Does a short-term exposure to atrazine provoke cellular senescence in Chlamydomonas reinhardtii?

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ABSTRACT

The aim of this study was to investigate the impacts and modes of action of a chemical and nutrient deprivation on the cellular senescence process of Chlamydomonas reinhardtii. Several molecular and cellular parameters related to senescence phenomena were monitored in C. reinhardtii cells exposed for 24 h to a sublethal concentration (0.25 μM) of the herbicide atrazine and in unexposed 96 h cells in an early stationary phase of growth. All endpoints showed the same pattern of response between treatments, except for the intracellular level of calcium, where a significant increase was observed in 24 h-exposed cultures compared to 24 h-log controls. Results also indicated that cell viability remained above 98% for all conditions. Reactive oxygen species (ROS) levels and caspase activity increased in all experimental cultures with respect to 24 h-log controls and alterations in the nuclear morphology and cells with auto-phagosomes were observed in all treatments. However, a decrease in lipid peroxidation was detected which could be related to the observed increment of autophagic vacuoles that recycle damaged material, such as altered lipids in microalgal membranes. Furthermore, responses at the molecular level were also investigated. Gene transcription analyses, carried out by RT-qPCR technique, indicated an increase in transcripts for genes encoding glutathione S-transferase (GST), ascorbate peroxidase (APX I) and Mn-superoxide dismutase (SOD-1) and a decrease for those encoding catalase (CAT), glutathione peroxidase (GPX) and Mn-superoxide dismutase (SOD-1) in both experimental treatments. Overall molecular and cellular results suggest that a short-term exposure to a sublethal concentration of atrazine may induce senescence features in microalgal cells which are the base of aquatic food webs.

1. Introduction

Unicellular and multicellular organisms live in a constant struggle to cope with extrinsic and intrinsic damaging agents. The inevitable accumulation of damage throughout the life cycle leads to the deterioration of cell components and the impairment of cellular functions (Campisi, 2007) provoking cellular senescence. Many cellular challenges for organisms originate from limitations in the maintenance and repair mechanisms of DNA or by anomalies in the antioxidant mechanisms that contribute to the detoxification of reactive oxygen species (ROS) (Van Heemst et al., 2007). In fact, many features of senescence result from the incapacity of cells to adapt to stress conditions and when damage accumulates irreversibly, mitotic cells rely on either of two mechanisms to avoid replication: they can permanently arrest the cell cycle or trigger cell death programs (Vicencio et al., 2008). On the one hand, apoptosis is the best-described form of programmed cell death (PCD) that can occur through processes such as chromatin condensation and margination, DNA cleavage with unchanged cytoplasm and organelles, and an increase in cytosolic calcium concentration and in the activity of a family of cysteine proteases named caspases (Danon et al., 2000; Hoeberichts and Woltering, 2003). On the other hand, autophagy (i.e., self-eating), which is a lysosomal degradation pathway essential for homeostasis, may contribute to cell death as well (Vicencio et al., 2008); the typical hallmark of this phenomena is the presence of auto-phagosomes in the cytoplasm of cells (Pérez-Pérez et al., 2010, 2012).

In aquatic ecosystems, unicellular organisms, such as microalgae, are exposed to a heterogeneous natural environment and have to deal
with nutrient patchiness. Thus, when unicellular populations increase, nutrients become depleted leading to senescence or cell death. This phenomenon is analogous to cultures grown in closed systems (batch culture) under laboratory conditions (Humby et al., 2013). Batch culture growth is characterized as having a logarithmic growth phase where nutrients are sufficient to support high rates of growth. Then, as cell number increases and nutrients become depleted, there is a transition to a stationary phase where division ceases and cell numbers are maintained, which is generally referred to as cellular senescence (Franklin et al., 2006; Fredriksson and Nystrom, 2006). Cellular senescence is a stable form of cell cycle arrest that limits the proliferation of damaged cells (White and Lowe, 2009); it is under this phase that cells undergo metabolic and structural changes that can lead to death and the loss of cell numbers (Fogg and Thake, 1987; Wanner and Egli, 1990). Even if new nutrients were added in the medium many cells are not capable of returning to its growth state, i.e., the cellular senescence process will not be reversible and cells will die (Franklin and Berges, 2004; Fredriksson and Nystrom, 2006). In particular, PCD has been observed in microalgae exposed to several stressors, such as natural toxicants (i.e., ochratoxin) (Sorrenti et al., 2013), metals (Bahadar et al., 2014; Gid et al., 1996), radiation (Sanches Silveira and Myaki Pedroso, 2014) and pesticides (Zhang et al., 2012); however the link between these stresses and PCD remains unknown.

Atrazine (ATZ) is one of the triazine herbicides most extensively applied in agriculture all over the world and used as a chemical stressor in this work. Atrazine inhibits photosynthesis blocking the photosynthetic electron transport at photosystem II (Rutherford and Krieger-Liszkay, 2001) and thereby energy production, preventing CO2 fixation in target and non-target organisms. Detrimental effects of atrazine on aquatic ecosystems and ATZ-induced alterations of aquatic community structures have been previously reported (Choi et al., 2012; Didur et al., 2012; Sjollema et al., 2014 and Weiner et al., 2004). Due to environmental concerns, some triazines, including atrazine, have been banned in Europe (European Commission (SANCO/10496/2003-final), 2003). However, atrazine is still detected in the environment together with the triazines in use nowadays (Loos et al., 2009 and Ma et al., 2006). This is due to their long retention time in the soil and aquifers, which leads to perdurable leaching and long-lasting levels in different ecosystems even years after their prohibition (Lorente et al., 2014) at concentrations exceeding 10 μg L−1 (USEPA, 2012).

Among aquatic microalgae, *Chlamydomonas reinhardtii* was used in this work. This freshwater species is currently used extensively in biological research and in molecular genetic studies as a model organism because of its ease of cultivation, rapid growth, haploidy and possibility of inducing sexual reproduction (Harris, 1989, 2001), as well as the knowledge of its sequenced genome (Merchant et al., 2007). Previous studies using transcriptomic, proteomic and cytomic analyses showed that atrazine altered the energy status of *C. reinhardtii* cells and provoked an inhibition of photosynthesis that affected the entire physiological and biochemical state of microalgal cells (Esperanza et al., 2016). Despite these alterations, cells were able to overcome stress and maintain cellular viability after 3 and 24 h of atrazine exposure (0.25 μM), suggesting that *C. reinhardtii* shows an important adaptability to the adverse conditions (Esperanza et al., 2015a). The potential stimulation of the antioxidant defence mechanisms could be involved in this adaptation to oxidative stress.

The aim of the present work was to evaluate the effects of chemical exposure and nutrient deprivation on the molecular and cellular functions of green unicellular algae in order to study the potential occurrence of cellular senescence processes in *C. reinhardtii*. To do so, microalgal cultures in exponential phases were exposed to a sublethal concentration of the herbicide atrazine (0.25 μM) for acute 24 h. Unexposed cultures were also monitored over time to evaluate potential senescence features in stationary cultures (96 h). Several molecular and cellular parameters related to senescence process were studied: cytotoxic levels of Ca2+ were evaluated to study signal transduction, and ROS production and lipid peroxidation measured to determine the oxidative stress status in microalgal cells. Moreover, the transcriptional responses of oxidative stress-related genes were measured using RT-qPCR for studying the potential activation of antioxidant enzyme system. Finally different hallmarks of PCD, such as caspase activity, nuclear morphological alterations and presence of auto-phagosomes were also investigated.

2. Materials and methods

2.1. Microalgal cultures

The unicellular green alga *Chlamydomonas reinhardtii* Danegard (strain CCAP 11/32A mt +) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK) and Canadian phycological culture center (Toronto, Canada). *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. Initial cell density for each experiment was 2 × 105 cells mL−1, with the aim of having stationary cultures at 96 h (Fig. 1).

The atrazine (ATZ) concentration used (0.25 μM) in the present study was based on 96 h EC50 determined by previous growth toxicity tests (Esperanza et al., 2016). Before each experiment, fresh stock solutions of atrazine were prepared by dissolving the pure compound (Sigma-Aldrich, MW: 215.68, purity: 98.8%) in methanol and filtering through 0.2 μm membrane filters. To achieve this nominal concentration, stock solutions volume added to the microalgal cultures never exceed 0.05% of final volume. A statistical comparison between 0.05% methanol controls and no-solvent controls revealed no significant difference in algal growth (t-test; p > 0.05), and all subsequent tests were performed using only the solvent control. Nominal and effective concentrations of atrazine were found to be similar following a gas chromatography/mass spectrometry analyses (Fernández-Naveira et al., 2016) indicating stable concentrations through time in microalgal cultures.

All cultures were carried out in triplicate and at least two independent experiments were conducted for each parameter analysed. Microalgal cultures were incubated without herbicide (control log culture) for 24 h, corresponding to a complete light-dark cycle where cells were in logarithmic phase. Other cultures were incubated for 24 h with 0.25 μM of atrazine (ATZ exposed culture) to analyse an acute exposure of this herbicide. Furthermore, microalgal cultures without atrazine (unexposed stationary cultures) were kept for 96 h in the incubator for doing the analysis when cells are in stationary phase (Fig. 1). All cultures were incubated under controlled light, temperature and agitation conditions.

2.2. Flow cytometric analyses

Cell suspensions (2 × 105 cells mL−1) were incubated with the appropriate fluorochrome at room temperature (RT) 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. 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). All FCM determinations were performed at least twice and duplicate samples were run on the flow cytometer.

### 2.2.1. Growth measurement and cell viability determination

Growth of microalgal cultures was measured by counting culture aliquots with a flow cytometer using a suspension of fluorochrome-containing micro-spheres for its calibration (FlowCount Fluorospheres, Beckman Coulter Inc.). Daily growth rates (DGR) (day\(^{-1}\)) for unexposed and exposed (0.25 μM of atrazine) cultures were calculated for periods of 24 h using the following formula: 

\[
\text{DGR} = \frac{\ln(N_t) - \ln(N_0)}{t},
\]

where \(N_t\) is the cell density at time \(t\) and \(N_0\) is the cell density at time 0.

FCM assay based on dye exclusion of the probe propidium iodide (PI) was used to identify cells with intact plasma membrane. 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 analysed by FCM (Prado et al., 2009). 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.

### 2.2.2. Intracellular free calcium

The FCM assay based on dye exclusion of the probe Calcium Green-1 acetoxyethyl ester was used to detect changes in cytosolic free Ca\(^{2+}\) levels. The cell-permeant acetoxyethyl ester, which is non-fluorescent and Ca\(^{2+}\) insensitive, can be passively loaded into cells where it is cleaved by ubiquitous intracellular esterases to the cell-impermeant fluorescent product Calcium Green-1. This cellular product exhibits an increase in fluorescent emission intensity (530 nm) upon binding to Ca\(^{2+}\). As previously described in Prado et al., 2012b; *C. reinhardtii* cell suspensions were incubated with the fluorochrome (final concentration: 8 μM) at 30 °C for 2 h. The fluorochrome green fluorescent emission was collected in the FL1 channel. Data were collected as the mean fluorescence value of the cell population since data showed a normal distribution (Prado et al., 2012b).

### 2.2.3. Oxidative stress determination

Oxidative stress in *C. reinhardtii* was evaluated by FCM using determinations of intracellular levels of hydrogen peroxide (H\(_2\)O\(_2\)) with an oxidation-sensitive fluorescent dye, dihydrorhodamine 123 (DHR123). DHR123 was added at a final concentration of 29 μM for 40 min to the cell suspensions (2 × 10\(^5\) cells ml\(^{-1}\)). Since data were normally distributed, mean fluorescence values of orange-ethidium bromide (EB) were collected in the FL3 channel (Prado et al., 2012a).

### 2.2.4. Caspase activity

A flow cytometric assay was performed following the manufacturer’s instructions using CellEvent Caspase-3/7 Green kit (Molecular Probes) to study the caspase activity in *C. reinhardtii* cells (González-Pleiter et al., 2017). The CellEvent Caspase-3/7 Green Detection Reagent is a four-amino acid peptide (DEVD) conjugated to a nucleic acid-binding dye with absorption/emission maxima of 502/530 nm. DEVD peptide inhibits the ability of the dye to bind to DNA. However, after activation of caspase-3/7 in damaged cells, the DEVD peptide is cleaved, enabling the dye to bind to DNA and produce a bright fluorogenic response. The green fluorescent emission of the dye was collected in the FL1 channel. Data were collected as the mean fluorescence value of the cell population since data showed a normal distribution.

### 2.3. Spectrophotometric analyses

Lipid peroxidation, catalase activity and protein content were measured in *Chlamydomonas reinhardtii* cells.

Algae were harvested (5 × 10\(^6\) cells) by centrifugation and dissolved in 800 μL phosphate buffer (50 mM) containing trichloro-acetic acid (TCA) (0.67%, w/v) in the lipid peroxidation protocol followed by cell disruption using ultra-sonication at 400 mA for 180 s. Homogenates were centrifuged and the supernatant was collected and stored at −80 °C. Total protein content of each supernatant was measured by the Bio-Rad protein assay (Bio-Rad) using the Coomassie Brilliant Blue G-250 dye (absorbance was read at 600 nm) (Bradford, 1976).

#### 2.3.1. Lipid peroxidation

Lipid peroxidation (LP) was measured as an indicator of ROS-mediated damage. Levels of lipid peroxidation products were measured as thiobarbituric acid reactive substances (TBARS) according to methods described by Heath and Packer (1968) with modifications. The method was based on the malondialdehyde (MDA) production during the oxidation of polyunsaturated fatty acids. 150 μL of supernatant was mixed with 450 μL of 0.5% thioarbituric acid (TBA) (w/v) in 20% TCA.
stationary cells (5 × 10⁶ cells) were stained with 7.2 μM of DAPI and MDC ware (Bio-Rad). QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer s protocol. For each replicate (n = 3) select reference genes.

Relative gene transcription was calculated using reference genes (CBLP, BTUB and UBCLX) and the Cq values were corrected with the estimated PCR efficiency. The comparative threshold method (ΔΔCt) (Livak and Schmittgen, 2001) was used for relative quantification. Each reaction was run in technical triplicate and three independent biological replicates were calculated. The control samples (non-exposed microalgae) at 24 h were chosen to represent 1 × transcription of the genes of interest. Treated samples were expressed relative to the corresponding control to determine the difference in transcription.

2.6. Data analyses

For data analyses mean values ± standard deviation (SD) of experiments were calculated. Results were then expressed as a percentage of variation with respect to control at 24 h, for which a value of 100% was assigned. These data were statistically analysed by an overall one-way analysis of variance (ANOVA) using IBM SPSS Statistics software 21.0. A p-value < 0.05 was considered statistically significant. When significant differences were observed, control cultures at 24 h vs. exposed and stationary cultures means were compared using the Dunnett post hoc test (p < 0.05).

3. Results and discussion

3.1. Cell viability and daily growth rates

The percentage of viable cells of C. reinhardtii with intact plasma membrane (PI- cells) remained close to 100% in all tested conditions; no significant differences (p > 0.05) were detected in comparison to 24 h-control (data not shown), indicating that 0.25 μM of atrazine represents a sublethal concentration for these unicellular algae.

Regarding growth rates, results obtained in unexposed cultures showed a significant decrease (p < 0.05) in the 72–96 h DGR (0.55 ± 0.02) with respect to 0–24 h-control cultures (1.96 ± 0.05), suggesting that after 96 h cells enter stationary phase, probably due to the depletion of nutrients in the medium that are insufficient to support high rates of growth. Moreover, cells were affected by herbicide stress (0.25 μM of atrazine), exposed cultures showed a significant decrease (p < 0.05) in the 0–24 h DGR (1.66 ± 0.08) and in the 72–96 h DGR (0.40 ± 0.02) with respect to 0–24 h-control cultures.

Similar results were detected in Esperanza et al. (2015a) where growth data indicated that atrazine treatment after 24 h caused a significant (p < 0.05) inhibition of growth of 45.6% in C. reinhardtii cells. It has also been reported that other microalgal cells exposed to several concentrations of atrazine after 48 and 96 h were not able to complete their cell division but maintained their metabolic activity and cell viability (González-Barreiro et al., 2004).

3.2. Calcium signalling

Ca²⁺ is a ubiquitous intracellular second messenger in the signal transduction of environmental stimuli in plants (Sun et al., 2006). The acute exposure to atrazine caused a significant increase (113.60 ± 4.59%, p < 0.05) of the intracellular free Ca²⁺ levels in C. reinhardtii with respect to control log cultures. However, in 96 h-stationary unexposed cultures a significant decrease (82.47 ± 8.94%, p < 0.05) of the intracellular free Ca²⁺ was observed (Fig. 2).

The increase in the cytosolic Ca²⁺ level has been previously observed in plant cells in response to different stress factors such as heat and aluminium (Gong et al., 1998; Sivaguru et al., 2005). When plants are forced to respond to environmental stimuli, the Ca²⁺ level increases rapidly and transiently in the cytoplasm as a result of either Ca²⁺ uptake from the extracellular space through the plasma membrane channels or Ca²⁺ release from internal stores, such as the endoplasmic reticulum or vacuoles (Sun et al., 2006). In this study, the fast
significant increase in cytosolic Ca\(^{2+}\) level in cells acutely exposed to atrazine could indicate that this ion acts as a messenger in response to chemical stress. The fact that Ca\(^{2+}\) level raises rapidly and transiently could explain the results obtained in unexposed stationary cultures where analyses were realized after 96 h (Fig. 2). Several studies showed an increase in intracellular free Ca\(^{2+}\) levels in microalgae in response to different stress factors, i.e., nitrogen starvation, triclosan and paraquat (Chen et al., 2014; González-Pleiter et al., 2017; Prado et al., 2012b).

Since the role of Ca\(^{2+}\) as a secondary messenger in the cell response to oxidative stress has been suggested (Price et al., 1994), intracellular levels of reactive oxygen species (ROS) were studied in both experimental treatments with respect to control log cultures.

### Table 1

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Abbreviation</th>
<th>Primer sequence</th>
<th>Efficiency (%)</th>
<th>Amplicon length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>G protein beta subunit-like polypeptide</td>
<td>CBLP</td>
<td>Forward 5′ GCC ACT CCC TGT AAA TGC C 3′ Reverse 5′ CTC CTC CTA AAC CCC TCC AG 3′</td>
<td>93.2</td>
<td>88</td>
<td>Sanchez et al. (2015)</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2</td>
<td>UBCX</td>
<td>Forward 5′ ATA CAC AAC GAC GAT GAT 3′ Reverse 5′ TGG GTG GTG TCC TCT CAT CA 3′</td>
<td>96.6</td>
<td>95</td>
<td>Sanchez et al. (2015)</td>
</tr>
<tr>
<td>Beta tubulin 2</td>
<td>BTUB</td>
<td>Forward 5′ CCC CGG CCG GCA CTT CTT C 3′ Reverse 5′ GTC GCC GGC GCA CAT CAT 3′</td>
<td>94.3</td>
<td>133</td>
<td>Idione et al. (2014)</td>
</tr>
<tr>
<td>Ascorbate peroxidase</td>
<td>APX-1</td>
<td>Forward 5′ AGT GTG GTA GTG GGT GGC AG 3′ Reverse 5′ CGG TAT GAT GAT AAG GGT TCG CA 3′</td>
<td>103.2</td>
<td>100</td>
<td>Sanchez et al. (2015)</td>
</tr>
<tr>
<td>Catalase</td>
<td>CAT</td>
<td>Forward 5′ CAG GAG GCT GCA GGA AAA CT 3′ Reverse 5′ ATG ACA ACA TGT ACA TTA CCG GG 3′</td>
<td>93.4</td>
<td>109</td>
<td>Sanchez et al. (2015)</td>
</tr>
<tr>
<td>Mn-superoxide dismutase</td>
<td>SOD-1</td>
<td>Forward 5′ TGT CAG TTT TGT TTC OCT GGT 3′ Reverse 5′ CTT CTA TGT TCC TGC CCA GC 3′</td>
<td>92.3</td>
<td>108</td>
<td>Sanchez et al. (2015)</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>GPX</td>
<td>Forward 5′ CTG TTG GGT TTG TCC TGC TC 3′ Reverse 5′ GCC AGC CCC TAC GAT ACA AG 3′</td>
<td>95.1</td>
<td>119</td>
<td>Sanchez et al. (2015)</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>GST</td>
<td>Forward 5′ GCT AGC AAC GAA AAA CTC TTT 3′ Reverse 5′ GGC TCA GAA TGT CAA TCT GG 3′</td>
<td>97</td>
<td>106</td>
<td>Sanchez et al. (2015)</td>
</tr>
</tbody>
</table>

**Fig. 2.** Variations in intracellular free calcium and H\(_2\)O\(_2\) levels, caspase activity, lipid peroxidation and catalase activity in *C. reinhardtii* cells exposed to 0.25 μM of atrazine for 24 and 96 h-unexposed stationary cells. Results for parameters are shown as a percentage of variation with respect to 24 h-control (for which the value of 100 was assigned, indicated by the dashed line). Significant differences with respect to 24 h-log control are represented by an asterisk (*). Different letters indicate significant differences between treatments (p < 0.05) according to the Dunnett post hoc test.

In algae, the excess ROS content may affect many cellular functions through modification of nucleic acids, oxidation of proteins, deterioration of chlorophyll, and induction of lipid peroxidation (Ledford and Niyogi, 2005; Mittler et al., 2004; Vavilala et al., 2016). Particularly, MDA is one of the several low-molecular-weight end products formed by the decomposition of primary hydroperoxides and lipid polymers (Gill and Tuteja, 2010). Measurement of MDA level is routinely used as an index of lipid peroxidation in algae, and has been documented to vary in the algae *C. pyrenoidosa* under many stressful conditions such as high temperatures (Vavilin et al., 1998), exposure to wide range of trace elements in the alga *P. viridis* (Li et al., 2006), exposure to organic pollutants in the alga *E. gracilis* (Li et al., 2009) and in *C. reinhardtii* (Elbaz et al., 2010; Sanchez et al., 2015). Contrary to expectations, the levels of MDA decreased in acutely exposed cultures and in 96 h-unexposed stationary cultures (95.57 ± 3.31%, 81.23 ± 6.64%, respectively). These differences are significant (p < 0.05) only in stationary cultures (Fig. 2). No significant differences (p > 0.05) were detected in algae acutely exposed and stationary cells, showing similar levels of MDA (Fig. 2). MDA results did not indicate lipid peroxidation in algal cell membranes after exposure to atrazine and depletion of nutrients in the culture medium on a potential oxidative stress of *C. reinhardtii*, FCM analyses of intracellular ROS generation was evaluated. Results obtained showed a significant increase (p < 0.05) in the intracellular level of hydrogen peroxide (H\(_2\)O\(_2\)) in cultures acutely exposed to the chemical (187.83 ± 8.99%) and in 96 h-unexposed cultures (181.75 ± 19.73%) with respect to 24 h-log control cultures (Fig. 2). Data showed similar levels of impact on this oxidative stress marker for both treatments groups (Fig. 2), indicating that the production of ROS may be a common action mechanism between atrazine toxicity stress in exposed cultures and starving stress in stationary cultures.
to atrazine or in 96 h-stationary cultures but FCM analysis showed an increase of ROS for these two treatments (Fig. 2). Although the measurement of functional responses of individual cells by FCM is more sensitive than bulk analyses using spectrophotometry, this absence of measurement of functional responses of individual cells by FCM is more instrumental. To prevent damages of cellular components, leading to a disruption of crucial metabolic processes (Mittler, 2002; Vega et al., 2006). To prevent the overproduction of ROS in cells is often related to unspecific damages of cellular components, leading to a disruption of crucial metabolic processes. The overproduction of ROS in cells is often related to unspecific damages of cellular components, leading to a disruption of crucial metabolic processes (Mittler, 2002; Vega et al., 2006). 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Fig. 4. Cell alterations evaluated in *C. reinhardtii* cells in 24 h log control cultures, 24 h exposed cultures to 0.25 μM of atrazine and 96 h stationary cultures. (A) Chromatin alterations or nuclear morphological alterations and presence of autophagic vacuoles, evaluated by DAPI and MDC stainings, respectively, using epifluorescence microscopy. Results are shown as percentage of variation of DAPI-positive and MDC-positive with respect to 24 h-control (for which a value of 100 is assigned, indicated by the dashed line). Significant differences (p < 0.05) are represented by an asterisk (*). Different letters indicate significant differences between treatments at the level of 0.05 (p < 0.05) according to the Dunnett post hoc test. Representative pictures were taken with a high-definition cooled colour camera Nikon DS-5Mc at ×1000 magnification, (B) DAPI-negative cell and (C) DAPI-positive cell and (D) MDC-positive (the right cell) and MDC-negative (the left cell).

3.5. Caspase activity and other hallmarks of PCD

Programmed cell death (PCD) is a cellular process that can occur in response to various biotic or abiotic stresses. PCD may be of high ecological relevance in a unicellular alga, as it may guarantee survival of the population under unfavourable environmental conditions (Darehshouri et al., 2008). Several PCD processes are mediated by a set of enzymes called caspases that have the unusual characteristic of cleaving peptide chains after aspartate residue. Caspase-dependent PCD is regulated by two pathways (Elmore, 2007). These include the extrinsic ‘death-receptor-mediated’ and an intrinsic ‘mitochondrion-mediated’ pathway. These pathways converge with caspase activation.

In this study, a significant (p < 0.05) increase in the caspase activity was observed in 24 h-exposed and 96 h-stationary cultures (113.60 ± 4.59%, 126.23 ± 3.85%, respectively) (Fig. 2). These enzymes are involved in the activation of a caspase activated DNase and subsequently, it generates the classical oligonucleosomal DNA ladder (Sprick and Walczak 2004).

Based on this, cells were analysed by DAPI staining to study the DNA damage in *C. reinhardtii* nuclei. A significantly increased number (p < 0.05) of DAPI-positive (Fig. 4c) cells with intense fluorescence stained nuclei were observed in both treatments compared to 24 h-log control (Fig. 4a). The percentage of DAPI-positive cells reached 146.60 ± 10.10% and 163.79 ± 11.77% in ATZ-exposed cultures at 24 h and in stationary cultures at 96 h, respectively, with similar levels of response (Fig. 4a) as compared to control algae. Obtained results showed the activation of caspase cascade (Fig. 2) that may be related to the morphological changes displayed in the nuclei of *C. reinhardtii* cells (Fig. 4a) with regard to control cultures.

Moreover, to study the potential presence of autophagic vacuoles, cells were analysed by MDC staining, a fluorescent dye that specifically stains auto-phagosomes (Contento et al., 2005; Xiong et al., 2007). A significantly increased (p < 0.05) number of MDC-positive cells were observed in algae exposed for a short period to atrazine and in unexposed cells after 96 h (Fig. 4a). The percentage of MDC-positive cells reached 134.97 ± 2.31% and 136.70 ± 0.57% in exposed cultures at 24 h and in control cultures at 96 h, respectively compared to 24-h control cultures (Fig. 4a). No significant differences (p > 0.05) were detected between treated cultures in DAPI and MDC analyses (Fig. 4a); cells exposed to both treatments (chemical stress and depletion of nutrients) were suffering the same level of alteration. In summary, results suggest an activation of the caspase cascade (Fig. 2), DNA damage in *C. reinhardtii* nuclei and auto-phagosomes in atrazine exposed cells (Fig. 4). These senescence-related parameters were also detected in cells in 96 h-stationary cultures (Figs. 2 and 4). This suggests that a short-term exposure to atrazine provoked a cellular senescence in *C. reinhardtii*, since both treatments have suffered same type and level of alteration.

Regarding DAPI-positive cells, several authors have related these changes in nuclear morphology in plants and microalgae to chromatin condensation, which is frequently described as an apoptotic endpoint (Darehshouri et al., 2008; Elmore, 2007; Ferrandás et al., 2014; Giri et al., 2013; Jimenez et al., 2009; Zappini et al., 2009, 2010). In accordance to this, *C. reinhardtii* cells exposed to atrazine for 24 h and unexposed stationary cells at 96 h presented irregular DAPI staining as well as a slight degree of chromatin clumping in their nuclei (Sprick and Walczak 2004). Since DAPI can stoichiometrically bind to DNA, an increase of dye fluorescence could exclusively be related to the different DNA content in the cells. However, in DAPI-positive cells (Fig. 4c), chromatin aggregation was not only restricted to the nuclear area, with blue-stained granules also observed in the center of the cell. These observations suggest some degree of karyolysis (Jiménez et al., 2009). Esperanza et al. (2015b) observed that a known prooxidant herbicide (paraquat) also provoked similar nuclear morphological alterations in (DAPI-positive) cells in *C. reinhardtii*, supporting again the central role of ROS generation as a trigger of the PCD-related alterations reported in aged cells in this work.

Autophagy has been associated with cell death phenomena. But also as a catabolic process, autophagy allows eukaryotic cells to recycle intra-cellular components including entire organelles during development or under stress conditions such as nutrient limitation (He and Klionsky, 2009; Liu and Bassham, 2012; Mizushima et al., 2011). In addition to recycling, autophagy is required for the degradation of damaged or toxic material that can be generated as a result of ROS...
accumulation during oxidative stress (Pérez-Pérez et al., 2012). The treatment of *C. reinhardtii* cells with H₂O₂ or methylvioleogen results in autophagy activation (Pérez-Pérez et al., 2010, 2012). Interestingly in this study, a decrease in lipid peroxidation was observed (Fig. 2). These data could be related to the presence of autophasic vacuoles that would recycle damaged material, such as altered lipids in microalgal membrane. This could explain the absence of damage in plasma membrane observed in this work reported by PI analyses using FCM. Several authors used MDC staining to investigate the presence of auto-phagosomes in *Arabidopsis* cells (Contento et al., 2005; Xiong et al., 2007). However, this is the first study, to the authors’ knowledge, to use MDC staining in microalgae.

Overlaps between the processes of PCD are detected in this study, such as caspase activity, typical hallmark of apoptosis and auto-phagosomes, typical of autophagy. Several studies have claimed that the two are fully synchronous and finally proceed towards death (van Doorn and Woltering, 2004). In recent years, many researchers have indicated the occurrence of PCD in unicellular algae (Franklin et al., 2006; Pérez-Pérez et al., 2010, 2012), such as *C. reinhardtii*, exposed to various stimuli, i.e. acetic acid (Zuo et al., 2012), ultraviolet light C (UV-C) radiation (Moharikar et al., 2006, 2007), heat (Durand et al., 2011; Nedelcu, 2006) and mastoparan (Yordanova et al., 2013).

As commented before, the use of senescence in microalgal work is problematic since the process of growing old is difficult to define in unicellular organisms. Senescence has often been used to refer to the appearance of cells after exponential growth, i.e. when many cells are probably dead due to natural cell death (Franklin et al., 2006). In the present work, the occurrence of several PCD hallmarks is reported in *C. reinhardtii* cells after a short-term exposure to atrazine as well as in nutrient-deprived cells. In this sense, algal senescence could be functionally equivalent to multicellular plant senescence (which is controlled via PCED) with protease expression and cellular dissolution occurring in order to fulfil a functional role in natural populations (Franklin et al., 2006).

4. Conclusions

In the present study, the response of the freshwater microalga *C. reinhardtii* exposed to a sublethal concentration of the herbicide atrazine (0.25 μM) for 24 h and in 96 h-unexposed stationary cultures was studied to investigate the effect of a chemical (herbicide) and nutrient deprivation on several senescence-related parameters.

Results of intracellular levels of calcium suggest that this ion can act as a transitory messenger in the signal transduction of *C. reinhardtii* cells exposed to stress conditions, since an increase was detected after short-term exposure to atrazine. Both experimental treatments provoke an increment in ROS generation, suggesting that the common action mechanism between both treatments (chemical and starvation) may be oxidative stress. Moreover, the modification of the expression of antioxidant-related genes observed in this work, either increase in GST and APX 1 or decrease in CAT, GPX and SOD 1 (Fig. 3), suggest a versatile and flexible antioxidant system in response to ROS overproduction. This intracellular increment of ROS levels could be related to the occurrence of typical PCD hallmarks in both experimental treatments. Unexposed cultures in stationary phase showed an activation of caspase cascade, as well as nuclear morphological alterations and auto-phagosomes in *C. reinhardtii* cells, indicating cellular senescence in these cultures. These senescence characteristics with a similar level of alteration with a similar level of alteration also appear in cultures exposed to atrazine. Finally, the decrease observed in lipid peroxidation in both treatments could be also related to the increment of autophasic vacuoles that recycle damaged material, such as altered lipids in microalgae membranes.

Microalgae are known to be sensitive to chemicals and, because of their short life cycle, often provide one of the first signals of ecosystem impacts. Their primary production and their essential roles in nitrogen and phosphorus cycling are critical to aquatic ecosystems. Molecular and cellular alterations reported in the present work suggest that exposure to chemical substances, such as atrazine, induces ROS overproduction leading to the occurrence of cellular senescence alterations in the microalgal populations, potentially compromising biodiversity, structure and function of higher trophic levels in freshwater ecosystems.

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