PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: Miles Minio

Title of PhD research: Impact of light and nutrients availability on photosynthesis and resources allocation in algae.

Name of PhD supervisor: Caterina Gerotto
Research lab name: Laboratorio di fisiologia delle piante e delle alghe

Cycle:
[ ] XXXVI
[x] XXXVII

PhD Curriculum::
[x] Marine biology and ecology
[ ] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[ ] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: .....

ABSTRACT (1000 characters, including spaces):
Microalgae occupy a variety of ecological nieces and show a great diversity in their physiology and their responses to environmental factors. Despite their physiological flexibility, little is known about the physiology and photosynthetic regulation in response to stress of many algal species. In regards to macronutrient deprivation, few studies have been performed on growth in limiting sulphur concentrations. We are investigating how various marine microalgal species (*D. salina*, *T. suecica* and *P. tricornutum*) respond to low sulphate availability and how such changes impact photosynthesis, the source of energy for microalgal cells. Pigment quantification and *in vivo* chlorophyll fluorescence analysis allowed to characterize photosynthetic activity. Analysis of the allocation of resources is also being performed through (i) FT-IR spectroscopy, to assess changes in the carbon allocation, and (ii) elemental analysis, to check for shifts in the ratios between macroelements in the cells.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Microalgae play a vital role in the ecology of the planet, contributing to roughly 45% of global productivity, they play a fundamental role in the biogeochemical cycles and can occupy a variety of ecological nieces showing a great physiological plasticity in response to a multitude of environmental factors such as irradiance, nutrient availability, salinity, and temperature. In the past decade, research has shown a greater interest for these organisms as a source of human and animal food, for their ability to produce various bioactive compounds relevant to the production of both industrial and pharmaceutical products, and as a renewable source for biofuel production. Thanks to their physiological plasticity, the study of microalgae provides also great insight on the evolution and adaptations of photosynthesis, physiological responses, and nutrient allocation under to stressful conditions in photosynthetic organisms. For our study we choose three microalgal specie: (i) *Dunaliella salina*, a wall-less Chlorophyta that dominates hypersaline waters. Thanks to its ability to thrive in such environments, where nutrient depletion due to salts precipitation may occur, *D. salina* has become a model organism for the study of stress responses. *D. salina* is of some biotechnological interest, like being intensively cultured for the commercial production of glycerol, and β-carotene (Giordano et al., 2000; Monte et al., 2020; Polle et al., 2020), (ii) *Tetraselmis suecica*, a marine green alga part of the prasinophyte class mainly used as feed in aquaculture and as a vitamin E and antioxidant source for human and animals (Sansone *et al*., 2017;Abiussi *et al*., 2014), (iii) *Phaeodactylum tricornutum*, a diatom used for the production of polyunsaturated fatty acids, pigments and antioxidants (Fajardo *et* al., 2007; Daboussi *et al*., 2014).

- SCIENTIFIC AIMS

The purpose of our study is to investigate how the acclimation to growth limiting sulphur (S-lim) concentrations and different light regimes affect the physiology and the regulation of photosynthesis in various microalgal species through qualitative and quantitative assessment of pigments, *in vivo* chlorophyll fluorescence analysis, biochemical analyses on both total and thylakoidal protein extracts and characterization of the allocation of resources through FT-IR spectroscopy and elemental analysis. A genomic investigation of the main genes associated with the photosynthetic apparatus and the sulphur metabolism is also being performed. Deepening our knowledge on the physiological responses of photosynthetic species to environmental challenges is pivotal to the comprehension of factors controlling the fitness of species in their natural habitats, but also to improve their productivity in more applied research.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Effects on growth, cell composition and photosynthesis of sulphur-limiting conditions in *D. salina, T. suecica* and *P. tricornutum*.

Although both nitrogen (N) and sulphur (S) are macronutriens, essential for protein, vitamin biosynthesis and chlorophyll (Chl) production, physiological responses to S limitation are far less studied compared to those of N. The ability of the cell to assimilate nutrients depends on both the concentration of the single nutrient and it's balance with other components in the medium, as such, study of how the change in S concentration influences the cell quota of other nutrients and macromolecules can give insights in to the sulphur metabolism and how different species regulate it, the rest of their metabolism and photosynthesis in response to S deficiency (Fernandes *et al*., 2020). For this experiment we acclimated cultures to growth in S-lim conditions and investigated the biochemical compositions, growth and elemental stoichiometry of three species, *D. salina*, *T. suecica* and *P. tricornutum.*

Methods: *D. salina, T. suecica* and *P. tricornutum* were grown in the AMCONA artificial sea water medium (Fanesi et al., 2014) (specifically, 0.5 M of NaCl and 25 mM of Na_2SO_4) and acclimated to growth in 50 µM Na_2SO_4 (S-lim condition). *D. salina* was grown also in 1.5 M NaCl both in normal AMCONA and S-lim conditions. The cultures were grown in controlled conditions, 20°C, 24h continuous light of 50 µmol photons m^{-2} s^{-1} (*T. suecica* and *P. tricornutum*) or 100 µmol photons m^{-2} s^{-1} (*D. salina*). All analysis were performed

on samples from at least three independent biological replicates and growth curves were derived from daily cell counts with a CASY TT Cell Counter. Samples were then taken during the exponential phase and analysed for (i) C and N content with an element analyser also connected to ID Micro EA isotope ratio mass spectrometer to obtain carbon stable isotope (δ13C) ratios. (ii) Other elements such as P, S, Cl, K, Ca, Fe and others were analysed through total reflection X-ray fluorescence spectrometry, (iii) FTIR spectroscopy was performed to obtain the ratios between the macromolecular pools to determine C allocation, (iv) protein and pigment content were evaluated spectrophotometrically (V) The pigment composition was also analysed through HPLC analysis in collaboration with prof. Nicoletta La Rocca (Unipd).

Expected/Obtained Results.

The control and S-limited growth conditions were already optimized in the lab for *T. suecica* and *D. salina* (0.5M AMCONA) allowing to proceed with cells characterization, while for *D. salina* growth at higher salinity (1.5M NaCl) and for *P. tricornutum* my work started from the characterization of growth in S-limited conditions. Overall, The S-lim condition affected differently the three species. In *D. salina*, both in 0.5M and 1.5M of NaCl, S-lim cultures grew slower during the first week and then recovered toward the end of the growth curve, with a cellular density similar to that of the control (CTR) in 0.5M NaCl but not in 1.5M NaCl. This was different from *T. suecica* where the first days of growth weren't affected, and from *P. tricornutum*, the species most impacted by the S-lim conditions with stressed cultures doubling the cell number in two days and then entering a stationary phase while the CTR cell number was twenty times higher in the stationary phase. Regarding the biochemical composition, in CTR conditions, the C represented about 50% of the cells dry weight in all three species while %N varied according to the species. Further, S-limitation impacted %C and %N in a species-specific manner, with only *D. salina* showing a decrease in %C in S-lim samples (Figure 1). All species, however, displayed a more negative δ13C in S-lim, indicating the occurrence of changes in C metabolism.

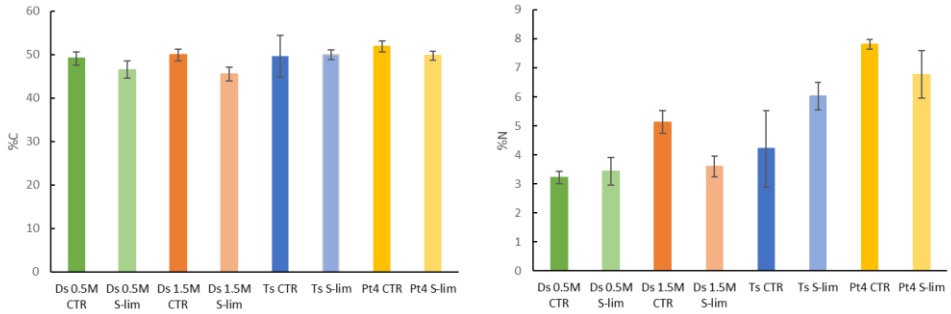

Figure 1: %C and %N in *D. salina (DS) 0.5M and 1.5M NaCl, T. suecica (TS)* and *P. tricornutum (PT4).*

In *D. salina*, between conditions, protein contend remains the same while FTIR analyses revealed a relative increase in carbohydrates and lipids in cells in S-lim conditions. Analyses of photosynthetic pigment composition showed that in response to S-lim conditions there was a decrease in photosynthetic pigments per cell. *D. salina* also varied the Chl a/Chl b ratio, which was higher in S-lim cells while in *T. suecica* this parameter was almost unaffected. Similarly, preliminary HPLC analyses of the carotenoid profile showed a variation in the accumulation of specific carotenoids in *D. salina* but not in *T. suecica*. These suggest the two green algae modulated differently the composition of photosynthetic apparatus in response to the stressful conditions. Additional analyses are planned to better elucidate this point, as described in WP2.

WP 2. Characterization of the photosynthetic regulation in *D. salina* and *T. suecica*

Microalgae are able to live in a wide range of habitats and to do so, they have evolved a wide range of physiological responses to cope with their constantly changing environment. Even within the same phylum, microalgae have evolved diversified regulatory processes in order to acclimate photosynthesis and reduce photooxidative damage. Despite physiological variety of microalgae, our knowledge of their regulatory processes is limited to few species. The scope of this study is the biochemical and *in vivo* characterization of the photosynthetic regulation in *D. salina* and *T. suecica*.

Methods. Cultures and experiments were set up in the same conditions as in WP1. Three main methodologies were exploited. (i) We analysed the *in vivo* chlorophyll fluorescence analysis at the Dual PAM fluorometer. After a 40 minute dark acclimation, algal cells were subject to either Light curve (LC) experiments, in which we expose the cells to gradually higher light intensities that suggest at what light intensities the photosynthetic apparatus is saturated or Induction Curves (IC), in which we observe the changes in time to how the sample responds to a specific light intensity and how the photosystems recover to a dark acclimated state. The main parameters we analysed were YII (quantum efficiency of the photosystem II) and the NPQ (non-photochemical quenching) which is related to photoprotection. (ii)Total and thylakoidal protein extraction protocols were optimised to characterize the photosynthetic apparatus by SDS-PAGE and Western Blotting. (iii) To the genome of *D. salina* available on Phytozome, we aligned through the BLAST tool the protein sequences of known proteins, pertaining to the photosynthetic apparatus, deriving from annotated plant and algal genomes. From these sequences we then built phylogenetic trees in order to verify that the protein sequences clustered with the sequences we expected. The multiple sequence alignments and phylogenetic trees were constructed in MEGA11 with the ClustalW algorithm and Maximum Likelihood method respectively.

Expected/Obtained Results.

From the *in vivo* Chl fluorescence analysis, ICs at saturating lights showed differences in the regulation of NPQ between the two species (Fig.2) with *T. suecica* showing a kinetic similar to that found in plants while in *D. salina* the recovery to the dark acclimated conditions seams to differ from the typical relaxation kinetics of plants and other green algae suggesting a difference in the regulation of NPQ or the electron transport chain. In order to investigate possible differences in the genome and protein profile that could explain these differences, we are performing biochemical and bioinformatic analyses. The Coomassie blue stained gels deriving from the SDS-PAGEs showed differences in the protein profile of *D. salina* and *T. suecica* in bands were proteins of the photosynthetic apparatus are known to migrate and western blots are being performed to verify the location of various photosynthetic proteins. Through the construction of phylogenetic trees of various protein sequences we were able to identify the sequences of proteins of the photosynthetic apparatus in *D. salina* such as PTOX, involved in circular electron flow and chlororespiration, and PSBS which is known in plants to regulate NPQ but is also present in algae were it seems to be expressed only during acclimatation to high light intensities (Fig.3). Regarding genes encoding for various Light harvesting Complexes, the genes encoding for the LHCSR protein that regulates NPQ in green algae weren't found, this could be because of the quality of the available genome or because of a different regulation of NPQ.

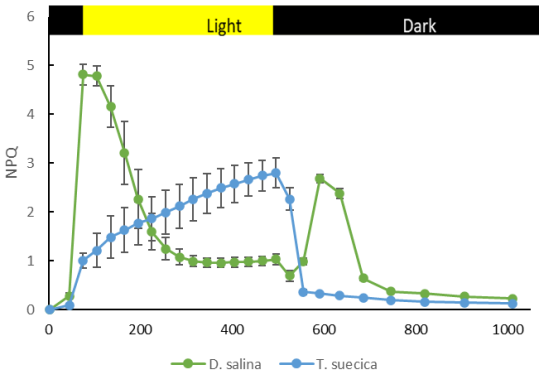

Figure 2: NPQ in *D.salina* and *T. suecica* during an IC. The yellow and black bands indicate the phases of the kinetic in wich the actinic lite is switched on or off respectively.

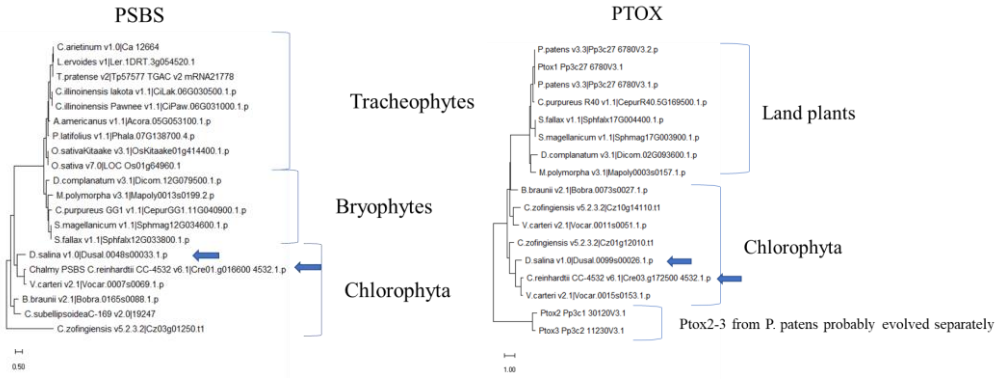

Figure 3: Maximum Likelihood trees of the PSBS and PTOX genes. Blue arrows indicate the *D. salina* genes and of the model green alga *C. reinhardtii*.

- REFERENCES

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Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Innovazione e trasferimento tecnologico. Prof. Donato Iacobucci

2. Analisi di regressione mediante Microsoft Excel (Prof. Francesca Beolchini)

List of conferences/workshops attended and of contributions eventually presented

1. Green Christmas Session "Photosynthetic microorganisms for sustainable development" of the "Institute for Biological Systems" (6-7 december 2022) (attended online)

2. Riunione annuale dei gruppi di "Biologia cellulare e molecolare" e "Biotecnologie e differenziamento" della Società Botanica Italiana (Ancona, 21-23 june 2023) (Attended; co-author of an abstract presented by Caterina Gerotto)

3. XIX PhD day for "Consorzio interuniversitario Reattività Chimica e Catalisi" (Bari, 30 june 2023) (oral presentation entitled: Acclimation to salinity and sulphur limitation in *Dunaliella salina*: focus on photosynthesis and cell composition)

4. **Forthcoming:** Riunione del Gruppo di algologia of the "Società Botanica Italiana" (Napoli, 27-28 october 2023) (Abstract entitled: Effects of salinity and sulphur limitation on photosynthesis and cell composition in *Dunaliella salina* has been submitted for oral presentation)

Part 3. PhD student information on publications

Manuscripts in preparation

1. **Minio M**, Battistuzzi M, La Rocca N, Norici A, Gerotto C. Effects of sulfate limitation on photosynthesis and cell composition of two unicellular marine microalgae. This *manuscript in preparation* is based on the work described in WP1.

2. Trotti J, Gulino F, Aceto M, **Minio M**, Gerotto C, Mica E, Valè G, Barbato R, and Pagliano C. Physiological responses to salt stress at seedling stage in wild (*Oryza rufipogon* Griff.) and cultivated (*Oryza sativa* L.) rice. This *manuscript in preparation* is based on a research work lead by Prof. Cristina Pagliano (Università del Piemonte Orientale) in which we have been asked to characterize the C and N composition in rice samples.

14 October 2023

Student signature

Supervisor signature