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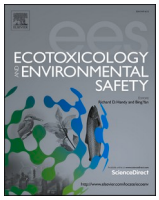

Exposure of the alga *Pseudokirchneriella subcapitata* to environmentally relevant concentrations of the herbicide metolachlor: Impact on the redox homeostasis

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ABSTRACT

This study investigated the effect of the herbicide metolachlor (MET) on the redox homeostasis of the freshwater green alga *Pseudokirchneriella subcapitata*. At low MET concentrations (≤40 μg L^{-1}), no effects on algal cells were detected. The exposure of *P. subcapitata* to 45–235 μg L^{-1} MET induced a significant increase of reactive oxygen species (ROS). The intracellular levels of ROS were particularly increased at high (115 and 235 μg L^{-1}) but environmentally relevant MET concentrations. The exposure of algal cells to 115 and 235 μg L^{-1} MET originated a decrease in the levels of antioxidants molecules (reduced glutathione and carotenoids) as well as a reduction of the activity of scavenging enzymes (superoxide dismutase and catalase). These results suggest that antioxidant (non-enzymatic and enzymatic) defenses were affected by the excess of MET. As consequence of this imbalance (ROS overproduction and decline of the antioxidant system), ROS inflicted oxidative injury with lipid peroxidation and damage of cell membrane integrity. The results provide further insights about the toxic modes of action of MET on a non-target organism and emphasize the relevance of toxicological studies in the assessment of the impact of herbicides in freshwater environments.

1. Introduction

Herbicides are used to prevent, destroy, or mitigate undesirable plants or weeds. However, the large use of these compounds has, as a consequence, the contamination of soil and water. Herbicides arrive in waters mainly due to agricultural runoff and leaching being its dissipation in aquatic systems dependent on the applied quantity, method of application and mobility (OECD, 2001). Some herbicides are very persistent, with a prolonged half-life, which raises concerns due to potential problems associated with water quality, ecosystems safety and human health (Kim et al., 2017).

Metolachlor (MET), together with glyphosate and atrazine, is an herbicide widely used in large-scale crops (Helmer et al., 2015). MET is a relatively mobile and persistent broad-spectrum herbicide belonging to chloroacetamide group (Liu et al., 1995); it is one of the most frequent class of herbicides found in USA and European surface waters (Battaglin et al., 2000; Roubeix et al., 2012; Székács et al., 2015). More specifically, MET has been detected in surface waters, reaching concentrations as high as 143 μg L^{-1} during crop growing season (Battaglin et al., 2000). Additionally, US-EPA reported, in its Water Quality Portal, 77,768 detections of MET out of 164,928 surface waters, between January 1998 and June 2018, with a maximum concentration of 464 μg L^{-1} (US-EPA, 2019).

MET is applied for the control of annual grass weeds, yellow nutsedge (*Cyperus esculentus*), and some broadleaf species (Heydens et al., 2010). It acts via inhibition of the enzyme elongase (Gotz and Böger, 2004). In plants, this herbicide abolishes the synthesis of chlorophyll, proteins, fatty acids, isoprenoids and flavonoids which, in the last instance, impairs cell division and plant elongation (Deal and Hess, 1980; Singh and Singh, 2016). Most of the herbicides, including chloroacetamides, are non-specific to its main target (weeds). Thus, it is expectable that the effect of these compounds can be extended to aquatic, non-target, organisms and in particular to algae (primary producers) due to their similarity with plants (both present photosynthetic

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capacity) with implications to higher trophic levels and all food chain. In this sense, some studies have been reported the toxicity of MET in aquatic organisms like cyanobacteria (Chen et al., 2019), algae (Deng et al., 2015; Ebenezer and Ki, 2013; Fairchild et al., 1998; Thakkar et al., 2013), aquatic plants (Diepens et al., 2017; Fairchild et al., 1998), amphibian (Quintaneiro et al., 2018), crustaceans (Velisek et al., 2019) and fishes (Quintaneiro et al., 2017). MET inhibits algae (Ebenezer and Ki, 2013; Machado and Soares, 2019; Thakkar et al., 2013; Vallotton et al., 2008) and cyanobacteria growth (Chen et al., 2019), and induces the damage of the photosynthetic apparatus in algae (Fairchild et al., 1998; Korkaric et al., 2015; Machado and Soares, 2020), cyanobacteria (Chen et al., 2019) and aquatic plants (Diepens et al., 2017).

Reactive oxygen species (ROS) are by-products of normal cellular metabolism in all aerobic organisms (Das and Roychoudhury, 2014). However, physical-chemical stress can lead to excessive ROS production. For maintaining redox homeostasis, cells activate a complex ROS scavenger system composed of non-enzymatic [such as reduced glutathione (GSH) and carotenoids (CAR)] and enzymatic antioxidant defenses [for instance, superoxide dismutase (SOD) and catalase (CAT)] (Mallick and Mohn, 2000), in an attempt to avoid oxidative stress (OS), i.e., the imbalance between ROS formation and antioxidant responses (Ayala et al., 2014). GSH is of huge importance in the management of oxidative stress. In photosynthetic organisms, it is found in most cellular compartments, including mitochondria and chloroplasts, where prevents thiol groups oxidation and reacts directly with free radicals (Das and Roychoudhury, 2014). CAR are involved in the harvesting of light, in photosynthesis, and in the protection of chloroplasts of plants and algae against photo-oxidation, through the quenching of singlet oxygen (Varela et al., 2015). SOD is the first enzymatic defense against ROS, catalyzing the conversion of superoxide anion into hydrogen peroxide and molecular oxygen in organelles (chloroplasts, mitochondria, and peroxisomes) and in the cytoplasm (Das and Roychoudhury, 2014). Then, CAT acts by removing the hydrogen peroxide resulting from SOD activity and converts it into the water and molecular oxygen. CAT usually acts when hydrogen peroxide is present at high concentrations (Gill and Tuteja, 2010).

It has been described that herbicides can lead to the generation of ROS and origin OS in aquatic organisms (Lushchak, 2011). In this context, some studies reported that MET induced ROS generation in crustacean *Procambarus virginalis* (Velisek et al., 2019) and in green algae *Chlorella pyrenoidosa* (Liu and Xiong, 2009), *Scenedesmus obliquus* (Liu et al., 2017) and *Parachlorella kessleri* (Maronic et al., 2018; Spoljaric et al., 2011). As a consequence of ROS overproduction, it was described the modification of the activity of enzymatic defenses (namely SOD and CAT) in crustaceans and algae (Liu et al., 2017; Liu and Xiong, 2009; Velisek et al., 2019), the reduction of GSH level in crustaceans (Velisek et al., 2019) and the lipid peroxidation in algae (Maronic et al., 2018; Spoljaric et al., 2011). However, the mechanisms involved in the oxidative stress induced by MET in algal cells remain largely unknown.

Pseudokirchneriella subcapitata is a microalga present in freshwater bodies and has been used as a cell model in toxicity studies, being very sensitive to a large variety of compounds (heavy metals and emerging contaminants, namely, antibiotics and herbicides) (Aderemi et al., 2018; Hasenbein et al., 2017; Machado and Soares, 2016). Due to its environmental significance, this alga has been recommended for ecotoxicity assessment of chemicals by International organizations such as Organization for Economic Co-operation and Development (OECD, 2011) and the United States Environmental Protection Agency (US-EPA, 2012).

In previous work, it was found that MET induced a perturbation of *P. subcapitata* physiology and impaired the normal progression of the reproductive cycle (Machado and Soares, 2020). The present work aimed to further investigate modes of action (MoA) of MET on *P. subcapitata*, namely the role of OS. With this end, algal cells were exposed to environmentally relevant MET concentrations, for 72 h, and the intracellular ROS accumulation was evaluated. Cellular mechanisms of protection, namely non-enzymatic (GSH and CAR levels) and enzymatic (SOD and CAT activity) defenses, as well as cellular oxidative damage (lipid peroxidation and disruption of cell membrane integrity), as additional OS biomarkers, were also assessed. An overview of the observable adverse effects of MET on *P. subcapitata* is presented.

The information here obtained regarding the MoA of MET on *P. subcapitata,* an ecologically relevant organism, can be further used in developing of: i) theoretical models for predicting algal sensitivity to toxics; ii) more sensitive assays to assess chemical toxicity and iii) new herbicides more eco-friendly, ie, with lower impact on non-target organisms.

2. Material and methods

2.1. Algal strain and culture conditions

The axenic strain of the freshwater alga *Pseudokirchneriella subcapitata* (278/4) obtained from the Culture Collection of Algae and Protozoa (CCAP, UK) was used in this study. The alga was maintained in OECD medium (OECD, 2011) with 2% (w/v) of agar (Merck) and stored at 4 °C. Pre-cultures and cultures (initial cell concentration of ~5×10^4 mL^{-1}) were grown in 20 and 40 mL of OECD medium, respectively, on an orbital shaker at 100 rpm and 25 °C, under continuous "cool white" fluorescent light (fluorescent lamps with a color temperature of 4300 K), with an intensity of 4000 *lux* at the surface of the flasks for 3 and 2 days, respectively (Machado and Soares, 2012a). Then, cultures were harvested by centrifugation at 2500×*g* for 5 min and resuspended in deionized water at 5×10^6 cells mL^{-1} for MET exposure assays. Algal cell concentration was measured by an automatic cell counter (TC10, Bio-Rad).

2.2. Exposure of the alga P. subcapitata to MET

Algal cells in the exponential phase of growth (2 days) were exposed for 72 h to 40, 45, 115 and 235 μg L^{-1} of the herbicide MET. These concentrations corresponded to no observed effect (NOEC) and 10, 50, and 90% of algal growth inhibition (72h-EC_{10}, EC_{50} and EC_{90}), when compared with control (cells not exposed to MET), respectively, after an exposure of 72 h. These effect concentrations were previously determined by OECD growth inhibition test using a geometric series of eight MET concentrations in a range of 16–400 μg L^{-1} (Machado and Soares, 2019). The herbicide MET (PESTANAL®, analytical standard, purity 97.6%) was purchased from Fluka Analytical (Sigma-Aldrich, Seelze, Germany). MET stock solution (11.8 mg L^{-1}) was prepared in water and stored in the dark at 4 °C. 1 L Erlenmeyer flasks containing OECD medium and different MET concentrations were inoculated with 5×10^4 cells mL^{-1} of *P. subcapitata* (from a 2 days culture), in a final volume of 400 mL, and incubated in the conditions described above for pre-cultures and cultures. As control, algal cells were incubated under the same conditions but without MET.

After 72 h of incubation, cells were harvested by centrifugation (2500×*g*, 5 min) and resuspended in: 100 mmol L^{-1} phosphate-buffered saline (PBS), at pH 7.0, for the quantification of ROS production and visualization of lipid peroxidation; OECD medium for the determination of cell viability, reduced glutathione and carotenoids content; lysis buffer (please see composition in section 2.8) for the determination of the activity of antioxidant enzymes.

2.3. Analysis of ROS accumulation

Intracellular ROS production was monitored with 2′,7′–dichlorodihydrofluorescein diacetate (H_2DCFDA). Algal cells (1×10^6 mL^{-1}) suspended in 100 mmol L^{-1} PBS (pH 7.0) were incubated, in the dark, with H_2DCFDA in a final concentration of 10 μmol L^{-1}, for 90 min, as previously detailed (Machado and Soares, 2016). Fluorescence intensity was measured, in quintuplicate, as relative fluorescence units

(RFUs) in a microplate reader (Victor 3, Perkin-Elmer) at a fluorescence excitation wavelength of 485/14 nm and an emission of 535/25 nm. Fluorescence was corrected by subtracting the autofluorescence of cells, PBS buffer and dye and normalized considering cell biovolume, previously determined (Machado and Soares, 2020). ROS production was expressed as the ratio of fluorescence of the assay/fluorescence of the control (cells not exposed to MET).

To determine the impact of MET on H_2DCFDA, an abiotic control (without algal cells) was performed using the highest MET concentration tested (235 μg L^{-1}). Thereby, 100 μL of MET (470 μg L^{-1}) were placed in quintuplicate, in a 96-well microplate, and combined with 100 μL of H_2DCFDA (20 μmol L^{-1}). Positive and negative controls were performed by substituting MET by equal volume of 1.27 mmol L^{-1} H_2O_2 or 100 mmol L^{-1} PBS, respectively. Fluorescence intensity was measured after an incubation of 45 min, in a microplate reader, as reported above for ROS determination. It was verified that MET did not interfere with H_2DCFDA assay, since it could not able to oxidize the redox-sensor (data not shown).

2.4. Visualization of lipid peroxidation

Lipid peroxidation was visualized using the fluorescent dye 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoicacid (C11-BODIPY$^{581/591}$, Invitrogen), which applicability in microalgae was demonstrated by Almeida et al. (2017) and Cheloni and Slaveykova (2013). Algal cells (1×10^6 mL^{-1}) were suspended in 100 mmol L^{-1} PBS (pH 7.0) and incubated, in the dark, with C11-BODIPY$^{581/591}$ in a final concentration of 5 μmol L^{-1}, for 4 h. Subsequently, cells were observed using an epifluorescence microscope equipped with an HBO-100 mercury lamp and a filter set GFP, from Leica. The images were acquired with a Leica DC 300 F camera and processed by Leica IM 50- Image manager software.

2.5. Evaluation of cell membrane integrity

Cell membrane permeability was assessed using the non-permeant SYTOX green (SG) probe, as previously described (Machado and Soares, 2012a). SG is excluded from cells with intact plasma membrane (SG negative cells) but penetrates cells with compromised (damaged) membrane (SG positive cells) (Machado and Soares, 2012a).

Algal cells resuspended in OECD medium (1×10^6 mL^{-1}) were stained with 0.5 μmol L^{-1} of SG (Molecular Probes) for 20 min, at 25 °C, in the dark. Then, cells were observed using an epifluorescence microscope equipped with a mercury lamp and a filter set GFP from Leica. As positive control (i.e. cells with permeabilized cell membrane), heat-treated cells at 65 °C for 1 h were used. Algal cells in the exponential phase of growth (with intact cell membrane) were used as negative control. In each assay and for each MET concentration, at least three samples of 100 cells ($n \geq$ 300 cells) were scored in randomly selected microscope fields.

2.6. Assessment of intracellular reduced glutathione content

Intracellular GSH was monitored with monochlorobimane (mBCl, Sigma-Aldrich) as previously described (Machado and Soares, 2012b). Algal cells were resuspended in OECD medium (1×10^6 mL^{-1}) and incubated with 50 μmol L^{-1} of mBCl for 90 min in the dark. Treated cells with 1 mmol L^{-1} of iodoacetamide, for 1 h, were used as negative control. Fluorescence intensity was measured as RFUs, in quintuplicate, using a microplate reader at fluorescence excitation of 355/40 nm and an emission of 460/25 nm. Fluorescence was corrected by subtracting autofluorescence of cells, OECD medium and dye and normalized considering cell biovolume, as reported above. The results were expressed as the ratio of fluorescence of the assay/fluorescence of the control (cells not exposed to MET).

2.7. Quantification of carotenoids

Carotenoids were extracted from algal cells (3×10^6 mL^{-1}) with 90% of acetone (v/v) (VWR Chemicals) at 4 °C for 20 h. The content of carotenoids were determined spectrophotometrically at 480 nm, as described by Strickland and Parsons (1972) and normalized by cell biovolume, as reported above. Carotenoids content was expressed as the ratio of pigment content in the assay and the pigment content in the control (cells not exposed to MET).

2.8. Antioxidant enzymes activity determination

For cellular disruption, algal cells were harvested by centrifugation at 5000×*g*, for 5 min, at 4 °C, and resuspended (1–2 x 10^7 mL^{-1}) in iced lysis buffer [100 mmol L^{-1} of PBS with 1 mmol L^{-1} of ethylenediaminetetraacetic acid (EDTA) and 0.5 mmol L^{-1} of phenylmethylsulfonyl fluoride (PMSF), at pH 7.0]. Then, cell suspension (1 mL) was placed in a 2 mL microtube with 0.6 g of glass beads (425–600 μm of diameter) and lysed in a FastPrep-24 bead beater (MP Biomedicals, USA), using 10–12 cycles of 6.5 m s^{-1} for 45 s, with 1 min intervals between each cycle for cooling the samples on ice. After 10 cycles, the efficiency of cell lysis was confirmed by microscopy using a Neubauer counting chamber. Aliquots of 10 μL were taken, from the samples, and undisrupted cells were counted and compared with the initial number of cells. The counting process was carried out as quickly as possible. During the process, all samples were kept cool by placing them on crushed ice. When cell lysis efficiency was higher than 90%, cell lysates were centrifuged at 3220×*g* for 15 min, 4 °C, and the supernatants stored for enzymes determination.

Catalase (CAT) activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm, i.e. the breakdown of H_2O_2 by catalase, according to the procedure described by Aebi (1984). The disappearance of H_2O_2 is recorded by 5 min in a 3 mL reaction mixture consisting of 1 mL of 50 mmol L^{-1} PBS with 0.1 mmol L^{-1} EDTA, at pH 7.0, 1 mL of 30 mmol L^{-1} H_2O_2 (in PBS 50 mmol L^{-1}, pH 7.0) and 1 mL of cell extract. The reaction was initiated by the addition of H_2O_2. CAT activity was expressed as Units (U) per 10^9 cells and calculated using the molar extinction coefficient of 43.6 L mol^{-1} cm^{-1}; results were normalized considering cell biovolume, as described above.

Superoxide dismutase (SOD) activity was measured using xanthine-oxidase-cytochrome *c* method, as described by McCord and Fridovic (1969). This method is based on the ability of superoxide radical to inhibit the reduction of cytochrome *c*. The reaction mixture contained 50 mmol L^{-1} PBS (pH 7.8), 0.1 mmol L^{-1} EDTA, 0.01 mmol L^{-1} cytochrome *c*, 0.05 mmol L^{-1} xanthine, 0.005 U of xanthine oxidase and cell extract. As control, an uninhibited reaction (without cell extract) was performed for obtaining the higher rate of reduction of cytochrome *c*. The reaction was initiated by the addition of xanthine-oxidase and the increase of absorbance at 550 nm was followed for 5 min. One unit of SOD activity is defined as the amount of enzyme that gave 50% inhibition of the control rate of cytochrome *c* reduction. SOD activity was expressed as Units (U) per 10^9 cells; results were normalized considering cell biovolume, as described above.

2.9. Reproducibility of the results and statistical analysis

Experiments were repeated, independently, under identical conditions, at least three times. The data are presented as the mean ± standard deviation (SD). Statistical analyses were performed comparing the difference between control and each MET concentration, using unpaired *t*-test; P values < 0.05 were considered statistically significant.

3. Results

3.1. MET exposure induced ROS production in algal cells

This study started with the evaluation of the intracellular ROS accumulation by the alga *P. subcapitata* exposed for 72 h to 40, 45, 115 and 235 μg L^{-1} MET; these concentrations correspond to NOEC and 72h-EC_{10}, EC_{50} and EC_{90}, respectively (Machado and Soares, 2019). MET at 45–235 μg L^{-1} induced a significant increase of ROS, comparatively to control (Fig. 1). ROS production was particularly increased at high (115 and 235 μg L^{-1}) but environmentally relevant MET concentrations.

3.2. MET lead to lipid peroxidation and plasma membrane damage

ROS are highly reactive with lipids, especially polyunsaturated fatty acids of lipid membranes, inducing their oxidative degradation (Ayala et al., 2014). Considering that lipids are responsible for maintain the integrity of cellular membranes, its large peroxidation can modify the composition, structure, and dynamics of lipid membranes leading to membrane damage. The increase of intracellular ROS levels observed in algal cells exposed to MET prompted us to evaluate the impact of MET on the lipid peroxidation and the plasma membrane integrity of these cells.

Algal cells exposed for 72 h to 115 or 235 μg L^{-1} MET and subsequently stained with C11-BODIPY$^{581/591}$ probe presented a green fluorescence, indicating a lipid peroxidation (Fig. 2A); the lipid peroxidation was accompanied by a loss of membrane integrity (SG positive cells) of 13 and 21%, respectively, of the cell population (Fig. 2B).

A kinetic approach allowed us to verify an increase of intracellular ROS levels at 4 h and 8 h of exposure to 235 and 115 μg L^{-1} MET, respectively. Lipid peroxidation of algal cells was detected at 8 h, for both MET concentrations (Figure S2 of Supplementary Material). The loss of membrane integrity was observed at 24 h and 48 h of exposure to 235 and 115 μg L^{-1} MET, respectively (Figure S1 of Supplementary Material). Together, these results indicate that ROS accumulation precedes the damage of cell membrane, probably, by lipid peroxidation.

3.3. MET caused reduction of non-enzymatic antioxidative defenses

To prevent injuries of important cellular components provoked by ROS, cells synthesize non-enzymatic antioxidants (Mallick and Mohn, 2000). Hence, the level of two important non-enzymatic antioxidants (GSH and CAR) in algal cells exposed to MET was evaluated. A decrease of GSH (Fig. 3A) and CAR content (Fig. 3B), comparatively to control, was observed in algal cells incubated for 72 h with 115 and 235 μg L^{-1} MET.

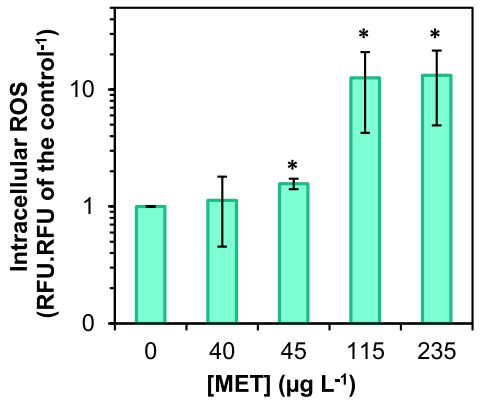

Fig. 1. ROS production in the alga *P. subcapitata* exposed to metolachlor. Cells were incubated with MET, in OECD medium, for 72 h and ROS were assessed using H_2DCFDA. The means with asterisk are significantly different of the control ($P < 0.05$).

3.4. Enzymatic antioxidant defenses under MET stress

ROS can also be neutralized by the action of a complex system involving enzymes, namely SOD and CAT (Mallick and Mohn, 2000). A decrease in the activity of these two enzymes was observed in algal cells exposed for 72 h to 115 and 235 μg L^{-} (Fig. 4). A reduction of CAT activity was also observed in algal cells exposed to 45 μg L^{-1} MET (Fig. 4B).

4. Discussion

MET is a chloroacetamide herbicide widely used on agricultural crops to control annual weeds. Commonly, herbicides are continuously and repeatedly applied resulting in its environmental prolonged persistence (Kaur and Bhullar, 2019). As this herbicide is designed to control plant growth, algae, due to their similarity with plants (for instance, both organisms are photosynthetic) are serious candidates to be affected.

P. subcapitata algal cells, when exposed to environmentally relevant concentrations (i.e., close to the concentrations that can be reached in the environment), presented an intracellular accumulation of ROS (Fig. 1). Similarly, ROS production in the microalgae *Parachlorella kessleri* and *Scenedesmus obliquus* exposed to MET was described (Liu et al., 2017; Maronic et al., 2018; Spoljaric et al., 2011).

In plant cells, ROS can be formed in the endoplasmic reticulum (Lesser, 2006); however, they are primarily originated in peroxisomes and in electron transport processes in mitochondria and chloroplasts (Del Río and López-Huertas, 2016; Mittler, 2017; Pospíšil, 2009). In previous work, it was shown that *P. subcapitata* exposed to MET presented a reduction of the photosynthetic activity of photosystem II (ϕ_{PSII}) and a decrease of the electron flow in the electron transport chain (ETC) (Machado and Soares, 2020). These results are compatible with the literature which describes a reduction of molecular oxygen by the electrons deflected from photosynthetic ETC, generating superoxide anion radicals which are dismutated to hydrogen peroxide with the subsequent formation of a hydroxyl radical completing the overall process of ROS formation on the PSII (Pospíšil, 2009). Although chloroplasts must contribute, however, they should not be the primary source of ROS in *P. subcapitata* exposed to MET, since the decrease of the electron flow in ETC and the reduction of photosynthetic activity was only detected after 48 h of cell exposure to the herbicide (Machado and Soares, 2020), while the increase of ROS accumulation was observed after 4–8 h (Figure S1 of Supplementary Material).

To cope with the raise of ROS accumulation, attain the redox homeostasis, and normal functioning, cells activate antioxidant defenses (Mallick and Mohn, 2000). Algal cells exposed to MET concentrations ≥115 μg L^{-1} presented a significant decrease in the non-enzymatic antioxidant defenses, namely CAR and GSH (Fig. 3). Our previous study also showed that MET induced a decrease of photosynthetic pigments chlorophyll *a and b* in *P. subcapitata* (Machado and Soares, 2020). It is described that chloroacetamide herbicides like alachlor and metolachlor damage plants destroying components of photosynthetic apparatus (Štajner et al., 2003). Thus, the reduction of non-enzymatic antioxidants (CAR and GSH) may be due to their biosynthesis inhibition and/or an excessive consumption owing to its involvement in the fight against OS. The reduction of GSH is also compatible with the alkylating properties of MET (Böger, 2000), namely the cysteine residue of GSH. In parallel, a decrease of the activity of antioxidant enzymes, namely of SOD and CAT (Fig. 4), was also observed, for MET concentrations ≥115 μg L^{-1}. The decline of SOD and CAT activity observed indicates that their scavenging function was reduced probably as a consequence of high stress and exhaustion of the antioxidant capacity. Similarly, Liu and Xiong (2009) also reported a decrease of CAT activity in the alga *Chlorella pyrenoidosa* exposed to MET concentrations ≥ 100

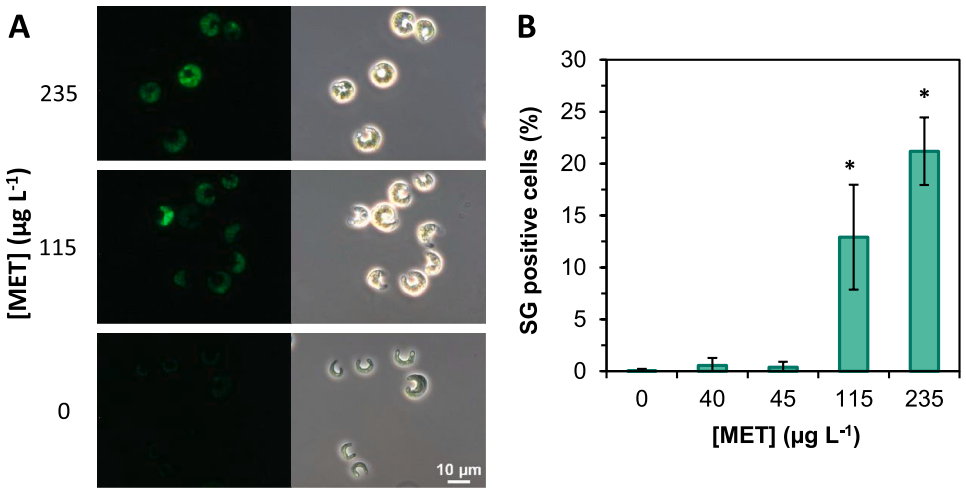

Fig. 2. Lipid peroxidation and cell membrane integrity of the alga *P. subcapitata* exposed to metolachlor. Cells were incubated with MET, in OECD medium, for 72 h. A – visualization of lipid peroxidation using C11-BODIPY$^{581/591}$ probe; cells with lipid peroxidation (green fluorescent cells) (left side) and respective phase contrast images (right side). B - membrane integrity; SYTOX Green (SG) positive cells: cells with permeabilized (damaged) plasma membrane. The means with asterisk are significantly different of the control ($P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

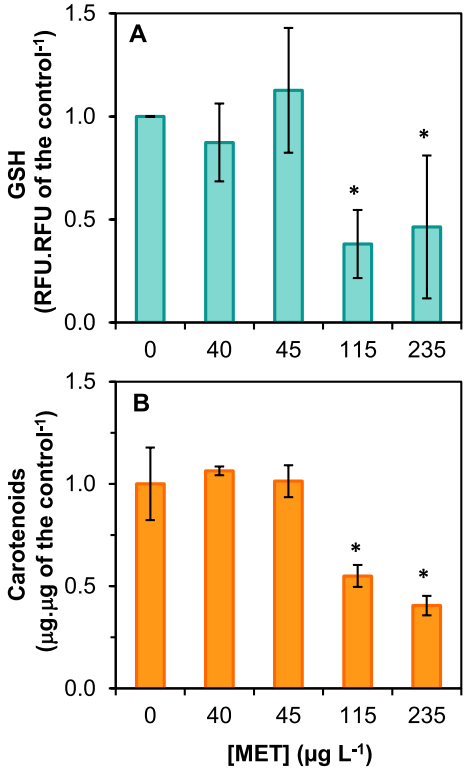

Fig. 3. Levels of non-enzymatic antioxidants in the alga *P. subcapitata* exposed to metolachlor. Algal cells were incubated in OECD medium, for 72 h, in presence or absence of MET. A – reduced glutathione (GSH) content determined by fluorimetry, after algal staining with monochlorobimane. B – carotenoids content evaluated by spectrophotometry. The means with asterisk are significantly different of the control ($P < 0.05$).

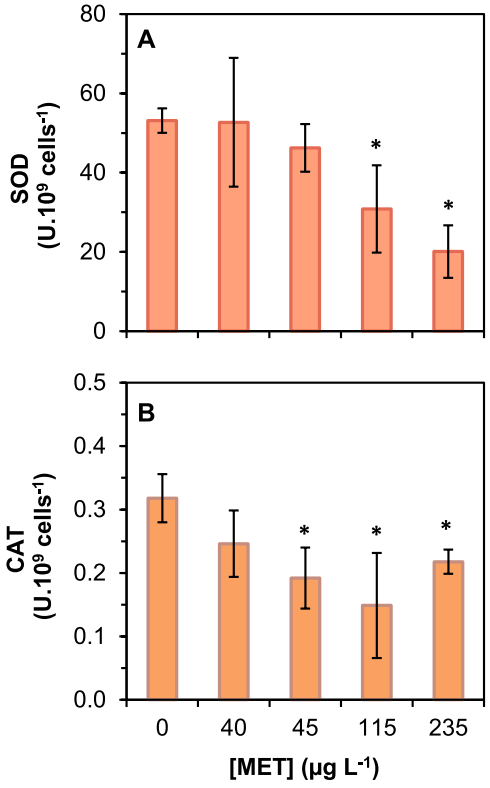

Fig. 4. Activity of antioxidant enzymes in *P. subcapitata* exposed to metolachlor. Cells were incubated with MET, in OECD medium, for 72 h and enzyme activity was determined, spectrophotometrically, in cells lysate. A - superoxide dismutase (SOD). B - catalase (CAT). The means with asterisk are significantly different of the control ($P < 0.05$).

µg L^{-1}. The decrease of SOD activity benefits the over accumulation of O_2^-.

It was described the inhibition of the starter enzyme of the elongase system in the alga *Scenedesmus acutus* and thus the biosynthesis of very-long-chain fatty acids (VLCFAs) having more than 18 C-atoms, after 2 h of incubation with MET (Böger, 2003); it was suggested that a plasma membrane poorly supplied with VLCFAs loses its barrier function. In *P. subcapitata,* the loss of membrane integrity (Fig. 2) should not be primarily attributed to the VLCFAs inhibition since a disturbance of the alga physiology was observed (raise of intracellular ROS at 4–8 h and lipid peroxidation at 8 h) before the damage of the plasma membrane was detected (24–48 h) (Figure S1 and S2 of Supplementary Material). Thus, the overproduction of ROS and the reduction of non-enzymatic and enzymatic antioxidants led to oxidative stress, which is probably the cause of plasma membrane damage of *P. subcapitata*, by lipid peroxidation (Fig. 2 and Figure S2 of Supplementary Material). The results here presented, together with those previously obtained (Machado and Soares, 2020), allow a mechanistic explanation of MET toxicity on a non-target organism, the freshwater alga *P. subcapitata* (Fig. 5).

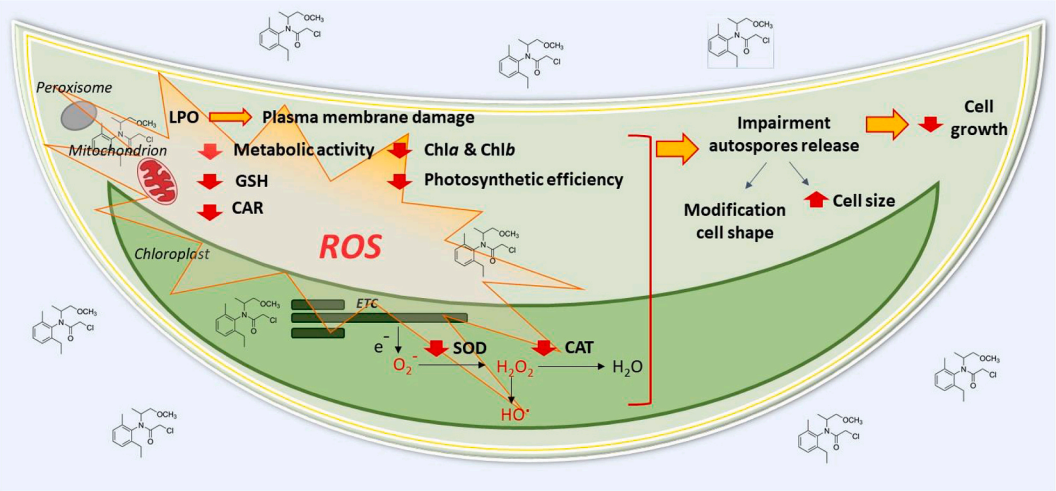

Fig. 5. Set of physiological indicators associated with the adverse outcomes induced by metolachlor (MET) on *P. subcapitata*. A proposal based on the results presented in this work and previous work (Machado and Soares, 2020). MET decreases metabolic activity, photosynthetic efficiency, and the electron transfer in ETC of chloroplasts. The redirection of electrons from ETC contributes for ROS production. MET origins a reduction of antioxidant defenses (GSH, CAR, SOD, and CAT), which intensify the OS and cause plasma membrane damage via lipid peroxidation. The impairment of autospores release can be on the basis of the increase of algal biovolume, modification of cell shape, and ultimately, a slowdown/arrest of growth. CAR – carotenoids; CAT – catalase; Chl*a* & Chl*b* – chlorophyll *a and b*; ETC – electron transport chain; GSH – reduced glutathione; LPO - lipid peroxidation; SOD – superoxide dismutase.

Thus, it was observed that MET induce a reduction of the concentration of photosynthetic pigments (chlorophylls *a and b*), a decrease of the photosynthetic efficiency, and a decline of the electron transfer in ETC in the thylakoid membranes of chloroplasts. Although chloroplasts should not be the main source of ROS, the redirection of electrons from photosynthetic ETC to ROS production contributes to the intracellular accumulation of ROS. It was also observed a reduction of non-enzymatic (GSH and CAR) and enzymatic (SOD and CAT) antioxidant defenses; combined, these effects contributed to the amplification of OS and can be seen as a failure of algal cells to maintain redox homeostasis and to avoid OS. The intracellular accumulation of ROS seems to be the cause of the loss of cell membrane integrity by lipid peroxidation. The inhibition of photosynthesis may lead to decreased cellular ATP levels, which, combined with the reduction of metabolic (esterase) activity and other physiological disturbances can be in the origin of the block of the normal progression of *P. subcapitata* reproductive cycle (Machado and Soares, 2020). The arrest of the algae reproductive cycle can be attributed to the incapacity of parent cells to release the autospores, which, in turn, originate an increase of algal biovolume and an alteration of cell shape and, finally, a slowdown/arrest of growth (Machado and Soares, 2020). An overview of MoA of MET on *P. subcapitata*, i.e., the set of physiological indicators associated with the adverse effect of MET, at environmentally relevant concentrations, on a freshwater alga is depicted in Fig. 5.

5. Conclusions

P. subcapitata seems to be tolerant to low MET concentrations (≤40 μg L^{-1}). At higher (although environmentally relevant) concentrations (45–235 μg L^{-1}) MET originated an increase of intracellular ROS accumulation. When exposed to 115–235 μg L^{-1} MET, for 72 h, algal antioxidants molecules (GSH and CAR) and scavenging enzymes (SOD and CAT) were not enough to counteract the excessive ROS production; as a consequence of this imbalance, ROS promote oxidative injury, such as lipid peroxidation and cell membrane damage.

The impact of MET on the redox homeostasis of a relevant freshwater alga, warns for the need to develop more specific and eco-friendly herbicides, with less environmental impact.

The results shown in the present study, combined with those previously found, shed light on the mechanisms underlying MET toxicity in a non-target organism, the green alga *P. subcapitata.*

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CRediT author statement

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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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111264.

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