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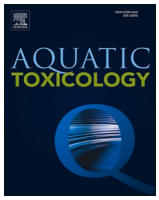

Unravelling joint cytotoxicity of ibuprofen and oxytetracycline on *Chlamydomonas reinhardtii* using a programmed cell death-related biomarkers panel

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ABSTRACT

Pharmaceutical active compounds (PhACs) are emerging contaminants that pose a growing concern due to their ubiquitous presence and harmful impact on aquatic ecosystems. Among PhACs, the anti-inflammatory ibuprofen (IBU) and the antibiotic oxytetracycline (OTC) are two of the most used compounds whose presence has been reported in different aquatic environments worldwide. However, there is still scarce information about the cellular and molecular alterations provoked by IBU and OTC on aquatic photosynthetic microorganisms as microalgae, even more if we refer to their potential combined toxicity. To test the cyto- and genotoxicity provoked by IBU, OTC and their binary combination on *Chlamydomonas reinhardtii*, a flow cytometric panel was performed after 24 h of single and co-exposure to both contaminants. Assayed parameters were cell vitality, metabolic activity, intracellular ROS levels, and other programmed cell death (PCD)-related biomarkers as cytoplasmic and mitochondrial membrane potentials and caspase-like and endonuclease activities. In addition, a nuclear DNA fragmentation analysis by comet assay was carried out.

For most of the parameters analysed (vitality, metabolic activity, cytoplasmic and mitochondrial membrane potentials, and DNA fragmentation) the most severe damages were observed in the cultures exposed to the binary mixture (IBU+OTC), showing a joint cyto- and genotoxicity effect. Both PhACs and their mixture caused a remarkable decrease in cell proliferation and metabolic activity and markedly increased intracellular ROS levels, parallel to a noticeable depolarization of cytoplasmic and mitochondrial membranes. Moreover, a strong increase in both caspase and endonuclease activities as well as a PCD-related loss of nuclear DNA integrity was observed in all treatments. Results analysis showed that the PhACs caused cell death on this non-target organism, involving mitochondrial membrane depolarization, enhanced ROS production and activation of PCD process. Thus, PCD should be an applicable toxicological target for unraveling the harmful effects of co-exposure to PhACs in aquatic organisms as microalgae.

1. Introduction

The presence of pharmaceutical active compounds (PhACs) in aquatic environments has gained increasingly concern in the last few years due to their high ecotoxic potential (Gunnarsson et al., 2019; Pereira et al., 2020; Brack et al., 2022). Because of all the daily-administered dosages in conjunction with their inefficient removal in wastewater treatment plants (WWTPs), cocktails of antibiotics, anti-inflammatories, antiseptics and other PhACs are currently released after human and veterinary consumption in freshwater and marine ecosystems worldwide, where organisms with environmental relevance, as microalgae, are subsequently exposed to mixtures of these emerging contaminants (Kovalakova et al., 2020; Nannou et al., 2020; Chen et al., 2021; González-González et al., 2022).

Abbreviations: ANOVA, analysis of variance; CCCP, carbonylcyanide m-chlorophenylhydrazone; DiBAC4(3), lipophilic anionic oxonol dye bis-(1,3-dibu- tylbarbituric acid) trimethine oxonol; DHR123, dyhydrorhodamine 123; FCM, flow cytometry; FDA, fluorescein diacetate; FS, forward scatter; HE, hydroethidine; HPLC–MS, high performance liquid chromatography-mass spectrometry; IBU, ibuprofen; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; NSAID, non-steroidal anti-inflammatory drugs; OTC, oxytetracycline; PBS, phosphate-buffered saline; PCD, programmed cell death; PhACs, pharmaceutical active compounds; PI, propidium iodide; ROS, reactive oxygen species; SD, standard deviation; SS, side scatter.

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Among PhACs, ibuprofen (IBU) and oxytetracycline (OTC) represent the two most frequently reported therapeutical classes in aquatic ecosystems, namely, non-steroidal anti-inflammatory drugs (NSAIDs) and antibiotics, respectively (dos Santos et al., 2021). IBU is the third most popular NSAID worldwide, dispensed as analgesic, antirheumatic and antipyretic agent for human and veterinary medicine (Moro et al., 2014; Maamar et al., 2017). Regarding OTC, this antibiotic belongs to the tetracycline family, the most concentrated antibiotic group in European WWTPs influents, with a value of 48 μg L^{-1} (Gavrilescu et al., 2015; Osorio et al., 2016). Due to their ubiquitous presence in European freshwater ecosystems, IBU and OTC are currently listed as substances of emerging environmental concern by the NORMAN compilation, being detectable at elevated concentrations (μg L^{-1}) in 86% and 60% of European WWTPs effluent samples, respectively (Rodil et al., 2012; Zhou et al., 2019). Besides, IBU has been recently identified as one of the compounds of emerging Arctic concern by the Arctic Monitoring and Assessment Programme (AMAP) (AMAP, 2017; Olalla et al., 2020), while OTC is also included in the Joint Research centre (JRC) watch list of marine-relevant contaminants due to its common use as bacteriostatic agent in mariculture activities (Tornero and Hanke, 2017). Furthermore, the study of this antibiotic is justified by the European One Health Action Plan against Antimicrobial Resistance (AMR), which supports the need of more environmental research to "improve knowledge of the occurrence and spread of antimicrobials in the environment" linked to the EU's "Strategic Approach to Pharmaceuticals in the Environment" (European Commission, 2019).

Unlike conventional environmental contaminants, PhACs are bioactive compounds designed to pass through the biological membranes and interact with specific physiological pathways at extremely low concentrations. Thereby, understanding their potential mechanism of toxicity to non-target organisms has become a research priority. In mammalian cells, IBU therapeutic target is the blockage of cyclooxygenases, promoting the prostaglandins biosynthesis route disruption (Saad et al., 2016), while OTC blocks the first step of the elongation phase in the bacterial translation, inhibiting the binding of the aminoacyl-tRNA to the A site or acceptor domain of mRNA-ribosome complex in the minor prokaryotic ribosome subunit (30S) (Kolar et al., 2014). Regarding the toxicity exerted by these compounds on microorganisms in aquatic environments, several works have recently reported that IBU and OTC block cell division in cultures of freshwater and marine microalgae (Moro et al., 2014; Seoane et al., 2014; Aguirre-Martínez et al., 2015; Li et al., 2016; Wu and He, 2019; Siedlewicz et al., 2020; Wang et al., 2020; Moro et al., 2021). However, the cellular and subcellular alterations provoked by IBU and OTC towards these photosynthetic microorganisms have not been described (Sharma et al., 2021; Xin et al., 2021). This gap in toxicity data is even more pronounced considering a more realistic scenario where the contaminants in aquatic ecosystems are usually detected together with other compounds of similar characteristics. The co-occurrence of PhACs is of great concern because of the potential interactive effects that they may induce in exposed non-target organisms. Based on this, focus on the impact of PhACs on environmental systems has recently been shifted to the assessment of combination effects, but again using population growth parameter as the unique toxicity endpoint in microalgal bioassays (Rodea-Palomares et al., 2010; González-Pleiter et al., 2013; Teixeira and Granek, 2017; Xiong et al., 2019).

Several studies have reported that, regardless of their mode of action, mitochondrial membranes may be a relevant toxicological target of PhACs on aquatic organisms (Burgos-Aceves et al., 2018; Jiang et al., 2019), especially in the case of exposure to antibiotics (Seoane et al., 2014). Alterations in the normal functioning of mitochondria can trigger programmed cell death (PCD) processes through the overproduction of ROS and the activation of certain enzymes such as caspases or endonucleases (Farkhondeh et al., 2020). The concept of PCD refers to a natural and highly conserved cellular process of self-destruction, which is genetically decided and regulated by the balance between survival induction and negative or death signals (Lockshin and Williams, 1964; Jiménez et al., 2009; Durand and Ramsey, 2019). PCD-related phenomena have already been described in microorganisms as eukaryotic microalgae (Zuppini et al., 2010; Yordanova et al., 2013; Berges and Choi, 2014; Bidle, 2016; Barreto Filho et al., 2022); but little knowledge is currently available on the activation of these events on these primary producers in response to PhACs exposure.

On this basis, this work focused on the potential negative impact of the NSAID IBU and the antibiotic OTC, on the aquatic photosynthetic microorganism *Chlamydomonas reinhardtii* upon single and co-exposure to both compounds. A cytomic panel of PCD-related biomarkers was carried out including cell vitality, metabolic activity, cytoplasmic and mitochondrial membrane potentials, cytoplasmic ROS levels, caspase-like activity, and endonuclease activity assessed by TUNEL assay, in addition to nuclear DNA fragmentation analysis through comet assay. Overall, this study attempts to provide information about the potential joint cytotoxicity of both PhACs as well as to evaluate the occurrence of PCD as a relevant toxicological target for risk assessment of aquatic contaminants on microalgae.

2. Materials and methods

2.1. Microalgal species

The green microalga *Chlamydomonas reinhardtii* (strain CCAP 11/32A mt+) was cultured in Tris-minimal phosphate medium (Harris, 1989) at 18 ± 1 °C, irradiated at 100 μmol photon m^{-2} s^{-1} with a 12/12 h light/dark period and continuous aeration with filtered air (0.22 μm).

2.2. Chemicals and experimental setup

The emerging contaminants studied were the NSAID ibuprofen (IBU; CAS No. 15,687–27–1) and the antibiotic oxytetracycline (OTC; CAS No. 79–57–2). Both were reagent-grade chemicals purchased from Sigma-Aldrich with purity higher than 95%. Before the bioassays, concentrated solutions of both contaminants were freshly set up in autoclaved Milli-Q water. Exposures were performed for 24 h in triplicates in Pyrex tubes with 60 mL of culture under the same environmental conditions above described. Cells in log growth phase were adjusted to 2×10^5 cells mL^{-1}. Control cultures without chemicals were also carried out.

A previous toxicity screening based on growth inhibition was performed to determine the concentrations used for the experiments. Microalgal growth toxicity bioassays using a range of concentrations of each test substance (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mg L^{-1} for OTC; 50, 100, 200, 250, 300, 400 and 500 mg L^{-1} for IBU) were carried out. Finally, concentrations assayed in this study for both PhACs were determinated considering the 24 h-EC_{50} values for growth obtained with the CompuSyn software (Fig. S1; Chou and Martin, 2005). The 24 h-EC_{50} was 250 mg L^{-1} for IBU and 1.5 mg L^{-1} for OTC. These concentrations were selected for each compound to perform the different assays (Table 1). Effective EC_{50}-concentrations of IBU and OTC at 0 and 24 h were confirmed by a high performance liquid chromatography-mass spectrometry (HPLC–MS) analysis (Table S1). To allow a standardized exposure to both compounds, assayed concentrations were referred to toxic units (TU) as follows (Bundschuh et al., 2014):

$$TU = C_i / EC_{50i} \quad (1)$$

Table 1
Concentrations selected for the assays (mg L^{-1}) and the value in toxic units (TU) for IBU, OTC and IBU+OTC mixture.

Chemicals	Toxic Units (TU)	Assayed concentrations (mg L^{-1})
IBU	1 TU_{IBU}= $EC_{50\ IBU}$	250
OTC	1 TU_{OTC}= $EC_{50\ OTC}$	1.5
IBU+OTC	1 $TU_{IBU+OTC}$= ½ TU_{IBU} + ½ TU_{OTC}	125 IBU + 0.75 OTC

where C_i is the concentration of contaminant i and EC_{50} is the median effective concentration of the contaminant i.

2.3. Flow cytometric assays

Flow cytometric (FCM) assays were analysed on a Gallios cytometer (Beckman Coulter Inc.) with a 488 nm laser, detectors of forward (FS) and side (SS) scattering light and fluorescence detectors: 505–550 nm (FL1), 550–600 nm (FL2), 600–645 nm (FL3) and >645 nm (FL4).

2.3.1. Cell density and growth rates

Cell density of control and treated cultures was determined by FCM and then, growth rates were calculated (Rioboo et al., 2009).

2.3.2. Analysed parameters and FCM protocols

Vitality, metabolic activity, cytoplasmic and mitochondrial membrane potentials, intracellular ROS levels, and caspase and endonuclease activities were analysed by FCM after 24 h of single and co-exposure to both pollutants. Samples of the cultures were prepared with 2×10^5 cells mL^{-1} in phosphate-buffered saline (PBS, pH 7.4). Then, cell samples were marked with the pertinent fluorochrome, in darkness and room temperature prior to analysing by FCM. Detailed information about each fluorochrome used, its application, mode of action, final concentration and incubation time is shown in Table S2. A total of 10,000 cells were gated by their size (FS) and chlorophyll *a* fluorescence (FL4) (Fig. S2).

2.4. Comet assay

Comet assay was used to study the genotoxicity of the contaminants tested on *C. reinhardtii,* by detecting breaks in the DNA strands (Singh et al., 1994). The protocol was applied as reported in Prado et al. (2009) and Esperanza et al. (2015a). The percentage of DNA in the tail of comets (% tDNA) of 100 cells per replicate was calculated with OpenComet plug-in (Image J). This% tDNA is directly related to DNA fragmentation (Gyori et al., 2014).

2.5. Results analysis

Results were expressed as mean and standard deviation (SD) values of three replicates for all the treatments. These data were analysed using SPSS Statistics software (v.25.0, IBM). Hypothesis that each PhAC, and their combination, does not affect the biomarker was statistically analysed by a one-way variance analysis (ANOVA). When this hypothesis was rejected, Tukey's *post hoc* test was applied. A p-value < 0.05 was considered for all the analyses.

Graphs were created with the software SigmaPlot 12.0 version. Representative cytograms, obtained and analysed with Kaluza Analysis v.1.1. software (Beckman Coulter Inc.), are reported in Fig. S3.

3. Results and discussion

3.1. Cytotoxic impact of IBU, OTC and IBU+OTC on Chlamydomonas reinhardtii

After 24 h of treatment with 1 TU of IBU, OTC and IBU+OTC, both compounds and their mixture displayed a similar perturbation on *C. reinhardtii* proliferation, finally provoking a 50% decrease ($p < 0.05$) in microalgal growth rates compared with the control (Fig. 1A). Regarding to OTC, it has been reported that exposure to tetracycline family antibiotics induced growth inhibition in different microalgae (Kolar et al., 2014; Seoane et al., 2014; Li et al., 2016; Wu and He, 2019; Moro et al., 2020; Siedlewicz et al., 2020). Reported EC_{50} values for the microalga *Chlorella vulgaris,* the microalga *Tetraselmis suecica*, and for the cyanobacteria *Aphanizomenon flos-aquae* were 10, 17.7 and 7.7 mg L^{-1} of OTC, respectively (Kolar et al., 2014; Seoane et al., 2014; Siedlewicz et al., 2020). In the present study the EC_{50} value after 24 h of exposure was 1.5 mg L^{-1} (1 TU), being the lowest value described to date. These data indicate that *C. reinhardtii* is a species particularly sensitive to the toxic action of this antibiotic. Regarding to IBU, EC_{50} values for growth equal or higher than 5 mg L^{-1} have been reported previously on freshwater microalgae such as *Scenedesmus rubescens* (Moro et al., 2014) or *Pseudokirchneriella subcapitata* (Aguirre-Martínez et al., 2015), and on marine microalgae as *Isochrysis galbana* (Aguirre--Martínez et al., 2015). However, other studies with freshwater green microalgae have reported EC_{50} values like the one described in this study: 342.2 mg L^{-1} for *Desmodesmus subspicatus* (Cleuvers, 2004) and 123.29 mg L^{-1} for *Scenedesmus obliquus* (Wang et al., 2020). This remarkable dispersion of values indicates that the toxicity exerted by this NSAID on microalgal growth is highly variable depending on the species tested. Interestingly, in a previous study where the growth of *C. reinhardtii* exposed to several concentrations of IBU was not affected, Moro et al. (2021) suggested that this PhAC can be a secondary toxic agent, causing growth inhibition of aquatic organisms in combination with other contaminants of the aquatic ecosystems, as reported for *Lemna gibba* (Renner, 2002). However, in this work, exposure to the PhACs mixture did not significantly increase the damage exerted individually by OTC or IBU on *C. reinhardtii* population growth.

Previous works reported that the exposition to tetracycline antibiotics induced vitality inhibitory effect in *Chlamydomonas reinhardtii* (Li et al., 2016), *Pseudokirchneriella subcapitata* (Kolar et al., 2014), *Tetraselmis suecica* (Seoane et al., 2014) and in the cyanobacteria *Aphanizomenon flos-aquae* (Kolar et al., 2014). Toxic effects of IBU in microalgae vitality were also reported in *Scenedesmus rubescens* (Moro et al., 2014), *Pseudokirchneriella subcapitata* (Aguirre-Martínez et al., 2015), *Isochrysis galbana* (Aguirre-Martínez et al., 2015) and *Desmodesmus subspicatus* (Cleuvers, 2004). Interestingly, in the present study, the analysis of cell vitality showed a remarkable increase ($p < 0.05$) in the percentage of

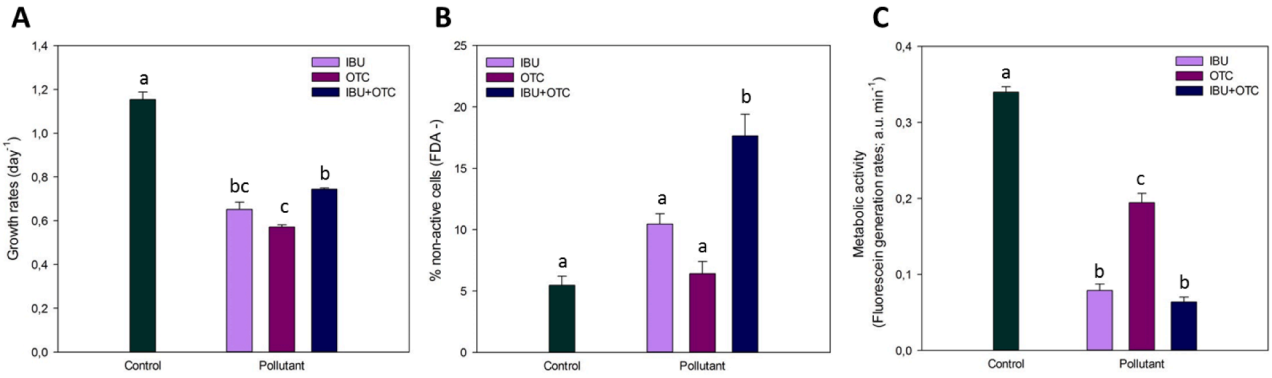

Fig. 1. Variations in growth rates (**A**), vitality (**B**), and metabolic activity (**C**) of *C. reinhardtii* cells in control cultures and cultures exposed to 1TU of IBU, OTC and IBU+OTC for 24 h. Different letters represent significant differences ($p < 0.05$) among experimental conditions after Tukey's *post hoc* test.

non-active cells (FDA -) exclusively on cultures exposed to the mix (IBU+OTC), being this increase around 3-fold with respect to control cultures (Fig. 1B). In addition, real time analysis of metabolic activity (RT-esterase activity) on active cells (FDA +) indicated a significant reduction ($p < 0.05$) in esterase activity in all tested treatments (Fig. 1C) but being the most affected the cultures exposed to IBU and IBU+OTC that displayed a decrease of approximately 20% with respect to the control (Fig. 1C). Taking these results into account, loss of metabolic activity could be an early sensitive biomarker of the joint cytotoxicity provoked by the co-exposure to these PhACs on microalgal cells.

3.2. Overproduction of ROS and depolarization of cytoplasmic and mitochondrial membranes on C. reinhardtii cells after PhACs exposure

An increase in the intracellular generation of ROS has been reported as a common toxic outcome to exposure to contaminants on microalgal cells (Lushchak, 2011; Cirulis et al., 2013; Pulido-Reyes et al., 2015; Rodea-Palomares et al., 2012; Almeida et al., 2017). In this study, generation of superoxide anion (O_2^-) in control and exposed cells of *C. reinhardtii* was analysed by FCM. Results evidenced a drastic ($p < 0.05$) increase in ROS levels in all treatments, showing a percentage of cells with high levels of O_2^- (HE+) of 94% for IBU, 72% for OTC and 83% for IBU+OTC mixture (Fig. 2A). Considering these data, IBU exposure provoked the highest oxidative toxicity on *C. reinhardtii* cells. In animal cells, IBU and OTC also trigger cellular oxidative stress, generating the overproduction of O_2^- and H_2O_2, altering the maintenance of basal ROS levels (Milan et al., 2013; Feng-Jiao et al., 2014; Afzal et al., 2017). These studies in animal eukaryotic cells agree with the results obtained here, demonstrating that IBU and OTC unbalanced the redox state in *C. reinhardtii* cells. In fact, regarding to *C. reinhardtii* exposed to OTC, Míguez et al. (2021) recently showed an overexpression of some oxidative stress genes by quantitative PCR in this photosynthetic microorganism. Other studies also observed an increase in the production of ROS in *C. reinhardtii* treated with herbicides (Esperanza et al., 2017), personal care products (González-Pleiter et al., 2017) or silver nanoparticles (Sendra et al., 2017). According to these data, microalgae exposure to certain compounds such as PhAC triggers an increase in the production of ROS, generating an oxidative stress that could provoke nuclear, mitochondrial and chloroplastic DNA modifications, protein oxidation associated with loss of functionality, cell depigmentation and lipid alterations associated with damage to the cellular membranes (Prado et al., 2015; Mittler, 2017; Esperanza et al., 2017; Almeida et al., 2017).

Therefore, fast-stress response biomarkers related to membrane permeability constitute some of the most sensitive parameters to study the potential effect of one or more compounds on unicellular organisms. In the present study, the cytoplasmic membrane potential indicates that exposure to both compounds and to their binary mixture induced a notable ($p < 0.05$) depolarization of microalgal cells, showing a population of depolarized cells ($DiBAC_4(3)^+$) of 11%, 15% and 21% for IBU, OTC and IBU+OTC cultures, respectively (Fig. 2B). Comparable results were obtained in relation with the mitochondrial membrane potential, since a significant ($p < 0.05$) increase in the population of cells with depolarized mitochondrial membrane was also showed for all the treatments assayed (Fig. 2B). In this case, the percentage of cells with mitochondrial membrane depolarization (JC-1-) was even greater, with a population of JC-1- cells of 29%, 41% and 53% for IBU, OTC, and IBU+OTC cultures, respectively (Fig. 2C). In both cases, the antibiotic OTC was more toxic than the NSAID IBU and the mixture of both PhACs was the treatment that caused greater alterations (Fig. 2B, C), showing a notable joint cytotoxicity on cellular membranes.

Since these PhACs induce depolarization of the cytoplasmic membrane, they can cause important physiological changes on microalgal cells that can lead to other functional alterations, compromising their viability (Melegari et al., 2013; Esperanza et al., 2015b). Accordingly, microalgal cultures exposed to the binary combination of these PhACs also showed the greatest affectation values in the vitality assayed biomarker (Fig. 1B).

With respect to the reported mitochondrial membrane disturbances, the antibiotic OTC and the mixture IBU+OTC caused the greatest damage in the potential of these microalgal membranes (Fig. 2C). Some antimicrobials have also been shown to suppress mitochondrial protein synthesis in eukaryotic cells (McKee et al., 2006). Based on this, OTC could also damage mitochondrial ribosomes, which are fundamental for cell respiration and energy metabolism. Moreover, OTC may also provoke perturbances at chloroplast level. In fact, Míguez et al. (2021) observed a drastic decrease of 80% in the photosynthetic efficiency of *C. reinhardtii* after 24 h of exposure to OTC, whereas Sendra et al. (2018) reported a drop of photosynthesis activity in *C. reinhardtii* treated with erythromycin. Other studies with microalgae and cyanobacteria also support that the toxicity induced by antibiotics is clearly related to prokaryotic origin of these cell organelles (González-Pleiter et al., 2013; Seoane et al., 2014).

3.3. IBU, OTC and IBU+OTC induce programmed cell death (PCD) on C. reinhardtii

Nowadays, it is known that many processes that were originally directly attributed to the disturbances exerted by ROS such loss of membrane integrity or mitochondrial functionality, are also part of the PCD pathways (Mittler, 2017). PCD is a cellular process of great importance for the maintenance of homeostasis and for the response to biotic and abiotic stress in unicellular organisms (Bidle, 2016; Kabbage et al., 2017). In fact, marine and freshwater microalgae activate apoptotic pathways under osmotic stress (Jiménez et al., 2009), UV exposure (Moharikar et al., 2006), heat shock (Zuppini et al., 2007),

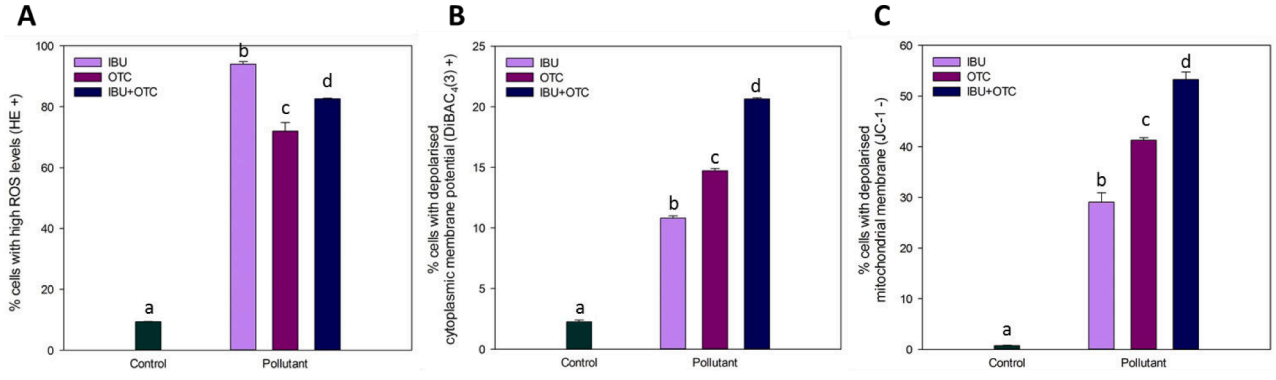

Fig. 2. Variations in intracellular superoxide anion levels (**A**), cytoplasmic membrane potential (**B**), and mitochondrial membrane potential (**C**) of *C. reinhardtii* cells in control cultures and cultures exposed to 1TU of IBU, OTC and IBU+OTC for 24 h. Different letters represent significant differences ($p < 0.05$) among experimental conditions after Tukey's *post hoc* test.

carbon stress (Orellana et al., 2013), senescence (Jiménez et al., 2009; Esperanza et al., 2017), or nitrogen starvation (Jiménez et al., 2009).

Among the classical biomarkers used to characterize the activation of PCD on cells (Van Aken and Van Breusegem, 2015; Bidle, 2016; Kabbage et al., 2017), the caspase and endonuclease activities as well as the fragmentation of nuclear DNA are commonly studied, in addition to the disturbances in the mitochondrial potential already reported above (Fig. 2C). Caspase 3/7 and endonuclease activities were tested on *C. reinhardtii* after 24 h of exposure to 1 TU of IBU, OTC and their mixture, to determine the potential activation of these enzymatic activities. Results showed a significant increase ($p < 0.05$) in the activation of caspases and endonucleases in all treatments tested (Fig. 3A, B).

Similar results of caspase activation have been reported in previous works with this microalga exposed to atrazine (Esperanza et al., 2017) and triclosan (González-Pleiter et al., 2017) or to Benzophenones 3 and 4 (Anido-Varela et al., 2022). However, to date, the activation of caspases in the presence of IBU had only been shown in animal cells (Thirunavukkarasu et al., 2003). In relation to the assayed antibiotic OTC, Zhang et al. (2015) observed that the exposure to tetracycline family, also induces PCD mediated by ROS stress in *Danio rerio* embryos, causing the activation of effector caspases, including caspases 3 and 7, which agrees with the results obtained in microalgae in the present study. But, although caspase enzymes are directly related to the activation of DNases endonucleases, to the authors' knowledge, this is the first study to successfully report the endonuclease enzymatic activation in cytotoxicity bioassays with microalgae.

Endonucleases enzymes are directly related to the degradation of nuclear DNA into fragments approximately 200 bp in length, which is a PCD-linked marker (Widlak and Garard, 2005; Kabbage et al., 2017; Sergeeva et al., 2017). In order to complete the endonuclease activation analysis, a comet assay was performed to quantify the stability of nuclear DNA in response to both PhACs action. In line with the previous results regarding the activation of endonucleases, a noteworthy increase ($p < 0.05$) in% tDNA was recorded in all the treatments tested, with the greatest damage observed in the cultures exposed to the binary mixture (Fig. 3C), showing a joint genotoxicity effect on *C. reinhardtii* cells. Representative images of undamaged cells and comets are shown in Fig. S4. In previous studies with animal cells, OTC and IBU have shown similar toxicity mechanisms to those showed in this study. In the specific case of OTC, this drug induces DNA damage and may even cause changes at the epigenetic level, altering gene expression and inducing nuclear DNA fragmentation (Odore et al., 2015; Gao et al., 2014; Gallo et al., 2017).

Overall, all the cytotoxicity biomarkers studied suggest the activation of PCD processes in *C. reinhardtii* because of exposure to the antibiotic OTC, the anti-inflammatory IBU and their binary mixture, finally causing severe alterations at nuclear DNA level. The different alterations that cells undergo during the PCD process occur in an organized way, in fact, the increase in ROS levels observed for all treatments tested (Fig. 2A) and the activation caspases and endonucleases (Fig. 3A, B) can be related to the depolarization of mitochondrial membranes detected in *C. reinhardtii* exposed to the two PhACs and their combination (Fig. 2C). This induction of the PCD program, triggered by oxidative stress, causing the permeabilization of the mitochondrial membrane has already been observed in animal cells (Saelens et al., 2004; Ly et al., 2003; Kim et al., 2013; Kabbage et al., 2017).

A summary of the differences in toxicity of the treatments tested in relation to the parameter analysed is shown in Table 2. In general, the mixture of both PhACs increased the toxicity exerted by these compounds on the photosynthetic microorganism *C. reinhardtii*. Although the treatment that induced the highest ROS overproduction was 1 TU of IBU, for most of the parameters assayed (vitality, metabolic activity, cytoplasmic and mitochondrial membrane potentials, and DNA fragmentation) the greater alterations were recorded in the cultures treated with the binary mixture (IBU+OTC), showing a joint cyto- and genotoxic effect. Based on the statistical analysis of the results, growth rate and caspase and endonuclease activities were equally affected by all the treatments.

4. Conclusions

Exposure to IBU, OTC and their binary mixture (IBU+OTC) induced ROS overproduction on microalgal cells, causing several perturbations at cellular level such as depolarization of cytoplasmic and mitochondrial membranes. These alterations affected the cellular metabolic activity and ultimately lead to the occurrence of PCD phenomena, triggering the activation of caspases and endonucleases, which led to nuclear DNA fragmentation. In addition, for most of the parameters analysed the co-exposure to IBU+OTC showed a joint toxicity effect.

In view of the obtained results, PCD may be one of the relevant toxicological targets for evaluating the deleterious effects provoked by PhACs exposure in unicellular aquatic organisms, especially in photosynthetic ones. If the occurrence of this type of contaminants in aquatic

Table 2
Differences in the toxicity of PhACs according to the analysed parameter.

PARAMETERS	TOXICITY
Growth rates	OTC ≥ IBU ≥ IBU+OTC
Vitality	IBU+OTC > IBU = OTC
Metabolic activity	IBU+OTC = IBU > OTC
ROS levels	IBU > IBU+OTC > OTC
Cytoplasmic membrane potential	IBU+OTC > OTC > IBU
Mitochondrial membrane potential	IBU+OTC > OTC > IBU
Caspase activity	IBU+OTC = IBU = OTC
Endonuclease activity	OTC = IBU = IBU+OTC
DNA fragmentation	IBU+OTC > OTC = IBU

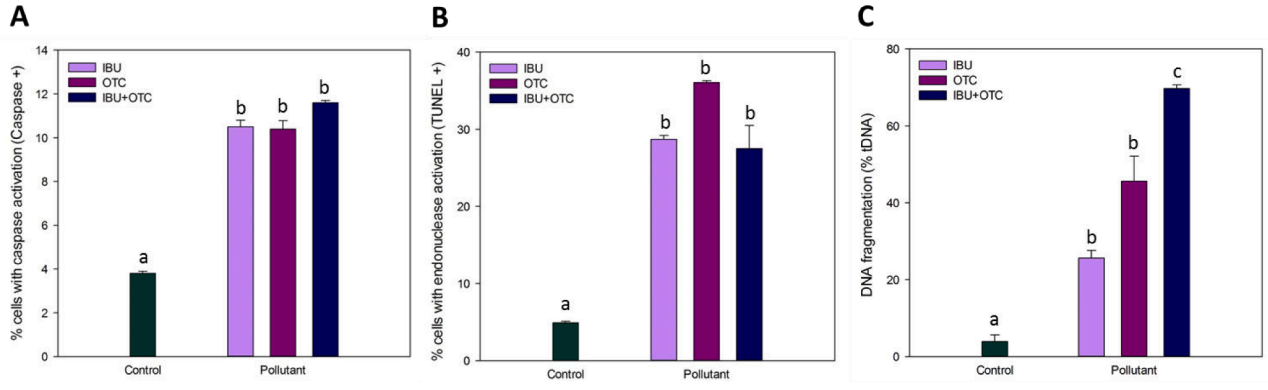

Fig. 3. Variations in caspase activity (**A**), endonuclease activity (**B**), and DNA fragmentation (**C**) of *C. reinhardtii* cells in control cultures and cultures exposed to 1TU of IBU, OTC and IBU+OTC for 24 h. Different letters represent significant differences ($p < 0.05$) among experimental conditions after Tukey's *post hoc* test.

environments induces PCD in microalgae, they may have a direct effect on the regulation of the populations of primary producers, potentially disrupting the trophic structure of aquatic communities, as well as the global carbon fixation.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106455.

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