Sensitivity of the green algae *Chlamydomonas reinhardtii* to gamma radiation: photosynthetic performance and ROS formation

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Abstract

The aquatic environment is continuously exposed to ionizing radiation from both natural and anthropogenic sources, making the characterization of ecological and health risks associated with radiation of large importance. Microalgae represent the main source of biomass production in the aquatic ecosystem, thus becoming a highly relevant biological model to assess the impacts of gamma radiation. However, little information is available on the effects of gamma radiation on microalgae species, making environmental radioprotection of this group of species challenging. In this context, the present study aimed to improve the understanding of the effects and toxic mechanisms of gamma radiation in the unicellular green algae *Chlamydomonas reinhardtii* focusing on the activity of the photosynthetic apparatus and ROS formation. Algae cells were exposed for 6 hrs to gamma radiation (0.49-1677 mGy/h) and chlorophyll fluorescence parameters obtained by PAM fluorometry, while two fluorescent probes carboxy-H_2DFFDA and DHR 123 were used for the quantification of ROS. The alterations seen in *C. reinhardtii* PSII functional parameters after 6 hrs of exposure to gamma radiation showed modifications of PSII energy transfer associated with electron transport and energy dissipation pathways, especially at the higher dose rates used. Results also showed that gamma radiation induced ROS in a dose-dependent manner under both light and dark conditions. The observed decrease in photosynthetic efficiency seems to be connected to the formation of ROS and can potentially lead to oxidative stress and cellular damage in chloroplasts. To our knowledge, this is the first report on changes in several chlorophyll fluorescence parameters associated with photosynthetic performance and ROS formation in microalgae after exposure to gamma radiation.

Keywords: Gamma radiation; microalgae; photosynthesis; PAM fluorometry; reactive oxygen species; fluorescent probes.

1. Introduction

Ecosystems are continuously exposed to ionising radiation from natural sources such as cosmic radiation and natural radionuclides found in soil and rocks. However, anthropogenic activities (e.g. nuclear power production and accidents, weapon production and testing, radioactive waste storage and medical use of radionuclides) can lead to increased doses of radiation in the environment (Unscear 2008). Ionizing radiation such as α-particles, β-particles and γ-rays (photons) that are emitted by atomic nuclei as a result of their decaying process, are known to induce adverse effects on organisms including DNA damage, growth reduction, reproduction impairment and morphological alterations (Reisz et al., 2014). Gamma radiation in particular is composed of high-energy penetrative photons with high frequency and short wavelength that can interact with biological matter and cause ionization or excitation of endogenous molecules and instigate various types of damage in a number of organisms. Most of the published literature on the effects of gamma radiation in non-human biota has focused on mammals, invertebrates, fish and plant species, leaving a significant gap of information for species habiting the lower levels of the trophic hierarchy (Dallas et al., 2012; Real et al., 2004; Vanhoudt et al., 2012). As primary producers, microalgae play an important role in the functioning of aquatic ecosystems, producing oxygen and energy and recycling nutrients on which many organisms within the ecosystem rely on. For this reason, the effects of gamma radiation on algae can directly affect the structure and function of this ecosystem resulting in oxygen depletion and decreased primary productivity and thus affect not only the organisms themselves but also impact other organisms in the food chain (Daam et al., 2009; Prado et al., 2011; Rioboo et al., 2007). Based on its important ecological function in freshwater ecosystems, the microalgae *Chlamydomonas reinhardtii* was chosen as model species in this study. *C. reinhardtii* is an unicellular green algae frequently used in ecotoxicity testing due to its rapid growth rate, its ease of use in testing and culturing in controlled laboratory conditions and its sensitivity to several contaminants. Moreover, its genome has been sequenced with several molecular and genomic tools available (Merchant et al., 2007) and the complete biology has been described in detail (Harris, 2009), making this microalgae extremely useful to study adaptive responses at cellular, physiological, biochemical and molecular levels upon exposure to gamma radiation (Mendez-Alvarez et al., 1999). The biological effects of ionizing radiation has not been extensively studied in algae, though decreased growth and survival, gene recombination, increased DNA damage and repair and disruption of the photosynthetic process have been reported in *C. reinhardtii* after acute and chronic exposure to gamma radiation (Boreham and Mitchel, 1993; Chankova and

Bryant, 2002; Chankova et al., 2005, 2007; Davies, 1966; Kohn et al., 1967; Lawrence and Holt, 1970; Posner and Sparrow, 1964; Rea et al., 2008).

Photosynthesis is a complex and highly integrated series of redox and enzymatic processes that regulate the survival of photosynthetic organisms such as microalgae. The light energy absorbed by chlorophyll molecules (e.g. photosystem antenna) can be used in three competitive processes: a) be transferred through the electron transport chain to fixate carbon (photochemical quenching), b) dissipate as heat (non-photochemical quenching) or c) be re-emitted at a slightly longer wavelength as light (chlorophyll fluorescence). Thus, by monitoring chlorophyll *a* fluorescence it is possible to assess the response of the photosynthetic apparatus and correspondent photochemical processes under normal and stressful conditions (Maxwell and Johnson, 2000; Ralph et al., 2007). Some studies have also reported inhibition of chlorophyll *a* and *b* synthesis and loss of photosystem functionality and the activity of the oxygen-evolving apparatus in algae species after exposure to gamma radiation (Kohn et al., 1967; Rea et al., 2008). Despite some data demonstrating effects, the impact of gamma radiation on the photochemical processes involved in photosynthetic activity of *C. reinhardtii* is still limited.

Under normal cellular conditions, reactive oxygen species (ROS) are formed by the inevitable leakage of electrons from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a by-product of various metabolic pathways localized in different cellular compartments. Moreover, exposure to environmental stresses (e.g. drought, salinity, metal toxicity, UV-radiation, etc.) can stimulate the production of ROS due to disruption of cellular homeostasis (Halliwell and Gutteridge, 2007). It is believed that one of the main impacts of gamma radiation is due to the interaction with atoms or molecules in the cell to produce highly reactive species (superoxide radicals, hydroxyl radicals and hydrogen peroxide); either directly by oxidation of water or indirectly by the formation of secondary, partially generated ROS. This over-production of ROS will react rapidly with critical components of the cell such as lipids, proteins and DNA, causing disturbance of cellular mechanisms (Apel and Hirt, 2004; Jan et al., 2012; Reisz et al., 2014). While some studies have been conducted on oxidative stress responses under irradiation conditions, studies on gamma radiation-induced ROS and oxidative stress in algae species are scarce.

The goal of this study was to evaluate the impact of gamma radiation produced by a 60-cobalt irradiation source on the photosynthetic activity of *C. reinhardtii* using PAM fluorometry. Additionally, the intracellular production of ROS in response to gamma radiation was also investigated using two fluorescent probes, the 5-(and-6)-carboxy-2’7’-

difluorodihydrofluorescein diacetate (H_2DFFDA) and the dihydrorhodamine 123 (DHR 123). Cellular ROS levels were determined under light and dark conditions and linked to effects on photosynthetic parameters (maximum quantum efficiency of photosystem II (PSII), efficiency of the oxygen-evolving complex, effective quantum efficiency of PSII, non-photochemical quenching, coefficients of photochemical and non-photochemical quenching and photosynthetic electron transport rate) to provide a better understanding on the effects of gamma radiation in the microalgae *C. reinhardtii*.

2. Material and Methods

2.1. Microalgae cultures

Exposure experiments were performed with the unicellular freshwater algae *C. reinhardtii* (NIVA-CHL153; Norwegian Institute for Water Research, Oslo, Norway), grown in high salt growth medium (HSM; Harris, 2009) with an initial number of 10^7 cells/L. The *C. reinhardtii* cultures were kept for 3 to 4 days in 1 L of HSM media at 20 ± 2ºC, with orbital shaking at 90 rpm and under continuous illumination (93 ± 6 μmol/m^2/s^1) provided by cool–white fluorescence lamps (TLD 36W/950, Philips, London, UK) in an Infors Multitron 2 incubator (Infors AG, Bottmingen, Switzerland) to ensure that cultures were in the exponential growth phase. Immediately before each exposure experiment, algal cells were collected by centrifugation at 7000 rpm for 15 minutes (Avanti J-265 XP Centrifuge, Beckman Coulter, California, USA) for removal of growth medium. The HSM medium was decanted and cells washed with 50 mL of deficient HSM medium (medium lacking EDTA and trace metals) and centrifuged again at 7000 rpm for 10 minutes. Washed cells were re-suspended in 10 mL of deficient HSM and centrifuged at 3000 rpm for 10 minutes and the resulting pellet was re-suspended in 2 mL of deficient HSM. Cell number was counted with a multisizer counter (Beckman Coulter Counter Multisizer 3, Miami, USA) and adjusted to an initial cell density of 18 × 10^7 cells/mL in 6 mL of deficient HSM. A deficient HSM was chosen as the exposure media to avoid interaction of the fluorescent probes used to assess ROS formation with the metal components present in the HSM media used for algae culturing. All glass material used for media preparation and experiments was appropriately washed and autoclaved prior to use to avoid microbial contamination. Culture samples were also regularly observed under the microscope to detect microbial contamination.

2.3. Gamma radiation exposure

Gamma radiation exposures were conducted at the FIGARO experimental facility (Norderås, Norway) at the Norwegian University of Life Sciences (NMBU, Ås, Norway). *C. reinhardtii* was exposed for a total of 6 hours to external ^{60}Co gamma radiation in the front row of 96-well microplates (FalconTM, Oslo, Norway), after which Photosystem II (PSII) efficiency and ROS formation were determined. Exposure was conducted for a short period to avoid fluorescence artefacts arising due to substantial microalgal growth. A total of thirteen gamma doses were tested, divided in two exposure experiments: the first experiment applied dose rates to water from 235 to 1677 mGy/h and the second applied dose rates to water from 0.49 to 114 mGy/h. Each experiment had a corresponding control and was run three times with four replicates per dose rate. For both exposure experiments, microplates were positioned at distances away from the gamma source corresponding to the air kerma rates and dose rates to water ($D_{(water)}$) presented in Table I.

Field dosimetry (air kerma rates measured with an ionization chamber) was traceable to the Norwegian Secondary Standard Dosimetry Laboratory (Norwegian Radiation Protection Authority, NRPA, Oslo, Norway) (Bjerke and Hetland, 2014). Dose rates to water in the center of microplate wells (front row) were estimated according to Bjerke and Hetland (2014) and used as a proxy for dose rates to the microalgae. Actual air kerma rates were measured using an Optically Stimulated Luminescence (OSL) based dosimetry system from Landauer, i.e., nanoDots dosimeters and InLight microSTAR reader, as follows. For the highest dose rate (D_{water}=1677 mGy/h), nanoDots with 5 mm polypropylene build up caps were exposed in the same position as the front row of the 96-well microplates used for algae exposure. For the second experiment, nanoDots positioned at the front of the microplates were exposed without buildup caps for the dose rates tested, applying a conversion factor for calculation of air kerma dose rates when not using buildup caps (Hansen and Hetland, 2015). Total doses were calculated from measured dose rates (mGy/h), multiplied by total exposure time (h) (see Table I for more information on total doses).

Table I – Dose rates and total doses used in the gamma radiation exposures with *Chlamydomonas reinhardtii*.

Dose rate D_{water} (mGy/h)*	**Total dose D_{water} (mGy)**	**Air kerma rate (mGy/h)**
0.49	2.9	0.4 ± 0.03**
1.2	7.1	1.1 ± 0.7**
4.5	27.0	3.7 ± 0.2**
11.2	67.3	9.9 ± 0.5**
45.4	273	36.9 ± 1.2**

114	686	103.3 ± 5.2**
235	1408	237.4 ± 14.6**
663	3978	554***
788	4728	659***
943	5658	788***
1134	6804	947***
1374	8244	1147***
1677	10062	1448****

*Uncertainty (K=2) for dose rate estimates is ~10% (Bjerke and Hetland, 2014).

**Measured with nanoDots. Uncertainty based on standard deviation of 3 nanoDots.

***Estimated based on a combination of nanoDots measurements and field dosimetry, including attenuation of intensities according to Bjerke and Hetland (2014). Uncertainty (K=2) for dose rate estimates in ~10%.

****Uncertainties (K=2) for nanoDot measurements are ~5%.

2.3. Photosystem II efficiency

PSII efficiency of *C. reinhardtii* exposed to gamma radiation was determined following Herlory et al. (2013) and Juneau et al. (2002) and adapted to the experimental conditions used in this study. Algae cells were exposed to ^{60}Co radiation at a final concentration of $3x10^5$ cells in 200 µl of deficient HSM under ambient light and at room temperature (20°C). Chlorophyll *a* fluorescence was determined with a PAM fluorometer (PAM-2000, Heinz Walz GmbH, Effeltrich, Germany) and fluorescence parameters calculated according to the formulas expressed in Table II. Upon exposure, *C. reinhardtii* cells were dark adapted for 30 min to allow complete oxidation of PSII reaction centres and the minimum and maximum fluorescent yields of PSII in the dark adapted state (F_0 and F_m, respectively) were determined for each dose rate and corresponding control. Both yields were used to calculate the maximum quantum yield (F_v/F_m) and the efficiency of oxygen-evolving complex (OEC) of PSII. Subsequently, dark-acclimated cells were illuminated by an actinic light at intensity equivalent to the incubation light (~100 µmol/m^2/s^1) and the current fluorescence yield Ft, the minimum fluorescence yield in the light F'_0 and the maximum fluorescence yield in the light (F'_m) values recorded. With the light adapted yields, the effective quantum yield (Φ_{PSII}), the coefficients of photochemical (qP) and non-photochemical quenching (qN), non-photochemical quenching (NPQ), relative photosynthetic electron transport rate (ETR) and relative photochemical ($qP_{(rel)}$) and non-photochemical quenching $qN_{(rel)}$ were calculated. 0.01–300 µM atrazine (CAS number: 1912- 24-9, purity ≥97%) and 0.001–30 µM diuron ([3-(3-4-dichlorophenyl)-1,1-dimethylurea (DCMU)], CAS number: 330-54-1, purity ≥98%) obtained from Sigma-Aldrich (United Kingdom) were dissolved in dimethylsulfoxide (Sigma-Aldrich, UK, purity ≥99%) and used as positive controls in the assay.

Table II – Fluorescence parameters calculated from PAM fluorometry measurements in *Chlamydomonas reinhardtii* exposed to gamma radiation.

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 Bjorkman, 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

*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).

2.4. ROS assay

ROS formation was detected using the probes carboxy-2',7'-difluorodihydrofluorescein diacetate (H_2DFFDA, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) and dihydrorhodamine 123 (DHR 123, Invitrogen, Molecular Probes Inc., Eugene, OR, USA), both being stored as 50 mM stock solution in DMSO at -20°C before use. H_2DFFDA is a non-polar, non-fluorescent probe that enters the cells freely, which presents an improved photostability compared to the chlorinated fluorescein derivatives commonly used (Szivák et al., 2009). Once inside the cell, H_2DFFDA is hydrolysed by cellular esterases to non-fluorescent carboxy-2,7-difluorodihydrofluorescein (H_2DFF), which is retained in the cell. In the presence of a variety of ROS, H_2DFF is converted by oxidation to carboxy-2,7-difluorofluorescein (DFF), a highly fluorescent final product localized in the cytosol (Nestler et al., 2012; Winterbourn, 2014). DHR 123 is the non-fluorescent dihydroderivative of rhodamine 123, a fluorescent, lipophilic and positively charged compound used as a

mitochondria-specific dye. After cellular uptake, DHR 123 is oxidized back to rhodamine 123 after reacting with an oxidant, which moves (after oxidation) to the mitochondria, where it is sequestered. Both H_2DFFDA and DHR 123 have similar structural features and are oxidised by similar oxidative/radical mechanisms, where their final fluorescent products can be quantified by relative fluorescence (Negre-Salvayre et al., 2002; Winterbourn, 2014). ROS production in *C. reinhardtii* exposed to gamma radiation was determined as described by Jamers et al. (2009), Stoiber et al. (2011) and Szivák et al. (2009), and adapted to the experimental conditions used in this study. Briefly, for each dose rate, a final concentration of $3x10^5$ algal cells in 100 μL of deficient HSM was added to 100 μL of deficient HSM with either H_2DFFDA or DHR123 probes (5 μM final concentration). Algal cells were exposed under ambient light for H_2DFFDA probe and total darkness for DHR123 at room temperature and the fluorescence recorded hourly on a microplate fluorescent reader (Fluoroskan Ascent 2.5, ThermoFisher Scientific, USA) with excitation/emission of 485/538 nm. Natural fluorescence of irradiated deficient HSM in combination with the probes (without presence of algae) for each dose rate was also analysed and the resulting fluorescence subtracted to eliminate interference of non-algal ROS production. The relative fluorescence obtained for both probes at each dose rate was expressed as fold induction comparative to the control. 0.1–3000 μM hydrogen peroxide (H_2O_2, CAS number: 7722-84-1, purity ≥30%) and 0.01–1000 μM paraquat dichloride hydrate (CAS number: 75365-73-0, purity ≥30%) obtained from Sigma-Aldrich (United Kingdom) were used as positive controls in the assay.

2.5. Statistical analysis

Statistical analyses were performed using XLStat2016® (Addinsoft, Paris, France) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Results are presented as mean ± standard error. No significant differences were found between controls for both exposure experiments, so data for all dose rates was combined to assess statistical differences. Significant differences in PSII efficiency parameters and ROS formation between dose rates were calculated using one-way analysis of variance (ANOVA) or Kruskal–Wallis One Way Analysis of Variance on Ranks. If significant, pairwise multiple comparison procedures were conducted, using the Tukey test or the Dunn's method. Statistical significance was set at $p<0.05$.

3. Results

3.1. Photosynthetic performance

Atrazine and diuron were used as positive controls to evaluate the photosynthetic performance of *C. reinhardtii* using PAM fluorometry. Results obtained for both compounds (Supplementary Figures A1 and A2) showed significant impact on several chlorophyll fluorescence parameters, especially Φ_{PSII} and ETR. The impact of gamma radiation on the photosynthetic activity of *C. reinhardtii* after 6 hrs of exposure is shown in Figures 1, 2 and 3 for dark- and light-acclimated states.

3.1.1. Dark-acclimated fluorescence parameters

In the dark-acclimated state, no significant effects were observed for the minimum (F_0) and maximum (F_m) fluorescence signals from 0.49 to 114 mGy/h ($p>0.05$). At higher dose rates, both fluorescence yields were significantly higher than the control ($p<0.05$). Both F_0 and F_m increased 1.1-, 1.2- and 1.3-fold after exposure to 235, 663 and 788 mGy/h ($p<0.05$), while at higher dose rates the values remained stable ($p>0.05$). No effect of gamma radiation was observed on the maximum quantum efficiency of PSII (F_v/F_m) after 6 hrs of exposure, whatever the dose rate used (Figure 1 E, F). The efficiency of the oxygen-evolving complex (OEC) only decreased compared to the control value for 0.49, 1.2, 11.2 and 45.4 mGy/h, even though to a lower extent ($p<0.05$). No effects were detected at higher dose rates ($p>0.05$).

3.1.2. Light-acclimated fluorescence parameters

Light-acclimated parameters were only determined at dose rates higher than 235 mGy/h, since no significant differences were found for dark-acclimated parameters at lower dose rates (Figure 1 A, C, E). In the light-acclimated state, algae cells showed a decrease in the quantum efficiency of PSII photochemistry (Φ_{PSII}) with increasing dose rate (Figure 2A). Φ_{PSII} decreased from 1.1-fold of the control at 943 mGy/h to 1.4-fold of the control at 1677 mGy/h ($p<0.05$), suggesting the activation of non-photochemical processes. In fact, non-photochemical quenching (NPQ) increased with increasing dose rate, up to 1.7-fold higher than the control at the highest dose (1677 mGy/h). This increase in NPQ appeared to be accompanied by an increase in the coefficient of non-photochemical quenching (qN), even though at a lower extent (Figure 2C). No significant differences were found for the photochemical quenching coefficient (Figure 2D) between control and irradiated algae cells for any of the dose rates applied ($p>0.05$). Exposure to gamma radiation also altered the photosynthetic electron transport process of algae cells (Figure 2E), as expressed by the decrease in the electron transport rate (ETR). The ETR decreased 1.1-, 1.2-, 1.3- and 1.4-fold

compared to control after exposure to 943, 1134, 1374, 1677 mGy/h, respectively (p<0.05), while at lower dose rates no significant differences were found between the groups (p>0.05).

The relative distribution of the energy dissipation processes through the PSII of control and irradiated *C. reinhardtii* is shown in Figure 3. Algae cells exposed to gamma radiation showed a decrease in the relative photochemical quenching $qP_{(rel)}$ with increasing dose rate, up to 2–fold lower than the control at the highest dose rate tested (1677 mGy/h, p<0.05). On the other hand, a significant increase was detected for the relative non-photochemical quenching $qN_{(rel)}$ in irradiated algae cells from 788 to 1677 mGy/h (1.1- to 1.2- fold, p<0.05).

3.2. ROS formation

H_2O_2 and paraquat were used to evaluate the performance of the ROS formation bioassay in *C. reinhardtii* using the two fluorescent probes H_2DFFDA and DHR 123. The results obtained for both compounds showed a significant concentration and time-dependent increase in ROS formation after incubation with both probes (Supplementary Figures A3 and A4).

The exposure of *C. reinhardtii* cells to gamma radiation resulted in a significant production of ROS over time for both the H_2DFFDA and DHR 123 probes (Supplementary Figure A5), where 6 hrs exposure provided the best response, as seen in previous experiments (Almeida, 2015). ROS levels produced by gamma radiation after 6 hrs of exposure are presented as fold induction compared to the control in both light and dark conditions after incubation with H_2DFFDA and DHR 123 probes, respectively (Figure 4). In algae cells incubated with H_2DFFDA, a significant increase in ROS was observed at 4.5 mGy/h (1.3-fold, p<0.05), followed by a decrease to control levels at 11.2 mGy/h (p>0.05) and a dose dependent increase at higher doses, reaching a 2.8-fold increase at highest dose (1677 mGy/h, p<0.05). The pattern of ROS formation obtained after incubation with DHR 123 probe was different from that observed for H_2DFFDA, with higher ROS levels being formed at the highest doses used. The production of ROS exhibited a dose response with increasing dose rates from 4.5 mGy/h up to 663 mGy/h (1.1- to 7.6- fold, p<0.05). At higher dose rates, a steady state was reached (p>0.05) showing a maximum 8.2-fold induction at the highest dose (p<0.05).

4. Discussion

Microalgae represent important ecotoxicological models since they constitute the main source of biomass production and thus support the structure and function of the whole aquatic ecosystem. In this way, negative effects of ionizing radiation in algae species will likely have an impact not only on the algae itself, but potentially also impact other levels of the aquatic

food chain. Only a very limited number of studies have addressed the effects of gamma radiation on microalgae species, making this group of species one of the least studied in terms of environmental radioprotection. In this context, this study aimed to improve the understanding of the effects and toxic mechanisms of gamma radiation in the green algae *C. reinhardtii* by assessing interference with photosynthesis and the formation of ROS.

To date, the effects of ionizing radiation on the photosynthesis in algae are still poorly explored, and it remains unclear to which level algae are adversely impacted and which toxic mechanism or mode of action (MoA) is the most relevant. Currently, the development of pulse amplitude modulated (PAM) fluorometry to measure chlorophyll *a* fluorescence has been shown to be a simple, rapid and sensitive tool for testing of contaminants impact on photosynthetic activity (photosystem II) in *C. reinhardtii* (e.g. Juneau et al., 2001, 2002; Juneau and Popovic, 1999). The known PSII inhibitors atrazine and diuron were used as positive controls to evaluate the photosynthetic performance of *C. reinhardtii* using PAM fluorometry, whose results are in accordance with other studies with the same compounds in cultures of *C. reinhardtii* (e.g. Almeida, 2015; Nestler et al., 2012). Overall, the chlorophyll fluorescence parameters obtained by PAM fluorometry showed relevant effects of gamma radiation on *C. reinhardtii* photosynthesis, even though the total doses used were not sufficiently high to show complete inhibition of algae photosynthetic activity. Maximum quantum efficiency F_v/F_m, a measure of the maximal PSII photochemical efficiency after a period of dark-adaption, has been reported to be sensitive towards different environmental stressors and widely used to reflect impairment of PSII (Ralph et al., 2007). In this study, the F_v/F_m measured in *C. reinhardtii* remained unaffected after exposure to gamma radiation. This result indicates that the light harvesting capacity of PSII was intact during the range of dose rates used in this study, with more than 70% of the light absorbed by the photosystem II being used for photochemistry. This is in accordance with a previous study where a slight decrease in Fv/Fm was observed after exposure to a total dose of 8.4 Gy (4.2 Gy/h, 2 h exposure), while no alterations were seen at 3.1 Gy (Rea et al., 2008). F_v/F_m also remained unaltered in several plant species (e.g. *Arabidopsis thaliana*) after exposure to ^{137}Cs and ^{60}Co radiation (Kim et al., 2005; Vanhoudt et al., 2014), thus verifying that the photosynthetic capacity of PSII is not susceptible to perturbations by gamma radiation in these species.

The efficiency of the oxygen evolving complex (OEC) reflects the state of the water photo-oxidation process in photosynthetic organisms and is one of the most sensitive components of the photosynthetic electron transport chain on the oxidizing site of PSII (Kriedemann et al., 1985). For *C. reinhardtii* exposed to gamma radiation, no major changes were detected in the

OEC, suggesting that gamma radiation did not impact the water-splitting apparatus at the dose rates used. A study by Rea et al. (2008) showed a significant but small increase in the oxygen evolution of *C. reinhardtii* exposed to a total gamma doses of 8.4 Gy, as well as a significant reduction of OEC associated proteins at doses higher than 10 Gy. However, the direct effect of gamma radiation in the OEC of photosynthetic organisms is still not well documented.

Contrary to what was seen for Fv/Fm, a significant decrease was observed for the quantum efficiency of PSII (Φ_{PSII}) in light-exposed algae with increasing dose rates. Φ_{PSII} expresses the proportion of light absorbed by chlorophyll in PSII that is used for photochemistry at steady state of PSI-PSII electron transport (Genty et al., 1989; Juneau et al., 2002). A decreased Φ_{PSII} is expected to cause alterations within PSII photochemistry and electron transport associated with energy dissipation via regulated and non-regulated non-photochemical pathways (Juneau et al., 2001). In fact, a significant increase of NPQ with increasing dose rates was observed in this study indicating that non-photochemical processes were activated in gamma-exposed algae to dissipate excess light energy. Concomitantly, this decrease in Φ_{PSII} was also accompanied by an increase in qN with increasing dose rates, thus strengthening the idea that the quenching of PSII fluorescence by gamma radiation was not related to interference with the photochemical processes in *C. reinhardtii*. Algae and plants have evolved NPQ mechanisms to dissipate excess light absorbed energy as heat in order to protect the photosynthetic apparatus from oxidative damage (Niyogi, 1999). On the other hand, no effect was detected in the photochemical quenching parameter qP in all dose rates tested. Several studies have shown the insensitivity of this parameter to some environmental factors, thus rendering it not always appropriate to assess the overall photochemical activity of photosynthetic organisms under stressful conditions (Buschmann, 1999; Juneau et al., 2005). Even though this parameter measures approximately the fraction of open PSII reaction centres, it does not take into account their efficiency (Genty et al., 1989). A non-complementarity has also been shown between qP and qN due to the fact that both parameters do not refer to the same state of energy storage and dissipation *via* the photosynthetic apparatus (Buschmann, 1999). For this reason, the relative photochemical and non-photochemical quenching coefficients ($qP_{(rel)}$ and $qN_{(rel)}$, respectively) were proposed by Buschmann (1999) to complement the information given by qP and qN. Both $qP_{(rel)}$ and $qN_{(rel)}$ are normalized to the same reference signal, thus expressing the relative amount of photochemical and non-photochemical quenching as a fraction of the total quenching when going from a dark-adapted to a light-adapted state (Juneau et al., 2005). The dissipation energy pathways assessed through quenching analysis showed that in gamma-irradiated algae,

light energy was mainly dissipated by non-photochemical quenching, as illustrated by the increase in $qN_{(rel)}$, and not used in photochemistry as seen by the reduction in $qP_{(rel)}$ with increasing dose rates. So, the lack of alterations in qP seen in exposed algae showed that there was no change in the fraction of open PSII reaction centres, however, the efficiency of these open centres lowered with increasing dose rates, as indicated by the reduction in $qP_{(rel)}$. Therefore, when gamma irradiation inhibited the Φ_{PSII} of algae cells, the regulation of energy dissipation processes was due to an increase in non-photochemical energy dissipation processes, as illustrated by the increase in qN, NPQ and $qN_{(rel)}$.

The concomitant decrease in photochemical efficiency and increase in non-photochemical quenching seen in *C. reinhardtii* exposed to gamma radiation indicate the impairment of photosynthetic processes and seem to be associated with alterations in the electron transport process (Genty et al., 1989), as confirmed by the reduction in ETR with increasing dose rate. The relative ETR provides an empirical estimate of the flow rate of electrons through the photosynthetic chain, and the decrease seen in this study indicates that gamma radiation directly influences electron transport in algae. However, this effect in electron transport does not seem to be associated with the PSII donor site, as no major changes were detected in the OEC, but probably with other sites of the PSII-PSI electron transfer chain (e.g. electron acceptor side or PSI protein complex).

No studies exist on the effects of gamma radiation on these photosynthetic processes in algae species but when comparing these results with those obtained for plants, gamma radiation has a dissimilar MoA with NPQ being generally inhibited in plants when exposed to gamma radiation generated by ^{137}Cs and ^{60}Co (Kim et al., 2005, 2010; Moon et al., 2008; Vanhoudt et al., 2014). Alterations in NPQ can be connected to alterations in pigments, which for gamma radiation exposure have been partly linked to altered xanthophyll cycle activities in plant species (Moon et al., 2008). Only Kohn et al. (1967) addressed the impacts of gamma radiation in *C. reinhardtii* pigment synthesis, suggesting significant damage of a synthetic unit responsible for one or more steps in chlorophyll synthesis leading to inhibition of both chlorophyll *a* and *b*, but no connection was made to alterations in the photosynthetic activity of exposed algae. Studies following uranium and metal (Hg and Cu) exposure have shown similar impairment of the photochemical process in algae, and has been associated with an obstruction of electron transfer between photosystems as a consequence of the PSII reaction centres being in a reduced state due to limited re-oxidation of plastoquinone QA (Juneau et al., 2001, 2002, Herlory et al., 2013). In algae exposed to herbicides like atrazine and diuron (the positive controls used in this study), the blocking of the electron transport from the

primary to secondary plastoquinone (QA to QB) is normally associated with significant increases in the Fo and Fm signals (Kumar and Han, 2010), as also observed for *C. reinhardtii* exposed to gamma radiation in this study. However, it is still not clear by which mechanisms gamma radiation impairs the photosynthetic process in *C. reinhardtii*.

As reported earlier, Φ_{PSII}, qN, and NPQ are the most sensitive indicators of alterations in the photosynthetic performance of algae and highly dependent on the mechanism of action of the stressor/contaminant (Juneau and Popovic 1999; Juneau et al., 2002; Herlory et al., 2013; Ralph et al., 2007). The results obtained for algae exposed to gamma radiation showed that the Φ_{PSII} was more affected than F_v/F_m, a commonly observed phenomenon in algae (Ralph et al., 2007). In fact, the Φ_{PSII} is influenced by the stoichiometry of PSII:PSI, Calvin cycle activation and the activity of the xanthophyll cycle, being the most sensitive indicators of toxic impacts in the photosystem. On the other hand, F_v/F_m measures optimal photosynthetic activity and does not reflect the true nature of the PSII activity under normal light conditions, which can be of large importance when taking into consideration that some contaminants have increased impact in the dark (Juneau et al., 2007; Ralph et al., 2007). Similarly to Φ_{PSII}, changes in NPQ and qN were more responsive and sensitive than changes in F_v/F_m, as these parameters integrate all toxic effects on photosynthetic electron transport and its related processes, while F_v/F_m only indicates PSII charge separation and is not influenced by forward electron transport in PSII (Juneau et al., 2001, 2002). A non-complementarity between qP and qN was also detected in algae exposed to gamma radiation, similarly to responses seen for other environmental stressors such as temperature, low irradiance or the presence of contaminants as metals and pesticides (Juneau et al., 2005). Additionally, the use of the relative parameters $qP_{(rel)}$ and $qN_{(rel)}$ represented more adequately the proportion of the different quenching processes, with the photochemical part of the fluorescence quenching ($qP_{(rel)}$) being more affected than one would expect based on the evaluation of qP. $qP_{(rel)}$ and $qN_{(rel)}$ used in complement to the information given by qP and qN gave a better evaluation of the alterations in the photosynthetic process and energy dissipation processes in *C. reinhardtii* in response to gamma radiation.

In microalgae, ROS are mostly generated in the reaction centres of PSI and PSII, and in the chloroplast thylakoids as by-products of photosynthetic processes (Asada, 2006; Knauert and Knauer, 2008). The use of fast and direct bioassays using oxidation-sensitive fluorescent probes to monitor intracellular ROS formation has been widely used in algae species and proven effective in showing general oxidative stress after exposure to several contaminants (e.g. Nestler et al., 2012; Szivák et al., 2009). However, a limitation in ROS detection using

this type of probes is the necessity of low light conditions, as most of the commercialized probes are light sensitive (Szivák et al., 2009). Accordingly, a fluorinated derivative of fluorescein with improved photostability H_2DFFDA was used to assess intracellular ROS production in *C. reinhardtii* under normal light conditions. In parallel, DHR123, a light sensitive probe was also applied to investigate ROS formation upon gamma radiation exposure under dark conditions. H_2O_2 and paraquat were used to evaluate the performance of the ROS formation bioassay in *C. reinhardtii* using both fluorescent probes. The results obtained for both compounds showed a significant concentration and time-dependent increase in ROS formation after incubation with both probes. These results are consistent with the known toxic mechanism of H_2O_2 and paraquat (e.g. Jamers et al., 2009; Jamers and De Coen, 2010; Nestler et al., 2012; Prado et al., 2012) and showed the suitability of using both compounds as positive controls to study the intracellular generation of ROS in algae species.

As expected, results obtained for H_2DFFDA probe showed that gamma radiation caused an increase in ROS production with increasing dose rate in *C. reinhardtii* cells. The direct and indirect formation of ROS by gamma radiation can damage or modify essential proteins and lipid components present in the thylakoid membrane of chloroplasts, leading to alterations in the photosynthetic apparatus (Jan et al., 2012). These alterations can originate a cascade of reactions that leads to the disruption of the photosynthetic electron chain and consequently to the formation of additional ROS and oxidative stress (Stoiber et al., 2011). This is in accordance with the results obtained from the chlorophyll fluorescence parameters, which showed alterations of the photosynthetic process and consequently disturbance in the electron transport chain in response to gamma radiation. NPQ, in particular, is one of the regulatory mechanisms present in the photosystem to restrict the production of ROS (Roach and Krieger-Liszkay, 2014). In fact, NPQ increased in algae with increasing dose rates, highlighting the connection between the production of ROS and alterations in the photosynthetic apparatus of exposed algae.

In dark conditions, an increase in ROS production with increasing dose rate was also detected with the DHR 123 fluorescence probe, even though a plateau was reached at higher dose rates. These results suggest that the mechanism of ROS production by gamma radiation is independent of photosynthesis activity and light, as should be expected. Other mechanisms (not entirely dependent of light) can also be associated with the production ROS by gamma radiation, as for example the activation of other endogenous ROS-producing systems (e.g. mitochondrial electron transport chain) or the modulation and depletion of antioxidant capacity of cells (Jan et al., 2012; Reisz et al., 2014; Riley, 1994). Gamma radiation has been

linked to perturbations in the mitochondrial metabolism in the form of mitochondria-dependent ROS formation, increased mitochondria membrane potential and promoted respiration and ATP production (Reisz et al., 2014), processes that are not dependent of light and can lead to further propagation of ROS. Organisms, including microalgae, have developed several antioxidant defence mechanisms to counteract the production of ROS, which include ROS-scavenging enzymes such as catalase, ascorbate peroxidase, superoxide dismustase, glutathione-S-transferase, as well as non-enzymatic antioxidants as metallothionein, carotenoids as β-carotene, glutathione, etc. (Apel and Hirt, 2004; Halliwell and Gutteridge, 2007). However, when the formation of ROS exceeds the capacity of the antioxidant defence system, elevated levels of ROS can give rise to oxidative stress and consequently oxidative damage. It has already been shown that both cumulative and acute gamma radiation can disrupt the cellular redox balance through oxidation and inactivation of detoxifying enzymes, low GSH/glutathione disulphide ration and a decrease in the levels of low molecular weight antioxidants, subsequently leading to significant oxidative modifications (e.g. protein carbonylation, DNA damage and lipid peroxidation) (Jan et al., 2012; Reisz et al., 2014; Riley, 1994). Nonetheless, the underlying mechanisms of oxidative regulation and/or action in algae species are still unknown and need further study. In the case of plants, gamma radiation-induced oxidative stress has been shown in the form of enzymatic inactivation/activation, enhanced glutathione levels, lipid peroxidation and alterations in pigment synthesis (see review by Jan et al., 2012). However, these modifications were species-, dose- and duration-dependent in plants and a more thorough assessment is required in order to unravel how gamma radiation affects the antioxidant processes in algae.

5. Conclusions

The results obtained in this study demonstrated that short-term exposure to gamma radiation leads to an inhibition of photosynthetic performance and ROS formation in the algae *C. reinhardtii*. Gamma radiation affected the PSII photochemistry in algal photosynthesis at higher dose rates, with light energy mostly dissipated through non-photochemical processes potentially as countermeasures to protect the photosystem from ROS formation and oxidative damage. Modifications of PSII energy transfer associated with electron transport were also detected at higher dose rates, likely associated with ROS production by gamma radiation. The dose-dependent ROS production in *C. reinhardtii* exposed to gamma radiation seems to be influenced not only by photosynthesis but also by other non-light dependent physiological processes within algal cells, as alterations in the antioxidant defence system and perturbations

in the mitochondrial metabolism. Our findings provided the first insight into the mechanisms involved in gamma radiation toxicity on *C. reinhardtii* photosynthetic activity and ROS formation. Future studies should be directed towards a full characterization of the molecular mechanisms of gamma radiation induced damage to PSII and of the involvement of ROS in the oxidative damage to PSII reaction centres. Moreover, attention should also be paid to the gamma radiation ROS-induced mechanisms in algae cells, focusing on sites of production, propagation mechanisms and effects on the antioxidant defence system and consequent oxidative damage. Additional studies with longer exposure duration should also be conducted for a better assessment on how gamma radiation affects algae growth and propagation of adverse effects to the population level.

Conflict of interest

The authors declare the inexistence of any conflict of interest.

Acknowledgements

This work was supported by the Research Council of Norway through its Centres of Excellence funding scheme, project number 223268/F50.

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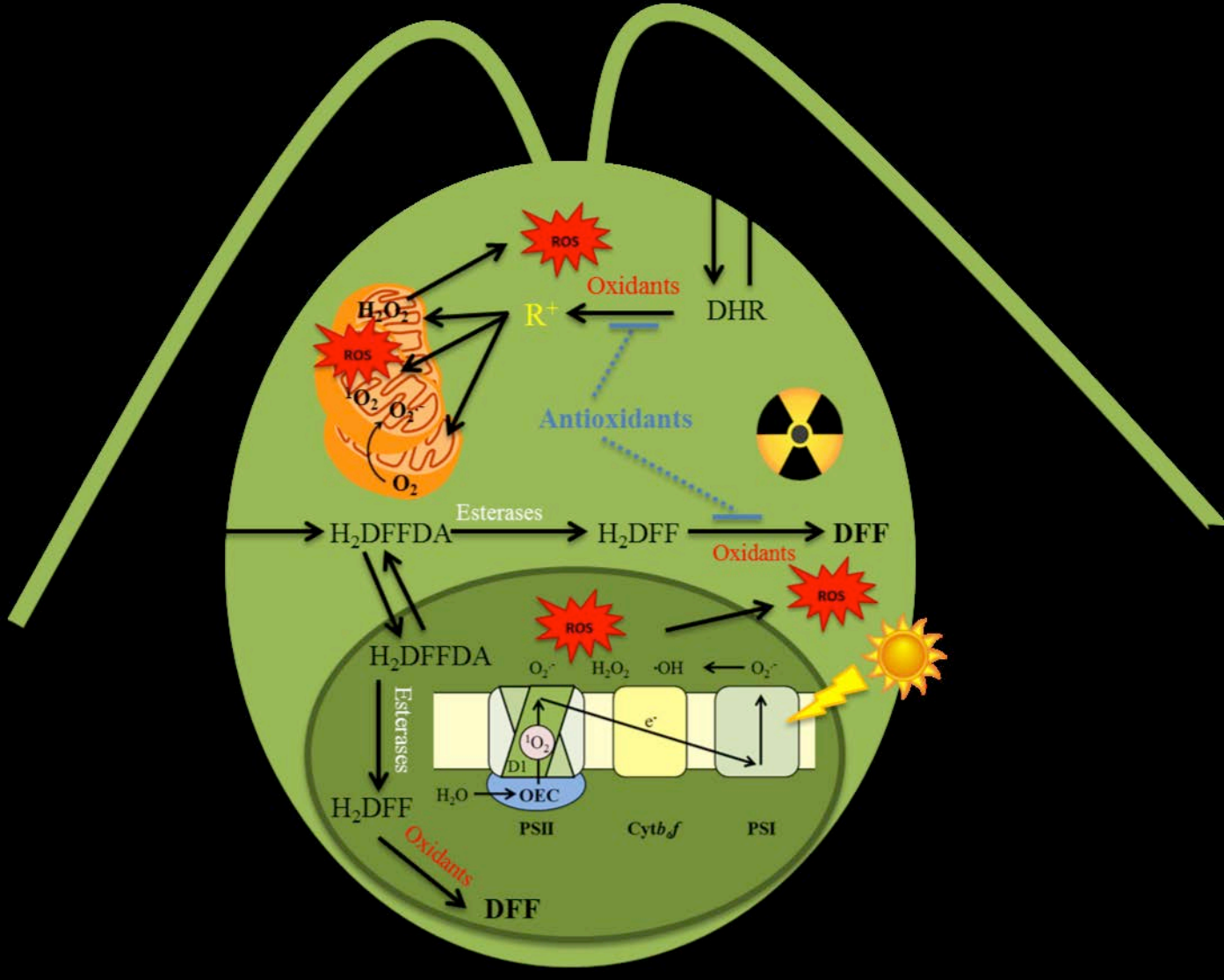
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Oxidants
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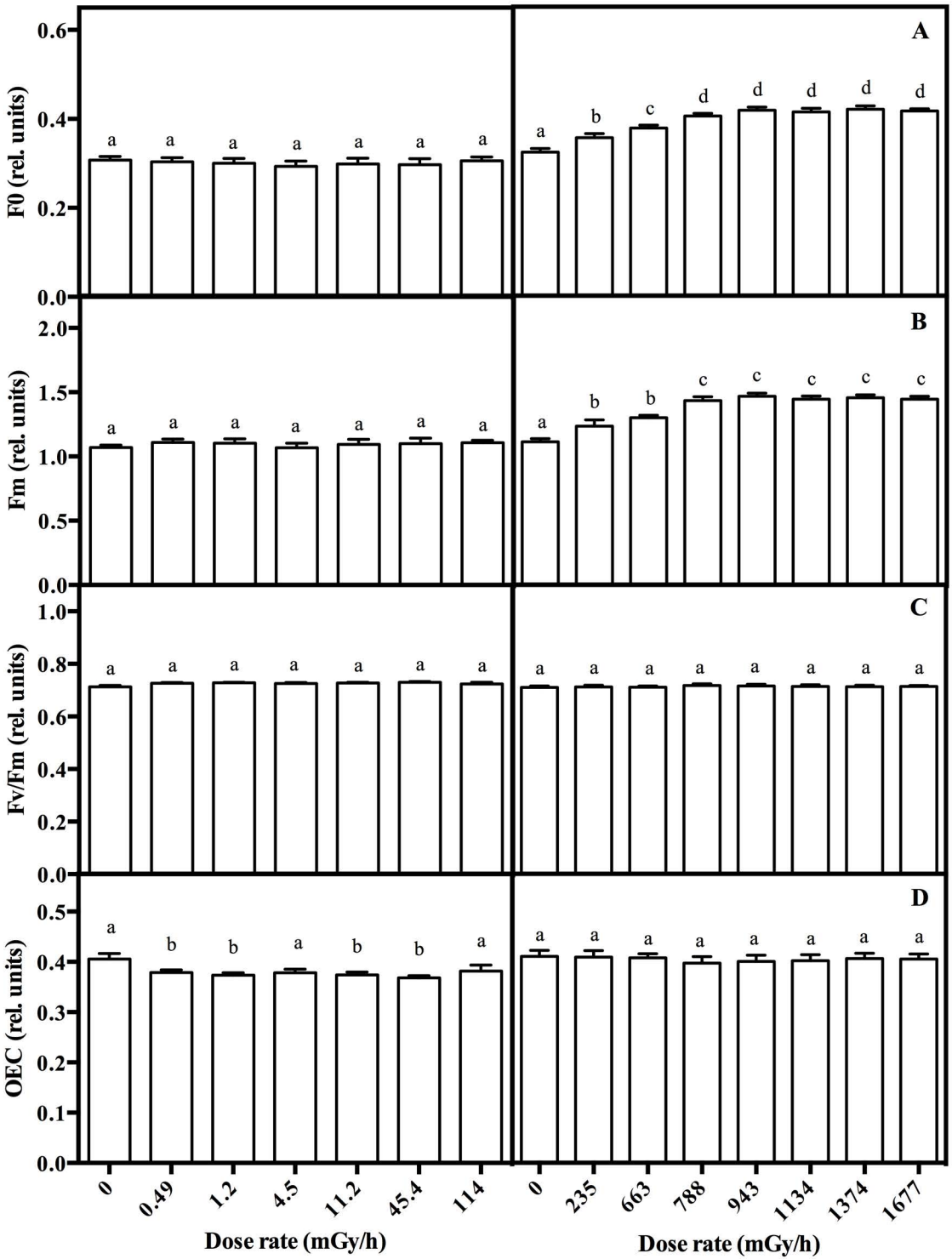
Figure captions:

Figure 1 –Variation in fluorescence parameters of *Chlamydomonas reinhardtii* in the dark-acclimated state exposed to gamma radiation for 6 hrs. A) F_0 – Minimum fluorescent yield, B) F_m – Maximum fluorescent yield, C) F_v/F_m – Maximum quantum yield, D) OEC – Efficiency of oxygen-evolving complex. The experimental results (Mean±SEM) represent 3 independent studies. Letters represent statistical differences between dose rates ($p < 0.05$).

Figure 2 – A) Quantum efficiency of PSII photochemistry (Φ_{PSII}), B) non-photochemical quenching (NPQ), C) coefficient of non-photochemical quenching (qN), D) coefficient of photochemical quenching (qP) and E) electron transfer rate (ETR) of PSII in *Chlamydomonas reinhardtii* exposed to high gamma radiation for 6 hrs. The experiment results (Mean± SEM) represented 3 independent studies. Letters represent statistical differences between dose rates ($p < 0.05$).

Figure 3 – Relative distribution of dissipation energy processes through the PSII of *Chlamydomonas reinhardtii* exposed to high gamma radiation for 6 hrs. A) Relative photochemical quenching ($qP_{(rel)}$), B) Relative non-photochemical quenching ($qN_{(rel)}$). The experiment results (Mean±SEM) represented 3 independent studies. Letters represent statistical differences between dose rates ($p < 0.05$).

Figure 4 – Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to gamma radiation for 6 hrs and measured in fluorescence H_2DFFDA (in the light) and DHR 123 (in the dark). The experimental results (Mean±SEM) represent 3 independent studies. Letters represent statistical differences between dose rates ($p < 0.05$).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F0 (rel. units)
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OEC (rel. units)
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0.5
1.0
1.5
2.0
0.8
0.1
0.3
a
b
c
d
0
0.49
1.2
4.5
11.2
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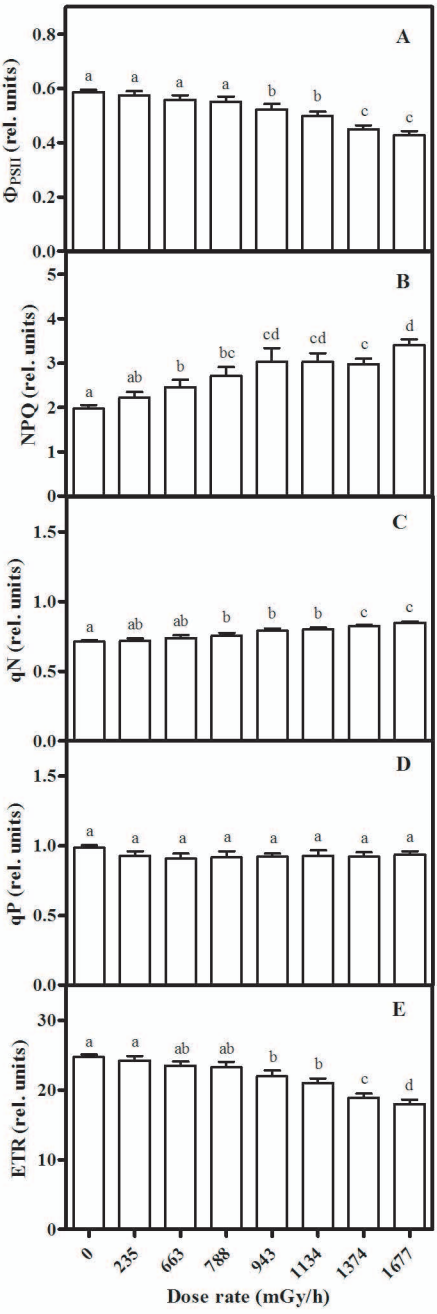
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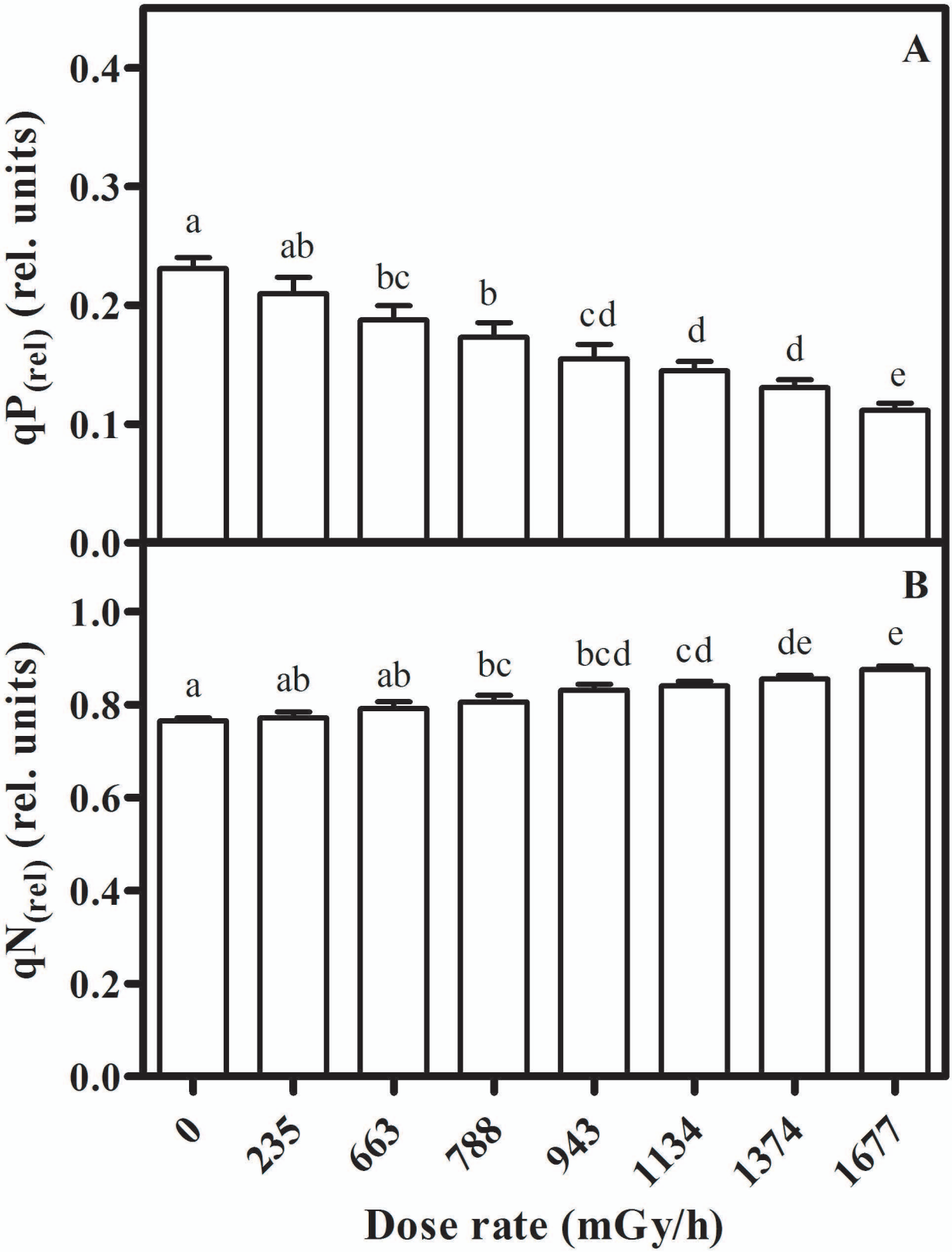
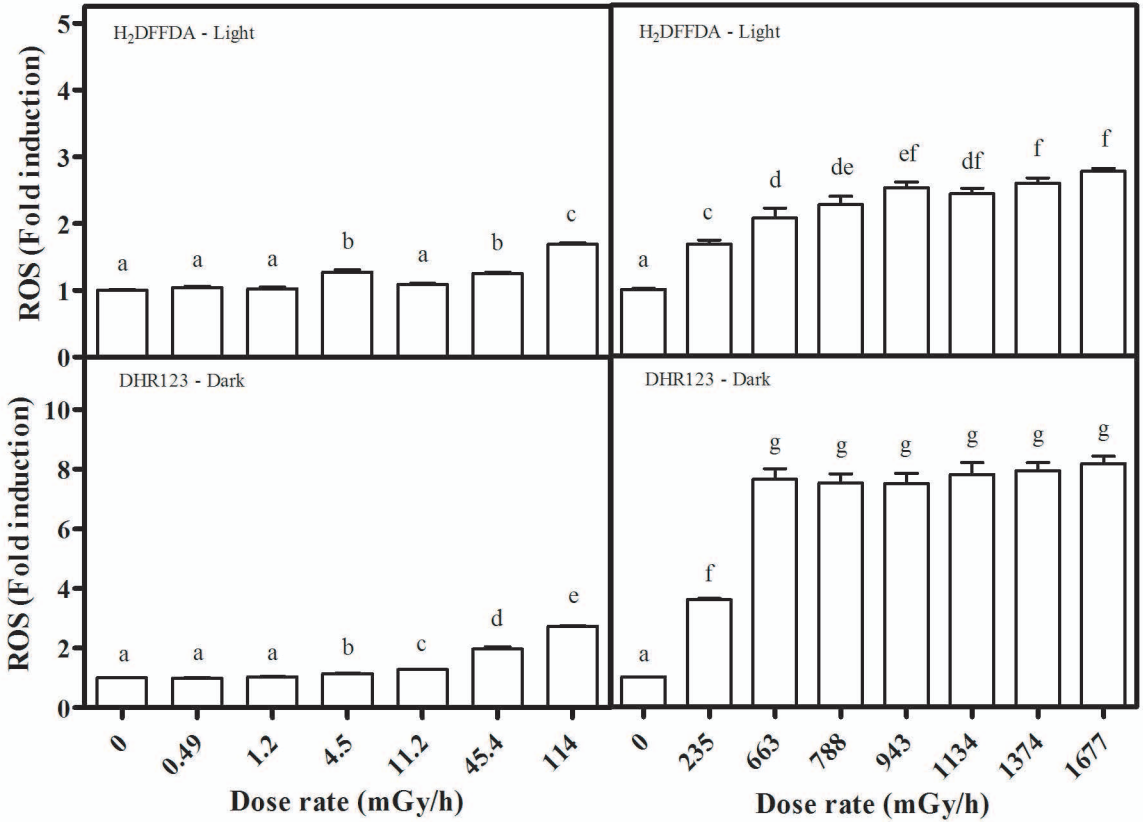
1677
Dose rate (mGy/h)


A
B
qP(rel) (rel. units)
qN(rel) (rel. units)
0.0
0.1
0.2
0.3
0.4
0.6
0.8
1.0
a
ab
bc
b
cd
d
d
e
a
ab
ab
bc
bcd
cd
de
e
0
235
663
788
943
1134
1374
1677
Dose rate (mGy/h)


H2DFFDA - Light
ROS (Fold induction)
a a a b a b c
H2DFFDA - Light
a c d de ef df f f
DHR123 - Dark
ROS (Fold induction)
a a a b c d e
DHR123 - Dark
a f g g g g g g
0 0.49 1.2 4.5 11.2 45.4 114
Dose rate (mGy/h)
0 235 663 788 943 1134 1374 1677
Dose rate (mGy/h)

Appendix A. Supplementary data

Sensitivity of the green algae *Chlamydomonas reinhardtii* to gamma radiation: photosynthetic performance and ROS formation

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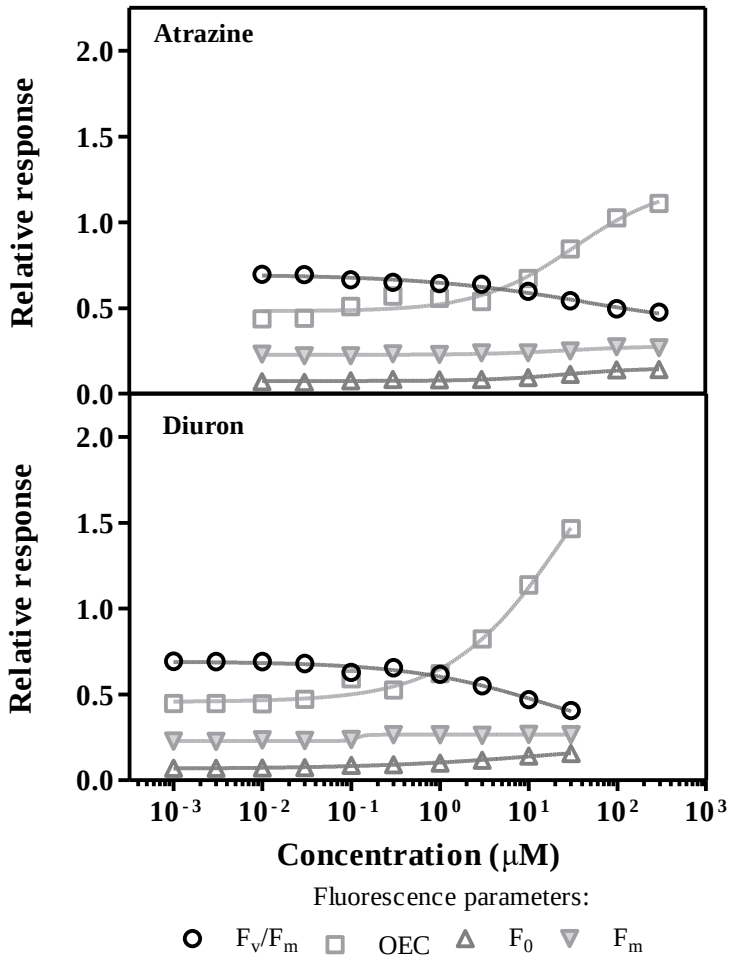

Figure A1 –Variation in fluorescence parameters of *Chlamydomonas reinhardtii* in the dark-acclimated state exposed to the positive controls atrazine and diuron for 6 hrs. F_v/F_m – Maximum quantum yield, OEC – Efficiency of oxygen-evolving complex, F_0 – Minimum fluorescent yield, F_m – Maximum fluorescent yield. The experimental results (Mean ± SEM) represent 3 independent studies.

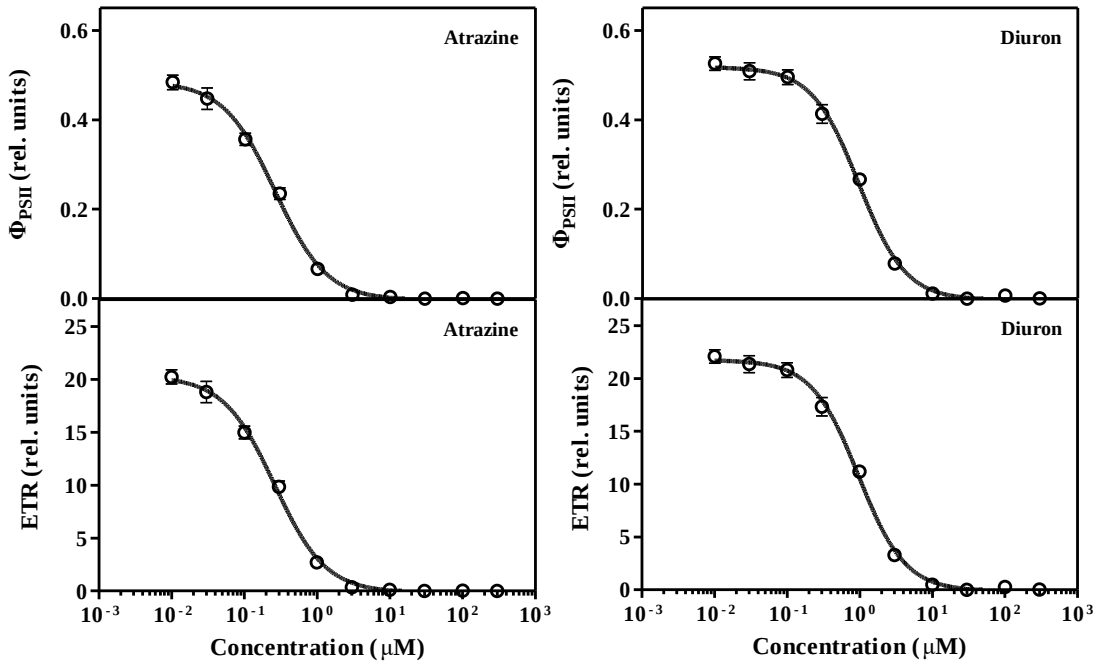

Figure A2 –Variation in fluorescence parameters of *Chlamydomonas reinhardtii* in the light-acclimated state exposed to the positive controls atrazine and diuron for 6 hrs. Φ_{PSII} – Effective quantum yield and ETR – Electron transfer rate. The experimental results (Mean ± SEM) represent 3 independent studies.

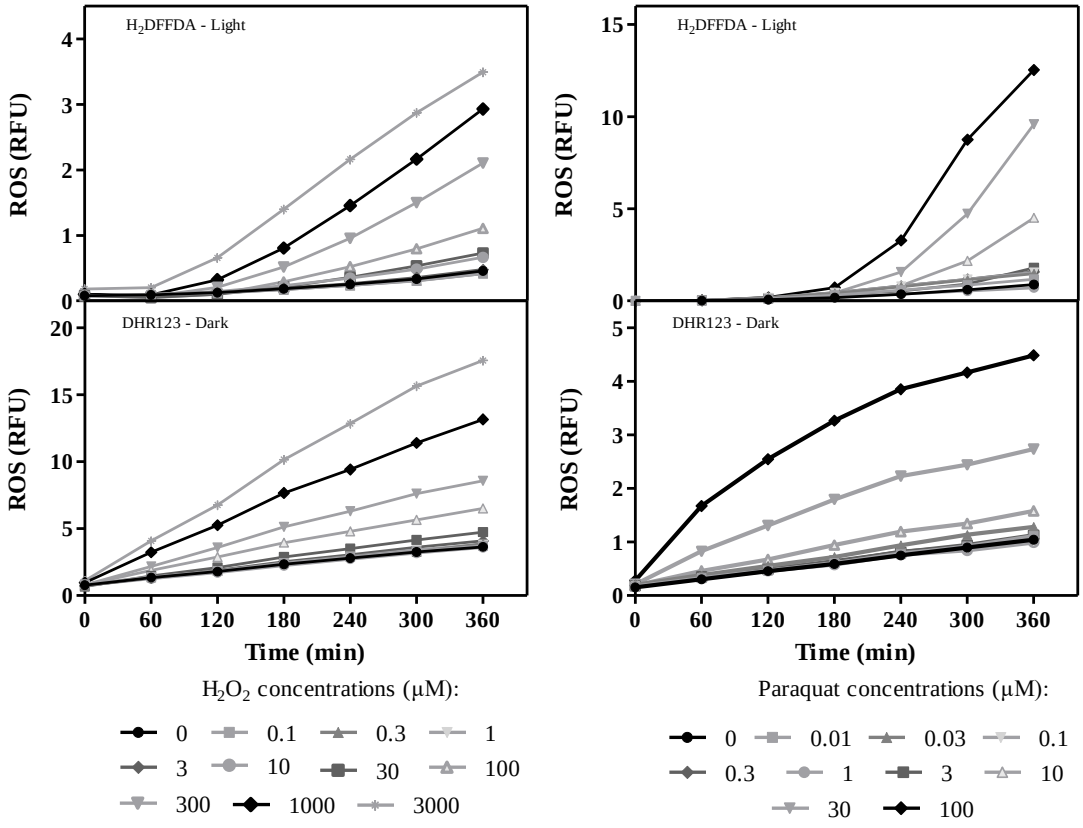

Figure A3 – Reactive oxygen species (ROS) as a function of time in *Chlamydomonas reinhardtii* exposed for 6 hrs in the light (probe H_2DFFDA) and the dark (probe DHR123) to the positive controls H_2O_2 and paraquat. The experimental results (Mean ± SEM) represent 3 independent studies. RFU – relative fluorescence units.

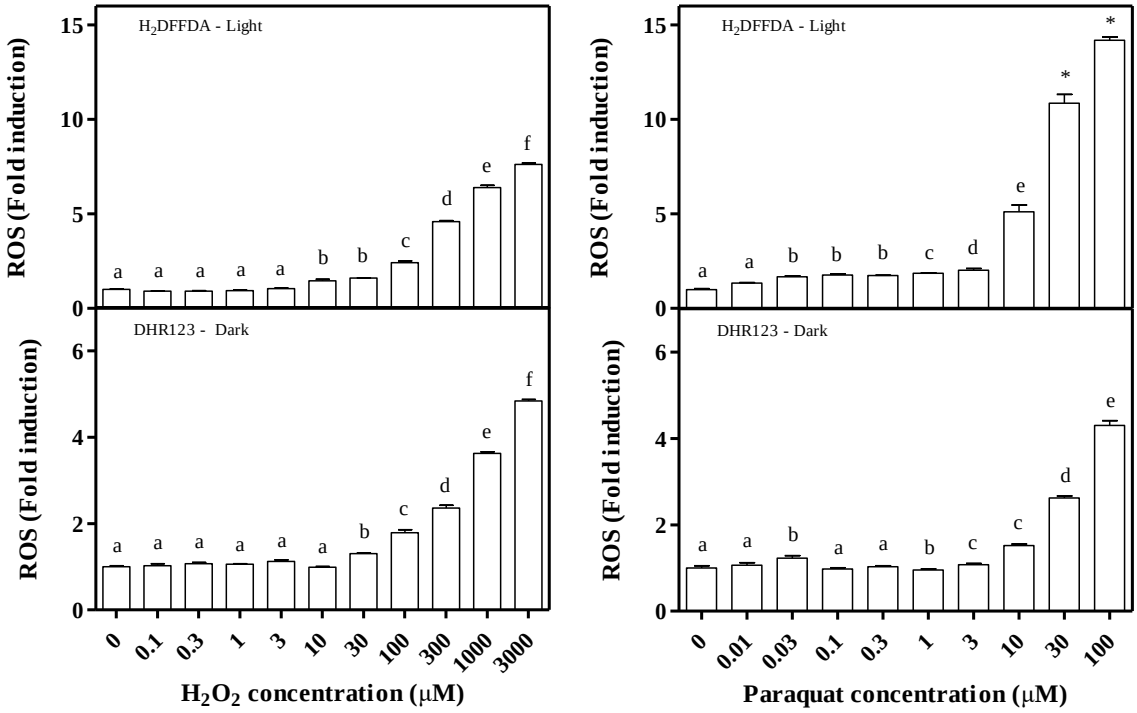

Figure A4 – Reactive oxygen species (ROS) formation in *Chlamydomonas reinhardtii* exposed to positive controls H_2O_2 and paraquat for 6 hrs and measured in fluorescence H_2DFFDA and DHR 123. The experimental results (Mean ± SEM) represent 3 independent studies. Letters represent statistical differences between dose rates ($p < 0.05$).

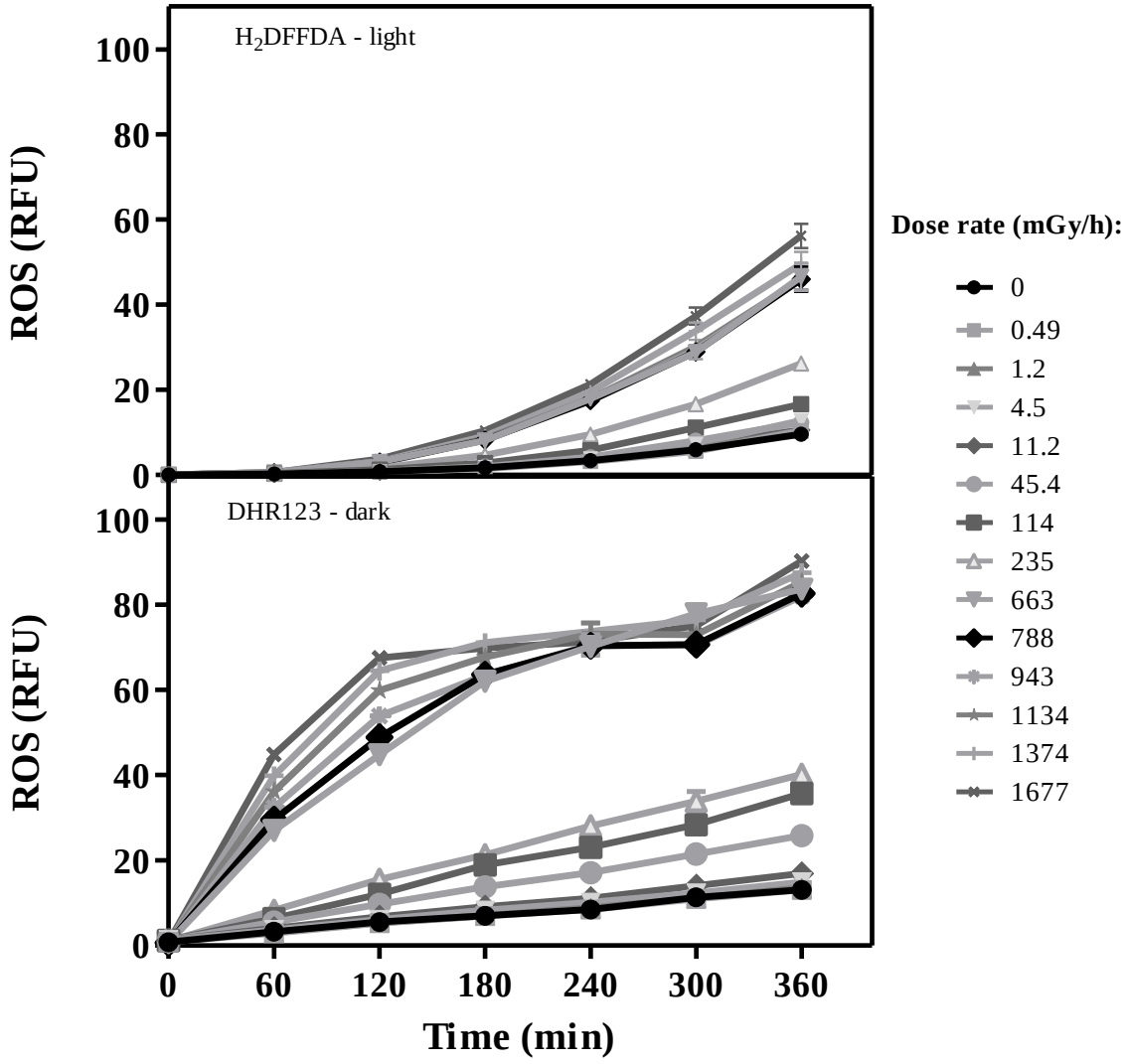

Figure A5 – Reactive oxygen species (ROS) as a function of time in *Chlamydomonas reinhardtii* exposed in the light and in the dark to gamma radiation for 6 hrs. The experimental results (Mean ± SEM) represent 3 independent studies. RFU – relative fluorescence units.