Oxidative stress potential of the herbicides bifenox and metribuzin in the microalgae *Chlamydomonas reinhardtii*

Ana Catarina Almeida[a]*, Tânia Gomes[a], Katherine Langford[a], Kevin V. Thomas[ab], Knut Erik Tollefsen[ac]

[a]Norwegian Institute for Water Research, Gaustadalléen 21, 0349 Oslo, Norway
[b]Current affiliation: Queensland Alliance for Environmental Health Sciences (QAEHS), The University of Queensland, 39 Keesels Road, Coopers Plains, 4108, Australia
[c]Centre for Environmental Radioactivity, Norwegian University of Life Sciences (NMBU), Post box 5003, N-1432 Ås, Norway

*Corresponding author: Norwegian Institute for Water Research (NIVA), Section of Ecotoxicology and Risk Assessment, Gaustadalléen 21, N-0349 OSLO, Norway; Tel: (+47) 98215483, Fax: (+47) 221852 00; E-mail address: aal@niva.no.

Abstract

The widespread presence of herbicides in the aquatic environment has raised awareness about the need to develop further in depth ecotoxicological risk assessments, more specifically on potential effects on photosynthetic organisms as microalgae. The majority of the information available regarding the toxicity of herbicides towards microalgae is related to traditional toxicological and regulatory-relevant endpoints such as growth inhibition, leaving a significant gap on knowledge regarding underlying interactions and damage to biological targets. In this context, this study aimed to supplement the general toxicity information of bifenox and metribuzin in the microalgae *Chlamydomonas reinhardtii* using a battery of selected high-throughput methods. This multiple-endpoint approach included the measurement of formation of reactive oxygen species (ROS), alterations in reduced glutathione (GSH) content, formation of lipid peroxidation (LPO), photosystem II (PSII) performance and loss of photosynthetic pigments after 24 hours exposure. Results obtained showed that both herbicides caused a concentration-dependent increase in ROS formation, with bifenox showing higher but less reactive ROS. This increase in ROS production by bifenox and metribuzin was followed by alterations in the antioxidant capacity of algae, oxidative damage in the form of LPO and alterations in pigment content. Furthermore, both herbicides impacted the photosynthetic activity of algae, as seen by alterations in the maximum and effective quantum efficiency of PSII, PSII photochemistry and energy dissipation pathways, impact in the water-splitting apparatus and reduction in the electron transport rate. The inhibitory effect of metribuzin on photosynthetic processes/components was larger than that seen for bifenox. The impact of bifenox and metribuzin in the photosynthetic processes of *C. reinhardtii* seems to be in close association with the formation of ROS and consequent oxidative stress and damage in algal cells. Overall, this study showed that the high-throughput methods developed could successfully characterise both potential Modes of Action and adverse effects of bifenox and metribuzin in *C. reinhardtii.*

Keywords: *Chlamydomonas reinhardtii*; Biocides; Reactive oxygen species (ROS); Reduced glutathione (GSH); Lipid peroxidation (LPO); Photosystem (PS) II efficiency; Photosynthetic pigments.

1. Introduction

Herbicides are specifically designed to control unwanted vegetation. These are continuously applied not only in agriculture, pasture systems and in forestry, but also in urban green areas and maintenance of roads and railways. Although their use is subjected to several regulations at the international level (e.g. EU, 2009, 2012, 2013), their constant and cumulative use can negatively affect non-target organisms present in the environment. Herbicides can be transported via surface runoff and contaminate nearby surface waters, causing hazardous effects in important organisms such as primary producers, that are at the basis of the aquatic food web (Lürling, 2011; Nestler et al., 2012a,b).

Herbicides bifenox and metribuzin have a widespread distribution in surface waters and are commonly found in the aquatic environment at concentrations considered toxic to aquatic organisms, including microalgae (EFSA, 2006, 2007; Almeida et al., 2017). Bifenox, with the ISO common name for methyl 5-(2,4-dichlorophenoxy)-2-nitrobenzoate (IUPAC), is a diphenyl ether (DPE) that belongs to the class of nitrophenyl ether herbicides such as fomesafen and lactofen (structural formula in supplementary data, Figure A1 (a); EFSA, 2007). Metribuzin, with the ISO common name for 4-amino-6-*tert*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one or 4-amino-6-*tert*-butyl-3-methylthio-1,2,4-triazin-5(4*H*)-one (IUPAC), belongs to the class of triazinone herbicides such as hexazinone and metamitron (structural formula in supplementary data, Figure A1 (b); EFSA, 2006). Both compounds are known to affect photosynthesis but by different modes of action (MoAs). Bifenox causes cellular membrane disruption, inhibition of photosynthesis and inhibits the protoporphyrinogen oxidase (Protox), an enzyme that catalyses the last common step in chlorophyll and heme synthesis (EFSA, 2007; Grossman, 2005). Metribuzin inhibits the electron transport in the Hill reaction due to its binding to the D1 protein in photosystem II (PSII) (Lürling and Roessink, 2006; Lürling, 2011).

In photosynthetic organisms, reactive oxygen species (ROS) are continuously formed by the inevitable leakage of reactive electrons from electron transport activities of chloroplasts, mitochondria and plasma membranes (Foyer et al., 1997). In microalgae, the interference of several herbicides such as atrazine, diuron, flumioxazin, isoproturon, norflurazon, paraquat and terbutryn with photosynthetic performance has been associated with the formation of ROS (Alscher et al., 1997; Geoffroy et al., 2004; Jamers and Coen, 2010; Nestler et al., 2012a,b; Prado et al., 2009; Ramel et al., 2009; Rioboo et al., 2002). Under normal cellular conditions, ROS are predominantly formed in the reaction centers of both Photosystems I (PSI) and II in chloroplast thylakoids. However, exposure to herbicides can result in a disequilibrium in these

reaction centres and consequently in chlorophyll, which can further enhance ROS production. If not strictly controlled, these highly reactive molecules can react with cellular components, trigger oxidative stress and ultimately cause oxidative damage to cellular macromolecules (Dayer et al., 2008). In photosynthetic organisms, ROS can also cause damage or modify vital proteins and lipid components of chloroplasts thylakoid membranes. One example is the formation of lipid peroxidation (LPO), which is associated with the peroxidation of membrane lipids. In addition, photosynthetic pigments such as chlorophylls and carotenoids can also suffer bleaching (i.e., the loss of photosynthetic pigments). This can occur either by the inhibition of chlorophyll or carotenoids biosynthesis, or by peroxidative destruction of the pigments already formed (Moreland, 1980; Wakabayashi and Böger, 2004). The destruction of photosynthetic pigments can originate a decrease in photosynthetic performance and ultimately the amount of energy available for cell metabolism, division and growth (Asada, 2006; Jones, 2005). While chlorophyll is the primary photosynthetic pigment (Hyka et al., 2013), carotenoids protect chlorophyll from photooxidation by dissipating the oxidative energy of singlet oxygen ($^1O_2$), acting as an antioxidant. If these are affected, chlorophyll is also indirectly destroyed (Wakabayashi and Böger, 2004). Plant cells also have other well-developed antioxidant defence mechanisms (Alscher et al., 1997). These can be enzymes that directly remove free radicals or molecules that decrease the formation of radicals, like proteins that minimize the availability of pro-oxidants (Livingstone, 2001; Nikinmaa, 2014). An important non-enzymatic antioxidant involved in the detoxification of ROS is glutathione (GSH) (Apel and Hirt, 2004).

Even though the main MoA of bifenox and metribuzin has been previously described in plants such as *Lemna* sp. (e.g. Fairchild et al., 1998; Grossman, 2005), little information still exists characterising their specific toxicity to microalgae. The present study aimed to verify if a battery of selected high-throughput methods could identify how these compounds specifically affect *Chlamydomonas reinhardtii*, a well-studied microalga commonly found in freshwater ecosystems. This alga has been identified as one of the most sensitive freshwater species to a range of contaminants including herbicides and PSII inhibitors, therefore being widely used in ecotoxicological studies (Alric et al., 2010; Chalew and Halden, 2009; Fischer et al., 2006; Juneau et al., 2007). A multiple-endpoint approach was used to analyse the potential of bifenox and metribuzin to induce the formation of ROS and consequent oxidative stress, by looking at alterations in GSH content, formation of LPO, PSII performance and loss of photosynthetic pigments. Given that both bifenox and metribuzin are known to impact photosynthesis, this multi-endpoint approach aimed to directly compare and differentiate their effects and the

sensitivity of *C. reinhardtii* and provide additional information on the underlying toxic mechanisms associated with their main MoA.

2. Materials and Methods

2.1. Microalgae cultures

Chlamydomonas reinhardtii (NIVA-CHL153; Norwegian Institute for Water Research, Oslo, Norway) was cultured in glass flasks, with an initial number of 1 x 10^3 cells/mL in Talaquil media, prepared with minor modification from Le Faucheur et al. (2005) by substituting $NaNO_3$ with NH_4Cl as in Szivák et al. (2009). Media was prepared at least 24 hours before use to allow the equilibrium of compounds. Flasks were incubated for 3-4 days in an Infors Multitron 2 incubator shaker (Infors AG, Bottmingen, Switzerland) at 20 ± 2 °C, with orbital shaking at 90 rpm and under continuous light (83 ± 6 µmol m^{-2} s^{-1}) using cool–white fluorescence lamps (TLD 36W/950, Philips, London, UK). Algal cells in exponential growth phase were used for each test, collected by centrifugation, washed and resuspended in the assay buffer. All glassware used was properly washed and autoclaved prior to use to avoid any microbial contamination. Culture samples were regularly observed under a microscope to notice the existence of any microbial contamination.

2.2. Test compounds and chemical analysis

The test compounds bifenox (CAS number: 42576-02-3) and metribuzin (CAS number: 21087-64-9) were acquired from Sigma-Aldrich (United Kingdom) with ≥ 97.0 % purity. Stock solutions were diluted in anhydrous dimethyl sulfoxide (DMSO, Sigma-Aldrich, United Kingdom) and stored at -20°C until use.

For the confirmatory analysis of bifenox and metribuzin, the stock standard solutions were diluted in dichloromethane and analysed by gas chromatography-high resolution mass spectrometry (GCT-Premier, Waters Corp, Milford MA, USA). The analytes were separated on a 30 m × 0.25 mm, 0.25 ìm film thickness DB-5MS column (Agilent Technologies) with helium carrier gas. Splitless injection at 250 °C was applied. The initial temperature of 60 °C was held for 2 min, followed by an increase of 5 °C/min to 310 °C and held for 5 min. The m/z used for quantification were 340.986 for bifenox and 214.088 for metribuzin. The results of the chemical analysis on the stock standard solutions are in Table A1 in supplementary data and confirmed the concentrations of the stock solutions used for the exposures. Therefore, as the measured concentrations did not exceed a ± 20 % change from nominal concentrations, the nominal concentrations were used herein.

2.3. Studied endpoints

The used assays were previously optimized for *C. reinhardtii* in terms of algal concentration, probe concentration and selection of microplates (Almeida et al., 2017). Three independent exposure experiments were executed in 96-well black microplates (Corning Incorporated, Costar®, NY, USA), with eight replicates for the control and four replicates for each tested concentration. A first assessment of the potential ROS formation of both bifenox and metribuzin was performed following 6 hours exposure. Then, all studied endpoints were measured after 24 hours exposure. Preliminary tests were performed to select the concentrations at which no actual decrease in algal density was observed. Accordingly, cell density was measured at the beginning and end of the 24 hours exposures by fluorescence using a Cary eclipse fluorescence spectrophotometer (Agilent Technologies, California, USA) at 485/685 nm excitation/emission. Results confirmed that no significant differences in growth occurred during the exposure (Table A2 in supplementary data). For all tests, an initial concentration of 3×10^6 algae cells per ml was used in each well. The selected concentrations for the compounds were between 1 nM and 1×10^5 nM (final concentration of DMSO 0.05% v/v). The same concentration of DMSO (0.05% v/v) was added to the controls, as preliminary tests were performed confirming no effect either in the toxicity of compounds or in algal growth (fluorescence of control cells at 0 and 24 hours in Table A3 of supplementary data). Exposures were run in the same incubator shaker as that used to maintain algal cultures, at 20 ± 2 °C, with orbital shaking at 90 rpm and under continuous light (83 ± 6 μmol m^{-2} s^{-1}) using cool–white fluorescence lamps (TLD 36W/950, Philips, London, UK).

2.3.1. ROS production

The production of ROS in *C. reinhardtii* was quantified as described in Almeida et al. (2017), using the non-fluorescent probe carboxy-H_2DFFDA (adapted from Stoiber et al., 2011; Szivák et al., 2009), following 6 hours and 24 hours exposure to bifenox and metribuzin. Stock solutions of 50 mM carboxy-H_2DFFDA (Invitrogen, Molecular Probes Inc., Eugene, OR, USA) were prepared in anhydrous DMSO and stored in aliquots at -20°C until use. An exposure solution was prepared by diluting 100 μl of stock solution in the assay buffer (final concentration 5 μM) along with the test compounds at the different tested concentrations. This exposure solution was then added to the microplate containing 100 μl of the algae suspension and incubated for 6 and 24 hours. Once taken up by the cells, cellular esterases hydrolyse carboxy-H_2DFFDA to non-fluorescent difluorodihydrofluorescein (H_2DFF). In the presence of

ROS, the non-fluorescence H_2DFF is oxidized to the green-fluorescent difluorofluorescein DFF, which is retained in the cytosol (Gunawan et al., 2013). The fluorescent product DFF was measured by fluorescence using the microplate reader 1400 Multilabel Counter, Victor 3 (Perkin Elmer) at an excitation of 488 nm and emission of 520 nm (Almeida et al., 2017). The natural fluorescence of both compounds in combination with the probe without algae was analysed and subtracted to eliminate any interference by non-algal ROS production. The formation of ROS was expressed as fold induction (% of control, % CT).

2.3.2. Quantification of GSH

The monochlorobimane (mBCl) assay was applied to quantify the GSH content (adapted from Machado and Soares, 2012), according to the method described in Almeida et al. (2017). This non-fluorescent probe reacts with GSH inside the cell forming fluorescent bimane-glutathione (B-SG) adducts, catalysed by glutathione S-transferase (GST; Haugland, 2005). Briefly, stock solutions of 50 mM mBCl (TermoFisher Scientific, Life Technologies Limited, Scotland) were prepared in anhydrous DMSO and kept in aliquots at -20°C until use. Stock solutions of equine GST (Sigma-Aldrich, United Kingdom) at 10 U/ml were diluted in the assay buffer just before usage. The exposure solution for each herbicide was added to the microplate containing the algae suspension and incubated under ambient light for 24 hours in the incubator. After exposure to both herbicides, a final working solution of mBCl probe (50 μM) and GST 10 U/ml (final concentration of 1U/ml) was pipetted to each well and plates were incubated in the dark for 1.5 hours with orbital shaking. Fluorescence was quantified using the microplate reader 1400 Multilabel Counter, Victor 3 (Perkin Elmer) at excitation/emission of 405/486 nm. Natural fluorescence of the compounds in combination with the probe without algae was analysed, with no interference obtained for the two tested compounds. The GSH content was expressed as fold induction (% of control, % CT).

2.3.3. LPO measurement

The probe C_{11}-BODIPY$^{581/591}$ (adapted from Cheloni and Slaveykova, 2013) was used to determine LPO after 24 hours exposure to bifenox and metribuzin (Almeida et al., 2017). This is a fatty acid analogue probe with specific fluorescence properties that can be oxidized by oxyl-radicals together with the endogenous fatty acids (Cheloni and Slaveykova, 2013). Stock solutions of 2.5 mM were prepared in anhydrous DMSO and stored in aliquots at -20°C until use. Exposure solutions were prepared by diluting the probe in the assay buffer (final concentration 5 μM) with the test compounds and added to the microplates containing algae.

Microplates were then incubated for 24 hours. The fluorescent product was quantified by fluorescence using the microplate reader 1400 Multilabel Counter, Victor 3 (Perkin Elmer) at excitation/emission wavelength of 488/535 nm. Natural fluorescence of both compounds in combination with the probe without algae was analysed and subtracted to eliminate any interference with the fluorescence readings. LPO was expressed as fold induction (% of control, % CT).

2.3.4. PSII performance

PSII efficiency was determined using a PAM fluorometer (Underwater Fluorometer DIVING-PAM, Heinz Walz GmbH, Effeltrich, Germany), as described in Almeida et al. (2017) (adapted from Herlory et al., 2013; Gomes et al., 2017). The fluorescence parameters maximum quantum yield (F_v/F_m) and the efficiency of oxygen-evolving complex (OEC) of PSII were determined after 30 minutes of dark acclimation of algal cells. Subsequently, dark-acclimated cells were illuminated by an actinic light at intensity equivalent to the incubation light (80 μmol/m^2/s^1) and the effective quantum yield (Φ_{PSII}), electron transfer rate (ETR), coefficients of photochemical (qP) and non-photochemical quenching (qN) and the non-photochemical quenching (NPQ) were determined. The relative dissipation of energy through PSII was also calculated through estimation of the relative photochemical ($qP_{(rel)}$), the relative non-photochemical quenching $qN_{(rel)}$ and the relative unquenched fluorescence ($UQF_{(rel)}$) parameters. All parameters were calculated according to the formulas expressed in Table I.

Table I – Fluorescence parameters calculated from PAM fluorometry measurements in *Chlamydomonas reinhardtii* exposed to bifenox and metribuzin.

Parameter	Definition	Equation	Reference
F_v/F_m	Maximum quantum efficiency of PSII	$(F_m - F_0)/F_m$	Schreiber, 2004
OEC	Efficiency of the oxygen-evolving complex	$F_0/(F_m - F_0)$	Kriedemann et al., 1985
Φ_{PSII}	Effective quantum efficiency of PSII	$(F'_m - F_t)/F'_m$	Genty et al., 1989
qP	Coefficient of photochemical quenching	$(F'_m - F_t)/(F'_m - F'_0)$	Schreiber et al., 1986; Juneau and Popovic, 1999

qN	Coefficient of non-photochemical quenching	$1-(F'_m-F'_0)/(F_m-F_0)$	Schreiber et al., 1986; Juneau and Popovic, 1999
NPQ	Non-photochemical quenching	$(F_m-F'_m)/F'_m$	Bilger and Björkman, 1990
ETR	Relative photosynthetic electron transport rate	0.5 x Φ_{PSII} x PAR x I_A*	Genty et al., 1989
$qP_{(rel)}$	Relative photochemical quenching	$(F'_m-F_t)/(F_m-F'_0)$	Buschmann, 1995
$qN_{(rel)}$	Relative non-photochemical quenching	$(F_m-F'_m)/(F_m-F'_0)$	Buschmann, 1995
$UQF_{(rel)}$	Relative unquenched fluorescence	$(F_t-F'_0)/(F_m-F'_0)$	Juneau et al., 2005

*Where 0.5 is a factor that assumes equal distribution of energy between PSII and PSI, PAR is the actinic photosynthetically active radiation generated by PAM2000 and I_A is the assumed absorbance by the photosynthetic organism (0.84).

2.3.5. Quantification of pigments

Pigments were analysed according to Almeida et al. (2017) (adapted from Arnon, 1949, Lichtenthaler, 1987), with both extraction and analysis performed in dim light to avoid photobleaching. After 24 hours exposure, algae cells were centrifuged at 16 000 x *g*, the pellet resuspended in cold 100 % acetone and incubated for 5 min on ice in dark conditions. A second centrifugation was made (16 000 x *g*) and the supernatants were transferred into a 96-well plate (FalconTM, Oslo, Norway). Absorption was measured in a VersaMax microplate reader (Molecular devices, California, USA) at 663 nm for chlorophyll *a*, 647 nm for chlorophyll *b* and 470 nm for total carotenoids. The concentrations of the pigments were determined according to Lichtenthaler (1987) and expressed as fold induction.

2.4. Statistical analysis

Data from three independent experiments were graphically displayed and statistical analyses performed by GraphPad Prism 8 software (GraphPad Software Inc., La Jolla, CA, USA). For data exhibiting an apparent normal distribution and homogeneous variance, parametric one-way ANOVA was applied, and the Tukey post hoc test was used for multiple comparisons. For data with apparent non-normal distribution and/or non-homogeneous variance, the non-parametric tests Kruskal-Wallis and Dunn's post hoc test were used. A non-linear regression using a sigmoidal dose-response curve with variable slope (four parameters) were fitted for the analysis of the PSII performance parameters according to:

$$f(x) = y\,\min + \frac{y\,\max - y\,min}{1 + 10^{((Log\ EC_{50} - x) \times Hill\ Slope)}}$$

where f is the effect, x the concentration of the herbicide, y min the bottom (variable), y max the top (variable) and EC_{50} the concentration of the herbicide with an effect of 50% when normalized to the top and bottom values.

XLStat2018.5® (Addinsoft, Paris, France) was used to calculate Pearson's correlation to measure the strength of association between the ROS formation at 24 hours and the other variables. The same software was used to calculate a Principal Component Analysis (PCA) for each compound to discriminate the main variables responsible for the variance of data. A *p*-value < 0.05 was considered significant for all statistical analyses.

3. Results

3.1. ROS production

After the first 6 hours exposure, both bifenox and metribuzin induced the formation of ROS in *C. reinhardtii* (for more information on the ROS production along the 6 hours see supplementary data Fig. A2 and A3). After 6 hours exposure, bifenox caused a concentration-dependent increase in ROS, with a significant induction compared to control at 10 x 10^3 nM and the largest response (3.6-fold increase) observed at the highest concentration (1 x 10^5 nM). Metribuzin presented a biphasic induction pattern, with a significant increase in ROS formation at 1 x 10^3 nM). After 24 hours exposure, the algae exposed to bifenox caused a significant increase in ROS compared to the 6 hours exposure at the 4 highest concentrations (2 x 10^3 nM to 1 x 10^5 nM). The same concentration-dependent pattern was observed, with significant induction of ROS formation at concentrations higher than 2 x 10^2 nM and with a maximum induction (5.2-fold) at the highest concentration (1 x 10^5 nM). For metribuzin there were no significant differences in the formation of ROS between 6 hours and 24 hours for any of the concentrations tested. The formation of ROS at 24 hours exposure was significantly higher than the control at the 4 highest concentrations (2 x 10^3 nM to 1 x 10^5 nM).

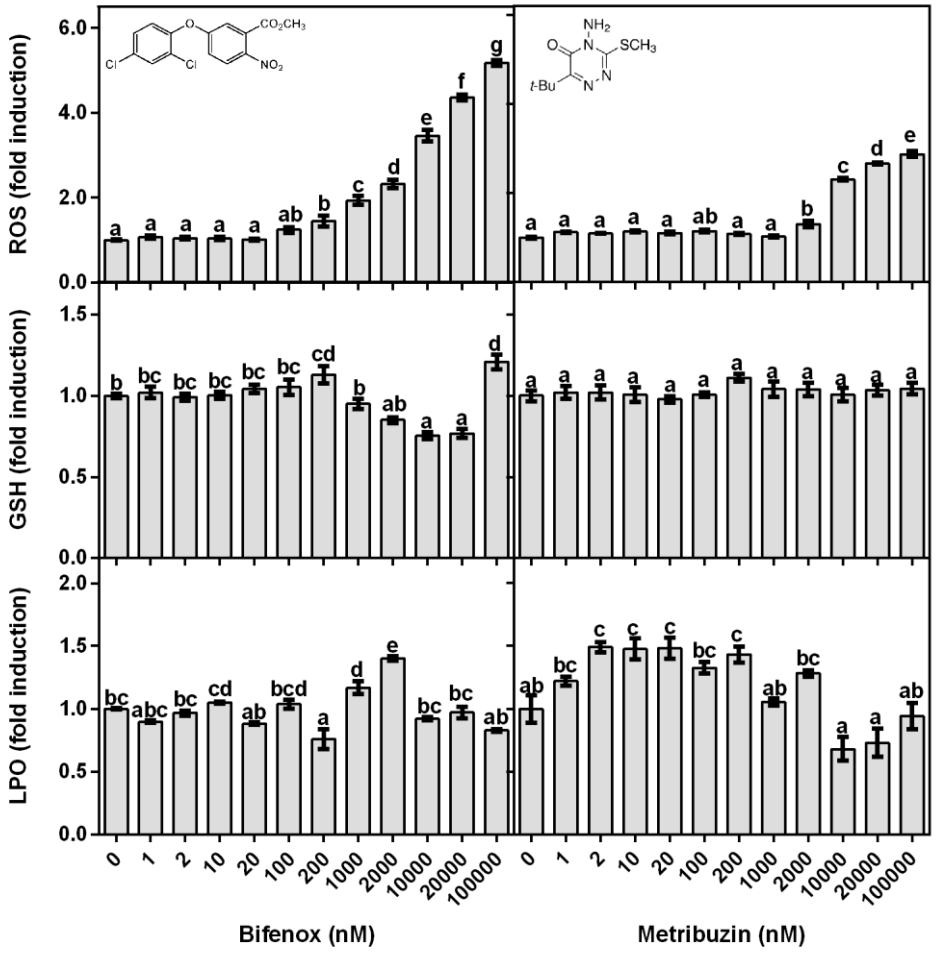


Figure 1 – Reactive oxygen species (ROS) formation, GSH content and lipid peroxidation (LPO) in *Chlamydomonas reinhardtii* exposed to bifenox and metribuzin for 24 hours. The data (mean ± SEM) represent 3 independent studies. Different letters indicate significant differences between concentrations ($p < 0.05$).

3.2. GSH content

After 24 hours exposure only bifenox influenced the GSH content in *C. reinhardtii* (Fig. 1). A small increase was induced at 2 x 10^2 nM and 1 x 10^5 nM (significantly different from control) and a decrease between these in the concentration range 10-200 µM (up to 0.8-fold).

3.3. LPO levels

The two compounds affected the LPO levels in *C. reinhardtii* after 24 hours exposure (Fig. 1). For bifenox significant differences were observed at intermediate concentrations, with a decrease at 2 x 10^2 nM and an increase at 1 x 10^3 nM and 2 x 10^3 nM. Metribuzin caused an

increase in LPO levels at several of the low concentration groups. A slight reduction in the LPO levels was also recorded for both compounds at the 3 highest tested concentrations (1 x 10^4 nM - 1 x 10^5 nM), however not significantly different from the control.

3.4. PSII performance

Results obtained for PSII performance showed that both compounds had a significant impact on several chlorophyll fluorescence parameters (Fig. 2; fitting values for the curves of PSII performance parameters are presented in supplementary data Table A4 for bifenox and Table A5 for metribuzin). Bifenox caused a 22.5% decrease in the F_v/F_m at 1 x 10^5 nM, indicating that more than 75% of the light absorbed by the algae could be used in photosynthesis (Fig. 2A). The efficiency of OEC was stimulated by bifenox, resulting in a 109% increase at the highest concentration used. After 24 hours exposure, bifenox caused a 32% reduction in the Φ_{PSII} at the highest concentration compared to the control, with no significant alterations at lower concentrations. The photosynthetic electron transport process (ETR) of algae cells was also altered by this compound, where a 32% decrease was observed at 1 x 10^5 nM. The quenching parameters decreased at the two highest concentrations tested, with 20.7%, 41.6% and 62.5% inhibition for qP, qN and NPQ at 1 x 10^5 nM, respectively. $UQF_{(rel)}$ was the dominant energy dissipation process for *C. reinhardtii* exposed to bifenox, with a 73.6% increase at 1 x 10^5 nM, while $qN_{(rel)}$ and $qP_{(rel)}$ decreased and increased 32.9% and 13.8%, respectively. As most of the parameters altered by bifenox did not reach a 50% effect, the EC_{50} values could not be calculated.

Metribuzin severely altered the photosynthetic parameters of PSII (Fig. 2B). F_v/F_m was reduced 74.5% at the highest concentration tested (1 x 10^5 nM). Accordingly, the EC_{50} was estimated to be 1140 nM (Table A6 in supplementary data). A significant alteration in the state of water-oxidation (OEC) was also observed, as reflected by the 442, 559 and 824% increase in the OEC compared to the control value for the three highest concentrations tested. However, the shape of the concentration-response curve obtained for this parameter did not allow a reliable assessment of the EC_{50}. In the light-acclimated state, exposed algae showed a concentration-dependent decrease in the Φ_{PSII} (EC_{50}= 180.1 nM) to achieving almost null photosynthetic activity at the highest concentration (1 x 10^5 nM). The ETR was also completely inhibited at concentrations higher than 1 x 10^3 nM, giving an EC_{50} of 193.9 nM. Metribuzin also caused a concentration-dependent change in qP, qN and NPQ, where qP was affected to the largest degree (EC_{50}= 196.9 nM). Similarly, at concentrations higher than 1 x 10^2 nM, $qP_{(rel)}$ and $qN_{(rel)}$ dramatically decreased and resulted in complete inhibition, indicating the complete shut-down

of the PSII reaction centres. On the other hand, the $UQF_{(rel)}$ yield increased 458% compared to the control, representing 100% of the fluorescence quenching at the highest concentrations tested (EC_{50}= 429.0 nM).

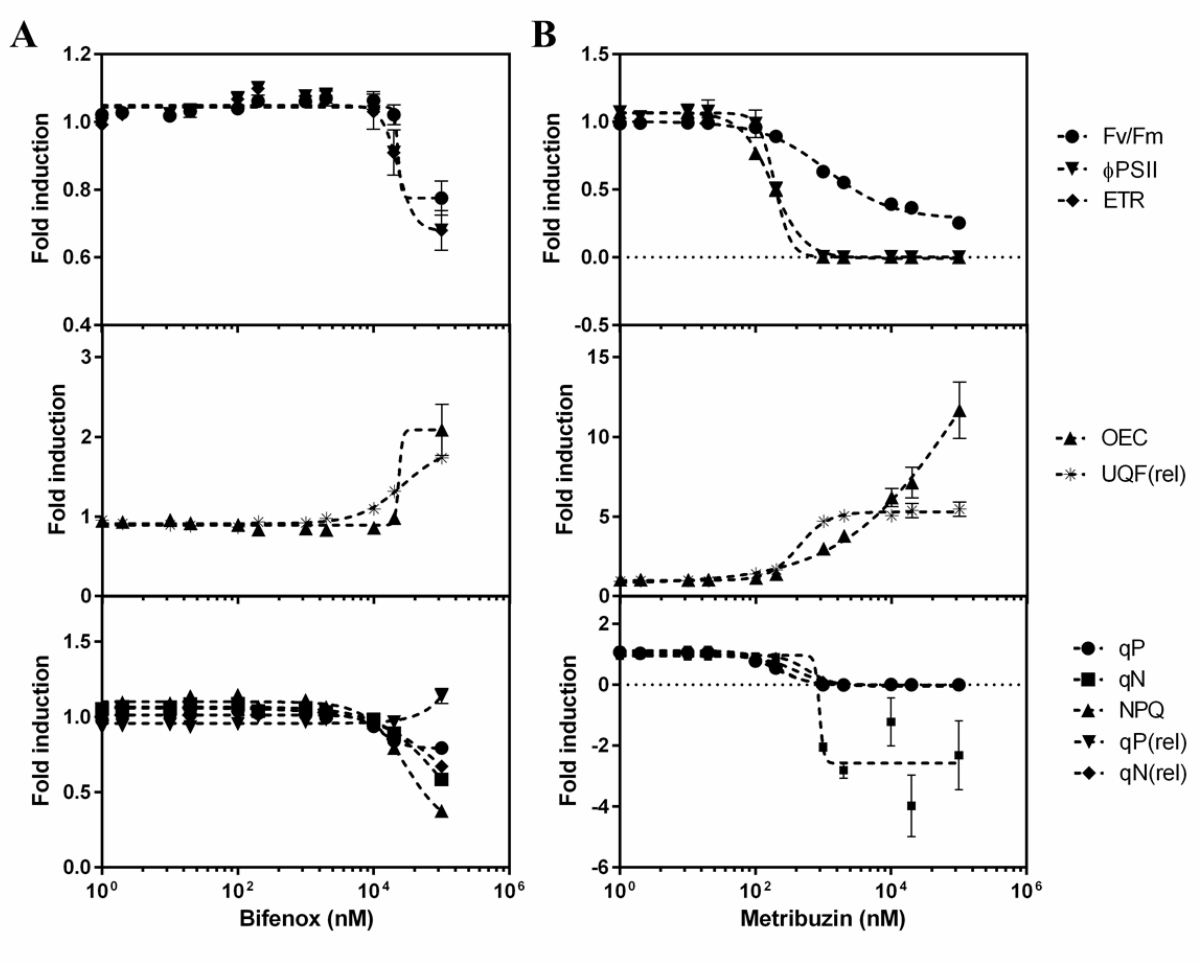


Figure 2 – Concentration-response curves obtained for the fluorescent parameters of *Chlamydomonas reinhardtii* exposed to bifenox (A) and metribuzin (B) for 24 hours. F_v/F_m – Maximum quantum yield, OEC – Efficiency of the oxygen-evolving complex, Φ_{PSII} – Quantum efficiency of PSII photochemistry, ETR – Electron transfer rate, qP – Coefficient of photochemical quenching, qN – Coefficient of non-photochemical quenching, NPQ – Non-photochemical quenching, $qP_{(rel)}$ – Relative photochemical quenching, $qN_{(rel)}$ – Relative non-photochemical quenching, $UQF_{(rel)}$ – Relative unquenched fluorescence. The data (mean ± SEM) represent 3 independent studies.

3.5. Pigments

Bifenox caused an increase in chlorophyll *a* at 2 x 10^3 nM, an increase in chlorophyll *b* at 2 nM and 20 nM and in chlorophylls *a* and *b* at 2 x 10^3 nM when compared to control (Fig. 3). For

carotenoids, no significant differences were found compared to the control ($p > 0.05$). Algae exposed to metribuzin only caused a significant decrease in chlorophylls *a* and *b* at 1 nM compared with the control (Fig. 3). No significant differences were found for chlorophyll *a*, chlorophyll *b* and carotenoids comparatively with the control.

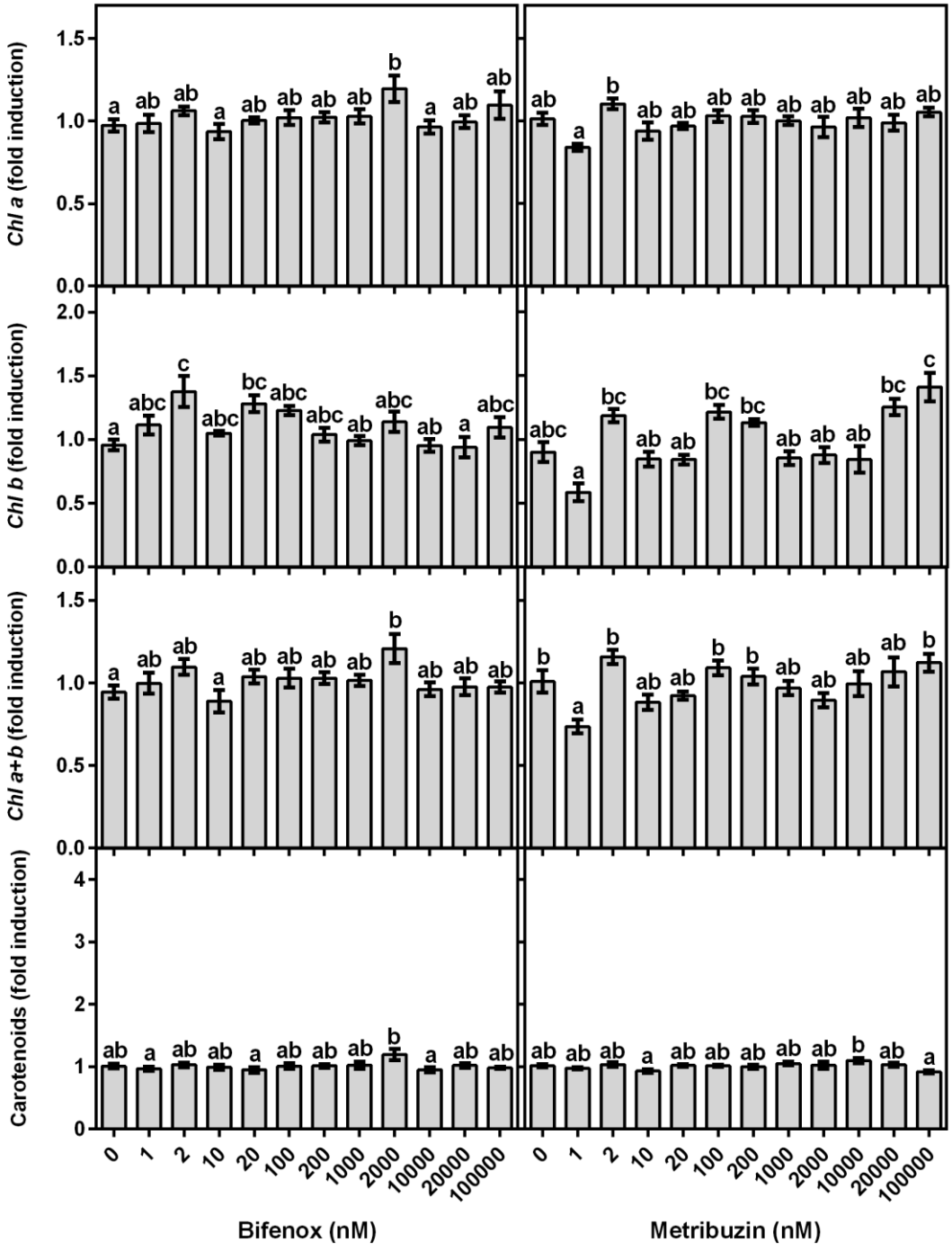


Figure 3 – Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), chlorophyll *a*+*b* (Chl *a*+*b*) and carotenoids content in *Chlamydomonas reinhardtii* exposed to bifenox and metribuzin for 24

hours. The data (mean ± SEM) represent 3 independent studies. Letters indicate significant differences between concentrations ($p < 0.05$).

3.6. Relation between endpoints

A PCA was applied to the overall data for each compound to better understand the global results and depict the association between the determined endpoints after 24 hours exposure (Figure 4). For bifenox (Fig. 4A), PC1 (60.4%) was best described by the variables associated with the PSII performance, along with ROS formation. PC2 (18.8%) was best explained by chlorophyll *a*, carotenoids, chlorophylls *a*+*b* contents and by LPO. Regarding metribuzin (Fig. 4B), the two principal components represented 79.9% of total variance, where PC1 represented 64.7% and PC2 15.3%. PC1 was again better explained by the PSII performance variables, along with ROS formation and LPO, while PC2 was best described by chlorophylls content (*a*, *b*, and *a*+*b*).

For bifenox, formation of ROS was positively correlated with $UQF_{(rel)}$, $qP_{(rel)}$ and OEC and negatively correlated with the other variables related with the PSII performance (qP, $qN_{(rel)}$, NPQ, qN, Φ_{PSII}, ETR and F_v/F_m) (Table A7 in supplementary data). For metribuzin the formation of ROS was positively correlated with OEC, and $UQF_{(rel)}$ and negatively correlated with all the other variables related with the PSII performance (F_v/F_m, $qN_{(rel)}$, NPQ, qN, $qP_{(rel)}$, qP, ETR and Φ_{PSII}), as well as with LPO (Table A8 in supplementary data).

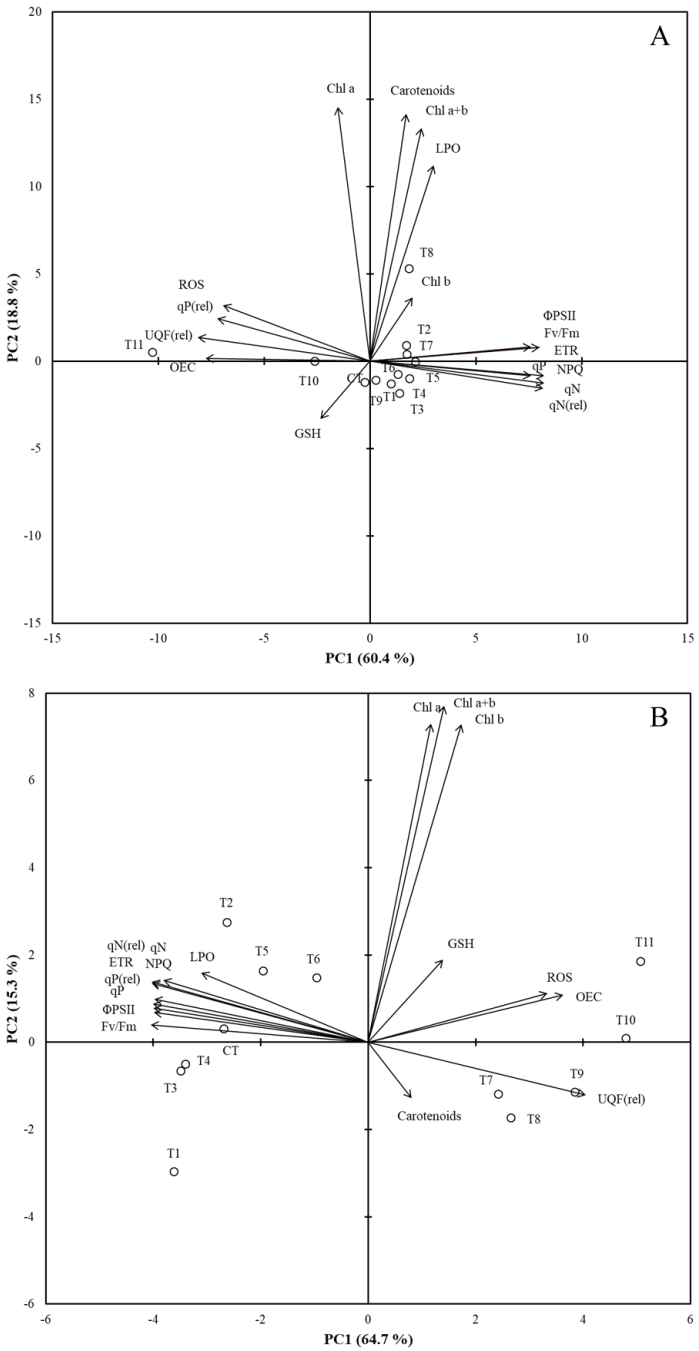


Figure 4 – Principal component analysis (PCA) of *Chlamydomonas reinhardtii* exposed to bifenox (A) and metribuzin (B) for 24 hours. CT – control, T1-T11 – Increasing concentrations of the compounds used, ROS – Reactive oxygen species, GSH – Glutathione, LPO – Lipid peroxidation, F_v/F_m – Maximum quantum yield, OEC – Efficiency of the oxygen-evolving complex, Φ_{PSII} – Quantum efficiency of PSII photochemistry, ETR – Electron transfer rate, qP – Coefficient of photochemical quenching, qN – Coefficient of non-photochemical quenching, NPQ – Non-photochemical quenching, $qP_{(rel)}$ – Relative photochemical quenching, $qN_{(rel)}$ – Relative non-photochemical quenching, $UQF_{(rel)}$ – Relative unquenched fluorescence, Chl *a* – Chlorophyll *a*, Chl *b* – Chlorophyll *b*, Chl *a*+*b* – Chlorophyll *a* and *b*.

4. Discussion

Herbicides are often detected in the aquatic environment namely in lakes, rivers, estuaries, ground water and coastal marine waters (Albanis et al., 1994; Arias-Estévez et al., 2008; Fatokio and Awofolu, 2004; Hela et al., 2005; Prado et al., 2009; Zhang et al., 2011). Moreover, if these compounds are specially designed to eradicate unwanted plants (pests), primary producers such as microalgae are particularly in danger. If these hazardous compounds affect primary producers, an impact further up in the trophic chain is expected to occur, either through changes in grazing community structure and diversity, or even loss of primary production as food for consumers higher in the food-web (Ralph et al., 2007).

Most of the toxicity information on herbicides is related to traditional toxicological and regulatory-relevant endpoints such as growth inhibition, which do not provide any information on how they interact and cause damage in biological targets (Worth et al., 2014). In this perspective, this study aimed to supplement the general toxicity information of bifenox and metribuzin in the microalgae *C. reinhardtii* with more specific physiological and biochemical endpoints. To achieve this, a set of high-throughput assays focussed on ROS formation, GSH content, LPO, photosystem II photosynthetic performance and pigments content was chosen.

Bifenox is known for inducing membrane disruption and inhibition of photosynthesis by Protox inhibition, originating light-dependent oxygen radical formation (Grossman, 2005; EFSA, 2007). By inhibiting the Protox activity, bifenox causes the accumulation of protoporphyrin IX inside the cells, which is then oxidized in the plasma membrane when reacting with O_2 formed in photosynthesis (Kilinc et al., 2009). In the present study, bifenox induced the highest levels of ROS, showing a concentration-dependent increase already at 6 hours. This is in accordance with literature, which refers that bifenox induces ROS not easily detoxified (Kilinc et al., 2009). These are possibly not highly reactive, being able to spread within cells from its site of origin and promoting oxidative stress, as observed for other compounds such as H_2O_2 (Ledford and Niyogi, 2005) and paraquat (Nestler et al., 2012a). The possible formed species include H_2O_2, HO•, NO, ROO•, O_2^- and peroxide-derived oxidants, as indicated by the fluorescent dye carboxy-H_2DFFDA (Gunawan et al., 2013). A concomitant increase in GSH content and ROS levels was observed at intermediate concentrations, suggesting that this molecule was triggered in response to bifenox exposure. GSH is one of the most important antioxidant molecules, with a known role in the detoxification of excess ROS in cells (Livingstone, 2001; Nikinmaa, 2014). However, a depletion in GSH levels at concentrations higher than 1×10^3 nM was observed, which resulted in an exponential increase in ROS formation. This decrease in GSH levels and increase in ROS resulted in a significant increase in LPO levels, indicating the increased

production of oxyl-radicals such as HO•, ROO•, RO• and ONOO- (indicated by the fluorescent dye C_{11}-BODIPY$^{581/591}$; Cheloni and Slaveykova, 2013). LPO levels decreased again to basal levels at the three highest tested concentrations (from 1 x 10^4 nM), even though the levels of ROS continued to increase, showing that other scavenging processes may have responded to the increase of radical species. In this case, enzymes that directly remove radicals (e.g. SOD, CAT, GPx) or other molecules such as proteins (e.g. protective pigments) may be involved in the antioxidant mechanisms to compensate for the GSH depletion (Livingstone, 2001; Nikinmaa, 2014). At the highest concentration tested (1 x 10^5 nM), GSH levels increased again probably to counteract the production of ROS, whose levels were highest at this concentration. The association between LPO levels and antioxidant mechanisms in exposed algae was also visible in the PCA, where LPO was accompanied by an increase in photosynthetic pigments and a decrease in GSH content. The low levels of oxy-radicals production measured by the LPO probe are also indicative that the type of ROS formed by bifenox are not highly reactive. As previously explained and seen for H_2O_2 (Ledford and Niyogi, 2005) and paraquat (Nestler et al., 2012a), the formed species may be able to travel further away from its site of origin, being the cell components more susceptible to ROS attack. This was further confirmed by the lack of correlation between ROS formation and LPO levels. The non-linearity observed between these two parameters may be explained by the specificity of the two fluorescent probes towards different reactive species. The absence and/or decrease of the oxyl-radicals detected by the C_{11}-BODIPY$^{581/591}$ probe can be explained by the formation of other end-products of LPO, as for example malondialdehyde and 4-hydroxyalkenals, which cannot be detected using this fluorescent probe. In addition, this non-linearity can also be connected to different unsaturation degrees presented by the different lipid components in cellular membranes modulated by the efficiency of local antioxidant defences (Halliwell and Gutteridge, 2007). In fact, additional information on a range of other cellular responses interconnected with these responses were also observed, including alterations in the antioxidant defense system (e.g. GSH) and photosynthetic pigments. This mechanism of toxic action is also seen for other compounds such as H_2O_2 and paraquat. H_2O_2 toxicity is related with the formation of hydroxyl radicals and consequent oxidation of biomolecules. This compound is very mobile and able to pass through membranes, entering vulnerable parts inside cells, thus becoming highly reactive and toxic (Russell, 2003). Other studies also suggest that compounds like paraquat cause an overall decline of the antioxidant defence activity (Nestler et al., 2012a,b). In this study, similarly to paraquat, it seems that the antioxidant defence mechanisms present in the algae cells were not able to deal with the ROS formed by bifenox at high concentrations. Paraquat is known to divert

electrons from the Photosystem I (PSI) to molecular oxygen, producing radicals such as superoxide radicals, H_2O_2 radicals, hydroxyl radicals (•OH), and other reactive species (Jamers and Coen, 2010). It exerts oxidative stress not only in the chloroplasts but also in mitochondria and nucleus, resulting in a depletion of the antioxidant capacity of cells (Nestler et al., 2012b), as seen for bifenox herein. It is also a known inducer of antioxidant enzymes such as glutathione-*S*-transferases, ascorbate peroxidases, dehydroascorbate reductase and glutamate-cysteine ligase (Jamers and Coen, 2010), which were not determined in the present study.

Similarly to bifenox, exposure to metribuzin also caused a significant increase in ROS formation in exposed algae, but to a lower extent. Metribuzin had a concentration-dependent biphasic increase in ROS formation at 6 hours, while at 24 hours exposure a higher formation of ROS was only detected at the four highest concentrations tested. Thus, metribuzin can generate oxidative stress through a concentration dependent formation of ROS, which accumulates over time. The change from a biphasic concentration-response relationship at 6 hours to a monotonic concentration-response relationship at 24 hours of exposure, possibly indicates an activation of algae protection mechanisms, as previously seen for bifenox. Results show that for metribuzin other antioxidant mechanisms rather than GSH are activated, as this antioxidant molecule was not triggered at any of the concentrations tested. LPO, on the other hand, quickly increased from low to median metribuzin levels, showing that even though the antioxidant defence system was efficient in counteracting ROS, it was not able to counteract the formation of this type of oxidative damage. At the highest concentrations, a decrease in LPO was accompanied by an increase in ROS formation, indicating the presence of other radical species rather than the oxy-radicals detected by the used probe, as previously explained for bifenox, probably associated with the formation of other end-products of LPO and/or damage to the photosynthetic apparatus. The MoA of metribuzin seems to be similar of that of atrazine, a well-studied triazine herbicide. Atrazine is known to inhibit the Hill reaction and the related noncyclic photophosphorylation in chloroplasts, and thus affect PSII activity (DeLorenzo et al., 2001). It instigates the generation of ^{3}Chl, which if not quenched by carotenoids may induce the formation of singlet oxygen that induces LPO (Fai et al., 2007). Damage of PSII due to photoinhibition occurs at the donor site (water-splitting complex) and the acceptor site (Q_A) and/or (Q_B) acceptors (Jones et al., 2003). Triazine herbicides are also known for significantly increasing F_0 and F_m due to the blocking of electron transport from primary to secondary plastoquinone (Q_A and Q_B), and thus causing an increase in chlorophyll *a* fluorescence. As this type of herbicides block the re-oxidation of Q_A, the absorbed energy

cannot be used in photochemistry (Conrad et al., 1993), interfering instead with other parts of the photosynthesis apparatus.

The PAM fluorometry results obtained for F_v/F_m for the controls showed that more than 70% of the light absorbed by the microalgae was used for photosynthesis (F_v/F_m = 0.71 ± 0.01 for bifenox exposure and 0.73 ± 0.04 for metribuzin exposure). This suggests that the microalgae were maintained in a good physiological state throughout the exposure period under the conditions used in this study. On the other hand, both bifenox and metribuzin negatively affected the photosynthetic apparatus of exposed algae, with inhibition of photosynthetic activity connected to damage to PSII reaction centers and consequent disruption of the electron transport chain. An inhibition of both F_v/F_m and Φ_{PSII} in algae cells exposed to the highest concentration of bifenox was accompanied by a simultaneous decrease in non-photochemical energy dissipation pathways (i.e., qN and NPQ), showing an impact in the photosynthetic capacity of algae by this herbicide. No significant alterations were detected in the qP in all the concentrations used in this study. The coefficient of photochemical quenching only measures the fraction of open PSII reaction centres not considering their efficiency, being not always suitable to evaluate the photochemical activity of photosynthetic organisms under stress (Genty et al., 1989; Buschmann, 1995; Juneau et al., 2005). The coefficients of photochemical and non-photochemical quenching (qP and qN, respectively) have also been shown to be non-complementary as they do not refer to the same state of energy storage and dissipation through the photosynthetic process (Buschmann, 1995). Accordingly, a new type of quenching parameters was proposed by Buschmann (1995), the relative photochemical ($qP_{(rel)}$) and non-photochemical quenching ($qN_{(rel)}$) coefficients, to give a better understanding of the energy distribution between chlorophyll fluorescence, photochemistry and heat production. The relative distribution of the energy dissipation processes through the PSII in algae exposed to bifenox showed that photochemical quenching was promoted to dissipate excess light energy, as illustrated by the slight increase in $qP_{(rel)}$ at the highest concentration tested, even though light energy was mainly dissipated by $UQF_{(rel)}$. No differences in qP after exposure to bifenox seems to indicate the lack of alteration in the fraction of open PSII reaction centres, however, their efficiency increased with increasing concentrations, as showed by the increase in $qP_{(rel)}$ at the highest concentration tested. This impairment of photosynthetic processes was further confirmed by the reduction in ETR and the increase in OEC, reflecting the capacity of bifenox to influence electron transport in algae.

The inhibitory effect of metribuzin on photosynthesis and related processes/components was observed in a higher severity than that seen for bifenox. As a triazinone herbicide, metribuzin

is a PSII herbicide analogue to plastoquinone, which reverses the binding to the Q_B binding site on the D1 protein of the PSII complex (Jones, 2005). This binding instigates the disruption of the electron flow, causing the re-emission of energy as fluorescence light rather than directing them to fuel the photochemical processes (Muller et al., 2008). The decrease in the algal F_v/F_m was found to be more pronounced when exposed to metribuzin than for bifenox, indicating a decrease in photosynthetic performance. Similarly, Φ_{PSII} also decreased with increasing metribuzin concentrations, reaching total inhibition at the highest concentration tested. This indicates significant alterations in PSII photochemical and non-photochemical pathways (regulated and non-regulated), as well as electron transport (Juneau et al., 2001). In fact, this decrease in Φ_{PSII} was accompanied by a simultaneous decrease in photochemical and non-photochemical pathways (i.e., qN, NPQ and qP) and the predominance of $UQF_{(rel)}$ as an energy dissipation process, thus reflecting a complete disruption of normal energy pathways in algae. This impairment in photosynthetic processes is also reflected by the decrease in ETR with increasing metribuzin concentrations, which visibly reflects its MoA.

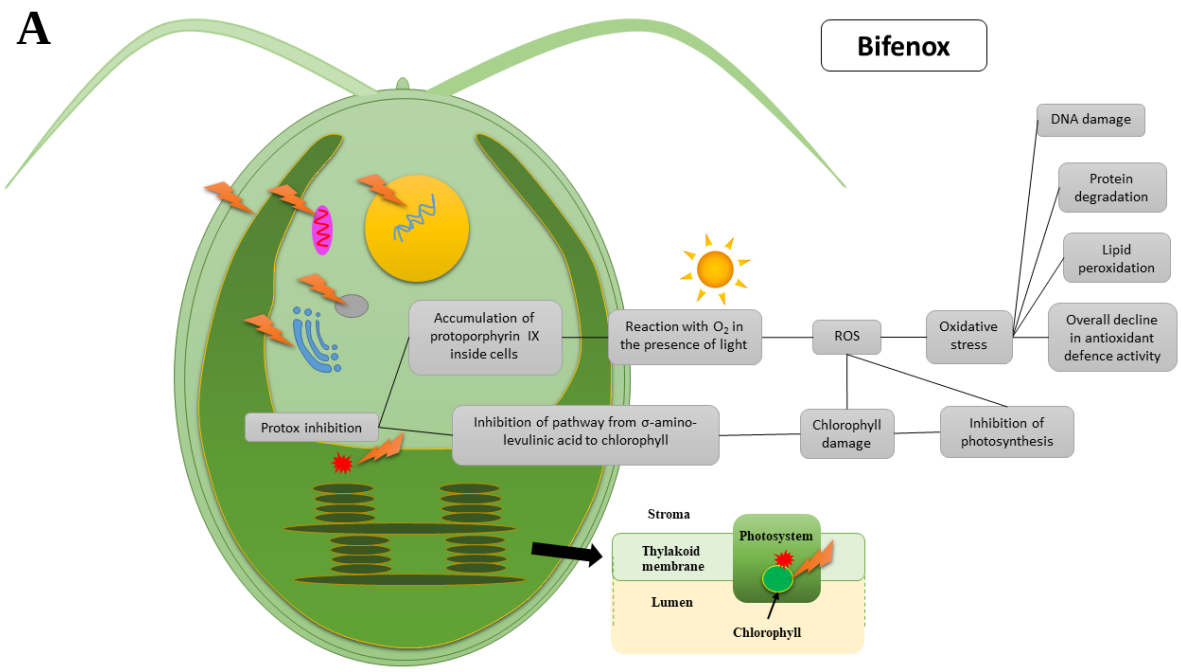
The inhibition of photosynthetic performance and more specifically the blockage of photosynthetic electron flow seen for bifenox and metribuzin can lead to additional ROS formation and oxidative damage in the form of LPO and membrane damage, due to the prevention of discharge of excitation energy collected by the PSII light harvesting complex (Hess, 2000). This close association between the impact of bifenox and metribuzin in the photosynthetic processes of *C. reinhardtii* and the formation of ROS and consequent oxidative damage was also evidenced by the PCA and correlation analysis. A significant correlation was detected between the chlorophyll fluorescence parameters and ROS formation for bifenox, indicating that the PSII reaction centers can potentially be damaged by ROS formation or vice-versa. In the case of metribuzin, a close association between PSII performance, ROS formation and LPO was also showed by the PCA, further evidencing this interconnection. In addition, a significant correlation was also detected between LPO and carotenoids, suggesting an involvement of these photosynthetic pigments to counteract ROS-mediated damage in light-harvesting pigments. Photosynthetic pigments were also slightly affected by metribuzin, especially chlorophyll *a* and *b*. Metribuzin is known to produce a ^{3}Chl state in PSII reaction centre that is capable of reacting with triplet oxygen ($^3O_2$) and forming the reactive singlet oxygen ($^1O_2$). This oxygen species can then damage adjacent chlorophyll-bearing proteins, separate the chlorophylls from their energy transfer systems and protective pigments such as carotenoids, causing further photogeneration by singlet oxygen (Jones, 2005). Inhibition of chlorophyll *a* fluorescence can be related to inhibition of electron flow in the PSII reaction

center and the donor site, while its increase can be observed if the inhibition occurs on the acceptor side if the PSII (Franklin et al., 2001), as previously indicated. The low levels of response obtained in the present study for carotenoids may be due to the used methodology for extraction and quantification that does not allow to differentiate which carotenoids were induced.

5. Conclusions

The high throughput methods used in this study as a screening tool for the MoA of herbicides successfully documented the effects of bifenox and metribuzin in *C. reinhardtii*, in which their known primary MoA of bifenox were clearly demonstrated. Both bifenox and metribuzin induced the formation of ROS in different parts of the photosynthetic apparatus and/or phases of the photosynthetic process, even though to different extents. In algae exposed to bifenox, ROS were formed not only inside the chloroplasts but also outside (protoporphyrin IX is oxidized in the plasma membrane). These reactive species seem to be less reactive and not easily detoxified, and not instantly detected by the antioxidant protective system, which could account for the higher ROS levels induced by this herbicide in comparison to metribuzin. Furthermore, the formed ROS seem to be able to travel along the cell and inflicting oxidative stress in different cell components. After a certain threshold level, the antioxidant protective system was triggered, being GSH involved in the process. This compound is known for directly affecting chlorophyll, initially by affecting its synthesis, and indirectly by altering photosynthetic processes associated with damage to PSII reaction centers and the electron transport process. In algae exposed to metribuzin, ROS were formed in thylakoid membrane of chloroplasts, initially disrupting the PSII complex and consequently photosynthesis. The ROS formed by metribuzin seem to be more reactive and instigating a quick response of the antioxidant protective system, where GSH does not seem to be involved. In this case, this system seems to be ready to counteract the oxidative stress until certain extent, as highly reactive species are already normally formed during photosynthetic processes. Therefore, lower ROS levels were obtained in response to this herbicide in contrast to bifenox. Metribuzin directly affected photosynthesis, as demonstrated by the clear inhibition of F_v/F_m, Φ_{PSII}, alterations in PSII photochemistry and energy dissipation pathways, impact in the water-splitting apparatus and reduction in the ETR rate. It also indirectly affected chlorophyll through the formation of ROS in the PSII complex. Based on the obtained results in combination with information collected from literature, the toxic mechanisms of bifenox and metribuzin in *C. reinhardtii* are proposed in Figure 5.

Future studies focusing on the ROS propagation mechanisms in response to bifenox and metribuzin should be performed, including a thorough assessment of which antioxidant defence mechanisms besides GSH are triggered to counteract oxidative damage. The process of LPO should also be further elucidated, in particular the saturation degrees presented by the different lipid components in cellular membranes, how they are modulated by the antioxidant defence system, as well as the formation of relevant end-products as for example malondialdehyde and 4-hydroxyalkenals. A more thorough pigment analysis should be performed to further discriminate which are directly affected by these compounds, as well as their specific role not only in the photosynthetic performance of exposed algae but also in the antioxidant defense system. Moreover, the use of a more comprehensive multi-endpoint analysis is also necessary to further elicited the toxic mechanisms of both herbicides. Additional studies with longer exposure duration should also be conducted for a better assessment on how bifenox and metribuzin affect algal growth and propagate adverse effects at the population level.



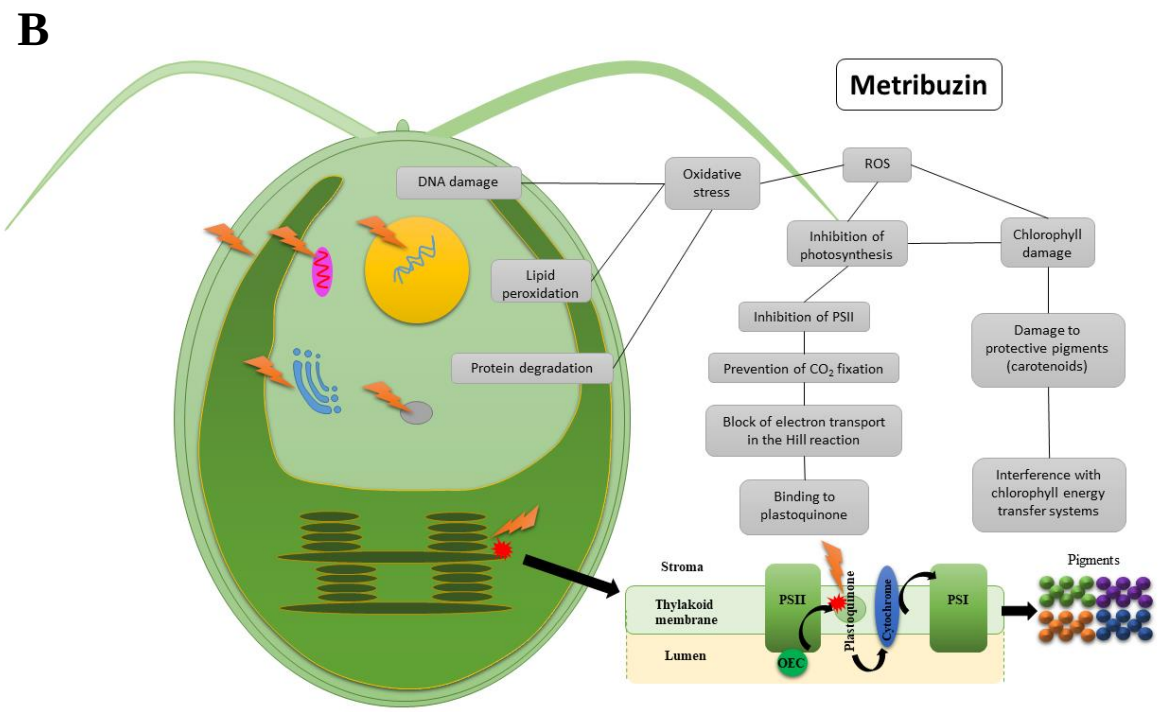


Figure 5 – Toxicity mechanisms of (A) bifenox and (B) metribuzin in *Chlamydomonas reinhardtii*.

Conflict of interest

The authors declare the inexistence of any conflicts of interest.

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