nitrogen source ( Miura et al., 2004 and Spalding, 2008). RNAi strains with reduced expression of both *LCIA* and *HLA3* grew poorly on low $CO_2$ (Duanmu et al., 2009).

The *ELI3* transcript encodes an early light inducible chlorophyll *a*/*b* binding protein (Table 2). *ELIs* such as *ELI3* encode polypeptides with high sequence similarity to the early light-induced proteins (Elips) characterized in vascular plants and to the Cbr protein from *Dunaliella bardawil*. Biochemical studies suggest that Elips bind both chlorophyll*a*nd lutein ( Adamska, 1997 and Adamska, 2001) and they might function in a photoprotective manner, possibly scavenging chlorophyll molecules during the turnover of light-harvesting complexes ( Adamska, 1997 and Adamska, 2001). Hutin et al. (2003)showed that Elips function in photoprotection, either by binding free chlorophyll or chlorophyll-degradation products, or by promoting the proper assembly of pigment protein complexes during exposure of cells to high light. It has been hypothesized that they play a protective role either by transiently binding the excited free chlorophyll molecules as transient pigment carriers and/or by binding xanthophyll pigments to dissipate the excess absorbed light energy ( Adamska, 2001, Havaux, 2003, Hutin et al., 2003, Król et al., 1999 and Montané and Kloppstech, 2000). Cbr, the *D. bardawil*homologue of Elips, is induced when algal cells are exposed to stressful conditions, such as high light treatment, sulfur starvation, norflurazon treatment (norflurazon blocks carotenoid biosynthesis) ( Lers et al., 1991 and Levy et al., 1993) or

atrazine treatment. This study also shows that atrazine causes an induction of *ELI3* transcript ( Table 2). More recently, ELIPS in *Arabidopsis* was proposed to physiologically function in the regulation of chlorophyll concentration in thylakoid membranes: it acts as a chlorophyll sensor that modulates 5-aminolevulinic acid biosynthesis to prevent accumulation of free chlorophyll and hence prevent photooxidative stress ( Tzvetkova-Chevolleau et al., 2007 and Zhang et al., 2012).

Transcriptomic results showed that the amounts of various transcripts related to photosynthesis decreased in the cultures exposed to atrazine with respect to control cultures (Table 2). Transcripts encoding for light-harvesting, carbon concentrating mechanism or other chlorophyll binding proteins were reduced in abundance. The general downregulation of genes associated with chlorophyll binding or biosynthesis is supported by the lower chlorophyll contents (Fig.1) (Chekounova et al., 2001 and Duanmu et al., 2013). Hemschemeier et al. (2013) also found this reduction in pigment content in dark-grown *C. reinhardtii* cells. Furthermore, the reduced abundances of transcripts related to photosynthesis would be reflected by the status of the photosynthetic apparatus and the PSII as we can see in FCM results, which show a decrease in the chlorophyll *a* fluorescence ( Fig. 2A). *C. reinhardtii* cells exposed to atrazine had lower chlorophyll contents, and exhibited reduced autofluorescence. However, there were a few photosynthesis-related transcripts whose amounts increased (*HLA3*, *LCIA* and *ELI3*; Table 2). In view of these data, it can be assumed that the few photosynthetic transcripts that were accumulated have a role in the acclimation of a photosynthetic organism to the stress provoked by the herbicide.

### *3.5. Proteomic analyses*

RNA-Seq technique allows the study of a rapid response to stress, but it is also important to consider the early alterations in the proteome profile. For this purpose, an iTRAQ peptide labeling experiment was made. Relative abundance of 12 proteins were significantly decreased ($p \leq 0.05$ and expression ratio ≤ 0.8) in cultures exposed to atrazine 0.25 μM, being 5 of them proteins related to photosynthesis (CA2, PSBO, PSB1, PRK1, ATPD) (Table 3).

Photosynthesis and carbon assimilation were also affected by atrazine at the proteomic level. Carbonic anhydrase (CA2 or CAH2), with carbonate dehydratase activity, was significantly decreased in treated cells (Table 3). This protein catalyzes the interconversion between carbon dioxide and bicarbonate, playing an important role in carbon fixation, metabolism and Calvin cycle. The interpretation of the physiological function of carbonic anhydrases (CAs) in unicellular algae is difficult due to the presence of multiple CA isozymes and different localizations, as commented above,

but at least two alpha CAs are found in the periplasmic space of *Chlamydomonas*, namely CAH1 and CAH2. These proteins facilitate the diffusion of Ci to the plasma membrane (Moroney et al., 2011). In microalgae that possess a carbon concentrating mechanism (CCM), the enzyme located in the chloroplast stroma that has carbonate dehydratase activity (CA2), is needed to convert the accumulated $H_2CO_3$ to $CO_2$, the substrate for Rubisco ( Badger and Price, 2003 and Huang et al., 2011). Uptake of inorganic carbon as $CO_2$ or bicarbonate is facilitated in *Chlamydomonas* across all membrane systems of the cell by CCM acting in concert with carbonic anhydrase ( Förster et al., 2006).

Table 3. Comparison of photosynthesis-related protein abundances in cultures exposed to 0.25 μM of atrazine for 3 h *vs.* cultures control of *C. reinhardtii* cells. For each comparison, a protein was deemed differentially downregulated if it met the following criteria: 1) significant ratio (≤ 0.8) and 2) significant *p*-value ($p \leq 0.05$).

| Accession number | Protein name | Description | 0.25 μM:control | |
|---|---|---|---|---|
| | | | Ratio | *p*-Value |
| P93109 | CA2 | Periplasmic carbonic anhydrase, alpha type | 0.2466 | 0.0032 |
| A8IYP4 | PRK1, 195910 | Phosphoribulokinase | 0.4613 | 0.0029 |
| A8J0E4 | PSBO, 130316 | Oxygen-evolving enhancer protein 1 of photosystem II | 0.5495 | 0.0010 |
| A8IYH9 | PSBP1, 33411 | Oxygen-evolving enhancer protein 2 of photosystem II | 0.4571 | 0.0055 |
| A8JF15 | ATPD, atpH, 132678 | Chloroplast ATP synthase delta chain | 0.4529 | 0.0430 |

The enzyme phosphoribulokinase (PRK1) also decreases in cultures exposed to atrazine (Table 3). PRK1 is recognized as an important regulatory protein in the process of carbon fixation. Unique to the Calvin cycle, PRK catalyzes the ATP-dependent phosphorylation of ribulose 5-phosphate, thereby regenerating ribulose 1,5-bisphosphate (RuBP), the $CO_2$ acceptor molecule and substrate for ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (McKay et al., 1991).

Moreover, the presence of both proteins encoding PSII components and subunits of the oxygen-evolving complex (OEC) of PS-II (PsbO, PsbP1) were also reduced following herbicide exposure. Furthermore, the levels of the chloroplastic ATP synthase subunit (ATPD), with proton-transporting ATP synthase activity was found to be reduced in cultures exposed to atrazine with respect to the control cultures (Table 3). Thus, under atrazine stress, the algal cells have diminished photosynthetic capacity due to the reduction of components of photosystem II, as well as the plastidic ATP-

generating system and this finding is in accordance with the observed significant decrease in the chlorophyll *a* fluorescence and pigments ( Fig. 1 and Fig. 2A).

Gene expression is controlled globally and at multiple levels in response to environmental stress (Lackner et al., 2012). As being indicated before, in the aim of this study, changes in the proteomic profile detected after only 3 h of exposure are mainly due to the stress directly caused by the herbicide on proteins, and not due to a change in the regulatory processes of gene expression.

In Esperanza et al. (2015), a significant decline in cellular activity was also observed in the *C. reinhardtii* cells exposed to atrazine and this may be also related to a reduction in the photosynthetic activity ( Fig. 1 and Fig. 2A). *C. reinhardtii* cells under atrazine stress conditions could change their photosynthetic metabolism to a heterotrophic one, because this herbicide exerts its mechanism of action on the photosystem II ( Marchetti et al., 2013 and 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.

To sum up, the presence of atrazine leads to an increase in the cellular ROS levels that can be related to a decrease in chlorophyll content and, hence, in a decrease in the photosynthetic activity that can enhance the ROS production, as a loop. Furthermore, this herbicide causes photosynthetic adjustments in *C. reinhardtii* cells based on alterations observed in transcriptomic profile as well as the disappearance of some photosynthesis-related proteins. On the one hand, levels of transcripts encoding for chlorophyll binding proteins, components of the PSII and carbonic anhydrases, which help increase the $CO_2$ concentration in the chloroplast, were reduced. On the other hand, some transcripts encoding for $C_i$ transporters were upregulated as a cell response to acclimation to stress provoked by the herbicide. Moreover, the amount of PSII component proteins, a chloroplast ATP synthase and a carbonic anhydrase, an important regulatory protein in carbon fixation process, decreased due to the stress caused by this herbicide.

## 4. Conclusions

A sublethal concentration of atrazine (0.25 μM) alters the transcriptomic and proteomic profile of *Chlamydomonas reinhardtii* cells. Using flow cytometry protocols a decrease in chlorophyll *a* fluorescence and an increase in intracellular ROS levels were detected in atrazine exposed cells with respect to control cells. A reduction in cellular pigment contents was also observed in exposed cultures. Transcriptomic analyses advance the understanding of photosynthetic adjustments that occur during herbicide

exposure. Inhibition of photosynthesis induced by atrazine toxicity will affect the entire physiological and biochemical states of microalgal cells. Protein profile was also altered by the atrazine stress. Taking into account these results, the increase in some pollutants in aquatic systems could lead to a dramatic decrease in $CO_2$ fixation at a global level.

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