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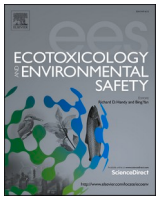

Research Paper

Assessment of cytotoxicity biomarkers on the microalga *Chlamydomonas reinhardtii* exposed to emerging and priority pollutants

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ABSTRACT

Contamination of aquatic ecosystems linked to anthropogenic activity is currently a major concern; therefore, ecotoxicological studies are needed to assess its effect on organisms. The main objective of this study was to investigate the effects of different pollutants on microalgae in search of sensitive biomarkers that can promote a common cytotoxic response regardless of the contaminant. Cultures of the freshwater microalga *Chlamydomonas reinhardtii* were exposed for 24 h to four chemicals, three emerging pollutants (benzophenone-3, bisphenol A and oxytetracycline) and one priority substance (atrazine). A cytometric panel was carried out to assess toxicity biomarkers including cellular growth, inherent cell properties, viability, vitality, cytoplasmic membrane potential and ROS levels. Lipid peroxidation, photosynthetic efficiency and transcriptional responses of photosynthesis- and oxidative stress-related genes using RT-qPCR were also studied. Some toxicity responses showed a similar pattern; a decrease in growth rate, vitality and photosynthetic efficiency and an increase in autofluorescence and in the number of cells with depolarised cytoplasmic membrane and were found for all chemicals tested. However, ATZ and OTC provoked a decrease in cell size, whereas BP-3 and BPA caused an increase in cell size, intracellular complexity and ROS levels and a decrease in cell viability. Assayed pollutants generally promoted an overexpression of genes related to cellular antioxidant defence system and a subexpression of photosynthesis-related genes. In addition to the traditional growth endpoint, cell vitality, autofluorescence and gene expression of catalase, glutathione peroxidase and Fe-superoxide dismutase were significantly affected for all chemicals tested, showing a common cytotoxic response. Among the tested substances, BP-3 provoked the strongest cytotoxic alterations on this microalga, pointing out that some emerging contaminants could be more harmful to organisms than priority pollutants.

1. Introduction

Water pollution and its effect on aquatic ecosystems are currently serious environmental issues to be faced. Over one-third of available freshwater on Earth is used for agricultural, industrial and domestic purposes, resulting in its contamination by various chemical compounds (Loos et al., 2009). The acute and chronic toxicity that these pollutants can exert on aquatic organisms (Sánchez-Avila et al., 2012) causes a continuous loss of biodiversity in aquatic ecosystems (Borgwardt et al., 2019; Lago et al., 2019).

The European Union (EU) established a framework for Community action in the field of water policy attaching a list of priority substances, which included heavy metals, hydrocarbons or pesticides, whose presence in the environment was regulated by law (European Commission, 2000). Atrazine (ATZ), an herbicide widely used in agriculture throughout the world, was included in that list. It acts on photosystem II (PSII), blocking the electron transport chain, inhibiting photosynthesis (Rutherford and Krieger-Liszkay, 2001). Previous studies have reported the negative effects of atrazine on aquatic ecosystems (Esperanza et al., 2017; Graymore et al., 2001; Kabra et al., 2014; Sjollema et al., 2014). A

Abbreviations: ANOVA, analysis of variance; a.u., arbitrary units; ATZ, atrazine; BP-3, benzophenone-3; BPA, bisphenol A; $DiBAC_4(3)$, lipophilic anionic oxonol dye bis-(1,3-dibu-tylbarbituric acid) trimethineoxonol; DHR123, dihydrorhodamine 123; FCM, flow cytometry; FDA, fluorescein diacetate; FS, forward scatter light; LP, lipid peroxidation; MDA, malondialdehyde, OTC, oxytetracycline, PAM, pulse amplitude modulation; PI, propidium iodide; PS, photosystem; QY, quantum yield; ROS, reactive, oxygen species; SS, side scatter light; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid; UV, ultraviolet.

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later European directive cited the so-called emerging pollutants, which were not included in the systematic monitoring programs of the EU, but which pose a significant risk as potentially harmful substances for the aquatic environment (European Union, 2013). This group of pollutants encompass a wide range of substances of different origin and chemical nature, as pharmaceuticals, personal care products (PCPs), illicit drugs, industrial chemicals, *etc*. Benzophenone-3 (BP-3) is used in sunscreens and various cosmetic products, as well as in other industries (Fent et al., 2010; Huo et al., 2016). It works as a broad-spectrum UV (ultraviolet) absorber and is one of the most abundantly UV filters used (Du et al., 2017). Bisphenol A (BPA) is a chemical additive widely used in the manufacture of polycarbonate and epoxy resins and as flame retardant (Juan-García et al., 2015; Staples et al., 1998). Its use was recently limited, due to the potential harmful effects on human health as an endocrine disruptor (Almeida et al., 2018; ECHA, 2017; European Union, 2016). Oxytetracycline (OTC) is a broad-spectrum antibiotic widely used in both human and veterinary medicine. Its mechanism of action is the inhibition of bacterial protein synthesis (Chopra and Roberts, 2001). Antibiotics are also commonly applied as a prophylactic and therapeutic tool to combat pathogens in aquaculture; thus, they can easily reach the environment (Cabello, 2006).

The toxicity evaluation of these pollutants must be carried out according to REACH Regulation (European Commission, 2006). Therefore, it is important to assess the potential harmful effects of these compounds on exposed aquatic organisms. Several studies have shown their toxicity on different phytoplankton species (Esperanza et al., 2020; Tato et al., 2018; Du et al., 2017; Mao et al., 2017; Seoane et al., 2014; Gonçalves et al., 2007). Unicellular algae are frequently chosen for *in vitro* toxicity bioassays, because of their importance at ecological level. As primary producers, microalgae fulfil essential functions in the food chain and any prejudicial effect on them could have a negative impact on higher trophic levels (Esperanza et al., 2020; Rioboo et al., 2007). The freshwater green microalga, *Chlamydomonas reinhardtii*, is widely used as a model for environmental toxicological analyses, since it is commonly found in soils and freshwater and it presents sensitivity to different pollutants (Esperanza et al., 2015a). It is also used in molecular biology and genetic studies since its genome is sequenced (Merchant et al., 2007).

The aim of the study was to evaluate the cytotoxic effects of four contaminants, three emerging pollutants (BP-3, BPA and OTC) and a priority substance (ATZ), on the microalga *C. reinhardtii*. The main objective was to find out if this microalga has a general response against all compounds or if the response is specific to each one. Different parameters were analysed to detect possible early alterations in microalgal cells, in search of sensitive biomarkers of general response on microalgae. Analyses were carried out after 24 h of exposure, since after this time an entire cell cycle and a light:dark cycle were completed.

2. Materials and methods

2.1. Microalgal cultures

The freshwater microalga *Chlamydomonas reinhardtii* Dangeard (strain CCAP 11/32 A mt+) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK). *C. reinhardtii* was cultured in Tris-minimal phosphate medium (Harris, 1989) in flasks filled with 50 mL of culture, in a rotary shaker set at 150 rpm, under controlled conditions: 20 ± 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 toxicity tests and an initial cell density was 2×10^5 cells mL^{-1}. All cultures were carried out in triplicate and cultures without tested chemicals were included as controls.

2.2. Chemicals

The compounds tested, benzophenone-3 or oxybenzone (2-hydroxy-4-methoxybenzophenone, BP-3), bisphenol A (4,4'-dihydroxy-2,2-diphenylpropane, BPA), oxytetracycline ((4S, 4aR, 5S, 5aR, 6S, 12aS) - 4 - (dimethylamino) - 3,5,6,10,11,12a –hexahydroxy-6-methyl-1,12–dioxo-1,4, 4a, 5,5a,6,12,12a-octahydrotetracen-2-carboxamide, OTC) and atrazine (2-chloro-4-ethylamine-6-isopropylamine-1,3,5-triazine, ATZ), were purchased from Sigma-Aldrich and were reagent-grade chemicals with purity higher than 95%. Concentrations of each pollutant were fixed considering the 96 h-EC_{50} values for growth reported for *C. reinhardtii* in previous studies and were also expressed as cellular quota: 5 mg L^{-1} for BP-3 which corresponds to 25 pg $cell^{-1}$ (Esperanza et al., 2019), 30 mg L^{-1} for BPA which corresponds to 150 pg $cell^{-1}$ (Esperanza et al., 2020), 2.5 mg L^{-1} for OTC which corresponds to 12.5 pg $cell^{-1}$ (unpublished data) and 0.054 mg L^{-1} for ATZ which corresponds to 0.27 pg $cell^{-1}$ (Esperanza et al., 2015b). Effective concentrations of each pollutant were confirmed by a high performance liquid chromatography-mass spectrometry (HPLC–MS) analysis (Supplementary Table S1). Before each experiment, stock solutions of each chemical were prepared by dissolving each compound in methanol. The stock solution volume added to each microalgal culture never exceeds 0.05% of final culture volume. The same volume of methanol, without contaminant, was added to the controls. In previous studies it was verified that there were no significant differences ($p > 0.05$) in algal growth between cultures with and without the addition of methanol (Esperanza et al., 2019).

2.3. Flow cytometric (FCM) analyses

FCM analyses were performed on a Gallios flow cytometer (Beckman Coulter Inc.), fitted with a 488 nm excitation laser, detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors: FL1 (505–550 nm), FL2 (550–600 nm), FL3 (600–645 nm) and FL4 (> 645 nm). All the analyses were carried out after 24 h of exposure. Aliquots of each culture were re-suspended in phosphate buffered saline (PBS) solution to obtain a final density of 2×10^5 cells mL^{-1}. Cell suspensions were incubated with the appropriate fluorochrome, in darkness following the proper protocol. For each parameter, 10^4 cells were analysed per culture and fluorescence measurements were obtained in logarithmic scale.

2.3.1. Cellular growth

Cell density was determined after 24 h by counting culture aliquots in the FCM using a suspension of fluorospheres, Flow-Count Fluorospheres (Beckman Coulter Inc.), with a known concentration. 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.

2.3.2. Inherent cell properties

Relative cell size or volume, intracellular complexity or granularity and chlorophyll *a* fluorescence were analysed by FCM directly from the cells in suspension, without staining. Cell size is related to the FS signal and complexity to the SS signal (Shapiro, 1995). Chlorophyll *a* fluorescence was detected in the FL4 channel, since it was excited at 488 nm, emitting in the range of 680–720 nm (Prado et al., 2011). Results were expressed in arbitrary units (a.u.) as the mean of the FS, SS and FL4 fluorescence intensities, respectively.

2.3.3. Cell viability

Propidium iodide (PI) was used to discriminate between viable and non-viable cells based on their permeability to this intercalating agent. Culture aliquots were incubated with 4 μM of PI for 10 min (Prado et al., 2009). Orange PI fluorescent emission was collected in the FL3 detector. Results were expressed as the percentage of viable cells (PI -) *vs.* the total amount of cells analysed.

2.3.4. Cell vitality

Cellular esterase activity was measured using fluorescein diacetate (FDA). Once inside the cell, FDA-acetate residues are cleaved off by intracellular esterases and the fluorescent free fluorescein obtained remains trapped inside viable cells. Metabolic activity or vitality can be estimated by measuring the fluorescence intensity emitted by cells in function of the fluorescein accumulated. *C. reinhardtii* cultures were incubated with 0.12 μM of FDA for 15 min (Prado et al., 2009). Results were expressed as the mean green fluorescence (FL1) intensity in a.u.

2.3.5. Cytoplasmic membrane potential

Alterations on cytoplasmic membrane potential were studied using bis-(1,3-dibutylbarbituric acid) trimethineoxonol ($DiBAC_4(3)$). This fluorochrome can enter depolarized cells where it binds to intracellular proteins or membranes, showing green fluorescent emission (Wolff et al., 2003). The experimental cultures were incubated with 0.97 μM of $DiBAC_4(3)$ for 10 min (Prado et al., 2012b). Results were expressed as the mean green fluorescence (FL1) intensity in a.u.

2.3.6. Intracellular levels of hydrogen peroxide

Intracellular hydrogen peroxide (H_2O_2) levels, included in the group of reactive oxygen species (ROS) were assayed using dihydrorhodamine 123 (DHR 123). Once inside the cell, this fluorochrome is oxidized, mainly by H_2O_2, producing rhodamine 123, a fluorescent probe that remains trapped in the intracellular space (Gomes et al., 2005; Yazdani, 2015). Microalgal suspensions were incubated with 29 μM DHR123 for 40 min (Prado et al., 2012b). Intracellular levels of hydrogen peroxide were expressed as the mean green fluorescence (FL1) intensity in a.u.

2.4. Lipid peroxidation

Lipid peroxidation (LP) was measured as an indicator of ROS-mediated damage (Wei et al., 2000). Malondialdehyde, product of the peroxidation of polyunsaturated fatty acids, was measured according to methods described by Heath and Packer (1968), with some modifications. The malondialdehyde (MDA), reacts with thiobarbituric acid (TBA), yielding a pink coloured species (Ersan et al., 2006). 45 mL of *C. reinhardtii* cultures were centrifuged; cells were resuspended in 1 mL of PBS and disrupted in a Mini-Beadbeater, with a mixture of glass and zirconium beads and with sonication. 400 μL of supernatant were mixed with 500 μL of 10% trichloroacetic acid (TCA) (w/v) and 250 μL of 0.67% thiobarbituric acid (TBA) (w/v). The mixture was heated at 100 °C for 30 min and cooled on ice for 10 min. Absorbance was measured in a spectrophotometer (Shimadzu UV-160) at 532 nm and corrected for unspecific turbidity by subtracting the absorbance of the same sample at 600 nm. The blank solution was prepared with TBA and TCA at the same concentration w/v.

2.5. Photosynthetic efficiency

The quantum yield (QY), which provides information about the photochemical energy conversion in photosystem II (PSII), was measured in 30 min dark-adapted samples (3 mL) by Pulse Amplitude Modulation (PAM) fluorometry, using an AquaPen-C-AP-C100 fluorometer (Photon Systems Instruments).

2.6. RT-qPCR

Total RNA was isolated from 8 x 10^6 cells of the three biological replicates of each treatment, using the RNeasy® Plant Mini Kit (Qiagen). RNA concentration was determined using NanoDrop™ (Thermo Scientific). Reverse transcription was carried out with the QuantiTect® Reverse Transcription Kit (Qiagen). Genes selected for this study with names, abbreviations, primer sequences and primer-specific efficiency (%) are shown in Supplementary Table S2. RT-qPCR was performed using CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and iQ™ SYBR® Green supermix as previously described in Esperanza et al. (2017), but in this case, each reaction was run in technical triplicates. Data acquisition and analysis were performed by CFX Maestro™ Software (Bio-Rad). 18S and CBLP were selected as reference genes for calculating the relative gene transcription; Cq values were corrected with the estimated PCR efficiency. The comparative threshold method (ΔΔCt) (Livak and Schmittgen, 2001) was used for relative quantification. Control samples represented 1 x transcription of the selected genes and treated samples were expressed relative to control.

2.7. Data analysis

Results were expressed as percentage of variation with respect to control, for which a value of 100% was assigned. Mean and standard deviation (SD) values of the three replicates were determined for each treatment and for control cultures. These data were statistically analysed using IBM SPSS Statistic software 25.0. Data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene test). Log transformation was performed when necessary to fit the normality and homoscedasticity criteria. Then, these data were analysed by overall one-way analysis of variance (ANOVA). When significant differences were observed, controls *vs.* treated means were compared using the Dunnett *post hoc* test. A p-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Cell growth and morphological properties

After 24 h of exposure to the pollutants, *C. reinhardtii* cultures showed a decrease in growth besides alterations in cell size and complexity (Fig. 1). Regarding growth, a significant ($p < 0.05$) decrease in growth rates of *C. reinhardtii* cultures exposed to all the tested compounds was observed with respect to control cultures; these results were expected since microalgae were exposed to concentrations that did affect cell proliferation causing a 50% decrease in the growth rate at 96 h. The most remarkable effect on growth after 24 h was caused by BP-3 (5 mg L^{-1}); with a 90% decrease in the growth rate (Fig. 1). Negative impact of BP-3 on microalgal growth has been reported in other studies with *C. reinhardtii,* showing a concentration-dependent growth inhibition in cells exposed for 24 h to 2.5, 5 and 10 mg L^{-1} (Esperanza et al.,

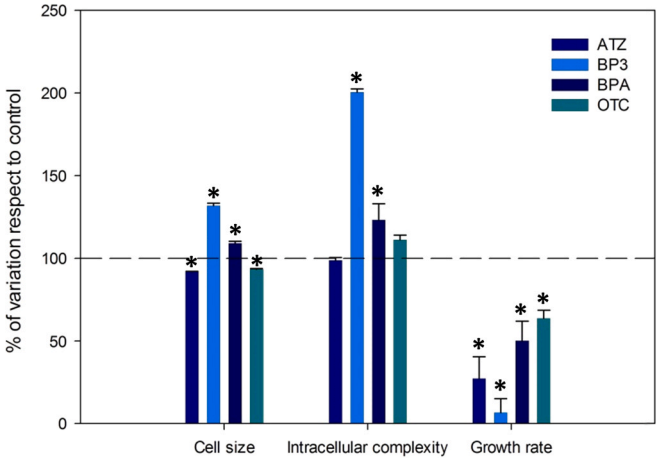

Fig. 1. Variations in relative cell size, intracellular complexity and growth rate of *Chlamydomonas reinhardtii* cells exposed to the 96 h EC_{50} for growth of the pollutants atrazine (ATZ), benzophenone-3 (BP3), bisphenol A (BPA) and oxytetracycline (OTC) for 24 h. Results are shown as percentage of variation of each parameter with respect to control (for which a value of 100 is assigned, indicated by the dashed line). Significant differences with respect to control ($p < 0.05$) are represented by an asterisk (*).

2019), and a 72 h-EC_{50} of 1.85 mg L^{-1} (Mao et al., 2017). For other microalgal species, it was reported a 96 h-EC_{50} of 2.98 mg L^{-1} in *Chlorella vulgaris* (Du et al., 2017) and 1 mg L^{-1} in *Tetraselmis suecica* (Seoane et al., 2017b). In cultures exposed to ATZ (0.054 mg L^{-1}), a decrease of 70% in growth rate was observed (Fig. 1). Similar concentrations of ATZ provoked an alike reduction in previous studies on *C. reinhardtii* (Esperanza et al., 2016, 2015b; Fernández-Naveira et al., 2016) and on other green microalgal species as *Chlorella* sp. with a 96 h-EC_{50} of approximately 0.12 mg L^{-1} (Sun et al., 2020) and *Nannochloropsis* sp. with a 72 h-EC_{10} of 0.28 mg L^{-1} (Stone et al., 2019). Growth rates were also affected in cultures exposed to BPA (30 mg L^{-1}) and OTC (2.5 mg L^{-1}) with a 50% and 35% decrease, respectively, with respect to the control cultures (Fig. 1). Different responses for growth inhibition in microalgal species exposed to BPA have been reported. The 96 h-EC_{50} in *Scenedesmus quadricauda* was approximately 13 mg L^{-1} (Xiang et al., 2018) and a significant toxicity was observed even at very low concentrations of BPA (0.57 mg L^{-1}) on *Tetraselmis chuii* (Falcão et al., 2020). However, in *Picocystis* sp, growth inhibition did not exceed 43% after five days of exposure to much higher concentrations of BPA (75 mg L^{-1}) (Ben Ouada et al., 2018). Regarding OTC, 50% growth inhibition has been reported at different concentrations in several microalgal species: *Chlorella vulgaris* (at 7 mg L^{-1}) (Eguchi et al., 2004), *Tetraselmis chuii* (at 11.18 mg L^{-1}) (Ferreira et al., 2007), *Tetraselmis suecica* (at 17.25 mg L^{-1}) (Seoane et al., 2014) and *Raphidocelis subcapitata* (0.17–4.5 mg L^{-1}) (Miazek and Brozek-Pluska, 2019).

Cell size and complexity are inherent characteristics influenced by culture conditions and cell density (Umorin and Lind, 2005). Cultures exposed to OTC and ATZ showed a slight but significant ($p < 0.05$) decrease in the FS signal, related to a decrease in cell volume (Fig. 1). This data is in accordance with the decrease reported in cell size of *T. suecica* cells exposed to OTC from concentrations of 2.5 mg L^{-1} (Seoane et al., 2014). On the other hand, microalgal cells exposed to BP-3 and BPA showed a significant ($p < 0.05$) enhancement of the FS signal, related to an increase in cell volume respect to the control cells. This increase was more pronounced in the case of BP-3-exposed cells, probably due to their incapacity to complete cell division and finish the cytokinesis process, as evidenced by growth data (Fig. 1). Cells treated with BP-3 and BPA also showed a significant ($p < 0.05$) increase in the SS signal (Fig. 1). Active cells enlarged before dividing because of the increase in cellular constituents and this active metabolism could be reflected by the increase in cellular complexity or SS signal. BP-3 was the pollutant that most affected the morphological properties of *C. reinhardtii* cells, showing a 2-fold increase in complexity with respect to control cells (Fig. 1). Similar results were found in *Tetraselmis suecica* exposed to omeprazole (Seoane et al., 2017a) and in *Chlamydomonas moewusii* exposed to paraquat (Prado et al., 2011).

3.2. Cell viability, vitality and cytoplasmic membrane potential

After 24 h of exposure, the percentage of *C. reinhardtii* viable cells with intact plasma membrane remained close to 100% for all assayed pollutants. However, cultures exposed to BP-3 and BPA showed a slight but significant ($p < 0.05$) decrease in cell viability (Fig. 2). Similar results were obtained in previous studies with these pollutants (Esperanza et al., 2015b; Seoane et al., 2014; Soares et al., 2020). Esperanza et al. (2019) also observed a reduction in viability in *C. reinhardtii* cells exposed to BP-3.

Regarding cell vitality, all treatments caused a significant ($p < 0.05$) decrease in the cellular metabolic activity compared to control cells (Fig. 2), which is related to the observed decrease in growth (Fig. 1). Esterase activity is considered a good indicator of cellular metabolic status and its decrease is a sign of environmental pressure and cellular stress (Regel et al., 2002). Cultures more affected were, again, those exposed to BP-3 and BPA, with a 65% and 45% decrease in metabolic activity, respectively (Fig. 2). Several studies have documented the reduction in metabolic activity of microalgal cells in the presence of various contaminants (Debenest et al., 2010; Esperanza et al., 2019; Li et al., 2019; Prado et al., 2009). FDA assay showed more significant results than PI assay for all analysed pollutants. This can be explained because both bioassays can distinguish between cells with an intact plasmatic membrane (viable cells) and cells with a damaged membrane (non-viable), but FDA assay could include as non-viable cells those cells with an intact membrane but without esterase activity.

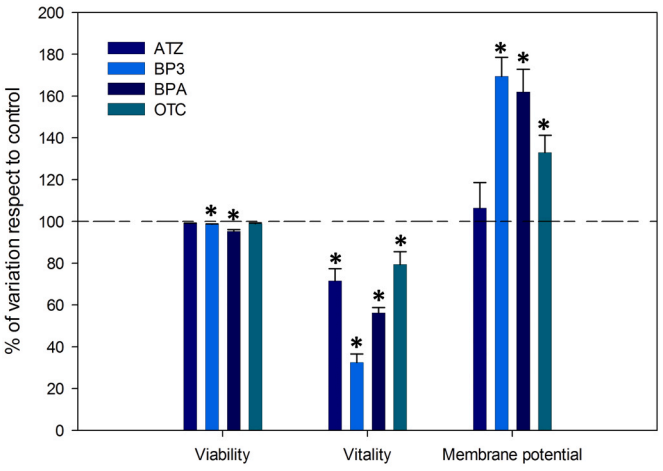

Fig. 2. Variations in cell viability, vitality and cytoplasmic membrane potential of *Chlamydomonas reinhardtii* cells exposed to the 96 h EC_{50} for growth of the pollutants atrazine (ATZ), benzophenone-3 (BP3), bisphenol A (BPA) and oxytetracycline (OTC) for 24 h. Results are shown as percentage of variation of each parameter with respect to control (for which a value of 100 is assigned, indicated by the dashed line). Significant differences with respect to control ($p < 0.05$) are represented by an asterisk (*).

All the assayed compounds affected cytoplasmic membrane potential of *C. reinhardtii* cells, leading to a depolarization of cell membrane. A significant increase ($p < 0.05$) in the number of cells with a depolarised cytoplasmic membrane was detected in cultures exposed to BP-3, BPA and OTC with respect to control cultures; this increase was around 1.7-, 1.6- and 1.3-fold, respectively (Fig. 2). Similar results were observed in *C. reinhardtii* cells exposed to BPA (Esperanza et al., 2020), BP-3 (Esperanza et al., 2019), and triclosan (González-Pleiter et al., 2017) and in *Tetraselmis suecica* exposed to BP-3 (Seoane et al., 2017b). Changes in cytoplasmic membrane potential are considered a sensitive response to physicochemical changes in the extracellular environment (Lloyd et al., 2004). Taking into account the previous results, membrane potential and metabolism were more affected by the tested emerging contaminants than by the priority contaminant (ATZ) tested. It can be due to the different mode of action of each compound on the microalga. In addition, results of cell vitality and cytoplasmic membrane potential indicated that these biomarkers were one of the most sensitive and resulted in the same cytotoxic response on the microalga regardless of the pollutant analysed.

3.3. Photosynthesis-related parameters

Environmental changes produced by the presence of contaminants can cause alterations in the photosynthetic capacity of microalgae (Choi et al., 2012; Suresh et al., 2014). The exposure to all the pollutants tested provoked a significant ($p < 0.05$) increase in chlorophyll *a* fluorescence of cells. The increase in the autofluorescence could be explained by a blockage of the electron transport chain at the PS II level to inactivation of some reaction centres (Cid et al., 1995; Murthy et al., 1990; Samson and Papovic, 1988; Singh and Singh, 1987). *C. reinhardtii* cells exposed to BP-3 showed the most noticeable difference, with a 150% increase in their autofluorescence with respect to control cells (Fig. 3). This result could be related to the observed increase in size and complexity (Fig. 1).

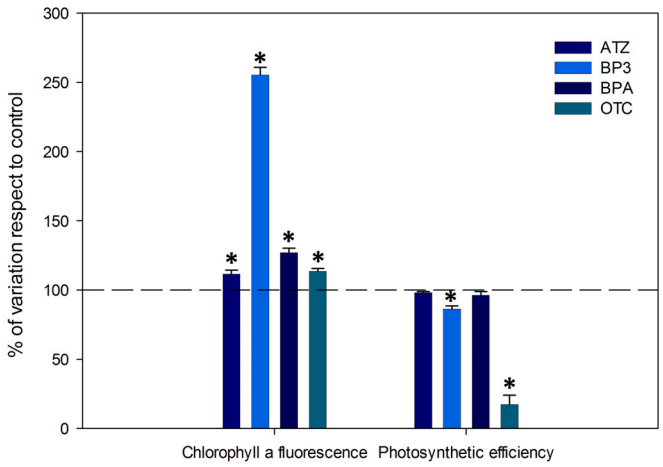

Fig. 3. Variations in chlorophyll *a* fluorescence and photosynthetic efficiency of *Chlamydomonas reinhardtii* cells exposed to the 96 h EC_{50} for growth of the pollutants atrazine (ATZ), benzophenone-3 (BP3), bisphenol A (BPA) and oxytetracycline (OTC) for 24 h. Results are shown as percentage of variation of each parameter with respect to control (for which a value of 100 is assigned, indicated by the dashed line). Significant differences with respect to control ($p < 0.05$) are represented by an asterisk (*).

An increase in autofluorescence in *C. reinhardtii* and *T. suecica* cells exposed to BP-3 were reported previously (Esperanza et al., 2019; Seoane et al., 2017b). Cell autofluorescence results indicated that this endpoint was one of the most altered and the same pattern of response on the microalga was observed regardless of the tested contaminant.

Although chlorophyll *a* fluorescence provides useful information on the efficiency of energy conversion in photosystem II (PSII) reaction centres (Buonasera et al., 2011), the photosynthetic efficiency or effective quantum yield (QY) was also measured in dark-adapted samples. After 24 h, a significant ($p < 0.05$) decrease in QY was observed in cultures exposed to BP-3 and OTC. The most remarkable effect was obtained in cultures exposed to OTC, showing a QY reduction of more than 80% with respect to the control (Fig. 3). Sendra et al. (2018) also observed a reduction of the QY in *C. reinhardtii* cells exposed to the antibiotic erythromycin. Although, microalgae are not the target organisms for antibiotics, the observed toxicity could be explained by the prokaryotic origin of chloroplasts (González-Pleiter et al., 2013; Seoane et al., 2014). On the other hand, is noteworthy that ATZ, did not show a significant decrease in photosynthetic efficiency and showed the lower increase in cell autofluorescence (Fig. 3), in spite of its primary target is the inhibition of photosynthetic electron transport, displacing plastoquinone (QB) from its binding site in the D1 protein of photosystem II (PS II), as referred in Cremlyn (1991).

Several studies reported alterations in photosynthesis-related parameters in microalgae exposed to different pollutants, *e.g.* heavy metals as cadmium in *Koliella antarctica* (la Rocca et al., 2009) or mercury in *C. reinhardtii* (Kukarskikh et al., 2003) and *Thalassiosira weissflogii* (Wu et al., 2012); herbicides as diuron in *Tetrallantos lagerheimii*, *Coelastrum sphaericum* and *Pediastrum boryanum* (Figueredo et al., 2009) and atrazine and isoproturon in *Scenesdesmus vacuolatus* (Vallotton et al., 2008) and antibiotics as kanamycin and tetracycline in *Dictyosphaerium pulchellum* and *Micractinium pusillum* (Bashir and Cho, 2016).

3.4. Oxidative stress: ROS production and lipid peroxidation

Oxidative stress is directly related to ROS production. ROS are continuously produced during normal aerobic metabolism. When there is an imbalance between the antioxidant mechanisms and the production of these molecules, a stress situation occurs, causing damages to the cellular components (Gutteridge, 1995; van Creveld et al., 2014). Results showed a significant ($p < 0.05$) increase in the fluorescence related to the intracellular H_2O_2 levels of 2- and 1.5-fold in cultures treated with BP-3 and BPA, respectively (Fig. 4). The excess of these compounds in the intracellular space can damage cell membranes by lipid peroxidation (LP), losing permeability and integrity (Ben Ouada et al., 2018; Valavanidis et al., 2006). Indeed, results revealed an increase in lipid peroxidation levels of more than 2.7-fold in cultures exposed to BP-3. However, the other treatments did not show significant changes ($p > 0.05$) with respect to control cultures (Fig. 4).

The increase in the levels of ROS in microalgal cultures treated with BP-3 and BPA could be directly related to the decrease in growth rates (Fig. 1), the loss of stability of the membranes and cell viability (Fig. 2) and to the increase in the intracellular complexity (Fig. 1). The negative effect of ROS and oxidative stress was also observed in previous studies with microalgae exposed to different pollutants, as herbicides, heavy metals and personal care products (Almeida et al., 2019; Jamers et al., 2009; Esperanza et al., 2019; González-Pleiter et al., 2017; Mofeed and Mosleh, 2013; Prado et al., 2012b, 2012a; Soto et al., 2011; Xiang et al., 2018).

3.5. Effects on transcription of selected genes involved in oxidative stress defence and photosynthesis

The induction of antioxidant enzymes plays an important role to prevent unspecific damages of cellular components and metabolism disruption originated by the overproduction of ROS in cells (Mittler, 2002; Pokora and Tukaj, 2010). To evaluate the effects of the pollutants on the cellular antioxidant defence system, the expression of *CAT* (encoding a catalase), *Fe-SOD* and *Mn-SOD* (encoding superoxide dismutases), *GST* (encoding a glutathione S-transferase), *GPX* (encoding a glutathione peroxidase) and *APX-1* (encoding an ascorbate peroxidase) genes was studied (Fig. 5). After 24 h of exposure, ATZ provoked a significant ($p < 0.05$) increment in the expression of *CAT, Fe-SOD* and *GPX* with respect to control (Fig. 5). In microalgal cells exposed to BP-3, all genes encoding for antioxidant enzymes were significantly ($p < 0.05$) upregulated (Fig. 5). Indeed, BP-3 cultures showed the highest variations in the expression of most of these genes, being *APX-1* the most affected one, with an increase of around 5.2-fold (Fig. 5). Exposure to BPA also prompted a significant ($p < 0.05$) upregulation of *CAT*, *Fe-SOD, Mn-SOD, GPX* and *GST* (Fig. 5). Finally, cultures exposed to OTC showed a significant ($p < 0.05$) increase in the expression of *CAT*, *Fe-SOD* and *GPX* and a reduction in the expression of *Mn-SOD*, the only

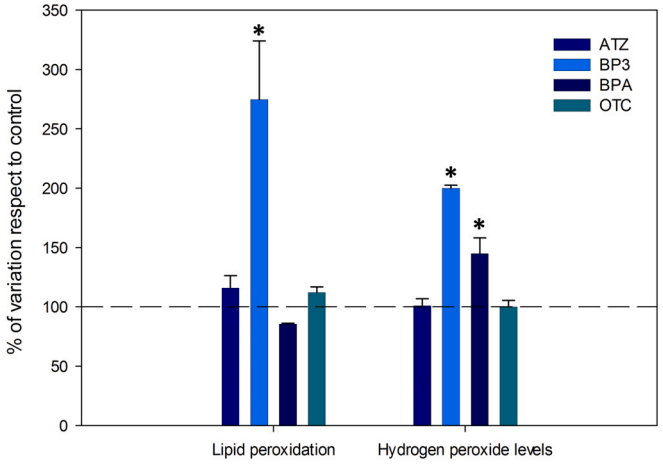

Fig. 4. Variations in lipid peroxidation and fluorescence related to hydrogen peroxide levels of *Chlamydomonas reinhardtii* cells exposed to the 96 h EC_{50} for growth of the pollutants atrazine (ATZ), benzophenone-3 (BP3), bisphenol A (BPA) and oxytetracycline (OTC) for 24 h. Results are shown as percentage of variation of each parameter with respect to control (for which a value of 100 is assigned, indicated by the dashed line). Significant differences with respect to control ($p < 0.05$) are represented by an asterisk (*).

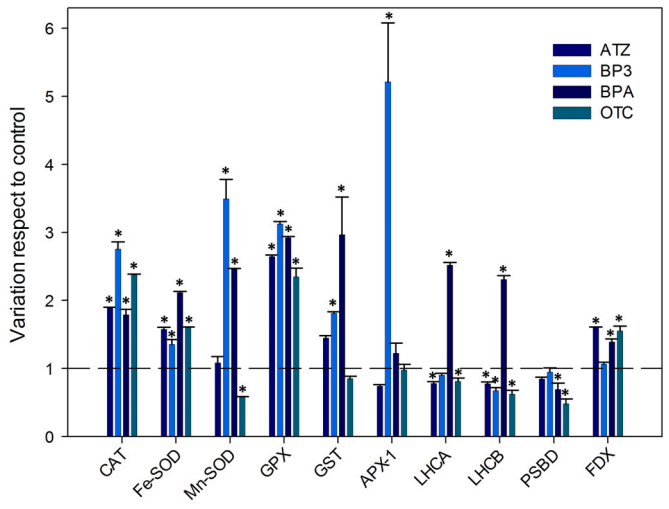

Fig. 5. Relative gene transcription levels measured by RT-qPCR in *Chlamydomonas reinhardtii* cells exposed to the 96 h EC_{50} for growth of the pollutants atrazine (ATZ), benzophenone-3 (BP3), bisphenol A (BPA) and oxytetracycline (OTC) for 24 h. Results are shown as the relative expression of selected genes with respect to control (for which a value of 1 is assigned, indicated by the dashed line). Significant differences ($p < 0.05$) are represented by an asterisk (*).

gene related with oxidative stress defence significantly ($p < 0.05$) downregulated (Fig. 5). Regarding photosynthesis-related genes, ATZ caused a significant ($p < 0.05$) downregulation of *LHCA* and *LHCB* genes (encoding proteins involved in the light-harvesting complex) and a significant ($p < 0.05$) upregulation of *FDX* (encoding a Ferredoxin) (Fig. 5). On the other hand, BP-3 did not have any significant effect on the expression of *LHCA*, *FDX* and *PSBD* (encoding a photosystem II protein D2), but significantly ($p < 0.05$) downregulated the expression of *LHCB* gene (Fig. 5). Cells cultured in the presence of BPA and OTC showed significant ($p < 0.05$) alterations in all the photosynthesis-related genes studied. BPA significantly ($p < 0.05$) increased the expression of *LHCA*, *LHCB* and *FDX*, and significantly ($p < 0.05$) decreased the expression of *PSBD* (Fig. 5). In fact, cultures exposed to this pollutant showed the highest induction of the expression of the genes encoding for light-harvesting proteins. Finally, the antibiotic OTC showed a significant ($p < 0.05$) reduction in the expression of *LHCA*, *LHCB* and *PSBD* and a significant ($p < 0.05$) increment in *FDX* (Fig. 5). Of all the genes analysed, only three, *CAT*, *GPX* and *Fe-SOD*, showed a common cytotoxic response for all tested contaminants, indicating that they could be useful to study the toxicity of different pollutants in microalgae.

Several studies have reported the overproduction of ROS in microalgae exposed to the priority pollutant ATZ (Esperanza et al., 2016; Mofeed and Mosleh, 2013). However, the increase in ROS levels is a common response to environmental stress, as the presence of emerging pollutants (Lushchak, 2011; Pinto et al., 2003; Pulido-Reyes et al., 2015; Seoane et al., 2017a). To prevent damages, cells activate protective mechanisms including the induction of antioxidative enzymes (Mittler, 2002; Pokora and Tukaj, 2010). An imbalance between ROS production and the cellular antioxidant defence mechanisms could instigate an oxidative stress leading to membrane integrity disruption, deterioration of chlorophyll, induction of lipid peroxidation, mitochondrial malfunction, apoptosis and eventually cell death (Dan Hess, 2000; Vavilala et al., 2016). After 24 h of exposure, BP-3 and BPA strongly triggered the formation of hydrogen peroxide (H_2O_2) as revealed by FCM data (Fig. 4). These results correlate well with the significant increase in the expression of all the antioxidant enzymes (Fig. 5). CAT, GPX and APX-1 are considered essential enzymes to maintain the antioxidant system, since these molecules can regulate the intracellular hydrogen peroxide levels *i.e.*, transformation of H_2O_2 to H_2O (Livingstone, 2001; Nakano and Asada, 1981) and are responsible for the protection form the oxidation of lipids in cell membranes (Epp et al., 1983; Shigeoka et al., 2002). The production of GST was only upregulated in cells exposed to BP-3 and BPA, the two pollutants more toxic for *C. reinhardtii* in this study. This enzyme is known for its ability to bind toxins and its function as transport proteins, being important for the detoxification process inside the cells (Udomsinprasert et al., 2005). However, the overproduction of ROS seemed to overwhelm the detoxifying mechanisms of the microalga and these two pollutants compromised algal membrane integrity in exposed cells (Fig. 2). This damage in the lipid bilayer could also lead to non-specific membrane ion permeability which is related to the observed depolarization of the membrane, changes in cell volume and the general decrease in cellular activity (Figs. 1 and 2). Moreover, overproduction of ROS in microalgae exposed to BP-3 and BPA could provoke the observed increase in intracellular complexity (Fig. 1) due to degradation and disorganisation of cell organelles as a result of the interaction between these highly toxic agents and unsaturated lipids of membranes (Bray et al., 1993; Suntres, 2002). Most specifically, BP-3 was the pollutant that produced the strongest increase in H_2O_2 production, as well as the most overexpression of *APX-1* (Figs. 4 and 5). In fact, the microalgal defence mechanisms against oxidative stress could not be able to regulate the overproduction of ROS induced by BP-3 and this pollutant would damage cell membrane integrity by lipid peroxidation (Figs. 2 and 4). However, in the case of BPA, OTC and ATZ, non-significant changes were detected in lipid peroxidation and ATZ and OTC also did not provoke a significant increase in intracellular peroxide levels (Fig. 4). A potential explanation could be that the stimulation of the antioxidant enzymes observed in this case was effective enough to control ROS overproduction (Fig. 4). Nevertheless, damages to the photosynthetic process, cytoplasmic membrane and cellular metabolic activity were detected in cells exposed to these emerging pollutants (Figs. 2 and 3). SOD overexpression in the presence of the pollutants also suggests that other ROS species (*e.g.* superoxide radicals) are being produced. A significant decrease in the expression of *Mn-SOD* was only observed in cells exposed to OTC. However, this enzyme is only one of the three isoforms described in microalgae (Zbigniew and Wojciech, 2006) and the other isoform analysed (Fe-SOD) did show a significant increase in its expression.

Oxidative damage induced by ROS could also impact negatively photosynthetic machinery as the effect of ROS on photosynthesis is well documented and has been found to damage PSII (Ekmekci and Terzioglu, 2005). All the pollutants tested altered the expression of some of the genes involved in the photosynthetic process (Fig. 5). Environmental stressors could affect the function of photosynthetic systems (Geoffroy et al., 2007; Juneau et al., 2002), thereby altering the fluorescence emission as it was observed for all the pollutants tested (Fig. 3). The more fluorescence increases, the less energy is used in the photosynthesis reaction. One of the most significant result was the overexpression of *LHCA* and *LHCB*, encoding for proteins of the light harvesting complex, in cells cultured in the presence of BPA. These proteins are used to harvest more of the incoming light optimizing photosynthetic function and minimizing photooxidative damage in response to light quantity and quality (Stauber et al., 2003). Light-harvesting proteins promote efficient light energy dissipation and energy quenching and they also have an important protective function during stress conditions (Grewe et al., 2014). The overexpression of these genes may be a response of cell to the oxidative damages caused by BPA and it could be related to the increase in microalgal autofluorescence (Fig. 3). It was also known that ROS damage PSII by inhibiting the repair cycle involving the D proteins (Latifi et al., 2009). In the present study, BPA and OTC provoked a downregulation in the expression of PSBD, encoding PSII D2 protein. *LHCA*, *LHCB* and *PSBD* genes were significantly downregulated in OTC-exposed cells (Fig. 5). As mentioned above, it was reported that antibiotics could produce adverse effects on non-target microalgae. Due to their cyanobacterial nature, the prokaryote-like elements of

chloroplasts make these plastids potential antibiotic targets (González-Pleiter et al., 2013; Seoane et al., 2014). In fact, it is remarkable the drastic decrease observed in the photosynthetic efficiency of cells exposed to OTC (Fig. 3). The photosynthetic efficiency decrease produced by BP-3 and especially OTC could, in turn, enhance ROS production causing oxidative damages and function abnormalities (Liu et al., 2012). Regarding *FDX*, ATZ, BPA and OTC caused a significant upregulation of this gene, which could be a response of the microalgae, making an attempt to increase photosynthetic efficacy.

Alterations in genes involved in photosynthesis and oxidative stress defence were reported in other studies on *C. reinhardtii* exposed to diverse emerging and priority pollutants as perfluoroalkyl phosphonic acids (Sanchez et al., 2015), atrazine (Esperanza et al., 2017) or triclosan (González-Pleiter et al., 2017; Pan et al., 2018); on *Chlorella vulgaris* exposed to paraquat (Qian et al., 2009); and on *Phaeodactylum tricornutum* exposed to atrazine (Bai et al., 2015) and nonivamide (Zhou et al., 2013).

Biomarkers should be simple to use and suitable for different species and ideally, obtained by non-destructive sampling procedures. Thus, among the different subcellular endpoints assessed in this study by FCM, chlorophyll *a* fluorescence and esterase activity were selected as the most sensitive parameters. Chlorophyll *a* fluorescence would be a good biomarker because no commercial fluorochrome is necessary to analyse this microalgal inherent property and have considerable potential for measuring effects of chemicals under field conditions. FDA assay is also appropriate to detect changes in metabolic activity on a day-to-day or even shorter basis, which makes it well suited to monitor short-term phytoplankton responses to environmental changes or to diverse pollutants. Regarding results obtained by RT-qPCR, the most affected biomarkers were catalase, glutathione peroxidase and Fe-superoxide dismutase, antioxidant enzymes commonly analysed in ecotoxicity studies.

4. Conclusions

Although the compounds tested have a different chemical nature, all of them provoked a similar pattern of response on *C. reinhardtii* cells, in varying degrees, in the following parameters: growth, cellular vitality, membrane potential, cellular autofluorescence and photosynthetic efficiency. The most affected biomarkers were vitality and autofluorescence analysed by FCM and the gene expression of catalase, glutathione peroxidase and Fe-superoxide dismutase analysed by RT-qPCR. Molecular and cellular results suggest that emerging pollutants can cause equal or higher toxicity than priority contaminants. This kind of ecotoxicological studies enhances the need for new legislation to regulate the entry into the ecosystems of potentially harmful substances.

CRediT authorship contribution statement

Laura Míguez: Investigation, Formal analysis, Writing - original draft, Visualization. **Marta Esperanza:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Visualization. **Marta Seoane:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Visualization. **Ángeles Cid:** Conceptualization, Methodology, Resources, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111646.

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