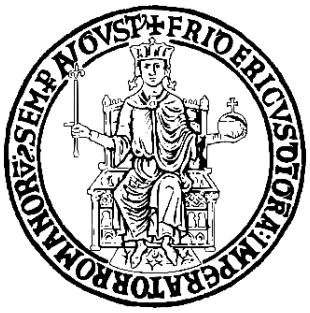
UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"



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Trophic transitions from dark to light in the unicellular red algae *Galdieria phlegrea*

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Abstract

Galdieria phlegrea (strain 734) is a unicellular and polyextremophilic red microalgae, endowed with high metabolic plasticity, which allows it to alternate trophic modalities (autotrophic, heterotrophic, and mixotrophic) and to thrive in extreme environmental conditions.

The present study investigates the physiological and functional mechanisms of photosynthetic reactivation during the trophic transition from heterotrophy to autotrophy, to define temporal dynamics, reorganization of photosystems, and strategies of use of intracellular reserves.

The results show that heterotrophic cells accumulate energy reserves in the form of lipids and carbohydrates, keeping the functional core of the photosystems unchanged. Cell density increases, while cell size decreases in the transition to autotrophy. Exposure to light initiates a coordinated process of reactivation of photosynthesis. In fact, following the transition in five days, it is observed that the maximum quantum yield of Photosystem II (Fv/Fm), together with the effective quantum yield of Photosystem II (Φ_{PSII}), increases within five days, indicating the restoration of electronic transfer and oxygen production. The results are confirmed by spectroscopic, 77K fluorescence, and confocal microscopy analyses, which show that Photosystem I (PSI) maintains structural and functional integrity even in the absence of light, acting as a center for reorganization, while the antennal complexes and Photosystem II (PSII) recompose rapidly.

In conclusion, we can argue that photosynthesis recovery takes place in three distinct phases: (i) latency; (ii) activation, and (iii) stabilization, optimizing the use of stored energy resources, which reflect an optimization of the deployment of intracellular energy reserves.

These results highlight the exceptional resilience and versatility of *G. phlegrea* and suggest its potential as a model organism for trophic adaptation studies and for biotechnological applications.

Riassunto

Galdieria phlegrea (ceppo 734) è una microalga rossa unicellulare e poliestremofila, dotata di un'elevata plasticità metabolica, che le permette di alternare modalità trofiche (autotrofa, eterotrofa e mixotrofa) e di prosperare in condizioni ambientali estreme.

Il presente studio indaga i meccanismi fisiologici e funzionali della riattivazione fotosintetica durante la transizione trofica da eterotrofia ad autotrofia, con l'obiettivo di definire dinamiche temporali, riorganizzazione dei fotosistemi e strategie di utilizzo delle riserve intracellulari.

I risultati mostrano che le cellule eterotrofe accumulano riserve energetiche sotto forma di lipidi e carboidrati, mantenendo il nucleo funzionale dei fotosistemi inalterato. La densità cellulare aumenta, mentre le dimensioni cellulari diminuiscono nel passaggio all'autotrofia. L'esposizione alla luce avvia un processo coordinato di riattivazione della fotosintesi; difatti, seguendo la transizione in 5 giorni, si osserva che la resa quantica massima del Fotosistema II (Fv/Fm) unitamente alla resa quantica effettiva del Fotosistema II (Φ_{PSII}) aumentano entro 5 giorni, indicando il ripristino del trasferimento elettronico e della produzione di ossigeno. I risultati sono confermati dalle analisi spettroscopiche, di fluorescenza a 77 K e di microscopia confocale, che mostrano che il Fotosistema I (PSI) mantiene integrità strutturale e funzionale anche in assenza di luce, fungendo da centro per la riorganizzazione, mentre i complessi antennali e il Fotosistema II (PSII) si ricompongono rapidamente. In conclusione, possiamo sostenere che il recupero della fotosintesi si svolge in tre fasi distinte: (i) latenza; (ii) attivazione; e (iii) stabilizzazione, ottimizzando l'uso delle risorse energetiche immagazzinate che riflettono un'ottimizzazione dell'impiego delle riserve energetiche intracellulari.

Questi risultati evidenziano l'eccezionale resilienza e versatilità di *G. phlegrea* e ne suggeriscono il potenziale come organismo modello per studi sull'adattamento trofico e per applicazioni biotecnologiche.

Chapter 1 – Introduction

1.1 Extremophilic microalgae: adaptations and scientific interest

Microalgae represent an incredibly diverse group of photosynthetic microorganisms that inhabit virtually every ecosystem on our planet (Barsanti & Gualtieri, 2014). Their ability to survive is remarkable, as they thrive in various environments, including marine and freshwater habitats, desert sands, snow, ice, and even hot springs (Arsad et al., 2022; Rojas-Villalta et al., 2024).

Along with cyanobacteria, microalgae are considered among the first photosynthetic organisms to produce oxygen (Rojas-Villalta et al., 2024; Sánchez-Baracaldo & Cardona, 2020). This ancient evolutionary history has enabled them to develop extraordinary ecological and metabolic diversification for adaptation and environmental modeling (Rojas-Villalta et al., 2024; Sánchez-Baracaldo & Cardona, 2020).

Within this vast kingdom, extremophile microalgae are of particular interest, defined by their ability not only to survive, but also to thrive in extreme environmental conditions that would be lethal to most other forms of life (Marzban & Tesei, 2025; Rekadwad et al., 2023; Rojas-Villalta et al., 2024). These microorganisms attract growing scientific attention due to their adaptability to high or very low temperatures, extreme pH levels, high salinity, heavy metal contamination, intense radiation, or limited nutrient availability (Marzban & Tesei, 2025; Rojas-Villalta et al., 2024; Varshney et al., 2015). Furthermore, some species are classified as poly-extremophiles, capable of surviving and flourishing under multiple extreme conditions simultaneously, such as high temperatures and acidic pH (Ciniglia et al., 2004; Rappaport & Oliverio, 2023; Rinaldi et al., 2024).
A prime example is the genus *Galdieria*, a thermophilic and acidophilic red alga that adapts to geothermal habitats characterized by high temperatures and strong acidity (Albertano et al., 2000; Schönknecht et al., 2013; Stadnichuk & Tropin, 2022). Extremophile microalgae are able to maintain active metabolism and reproduce in hostile environments thanks to unique biochemical, physiological, and structural adaptations (Arora et al., 2025; Gross & Schnarrenberger, 1995; Rinaldi et al., 2024). Unlike many extremophile bacteria and archaea, extremophile microalgae integrate photosynthetic capability with flexible metabolic strategies, making them a valuable model for studying the limits of life and developing innovative biotechnological applications (Curien et al., 2021; Marzban & Tesei, 2025; Rojas-Villalta et al., 2024). Environmental factors such as temperature, salinity, light, and pH strongly influence the growth and biochemical composition of these microalgae, also determining specific adaptations (Villegas-Valencia et al., 2023). For example,

both psychrophiles and thermophiles have developed specific adaptations that allow them to maintain metabolic function in opposite environmental conditions (Kohli et al., 2020).

In psychrophiles, low temperatures would slow down biochemical reactions, but these organisms compensate for this with enzymes that are highly efficient even in cold conditions and cell membranes enriched with polyunsaturated fatty acids (PUFAs), which ensure lipid fluidity (Gupta et al., 2025; Rojas-Villalta et al., 2024; Varshney et al., 2015). Some psychrophilic microalgae, including *Chlamydomonas nivalis, Chloromonas,* and *Microglena,* accumulate essential fatty acids such as eicosapentaenoic acid (EPA), arachidonic acid (AA), and docosahexaenoic acid (DHA), making them a promising resource for the nutraceutical and pharmaceutical industries (Suzuki et al., 2023). These compounds are used in the prevention of cardiovascular disease, in supporting neurological development, and in the formulation of dietary supplements with high biological value (Gupta et al., 2025; Suzuki et al., 2023; Varshney et al., 2015).

In contrast, thermophiles have developed thermostable enzymes with reinforced protein structures, capable of maintaining high catalytic activity even at very high temperatures, and cell membranes with a high content of saturated and branched fatty acids, which limit excessive heat-induced fluidity (Imanaka, 2011; Kohli et al., 2020; Sterner & Liebl, 2001). In addition, they possess molecular chaperones and heat stress proteins that prevent protein denaturation and aggregation, along with metabolic and photosynthetic modifications that ensure survival in extreme environments (Burkhardt et al., 2024; Saini et al., 2021). Significant examples of thermophiles are algae such as *Cyanidioschyzon merolae* and *Galdieria sulphuraria,* both belonging to the order of *Cyanidiophiceae*, capable of growing in acidic hot springs (Abiusi, Trompetter, et al., 2022; Barbier et al., 2005). These algae produce pigments (such as phyco-biliproteins and carotenoids) and secondary metabolites that find application in biotechnology (Hirooka & Miyagishima, 2016; Saini et al., 2021; Van Etten et al., 2023).

Some extremophilic microalgae are distinguished by their capacity to thrive in environments characterized by osmotic imbalances or extreme pH values (Ciniglia et al., 2004; Gross & Schnarrenberger, 1995; Sydney et al., 2019). Alkaliphiles colonize basic environments, such as carbonate-rich saline lakes (Horikoshi, 1999; Varshney et al., 2015). Halophiles tolerate very high salt concentrations, with *Dunaliella salina* being a typical example in hypersaline waters (Barbosa et al., 2023). From a physiological and molecular standpoint, acidophilic algae have developed sophisticated strategies to maintain cytoplasmic pH homeostasis (Arora et al., 2025; Hirooka & Miyagishima, 2016). Among these, acidophiles, such as the representative species *Galdieria* spp.,

grow under extremely low pH conditions (capable of development between pH 0 and 4) (Abiusi, Trompetter, et al., 2022; Albertano et al., 2000). These strategies include high activity of proton pumps (P-type and V-type ATPases, as well as V-PPases), modifications in membrane lipid composition that reduce H^+ permeability, and the production of compatible solutes and antioxidant systems (ascorbate, glutathione, superoxide dismutase) (Abinandan et al., 2020; A. Liu et al., 2023). During phases of acid stress, photosynthetic activity is temporarily reduced, and cellular energy is reallocated towards defense systems (Arora et al., 2025; A. Liu et al., 2023). In certain strains, such as *Chlamydomonas eustigma*, a basal increase in the gene expression of proton pumps and heat shock proteins (Hsp70, Hsp60) has been observed, testifying to particularly high molecular stability under extreme conditions (Arora et al., 2025; Hirooka & Miyagishima, 2016). Conversely, alkaliphilic algae must address the challenge of generating an effective proton gradient in high-pH environments (Soontharapirakkul & Incharoensakdi, 2010). They utilize Na^+/H^+ antiporters and H^+-ATPases to re-establish the electrochemical potential, expel excess intracellular Na^+, and, in some cases, accumulate glycerol from starch as a protective mechanism (Gimmler, 2000; Soontharapirakkul & Incharoensakdi, 2010). They also exhibit cell wall thickening and an enhancement of carbon-concentrating mechanisms, featuring increased carbonic anhydrase activity and upregulated expression of photosynthetic and RuBisCO genes (Moroney & Ynalvez, 2007; Qu & Miao, 2021). This allows them to effectively utilize bicarbonate (HCO_3^-) as a carbon source under alkaline conditions (Moroney & Ynalvez, 2007).

Scientific interest in these microalgae lies not only in understanding their adaptive mechanisms but also in their biotechnological potential (Patel et al., 2019; Vadlamani et al., 2017). They offer significant advantages for large-scale cultivation: they reduce contamination by neutrophilic microorganisms, limit culture crashes, and lower operational costs associated with pH control (Arora et al., 2025). Certain acidophilic strains, like *G. sulphuraria* and *G. phlegrea*, have been tested for municipal and mining wastewater treatment, showing promising results in both nutrient removal and the production of lipid-rich biomass and high-value pigments (Arashiro et al., 2020; di Cicco et al., 2021; Raheem et al., 2018). Alkaliphilic algae, on the other hand, find applications in the treatment of high-pH industrial effluents, CO_2 sequestration from biogas, and the production of bioactive metabolites such as carotenoids and antioxidants (Arora et al., 2025; Granada-Moreno et al., 2017; Kikuchi et al., 2024).

As for halophilic algae, they thrive in environments characterized by high salt concentrations, such as hypersaline lakes, industrial salt pans, and natural evaporites (Cycil et al., 2020; Oren, 2024). Among the best-known examples are *Dunaliella salina* and *Aphanothece halophytica*, which develop

adaptation strategies such as intracellular accumulation of glycerol and other osmoprotectants, alteration of membrane composition, and production of enzymes and proteins resistant to osmotic stress (Keil et al., 2023; Varshney et al., 2015). These mechanisms allow them to maintain cellular homeostasis, metabolism, and photosynthesis even under conditions of high osmotic pressure, making them useful for the production of β-carotene, biomass for bioenergy, and industrial metabolites (Barbosa et al., 2023; Patel et al., 2019).

Another group of extreme microalgae includes species capable of tolerating high concentrations of heavy metals or intense UV radiation, conditions typical of contaminated environments or those with high solar exposure (Manfredi et al., 2025; Retta et al., 2024). These microalgae develop advanced defense mechanisms, including the synthesis of metallothioneins and intracellular chelators to sequester toxic metals, as well as photoprotective pigments such as carotenoids, scytonemins, and amino acids derived from mycoalgic acids, which are capable of absorbing and dissipating UV energy (Chakravorty et al., 2023; Manfredi et al., 2025). In addition, antioxidant systems and DNA repair mechanisms reduce oxidative damage and protect cell function (Chinjoo & Golzary, 2025; Pereira et al., 2024). These characteristics have made these microalgae promising for innovative biotechnological applications, such as the bioremediation of contaminated water, the production of bioactive metabolites, and use in natural cosmetics as sunscreens, in addition to their potential as environmental biomarkers (Martínez-Ruiz et al., 2022; Rojas-Villalta et al., 2024; Shanab et al., 2012).

1.2 The genus *Galdieria*: ecology, physiology, and metabolism

The genus *Galdieria* (Cyanidiophyceae, Rhodophyta) comprises unicellular red algae that inhabit extreme environments, strongly associated with geothermal sites characterized by high acidity and temperature (Čížková et al., 2019; Miyagishima et al., 2017). Historically, *Galdieria* was established by Merola et al. (1981) to distinguish thermophilic and acidophilic red algae isolated, previously assigned to *Cyanidium*, based on a combination of morphological, physiological, and ecological traits. The genus belongs to the class Cyanidiophyceae (or Cyanidiales), a lineage of early-diverging red algae that includes three main genera: *Cyanidium*, *Cyanidioschyzon*, and *Galdieria*. Classical taxonomy, relying on morphology and pigment composition, initially grouped all thermoacidophilic red algae within *Cyanidium*, but subsequent ultrastructural and molecular analyses (rDNA and plastid markers) revealed deep phylogenetic divisions justifying the separation of *Galdieria* as an independent genus (Albertano et al., 2000; Ciniglia et al., 2004).

Modern phylogenomic analyses have further refined this framework. Comparative nuclear, plastidial, and mitochondrial genome studies have demonstrated that the Cyanidiophyceae are monophyletic but internally structured into distinct lineages (Iovinella et al., 2022; Park et al., 2023). The most recent taxonomic revision, based on plastid genome data, has raised the classification level of the class Cyanidiophyceae, which is now divided into four Orders, including the new order Galdieriales (Park et al., 2023). This classification formally recognizes that the four main lines (*Galdieria, Cyanidium, Cyanidioschyzon,* and the mesophilic line "C*ave Cyanidium*") are distinct at a higher phylogenetic level (Park et al., 2023). *Galdieria* forms a robust clade that is sister to *Cyanidioschyzon* (Iovinella et al., 2022). The genus *Galdieria* is now formally placed in the order Galdieriales nov., while the mesophilic line has been classified in the new order Cavernulicolales ord. nov. (Park et al., 2023).

Nevertheless, incongruences among organellar and nuclear phylogenies suggest a complex evolutionary history, possibly involving ancient hybridization events, independent organellar evolution, or Horizontal Gene Transfers (HGT) among sympatric lineages (Rossoni et al., 2019; Schönknecht et al., 2013). Recent multilocus and genomic studies propose that *Galdieria* encompasses several cryptic species, with *G. sulphuraria*, the type species, representing a species complex comprising genetically and ecologically divergent lineages (Curien et al., 2021; Hirooka & Miyagishima, 2016). Other recognized species include *G. maxima*, *G. partita*, and *G. phlegrea*, each characterized by specific ecological niches and physiological capacities (Park et al., 2023; Stadnichuk & Tropin, 2022). Phylogenetic data support a cosmopolitan distribution of these lineages, with biogeographic patterns reflecting both local adaptation to geothermal microhabitats and dispersal events across continents (Iovinella et al., 2018, 2022).

Ecologically, members of *Galdieria* colonize hot springs, fumaroles, and volcanic soils, where pH ranges from 0.5 to 3.0 and temperatures fluctuate between 40 and 56 °C (Ciniglia et al., 2004; Curien et al., 2021; Gross & Schnarrenberger, 1995). Their cosmopolitan distribution, reported in Europe, Asia, and the Americas, highlights their remarkable capacity to thrive in ecosystems highly unfavorable for photosynthetic eukaryotes (Fig. 1) (Castenholz & McDermott, 2010; Pinto et al., 2007; Stadnichuk & Tropin, 2022)**.**

Figure 1. *Galdieria* in extreme geothermal habitats. A. Geothermal sites in Iceland, such as the Grensdalur hot spring, host *Galdieria* populations adapted to extreme acidity and heat. **B.** Close-up of a rock in an Icelandic hot spring near Reykjavik with sulfur and *Galdieria sulphuraria* (Image credit: Christine Oesterhelt).

Physiologically, *Galdieria* exhibits exceptional metabolic flexibility. The species *G. sulphuraria, G. phlegrea, and G. maxima* can grow photoautotrophically, utilizing chlorophyll *a* (Chl *a*) and a complex phycobiliprotein system (phycocyanin, allophycocyanin, phycoerythrin), to produce energy from light (Portillo et al., 2022; Thangaraj et al., 2011). However, these same species are also able to develop heterotrophically, taking advantage of a broad spectrum of organic substrates such as carbon and energy sources independently of light (Occhipinti et al., 2023; Retta et al., 2024; Salbitani et al., 2021). Furthermore, under variable environmental conditions, they can adopt a mixotrophic metabolism, combining photosynthesis with the assimilation of organic compounds, thus optimizing growth and survival in their extreme habitats (Abiusi, Moñino Fernández, et al., 2022; Curien et al., 2021).

In contrast, other members of the Cyanidiophyceae, such as *Cyanidium caldarium* and *Cyanidioschyzon merolae*, are predominantly photoautotrophic and have limited or no heterotrophic abilities, highlighting how *Galdieria* represents the most versatile genus of the group (Krupnik, 2023).

Analyses of *G. sulphuraria* and various *G. phlegrea* strains have revealed a substantial number of genes acquired through HGT from coexisting bacteria and archaea, providing functions related to organic compound metabolism, detoxification, and stress tolerance (Qiu et al., 2013; Rossoni et al., 2019). Notably, the draft genome of *G. phlegrea* DBV 009 (~11.4 Mbp) demonstrated the acquisition of entire metabolic pathways via HGT, including the urea hydrolysis pathway (Qiu et al., 2013; Rossoni et al., 2019).

Recent integrative studies have further demonstrated that, in addition to HGT, ancient gene duplications and the haplodiplontic life cycle are critical for the genus's extraordinary adaptability (Hirooka et al., 2022). The alternation between wall-less haploid and cell-walled diploid stages enables *Galdieria* to withstand the dynamic and often unpredictable conditions of geothermal habitats (Hirooka et al., 2022). Overall, these insights establish *Galdieria* as a premier model for elucidating the evolutionary strategies that allow photosynthetic eukaryotes to persist in extreme environments (Hirooka & Miyagishima, 2016; Perez Saura et al., 2022).

1.2.1 *Galdieria phlegrea* (strain 734): species- and strain-specific traits

Within the genus *Galdieria*, *Galdieria phlegrea* has been studied for its adaptability to extreme environmental conditions and its distinctive metabolism (Carfagna et al., 2018; Giustino et al., 2025; Qiu et al., 2013). Strain 734 (Fig. 2), considered in this study, was isolated from the geothermal springs of Agri Diyadin (Turkey), a thermal area characterized by high acidity and extreme temperatures, typical of Cyanidiophyceae habitats (Carfagna et al., 2018; Iovinella et al., 2018).

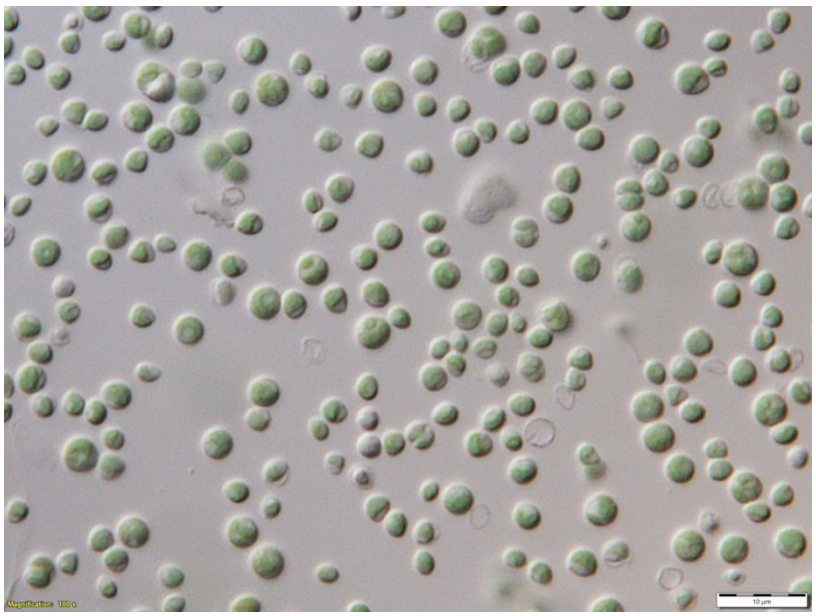

Figure 2. Microscopic image of the polyextremophilic red microalga *Galdieria phlegrea* (strain 734), exhibiting its characteristic spherical morphology. The image was captured at 1000x magnification using a Zeiss light microscope. The scale bar represents 10 µm.

Like other related species, *G. phlegrea* possesses a complete photosynthetic apparatus, supplemented by accessory pigments that optimize light capture under limiting conditions (Giustino et al., 2025;

Imbimbo et al., 2020). However, this species is particularly notable for its pronounced heterotrophic and mixotrophic capacities (Salbitani et al., 2021). Although the complete genome of strain 734 is not yet available, genomic studies on other *G. phlegrea* strains (DBV 009 and "Soos") have revealed a genome significantly enriched with prokaryotic-derived sequences acquired through HGT (Rossoni et al., 2019; Schönknecht et al., 2013).

Overall, the combination of its metabolic plasticity, photosynthetic competence, and likely genomic adaptations makes strain 734 a promising model for studies on adaptation to extreme environmental conditions and metabolic strategies of thermo-acidophilic microalgae.

1.3 The Oxygenic Photosynthesis: a brief description

Photosynthesis is a fundamental biological process through which organisms such as cyanobacteria, algae, and plants convert solar energy into chemical energy, sustaining almost all life on Earth (Junge, 2019; Kallio et al., 2025; Stirbet et al., 2019). Photosynthesis involves pigments such as chlorophylls and carotenoids that absorb photons and use that energy to facilitate the transformation of water and carbon dioxide into energy-rich organic compounds (simple and complex sugars), releasing molecular oxygen as a byproduct (Junge, 2019; Kallio et al., 2025; Stirbet et al., 2019).

Depending on the organism and the nature of the electron/proton donor (e.g., H_2O, H_2S, H_2), two types of photosynthesis are known: anoxygenic and oxygenic (Junge, 2019; Kushkevych et al., 2021).

The overall reaction of oxygenic photosynthesis can be written as,

$$\mathbf{6\ CO_2 + 6\ H_2O + light\ energy \rightarrow C_6H_{12}O_6 + 6\ O_2}$$

The evolution of anoxygenic photosynthesis predates that of oxygenic photosynthesis and is estimated to have occurred approximately 3.8 billion years ago (Cardona, 2019; Crowe et al., 2013). Anoxygenic photosynthetic bacteria still existing today include purple, sulfur, and non-sulfur green, and heliophilic bacteria (Adessi & De Philippis, 2013; Olson, 2006). These organisms use electrons from easily oxidizable sources such as H_2 and H_2S, but these are limited to certain environments such as volcanic ones (Cardona, 2019; Olson, 2006).

The need for a more abundant and stable source of electrons led to the evolution of oxygenic photosynthesis (Crowe et al., 2013; Stirbet et al., 2019). Cyanobacteria, which appeared about 2.5 billion years ago, produced oxygen thanks to their ability to use water as an electron donor,

transforming the Earth's atmosphere from reducing to oxygenated, with the first significant levels of oxygen appearing about 2.2 billion years ago (Crowe et al., 2013; Junge, 2019). This innovation had profound effects on ecosystems, atmospheric composition, and the carbon cycle, highlighting the importance of understanding photosynthetic mechanisms across different organisms (Kallio et al., 2025; Stirbet et al., 2019).

Almost all life on Earth depends on photosynthesis since it provides food and oxygen essential for respiration and fundamental for our survival (Masojídek et al., 2013).

Oxygenic photosynthesis occurs within the thylakoid membranes, which are located in the chloroplasts of eukaryotic photoautotrophs or in the cytoplasmic membrane systems of cyanobacteria (Johnson, 2016; Shimoni et al., 2005). In chloroplasts (Fig. 3), the thylakoid membranes are organized into *grana* (stacked regions) and *stroma lamellae* (unstacked regions), both surrounded by the stroma, whereas the internal compartment of the thylakoids is referred to as the *lumen* (Johnson, 2016; Shimoni et al., 2005). According to the endosymbiotic theory, chloroplasts are the result of the absorption of ancient cyanobacteria by an eukaryotic cell (Sato, 2020; Zimorski et al., 2014).

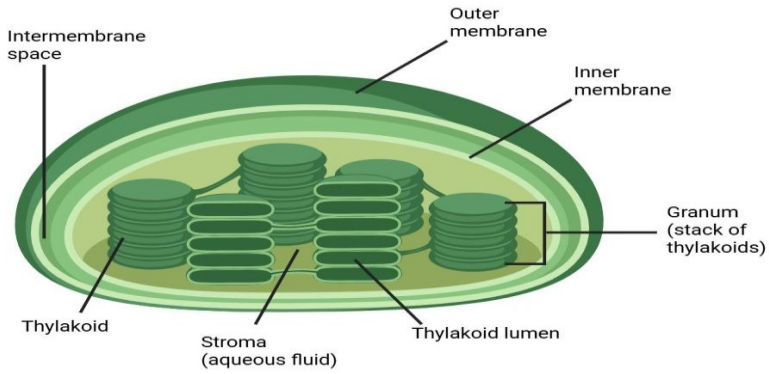

Figure 3. Schematic structure of a chloroplast. Cross-section illustrating the main compartments of the organelle responsible for photosynthesis. The structure is defined by the outer membrane and the inner membrane, separated by the intermembrane space. The internal space, a dense fluid called the stroma. Within the stroma are the thylakoids, flattened sacs containing chlorophyll; stacks of thylakoids form the grana. The thylakoid lumen is the space enclosed by the thylakoid membranes.

The photosynthetic process can be divided into two distinct yet interconnected phases (Fig. 4): the light-dependent reactions and the light-independent reactions (Calvin–Benson cycle) (Calvin & Benson, 1948; Martin et al., 2000).

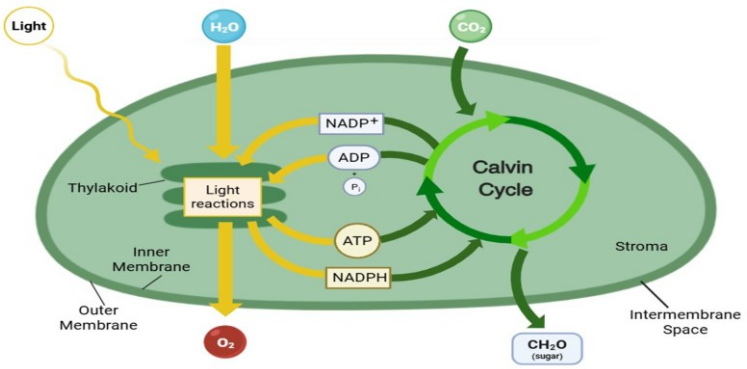

Figure 4. Overview of oxygenic photosynthesis. Schematic diagram illustrating the two interconnected stages of photosynthesis within the chloroplast. The light reactions occur in the thylakoid, utilizing light and H_2O to produce ATP, NADPH, and O_2. These energy carriers then power the Calvin-Benson cycle in the stroma, which fixes CO_2 to synthesize CH_2O (sugar).

Light-dependent reactions occur in the thylakoid membranes and are responsible for the conversion of solar energy into chemical energy in the form of ATP and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) (Stirbet et al., 2019). In this phase, photosynthetic pigments absorb photons, promoting electrons to higher energy states. These excited electrons are transferred through a series of redox carriers embedded in the thylakoid membrane (Johnson, 2016).

Dark reactions or Calvin-Benson cycle take place in the stroma of the chloroplast and do not require light directly (Calvin & Benson, 1948; Martin et al., 2000). The ATP and NADPH produced in the light reactions are used to reduce carbon dioxide and synthesize carbohydrates according to the following overall reaction:

$$\mathbf{6CO_2+12NADPH+18ATP \rightarrow C_6H_{12}O_6+12NADP^+ +18ADP+6H_2O+18P_i}$$ (Hill & Bendall, 1960).

Photosynthesis uses only photons within a wavelength range called Photosynthetically Active Radiation (PAR), corresponding to wavelengths from 400 to 700 nm (McCree, 1971).

Photon absorption occurs through photosynthetic pigments, which transfer the absorbed energy to the Reaction Centers (RC) where photosynthesis occurs; photons must possess sufficient energy to excite an electron in the pigment molecule and thus initiate charge separation (Hohmann-Marriott & Blankenship, 2011). According to Richmond (2008), the pigments most commonly found in microalgae are chlorophylls, phycobilins, and carotenoids (Fig. 5).

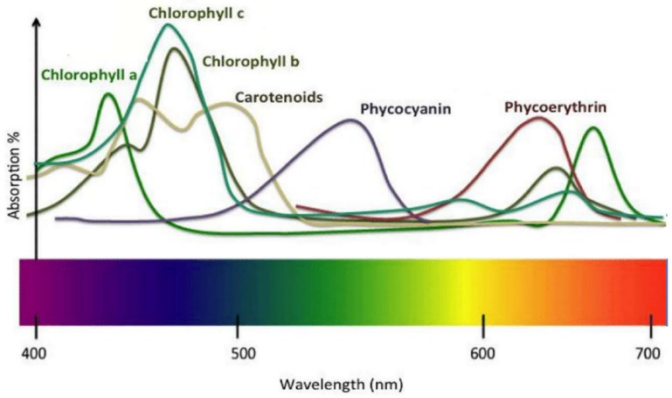

Figure 5. Absorption spectra of the main pigments found in microalgae: chlorophylls, phycobilins, and carotenoids. Adapted from Vieira et al. (2025).

Two classes of ubiquitous pigments can be distinguished in photosynthesizing organisms: chlorophylls and carotenoids. Another specific class of pigments, phycobiliproteins, is also present in cyanobacteria and some algal groups (red algae) (Chang et al., 2015; Kovaleski et al., 2022).

Chlorophylls (Fig. 6) are metalloporphyrin pigments belonging to the tetrapyrrole family. They are characterized by a cyclic tetrapyrrole ring, with a coordinated Mg atom at the center, and a long-chain aliphatic alcohol, phytol, esterified to a propionic acid residue at position 7 (Kirk, 1987).

Cyanobacteria and red algae contain only chlorophyll *a*, while green eukaryotes contain two types of chlorophyll: chlorophyll *a* and chlorophyll *b*. These differ only in the presence of a methyl or aldehyde group at the C3 position of the second pyrrole ring, respectively (Beale, 1999).

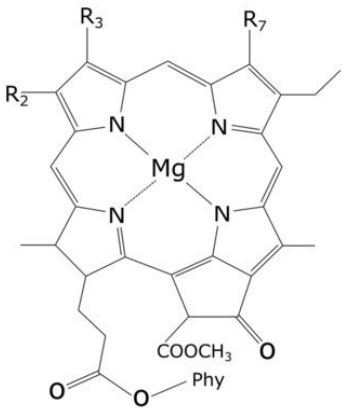

Figure 6. Structure of chlorophyll (Wolf & Blankenship, 2019).

1.3.1 Photosystem II (PSII) and water splitting

Photosystems are multi-subunit transmembrane pigment–protein complexes that catalyze electron transport from donors on the inner surface of the thylakoid membrane to acceptors on the outer surface (Ballottari et al., 2012; Boekema et al., 1995). Each photosystem consists of a central complex, which contains the RC where light-driven charge separation and the initial electron transfer occur, and a peripheral antenna that captures light and transfers excitation energy to the RC, enhancing photon absorption efficiency (Ballottari et al., 2012; Boekema et al., 1995). While the core subunits are highly conserved through evolution, the peripheral antenna proteins show greater diversity and adaptability (Alboresi et al., 2008).

Photosystem II (PSII) (Fig. 7) is a dimeric complex composed of multiple proteins, present in the thylakoidal membrane of microalgae (Cardona, 2019; Kok et al., 1970). It plays a crucial role in the first thermodynamic reaction of oxygenic photosynthesis, specifically the oxidation of water (Barber, 2008; Renger & Renger, 2008). Thanks to the energy of light, PSII facilitates the separation of two water molecules, producing electrons, protons, and molecular oxygen, which are fundamental for the subsequent phases of photosynthesis (Arnon et al., 1954; Barber, 2008; Bhattacharjee et al., 2024).

The RC represents the functional heart of the PSII and is made up of a heterodimer of the D1 and D2 subunits (Fig. 7), which manage the main cofactors that participate in electron transfer: the electron-donating pigment (P680), pheophytin, and the quinones plastoquinone QA and QB (Barber, 2008; Nelson & Yocum, 2006). Between these parts, the D1 subunit is particularly vulnerable to light damage, so it is continually replaced and repaired, an essential process for maintaining the photochemical efficiency of PSII (Barber, 2008; Nelson & Yocum, 2006).

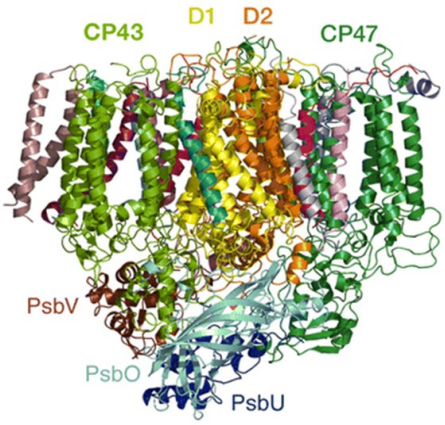

Figure 7. Structure of the Photosystem II (PSII). This complex is embedded in the membrane, driving water splitting, with core subunits (D1, D2, CP43, CP47) and accessory proteins (PsbO, PsbU, PsbV) oriented between the stroma and the lumen (Bhattacharjee et al., 2024).

In the part of the thylakoidal lumen, near the RC, there is the Oxygen Evolving Complex (OEC), the place where water splitting occurs (Barber, 2008; Chrysina et al., 2023; Vogt et al., 2015). The OEC is a heterometallic structure formed by an Mn_4CaO_5 cluster, which is responsible for accumulating four oxidative equivalents during the cycle of S (S_0-S_4) states (Barber, 2008; Chrysina et al., 2023; Vogt et al., 2015). At the end of this process, a molecule of oxygen is released (Barber, 2008; Chrysina et al., 2023; Vogt et al., 2015). Structural analyses on the PSII of microalgae, such as *Cyanidium caldarium*, have highlighted notable differences in the composition of extrinsic proteins compared to those present in cyanobacteria, suggesting the existence of specific stabilization strategies to optimize the performance and protection of the OEC (Ago et al., 2016; Bricker et al., 2012; Ifuku & Noguchi, 2016). Diatoms, like other microalgae, have different adaptations: PSII includes the extrinsic protein Psb31, not present in cyanobacteria or green plants, which binds directly to the core of the complex and contributes to the stability of the OEC (Levitan et al., 2019; Okumura et al., 2008). Cryptophytes, for example, *Chroomonas placoidea*, maintain an extrinsic protein set similar to that of red algae (PsbO, PsbU, PsbV, PsbQ′) but lacking Psb31, suggesting alternative OEC stabilization strategies (Zhang et al., 2024). Finally, in green algae, such as *Chlamydomonas reinhardtii*, the extrinsic subunits PsbO, PsbP, and PsbQ show peculiar organization and interactions compared to those of higher plants, while accessory factors such as Psb2 contribute to the protection of the complex during assembly (Fadeeva et al., 2023; Umena et al., 2011). The collection of solar energy is carried out by antenna complexes (Light-Harvesting Complex II, LHCII), which transfer the absorbed energy to the PSII RC (Mekala et al., 2015; Minagawa, 2011; Zhang et al., 2024). In microalgae, these complexes can be subject to phosphorylation and subsequently repositioned towards Photosystem I (PSI) during

State Transitions processes (Minagawa, 2011; Zhang et al., 2024). This dynamic adaptation mechanism allows light absorption to be regulated and photosynthetic efficiency to be improved in response to environmental variations (Minagawa, 2011; Zhang et al., 2024).

1.3.2 Photosystem I (PSI) and NADPH formation

Photosystem I (PSI) (Fig. 8) is one of two large photosynthetic complexes responsible for converting light energy into chemical energy in microalgae (Cardol et al., 2011; Duysens & Amesz, 1962; Thangaraj et al., 2011). After absorbing photons, PSI transfers electrons to stromal acceptors, mainly ferredoxin, which then carries the electrons to ferredoxin–$NADP^+$ reductase (FNR), allowing the final reduction of $NADP^+$ to NADPH (Cardol et al., 2011). This electron transfer process is at the heart of Linear Electron Flow (LEF), providing the reducing power necessary for carbon fixation in the Calvin-Benson cycle and other chloroplast anabolic pathways (Cardona, 2019; Johnson, 2016).

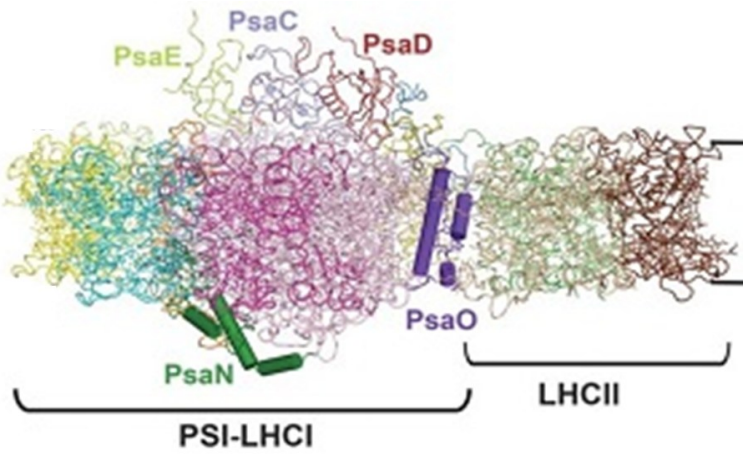

Figure 8. Structure of the Photosystem I (PSI). Light-Harvesting Complex I (LHCI) supercomplex with associated LHCII. The views show the circular organization of the subunits and the transmembrane, highlighting some key external subunits such as PsaC, PsaD, PsaE, PsaN, and PsaO, orientation between the stroma and the lumen, essential for photosynthesis (Miller, 2018).

In many unicellular microalgae, such as *Chlamydomonas reinhardtii*, the interaction dynamics between PSI, ferredoxin (Fd), and Ferredoxin-$NADP^+$ Reductase (FNR) are highly flexible and regulated as a function of the availability of stromatic acceptors and environmental conditions, thus modulating the net production of NADPH (Milrad et al., 2024; Mosebach et al., 2017). Microalgae, such as *C. reinhardtii*, also have alternative (pseudocyclic) electron drainage pathways, such as oxygen photoreduction or transfers to enzymes such as hydrogenase or plastoquinone-reducing type II NAD(P)H dehydrogenase (Nda2), which reduce the formation of NADPH but protect the

photosynthetic system from overreduction phenomena and oxidative stress (Peltier et al., 2023). Besides, in *C. reinhardtii,* the dynamic between PSI, Fd, and FNR is highly regulated as a function of environmental conditions, the availability of stromatic acceptors, and the need to balance ATP and NADPH (Huang et al., 2021; Takahashi et al., 2006).

Other microalgae show similar or alternative strategies to regulate NADPH production. In *Synechocystis sp.* PCC 6803, PSI can be engineered into *in vitro* systems to regenerate NADPH through PSI-Fd-FNR protein spindles, highlighting the versatility and efficiency of PSI in electron transfer (Medipally et al., 2023). In *Nannochloropsis sp.* and *Phaeodactylum tricornutum*, PSI can form dimers or interact with additional LHC complexes, increasing the stability of electron transfer and optimizing the production of NADPH under variable light conditions (Naschberger et al., 2022; Nikkanen et al., 2021).

1.3.3 Electron transport chain (ETC) and ATP synthesis

The photosynthetic Electron Transport Chain (ETC) (Fig. 9) connects the two photosystems and is responsible for transferring electrons from water to $NADP^+$, coupling this process with the translocation of protons across the thylakoid membrane and the generation of a proton motive force (ΔpH) used by ATP synthase (Arnon et al., 1954; Hill & Bendall, 1960; Nikkanen et al., 2021; Peltier et al., 2010). This process, known as LEF (Fig. 9 and Fig. 10A), involves the movement of electrons in a unidirectional path from water in PSII to $NADP^+$ via PSI, leading to the production of both ATP and NADPH (Arnon et al., 1954; Hill & Bendall, 1960). The ETC is composed of several protein complexes and mobile electron carriers: PSII, plastoquinone (PQ), cytochrome b_6f (Cyt b_6f), plastocyanin (PC), PSI, Fd, and FNR (Hill & Bendall, 1960; Nikkanen et al., 2021; Peltier et al., 2010).

After water oxidation in PSII, electrons are transferred to the primary quinone acceptor QA and then to the secondary quinone QB, which, once fully reduced and protonated, diffuses into the thylakoid membrane as plastochinol (PQH_2) (De Causmaecker et al., 2019; Tikhonov, 2018). PQH_2 donates electrons to the Cyt b_6f complex, which manages the so-called Q cycle, a process that recycles electrons and moves additional protons from the stroma to the lumen, increasing the proton gradient (De Causmaecker et al., 2019; Tikhonov, 2018).

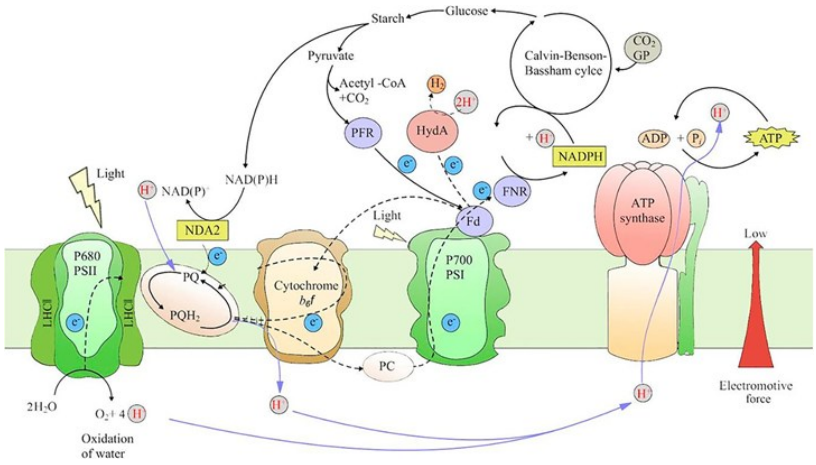

Figure 9. Schematic representation of the photosynthetic electron transport chain. Fd: ferredoxin; FNR: ferredoxin–NADP$^+$ reductase; NADP: nicotinamide adenine dinucleotide phosphate; PC: plastocyanin; PQ: plastoquinone; PSI: photosystem I; PSII: photosystem II; LHCII: light-harvesting complex II; Nda2: NADPH dehydrogenase; ATP: adenosine triphosphate (Grechanik & Tsygankov, 2022).

However, LEF alone is often insufficient to meet the ATP demand for carbon fixation and other metabolic processes (Laughlin et al., 2020).

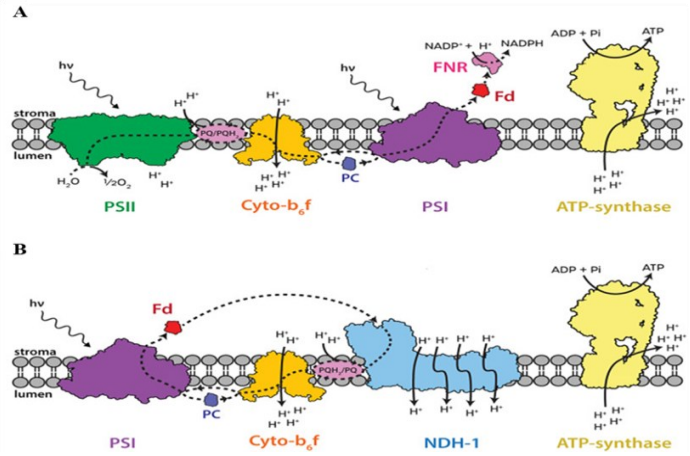

Figure 10. Light reactions of oxygenic photosynthesis. (A) linear/non-cyclic photophosphorylation (LEF), where electrons move from Photosystem II (PSII) to Photosystem I (PSI), reducing NADP$^+$ to NADPH and generating ATP; and **(B)** cyclic photophosphorylation (CEF), where electrons from Photosystem I (PSI) are recycled back through the cytochrome b6f complex and NDH-1 to generate ATP but no NADPH. Adapted from Laughlin (2020).

In addition to LEF, photosynthetic organisms and microalgae are capable of performing a Cyclic Electron Flow (CEF) around PSI (Fig. 10B) (Peltier et al., 2010; Ruben, 1943), where electrons are

recycled from Fd back to the cytochrome b_6f complex, enhancing the proton motive force (pmf) and ATP production without generating NADPH (Nawrocki et al., 2019; Peltier et al., 2023).

These cyclic routes, mediated by proteins such as PGR5/PGRL1 or the Nda2 enzyme, are crucial for balancing the ATP/NADPH ratio under variable light or nutrient conditions (Johnson et al., 2014; Peltier et al., 2024). Unlike the linear pathway, CEF does not produce NADPH or oxygen, thus serving as a regulatory mechanism to balance the ATP/NADPH ratio required by the Calvin–Benson cycle and other metabolic processes (Shikanai, 2014). The dynamic regulation of LEF and CEF enables photosynthetic organisms to adapt to changing environmental and energetic conditions, ensuring efficient energy conversion and photoprotection (Nikkanen et al., 2018).

Microalgae employ CEF around PSI in addition to linear electron flow (Milrad et al., 2024), but, in addition, they possess Pseudocyclic Electron Flow (PCEF) pathways, such as flavodiiron protein (FLV)-mediated oxygen photoreduction, which act as alternative electron sinks when $NADP^+$ reduction is limited (Chaux et al., 2017). These pathways are essential for maintaining redox balance and protecting the photosynthetic apparatus from over-reduction and oxidative stress (Gerotto et al., 2016).

Interestingly, although the NAD(P)H dehydrogenase complex (NDH complex) is important in the CEF of higher plants, many green algae do not use this mechanism or have it only in a limited form. These algae depend mainly on Nda2 for the electron cycle (Peltier et al., 2024). In *Chlamydomonas reinhardtii*, it has been observed that CEF, PCEF, and electron flow from chloroplasts to mitochondria collaborate and balance each other to enhance CO_2 assimilation (Peltier et al., 2024).

Moreover, in extremophilic algae such as *Chlorella ohadii*, a unique PSII-associated cyclic electron flow (PSII-CEF) mechanism contributes to photoprotection and high photosynthetic performance under intense light, mitigating PSII photoinhibition (Ananyev et al., 2017; Fadeeva et al., 2023).

In *C. reinhardtii*, the formation of the PSI–LHCI–LHCII supercomplex during state 2 transition (Huang et al., 2021) can also influence ETC organization and pmf distribution. The addition of two LHCII trimers increases PSI light-harvesting capacity and modifies the balance between linear and cyclic flows, potentially affecting Fd reduction rates and ATP synthesis efficiency (Huang et al., 2021; Joliot et al., 2022). Pigments at the PSI–LHCII interface (chlorophylls and carotenoids) act as energy bridges and quenching sites, promoting controlled dissipation of excess excitation and stabilizing electron transport (Huang et al., 2021; Joliot et al., 2022).

The synergistic functioning of LEF, CEF, PCEF, and alternative electron transfer pathways provides microalgae with significant bioenergetic adaptability, allowing them to rapidly align to environmental changes and optimize the balance between ATP and NADPH generation, which is essential for metabolism, growth, and photoprotection (Cardol et al., 2011; Milrad et al., 2024).

1.4 Photosynthesis in red algae

Photosynthetic organisms have evolved diverse strategies to optimize light capture, adapting to variable environmental conditions (Miyake, 2020; Sonoike, 2025).

Terrestrial plants and many green algae employ complex antenna complexes integrated into the thylakoid membrane (LHCs) that absorb in the blue (~430-470 nm) and red (~660-680 nm) bands thanks to chlorophylls *a* and *b* (Fig. 11) (Anderson, 1986; Kosugi et al., 2024). In contrast, cyanobacteria and red algae (Rhodophyta) possess external soluble antennas called phycobilisomes (PBS), located on the stromal side of PSII (Satpati & Pal, 2020).

Besides the PBS, red algae have evolved additional pigment adaptations, such as chlorophyll *d* or other shifted chlorophyll variants, allowing absorption up to approximately 710 nm and broadening the light spectrum they can utilize (Cheng et al., 2019; Larkum & Kühl, 2005). The use of pigments that operate at different wavelengths allows certain red algae to thrive in environments with filtered light (Fig. 11) (Cheng et al., 2019; Larkum & Kühl, 2005).

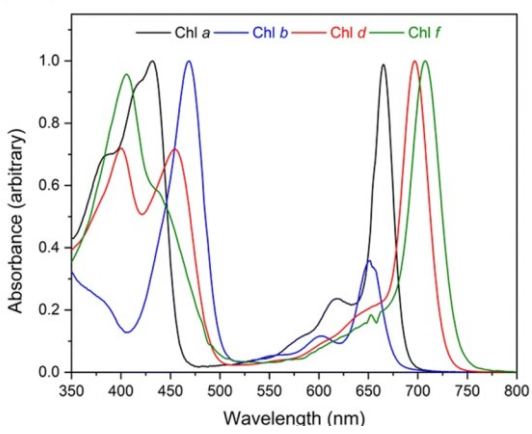

Figure 11. Absorption spectra of chlorophylls a, b, d, and f in red algae. The spectra illustrate the wavelength range of light absorbed by each type of chlorophyll, showing how chlorophylls *d* and *f* extend the usable light spectrum beyond that of chlorophylls *a* and *b* (Wolf & Blankenship, 2019).

Phycobilisomes consist of phycobiliproteins such as phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC), which absorb light in the ~500-650 nm region, less effectively captured by Chl *a* alone (Puzorjov & McCormick, 2020). The typical PBS structure (Fig. 12) features a central core of APCs from which PC and PE "rods" extend and converge in an energy funnel towards the core (Li et al., 2023). These structures efficiently capture light and transfer it to the chlorophyll *a* of RC (Dagnino-Leone et al., 2022; Grossman et al., 1993). This arrangement allows photosynthetic organisms, such as red algae and cyanobacteria, to utilize wavelengths of light that are not efficiently absorbed by chlorophyll, enabling adaptation to variable light conditions, including low-light or deep-water environments (Li et al., 2023; Mascoli et al., 2022).

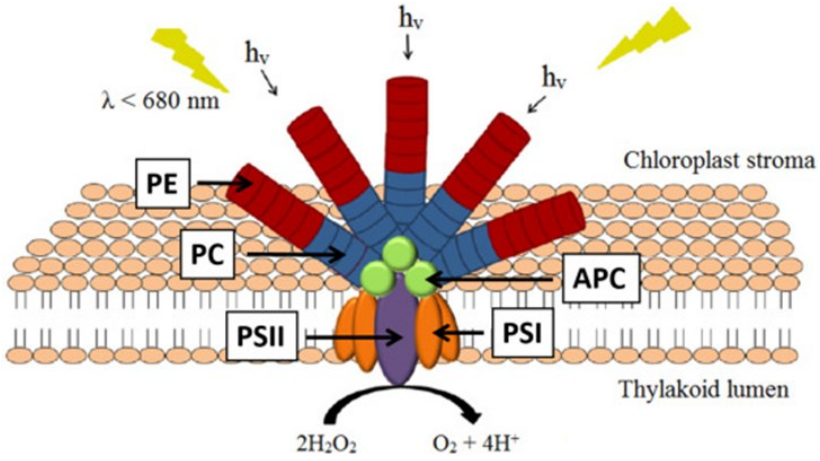

Fig. 12. Structure of the phycobilisome (PBS). This light-harvesting complex is organized from different phycobiliproteins (PBP) to efficiently transfer energy unidirectionally from phycoerythrin (PE, red) to phycocyanin (PC, blue), allophycocyanin (APC, green), and finally to the RC of PSII (purple) and PSI (orange) (Fernández-Rojas et al., 2014).

In the genus *Galdieria*, a red extremophilic alga, studies have detected the presence of PC and APC, while PE is found in variable amounts depending on the species and environmental conditions (Chini Zittelli et al., 2023; Retta et al., 2024). This composition enables *Galdieria* to thrive under a wide range of light conditions, including extreme habitats such as hot springs and acidic environments (Perez Saura et al., 2022).

Linker polypeptides maintain the structural integrity of PBS, connect the rods to the nucleus, and mediate energy transfer between chromophores and cores (Li et al., 2023; Puzorjov & McCormick, 2020). Chromophore-linker networks favor ultra-rapid transfers (in the order of a few ps) and maintain an efficient energy flow towards the photosystem RC (Dodson et al., 2023). Experimental

studies indicate that the transfer efficiency of PBS towards RC can reach values of the order of 90-95 % (Dodson et al., 2023).

In extremophiles such as *Galdieria sulphuraria*, PBS shows structural adaptations to ensure thermal and acid stability, with subunits capable of withstanding high temperatures and low pH (Puzorjov & McCormick, 2020). For example, phycobiliproteins isolated from *Galdieria* are more stable at 56 °C and in pHs between 1 and 5 than other species (Carfagna et al., 2018; Puzorjov & McCormick, 2020). Transfer mechanisms have also been observed in *Galdieria* in "far-red" light conditions, suggesting flexibility in the energy exchange between PBS, PSI, and PSII (Stadnichuk et al., 2011).

From an evolutionary perspective, the red algal lineage has retained phycobilisomes (PBS), large soluble/peripheral antenna complexes associated with the stromal surface of the thylakoid membrane, whereas the green lineage has evolved membrane-integrated chlorophyll-based light-harvesting complexes (LHCs) (Bag, 2021). In algae species that grow in hot or acidic environments, more resistant subunits and connectors have been discovered, which protect functionality in extreme conditions (Puzorjov & McCormick, 2020). The mobility of PBS varies between species, and in many extreme red algae, PBS is relatively fixed, influencing photoprotection mechanisms and state transitions (Kaňa et al., 2014).

Beyond light capture, red algae have also evolved specialized mechanisms for carbon fixation. During the photosynthesis cycle, many red algae have forms of RuBisCO referred to as “red type,” which may have superior kinetic characteristics compared to those of RuBisCO in green plants (Oh et al., 2023). In practical terms, this interest in red RuBisCO has encouraged engineering research for more efficient versions for introduction into green plants (Oh et al., 2023).

In addition to pigments and carbon fixation, red algae possess specialized PSII components that enhance stability in extreme conditions. In red algae, such as *Cyanidioschyzon merolae, Porphyridium purpureum*, and *Cyanidium caldarium*, PSII includes the proteins PsbO, PsbU, PsbV, and PsbQ′, the latter derived from CyanoQ, which play a key role in protecting the complex under extreme environmental conditions (Ifuku & Noguchi, 2016; Yamada et al., 2018). In particular, the PsbQ′ protein, which is unique to red algae, represents an evolutionary intermediate form between the complexes found in cyanobacteria and those in higher plants (Ago et al., 2016; Ifuku & Noguchi, 2016). It is essential for the assembly and stability of PSII, although it alone is not sufficient to restore full photosynthetic activity in the absence of the other subunits (Ago et al., 2016; Enami et al., 2000). Overall, these extrinsic proteins give PSII greater stability, allowing red algae to adapt effectively to acidic environments and high temperatures, thus distinguishing themselves from other microalgae

(Enami et al., 2000; Ifuku & Noguchi, 2016). Fourier Transform InfraRed Spectroscopy (FTIR) structural and spectroscopic analyses reveal that removing the extrinsic proteins severely alters the conformation of the oxidizing center, impairing oxygen evolution; reconstitution with PsbV and the other subunits restores both the native-like structure and catalytic activity, highlighting their strong functional interdependence (Ifuku & Noguchi, 2016). From an evolutionary perspective, the coexistence of cyanobacterial proteins (PsbO, PsbU, PsbV) together with the innovative element PsbQ′ makes red algae an intermediate example in the evolution of PSII, demonstrating both structural and ionic adaptation to photosynthesis in extreme aquatic conditions (Enami et al., 2000; Ifuku & Noguchi, 2016; Yamada et al., 2018).

In conclusion, photosynthesis in red algae is characterized by the use of highly effective PBS, red-type RuBisCO, and PSII with particular extrinsic subunits, factors that offer these organisms great ecological and metabolic flexibility (Kaňa et al., 2014; Oh et al., 2023). These adaptations allow them to live and develop in extreme conditions, serving as an evolutionary and biotechnological example for optimizing photosynthesis in other contexts (Rojas-Villalta et al., 2024).

1.4.1 Chlorophylls and phycobilins

The chlorophyll biosynthesis pathway is a multi-step process that creates chlorophyll molecules for photosynthesis. It begins with the amino acid glutamate, which is converted into 5-aminolevulinic acid (ALA), the universal precursor for all tetrapyrroles (Wang et al., 2022). This precursor is then used to form the tetrapyrrole ring system, and after several enzymatic steps, including the insertion of magnesium into protoporphyrin IX catalyzed by chlorophyll synthetase, and the subsequent addition of a phytol tail to generate functional chlorophyll (Sarian et al., 2016; Walker & Weinstein, 1994; Willows, 2019). Therefore, chlorophylls are cyclic derivatives of porphyrins, tetrapyrroles that contain a chelated Mg^{2+} and a characteristic fifth ring (Porra, 1997; Sarian et al., 2016).

Phycobiliproteins, in contrast, are water-soluble chromoproteins containing phycobilins as chromophores (MacColl, 1998). These linear tetrapyrroles are derived from the same biosynthetic pathway as heme and chlorophyll but do not incorporate a metal ion (Brown et al., 1990).

Tetrapyrroles in general are macrocyclic molecules with distinct structural and functional properties (Brown et al., 1990; Brzezowski et al., 2015). The different chemical and physical properties of tetrapyrroles are defined by their molecular structure of conjugated double bonds, the difference of substituted side chains, and the chelation of metal ions (Brzezowski et al., 2015). Thus, depending on

their molecular structure, tetrapyrroles are specialized to absorb visible light or to accept various redox states (Hu et al., 2024). In plants, the tetrapyrrole biosynthesis pathway occurs in plastids, branched towards the formation of different tetrapyrrole end-products through several enzymes (Tanaka & Tanaka, 2007). A wide range of regulatory mechanisms ensures adequate synthesis of tetrapyrrole in plants, adapting them at any time of their development and to any change of environmental condition (Brzezowski et al., 2015; Tanaka & Tanaka, 2007; Wang et al., 2022). Tetrapyrroles biosynthesis is highly regulated to avoid the accumulation of intermediates (of their biosynthetic pathway) that can be photoactively oxidized, leading to the generation of highly reactive oxygen intermediates and subsequent cellular damage (Brzezowski et al., 2015; Wang et al., 2022).

1.4.2 Photosynthesis in *Galdieria*

To survive in extreme environments, photosynthesis in extremophilic microalgae must be tightly regulated to prevent excess energy that could lead to oxidative stress, particularly through the production of reactive oxygen species (ROS) generated by PSI (Miyake, 2020; Shimakawa & Miyake, 2018; Sonoike, 2025).

The photosynthetic systems of *Galdieria* spp. and *Cyanidioschyzon merolae* possess unique molecular adaptations that enhance both photoprotection and metabolic flexibility. These include the ability to regulate CEF around PSI and to modulate antenna complexes, allowing an optimal balance between light absorption and photosynthetic efficiency (Joliot et al., 2022; Klughammer & Schreiber, 1994; Oesterhelt, Schmälzlin, et al., 2007). Moreover, *Galdieria* is able to modulate its photosynthetic activity in response to the availability of organic substrates (Gross & Oesterhelt, 1999; Oesterhelt, Schmälzlin, et al., 2007). The presence of glucose, for example, has been shown to suppress the biosynthesis of Chl *a* and phycocyanobilin at the level of coproporphyrinogen III formation, indicating a close link between carbon metabolism and pigment production (Gross & Oesterhelt, 1999). Furthermore, exogenous glucose has been shown to reduce oxygen evolution and PSII activity, accompanied by a decrease in D1 and RuBisCO protein levels (Oesterhelt, Schmälzlin, et al., 2007). These results have established that *Galdieria* can downregulate photosynthesis under mixotrophic or heterotrophic conditions, using organic carbon sources to minimize photo-oxidative stress (Gross & Oesterhelt, 1999; Oesterhelt, Schmälzlin, et al., 2007).

On the molecular level, Marquardt et al. (2001) in *Galdieria sulphuraria* identified a multigene family encoding the light-harvesting polypeptides associated with PSI (LHCI), revealing

evolutionary divergence from both green algal and cyanobacterial antenna proteins. The gene family displayed sequence and compositional features consistent with adaptation to extreme light and temperature conditions. Subsequent work by Thangaraj et al. (2011) provided a structural and spectroscopic characterization of the PSI–LHCI supercomplex in *G. sulphuraria*, demonstrating that it consists of seven to nine LHCI subunits that are tightly coupled to the PSI core. Optical spectroscopy and ultrafast fluorescence measurements have shown that these LHCI subunits are functionally very well coupled to the PSI core, allowing highly efficient excitation energy transfer with minimal loss, an arrangement more integrated than that found in most other eukaryotic photosynthetic systems. These observations set the stage for recent advances in structural biology. Cryo-electron microscopy further revealed that *Galdieria*'s PSI lacks phylloquinones, which normally act as secondary electron acceptors, and instead contains a benzoquinone-like molecule, indicating a unique evolutionary adaptation within the red algal lineage (Nagao et al., 2025). Moreover, the PSI–LHCI complex also includes RedCAP, a red-lineage chlorophyll-binding protein that stabilizes the supercomplex, as well as carotenoids such as zeaxanthin, β-cryptoxanthin, and β-carotene, which contribute to photoprotection and Non-Photochemical Quenching (NPQ) (Nagao et al., 2025). Moreover, the blue-shifted absorption spectra of the LHCI subunits enhance photon capture in the short-wavelength region, optimizing energy utilization under the fluctuating and intense light conditions typical of *Galdieria* habitats (Nagao et al., 2025).

Complementary to PSI adaptations, studies on redox regulation of chloroplast enzymes revealed that enzymes of the Calvin–Benson cycle (such as PRK, GAPDH, and FBPase) are subject to redox control in *G. sulphuraria* (Oesterhelt, Klocke, et al., 2007), suggesting an integrated system that coordinates energy flow and carbon fixation with environmental fluctuations.

As described, photosynthesis in *Galdieria* must be tightly regulated to avoid oxidative stress (Miyake, 2020). This regulation is intimately linked to light, which acts not only as an energy source but also as a signal that modulates gene expression and protection pathways from intense light damage (Albertano et al., 2000; Castenholz & McDermott, 2010; Stadnichuk & Tropin, 2022). In the extreme environments inhabited by some microalgae, such as the *Cyanidiales* (*Galdieria* spp., *Cyanidioschyzon merolae*, and *Cyanidium* spp.), light management is of fundamental importance, as these species often thrive under high irradiance, for example, in hot springs or on acidic surfaces exposed to solar radiation (Abram et al., 2022; Stadnichuk & Tropin, 2022; Villegas-Valencia et al., 2023).

However, in certain ecological niches, *Galdieria* species also colonize endolithic environments, where less than 1% of surface photon flux reaches the cells (Thangaraj et al., 2011). In these dimly lit habitats, *Galdieria sulphuraria* must finely regulate its photosynthetic machinery and gene expression to efficiently use the limited light available. Such regulation includes modifications in antenna protein expression, pigment composition, and energy dissipation pathways to optimize light harvesting while preventing photodamage when light intensity suddenly increases (Nagao et al., 2025).

In addition to photosynthetic regulation, light acts as a transcriptional signal, influencing the expression of genes involved in pigment biosynthesis, antioxidant mechanisms, and carbon metabolism (Abiusi, Moñino Fernández, et al., 2022; Curien et al., 2021). Under light stress or changes in light intensity, microalgae such as *Galdieria phlegrea* and *Galdieria sulphuraria* can activate protective pathways, including the accumulation of carotenoids and C-phycocyanin, which play roles both in photoprotection and nutrition (Aizpuru & González-Sánchez, 2024; Carfagna et al., 2018). These processes are closely linked to the species' metabolic flexibility, allowing them to shift from autotrophic to mixotrophic or heterotrophic modes depending on light and carbon availability, thereby maximizing the conversion of light energy into biomass and bioactive metabolites (Masson et al., 2025).

In conclusion, light in extremophilic microalgae is not merely an energy source for photosynthesis but also a key regulator that coordinates photosystem activity, oxidative stress protection, and gene expression, enabling these species to adapt to harsh environments characterized by fluctuating light and adverse conditions (Abram et al., 2022; Nagao et al., 2025; Stadnichuk & Tropin, 2022).

1.5 Biotechnological relevance of *Galdieria*

The ecological and physiological traits of *Galdieria* make it a microorganism of great biotechnological interest (Ambrosino et al., 2023; Perez Saura et al., 2022). Indeed, its ability to grow in environments where most photosynthetic organisms cannot survive opens the way to production processes with a low risk of microbial contamination and to the treatment of challenging aqueous matrices (Retta et al., 2024; Selvaratnam et al., 2014). This advantage implies that facilities cultivating *Galdieria* can drastically reduce sterilization requirements and the associated costs, thereby making certain pilot- and industrial-scale applications more economically feasible (Carone et al., 2024). Another key feature is its exceptional metabolic flexibility: species such as *G. sulphuraria* and *G. phlegrea* can grow autotrophically, heterotrophically, or mixotrophically, and

utilize a wide range of organic carbon sources, including lactose-rich substrates or dairy waste, as demonstrated in cultures on buttermilk (Occhipinti et al., 2023). This plasticity allows for the design of highly diverse processes, from illuminated photobioreactors to dark reactors for heterotrophic growth, which can be tailored to local resources (agro-industrial residues, effluents) and the desired economic strategy (maximizing pigments/proteins or treating pollutant loads) (Pleissner et al., 2023). Recent reviews highlight how this versatility makes *Galdieria* particularly suitable for circular bioeconomy chains, where biomass production can be combined with waste valorization (Anyaoha et al., 2024).

From a product perspective, *Galdieria* offers several high-value potentials (Retta et al., 2024; Rojas-Villalta et al., 2024). The microalga synthesizes phycobiliproteins (e.g., C-phycocyanin), carotenoids, and a variety of antioxidant compounds that are naturally more stable under thermal and acidic conditions compared to the corresponding products from mesophilic organisms, a feature of particular interest for food, cosmetic, and diagnostic applications (Carfagna et al., 2016; Occhipinti et al., 2023; Retta et al., 2024). Moreover, the biomass can serve as a feedstock for valorization processes, including pigment and protein extraction or thermochemical conversion to obtain bio-oils or energy intermediates in integrated processes (Cheng et al., 2019; Singh et al., 2023). The literature reports multiple valorization pathways, ranging from cold extraction of phycobiliproteins to thermal conversion processes applied to organic-rich matrices (Li et al., 2024; Tounsi et al., 2023).

One area in which *Galdieria* has received considerable attention is the remediation and resource recovery from contaminated streams (Sarma et al., 2024). Several studies have demonstrated the genus's ability to remove nutrients (N, P), reduce biological oxygen demand, and eliminate heavy metals such as lead and cadmium, making it useful in the treatment of challenging wastewater streams (Kharel et al., 2023). In parallel, *Galdieria* has proven effective in the biosorption and bioaccumulation of valuable elements: studies have reported selective recovery of gold and palladium as well as the accumulation and recovery of rare earth elements (REEs) from fluorescent lamp extracts and other matrices, offering opportunities for so-called "urban mining" (Ju et al., 2016; Singh et al., 2023). Despite its advantages, large-scale application of *Galdieria* is limited by lower biomass and lipid yields compared to industrial strains, and by the need to define efficient extraction and processing strategies, possibly coupled with wastewater treatment, to ensure economic viability (di Cicco et al., 2021; Somers et al., 2021). Compared to established algal models, molecular biotechnology in *Galdieria* remains less advanced: despite progress in genomics and basic tools, fine-tuned metabolic control and strain engineering for improved yields or carbon efficiency are still largely under investigation (Retta et al., 2024; Rojas-Villalta et al., 2024). Finally, pilot-scale

validation (techno-economic assessments and life cycle analysis) is relatively limited compared to more traditional microalgae, and issues such as mass transfer, energy management, and cost–benefit optimization must be addressed on a case-by-case basis (Carone et al., 2024).

In summary, *Galdieria* represents a promising biotechnological chassis, able to thrive under extreme conditions, utilize diverse substrates, and accumulate both contaminants and valuable elements, thus coupling wastewater treatment with resource recovery and high-value biomolecule production (Henkanatte-Gedera et al., 2017; Selvaratnam et al., 2014). However, translating this potential into industrial applications requires further advances in culture process optimization, molecular tools, and real-scale economic and environmental assessments, issues that justify the focus of the present thesis on strain-specific gene regulation and photosynthesis (Retta et al., 2024).

1.6 Gap of knowledge

Although recent studies have detailed how light regulates photosystem activity, photoprotection, and gene expression in *Galdieria*, significant gaps remain regarding the structural and functional dynamics of their photosynthetic apparatus during its metabolic transitions (Kato et al., 2024; Qiu et al., 2013; Rossoni et al., 2019). While genomic and physiological studies have characterized several strains, including *G. sulphuraria* and some *G. phlegrea* isolated (Qiu et al., 2013; Rossoni et al., 2019; Schönknecht et al., 2013), little is known about how photosystems and light-harvesting complexes reorganize specifically during the transition from heterotrophy to autotrophy (Oesterhelt, Schmälzlin, et al., 2007; Salbitani et al., 2021). Most existing research has focused on growth parameters, pigment production, or gene acquisition via horizontal transfer (Giustino et al., 2025; Perez Saura et al., 2022; Rossoni et al., 2019), leaving the dynamics of photosynthetic reactivation largely unexplored (Perez Saura et al., 2022; Stadnichuk & Tropin, 2022). In particular, the roles of phycobiliprotein rearrangement, non-photochemical quenching, and regulatory feedback between light perception and transcriptional responses remain poorly defined (Curien et al., 2021; Salbitani & Carfagna, 2020; Thangaraj et al., 2011). Furthermore, although the resilience of extremophile algae under high temperature and low pH is well recognized, the molecular mechanisms linking photoprotection, light-use efficiency, and metabolic flexibility are still insufficiently characterized (Arora et al., 2025; Curien et al., 2021). Addressing these knowledge gaps is essential to understanding how *Galdieria phlegrea* coordinates its photosynthetic apparatus with metabolic shifts and to fully exploit its potential both as a model organism and as a candidate for biotechnological applications (Hirooka et al., 2022; Schönknecht et al., 2013).

1.7 Aims of the thesis

The main purpose of this study was to investigate the physiological and biochemical changes that occur in *Galdieria phlegrea* during the transition from heterotrophic to autotrophic growth. This transition involves the reactivation of the photosynthetic apparatus and a profound reorganization of metabolic and energy mechanisms.

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Through this integrated approach, the thesis aims to provide a comprehensive and in-depth view of the mechanisms underlying the transition from heterotrophy to autotrophy in *G. phlegrea*, with particular attention to the reactivation of the photosynthetic apparatus, light use efficiency, and photoprotection strategies. The results obtained aim to contribute to the understanding of the adaptation and resilience processes of extremophile microalgae.

Chapter 2 – Materials and methods

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Chapter 3- Results

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Discussion and conclusion

Extremophile microalgae are of particular interest, defined by their ability not only to survive, but also to thrive in extreme environmental conditions that would be lethal to most other forms of life (Marzban & Tesei, 2025; Rekadwad et al., 2023; Rojas-Villalta et al., 2024). Furthermore, some species are classified as poly-extremophiles, capable of surviving and flourishing under multiple extreme conditions simultaneously (Rappaport & Oliverio, 2023; Rinaldi et al., 2024). Since these extreme environmental factors are not always stable but can vary in intensity, extremophilic organisms are not only interesting for their ability to survive in these environments but also display marked metabolic plasticity to cope with all the variations that arise from environmental factors (Varshney et al., 2015). Obviously, for extremophilic microalgae, the ability to reorganize and improve photosynthetic performance, whenever changes in environmental factors occur, is essential (Aguiló-Nicolau et al., 2025).

Thus, the main objective of this study was to investigate into the functional and physiological dynamics of *Galdieria phlegrea* (strain 734) during the transition from the heterotrophic (dark) to the autotrophic (light) state, filling the gaps relating to the complex reorganization of the photosynthetic apparatus that occurs in this period. This transition, crucial for the survival of the algae in extreme environments characterized by limited light, involves rapid and complete cellular and functional remodeling. *G. phlegrea* possesses high metabolic and physiological plasticity, which allows it to reversibly alternate between autotrophic and heterotrophic growth, representing a fundamental adaptive advantage in hostile contexts such as geothermal springs and environments with extreme physicochemical conditions, including high acidity and high temperatures (Náhlík et al., 2021; Salbitani et al., 2021).

Recent studies on *Galdieria partita,* a unicellular red alga belonging to the order Cyanidiales, have shown that the reversible transition between photoautotrophic and heterotrophic growth involves rapid cellular remodeling: during heterotrophic growth, the synthesis of cytoplasmic and mitochondrial proteins increases, while the production of plastidial and nuclear proteins for photosynthesis is suppressed (Yamashita et al., 2025). This adaptation does not involve active

degradation of photosynthetic structures, but rather their dilution due to transcriptional suppression combined with accelerated cell growth (Yamashita et al., 2025). This metabolic plasticity represents a competitive advantage in extreme environments where light availability is limited by mineral cover or seasonal factors (Perez Saura et al., 2022). Species of the genus *Galdieria* possess a fully functional photosynthetic apparatus; however, detailed investigations of the photosystems, particularly in *G. phlegrea*, remain limited (Stadnichuk & Tropin, 2022).

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Appendix
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