

## THE INTERACTION BETWEEN MICROALGAE AND GAMMA IRRADIATION AT LOW, NON-GROWTH INHIBITORY DOSES

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**Abstract.** The aim of this paper is to review the published results on the interaction between microalgae and gamma irradiation at low-doses, which does not inhibit cellular growth or cell proliferation. Particular emphasis is placed on the ability of gamma radiation to stimulate cellular metabolism, including the synthesis of useful compounds for biotechnological and medical uses. The paper also addresses aspects related to the mechanisms involved in changes in the cellular metabolism of irradiated cells.

**Keywords:** gamma radiation, non-inhibitory doses, microalgae, lipids, total proteins, ROS, carotenoid pigments.

**Rezumat. Interacțiunea între microalge și doze mici de radiație gamma care nu inhibă creșterea celulară sau multiplicarea celulelor.** Scopul acestei lucrări este de a revizui rezultatele publicate cu privire la interacțiunea dintre microalge și iradierea gama în doze mici, doze ce nu inhibă creșterea celulară sau proliferarea celulară. Un accent deosebit este pus pe capacitatea radiațiilor gama de a stimula metabolismul celular, inclusiv sinteza compușilor utili pentru uz biotehnologic și medical. Lucrarea abordează, de asemenea, aspectele legate de mecanismele implicate în modificările în metabolismul celular al celulelor iradiate.

**Cuvinte cheie:** radiație gamma, doze non-inhibitoare, microalge, lipide, proteine totale, ROS, pigmenți carotenoizi.

### INTRODUCTION

The interaction between microalgae as well as other microorganisms and gamma irradiation is a well-established topic for high doses, which inhibit cellular growth and cell multiplication, sometimes causing mutagenesis (CHOI et al., 2014; LIU et al., 2015). However, the work at low doses receives much less attention as compared with inhibitory doses (PLANEL et al., 1976). In recent years, there has been an increasing interest in using relatively low doses of gamma radiation to stimulate biological processes in microalgae RIVASSEAU et al. (2010), TALE et al. (2017), ERMAVITALINI et al. (2017a; b) MOISESCU et al. (2019) and ALMARASHI et al. (2020), as well as in other types of microorganisms (ARDELEAN et al., 2020, and references herein). Following our previous paper (ARDELEAN et al., 2020), the aim of this paper is to present the state of the art on the interaction between microalgae and low doses of gamma irradiation, in the general context of the effect of stressors on microalgal metabolism. The changes concerning the increased or decreased concentration of given compounds inside the algal cell could be important for understanding the mechanisms triggered by the interaction between gamma radiation and the cell, as well as for developing biotechnologies for increasing microalgae synthesis of chemicals useful for mankind.

**I. Gamma radiation at non-growth inhibitory doses, as a stressor to induce increased lipid production in microalgae.** To our best knowledge, RIVASSEAU et al. (2010) were the first to show that in a green Chlorophycean microalga grown under 450–2000 Gy irradiation, the carbohydrate concentration remains high and the pools of some free amino acids increased in the cell (i.e., particularly the most hydrophobic amino acids within cell membranes), as compared with the control.

TALE et al. (2017) used gamma irradiation (100, 300, 500, 700, 900, and 1100 Gy at dose rate of 3.097 kGy h<sup>-1</sup>) as a stressor to induce lipid hyper-accumulation in two strains of *Chlorella sorokiniana* (i.e., *C. sorokiniana* KMN2 and *C. sorokiniana* KMN3). The authors also showed that this lipid enhancement (up to 40% of biomass) is accompanied by accumulation in a higher proportion of shorter carbon chain fatty acid (i.e., C-16) compared to longer chain fatty acids, which is of significant potential in industrial biodiesel production.

JEONG et al. (2017) investigated the possibility of using low-dose rate gamma irradiation to increase lipid content in four microalgae species: *Tetraselmis suecica*, *Dunaliella tertiolecta*, *Phaeodactylum tricorutum*, and *Nannochloropsis oceanica*. Experimental results have shown that chronic LDR-type irradiation leads to increased cell densities, specific growth rates, and biomass of the four species. In *T. suecica*, *D. tertiolecta* and *P. tricorutum* there is also an increase in the amount of lipids, with a maximum in *T. suecica* when irradiated with a dose rate of 6 mGy h<sup>-1</sup>.

ERMAVITALINI et al. (2017a) showed that *Botryococcus* sp. irradiated at low doses (2, 4, 6, 8, and 10 Gy) changes the characteristics of their growth, biomass, percentage of total cell lipids and fatty acid profile. More precisely, the highest biomass (0.833 g) and lipid content (41 % total biomass) were found in the 10 Gy irradiated microalgae. Later, ERMAVITALINI et al. (2017b) analysed the fatty acid profile of *Botryococcus* sp. control cells and found only 6 types of fatty acids while in 10Gy irradiated microalgae cells found 12 types of fatty acids, with an increased proportion of long chain fatty acids and a low proportion of short chain fatty acids.

MOISESCU et al. (2019) demonstrated that the generation time of *Chlorella sorokiniana* UTEX 2130 decreases to 56% at 10 Gy, 60% at 50 Gy, and 77% at 100 Gy irradiation and the relative lipid content increases by 20% and 50% after 10 Gy and 100 Gy irradiation, respectively.

In our opinion, the interactions of photosynthetic microalgae with gamma radiation, at low, non-growth inhibitory doses is a new emerging but very important topic, both in terms of applications as well as for understanding the cellular and molecular mechanisms involved in these changes.

**II. Other stressors.** It is well known that other stressors are effective in enhancing lipid synthesis in microalgae. In a very recent review, ABO-STATE et al. (2019) summarize that the enhancement of lipid content in microalgae can be promoted by stressors such as nitrogen starvation, phosphate limitation, magnesium supplementation, carbon source, iron content in the culture medium, high salinity, high light intensities, low oxygen pressure, and dehydration. ABO-STATE et al. (2019) also performed several experiments with *Chlorella vulgaris* grown in BG<sub>11</sub> medium, with different concentrations of nitrogen (N) (i.e., 0, 380, 750, 1500, and 3000 mg L<sup>-1</sup>, using NaNO<sub>3</sub> as N source), phosphorus (P) (i.e., 0, 40, 80, 160, and 320 mg L<sup>-1</sup>, utilizing K<sub>2</sub>HPO<sub>4</sub> as P source), magnesium (Mg) (i.e., 19, 38, 75, 113, and 150 mg L<sup>-1</sup>, with MgSO<sub>4</sub>•7H<sub>2</sub>O as Mg source), and carbonate (CO<sub>3</sub>) (i.e., 0, 10, 20, 40, and 80 mg L<sup>-1</sup>, using Na<sub>2</sub>CO<sub>3</sub> as CO<sub>3</sub> source). Their results showed that the maximum growth was obtained at 1500, 160, 75, 20 mg L<sup>-1</sup> of N, P, Mg, and CO<sub>3</sub>, respectively. The lipid content increased significantly, as the concentration of Mg increased and the concentration of N, P and CO<sub>3</sub> decreased. Palmitic acid (C16:0) and linoleic acid (C18:2) were the dominant algal fatty acids (62.7% of total fatty acids), which strongly proved that *C. vulgaris* is a good candidate for biodiesel production (ABO-STATE et al., 2019).

In a very interesting paper, ALMARASHI et al. (2020) showed that biodiesel recovery from the green microalga *C. vulgaris* can be enhanced through a pre-treatment of the inoculum with low doses of cold atmospheric-pressure plasma (CAPP). A treatment of 30s resulted in the highest biomass productivity of 0.193 g L<sup>-1</sup> d<sup>-1</sup>. Moreover, short exposure times (30 and 60s) significantly increased the lipid content by 7.5% and 6.9%, respectively, over the control. Because 30s pre-treatment enhanced both growth and lipid content, the volumetric lipid productivity (i.e., 40.7 mg L<sup>-1</sup> d<sup>-1</sup>) increased by 16.6% and 17.6% over the control and 60s, respectively. Furthermore, the maximum volumetric fatty acid methyl esters (FAMES) production (i.e., 998.1 mg L<sup>-1</sup>) was recorded in the culture inoculated with 60s exposed cells, which was 43.5% and 15.7% higher than that of the control and 30s, respectively.

**III. Phytohormones.** Phytohormones control cell metabolism and, recently, have been found to be able to improve lipid productivity. GULDHE et al. (2019) reported optimized concentrations of different phytohormones, indole acetic acid, gibberellic acid, and kinetin showing an increase of 45.55%, 35.94% and 37.37% in biomass productivity in *Chlorella sorokiniana* as compared to nitrogen-limited control respectively. Overall, the highest lipid productivities of 50.24 and 49.43 mg L<sup>-1</sup> d<sup>-1</sup> were observed in optimized indole acetic acid and kinetin supplemented treatments respectively. Results from the present study illustrated the differential response of phytohormones from different classes on biomass and lipid enhancement, fatty acid composition and expression of related genes (GULDHE et al., 2019). Congruent results concerning the impact of exogenously supplemented auxins and cytokines on biomass, lipid content and fatty acid profile of *Desmodesmus* sp. JS07 and on enzymes involved in scavenging reactive oxygen species (ROS) are presented by SINGH et al. (2020).

**IV. Possible links between endogenous ROS and lipid accumulation.** In recent years, some research results indicated that ROS may be important mediators in lipid accumulation by different types of microorganisms, including microalgae (YILANCIUGLU et al., 2014; SHI et al., 2017; TALE et al., 2017). It is assumed that there may exist potential signal transduction mechanisms that trigger carbon partitioning and lipid accumulation in response to different environmental stresses, which serve to control homeostasis at the cellular level (YILANCIUGLU et al., 2014). TALE et al. (2017) showed that the exposure to ionizing radiation such as gamma rays is able to generate ROS, including OH• and H<sub>2</sub>O<sub>2</sub> which can affect the cellular structure and metabolism (i.e., morphology, biochemistry, and physiology of the cells) depending on the irradiation dosage. Their results showed that in response to gamma irradiation, there is an instant and huge build-up of ROS inside microalgal cells. Furthermore, in *C. vulgaris*, the specific intracellular ROS level can serve as a general quantitative marker for stress, irrespective of the type of stress induced (MENON et al., 2013).

**V. Lipid synthesis under exogenous ROS addition in the growth medium.** In connection with the above realities, relatively recently it was demonstrated that the proper exposure to exogenous ROS (e.g., H<sub>2</sub>O<sub>2</sub>) can trigger neutral lipid formation without any other separate or distinct stress (YU et al., 2015). For example, it was shown by BATTAH et al. (2014) that after 8 days of treating *C. vulgaris* with 2 mM and 4 mM H<sub>2</sub>O<sub>2</sub> the lipid content increased by 20 % and 87 % respectively, compared to the control, whereas the growth was inhibited by 15 % and 31 %, respectively (BATTAH et al., 2014). Furthermore, the same authors showed that among the used treatments, 12 μM of manganese chloride, 2.5 μM of cobalt nitrate, and 4 mM of H<sub>2</sub>O<sub>2</sub> were considered as feasible conditions for the over-production of lipids by *C. vulgaris*, recording a maximum increase of 12 %, 22 %, and 29 %, respectively over the corresponding controls.

Interestingly, the data revealed that high heavy metal stress (Mn<sup>2+</sup> and Co<sup>2+</sup>) and the oxidative stress of H<sub>2</sub>O<sub>2</sub> led not only to changes in lipid quantities but also to a great variation in the fatty acid composition of *C. vulgaris*. Whereas the dominant fatty acids recorded in the control culture of *C. vulgaris* were myristic (C14:0), palmitic (C16:0), oleic (C18:1), and linoleic (C18:2), in *C. vulgaris* treated with 12 μM manganese chloride and 2.5 μM cobalt nitrate a significant decrease to 15 % and 19 %, respectively, in total saturated fatty acids (SFAs) was recorded. These proportions were lower than their corresponding control with a noticeable increase in unsaturated fatty acids (UFAs). Their results suggested that Mn<sup>2+</sup>, Co<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> stressors are an effective approach to enhance lipid production in *C. vulgaris*. The authors claim that, despite inhibition of cellular growth by most of the treatments used, the increase in

lipid content led to an increase in lipid productivity up to 29 % higher than the control. YILANCIOGLU et al. (2014) confirmed that N limitation increased cellular lipid content up to 35% under 0.05 mM N concentration. In order to gain insight into the mechanisms of this phenomenon, they used fluorometric, flow cytometric, and spectrophotometric methods to measure oxidative stress and enzymatic defence mechanisms. Under N depleted cultivation conditions, they observed increased lipid peroxidation by measuring an important oxidative stress marker, malondialdehyde and enhanced activation of catalase, ascorbate peroxidase, and superoxide dismutase antioxidant enzymes. These observations indicated that oxidative stress is accompanied by an increased lipid content in the green alga. In addition, they showed that at optimum cultivation conditions, inducing oxidative stress by application of exogenous H<sub>2</sub>O<sub>2</sub> leads to increased cellular lipid content up to 44% when compared with non-treated control groups, arguing that oxidative stress and lipid overproduction are linked. Furthermore, SIVARAMAKRISHNAN & INCHAROENSAKDI (2017) showed that a UV pre-treatment followed by the application of H<sub>2</sub>O<sub>2</sub> can increase the total lipid production in *Scenedesmus* sp. They reported that at 2 mM H<sub>2</sub>O<sub>2</sub>, the mutant had an increase in the lipid content of 55 to 60% of dry cell weight compared to the wild type grown under the same conditions. Importantly, these results also suggest that oxidative stress mediates lipid accumulation.

**VI. Possible mechanisms.** The biological effect of gamma-rays is based on the interaction with atoms and molecules targets inside the cells, particularly water molecules (which represents approximately 90% of cell biomass), to produce free radicals which induce stress and damage to different important cellular components (KOVÁCS & KERESZTES, 2002; TALE et al., 2017). However, the cells have repairing mechanisms, including DNA-repairing mechanisms, inorganic scavengers (e.g., salts and Mn<sup>++</sup> ions) and organic scavengers (e.g., carotenoids and ROS scavenging enzymes) (SHI et al., 2017 and references herein). When the concentration of ROS exceeds the capacity of cellular antioxidants to maintain normal steady-state redox potential, the cell is under oxidative stress which, if prolonged, can lead to damaged biomolecules, mutagenesis, and ultimately cell death (YILANCIOGLU et al., 2014; SHI et al., 2017; TALE et al., 2017). The involvement of ROS in the increase of lipid content in microorganisms is experimentally demonstrated by recent results concerning the stimulating effects of exogenously added H<sub>2</sub>O<sub>2</sub> (YILANCIOGLU et al., 2014; SHI et al., 2017; TALE et al., 2017).

A study carried out by GOMES et al. (2017) 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 reactive oxygen species (ROS) formation. The microalgal cells exposed to different doses of gamma radiation (0.49–1677 mGy h<sup>-1</sup>) for 6h showed modifications of PSII energy transfer associated with electron transport and energy dissipation pathways, especially when higher dose rates were used. When it comes to the concentration of ROS, it was showed that gamma radiation induced ROS formation in a dose-dependent manner. The authors hypothesized that the decrease in photosynthetic efficiency seems to be connected to the formation of ROS. This appears to be the first report on changes in several chlorophyll fluorescence parameters associated with photosynthetic performance and ROS formation in microalgae, after exposure to non-growth inhibitory doses of gamma radiation.

In our opinion, based on the results reported in literature, ROS are categorically involved in the enhancement (under given conditions) of lipid synthesis in microalgae, and the proposed mechanism (Fig. 1) is based on this hypothesis (YU et al., 2015; YILANCIOGLU et al., 2014; SHI et al., 2017; TALE et al., 2017).

The next picture tries to synthesise the effect of ROS on gene expression in microalgal cells when the balance between generation of ROS and the general mechanisms involved in ROS inactivation is toward ROS generation. In this situation, there is an excess of ROS inside the cell compared to the so-called normal conditions, but this excess is not so high as to inhibit cells growth and multiplication, or to induce cell death. In this situation, some genes undergo change in their activity (YILANCIOGLU et al., 2014; YU et al., 2015; SHI et al., 2017; TALE et al., 2017). Theoretically, one can think that an enzyme involved in lipid synthesis, from an inactive state (not translated), started to be translated as shown by YILANCIOGLU et al. (2014), YU et al. (2015), SHI et al. (2017) and TALE et al. (2017), or from an already activated state goes further to a higher translation rate. We put forward that, for a given strain, at a given intracellular concentration of ROS acting for a given length of time, specific for each strain (but without exceeding the cell's capacity to protect itself) the ROS will induce metabolic changes linked to increased lipid synthesis. We stress our proposal that not only the intracellular concentration of ROS is important for triggering lipid biosynthesis, but also the period of time when triggering concentration really occurs inside the cell.

The picture illustrates the situation when the quantity of ROS exceeds the capacity of anti-ROS enzymes so ROS concentration is higher than in normal conditions (for a specific length of time for each strain), but lower than the concentration which induced the death of the cell. As one can see in figure 1, external stressors increase the intracellular quantities of ROS, in addition to already existing ROS in the so-called normal growth conditions. Depending on the already functional genes and corresponding anti-ROS enzymes, the disbalance in the ROS concentration emerges sooner or later (for a given strain) after the onset of external stressors. It is expected that, if, in a given strain, the anti ROS enzymes are already functional at time zero (when external stressor starts to act), the intracellular concentration of ROS will be lower as compared with the strain(s) where such enzymes are not (yet?) functional at the onset of the external stressor.

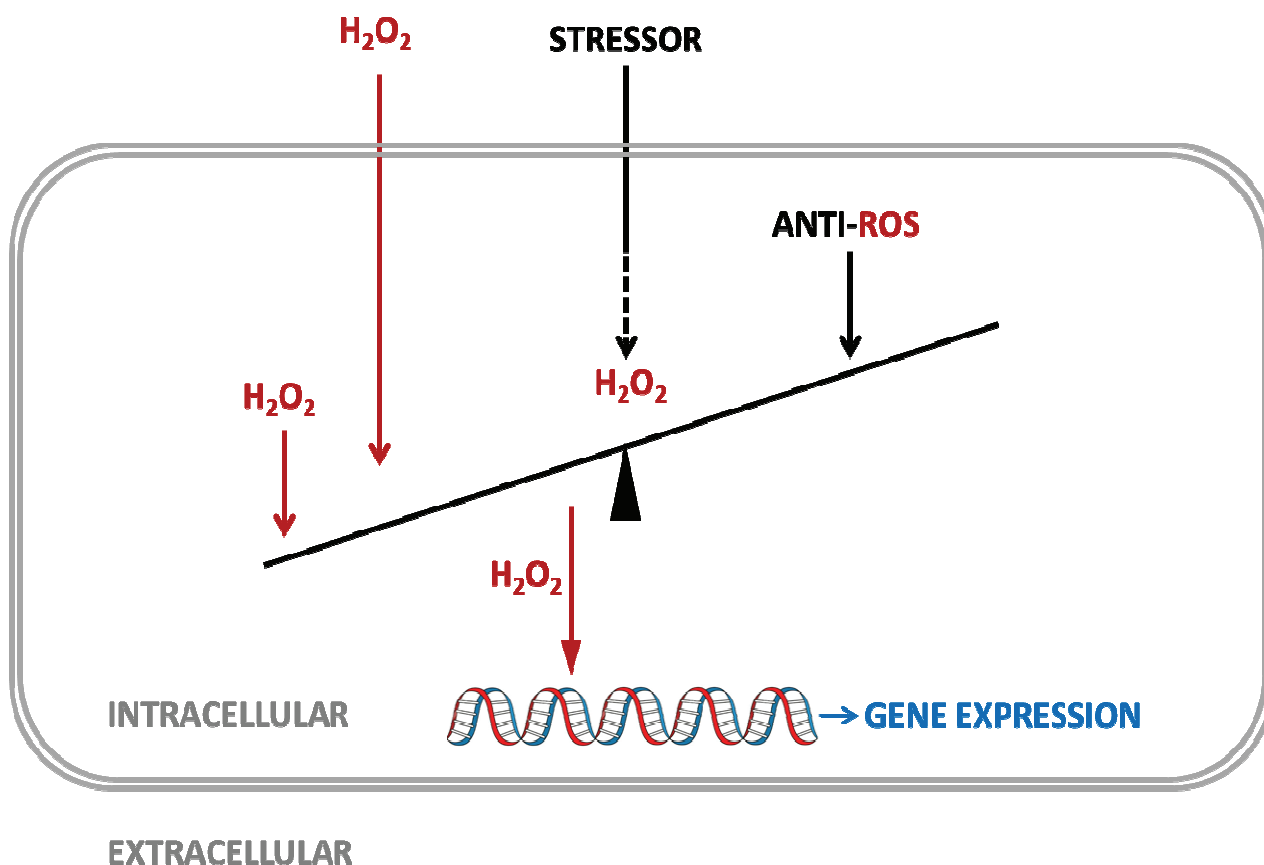


Figure 1. Diagrammatic picture concerning the proposed general mechanisms involved in the increase of lipid accumulation in microalgae induced by ROS species. Increased ROS can be achieved following the action of the external stressor, including gamma irradiation at low, non-growth inhibitory doses, external added ROS and by already existing intracellular ROS (in the so-called normal growth conditions). These intracellular ROS are substrate for anti – ROS mechanisms. The picture is based on the experimental results from YILANCIUGLU et al. (2014), YU et al. (2015), SHI et al. (2017) and TALE et al. (2017).

TALE et al. (2017) showed in their study that under gamma irradiation there are changes in the expression of some important genes for lipid metabolism, such as acetyl-CoA carboxylase (ACCase), and diacylglycerol acyl transferase (DGAT). They showed that these two genes are upregulated following gamma irradiation sustaining a possible mechanism by which gamma irradiation could induce increased lipid biosynthesis in *Chlorella sorokiniana* strain KMN3.

They also showed that the expression of the small subunit of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) catalyzes the first step of carbon fixation, decreases in response to gamma - irradiation, while the large subunit expression increases. It is an experimental result whose signification has not been identified so far (TALE et al., 2017). In conclusion, their results concerning RT-PCR mediated gene expression data clearly show that gamma-irradiation instantly up regulates two important regulatory enzymes in the lipid biosynthetic pathway (ACCase and DGAT). They stressed that a higher dose can completely kill the cell or induce apoptosis, while a small dose may give suboptimal or no effect at all. In the present work, after carefully studying cell survival and maximum ROS accumulation, it was concluded that 500 Gy could be a suitable dose to induce sufficient stress. The present gene expression data support that hypothesis (TALE et al., 2017). In view of the observed ROS accumulation and elevated lipid levels in response to gamma -irradiation, it is further hypothesized by the authors that gamma -irradiation-induced ROS accumulation may up regulate lipid biosynthetic pathway. According to TALE et al. (2017) this could be a possible mechanism by which gamma-irradiation may cause lipid accumulation.

Even with pioneering results concerning the signalling molecules (e.g., ROS) on genes which activities are modulated under gamma irradiation (YILANCIUGLU et al., 2014; YU et al., 2015; SHI et al., 2017; TALE et al., 2017) the knowledge of molecular mechanisms involved in these changes is only emerging (YILANCIUGLU et al., 2014; YU et al., 2015; SHI et al., 2017; TALE et al., 2017) as well as regarding the biological signification of these change in metabolites balance. Why, after such type of irradiation, the lipid content of the cell is increased? In the case of nutrient depletion (i.e., N, P), the increased lipid synthesis and deposition as lipid droplets are logically explained as changes in C metabolism when cellular growth is stopped or drastically decreased, as a way to redirect extra energy and C to intracellular inclusions (GULDHE et al., 2019).

**VII. Future prospects.** In our opinion, the interaction of microalgae, microorganisms in general, with gamma irradiation at doses which do not inhibit cell growth and multiplication is a topic of emerging significance both for fundamental research as well as for practical applications which could add new insights concerning epigenetics. Mechanism(s) involved in the interaction of low gamma irradiation with microalgae, microorganisms in general, are so far poorly understood and progress in this topic would allow us to better understand the complexity of cell metabolism and its regulation as well as the relationships between the effect of gamma irradiation and other stressors, but also phytohormones, within the framework of epigenetics. The advanced knowledge would, normally, sustain rationale design of protocols for biotechnological applications as well as help to understand the benefits for individual cell, and populations of cells.

The biological signification of lipid accumulation after low, no-growth inhibitory doses of gamma irradiation seems to be completely unknown so far. To our best knowledge there is no explanation or hypothesis in the literature concerning why gamma irradiation at low doses which do not inhibit cell growth and multiplication induces an increase in lipid content of the cells. This understanding needs more results and, together with the knowledge of molecular and cellular mechanisms involved in at least several species, it would help us not only reap economic benefits, but also deeper understand other forms of life.

According to TALE et al. (2017) gamma irradiation appears to have immense potential in commercial biodiesel production both by increasing the quantities of produced lipids and by having a higher proportion of short chain fatty acids useful for biodiesel production. We also should not forget that the lipids produced by microalgae can be useful not only for biodiesel production but also as a source of nutritional and medical significant fatty acids such as omega 3 and omega 6.

Compared to other stresses (such as nutrient limitation), from an operational point of view, gamma radiation can be much more easily applied to cells than other stresses such as nutrient limitation, which would require cell harvesting, that is an expensive additional step.

In a very recent paper, ARIF et al. (2020) originally coupled the increased accumulation of microalgal lipids for biodiesel production with advanced wastewater treatment, thus converting waste materials into useful metabolic products! In this paper there is no use of gamma irradiation, but we could put forward that linking their results with those showing the potential of low doses of gamma irradiation to increase lipid droplets accumulation in microbial cells, one can make a step ahead to an economically viable biotechnology.

Our results concerning the use of photosynthetic microorganisms – both cyanobacteria and microalgae – for (artificial) waste water purification (ARDELEAN et al., 2019) and simultaneous biomass accumulation as a source of lipids, proteins, chlorophyll *a* and carotene synthesis would thus be further coupled with gamma irradiation pretreatment of the inoculum.

The use of gamma irradiation is also expected to be important from an ecological point of view, not only strictly biotechnological as recently shown by GOLZ & BRADSHAW (2019) in their pioneering work. They studied the environmental effects of low doses of gamma irradiation, which do not inhibit the growth of microorganisms, on the biochemical composition (total protein content) of two primary producers and the further effect on the next trophic level (the primary consumer *D. magna*). They exposed two species of phytoplankton to 5 Gy and 25 Gy showing that gamma radiation had a significant effect on phytoplankton biochemical composition, namely to total protein content and an increased production of reactive oxygen species (GOLZ & BRADSHAW, 2019).

In our opinion, the study of interactions between low doses of gamma irradiation, which do not inhibit the growth, and living beings belonging to all three Kingdoms *Archaea*, *Bacteria* and *Eukarya*, is at the beginning of its exponential growth, having the potential of significant benefits for mankind and our Planet.

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