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

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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.

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 $\mu g\,L^{-1}$ (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

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.

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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 *C. reinhardtii* exposed to a sublethal concentration of the herbicide atrazine for 24 h, 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 *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 2×10^5 cells mL^{-1}.

Atrazine concentration used (0.25 μM) in the present study was based on 96 h 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 h of culture. These time points were selected based on previous cytometry studies where the effects of atrazine were analyzed every hour during 24 h; 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 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).

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-dibutylbarbituric 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 depolarized 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 homogenized 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 °C overnight in order to precipitate