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ALGAL RESPONSES TO ABIOTIC AND BIOTIC ENVIRONMENTAL CHANGES

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1. SUMMARY

The research I have conducted explored the interactions between algae and environment and investigated whether and how such interactions have an impact on the way algae interact with other organisms.

The heterogeneity of algal responses to environmental changes has the potential to drive modifications of the composition of natural phytoplankton communities, by altering the relationships among algal populations and between algae and predators. The comprehension of the physiological mechanisms that underlie such multiplicity of algal responses is expected to facilitate the development of scenarios aimed at describing the interaction among organisms in a changing ocean.

Key ecological issues, such as the establishment of symbioses, the occurrence of selective grazing and the competitive interaction among phytoplankton have been addressed in the present work by using a physiological approach. Some important conclusions have been reached; for instance, by confirming the importance of cell composition for the survival to grazing, I have given solid basis to the possibility that cell composition, and thus the ability to acclimate or remain homeostatic, are a major target for selection.

The novelty of my research lies not only in the study of a largely unexplored combination of algal species and environmental stimuli, but also in the development of new protocols and methods that, combined with the use of state-of-art technology, allowed me to test the experimental hypotheses.

The outcome of my work is discussed in relation to four different subtopics, which are mutually connected and correspond to as many chapters of my thesis. The outcomes of the various chapters are used as stepping stones toward a general understanding of the physiological processes and mechanisms that allow microalgae to cope with a changing environment.

2. INTRODUCTION

Algae (sensu Raven and Giordano 2014) occupy a multiplicity of habitats, which are characterized by different extents of fluctuations of their physicochemical properties. A very large variety of physiological responses to “abiotic changes” (e.g. changes of salinity, temperature, light, nutrient availability) and “biotic changes” (e.g. modifications in the number or type of competitors and predators) can be observed among algae. The modes through which different algae cope with changes in the surrounding environment determine the composition of natural algal assemblages to a great degree.
The heterogeneity of algal responses to environmental changes can be classified in two main categories: homeostatic and non-homeostatic responses. The term compositional homeostasis indicates the ability of organisms to keep their internal composition constant despite modifications in the chemical composition of the environment in which they live (Koojiman 1995; **Fig. 2.1**). Compositional homeostasis does not always match with functional homeostasis, i.e. the retention of balanced metabolic and energy fluxes. The maintenance of the growth rate after the onset of an environmental perturbation, for instance, often requires changes in the proteome (Giordano 2013).

In some cases, algal cell composition can change in response to environmental alterations. When one or more external stimuli induce changes either in the functioning of pre-existing catalysts, in the expressed proteome or in the genome, we deal with, respectively, regulation, acclimation and adaptation (Giordano 2013; **Fig. 2.2**). Acclimation, therefore, entails changes in the expression of genes that were not (or were more/less) transcripted/translated before the onset of the environmental perturbation; as a consequence of acclimation, the ratio between the rates of protein production and protein degradation can vary, so that the net amount of one or more protein(s) can ultimately change. During acclimation, new “set points” are established. Set points are limits within which a certain parameter can oscillate without consequences; deviations from the set points can be perceived by the cell and trigger the adoption of measures aimed at restoring the altered parameter within the ranges that are “suitable” in each specific set of conditions. The diversion of a cell parameter can be detected by different species with different degrees of sensitivity, which represent a target for selection (Giordano 2013).

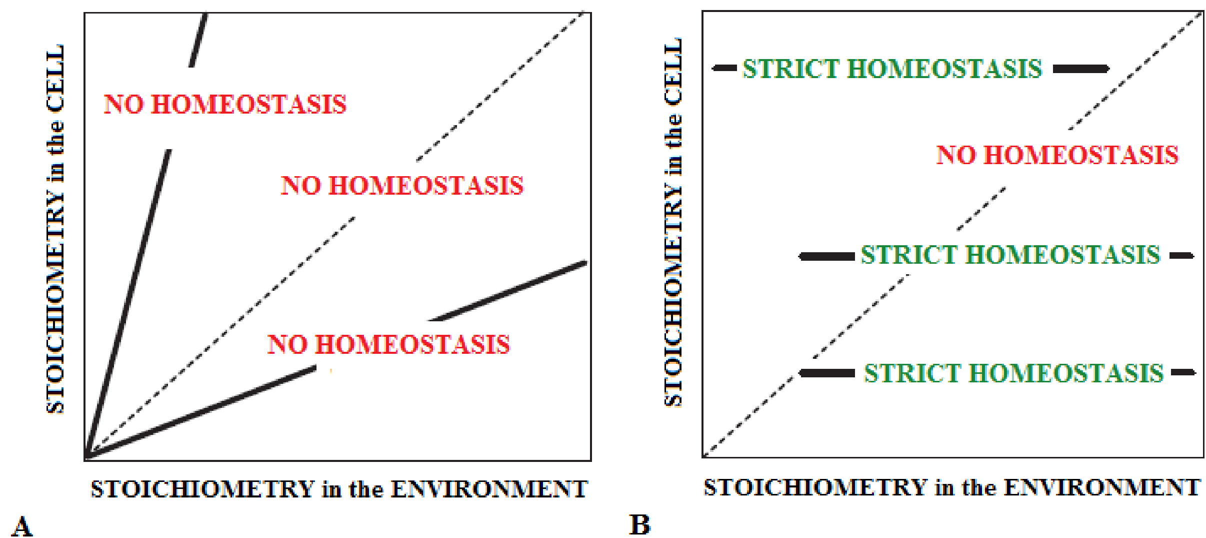


Fig. 2.1. Models that relate consumer stoichiometry and resource stoichiometry. The dashed line represents the "you are what you eat" model, according to which the elemental stoichiometry of the consumer matches exactly that of the food resource present in the environment. **A.** The solid lines represent a situation in which the retention of resources is constantly deviated from the total availability of resources. **B.** The horizontal lines represent a consumer whose stoichiometry is completely independent from the stoichiometry of the available resources. Modified from Sterner and Elser 2002.

What determines the adoption of either a homeostatic or an acclimatory response to a given environmental stimulus is not entirely understood. Major determinants can be enclosed in three categories:

- Genotype-related factors: different algal species have different potential for acclimation to external changes in each given set of conditions, depending on their phenotypic plasticity; the latter largely depends on the availability of metabolic routes that is ultimately determined by the genotype.

  Stimulus-related factors: the duration and the intensity of the environmental shift can have a great importance. The duration of the stimulus is perceived by cells in relation to their growth rate. Acclimatory responses, indeed, are not convenient for algae below and above certain ratios between the duration of the perturbation and the growth rate. The "upper limit" (above which homeostasis may be more convenient) depends on the fact that acclimation shall occur rapidly enough for the cells

to have the time to take advantage of its beneficial effects and to attain a positive energetic balance. The "lower limit" (below which homeostasis is more convenient) prevent cells from spending energy for the acclimation to very transient perturbations. It has been observed that when *Tetraselmis suecica* cultures growing at different rates were exposed to three environmental perturbations of fixed intensity and duration, cultures to which the lower growth rate was imposed were more homeostatic – in terms of cell composition – than cultures acclimated to the higher growth rate. This result was ascribed to the fact that a smaller portion of the doubling time is influenced by the environmental shift in cells growing at slower rates so that an acclimatory response is not justified (Fanesi et al 2013). It was also observed that the response of *Dunaliella parva* to a short term (few days) transition of the external N source was, as a whole, homeostatic (Giordano et al 2007), while the same species underwent acclimation after a long exposure to NH_4^+ (Giordano et al 2002). However, it is not clear whether such observations are attributable to the time required for changes in the expressed proteome to occur, or rather they represents a precise strategy to minimize the energy investment (see below) for the acclimation to a transient environmental alteration.

- Surrounding factors. Environmental conditions that are not directly related to the changing environmental factor (i.e. the stimulus) can interfere with the ability of algae to cope with the external change. For instance, the availability of energy can drive the adoption of a more or less "expensive" response to environmental changes. In the case of a homeostatic response, no additional costs are expected. Acclimation, instead, entails costs for changing the proteome and for bringing back the cell to the original conditions, once the perturbation ends (Giordano 2013; Woods and Wilson 2013). The energetic balance of the cell may thus drive the choice between acclimation and homeostasis in each given situation.

  Surrounding conditions also include the presence of competitors and predators. In the presence of competitors, algae are expected to opt for the strategy that gives them a reproductive advantage over the competitors themselves. Besides the cost in terms of ATP and reducing power, the costs in terms of growth rate shall thus be considered in the cell budget. Homeostatic responses may require a reduction of growth rate (i.e. less biomass produced per unit of time) during the exposure of algae to the environmental stimulus. However, the maintenance of a balanced cell composition can give an advantage to the cells once the original environmental conditions are restored, because it avoids reverse acclimation (Giordano 2013) and permits a quick recovery of the pre-stimulus growth rate.

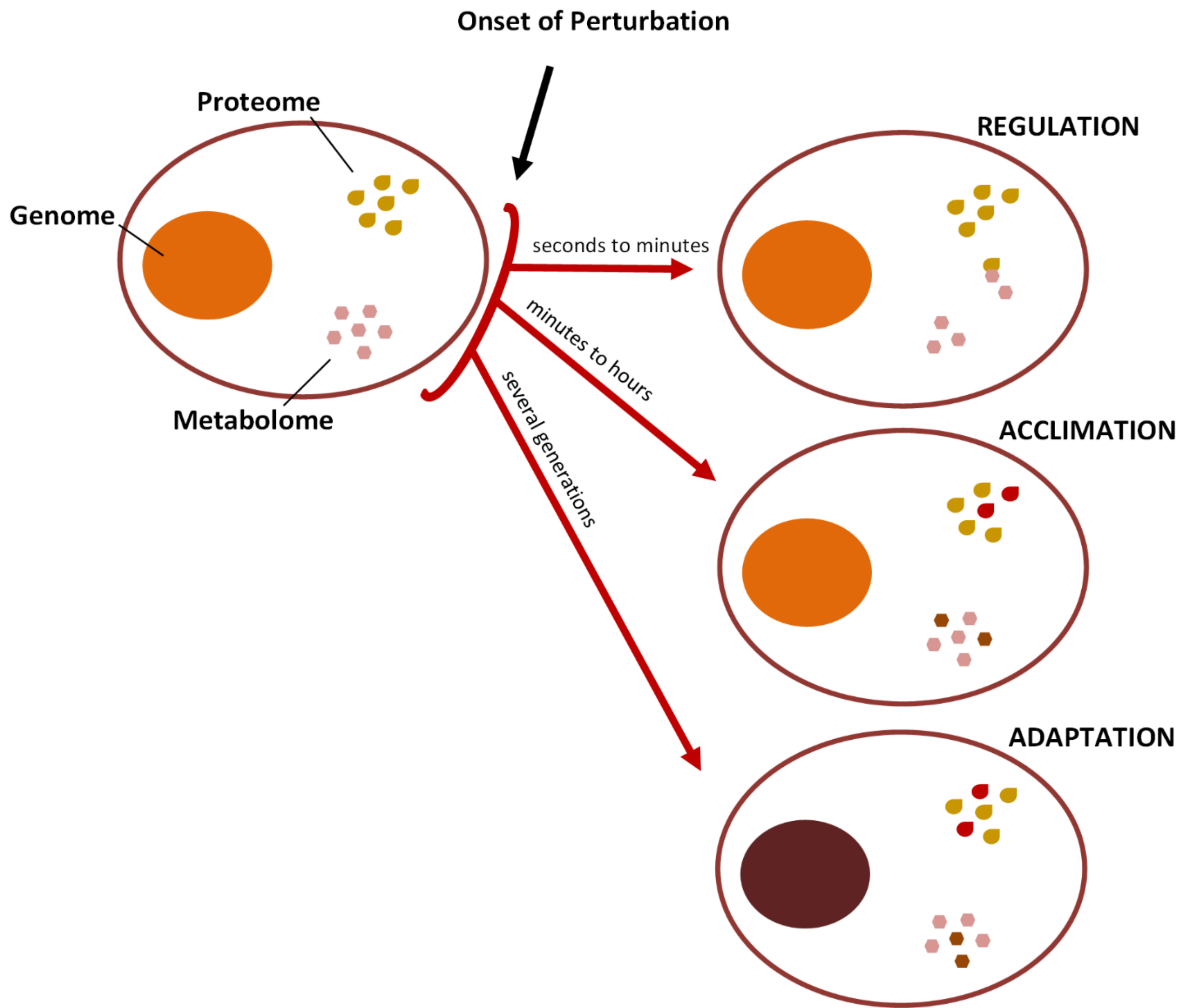


Fig. 2.2. The three main response modes to environmental changes in algae. Changes in the color of the symbols in the picture indicate changes in the cell components symbolized by those symbols. Regulation implies modifications of proteins already present in the cell. Acclimation entails quantitative and/or qualitative changes in the expressed proteome. Adaptation occurs when the genotype of the organism is altered by loss, mutation or acquisition of genes.

Regulation, acclimation and adaptation may be considered as processes that minimize the diversion from homeostasis in the presence of environmental perturbations (Giordano 2013). Algal responses will result from a compromise between the maximization of growth and the minimization of the costs for the adjustment of cell composition (Giordano 2013).

The inclination of algae towards an acclimatory or a homeostatic response has the potential to affect the interaction with their predators, insofar the cell constituents that are altered (or not) by an environmental shift represent discriminant factors for the predators' feeding. The mode through which and the extent by which this occurs are largely unknown. The comprehension of such processes are further complicated by the fact that the presence of the grazers itself can act as an environmental stimulus that triggers responses potentially affecting algal cell composition. The presence of predators, for instance, was shown to induce morphological changes in algae (Pondaven et al 2007), and some evidences suggest that modifications in algal cell composition are possible outcome of the coexistence with the grazers (Pondaven et al 2007; Ratti et al 2013). Acclimation of algae to the presence of their predators may implicate, for instance, the diversion of algal cell constituents towards a less attractive cell composition. If such diversion occurs at a different extent in different algae (or it only occurs in some of the algal populations that constitute a natural algal assemblage), their relative rate of mortality by feeding (and thus their relative abundance) can change.

A further unverified possibility is that the presence of competitors for resources (e.g. other algal populations) may in turn affect the interaction between algae and grazer.

The concepts described above are the focus of my research, which I addressed through the verification of simple and clear experimental hypotheses. The results of my investigation are described and discussed in the chapters 3, 4, 5, 6, which are connected as described in the paragraph 2.1 and contribute synergically to the understanding of the complex interaction between algae and environment.

2.1 WORKFLOW

The first goal of my study was to understand how common compositional homeostasis and acclimation are among microalgae and what drives the choice between the two in each given set of conditions. This is discussed in the chapter 3 of my thesis, named **"Acclimation and homeostasis in algae: two alternative response modes to external perturbations".** In this first set of experiments, I have examined the short-term and long-term responses of 10 species of microalgae to three different environmental perturbations (low to high CO_2, Light to Dark, NO_3^- to NH_4^+). The outcome of this work also gives a hint on the diverse disposition of algae belonging to different groups to acclimate to new external conditions. The observation that only the dinoflagellate *Amphidinium klebsii*, among the ten species under investigation, responded to changes in the CO_2 concentration by changing its organic composition pushed me to investigate whether the cell composition of a dinoflagellate-related alga, *Chromera velia*, varied at different CO_2 concentrations. This work is described, in the form of a research paper, in the chapter 4: "**Life at elevated CO_2 modifies the cell composition of *Chromera velia* (*Chromerida*)**".

After having dealt, in the first part of my thesis, with the interactions between algae and environment, I then wondered whether such interactions play a role in the relationships between algae and the biotic environment surrounding them. In order to address this issue, I considered the importance of the nutritional history of algal populations (insofar as it determines changes of their cell composition) in the relationships with their natural predators. The rationale and the outcome of this study are illustrated in the chapter 5 of this thesis, entitled "**The nutritional history of algae affects their interactions with grazers and with other algal populations**".

Some of the results that I have obtained in this part of my work turned out to be not only unexpected but also especially intriguing. Indeed, the observation that conspecific algal populations with different nutritional backgrounds could interact in a seemingly competitive way pushed me to take an interest in the relationships among algal populations of the same species and to conduct a critical evaluation of the literature on intraspecific communication in algae. This research made the shape of a review paper named "**Intraspecific chemical communication in microalgae**" (chapter 6).

3. ACCLIMATION AND HOMEOSTASIS IN ALGAE: TWO ALTERNATIVE RESPONSE MODES TO EXTERNAL PERTURBATIONS

3.1 INTRODUCTION

When a perturbation of the extracellular environment occurs, algal cells can detect the deviation of a certain parameter (e.g. the size of a cell organic pool) from a "set point" (chapter 2). This can obviously occur if such deviation is compatible with the sensitivity limits of the cells. A homeostatic response consists in the activation of processes aimed at bringing back the parameter to the set point value. When acclimation occurs, instead, new set points are established (Giordano 2013).
Acclimation to external changes is aimed at reaching a competitive advantage in terms of reproduction. If acclimation does not provide a reproductive advantage over competitors, homeostasis may be advantageous.
The "potential" for acclimation consists in the availability of metabolic routes and physiological systems that allow algae to change their cell composition in response to a given environmental stimulus. Algae that have this physiological plasticity may opt for either acclimation or homeostasis in response to environmental perturbations, as elucidated in the general introduction of this thesis (chapter 2), depending on the properties of the environmental stimulus, on the ecological context (i.e. presence of competitors and predators) and on species-specific biases. In this work, we tried to investigate the species-specific biases.
An additional issue to address is whether algae belonging to different groups respond to environmental changes in different ways and at different extents. Algal responses to environmental stimuli are somehow constrained by the metabolic pathways that are available for organisms, which largely depend on the genotype. We can expect algae that belong to the same group to share the main metabolic routes and thus to have a similar potential for acclimation to environmental changes. It has been demonstrated that the potential for acclimation to environmental stimuli can depend on one or few molecules, and thus on a limited number of metabolic pathways. For instance, *Synechococcus* cannot acclimate to UVB and low temperature in absence of the ClpP1 protein, having a proteolytic activity (Porankiewicz et al 1998); environmental changes that increase the probability of protein damage often stimulate the proteolytic

capacity of a cell (Parsell and Lindquist 1993). The differences among algal groups in the potential for acclimation to the environmental stimuli may have a great impact on the evolutionary trajectories of algae.
Cell organic composition is the result of the C allocation strategies that algae adopt in any given condition. If cyanobacteria, green algae, diatoms, dinoflagellates and coccolithophores have a different potential for acclimation to environmental changes, they also are expected to be able to respond to the same environmental perturbation in different ways.

To address these issues, the overall organic composition of ten species of marine microalgae, belonging to five different taxonomic groups, was evaluated before and after the application of the following three environmental perturbations (technical details are provided in the paragraph 3.2.1):

- Low to High CO_2: algae grown at the current atmospheric CO_2 concentration were transferred to a medium in equilibrium with a gas phase containing higher CO_2 concentration, similar to that expected for the end of the century considering a rapid economic and demographic boost and an intensive use of fossil energy sources (scenario A1F; IPCC 2000). Previous studies have shown that algae change their cell organic composition when exposed to high CO_2 in a species-specific way (e.g. Giordano and Bowes 1997; Li et al 2001). The possibility to predict the physiological responses of phytoplankton to high CO_2 concentrations is especially important in the light of the ongoing global changes.

- Light to Dark: algae grown in the light were transferred to dark. It seems likely that photoautotrophic organisms are not able to successfully acclimate to a prolonged exposure to darkness. Reports from the literature describe species-specific responses to this environmental perturbation, including the retention of a balanced cell composition (*Phormidium autumnale*; Montechiaro et al 2006) and the alteration of the internal cell composition (*Prorocentrum minimum*; Manoharan et al 1999).

- NO_3^- to NH_4^+: algae grown in the presence of NO_3^- were transferred to a medium containing NH_4^+ as the only N source. The reduction of NO_3^- to NH_4^+ is an important sink for the electrons generated by photosynthesis in algae; a NO_3^- to NH_4^+ swap in the culture medium is expected to free additional energy for the cells, which can be used for acclimation, in case it is possible to reach a new cell composition that is evolutionarily selected for cells in the new conditions.

My experimental hypothesis is that algae are able to respond to the "Low to High CO_2" and the "NO_3^- to NH_4^+" transitions, either homeostatically or acclimatively, with no constraint imposed by the lack of the acclimation potential, i.e. the physiological plasticity that allows cells to change their composition in response to environmental perturbations.

On the other hand, I expect the experimental algal species not to be able to acclimate to prolonged darkness. Algae belonging to the same group are expected to exhibit similar responses to the same environmental stimulus, since they share similar metabolic routes and thus possibly a similar "potential" for acclimation.

3.1.1 Experimental organisms

The algal species under investigation are marine organisms that represent the main (i.e. in terms of abundance and/or ecological importance) algal groups in ocean: cyanobacteria (Cyanophyceae), diatoms (Bacillariophyceae), dinoflagellates (Dinophyceae), green algae (Chlorophyta), coccolithophorids (Prymnesiophyceae). The list of the species under examination and of the codes that indicates the origin of each strain is provided in **Tab. 3.1**.

Cyanobacteria were the dominant photoautotrophs in the Proterozoic oceans. Still predominant, alongside the algae of the "green lineage", during the Paleozoic, they eventually made way for the algae of the "red lineage", which dominate the oceans since the Mesozoic (Falkowski et al 2004). Strains of the genera *Synechococcus* and *Phormidium* have been chosen for the present study. *Synechococcus* (Synechococcaceae, Synechococcales, Cyanophyceae) is a unicellular cyanobacterium present at all latitudes, in both open oceans and coastal regions, but mostly abundant in the surface-waters, where it gives a noteworthy contribution to the primary production (Scanlan and West 2002). The ecology, physiology and genomic of *Synechococcus* has been largely explored (Palenik et al 2006; Henley and Yin 1998; Agawin et al 2000). The genus *Phormidium* (Phormidiaceae, Oscillatoriales, Cyanophyceae) comprises freshwater and marine species with a wide geographical distribution (Marquardt et al 2007). The ability of *P. autumnale* to cope with environmental changes has been previously investigated, revealing its tendency towards the retention of a stable internal composition in the case of the exposure to prolonged darkness (Montechiaro et al 2006).

The eukaryotic branch of photoautotrophs originated from the engulfment of a cyanobacterium by a heterotrophic protozoan, occurred 1.5 billion years ago (Yoon et al 2004). Progressively, these

photosynthetic eukaryotes diverged into two lineages: green and red (Delwiche 1999). Algae of the green lineage, containing chlorophyll a and b, include **green algae** and those organisms that originated from endosymbiotic events that involved heterotroph organisms and green algae (e.g. euglenophytes). Among green algae, I have selected the species *Chlorella marina* (Chlorellaceae, Chlorellales, Trebouxiophyceae), *Dunaliella salina* (Dunaliellaceae, Chlamydomonadales, Chlorophyceae) and *Tetraselmis suecica* (Chlorodendraceae, Chlorodendrales, Chlorodendrophyceae).

Algae of the red lineage, containing chlorophyll a and c, include red algae and those algae that originated from endosymbiotic events that involved heterotroph organisms and red algae (e.g. diatoms, coccolithophorides). Despite **diatoms** appeared already 160-200 million of years ago, their diversification mostly occurred during the past 60 million years (Kooistra and Medlin 1996; Falkowski et al 2004). Diatoms contribute to a very large extent to the oceanic primary production (~40%) and to the export of C to the deep oceans, and have a primary role in the Si cycle in ocean (Nelson et al 1995). My diatoms of choice are *Phaeodactylum tricornutum* (Phaeodactylaceae, incerta sedis), *Thalassiosira pseudonana* and *Thalassiosira weissflogii* (Thalassiosiraceae, Thalassiosirales, Mediophyceae).

Coccolithophorids had a great diversification during Mesozoic era, which however ebbed with the ascent of diatoms' diversity (Katz et al 2004). Through the formation of coccoliths (i.e. calcium carbonate plates that enclose the cells), they contribute greatly to the oceanic calcium cycle. Calcification also lead to a decrease of the alkalinity, so that less atmospheric CO_2 moves to the surface oceans (Passow and Carlson 2012). Furthermore, coccolithophorids account for about 10% of the export of C to the deep ocean (Jin et al 2006). *Emiliania huxleyi* (Noelaerhabdaceae, Isochrysidales, Coccolithophyceae), which is the species I have selected for the present investigation, forms large blooms that extend across hundreds of thousands of km^2 (Brown and Podestá 1997).

The first **dinoflagellates** were already present ~ 200 million years ago (i.e. Triassic; Fensome et al 1996), but the greatest diversification of this algal group only occurred in the Cretaceous. Today, dinoflagellates are distributed in both marine and freshwater environments but their diversity and abundance is likely much lower than in the Cretaceous (Delwiche 2007). However, their ecological importance is undeniable. The photosynthetic species (which account for about half of the species) contribute importantly to the primary productivity in ocean (Delwiche 2007) while the heterotrophic species are important grazers of nanoplankton (Yang et al 2004). The toxin-producer dinoflagellates can threaten the survival of other organisms by direct consumption or – in top predators - bioaccumulation (Tindall and Morton 1988). Furthermore, dinoflagellates comprise species (e.g. *Symbiodinium*) that have greatly modified the oceans by establishing symbioses with corals, so allowing the development of the modern coral reefs (Lewis

and Smith 1971). Among dinoflagellates, I focused on *Amphidinium klebsii* (Gymnodiniaceae, Gymnodiniales, Dinophyceae), a photoautotrophic species that can establish symbioses with turbellarian worms (Schnepf and Elbrachter 1992).

3.2 MATERIALS AND METHODS

3.2.1 Cultures

The different algal species employed as model organisms for this study were obtained from different algal collections worldwide (**Tab. 3.1**). Algae were grown in AMCONA medium (Fanesi et al 2013) at 20°C and provided with continuous light at a photon flux density of 100 µmol photons m^2 s^{-1} (λ = 400-700 nm). Algae were cultivated in autoclaved glass tubes provided with cotton plugs and placed in growth chambers. Algae were cultivated semicontinuously and diluted daily. For each culture, the volume of algal suspension to be removed daily was calculated in order to impose a communal division rate to all cultures subjected to the same environmental shift (3 days for algae exposed to changes of N source or CO_2 partial pressure; 5 days for algae exposed to dark). After the beginning of the treatment, the growth rates of the different cultures were allowed to vary and be part of the response to the environmental stimuli.

For each species-treatment combination, three "control" cultures and three "treated" cultures were prepared. Cultures were allowed to acclimate to the imposed growth rate for 4 generations before the **T0** samples were collected. Then, after one division, the "treated" cultures were transferred to the new conditions:

- "Low to High CO_2" shift: cultures grown at 400 ppm CO_2 were bubbled with CO_2-enriched air (1000 ppm CO_2). The controls were bubbled with air only.
- "Light to Dark" shift: cultures grown at 100 µmol photons m^2 s^{-1} (λ = 400-700 nm) were transferred in the dark. The controls were kept in the light.
- "NO_3^- to NH_4^+" shift: cultures were centrifuged and resuspended in a medium in which $NaNO_3$ was replaced by an equal concentration of NH_4Cl. The controls were centrifuged and resuspended in fresh Amcona medium.

Algae were sampled 24 h after the beginning of the treatment (**T1**) and again after 12 (Low to High CO_2; NO_3^- to NH_4^+) or 20 (Light to Dark) days from the beginning of the treatment (**T2**).

Tab. 3.1. Name of the species and strains under investigation. **SAG**: Culture Collection of Algae, Göttingen University. **CCAP**: Culture Collection of Algae and Protozoan, Scottish Marine Institute. **UTEX**: Culture Collection of Algae, University of Texas. **CCMP**: now NCMA: National Center for Marine Algae and Microbiota, Bigelow Laboratory for Ocean Sciences.

Species	Strain
Amphidinium klebsii	SAG 36/80
Chlorella marina	CCAP 211/27
Dunaliella salina	CCAP 19/25
Emiliania huxleyi	CCAP 920/11
Phaeodactylum tricornutum	CCAP 1052/1A
Phormidium	CCAP 1462/8
Synechococcus	UTEX LB 2380
Tetraselmis suecica	CCAP 66/4
Thalassiosira pseudonana	CCMP 1335
Thalassiosira weissflogii	CCAP 1085/1

3.2.2 Cell number and growth

For each algal species, three independent cultures were grown in batch. Cell number and cell volume were determined with an automatic cell counter (CASY TT, Innovatis AG, Reutlingen, Germany). Aliquots of 100 µl were inoculated to 10 mL of an electrolyte solution (Casyton, Innovatis AG, Reutlingen, Germany); small volumes of this solution were suctioned at a constant rate through a 60 µm pore, to which a 1 MHz electric field was applied by means of an external platinum electrode. In order to assess the volume of the cells, the variation of the electrical resistance of the solution (which is increased by the passage of an intact cell) was recorded.

The specific growth rate (μ) of batch cultures was calculated from the portion of the growth curves in which the cell number increased exponentially. In this part of the curve, the relation between the variation of cell number (ΔN) over time (t) and the initial number of cell (N_0) can be described by the equation:

$$\Delta N = N_0\, e^{\mu t}$$

which is solved for μ as follows:

$$\mu = \ln\ (N_t/N_0) \cdot\ 1/t$$

After the imposition of a growth rate ($\mu = 0.14\ d^{-1}$), tiny aliquots of the semicontinuous cultures designed for the "Light to Dark" transition were harvested in order to assess the number of cell per unit of volume before the beginning of the treatment and 1, 4, 7, 11 and 20 hours after the beginning of the treatment. This was done in order to understand whether algal culture exposed to darkness were able to maintain a net growth in the absence of light and, in case, whether this growth followed the same rate that was imposed by dilution at the beginning of the experiment (**Fig. 3.14**).

3.2.3 Fourier-transformed infrared (FTIR) spectroscopy

The organic composition of algae subjected to the different environmental perturbations was determined by means of Fourier Transform Infrared (FTIR) spectroscopy.
In a FTIR spectrometer, the IR radiations emitted by an IR source are directed towards a beam splitter, which reflects 50% of the beam towards a fix mirror and 50% of the beam towards a mobile mirror. The two mirrors and the beam splitter constitute the "Michelson interferometer" (**Fig. 3.1**). The beams reflected by the two mirrors recombine and interfere in a constructive or destructive way, depending on the relative position of the mirrors. The resulting interferogram is a function of the position of the mobile mirror, which is converted to a function of the wavenumber (cm^{-1}) through the application of a Fourier transform. The resulting transmittance spectrum is ultimately converted to an absorbance spectrum.

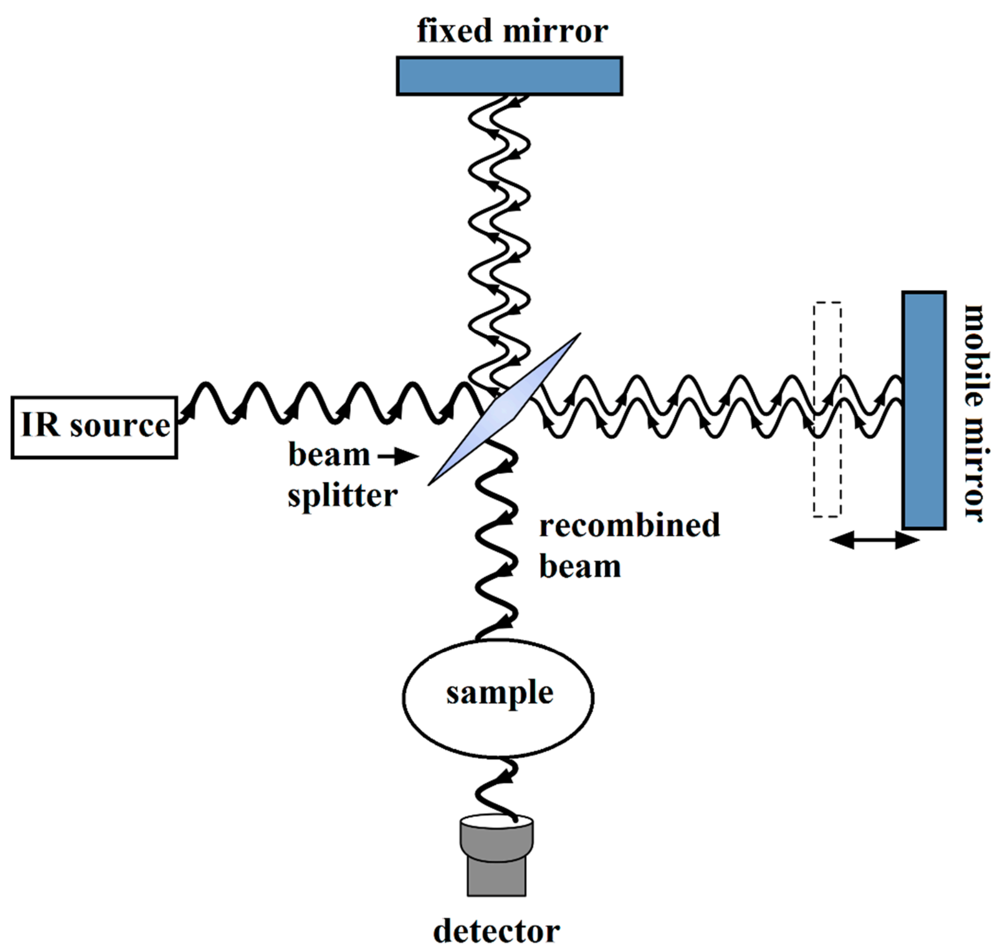


Fig. 3.1. Scheme representing a FTIR spectrometer. The fixed mirror, the mobile mirror and the beam splitter constitute the "Michelson interferometer".

The molecules of the samples that have a dipole moment can absorb the incident IR radiations at the wavelengths that correspond to the normal (i.e. vibrational) modes permitted to them; the normal modes of each molecule depend on the spatial distribution of the atoms in the functional groups. The signal that reaches the detector (downstream of the sample) will be impoverished in the frequencies that correspond to the normal modes of the molecules present in the sample. Peaks at different wavelengths can be used to identify different functional groups in the molecules and can therefore allow to attribute absorbances to the main organic pools present in the sample.

3.2.3.1 Sample preparation for FTIR analyses

Cells were harvested by centrifugation (700·g for 10 minutes) and washed twice in an ammonium formate solution isosmotic to the culture medium (0.5 M), in order to get rid of all traces of medium. The resulting pellets were resuspended in small volumes of ammonium formate, so that $2 \cdot 10^4$ to 10^5 cells were contained in 1 µl of cell suspension, depending on the desired number of cells for each species (see below); 50 µl of this cell suspension were pipetted onto a silicon window (Crystran, Ltd, Poole, UK). The optimal cell number per window was determined by preliminary tests aimed at obtaining the best possible signal to noise ratio for each species, and ranged from 10^6 (*T. suecica*) to $5 \cdot 10^6$ (*T. pseudonana*; *P. tricornutum*). Algal suspensions were deposited on the center of the silicon windows and then put in an oven at 60°C until total dehydration. Blanks consisted of 50 µl of ammonium formate deposited on the silicon windows.

3.2.3.2 Acquisition and analysis of the FTIR spectra

FTIR spectra were acquired with a Tensor 27 spectrometer (Bruker Optics GmbH, Ettlingen, Germany). Acquisition and analysis were conducted through the software OPUS 6.5 (Bruker Optics GmbH, Ettlingen, Germany). The acquisition parameters are listed in **Tab. 3.2**. Prior to any sample spectra acquisition, the blanks were checked for traces of ammonium formate.

Tab. 3.2. Parameters used for the acquisition of FTIR spectra.

Spectral resolution	4 cm^{-1}
Number of scans	32
Range of acquisition	4000 - 400
Apodization function	3-terms Blackman-Harris
Zero-filling factor	2
Pinhole aperture	3 mm

Spectra were baseline corrected by using the “Rubberband” algorithm (64 baseline points). CO_2-bands were excluded. Peaks in the spectra were attributed to the functional groups of the main organic pools in

the cells was done according to Giordano and coauthors (Giordano et al 2001 and references therein). The peaks at ~1650 cm^{-1} ("amide I"; C=O of amides associated with proteins) and ~1740 cm^{-1} (C=O of ester functional groups of triglycerides) were used as proxies for proteins and lipids, respectively. For carbohydrates, the areas of the peaks within the 900-1200 cm^{-1} (C-O-C of polysaccharides) were summed up; the peak at ~1074 cm^{-1} (Si-O of the siliceous frustules) was excluded from the carbohydrates determination.
The position of the peaks in the absorbance spectrum was identified after application of a second derivative with 9 smoothing points. The points of minimum in the second derivative indicated the position of peaks that constrained the spectra deconvolution. The "Curve fit" function of OPUS 6.5 allowed the reconstruction of an absorbance spectrum from the contributions of the single peaks. The relative ratios among proteins, carbohydrates and lipids were calculated by integrating the areas of the corresponding peaks in the FTIR spectra.

3.2.3.3 Similarity analysis

A reference library consisting of 1,566 spectra, representing the examined species subjected to the three environmental stimuli and harvested at three different sampling times, was created. FTIR spectra representing *E. huxleyi* cells subjected to the NO_3^- to NH_4^+ shift were excluded from the analyses, because of their low quality (low signal/noise ratio), which did not allow a proper deconvolution of the spectra.
The "Quick compare" algorithm of the software OPUS allowed calculating the similarity between each couple of spectra. The resulting values, which ranged from -1 to +1, were converted to percentages so that the interval $-1 < r < 0$ was associated to 0% and the interval $0 < r < 1$ was mapped linearly from 0 to 100%. A method that allowed to pre-process spectra before the assessment of the similarity was selected: the first derivatives of the FTIR spectra were calculated using the Savitzky-Golay algorithm and the spectral range from 1800 to 2830 cm^{-1} was excluded: this range of wavenumbers, in fact, is characterized by a strong absorbance band due to atmospheric CO_2.
For each species-treatment combination and each time interval, I calculated the similarity between the "control" spectra («s'») and between the "treated" spectra («s») at two sampling times. Whenever «s», was significantly (t-test; $p < 0.05$) lower than «s'», meaning that the "treated" cultures changed more than the "control" cultures in the considered time interval, I calculated a "change index": $CI = s'/s$.
The more "CI" is higher than 1, the more the treatment affected the overall cell composition.

3.3 RESULTS

3.3.1 Quantitative evaluation of overall changes in the organic composition of algal cells

An overview of the algal responses to the three environmental perturbations under examination, in terms of overall organic composition, is given in **Fig. 3.2**.

Different species were differently inclined to change their cell composition in response to environmental perturbations. Furthermore, the same species was homeostatic or not, depending on the type and duration of the stimulus. Changes of the overall organic composition occurred at different extents in different species-treatment-time combinations; the values relative to the "change index" (calculated as described in paragraph 3.2.3.3 and reported in **Tab. 3.3**) give a measure of the relative degrees of changes in the cell composition of algae.

The exposure to 1000 ppmv CO_2 significantly affected the cell composition of *A. klebsi* only; significant changes were observed in the cell organic composition of this species 24 h after the onset of the environmental perturbation; even larger modifications of cell composition were found after 12 days of exposure to high CO_2.

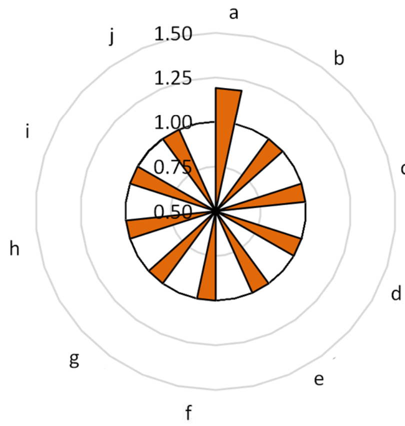
The long-term (20 days) transition from Light to Dark affected the overall cell composition of 7 out of 10 species: *A. klebsii*, *D. salina*, *E. huxleyi*, *P. tricornutum*, *Synechococcus*, *T. pseudonana*, *T. weissflogii*. The short-term exposure to dark (1 day), instead, caused significant changes in *D. salina* only, at a lower extent than observed in the same species after 20 days of exposure.

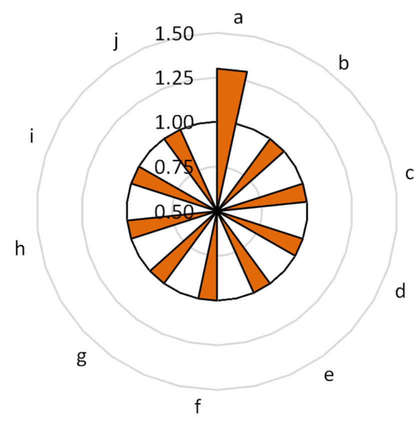
The change of N source in the culture medium did not affect the overall cell composition of any species within 24 h from the beginning of the treatment. Instead, the prolonged exposure to NH_4^+ triggered a reorganization of the cell organic composition in *Synechococcus* and *A. klebsii*.

Time after the onset of the perturbation

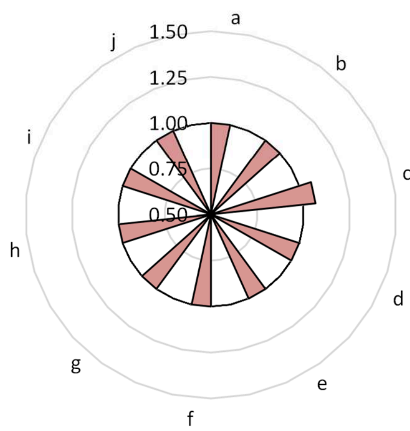
24 h **12-20 days**

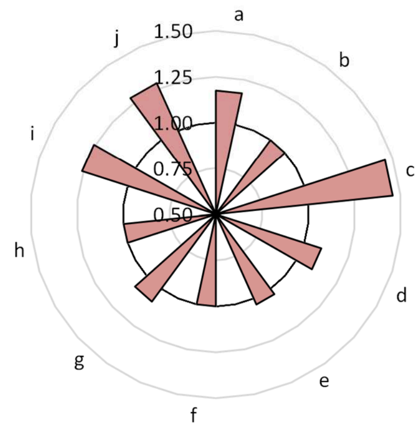
Low to High CO_2



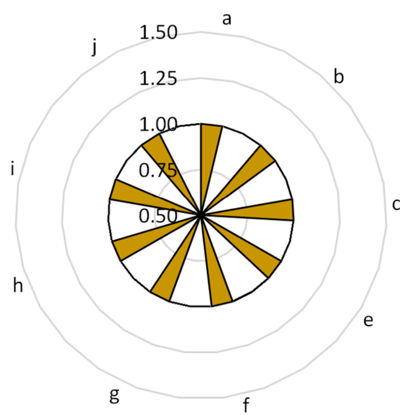


Light to Dark





NO_3^- to NH_4^+



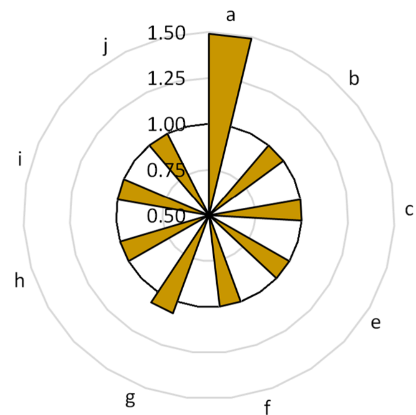


Fig. 3.2. (In the previous page). Bars within the inner black ring indicate that no significant changes were detected in cells exposed to the treatments; bars that extend beyond the black ring indicate that the treatment caused statistically significant compositional changes in the cells (CI > 1). Different letters represents different species, according to the following legend:

a = *Amphidium klebsii*; b = *Chlorella marina*; c = *Dunaliella salina*; d = *Emiliania huxleyi*; e = *Phaeodactylum tricornutum*; f = *Phormidium* sp.; g = *Synechococcus* sp.; h = *Tetraselmis suecica*; i = *Thalassiosira pseudonana*; j = *Thalassiosira weissflogii*.

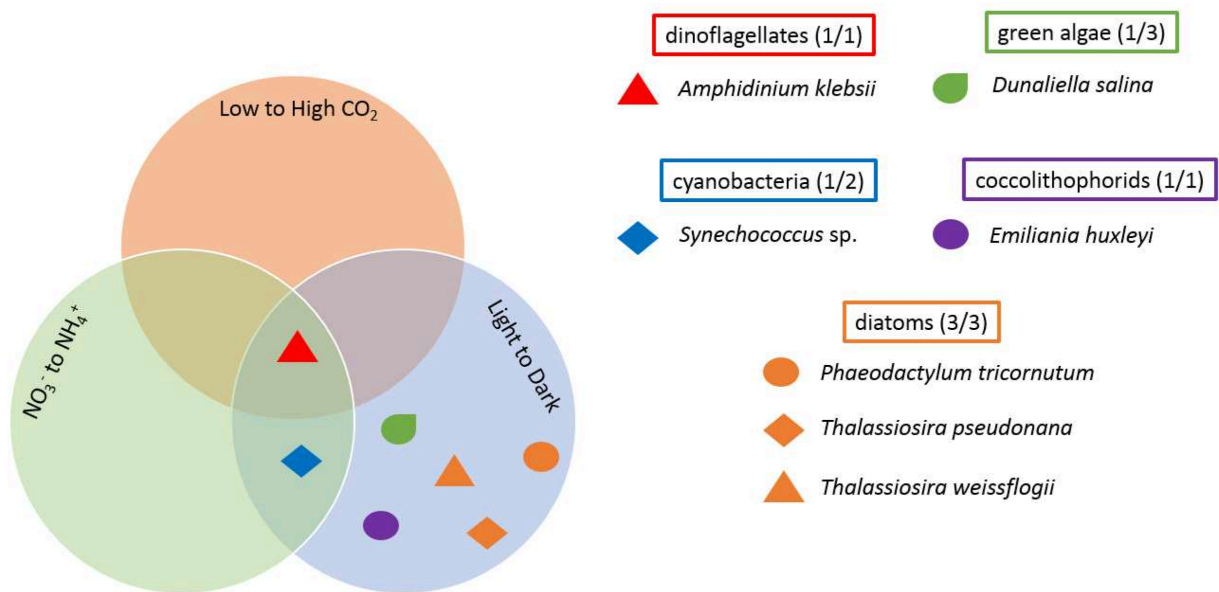


Fig. 3.3. Venn diagram representing the propensity of different algae (represented by different symbol/color combinations) belonging to different groups (represented by different colors) to respond to three different environmental transitions by changing their cell organic composition. In the legend, in parenthesis after the name of each algal group, the ratio between the number of species that changed their internal composition and the total number of examined species is reported (for instance, all examined diatoms – 3 over 3 – changed their cell composition).

Tab. 3.3. Summary of the algal species that changed their organic composition in response to a short-term and/or long-term environmental perturbation. In the reported species-treatment-time combinations, “s” (i.e. the similarity between treated spectra at two different sampling times) was significantly lower than s’ (i.e. the similarity between control spectra at two different sampling times); see paragraph 3.2.3.3 for further details. For each species-treatment-time combination, are shown: the s and s’ values; the p-values resulting from the comparison between s and s’; the change index ($C.I.= s'/s$).

Species-treatment-time combination	**s**	**s’**	**p-value**	**C.I.**
A. klebsii low to high CO_2 t0 vs t1	75.33 (3.487)	89.76 (4.909)	0.0143	1.192 (0.120)
A. klebsii low to high CO_2 t0 vs t2	66.86 (7.497)	86.93 (6.063)	0.0227	1.300 (0.236)
A. klebsii Light to Dark t0 vs t2	79.95 (0.17)	93.93 (2.21)	0.0004	1.175 (0.030)
D. salina Light to Dark t0 vs t1	93.15 (0.949)	99.36 (0.198)	0.0004	1.067 (0.013)
D. salina Light to Dark t0 vs t2	67.31 (2.84)	98.15 (0.41)	0.0001	1.458 (0.068)
E. huxleyi Light to Dark t0 vs t2	84.04 (2.708)	92.23 (1.711)	0.0114	1.097 (0.056)
P. tricornutum Light to Dark t0 vs t2	91.29 (1.87)	94.62 (0.52)	0.0411	1.036 (0.027)
Synechococcus Light to Dark t0 vs t2	91.00 (1.189)	98.89 (0.361)	0.0004	1.087 (0.018)
T. pseudonana Light to Dark	77.92 (2.08)	98.05 (0.52)	0.0001	1.258 (0.040)

t0 vs t2				
T. weissflogii Light to Dark t0 vs t2	74.52 (4.86)	95.64 (1.24)	0.0019	1.283 (0.100)
A. klebsii NO_3^- to NH_4^+ t0 vs t2	58.90 (5.58)	87.80 (1.08)	0.0009	1.491 (0.160)
Synechococcus NO_3^- to NH_4^+ t0 vs t2	79.06 (1.43)	84.39 (1.94)	0.0186	1.067 (0.044)

3.3.2 Qualitative evaluation of changes in the organic composition of algal cells

Cells of *A. klebsii* that were exposed to High CO_2 for 24 hours (T1) or 12 days (T2) had a higher lipid/protein ratio and a lower carbohydrate/lipid ratio than cells that were not subjected to this environmental shift (T0) (**Fig. 3.4**). Cells that were subjected to a long-lasting exposure to High CO_2 (T2) also had a significantly lower carbohydrates/protein ratio than the not exposed counterparts. The carbohydrate/lipid ratio was also significantly lower at T2 than at T1 (**Fig. 3.4**).

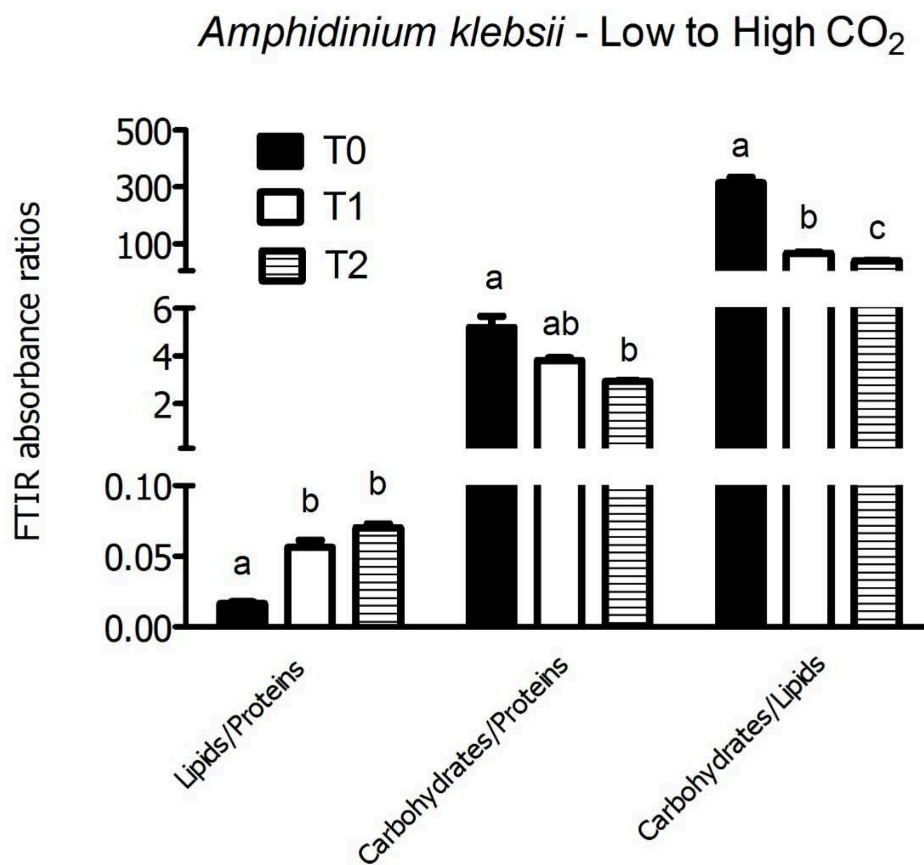


Fig. 3.4. FTIR absorbance ratios between the main organic pools in *A. klebsii* cells subjected to a Low (400 ppmv) to High (1000 ppmv) CO_2 shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; $n = 3$).

A significant difference was found between the carbohydrate/protein ratio at T0 and T2 in *A. klebsii* cells subjected to the Light to Dark transition; specifically, this ratio was lower after 20 days of exposure to darkness. No significant differences were observed between the other organic pools over time (**Fig. 3.5**).

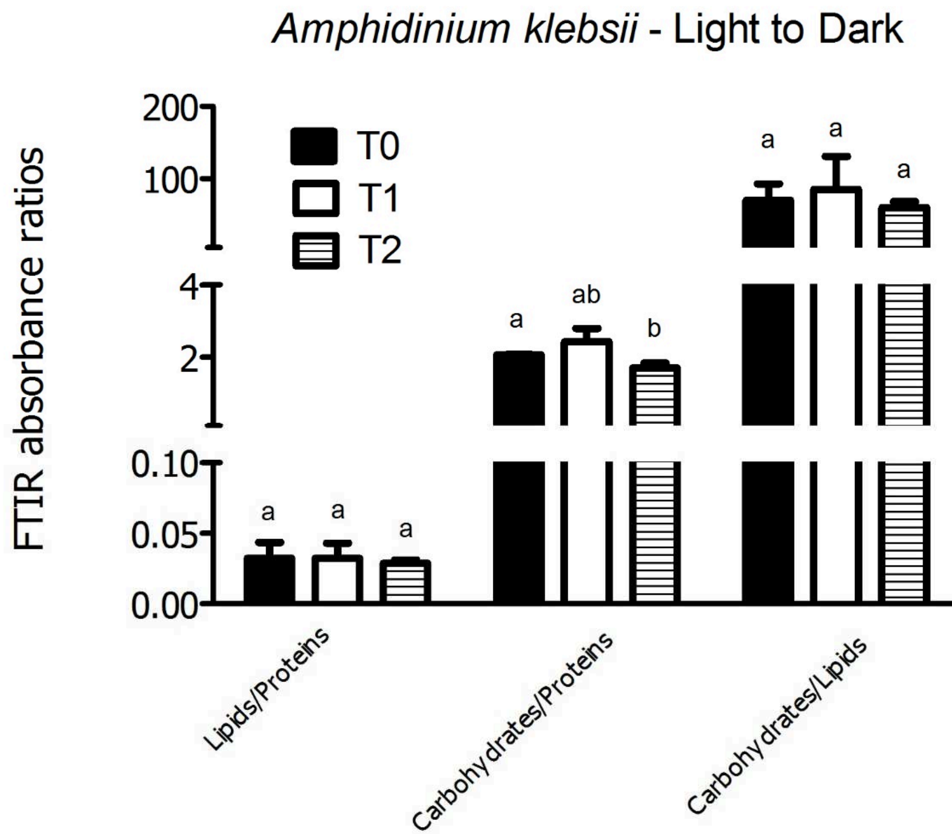


Fig. 3.5. FTIR absorbance ratios between the main organic pools in *A. klebsii* cells subjected to a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; n =3).

The exposure of *A. klebsii* to NH_4^+ for 20 days caused a significant increase of the lipid/protein ratio and a significant decrease of the carbohydrate/lipid ratio, while a shorter exposure (24 hours) to NH_4^+ did not elicit significant changes in the organic composition (**Fig. 3.6**).

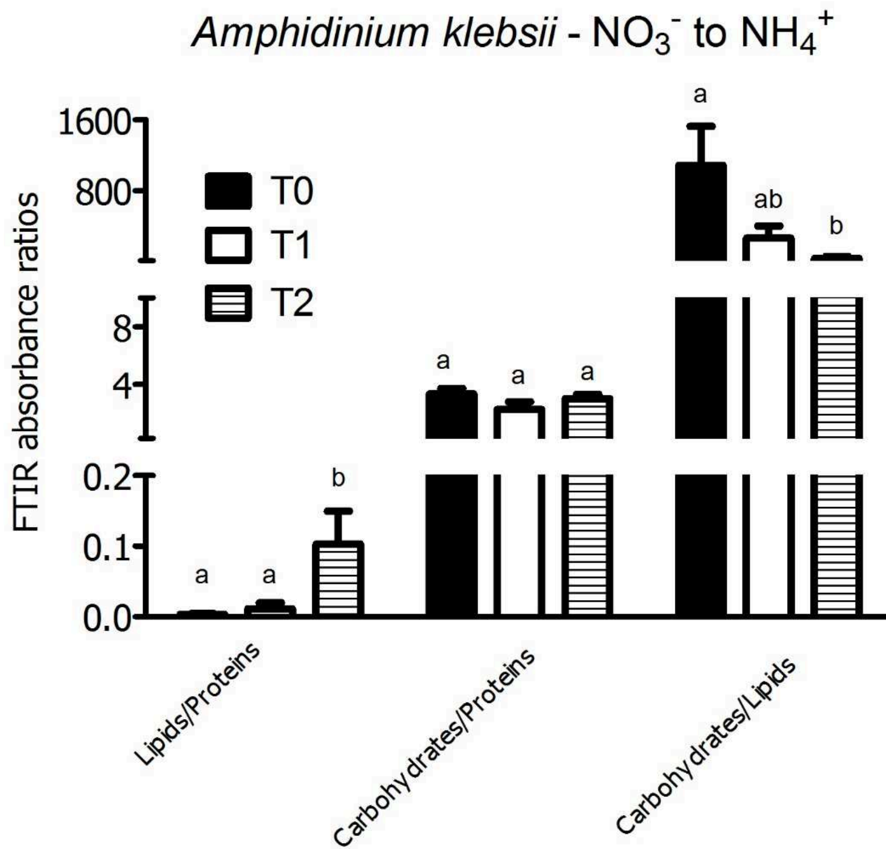


Fig. 3.6. FTIR absorbance ratios between the main organic pools in *A. klebsii* cells subjected to a change in the N source (from NO_3^- to NH_4^+). For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; $n = 3$).

The exposure to dark caused significant changes in the cell organic composition of *D. salina*, which were already evident 24 hours after the onset of the perturbation; specifically, the carbohydrate/protein and the carbohydrate/lipid ratios were lower at T1 than at T0, while the lipid/protein ratio was unvaried at the two sampling times (**Fig. 3.7**). At T2, cells of *D. salina* had a lower lipid/protein ratio and a higher carbohydrate/lipid ratio than cells at T0 and T1. The carbohydrate/protein ratio was equal to that of cells exposed for 24 hours to the dark (**Fig. 3.7**).

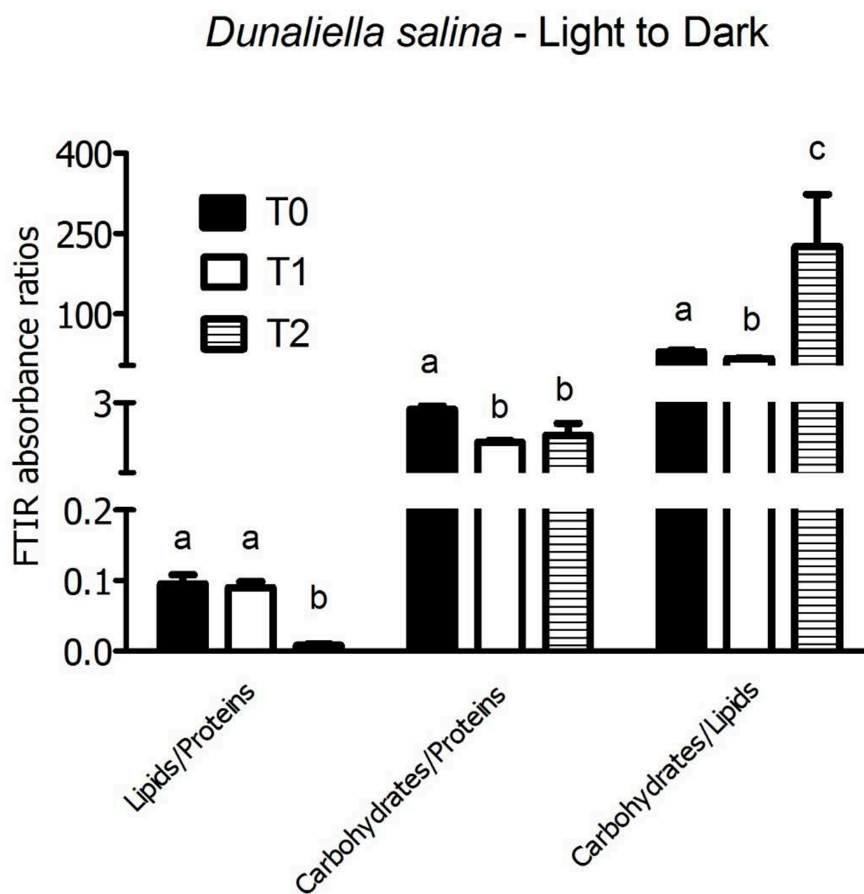


Fig. 3.7. FTIR absorbance ratios between the main organic pools in *D. salina* cells subjected to a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; $n = 3$).

The cell organic composition of *E. huxleyi* exposed to darkness for 24 hours was equal to that at T0. Cells exposed to dark for 20 days, instead, had a significantly lower lipid/protein ratio and a significantly higher carbohydrate/lipid ratio than cells at T0 and T1 (**Fig. 3.8**).

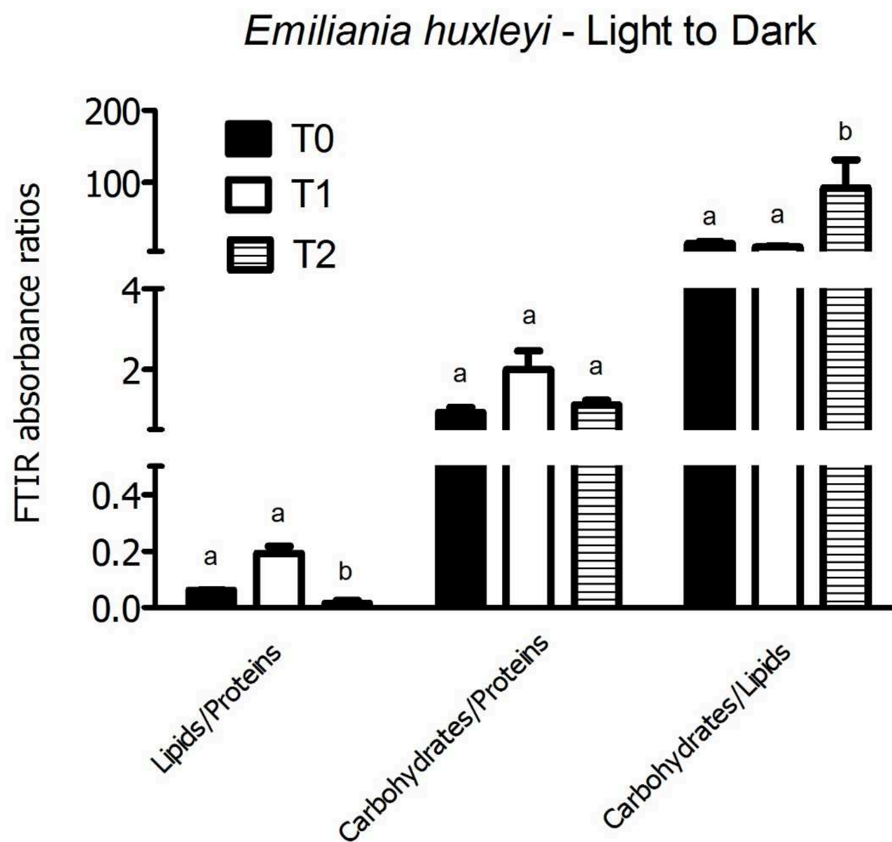


Fig. 3.8. FTIR absorbance ratios between the main organic pools in *E. huxleyi* cells subjected to a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; n =3).

The long-term (20 days) exposure to darkness triggered a reorganization of the cell organic composition of *P. tricornutum* cells. The carbohydrate/protein and the carbohydrate/lipid ratios were higher at T2 than at the other sampling times. No significant differences were found between cells at T0 and at T1 in terms of ratios among the main cell organic pools (**Fig. 3.9**).

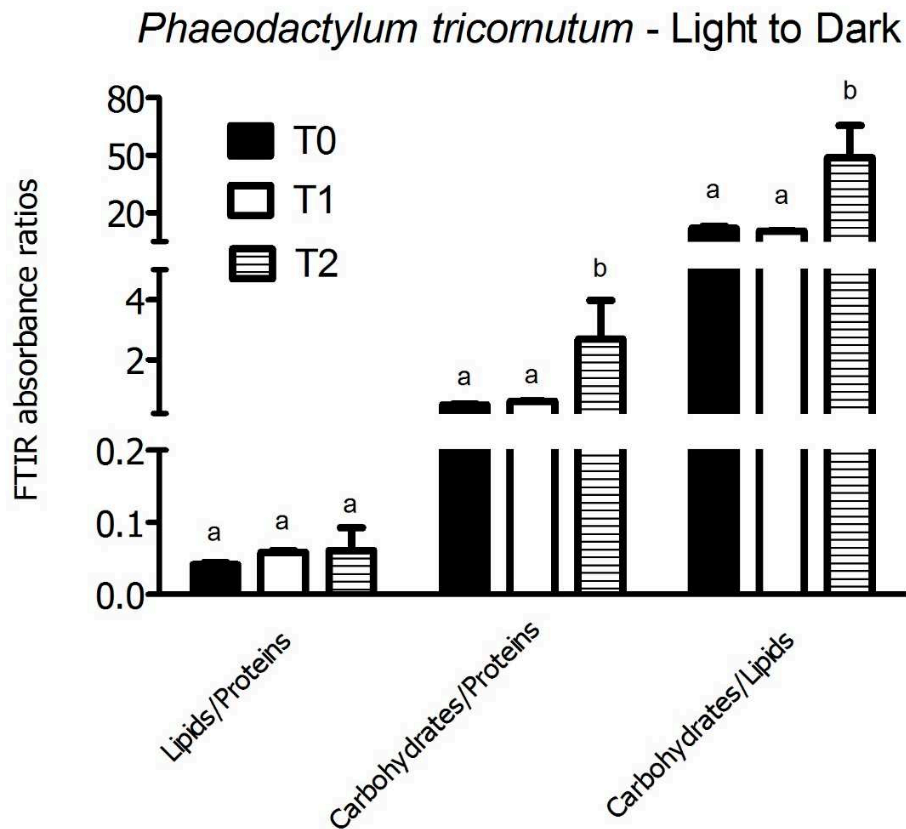


Fig. 3.9. FTIR absorbance ratios between the main organic pools in *P. tricornutum* cells subjected to a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; $n = 3$).

Significant differences were found between the organic composition of *Synechococcus* cells exposed to dark for 20 days and cells exposed for 24 hours; specifically, the lipid/protein ratio was higher at T2 than at T1, while the carbohydrate/lipid ratio was lower at T2 than at T1 (**Fig. 3.10**). The carbohydrate/protein ratio at T2 was lower than those at both T0 and T1. No differences were observed between not exposed cells and cells exposed to dark for 24 hours (**Fig. 3.10**).

Synechococcus also responded to a long-term (12 days) exposure to NH_4^+ by changing its cell organic composition. The lipid/protein ratio at T2 was lower than at T1, while the carbohydrate/lipid ratio at T2 was higher than at T1 (**Fig. 3.11**).

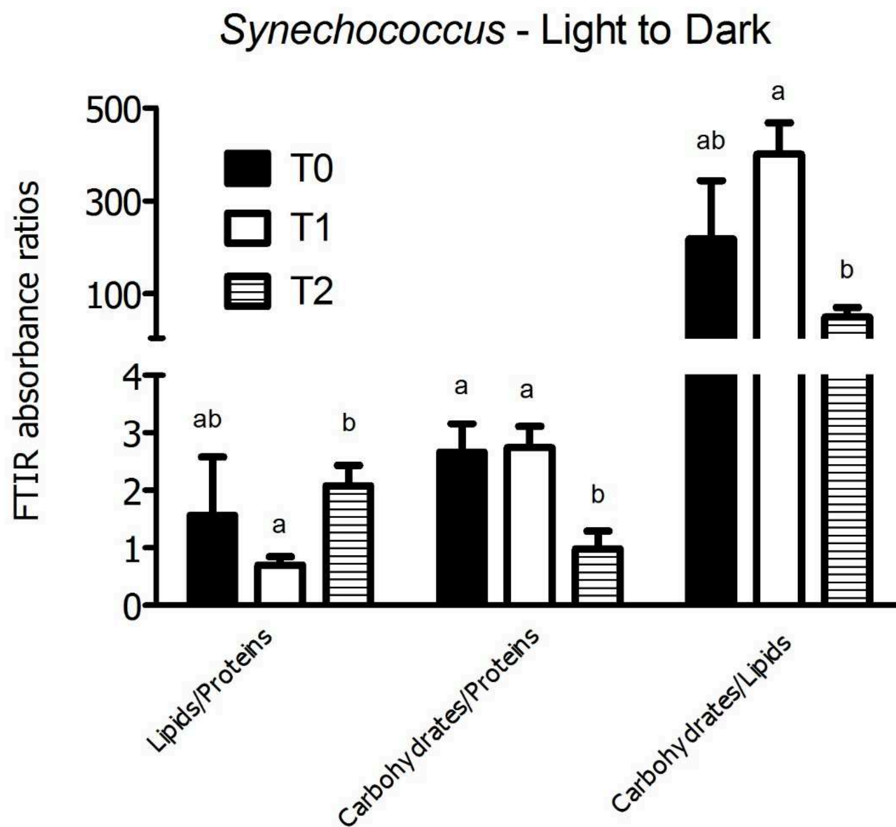


Fig. 3.10. FTIR absorbance ratios between the main organic pools in *Synechococcus* cells subjected to a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; n =3).

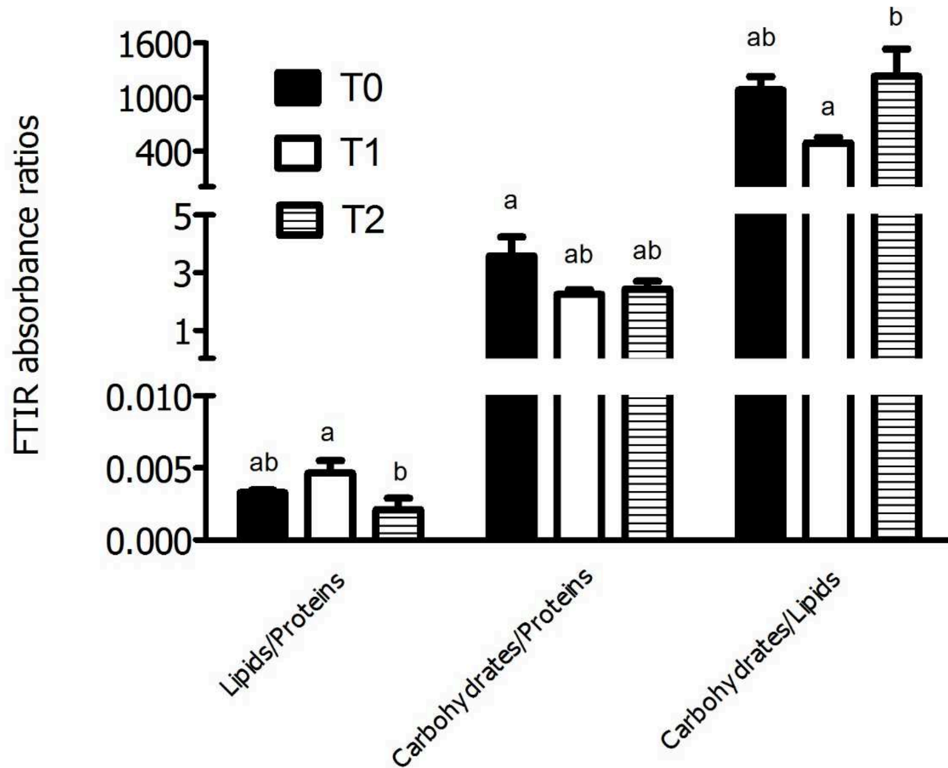


Fig. 3.11. FTIR absorbance ratios between the main organic pools in *Synechococcus* cells subjected a change in the N source (from NO_3^- to NH_4^+). For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; $n = 3$).

In *T. pseudonana* cells, the exposure to dark for 20 days elicited changes in the organic composition, while no significant changes were found between cells at T0 and cells exposed to dark for 24 hours only. The lipid/protein ratio was higher at T2 than at T1 and the carbohydrate/lipid ratio was lower than at the other sampling times (**Fig. 3.12**). The observed changes in the ratio among the main organic pools suggest that carbohydrates and proteins were consumed preferentially over lipids, or that lipids increased over time (or both). However, considering that lipids have a high energy content per unit of mass and volume (Pronina et al 1998; Raven 2005; Gushina and Harwood 2006), it seems unlikely that the second scenario occurred in the absence of light.

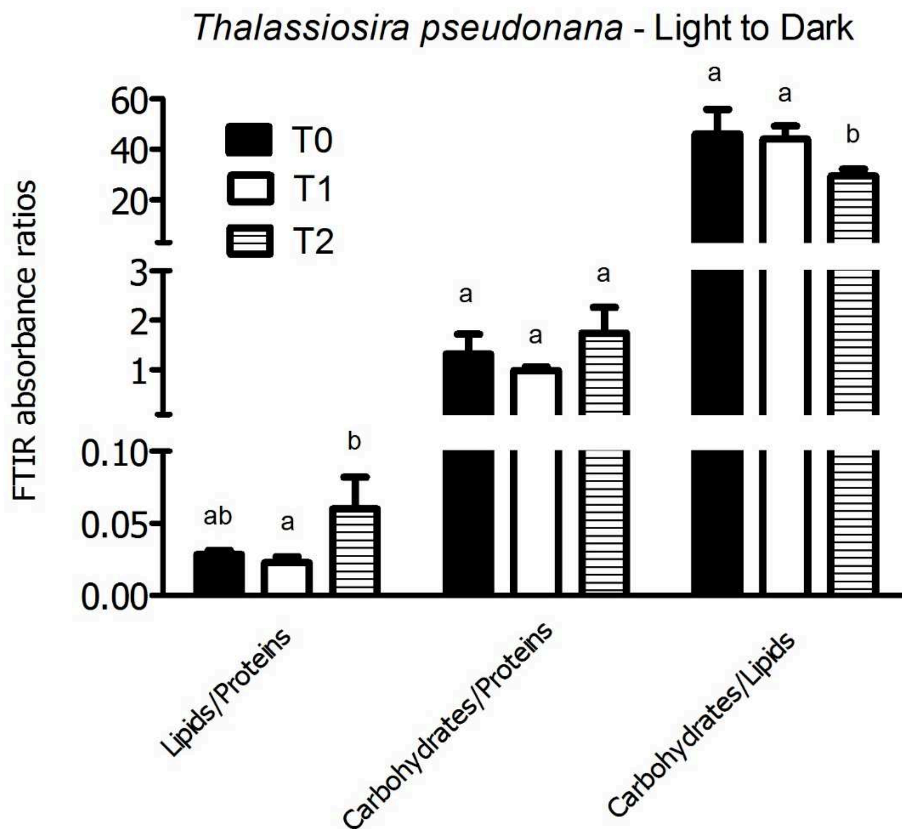


Fig. 3.12. FTIR absorbance ratios between the main organic pools in *T. pseudonana* cells subjected a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; $n = 3$).

T. weissflogii cells exposed for 20 days to dark (T2) had significantly lower lipid/protein and carbohydrate/protein ratios than cells at T0 and at T1. No differences in organic composition were found between cells at T0 and cells at T1 (**Fig. 3.13**).

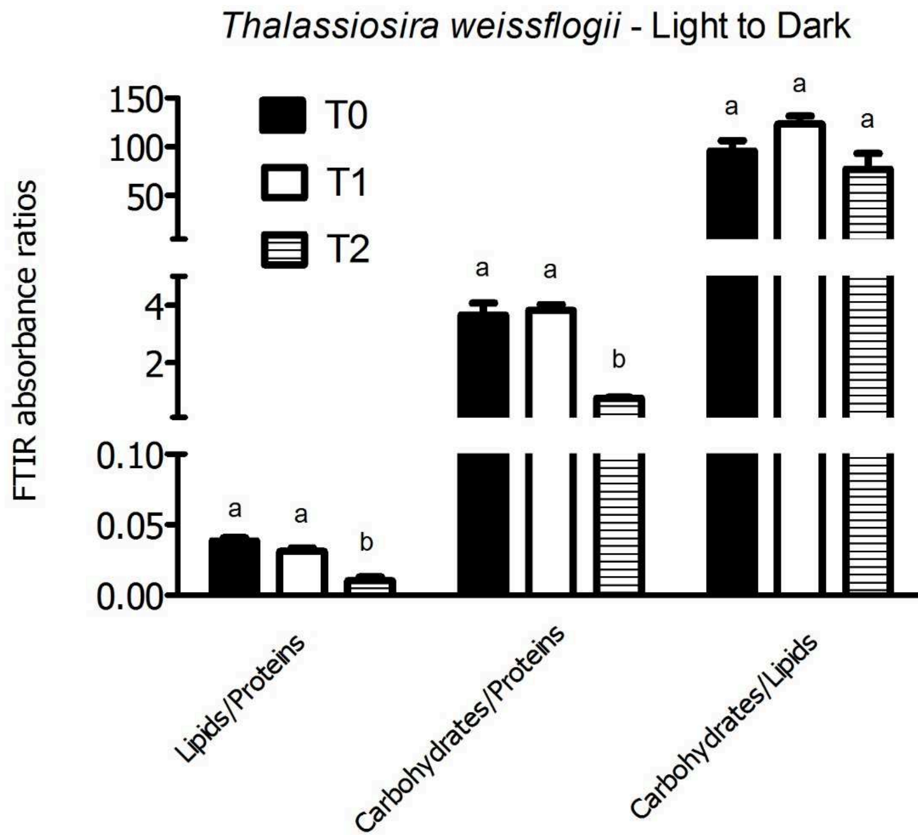


Fig. 3.13. FTIR absorbance ratios between the main organic pools in *T. weissflogii* cells subjected a Light to Dark shift. For each ratio, different letters above the bars indicate significant differences between the means ($p < 0.05$; n =3)

Tab. 3.4. FTIR absorbance ratios among the main organic pools in algae whose composition was affected by the indicated treatment(s).

Species/Treatment	Lipids/Proteins			Carbohydrates/Proteins			Carbohydrates/Lipids		
	T0	T1	T2	T0	T1	T2	T0	T1	T2
A. klebsii	**0.017**	**0.056**	**0.070**	**5.186**	**3.803**	**2.923**	**314.5**	**67.74**	**41.71**
Low to High CO_2	(0.001)	(0.005)	(0.003)	(0.466)	(0.113)	(0.027)	(19.11)	(4.163)	(2.087)
A. klebsii	**0.032**	**0.032**	**0.029**	**2.065**	**2.424**	**1.701**	**69.61**	**85.08**	**59.69**
Light to Dark	(0.011)	(0.011)	(0.002)	(0.024)	(0.376)	(0.141)	(22.86)	(45.79)	(8.475)
A. klebsii	**0.003**	**0.011**	**0.103**	**3.325**	**2.256**	**2.997**	**1084**	**263.9**	**36.34**
NO_3^- to NH_4^+	(0.001)	(0.008)	(0.047)	(0.367)	(0.532)	(0.322)	(440.6)	(137.3)	(24.16)
D. salina	**0.095**	**0.090**	**0.008**	**2.733**	**1.428**	**1.705**	**28.93**	**15.95**	**225.5**
Light to Dark	(0.013)	(0.008)	(0.002)	(0.143)	(0.079)	(0.466)	(3.204)	(1.160)	(97.54)
E. huxleyi	**0.061**	**0.192**	**0.015**	**0.929**	**1.992**	**1.115**	**15.27**	**10.30**	**92.17**
Light to Dark	(0.002)	(0.026)	(0.010)	(0.123)	(0.463)	(0.117)	(2.260)	(0.986)	(39.12)
P. tricornutum	**0.041**	**0.059**	**0.061**	**0.491**	**0.609**	**2.686**	**11.89**	**10.41**	**48.71**
Light to Dark	(0.003)	(0.002)	(0.032)	(0.033)	(0.011)	(1.289)	(1.155)	(0.419)	(16.80)
Synechococcus	**0.016**	**0.007**	**0.021**	**2.655**	**2.737**	**0.979**	**217.8**	**399.8**	**49.13**
Light to Dark	(0.010)	(0.001)	(0.004)	(0.502)	(0.368)	(0.312)	(125.8)	(68.23)	(21.36)
Synechococcus	**0.003**	**0.005**	**0.002**	**3.577**	**2.247**	**2.433**	**1081**	**492.1**	**1233**
NO_3^- to NH_4^+	(0.000)	(0.001)	(0.001)	(0.660)	(0.158)	(0.268)	(147.8)	(60.32)	(300.4)
T. pseudonana	**0.028**	**0.023**	**0.060**	**1.316**	**0.983**	**1.736**	**45.984**	**44.01**	**29.37**
Light to Dark	(0.003)	(0.004)	(0.022)	(0.404)	(0.075)	(0.527)	(9.787)	(5.171)	(2.728)
T. weissflogii	**0.038**	**0.031**	**0.010**	**3.652**	**3.808**	**0.770**	**95.38**	**123.2**	**76.57**
Light to Dark	(0.003)	(0.002)	(0.003)	(0.418)	(0.207)	(0.034)	(10.73)	(8.493)	(16.63)

3.3.3 Change of cell numbers in the dark

The cell concentration (cell ml^{-1}) measured in algal cultures exposed to the Light to Dark transition (**n**) (**Tab. 3.5**) was compared to:

1) The number of cells expected if cultures grew at the growth rate imposed by dilution (= $\mathbf{n_5}$);

2) The number of cells expected if the growth rate was zero (= $\mathbf{n_0}$).

The differences between **n** and $\mathbf{n_5}$ at six sampling times (**Δ1)** and the differences between **n** and $\mathbf{n_0}$ (**Δ2**) at six sampling times are summarized in **Fig. 3.14**, and shown in **Fig. 3.15** - **Fig. 3.24** for each different species.

After 4, 7, 11 and 20 days in the dark, the cell concentration of *A. klebsii* was significantly lower than that expected based on the growth rate imposed at the beginning of the treatment, while after 20 days in the dark it was significantly higher than the cell concentration expected assuming zero growth (**Fig. 3.15**). Similar results were obtained when cells of *C.marina*, *D. salina*, *Phormidium* sp., *Synechococcus*, *Tetraselmis suecica*, *Thalassiosira pseudonana* and *T. weissflogii* were exposed to darkness, except for the fact that their cell number per ml was significantly higher than those expected at zero growth also after 11 days in the dark (**Fig. 3.16**, **Fig. 3.17**; **Fig. 3.20**, **Fig. 3.21**; **Fig. 3.22**, **Fig. 3.23**; **Fig. 3.24**). In *P. tricornutum*, a similar trend was observed, except for the fact that the cell number per ml was significantly lower than those expected assuming growth = 0.14 d^{-1} already after 24 hours of exposure to darkness (**Fig. 3.19**).

E. huxleyi was able to grow for 11 days at the rate that was imposed at T0; at the end of the experiment, anyhow, its growth rate was significantly lower than that imposed by dilution ($\mu = 0.14$ d^{-1}) (**Fig. 3.18**).

Tab. 3.5. Cell concentration (cell ml^{-1}) in algal cultures subjected to the "Light to Dark" shift. The number of cell per unit of volume was assessed before the beginning of the treatment (0) and 1, 4, 7, 11 and 20 days after the beginning of the treatment. Number in parenthesis refer to standard deviations (n = 3).

Species	Time (days)					
	0	**1**	**4**	**7**	**11**	**20**
A. klebsii	4.09E+05 (1.53E+05)	4.68E+05 (4.66E+04)	8.15E+04 (1.66E+04)	6.15E+04 (1.53E+04)	3.46E+04 (3.23E+03)	6.51E+04 (1.22E+04)
C. marina	7.88E+05 (8.72E+04)	6.44E+05 (3.18E+04)	3.05E+05 (1.28E+05)	2.70E+05 (5.13E+04)	2.05E+05 (1.12E+05)	1.01E+05 (1.90E+04)
D. salina	1.73E+06 (2.05E+05)	1.68E+06 (1.47E+05)	6.24E+05 (3.29E+05)	4.43E+05 (1.65E+05)	3.33E+05 (6.65E+04)	1.89E+05 (5.84E+04)
E. huxleyi	4.03E+05 (2.78E+05)	3.80E+05 (6.67E+04)	6.62E+05 (1.38E+05)	6.93E+05 (1.44E+05)	1.29E+06 (2.59E+05)	4.28E+05 (1.58E+05)
P. tricornutum	5.04E+06 (6.67E+05)	3.59E+06 (3.82E+05)	1.97E+06 (4.81E+05)	1.61E+06 (1.87E+05)	5.94E+05 (9.52E+04)	1.02E+06 (3.23E+05)
Phormidium sp.	7.48E+05 (4.98E+04)	6.97E+05 (2.88E+04)	2.99E+05 (1.83E+05)	3.32E+05 (1.37E+05)	1.46E+05 (5.01E+04)	1.06E+05 (7.76E+03)
Synechococcus	2.03E+07 (1.44E+06)	2.21E+07 (1.59E+06)	7.74E+06 (4.87E+06)	8.93E+06 (1.22E+06)	6.55E+06 (1.21E+06)	4.34E+06 (9.24E+05)
T. suecica	1.20E+06 (1.44E+05)	1.14E+06 (1.64E+05)	8.94E+05 (8.46E+04)	8.03E+05 (6.76E+04)	4.76E+05 (3.69E+05)	3.96E+05 (2.51E+05)
T. pseudonana	5.79E+06 (1.73E+05)	5.89E+06 (5.05E+05)	2.09E+06 (2.77E+05)	1.30E+06 (3.08E+05)	9.45E+05 (4.32E+05)	3.85E+05 (7.46E+04)
T. weissflogii	7.50E+05 (7.22E+04)	6.63E+05 (4.13E+04)	3.24E+05 (1.54E+05)	3.10E+05 (1.40E+05)	2.07E+05 (1.16E+05)	1.06E+05 (2.04E+04)

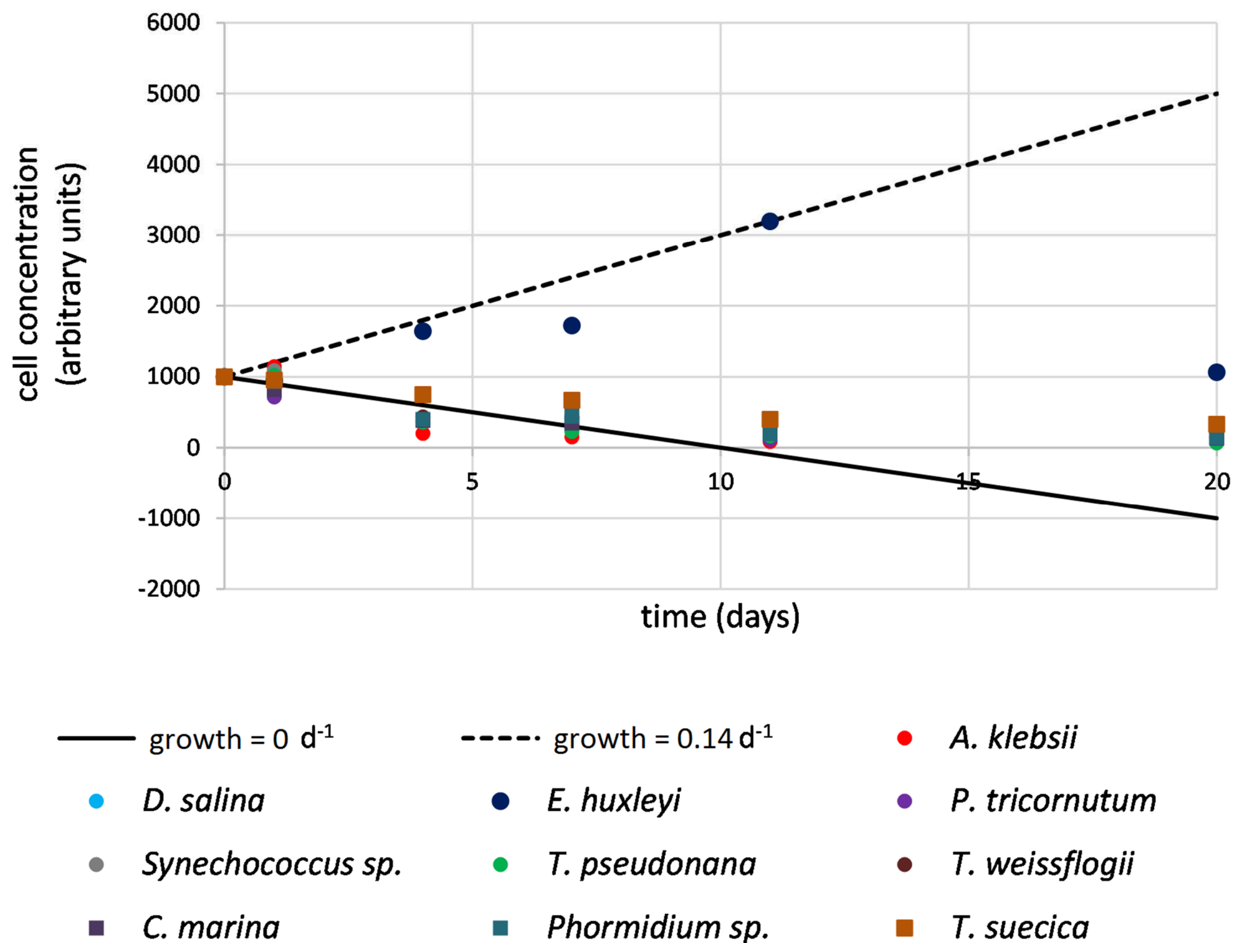


Fig. 3.14. For algae subjected to the "Light to Dark" transition, the number of cell per ml over time (normalized so that the initial density = 1000, for all species) is reported (colored dots and squares). These values are compared to the cell concentration expected if we assume zero growth and 10% daily dilution (black solid line) and to the cell concentration expected if I assume that the growth actually followed the growth rate we imposed at T0 (black dashed line).

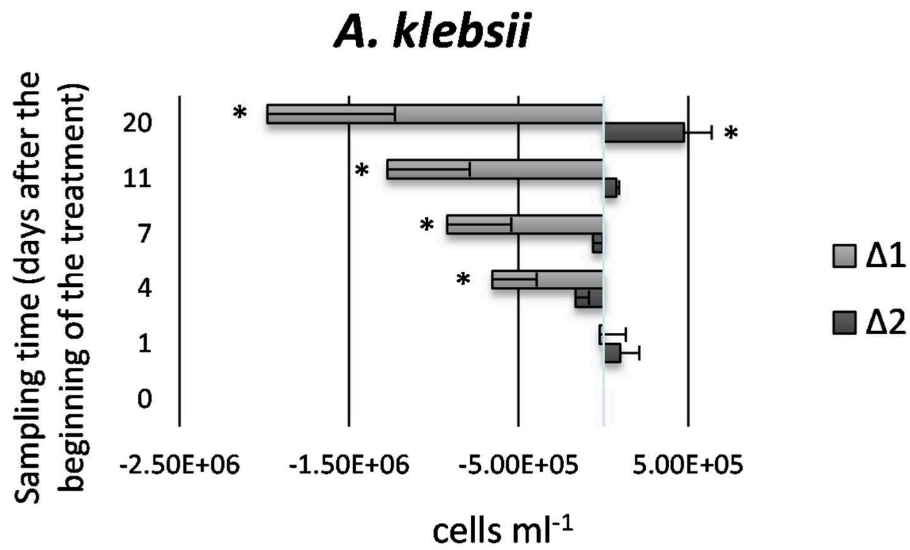


Fig. 3.15. For *A. klebsii* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate ($\mu = 0.14$ d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

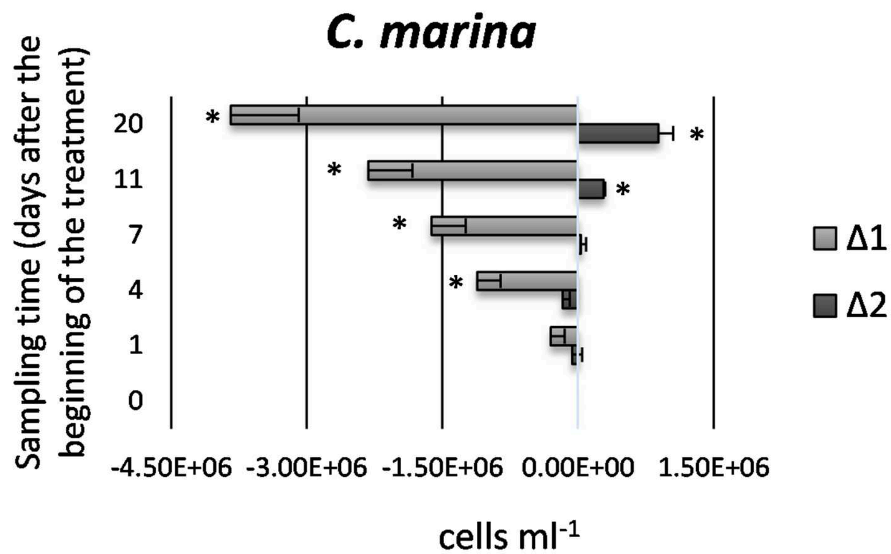


Fig. 3.16. For *C. marina* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate ($\mu = 0.14$ d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

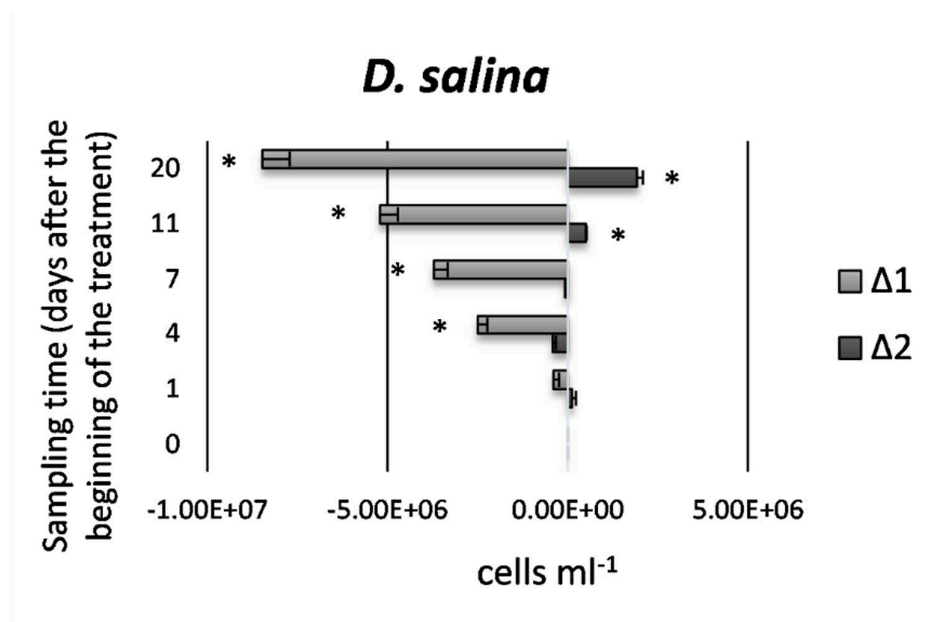


Fig. 3.17. For *D. salina* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate ($\mu = 0.14\ d^{-1}$), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

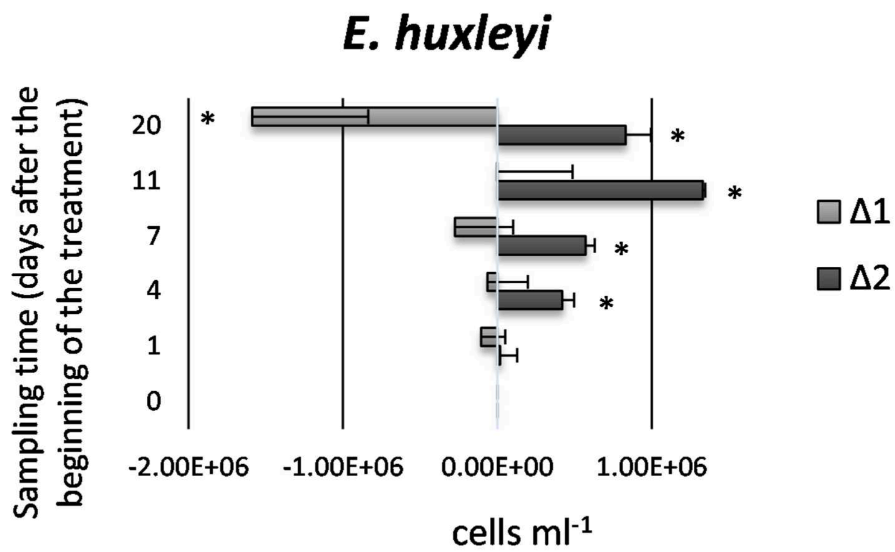


Fig. 3.18. For *E. huxleyi* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate ($\mu = 0.14$ d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

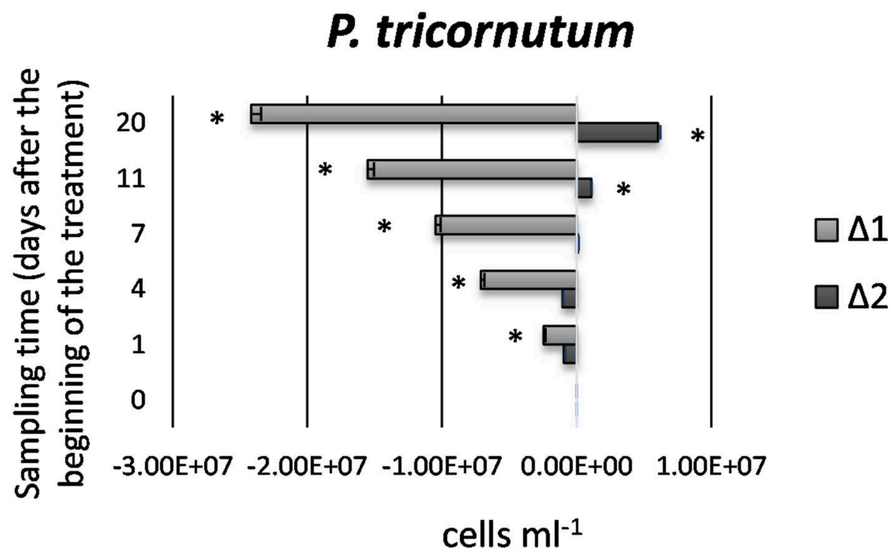


Fig. 3.19. For *P. tricornutum* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate (μ = 0.14 d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

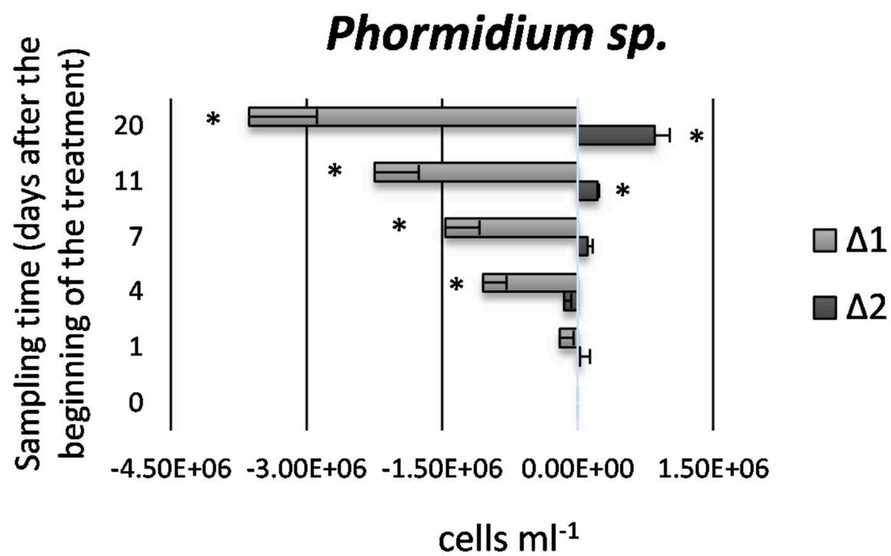


Fig. 3.20. For *Phormidium* sp. cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate (μ = 0.14 d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

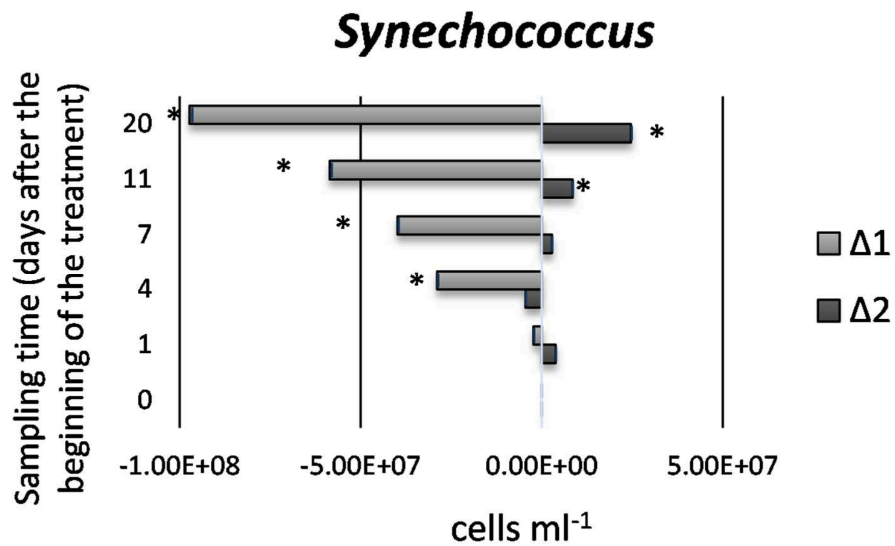


Fig. 3.21. For *Synechococcus* sp. cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate ($\mu = 0.14\ d^{-1}$), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

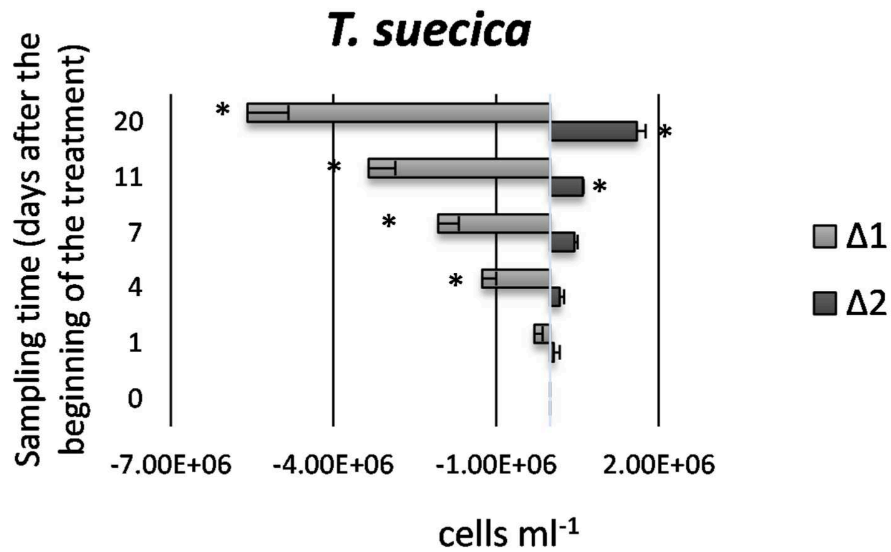


Fig. 3.22. For *T. suecica* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate ($\mu = 0.14\ d^{-1}$), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

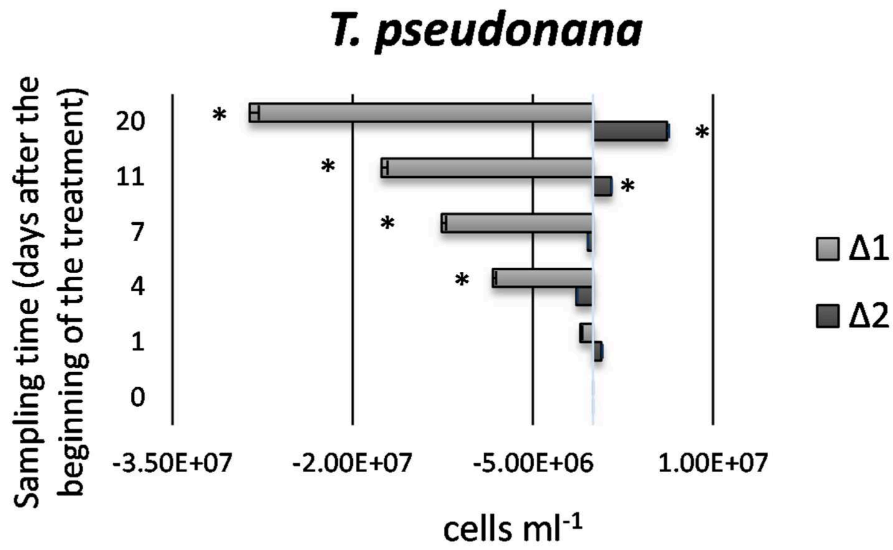


Fig. 3.23. For *T. pseudonana* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate (μ = 0.14 d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

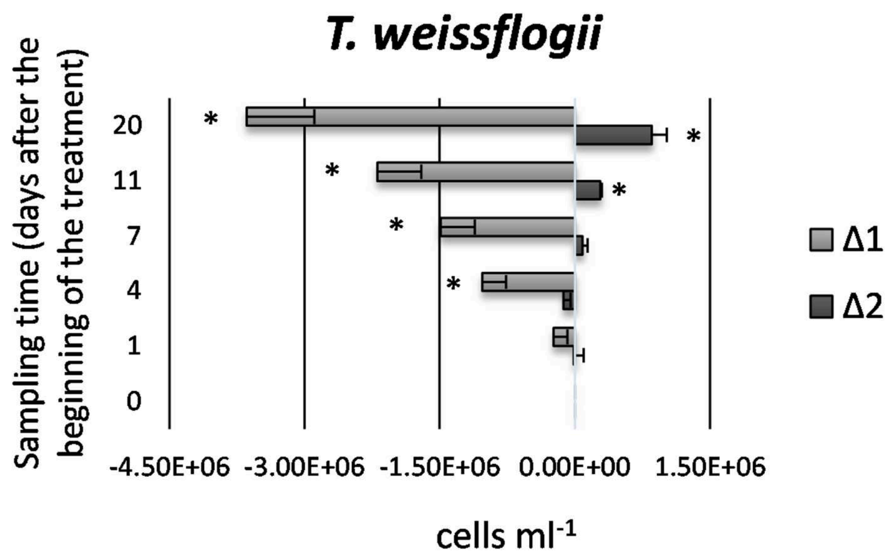


Fig. 3.24. For *T. weissflogii* cultures subjected to the "Light to Dark" shift, I report: the differences between the measured cell concentration and that expected assuming that cultures grew at the imposed rate (μ = 0.14 d^{-1}), at six sampling times (**Δ1**, grey bars); the differences between the measured cell concentration and that calculated assuming zero growth, at six sampling times (**Δ2**, black bars). Error bars represent standard deviations (n = 3).

3.4 DISCUSSION

3.4.1 Light to Dark shift

Among the three perturbations examined here, the “Light to Dark” transition triggered a cellular reorganization in a higher number of algal species compared to the other two environmental stimuli under examination. This treatment was selected because it would provide an instance in which phototrophic organisms cannot attain acclimation, although they may try. It is actually likely that several of our experimental organisms are capable of some degree of mixotrophy. In order to avoid that this ability makes data interpretation more difficult, we made sure that no appreciable amounts of organic matter were present in the growth medium (although absolute axenicity is hard to ensure for some species).

Previous studies showed that differences exist among algae belonging to different groups in the sensitivity to darkness. Diatoms were shown to bear prolonged periods of light deprivation; *Thalassiosira weissflogii* for instance, was able to survive 2 weeks in darkness (Berges and Falkowski 1998), while *T. gravida* could survive for 90 days in darkness (Smayda and Mitchell-Innes 1974). Several Antarctic diatoms have been shown to withstand many weeks in the dark in a vegetative state (Peters and Thomas 1996). Clorophytes, instead, appear unable to survive even shorter period of darkness (for instance, *Dunaliella tertiolecta* could not survive 6 days in the dark; Berges and Falkowski 1998). According to Berges and Falkowski, these differences may reflect ecological aspects. Diatoms, in fact, are very sensitive to the N deprivation, which can occur in surface water. In such cases, the ability to survive long-term exposures to darkness may allow diatoms to move to the deep waters, in which nutrients are more abundant (Smetacek 1985). These authors were aware that green algae of the genus *Scenedesmus* were reported to be able to survive in the dark for 3 months (Dehning and Tizler 1989). However, they pointed out that, in the case described by Dehning and Tizler, the majority of cells of the populations underwent lysis much before 3 months and that a drastic collapse of the protein content occurred in the first 5 days after the beginning of the exposure to darkness (Berges and Falkowski 1998). New reports of green algae capable of surviving in the dark with no organic C sources were published later on. For example, *Scenedesmus quadricauda* survived in the dark for 20 days (Furusato et al 2004) and *Koliella Antarctica* was able to withstand 90 days of darkness without any C source (Baldisserotto et al 2005).

My results show that in spite of the prolonged darkness, which should not allow growth, minimal proliferation of cells occurred in all species. No indications were found that supported the existence of differences in the degree of adaptation to darkness between diatoms and green algae. Since no new organic C could be generated under my experimental conditions, the observed proliferation must be associated to a decrease in the amount of organic matter per cell. The cells of all my experimental species, thus, opted for cell division, i.e. a multiplication of genotype copies, rather than for a fully homeostatic maintenance of existing biomass per cell, when exposed to prolonged darkness. Part of the biomass was likely employed by cells to generate the energy required for cell division and other potential metabolic activities. This was for instance observed in the case of the cyanobacterium *Phormidium autumnale*, whose cells tackled a long-term exposure to darkness (21 days) by consuming their organic pools (Montechiaro et al 2006).

In *P. autumnale*, the consumption of the main organic pools (proteins, lipids and carbohydrates) during 21 days in the dark occurred in a balanced way, so that the proportion among the main cell pools was retained (Montechiaro et al 2006), maintaining a homeostasis of pool ratios (but not of pool size). These authors proposed that the retention of a balanced cell composition is a strategy that was selected to cope with transient perturbations (Fanesi et al 2013) and to allow prompt resumption of growth when pre-perturbation conditions are restored. A similar situation was observed in my data set for a different strain of *Phormidium* and for the two green algae *T. suecica* and *C. marina* exposed to 20 days of darkness. A homeostatic response was also observed in all experimental species after 24 hours in the dark, with the only exception of *D. salina*. In the case of *A. klebsii*, *D. salina*, *E. huxleyi*, *P. tricornutum*, *Synechococcus*, *T. pseudonana* and *T. weissflogii*, this compositional homeostasis (in terms of ratio among the cell pools) was not maintained after the long-term exposure to darkness. In these species, the observed changes in the proportion among the main organic pools likely result from the uneven consumption of proteins, lipids and carbohydrates; differences among species can be linked to the different nature of the preferential resources. In most of these species, the biomass quality was modified through the consumption of either carbohydrates and/or lipids, as it is expected based on energetic considerations. According to Geider and La Roche, for instance, 1 g of polyglucan, 1 g of lipid and 1 g of protein require respectively, in order to be oxidized, 37 , 54.5 and 88 mmol O_2 (Geider and La Roche 2002). The preferential use of one and/or the other of these pools was species-specific, with no obvious link to taxonomy.

In *D. salina* a change of strategy has been observed over time: after 24 hours in the dark, this species had mostly consumed carbohydrates; after 20 days in the dark, instead, a greater consumption of

lipids than of the other cell pools was observed, likely due to the previous massive oxidation of carbohydrates.
All examined diatoms changed their cell organic composition after 20 days in the dark. Surprisingly and conversely to what observed in the other species, in the diatoms *P. tricornutum* and *T. pseudonana* also the protein pool was used to sustain growth during darkness, while *T. weissflogii* mostly consumed carbohydrates and lipids, in a proportional way. Thus, no correlation between the taxonomy of the species and the adopted strategy was found, since the two congeneric diatoms consumed the cell pools in a different way. The observed differences may be due to the fact that in *P. tricornutum* and *T. pseudonana* carbohydrates and lipid storages are constrained by the small size of cells. However, to the best of my knowledge, no solid experimental evidences supporting this argumentation are currently available; furthermore, no clear correlation between the size of algae and their specific growth rate has been found (Raven et al 2005; Raven et al 2013), which would suggest a similar resource allocation for algal cells of various size.

3.4.2 Low to High CO_2 shift; NO_3^- to NH_4^+ shift

Conversely to the "Light to Dark" transition, the "Low to High CO_2" and the "NO_3^- to NH_4^+" transitions have been selected for this study as environmental perturbations to which algae are expected to be able to acclimate. This does not mean that they are expected to always and exclusively acclimate, but rather to possess the potential for acclimation to these environmental stimuli. In fact, algae under investigation likely experience in their original habitat frequent changes of nutrient availability and CO_2 partial pressure. In nature, for instance, the relative concentrations of inorganic N compounds can vary rapidly, especially in coastal waters and in environments characterized by periodic fluctuation of the physicochemical parameters, such as the rockpools (Loder et al 1996). Algae are thus are expected to be adapted to "Low to High CO_2" and "NO_3^- to NH_4^+" environmental shifts, and to possess the physiological machinery for copying with them. A proof of such adaptation is the presence of systems for the management of both elevated and tiny concentrations of ammonium. Algae, for instance, are able to extrude NH_4^+ from the cytosol to the medium and/or to accumulate it into vacuoles (Britto et al 2001; Loqué et al 2005). Since NH_4^+ can passively move from the cytosol to the mitochondria in virtue of the negative electric potential of the mitochondrial matrix compared to the cytosol (Nobel 1999), mechanisms of NH_4^+ assimilation that limit the accumulation of NH_4^+ in the mitochondrial matrix have also evolved in green algae (Giordano et al 2003; Braun and Zabaleta 2007). Similarly, microalgae possess mechanisms for the management of CO_2 variations; the CO_2

concentrating mechanisms, for instance, can be modulated in many algal species, according to the CO_2 availability in environment (Beardall and Giordano 2002 and references therein).

The use of either NO_3^- or NH_4^+ requires that algal cells perform specific functional rearrangements to fulfil the distinct metabolic and energetic requirements linked to the management of one or the other N form (Huppe and Turpin 1994; Giordano and Bowes 1997). Responses of algae to a "NO_3^- to NH_4^+" transition have been investigated in *Dunaliella parva* (Giordano et al 2007). These authors observed a two-phase response: in the first phase (0-48 hours from the beginning of the treatment) changes in the cell organic pools and in cell function such as were detected, among which a 2-fold increase of the cell protein content; in the second phase (48-168 hours after the beginning of the perturbation), cell constituents were brought back within the original ranges. Giordano and coauthors interpreted this finding as a consequence of the late activation of system for a tight control of intracellular NH_4^+ by *D. parva*. I did not observe changes in the cell composition of the congeneric *D. salina* exposed to NH_4^+ for up to 20 days. This may indicate that different strategies are employed by taxonomically related species, but may also reflect some important disparities in the experimental design. For instance, conversely to my experiments, Giordano and coauthors did not completely replace the NO_3^--containing culture medium with a NH_4^+-containing medium, but rather tried to simulate a gradual change in the dominant N source by pumping the NH_4^+-containing medium into the culture vessel.

My results show that only *A. klebsii* and *Synechococcus* sp. responded to the "NO_3^- to NH_4^+" shift by changing their organic composition. The two species, however, adopted different strategies. In *Synechococcus*, the comparison between the ratios among the main organic pools at T1 and at T2 does not exclude that an increase in the cell protein content may have occurred; the diversion of a higher quota of C towards the assimilation of NH_4^+ into proteins or amino acids is a possible way to control the amount of free NH_4^+ and limit its accumulation within the cell. The results obtained for *A. klebsii*, instead, do not support the possibility of an increased assimilation of NH_4^+ into protein compounds; instead, the changes observed in the ratios among the main organic pools suggest that the dinoflagellate have increased its lipid quota and/or decreased, proportionally, the cell amount of carbohydrates and proteins.

The dinoflagellate *A. klebsii* was also the only species to respond to the Low to High CO_2 shift by changing its organic composition. Other dinoflagellate have been shown to acclimate to High CO_2. Cells of *Protoceratium reticulatum* grown at 1000 ppm CO_2, for instance, had a significantly higher protein an chlorophyll content, and a higher carbohydrate/lipid ratio, than cells grown at 380 ppm CO_2 (Montechiaro and Giordano 2009). Dinoflagellate can accumulate both carbohydrates in form

of starch and lipids (Seo and Fritz 2002), but the CO_2 availability in the external medium does not seem to affect the partitioning of C between carbohydrates and lipids in *P. reticulatum* (Montechiaro 2006). In my experiments, instead, the carbohydrate/lipid ratio of *A. klebsii*, decreased over the time of exposure to High CO_2; also the carbohydrate/protein ratio declined in a similar way, suggesting that C allocation was deviated towards compounds other than carbohydrates. The fact that the lipid/protein ratio increased during the exposure to High CO_2 suggests that such redefinition of the C allocation pattern could have occurred in favor of lipids.
Interestingly, in *A. klebsii*, all treatments elicited changes of cell organic composition in at least one time interval. Whether *A. klebsii* have a greater general propensity than other species to acclimate to environmental perturbations, and in case why, is hard to understand. It is also possible that *A. klebsii* requires less time to respond to the stimuli than the other algae under examination; in this case, the fact that no other species appeared to respond to the Low to High CO_2 shift by changing their organic composition may thus be ascribed to an insufficient time given to the cultures for acclimation. The comparison of the results obtained here for *P. tricornutum* (no changes in cell composition after the exposure to High CO_2 for 4 generations) with the results obtained after a longer exposure (8 generations, paragraph 5.3) suggest that a higher number of generations may occur for the output of an acclimatory response to emerge.

3.4.1 Similarity method

The output of the "similarity method" (described in paragraph 3.2.3.3) was compared to the results obtained from the deconvolution of FTIR spectra, followed by the attribution of peaks to different macromolecules within the cell and the calculation of the ratios among the main organic pools (paragraph 3.2.3.2). The occurrence of a match between the similarity method and the qualitative approach is strictly dependent on the extent of the qualitative investigation; cell components other than carbohydrates, proteins and lipids might also account for differences in the overall spectra. However, no changes in the overall organic composition of cells due to the environmental stimuli emerged from the similarity analyses that were not confirmed by corresponding qualitative changes at the same time interval. Vice versa, all changes of the ratio among the main cell compounds that were detected after exposure of algae to environmental stimuli coincided with the emergence of changes in the overall cell composition evaluated by means of the similarity analyses.
Therefore, the two methods seem to have a similar detection power. Previous studies showed that different organisms, cultured in different conditions, could be successfully represented by whole

FTIR spectra; cluster analyses conducted on whole spectra permitted not only to discriminate among species and culture treatments, but also to separate algae based on their phylogenetic distance (Domenighini and Giordano 2009).

Based on all above, the calculation of the similarity between spectra, followed by the comparison of the similarity between control spectra and between treated spectra acquired at two different sampling times, emerges as a powerful method for the rapid evaluation of possible shifts in the overall organic composition of algal cells.

3.1 CONCLUSIONS

The outcome of my experiments showed that different species were differently prone to change their composition in response to environmental changes. The type and duration of the perturbation influenced the choice between acclimation and homeostasis, so that the same species was homeostatic or not, depending on one or both these factors.

A general trend towards the retention of a stable internal composition was observed in algae exposed to the "Low to High CO_2" and to the "NO_3^- to NH_4^+" transitions. This might have heterogeneous causes:

1) algae did not have the "potential" for acclimation to the given environmental perturbation, i.e. the physiological plasticity that would allow them to modify the cell composition to achieve a reproductive advantage in the new conditions.

2) algae had the potential for acclimation but did not possess enough energy to acclimate to the given environmental stimulus.

3) algae could not acclimate to the given perturbation rapidly enough to be able to compensate the energy cost for acclimation and to exploit its beneficial effects in terms of increased division rate. In these conditions, homeostasis can be selected as a more convenient strategy.

The exposure to 20 days of darkness elicited changes in the organic composition of the majority of algae under examination. Changes in the proportion among the main organic pools likely resulted from the uneven degradation of cell constituents. The fact that the growth of these algae, even if always positive during the 20 days of treatment, could not match the growth rate imposed at T0 supports the idea that the observed changes in the organic composition of cells did not result from an acclimatory strategy. In fact, acclimation makes sense if it provides an advantage to algae in terms of reproduction rate; in a acclimatory response, after a rapid initial decline, the growth rate recovers (in some cases, completely) as a consequence of the adjustment of cell composition (Giordano 2013).

Considerations on the possible correlation between algal taxonomy and algal modes of response to the environmental perturbations would require the examination of responses of a higher number of species (ideally, a similar number of species for each algal group) than that examined in my study. Even if aware of this shortcoming, I believe my results can give a hint on the topic. All diatoms under examination changed their own cell composition in response to the "Light to Dark" transition, but none of them responded to the other environmental perturbations by modifying the proportion among the cell organic pools (**Fig. 3.3**). No correlation was found between the taxonomy of the species and

the direction of the changes observed after exposure to darkness (20 days): the two congeneric species *Thalassiosira pseudonana* and *T. weissflogii* did not adopt a common strategy.
Of the two species of cyanobacteria under examination, only *Synechococcus* responded to the environmental perturbations (specifically, to the "NO_3^- to NH_4^+" and to the "Light to Dark" transitions) by changing its cell composition, while *Phormidium* responded homeostatically to the stimuli, at least in terms of ratio among the cell organic pools. Among the three examined green algae, only *D. salina* modified its cell composition in response to environmental shifts, and particularly to the Light to Dark transition. Interestingly in *D. salina*, that was also the sole species to change its composition after only 24 hours without light, a two-phase strategy was observed in the dark consisting in a preferential consumption of carbohydrates first, and lipids then. For what concerns dinoflagellates and coccolithophorids, which are here represented by one species each, it is much more difficult to make generalizations.
In conclusion, it was not possible to recognize any pattern of response to environmental stimuli that was characteristic of a certain algal group. When algae are exposed to environmental changes in the N source and in the CO_2 availability, homeostasis appears a much more common and general strategy than usually believed.

4. LIFE AT ELEVATED CO_2 MODIFIES THE CELL COMPOSITION OF *CHROMERA VELIA* (*CHROMERIDA*)

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Running title: *Chromera* at elevated pCO_2

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4.1 **ABSTRACT**

We investigated the response to high CO_2 of *Chromera velia*, a photosynthetic relative of apicomplexan parasites, possibly involved in symbiotic associations with scleractinian corals. The inorganic C content in the proximity of the symbiotic algal cells within the tissues of scleractinians is disputed. According to some authors, it is very high. A higher C content in the endodermal tissues of scleractinians than in the external environment may have favoured the constitution of symbiosis with organisms that, like *Symbiodinium* and *Chromera*, have a type II Rubisco, which is intrinsically ill suited to low CO_2 environments. We thus cultured *C. velia* at the very high inorganic C estimated by some authors and assessed its growth and photosynthetic performance. We also evaluated whether these conditions affected C allocation and elemental stoichiometry in *C. velia* cells by state-of-the-art Fourier transform infrared spectroscopy and total reflection X-ray fluorescence spectrometry, in combination with more traditional biochemical and physiological techniques. Our results demonstrate that *C. velia* is perfectly capable of coping with very high CO_2, which even stimulates biomass production and increases N, P, Mn, Fe, and Zn use efficiency. Growth at elevated CO_2 changes the stoichiometric relationships among elements in *C. velia* cells, but has no effect on the relative abundance of the main organic pools. The high CO_2 in the animal tissue surrounding the photosynthetic cells may therefore facilitate *C. velia* life in symbiosis.

4.2 INTRODUCTION

Chromera velia is an alga (*sensu* Raven and Giordano 2014) isolated in 2001 in the Sydney Harbor (Moore et al 2008) and believed to live in symbiotic association with at least three different species of scleractinian corals (Moore et al 2008, Cumbo et al 2013). Recently, the new phylum Chromerida was proposed in order to accommodate *C. velia* and its relative *Vitrella brassicaformis* (Moore et al 2008; Obornik et al 2012). The genome of *Chromera* (Woo et al 2015) revealed that this alga represents a link between the parasitic Apicomplexa (e.g. *Toxoplasma* spp., *Plasmodium* spp.), non-photosynthetic organisms with a relic, unpigmented plastid (apicoplast), and their photosynthetic predecessors (Linares et al 2014; Woo et al 2015). It is believed that the chromerids share their photosynthetic ancestors with dinoflagellates, among which *Symbiodinium*, which, as it seems likely for *C. velia*, is a symbiont of scleractinians (Janouškovec et al 2010; Petersen et al 2014; Woo et al 2015). Differently from the symbiosis between *Symbiodinium* and scleractinians corals, which has been thoroughly studied since its first identification (Brandt 1881), the exact nature of the association between *C. velia* and stony corals has not been fully elucidated. The establishment of a symbiosis between *C. velia* free-living cells and larvae of the corals *Acropora digitifera* and *A. tenuis* (with the alga located both in the endoderm and in the ectoderm of the hosts) has been reported (Cumbo et al 2012), confirming the potential for an endosymbiotic behaviour of *C. velia*. Although thus the symbiotic relationship between *C. velia* and its hosts appears very likely, the information on its location in the animal tissue is scant. While *Symbiodinium* cells are well known to reside in the endodermal cells of the coral, within symbiosomes, the location of *C. velia* cells in the animal host is unclear. However, the fact that *C. velia* was firstly isolated from the cnidarium *Plesiastrea versipora* by using a isolation procedure similar to that used to extract *Symbiodinium* cells from their hosts (Moore et al 2008) is suggestive that the two organisms have a similar location within corals.

C. velia has been the object of genomic studies that have evidenced some of the peculiarities of this organisms. The chloroplast of *C. velia*, most likely inherited from red algae, contains genes also from the green lineage (Woehle et al 2011), although the extent of this contribution is disputed (Burki et al 2012). The interesting and yet unresolved evolutionary history of *C. velia* plastid also emerges from its somewhat unusual genome organization: genes that encode core photosynthesis protein (e.g. psaA, AtpB), in *C. velia* are fragmented and the fragments are independently processed; other plastidial genes are much larger than their counterparts in other organisms (Janouškovec et al 2013; Woo et al 2015).

The physiology of *C. velia* has been investigated with reference to only few processes. In this alga, the systems involved in light acquisition and utilization showed rather unusual characteristics (Moore

et al 2008; Kotabova et al 2011, 2014; Pan et al 2012; Tichy et al 2013; Bina et al 2014); for instance, Kotabova et al (2014) showed that *C. velia*, if exposed to red light, produces specific light harvesting complexes, with red-shifted absorption, but rapidly reverses to its normal light harvesting complexes when light quality changes. Also tetrapyrrole (thus also chlorophyll) biosynthesis is rather distinctive (Koreny et al 2011).

Little work has been conducted on the way *C. velia* fixes carbon. To the best of our knowledge the only paper addressing C fixation in *C. velia* is that by Quigg et al (2012), in which ^{14}C was used to asses CO_2 fixation. In this work, a strong dependence of CO_2 fixation on the light regime (continuous light versus a sinusoidal photoperiod) was observed. This study, however, was not designed to investigate *C. velia* responses to different CO_2 and total inorganic C (Ci) concentrations.

Various authors, (e.g. Beer et al 2000, Cai et al 2016) used microsensors/microelectrodes for spatially resolved determinations of solute concentrations in the coral tissue, but the complexity of the measurements did not afford conclusive results. Furla et al (2000) tried to assess the actual CO_2 concentration within the coral tissues from calculation and estimated that it is several orders of magnitude higher (147,000 µmol L^{-1} bicarbonate) than in the external medium; this would be the consequence of the activity of a CO_2 concentrating mechanism operated by the animal (Allemand et al 1998; Leggat et al 2002; Bertucci et al 2013). High CO_2 in the external environment has been proposed to exert a negative influence on coral symbioses (Pêcheux 2002; Anthony et al 2008). One may speculate that high Ci within coral endoderm favours symbiosis, as it is for instance the case for the green alga *Coccomyxa* sp., which thrives in the Ci rich environment provided by lichen symbiosis, but is unable to grow at ambient pCO_2 (Palmqvist et al 1997). It should be pointed out, however, that free-living strains of *Coccomyxa* seem to behave differently from those that are symbionts of lichens in respect to the Ci utilization (e.g. the polar *Coccomyxa subellipsoidea*, conversely to the symbiotic strains, appears to have a functional CCM; Blanc et al 2012).

In the assumption that a similar situation occurs in all scleractinian symbioses with photosynthetic organisms, also *C. velia* could be subject to elevated CO_2 concentrations when in its symbiotic stage. The fact that *C. velia*, like *Symbiodinium*, is equipped with a type II Rubisco (Janouškovec et al 2010), which is especially sensitive to changes in the CO_2/O_2 ratio (Raven et al 2012, 2014), can make these organisms especially responsive to the CO_2 enrichment associated with life within corals. Preliminary experiments suggest that a CO_2 concentrating mechanism (CCM; Giordano et al 2005) is present in free living *C. velia* cells (Giordano, Prasil and Pierangelini, personal communication). CCMs, although not very active, have also been detected in the evolutionarily related dinoflagellates (Leggat et al 2002; Ratti et al 2007). The influence of modulation of CCM activity on C allocation has been demonstrated in the model organism *Chlamydomonas reinhardtii* (Memmola et al 2014). The down

regulation of the CCM in the elevated CO_2 environment of the holosymbiont and the energy savings it brings may thus concur to change the C allocation pattern in *C. velia*. It has been shown for a number of organisms that life at elevated CO_2 may increase the use efficiency of light and nutrients such as N and Fe (i.e. less light, N and Fe are required for the same C fixation; see Beardall and Giordano 2002; Raven et al 2011; Giordano and Ratti 2013). This is expected to alter C allocation and elemental stoichiometry within the cell.

Based on all the above, we hypothesize that life at a CO_2 concentration similar to that found in coral symbiotic tissues would influence the organic and elemental composition of *C. velia* cells.

4.3 MATERIALS AND METHODS

4.3.1 **Cultures**

Chromera velia RM12 was obtained from the laboratory of O. Praŝil (Institute of Microbiology ASCR, Centrum Algatech, Třeboň, Czech Republic) and cultured semicontinuously in 250 mL Erlenmeyer flasks containing 150 mL of f/2 medium (Guillard and Ryther 1962). The cultures were maintained at 25°C and continuously illuminated with PAR light at an irradiance of 200 µmol photons m^{-2} s^{-1}. Two different CO_2 regimes were imposed by continuously bubbling the cultures with either atmospheric air (pCO_2 = 400 ppmv) or air enriched with CO_2 (pCO_2 = 25,000 ppmv), at a flow rate of 400 mL min^{-1}. The pCO_2 in the elevated pCO_2 treatment was sufficiently high to be in the approximate range suggested by Furla et al (2000) for the endoderm of corals, without having to change the amount of buffer and the growth pH used for the low CO_2 treatment. The gas mixtures were obtained with a flow-mass gas mixer (IN-FLOW, Bronkhorst Hi-Tech, Ruurlo, Netherlands); the flow rate was controlled through the software Flow-bus DDE 4.6 (Bronkhorst Hi-Tech, Ruurlo, Netherlands). In order to verify whether the growth media were at equilibrium with the gas phase, we measured the total dissolved inorganic carbon in the media (**Tab. 4.1**) with an infrared gas analyzer (Li-840A, Li-Cor Biosciences, Lincoln, USA), after conversion of all inorganic carbon to CO_2 by acidification. We then calculated the equilibrium values for CO_2 and the overall carbonate chemistry, for the pH and the salinity of the cultures, using the CO_2Sys free software (Pierrot et al 2006) (**Tab. 4.1**).

The dilution rates for the semicontinuous cultures were determined based on the growth rate of batch cultures maintained in otherwise equal conditions.

4.3.2 Cell numbers, cell size and dry weight

Cell numbers and mean cell volume were determined with an automatic cell counter (CASY TT, Innovatis AG, Reutlingen, Germany), as described in the paragraph 3.2.2. The cell dry weight was determined according to Ratti et al (2011).

4.3.3 Elemental stoichiometry

The elemental composition of *C. velia* was determined with an elemental analyzer (Costech International S.p.A., Pioltello, Milano, Italy) for C and N, and with a Total Reflection X-ray Fluorescence (TXRF) spectrometer (Picofox S2, Bruker Nano GmbH, Berlin, Germany) for P, S, Cl, K, Ca, Mn, Fe and Zn (Fanesi et al 2014).

4.3.4 Organic composition

The organic composition of *C. velia* was investigated by means of Fourier Transform Infrared (FTIR) spectroscopy, using a Tensor 27 spectrometer (Bruker Optics GmbH, Ettlingen, Germany). Preparation of samples, FTIR spectra acquisition and analysis were conducted according to Domenighini and Giordano (2009). Peterson's (1977) assay was used to determine the absolute amount of cell proteins. Since *Chromera* cells are known to be difficult to break, different lyses methods were compared (i.e. incubation in lysis media containing detergents, vetrification in liquid nitrogen followed by mechanical grinding, rapid N_2 decompression). Complete cell lysis and the highest protein recovery were attained with N_2 decompression, using a pressure bomb (Parr Instrument, Moline, Illinois, USA) (**Fig. 4.1**). This cell breakage procedure was therefore used.
The relative abundance of lipids and carbohydrates was determined semiquantitatively as in Palmucci et al (2011).

4.3.5 Carbonic anhydrase activity

The activity of carbonic anhydrases was assayed on intact cells and crude extracts, obtained according to Ratti et al (2007). The intracellular extract was further subdivided in a soluble and insoluble fraction following the procedure described in Karlsson et al (1995). Intact cells, soluble and insoluble fractions were assayed independently for the carbonic anhydrases by using the potentiometric method proposed by Wilbur and Anderson (1948) and later modified by Miyachi et al (1983). Intact cells or cell extracts were added to an assay mixture that contained 25 mM phosphate buffer (pH 8.36) and 10 µM $ZnCl_2$ (Giordano and Maberly 1989; Ratti et al 2007). After the addition of MilliQ water saturated with CO_2, we recorded the pH changes over time (Recorder 56, Perkin-Elmer, Waltham, USA) and then we calculated the time required for the pH to decrease of 1 unit. In the control assays,

intact cells were substituted with equal volumes of phosphate buffer, while crude extracts were substituted with equal volumes of boiled cell extract.

4.3.6 Statistics

All experiments were conducted on at least three distinct algal cultures. The statistical significance of mean differences was determined by t-tests using GraphPad Prism 5 (San Diego, USA), setting the significance level at $p < 0.05$.

4.4 RESULTS

4.4.1 Growth rates, cell size and cell dry weight

No differences in the specific growth rate were detected between low and high CO_2-grown cells in batch cultures (**Tab. 4.2**). The lowest growth rate measured in the batch cultures (0.16 d^{-1}) was imposed to all semicontinuous cultures by daily dilutions of the culture medium.

Semicontinuously cultivated *C. velia* cells exposed to a pCO_2 of 25,000 ppmv did not differ in the mean cell volume from the cells grown at 400 ppmv CO_2, but had a significantly higher dry weight ($p = 0.0079$; **Tab. 4.2**).

4.4.2 Elemental cell quotas and stoichiometry

The C content of *C. velia* was higher at high CO_2, regardless of the basis on which the results were expressed ($p = 0.0035$ on a cell-basis, **Tab. 4.3**; $p = 0.0002$ on a dry weight-basis, **Fig. 4.2**). The C production rate per cell was more than two times higher at 25,000 ppmv CO_2 ($p = 0.0176$, **Tab. 4.2**). The N and P contents, on a dry weight basis (pg element/pg dw), were significantly lower at high pCO_2 ($p < 0.0001$; $p = 0.0066$, respectively) (**Fig. 4.2**); the cell quotas of these elements, instead, were unaffected by the CO_2 treatment (**Tab. 4.3**). The S content was unaffected by pCO_2, both per cell and per unit of dry weight (**Tab. 4.3**; **Fig. 4.2**).

The contents of Cl, Ca, Mn, Fe and Zn were significantly higher at low pCO_2, on a dry weight basis (**Fig. 4.2**), but their content per cell was the same in the two growth regimes (**Tab. 4.3**).

The C/N, C/P, C/S and N/P atomic ratios were significantly lower at 400 ppmv CO_2 (**Fig. 4.3**), with the greatest difference between the two pCO_2 treatments (2.7-fold) for the C/P ratio. The ratio between P and S, instead, was lower at 25,000 ppmv CO_2 (**Fig. 4.3**).

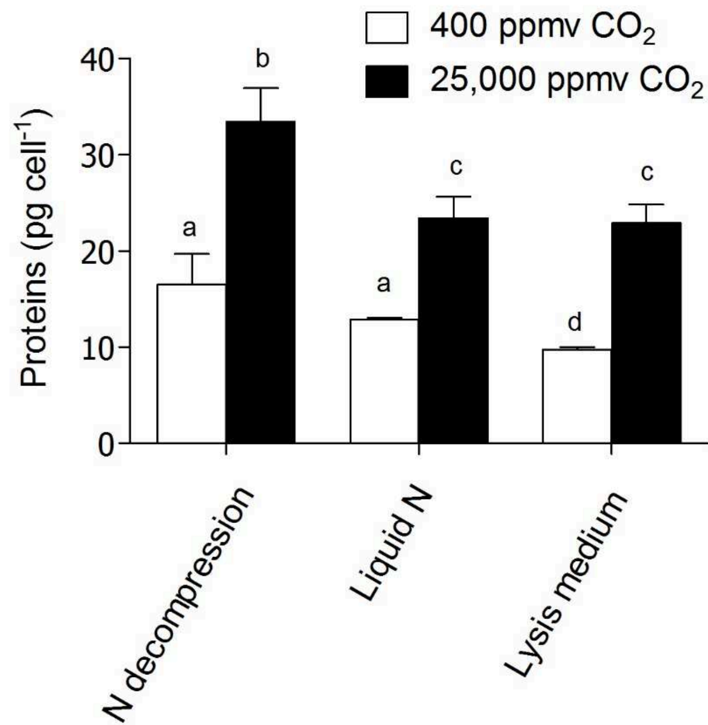


Fig. 4.1. Protein content in *Chromera velia* cells acclimated to either 400 or 25,000 ppmv CO_2. Three different methods of extraction are compared. Different letters indicate statistically significant differences between means (n = 3).

Tab. 4.1. Inorganic carbon speciation in media equilibrated with gas phases containing either 400 or 25,000 ppmv CO_2. The calculations were made with the CO_2SYS program, using mediua temperature, salinity, phosphate, pH and dissolved inorganic C (DIC) concentration as inputs. The equilibrium constant of Mehrbach et al (1973) refitted by Dickson and Millero (1987) were chosen. Data are shown as the means (± SD) of 3 replicates. Different superscript letters indicate significantly different means. TA = Total Alkalinity.

pCO_2	pH	DIC	HCO_3^-	CO_3^{2-}	CO_2	TA
µatm	*NBS*	*µmol kg^{-1}*	*µmol kg^{-1}*	*µmol kg^{-1}*	*µmol kg^{-1}*	*µmol kg^{-1}*
399^a (36.5)	8.11^a (0.09)	2,411^a (507)	2,108^a (390)	291^a (119)	11.5^a (1.05)	2,791^a (641)
24,795^b (2,247)	7.96^a (0.03)	101,933^b (2,665)	92,455^b (2,634)	8,766^b (539)	713^b (64.6)	110,063^b (2,728)

Tab. 4.2. Specific growth rate (µ), cell volume, cell dry weight, C production rate and cell protein content of *Chromera velia* acclimated to either 400 or 25,000 ppm CO_2 (v/v). The standard deviations are shown in parentheses. Means that are statistically different are identified by different superscript letters; equal letters indicate non significantly different means (n = 3).

Growth pCO_2	µ	Cell volume	Dry weight	C production rate	Protein content
ppmv	*d^{-1}*	*µm^3*	*pg cell^{-1}*	*pg cell^{-1} d^{-1}*	*pg cell^{-1}*
400	0.16^a (0.01)	203^a (19.8)	126^a (22.2)	9.82^a (1.91)	16.5^a (3.19)
25,000	0.17^a (0.02)	192^a (21.7)	212^b (20.8)	21.0^b (4.58)	33.4^b (3.50)

Tab. 4.3. Cell quotas of C, N, P, S, Cl, K, Ca, Mn, Fe and Zn in *Chromera velia* cultivated at either 400 (low CO_2) or 25,000 (high CO_2) ppmv CO_2. The mass and molar ratios between each of these elements and P are also shown. The standard deviations are shown in parentheses (n = 3, except for Cl, K, Ca, Mn, Fe and Zn cell quotas, for which 6 replicates were averaged). Different superscript letters indicate means that are statistically different; equal letters identify non significantly different means.

Element	Cell quota *pg cell^{-1}*		Mass ratio *g (g P^{-1})*		Molar ratio *mol (mol P)$^{-1}$*	
	Low CO_2	High CO_2	Low CO_2	High CO_2	Low CO_2	High CO_2
C	61.0^a (11.1)	125^b (14.3)	180^a (32.6)	393^b (56.8)	464^a (84.1)	1014^b (147)
N	14.0^a (2.51)	14.4^a (1.23)	41.3^a (7.40)	45.4^a (7.43)	91.4^a (16.4)	100^a (16.4)
P	0.41^a (0.17)	0.31^a (0.11)	-	-	-	-
S	0.26^a (0.04)	0.36^a (0.13)	0.82^a (0.06)	1.21^b (0.05)	0.79^a (0.06)	1.17^b (0.04)
Cl	0.14^a (0.07)	0.13^a (0.19)	0.48^a (0.14)	0.61^a (0.76)	0.42^a (0.12)	0.53^a (0.66)
K	0.15^a (0.05)	0.19^a (0.08)	0.57^a (0.12)	0.62^a (0.32)	0.45^a (0.10)	0.49^a (0.25)
Ca	0.20^a (0.10)	0.20^a (0.14)	0.71^a (0.26)	0.72^a (0.54)	0.55^a (0.20)	0.56^a (0.42)
Mn	0.02^a (0.01)	0.01^a (0.00)	0.04^a (0.00)	0.04^a (0.01)	0.02^a (0.00)	0.02^a (0.00)
Fe	0.22^a (0.13)	0.18^a (0.11)	0.54^a (0.08)	0.56^a (0.11)	0.30^a (0.05)	0.31^a (0.06)
Zn	0.05^a (0.02)	0.06^a (0.04)	0.12^a (0.01)	0.22^a (0.18)	0.06^a (0.00)	0.10^a (0.08)

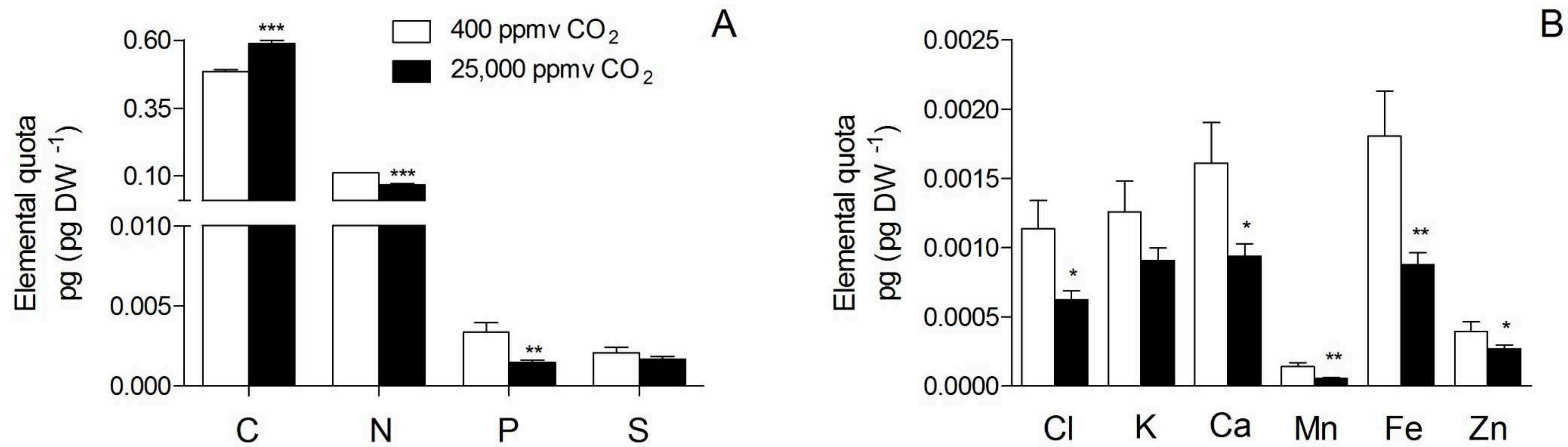


Fig. 4.2. Elemental composition of *Chromera velia* acclimated to either 400 or 25,000 ppm CO_2 (v/v). A) C, N, P and S content per unit of dry weight; B) Cl, K, Ca , Mn, Fe and Zn content per unit of dry weight. Asterisks indicate that the differences between means (n = 3) are significant (3 asterisks: p-value < 0.001; 2 asterisks: p-value from 0.001 to 0.01; 1 asterisk: p-value from 0.01 to 0.05).

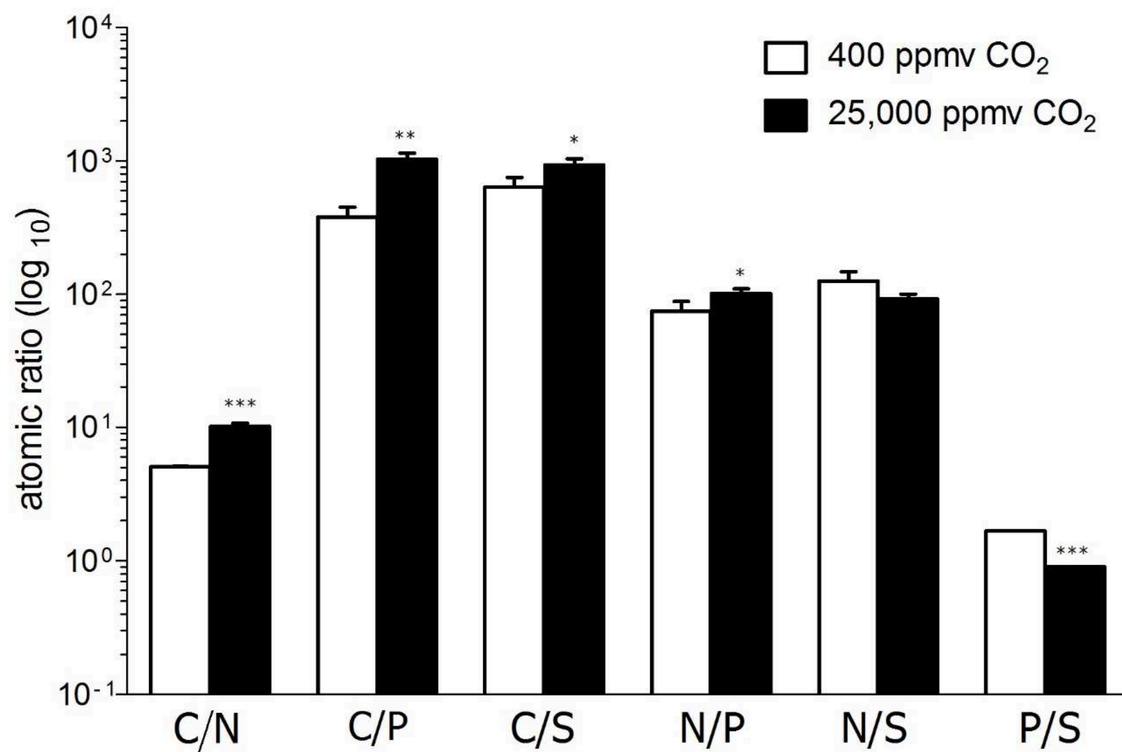


Fig. 4.3. C, N, P and S atomic ratios in *Chromera velia* cells acclimated to 400 or 25,000 ppm CO_2 (v/v). Asterisks indicate that he value at high CO_2 was significantly different from the corresponding value at low CO_2 (n = 3). (3 asterisks: p-value < 0.001; 2 asterisks: p-value from 0.001 to 0.01; 1 asterisk: p-value from 0.01 to 0.05).

4.4.3 Organic composition

All FTIR absorbance ratios (lipids/proteins, carbohydrates/proteins, carbohydrates/lipids) were unaffected by the pCO_2 in the growth medium (**Tab. 4.4**), indicating that all three pools did not vary or varied together as a function of growth conditions. The absolute protein content per unit of biomass (pg protein/pg of dry weight) and the carbohydrate/protein, lipid/protein and carbohydrate/lipid ratios were not statistically different between high CO_2- and low CO_2-grown cells (**Tab. 4.5**). The protein content per cell was significantly higher in high CO_2-grown cells than in their low CO_2-grown counterpart ($p = 0.0035$, **Tab. 4.2**).

4.4.4 Carbonic anhydrase activity

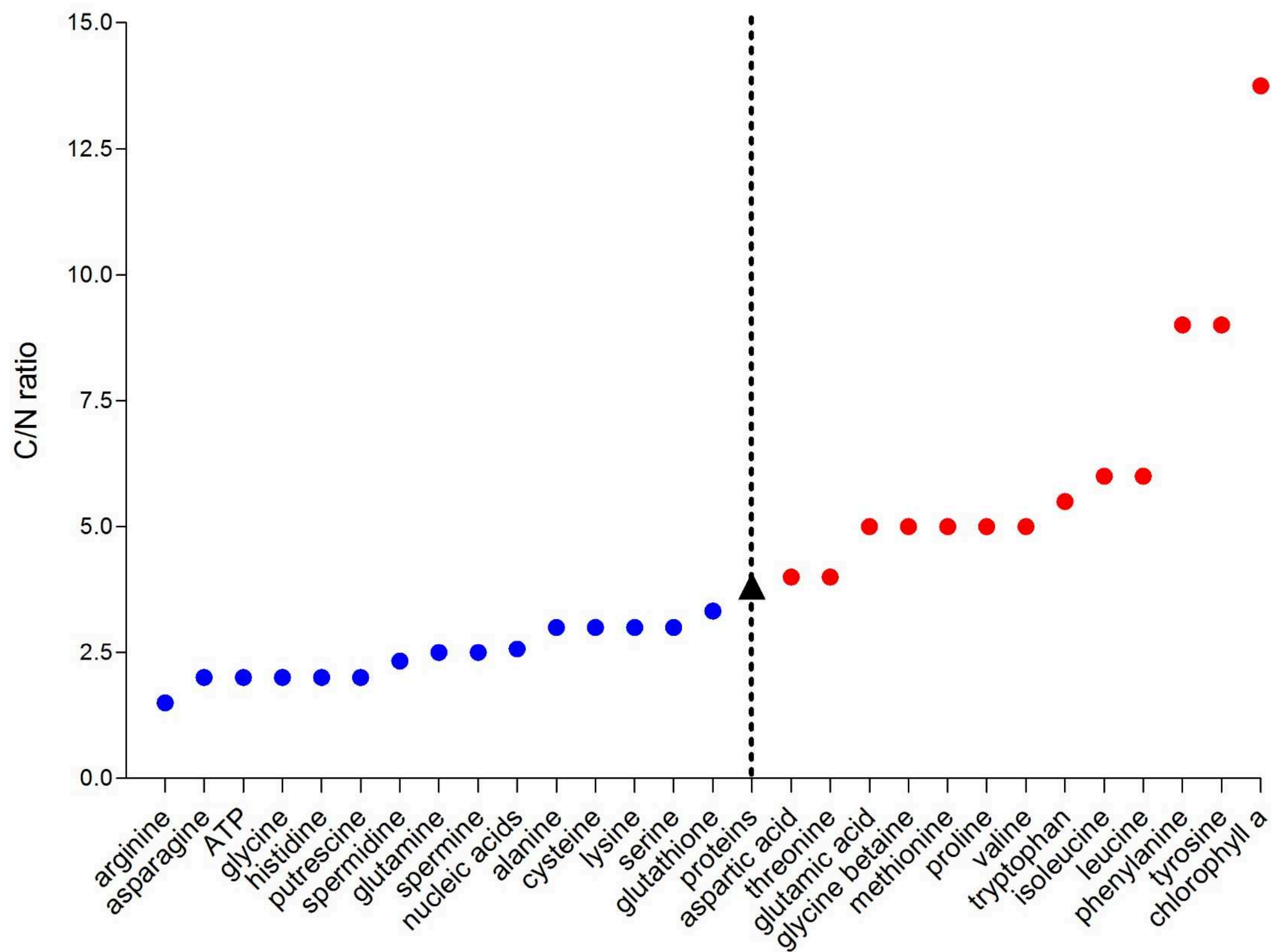
Although various carbonic anhydrase genes are present in the *C. velia* genome (Woo et al 2015), they have, when expressed, moderate activity. No periplasmic carbonic anhydrase activity was observed under any of the culture conditions. Intracellular carbonic anhydrase activity was detected solely in the soluble fraction of both low CO_2- and high CO_2-grown cells, with no significant differences between the two treatments.

Tab. 4.4. FTIR absorbance ratios for the main organic pools in *Chromera velia* cells acclimated to 400 or 25,000 ppmv CO_2. No significant differences were detected between the two culture conditions (n = 3).

Growth pCO_2	Lipids/Proteins	Carbohydrates/Proteins	Carbohydrates/Lipids
400	0.35 (0.07)	2.61 (0.27)	7.62 (0.75)
25,000	0.36 (0.14)	2.28 (0.46)	6.67 (1.32)

Tab. 4.5. Protein contents and relative abundances of lipids and carbohydrates in *Chromera velia* acclimated to either 400 or 25,000 ppm CO_2 (v/v), on a dry-weight basis. The standard deviations are shown in parentheses. Means that are statistically different are identified by different letters in the superscript; equal letters identify not significantly different values (n = 3).

Growth pCO_2	Proteins	Lipids	Carbohydrates
ppmv	*pg (pg DW^{-1})*	*relative units (pg DW^{-1})*	*relative units (pg DW^{-1})*
400	0.137^a (0.051)	1^a (0.268)	1^a (0.319)
25,000	0.158^a (0.013)	1.233^a (0.497)	1.019^a (0.198)



C/N ratio
15.0
12.5
10.0
7.5
5.0
2.5
0.0
arginine
asparagine
ATP
glycine
histidine
putrescine
spermidine
glutamine
spermine
nucleic acids
alanine
cysteine
lysine
serine
glutathione
proteins
aspartic acid
threonine
glutamic acid
glycine betaine
methionine
proline
valine
tryptophan
isoleucine
leucine
phenylanine
tyrosine
chlorophyll a

Fig. 4.4. (In the previous page). C/N atomic ratio for the main N-containing compounds in marine phytoplankton calculated from chemical formula. For compounds that have variable composition, the "mean" atomic ratio is reported (proteins from Geider and La Roche 2002, calculated from Laws 1991; nucleic acids from Sterner and Elser 2002). Blue and red dots indicate the stoichiometry of compounds with a C/N ratio, respectively, lower or higher than the mean C/N ratio in proteins (black triangle). The relative contribution of the C/N ratio of carbohydrates and lipids was calculated, but not included in the figure. [a]The pathway for polyamine metabolism in *Chromera velia* is reported by Woo et al 2015. [b] The plastid of *Chromera velia* contains chlorophyll a (but no other chlorophylls) and carotenoids (Quigg et al 2012).

4.5 DISCUSSION

4.5.1 Growth responses to elevated CO_2

Coral reefs became the most common type of bioconstruction only late in the evolutionary history of corals, probably in the Eocene (40-30 million years ago; Perrin 2002), after the global decline in the CO_2/O_2 ratio of the late Cretaceous (Royer et al 2001; Berling et al 2002; Berner et al 2003; Beerling and Berner 2005). Interestingly, this occurred in approximate concomitance with the spreading of C4 plants on land (Edwards et al 2001) and the radiation of microalgal taxa equipped with CCMs in the oceans (Giordano et al 2005). Coral symbioses almost exclusively involve dinoflagellates and, as it has been recently discovered (Moore et al 2008), chromerids; these are the only groups of eukaryotic photolithotrophs that contain type II Rubisco, an enzyme that is rather ineffective in discriminating in favour of CO_2 and against O_2 (Read and Tabita 1994). These organisms may have found refuge into the animal tissue, where the availability of inorganic carbon can be much higher than in the water column (Furla et al 2000), as an alternative to the development of CO_2 concentrating mechanisms (CCMs) that in many marine algae allow them to cope with the low CO_2 availability (Giordano et al 2005; Raven et al 2005). Consensus exists on the concomitance of the rise to prominence of coral reefs and the establishment of algal symbioses (see Stanley 2003 for a discussion on the origin of corals and coral reefs), although the presence of zooxanthellae in Palaeozoic corals of the order Rugosa has also been proposed, based on rather indirect evidence (Hill 1981) and in contrast with other authors (e.g. Coates and Jackson 1987).

The fact that the internal carbonic anhydrases of *C. velia*, according to our results, are not inducible by changes in the CO_2 environmental concentration is suggestive of the fact that *C. velia* does not possess a biophysical CCM, which usually involve CAs (Giordano et al 2005). However, the presence of a CCM that does not make use of inducible CAs cannot be excluded (Giordano et al 2005). Some evidences exist that support the presence, in the genome of *C. velia*, of genes encoding for proteins similar to those involved in biochemical CCMs in C3 and C4 plants; a plastid decarboxylase has also been found *in silico* (Giordano, Ewe and Prasil, personal communication). Genomic evidence, however, is hard to directly translate to function, especially considering the very common occurrence of potentially C4 genes in most aquatic autotrophs (Raven and Giordano 2017). The existence of rather weak CCMs has been observed

in the dinoflagellates, neighbouring taxon to Chromerida (Leggat et al 1999; Ratti et al 2007); the relative ineffectiveness of these CCMs (in terms of their ability to concentrate CO_2 in the proximity of Rubisco) may be another reason why dinoflagellates, and possibly chromerids, were prone to enter symbiotic associations.

Under our conditions, growth of free living *C. velia* was substantially (more than 2-fold) stimulated by the elevated CO_2 in terms of overall organic C production, but not in terms of cell division (**Tab. 4.2**). This observation is a further evidence of the fact that, at least in our growth conditions, if *C. velia* possess a CCM, it is not sufficient to saturate Rubisco at 400 ppmv CO_2. This contrasts with what found for the planktonic peridinin-containing dinoflagellate *Protoceratium reticulatum*, whose cell division rate increased with increasing pCO_2, with a decrease in the cell dry weight (Pierangelini et al 2016). If elevated Ci would strongly stimulate cell division *in hospite*, the stability of the symbioses would be threatened (Kinzie and Chee 1979; Taylor et al 1989; Baghdasarian and Muscatine 2000), although other mechanisms for the control of algal proliferation, needed to maintain a balanced symbiotic relationship, are likely operated by the host (Falkowski et al 1993; Gates et al 1995; Smith and Muscatine 1999). In this work, the use of free-living *C. velia* allowed us to isolate the effect of elevated Ci on growth from other animal-mediated mechanisms to control *C. velia* cell division. Whether this response to elevated Ci is an ancestral trait of *C. velia* or it was acquired after it became a symbiont of scleractinians is not known, at this stage.

The growth response of *C. velia* also has implications for the growth rate hypothesis (GHR; Sterner and Elser 2002), which assumes a relationship between P (as a component of ribosomal RNA) cell quota and growth rate. In *C. velia*, such a correlation exists in terms of cell division, but is absent if growth is considered in terms of biomass increase, suggesting that, in our case, more ribosomal P was required for cell division than for the observed biomass increase. The observed values of P content and growth rates also have a corollary: in the assumption that P is not accumulated in storage compounds at low CO_2, protein production (pg protein day^{-1}) requires less P at high CO_2 than at low CO_2. A thorough discussion of our data in relation to the GRH and its implications is well beyond the aim of this paper; for a critical discussion on the applicability of the GRH to microalgae, the reader may refer to Flynn et al (2010) and Giordano et al (2015).

4.5.2 Impact of elevated Ci availability on C allocation and elemental composition

C. velia cells, similarly to what observed for the dinoflagellate *Protoceratium reticulatum* (Montechiaro and Giordano 2010), exert a strict homeostatic control (Giordano 2013) on the relative abundance of the main organic components (**Tab. 4.5**). The protein to carbohydrates and to lipid ratios and the carbohydrate to lipid ratio are unaffected by the CO_2 used for growth, although, in absolute terms, high CO_2-grown cells contained more protein (**Tab. 4.2**), and thus more carbohydrates and lipids, than low CO_2-grown cells. This is surprisingly coupled with a substantially higher C content (**Tab. 4.3**) and C/N (and C/P and C/S) ratio at elevated pCO_2 (**Fig. 4.3**). This C allocation pattern and this elemental stoichiometry indicate that the extra C of high CO_2-grown cells is not preferentially allocated, as one may expect, to carbohydrates or lipids (they should increase more than protein, for this to be possible). One possible explanation could be the accumulation of inorganic N. However, ammonium accumulation has rarely been reported in algae (but see Pick et al 1991), while ammonium-related toxicity is a well recognized phenomenon (Zobell 1935); therefore, it is unlikely that ammonium accumulation accounted for an appreciable quota of the N at low CO_2. Consequently, these results must be ascribed to either the accumulation of nitrate (Conover 1975; Demanche et al 1979; Dortch 1982) and/or the allocation of some of the N of low CO_2-grown cells to compound with a lower C/N ratio than proteins (see **Fig. 4.4** for some examples of such molecules).

In addition, it is interesting to notice that the Fe, Mn and Zn cell content in *C. velia* cultured at both low and high CO_2 is much higher than that reported in other microalgae (Quigg et al 2011), while the K cell content is similar (especially when compared to microalgae of the green lineage; Quigg et al 2011).

What this means in physiological terms is hard to explain, especially due to the many anomaly of *C. velia*, which prevents easy comparisons to other algae. Future studies will hopefully cast further light on C allocation in *C. velia*.

5. THE NUTRITIONAL HISTORY OF ALGAE AFFECTS THEIR INTERACTIONS WITH GRAZERS AND WITH OTHER ALGAL POPULATIONS

5.1 INTRODUCTION

In aquatic systems, chemical communication is recognized as important for organisms to find partners and to get oriented during migratory events (Brönmark and Hansson 2000), but its importance in food supply has been underestimated for a long time. For instance, the classic models that described the relationships among trophic levels in oceans the aquatic food chain were almost entirely based on direct feeding of zooplankton on lower trophic levels and did not take into account any possible chemical communication. However, aquatic environment may became very hostile for visual interactions, especially in areas with high levels of turbidity and habitat complexity. Finally, already in the eighties but especially in the last couple of decades, an increasing number of studies began to investigate the role of chemical signaling in prey-predator dynamics. Most of these studies documented communication mechanisms based on the release and detection of cues between algae and grazers (Carr 1988; Ianora and Poulet 1993; Ianora et al 2003; Pohnert 2000, 2005; Fontana et al 2007; Poulet et al 2007).
Poulet and Marsot showed that the copepods *Acartia clausi* and *Eurytemora herdmani* were capable of discriminating between microcapsules enriched with an algal homogenate and non-enriched capsules; importantly, the algal homogenate was embedded in a membrane permeable to small molecules possibly originating from the homogenate. Most of the time, the zooplankters opted for the enriched particles. The rejection of the non-enriched particles occurred both before and after predators and prey had got in touch (Poulet and Marsot 1978). That copepods could catch and hold particles in the mouthparts and then repel them had already been documented; these animals can “taste” food particles through the “sensilla”, receptors present in the labrum and in the mandibles (Conover 1966; Ong 1969). What Poulet and Marsot proved was the zooplankters’ capability of sensing the content of the food particles without need of establishing a physical contact with them. Similar results were then obtained by testing the abilities of marine calanoid copepods to distinguish algae from plastic spheres of the same size (Donahay and Small 1979; Fernandez 1979) and the ability of freshwater cyclopoid copepods to discern flavored spheres from

non-flavored ones and flavored spheres from actual algae (DeMott 1986). Cladocerans of the genus *Daphnia* seem unable to discriminate food particles based on their smell (DeMott 1986). A similar inability has been reported for rotifers of the genus *Brachionus* (Rothhaupt 1990), despite their being equipped with chemoreceptors (in *B. plicatilis*, chemoreceptive pores are located in the anterior integument beneath the cingulum; Snell 1998). Such works provided evidences supporting the idea that some grazers – mostly copepods – but not others are able to "sniff" algal cell composition and consequently select the prey (Poulet and Marsot 1978; Ratti et al 2011; Sjoqvist et al 2013). Until that point, size was considered the main discriminating factor driving selective feeding by the zooplankters; the prey selection was intended as a passive process entirely dependent on the physical constraint imposed by the size of the feeding apparatus (Boyd 1976). Attempts were made to provide simple correlations between the size of algae and grazers that would be sufficient to predict the actual edibility of algae (e.g. Burns 1968).

Whether the ability of "smelling" (i.e. detecting the food quality with no physical contact) is more energetically favorable than "tasting", which implies that particles are hold, handled and then possibly rejected (Lehman 1976; Hughs 1980), in largely unknown. Certainly, chemical sensing appears fundamental in environments in which poorly nutritious particles dominate over algae, such as coastal waters (Porter 1977; Fernandez 1979). Therefore, algal cell composition is expected to be a major determinant of the selective feeding in combination with other factors such as the size (e.g. Peters 1984), the morphology and the structural properties of algae (e.g. Hamm and Smetacek 2007).

A number of evidences point towards a coevolution of microalgae and their grazers, with an interactive development of new defense and offense mechanisms (Porter 1977; Dybdal and Lively 1998; Hamm et al 2003; Assmy et al 2013). An example of this is provided by the diatoms' frustule. Even though the frustule is not an impenetrable barrier for herbivores (Smetacek 1999), it appears to represent a very good deterrent for some potential predators (Hamm et al 2003). A reciprocal influence of diatom frustules and specialized grazers could have occurred over evolutionary time scales and it is probably still in progress (Hamm et al 2003; Assmy et al 2013). Similarly, the chemical perception of algal cell composition by zooplankton may have become especially important in the Mesozoic, when nutritional changes are believed to have made algal biomass richer than in previous times and, consequently, to have fueled the appearance of more effective grazers (Olivieri 2008).

In some cases, algae exhibit systems for counteracting predation only when an actual risk exists; *Thalassiosira weissflogii*, for instance, is able to modulate the silicification of its frustule in the presence of copepods (Pondaven et al 2007; Ratti et al 2013). It is not known whether algae can modulate their

own cell composition based on the presence and the “taste” of the grazers, nor whether the food preferences of the predator had a role in the evolution of algae, for instance by driving the affirmation of physiological processes that allow cell composition to be more independent of external conditions. Indeed algal cell composition can be strictly linked to the environmental conditions, and especially to the absolute and relative amount of nutrients (Sterner and Elser 2002; see the general introduction of this thesis). The tendency and capability of algae to either acclimate or remain homeostatic in response to environmental stimuli may affect the food choice of the grazers. Let us take the example of algae living in a nutrient-replete environment, where they do not experience any energy limitation, and of grazers that preferentially feed on lipid-rich food particles. If an environmental change occurs that lead to a decrease of the C:N ratio within the algal cells, the latter may begin to allocate a larger amount of C in lipids, which do not contain N but have a higher energy content per unit of mass and volume than, for instance, carbohydrates (Pronina et al 1998; Raven 2005; Gushina and Harwood 2006). In a similar situation, algae with scarce ability to keep their own internal composition independent from that of the external medium can be disadvantaged, becoming a more pleasing food for the herbivore; algae that are able to regulate homeostatically their internal composition, for instance by keeping a lower C:N than that in the environment, would be advantaged.

Thus, the differential capability of uncoupling internal composition and external changes has the potential to determine which algae are more apt to counterbalance the grazing pressure, determining the phytoplankton structure in the presence of predators in a given environment. This capability being equal, as well as factors such as cell size and morphology, it is likely that biochemical profile, elemental composition and stoichiometry of algal cells (alone or combined) can actually determine which cells are eaten and which are not in a specific environment. Understanding which algal features affect the choice of selectively feeding grazers is expected to cast light onto the evolution of algal cells.

Considering all above, my experimental hypothesis is that algal cell composition has a primary role in the interaction between phytoplankton and grazers and has the potential to drive – by itself – selective feeding by zooplankton.

5.1.1 Experimental organisms

In order to test my experimental hypothesis (see above), I focused on the interactions between diatoms (Bacillariophyceae) and their grazers. Diatoms dominate many of today's aquatic environments and significantly contribute to the primary production (Smetacek 1999).

Among the large array of predators feeding on diatoms, I selected the marine copepod *Acartia tonsa* and the marine rotifer *Brachionus plicatilis*, well established zooplankton model organisms (Trommer et al 2012) and both potentially able to detect chemical information by means of chemoreceptors (Friedman and Strickler 1975; Snell 1998 and references therein).

Many zooplankton species selectively feed on algae depending on algal size and morphology (see above). In order to exclude factors such as size, morphology, strength of the algal cell wall and to investigate the predators' ability to choose their prey on the basis of algal cell composition only, I have employed populations of the same diatom species that differed solely in cell composition. The species I chose was *Phaeodactylum tricornutum* Bohlin, which is an important model organism for the study of diatoms' biology (Falciatore and Bohler 2002).

P. tricornutum is a coastal species that is also present in transition environments, such as tide pools and estuaries (De Martino et al 2007) and occurs at different depths in the ocean (Zhao et al 2014). It is a pennate diatom (order Bacillariales). Pennates are characterized by a bilateral symmetry and have evolved in the late Cretaceous, more recently than centric (order Biddulphiales) diatoms (Kooistra et al 2007), but have hugely and rapidly diversified since then (Bowler et al 2008).

P. tricornutum cells can have four morphologies: oval, fusiform, triradiate and cruciform, the latter being rarely reported (He et al 2014). Only oval cells have a silica valve (Francius et al 2008), while the cell wall of the other morphotypes are very poor in silica and mainly consists of organic compounds such as glucuromannans (Simpson and Volcani 1981; Lee 1999; Francius et al 2008). In most diatoms, about 50% of the cell dry weight is ascribable to the frustule (Smayda 1970); in *P. tricornutum*, however, the silica only constitutes 0.4-1% of the cell dry weight (Harvey 1955; Lewin et al 1958).

Different morphotypes also differ in their cell composition (e.g. in the protein, lipid and polysaccharide content) and in the lifestyle, so that fusiform and triradiate, more buoyant than oval cells, are prevalently found among plankton (Lewin et al 1958).

Many strains ("accessions") of *P. tricornutum* are available in the algal culture collections worldwide. A phenotypical and genotypical characterization of 10 of these strains has been conducted by De Martino and coauthors, which also provided an historical overview of the isolation and cultivation of this species

(De Martino et al 2007). For my study, I have selected the strain Pt1 8.6, which is becoming the reference strain because its genome has been fully sequenced and largely annotated (Bowler et al 2008; Ge et al 2014; Huysman et al 2014). Cells of this strain are reported as almost exclusively (>95%) fusiform, ranging from 19 to 24 µm in length and from 2.9 to 3.9 µm in width (De Martino et al 2007). The strain Pt1 8.6 derives from cells that were originally harvested in 1956 in Blackpool (UK) and is currently deposited in in CCAP (CCAP1055/1) and in CCMP (now NCMA; accession number: CCMP2561) (De Martino et al 2007). In my study, all cultures originated from a single clone. Even if pleiomorphy is sometimes observed when *P. tricornutum* cells experience a change in the culture conditions, suggesting that the morphotype is not genetically fixed in the different strains (De Martino et al 2007), I could not find oval, triradiate or cruciform cells in any of my cultures.

Great caution was used in order to ensure that the culture treatments selected for this study did not elicit morphological changes, including the appearance of different morphotypes, in *P. tricornutum* cells. The amount of silicic acid in the medium was reported to affect the proportion among morphotypes (in favor of the oval one) in the strains Pt3 and Pt9 of *P. tricornutum* (which consist of oval cells for up to 75%), but not in Pt1 (De Martino et al 2007), which was used for this study. Consistently, no oval cells were observed by periodic inspection of algae cultivated in the presence of GeO_2.

5.1.2 Experimental treatments

In order to obtain populations with different cell composition, the following five culture treatments were selected and imposed to *P. tricornutum* cultures (see the paragraph 5.2.1 for technical details on the treatments):

High CO_2

Low Light

GeO_2

0.5 mM NH_4^+

1 mM NH_4^+

These treatments were expected to cause changes in the cell composition of *P. tricornutum* in agreement with the information present in literature. The actual effect of the treatments on algal cell composition

was then verified by characterizing the cell composition of the single populations as described in the paragraph 5.2.

5.1.2.1 GeO_2 treatment

In view of the fact that less information is available in literature on the effect of GeO_2 on diatoms than on the effect of high CO_2, low light and NH_4^+ on diatoms, it seems opportune to provide a brief summary of what is known on the interaction between GeO_2 and these organisms.

Diatoms, in most cases, form a silicified cell wall, the frustule, containing amorphous silica ($[Si_nO_{2n-(nx/2)}OH_{nx}$] with $x \leq 4$). The progression of the cell cycle, in most diatoms, depends on the amount of silicon (Si) available in the environment: the cycle stops at the G1/S or at the G2/M transition if Si supply is scarce (Brzezinski et al 1990). Therefore, Si availability can be an important determinant of diatom growth in the oceans (Paasche 1980). A wide range of dissolved Si concentrations has been reported in the oceans (from below 1 µM up to 70 µM; Martin-Jezequel et al 2000); most marine surface waters are characterized by concentrations of dissolved Si ranging from 0 to 5 µM (Bainbridge 1980). Diatoms can use Si in two forms: $Si(OH)_4$ (undissociated silicic acid), which accounts for about 97% of total dissolved Si at pH 8.0, and $SiO(OH)_3^-$, which constitutes the rest of the dissolved Si (Stumm and Morgan 1981; Riedel and Nelson 1985).

Germanium (Ge) and Si are both included in group IV of the periodic table of elements and have similar chemical properties (Lewin 1966). Ge can be assimilated by all the organisms that are able to assimilate Si (Safonova 2007) but, conversely, have no known nutritional role. Germanium dioxide (GeO_2) negatively affects diatoms' growth already at a concentration of 1 mg L^{-1} (which is, however, far above the maximum concentration found in nature, 0.03 mg L^{-1}; Kuroda 1939); it is therefore widely used as diatom growth inhibitor (Lee 1999). The mechanism through with GeO_2 interferes with the use of Si by diatoms is not entirely understood. The degree of inhibition of diatoms growth by GeO_2 occurs is species-specific and only occurs in certain environmental conditions (Lewin 1966).

In *C. fusiformis* and *Amphiprora paludosa*, the inhibition of growth by 1 mg GeO_2 L^{-1} could be partially overcome by the addition of SiO_2 (6 to 20 mg SiO_2 L^{-1}; Lewin 1966), suggesting that, within certain limits, the Si/Ge ratio (estimated at about 10^{-5} in seawater) is more important than the absolute concentrations of Si and Ge. This was later supported by other works; for instance, the repression of

Synedra acus growth was observed when the Ge/Si molar ratio was higher than 0.05 but not at lower values (Safonova et al 2007).

Apart from growth, the treatment with GeO_2 is expected to modify the composition of diatom cells by affecting the amount of silica, which may be an important discriminating factor for the selection by grazers. The availability of Si (and thus likely the presence of GeO_2) has the potential to affect other aspects of algal physiology, which, most importantly for my investigation, can drive changes in algal cell composition. For instance, Zhao and collaborators showed that the cell content of Unsaturated Fatty Acid (UFA) was lower in Si-depleted cells cultivated at otherwise various conditions (Zhao et al 2014). Another instance is provided by Huang and coauthors, who reported that Si-starved *P. tricornutum* cultures differed from the replete counterparts in their content of miRNAs (Huang et al 2011), which act as post-transcriptional regulators in eukaryotes.

5.2 MATERIALS AND METHODS

5.2.1 Cultures

5.2.1.1 Algae

Phaeodactylum tricornutum Bohlin (strain Pt1 8.6) wild type and eYFP-tagged mutant were kindly provided by Angela Falciatore (CNRS, Laboratoire de Biologie Computationnelle et Quantitative, Paris). The strain Pt1 has been originally sampled in 1956 from the coastal waters off Blackpool, UK (De Martino et al 2007). The morphotype of this strain is reported to be mostly fusiform (95 to 100%); in our cultures, all cells had fusiform morphotype.

P. tricornutum "control" cultures were grown axenically in Artificial Multipurpose COmplement for the Nutrition of Algae (Amcona medium, thereinafter named "control" medium; Fanesi et al 2013) continuously bubbled with air at a flow rate of 400 mL min^{-1}; these cultures were kept at 20°C and provided with 24h light (λ = 400-700 nm; irradiance = 200 µmol photons m^2 s^{-1}).

The five selected culture treatments differed from the control conditions for the following characteristics:

- High CO_2: the culture medium was continuously bubbled with a gaseous mixture of air and 1% CO_2 v/v, obtained with a flow-mass gas mixer (IN-FLOW, Bronkhorst Hi-Tech, Ruurlo, Netherlands); the flow rate was set at 400 mL min^{-1} through the software Flow-bus DDE 4.6 (Bronkhorst Hi-Tech, Ruurlo, Netherlands).
- Low Light: light was provided with a lower photon flux density (50 instead of 200 µmol photons m^2 s^{-1}).
- GeO_2: Germanium oxide was added to the culture medium at a concentration of 1 mg L^{-1}. The Ge/Si molar ratio in the GeO_2-containing medium was equal to $4.66 \cdot 10^{-2}$ ($9.56 \cdot 10^{-3}$ mM Ge; $2.05 \cdot 10^{-1}$ mM Si).
- 0.5 mM NH_4^+: the culture medium had a different N source (NH_4^+ instead of NO_3^-) but the same N concentration.
- 1 mM NH_4^+: the culture medium differed in both the N source (NH_4^+ instead of NO_3^-) and the N concentration (1 mM instead of 0.5 mM).

For the determination of the specific growth rate in each culture condition, cells were cultivated in batch in 100 ml glass tubes containing 70 ml of algal suspension. For the analysis of cell composition, they were cultured semicontinuously in otherwise identical conditions.

Rhodomonas sp. and *Thalassiosira weissflogii* were cultured as a food for the copepods. They were kept in a growth chamber at 20°C and provided with continuous light (λ = 400-700 nm; irradiance = 200 μmol photons m^2 s^{-1}). *Rhodomonas* was grown in Enhanced Artificial Seawater (EASW) in which the N and P concentrations were, respectively, 2 and 4 times higher than in the original recipe.

Oxyrrhis sp. was cultivated and occasionally added to the copepod culture in order to get rid of the excess of food. It was grown in the light at 20°C in ASW and fed once a week with *Dunaliella salina*.

5.2.1.2 Grazers

The rotifers *Brachionus plicatilis* were provided by the laboratory of Ike Olivotto (DISVA, Università Politecnica delle Marche, Ancona, Italy). Rotifers were kept in homemade plastic containers having the shape of a truncated cone, filled with 1.5 L of ASW and continuously provided with gentle aeration (~ 1 bubble s^{-1}). The rotifers were fed daily with *Dunaliella salina*; the number of individuals per unit of volume was maintained approximately constant through weekly dilutions.

The calanoid copepod *Acartia tonsa* were kindly provided by the laboratory of Michael Steinke (University of Essex, UK). Copepods were kept in the dark, in glass bottles filled with 10 L of artificial seawater (ASW), under continuous gentle aeration (~ 1 bubble s^{-1}). Small evaporative water losses were compensated by adding autoclaved milliQ water, in order to maintain the salinity of the medium (34 on the practical scale).

Copepods were fed 2 times a week with a mixture of *Rhodomonas* sp. and *Thalassiosira weissflogii* at a 2:1 dry biomass ratio. Water in the bottles was changed every two weeks, but at least 1 week before the experiments; adult copepods and copepodites were collected by gently pouring the culture through a 200 μm mesh; then, they were transferred into clean containers with fresh ASW.

The conditions of the animals were checked routinely using a stero microscope. Adults that had no morphological and behavioral flaws (e.g. not swimming, damaged appendices) were sorted for the predation experiment. For the predation experiments, the algae:grazer biomass ratio was fixed in a narrow range: 1 rotifer per 80 μg of algal C; 1 copepods per 400 μg of algal C (Ratti et al 2013 and references therein).

5.2.2 Experimental design - workflow

The different populations of *P. tricornutum* were generated by culturing this alga in distinct growth media (Fig. 5.1A). Cells of each population were acclimated to the respective culture conditions for at least 8 generations, before any experiment was conducted. Characterization of each algal population was performed; this was especially important in view of a deep understanding of the results of the grazing experiments, in which grazers' predilection towards a certain algal cell composition would possibly emerge.

In the predation experiments, the different populations were mixed in pairs in the control medium and subjected to predation by either the rotifer *Brachionus* sp. or the copepod *Acartia tonsa*. A total of 20 experiments were carried out (10 for each grazer). For each couple of algal populations, 6 replicates in the absence of the predator and 6 replicates in the presence of the predator were analyzed. Each algal population in a mixture was represented alternately by either wild type or eYFP-labelled cells. For instance, in 6 replicates (3 in the absence and 3 in the presence of the predator), Low Light-grown wild type cells were mixed with High CO_2-grown eYFP-labelled cells; in the other 6 replicates, Low Light-grown eYFP-labelled cells were mixed with High CO_2-grown wild type cells.

Algae from two populations with different nutritional background were mixed in a 1:1 ratio.

The experimental set-up consisted of sterile 6-well plates in which algae and grazers were gently transferred; the plates were placed in a culture chamber, at 20°C, and illuminated with PAR light at a photon flux density of 200 μmol photons m^2 s^{-1}. The plates were gently shaken for the whole duration of the experiments. Preliminary tests were conducted to determine the time within which the transferring of each algal population to the control medium did not elicit changes in the respective cell composition (**Fig. 5.1B**). The duration of the experiment was then set at 6 hours.

At the end of the predation experiment, algal suspensions were separated from the grazers through a zooplankton net and then analyzed with a flow cytometer in order to verify whether possible shifts in the ratio between the two populations occurred after coexistence with the grazer (**Fig. 5.1C**). The discrimination between the two populations was based on the eYFP fluorescence.

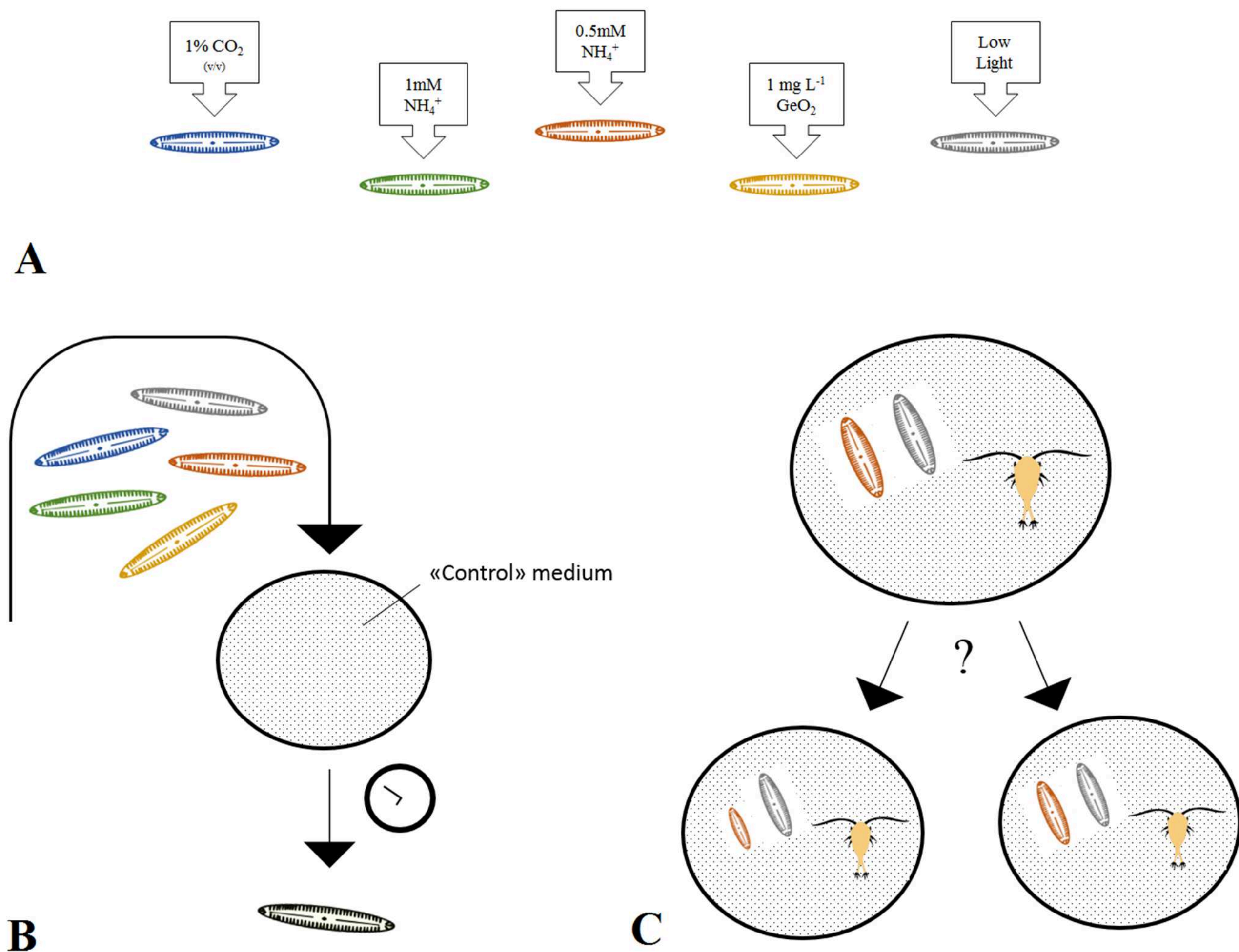


Fig. 5.1. Scheme of the workflow. **A.** Generation of the different populations of *Phaeodactylum tricornutum* by means of different culture treatments. **B.** Test aimed at setting the time of the predation experiment. **C.** Predation experiment: algal population were mixed in pairs in the absence and in the presence of the predators; the occurrence of selective feeding was then evaluated.

5.2.3 Cell number and cell volume

Cell number and cell volume were determined with an automatic cell counter (CASY TT, Innovatis AG, Reutlingen, Germany; see the paragraph 3.2.2 for technical details).
For each culture treatment, growth curves were created based on daily counts of the cell number ml^{-1}.
The specific growth rate (μ) of batch cultures was calculated from the portion of the growth curves in which the cell number increased exponentially (see the paragraph 3.2.2 for details).
All measurement were made in triplicates. In order to ensure similar growth to all cultures, a growth rate of 0.70 d^{-1} was imposed (by dilution) to the semicontinuously-growing cultures; this value was selected on the basis of the lowest growth rate calculated in batch (i.e. 0.71 d^{-1}, Low Light-grown cells).

5.2.4 Cell dry weight

For the cell dry weight determination, cells were washed with an ammonium formate solution isosmotic to the culture media (0.5 M). The cell suspension was then filtrated through pre-combusted GF/C glass fiber filters and dried at 80°C until weight stabilized. Cells on the filters were then put in a muffle furnace at 450°C for 4 h in order to get rid of the organic matter; the ash-free dry weight was then calculated by subtracting the ash weight from the dry weight.

5.2.5 Elemental composition

Samples for the determination of C and N contents in *P. tricornutum* cells were prepared as follows: cells were harvested by centrifugation (700·g for 7 minutes) and washed twice with an ammonium formate solution isotonic to the culture medium (0.5 M). The pellets were then dried in an oven at 60°C. The dry matter was transferred in tin capsules (0.5 to 2 mg of dry matter per capsule), which were closed very carefully; the samples were weighted before and after the closure of the capsules, in order to make sure that the capsules had no leaks.
Blanks consisted of empty tin capsules. Sulfanilamide (41.84% C; 16.27% N; 18.62% S; w/w) was used as standard for the preparation of a calibration curve. A tiny amount of V_2O_5 was put into the capsules

before the addition of either the algal pellet or the sulfanilamide, in order to facilitate the complete oxidation of the sample.

The amount of C and N in each sample was then determined with the elemental analyzer ECS 4010 (Costech International S.p.A., Pioltello, Milano, Italy). Samples were burnt in the combustion reactor in the presence of saturating oxygen and WO_3 as an oxidation catalyst; this allowed the conversion of total cell C into CO_2 and of total cell N into NO_x. In the remaining portion of the reactor, filled with copper wires, NO_x is converted to N_2. The gas mixture is transported by the carrier gas (helium) through a water trap, which ensure the removal of water vapor; it then reaches a chromatographic column (Poropak Q/S 0/80 mesh), where the gases are separated before reaching the thermal conductivity detector (TCD), which ultimately generates a signal proportional to the amount of each element in the sample (**Fig. 5.2**).

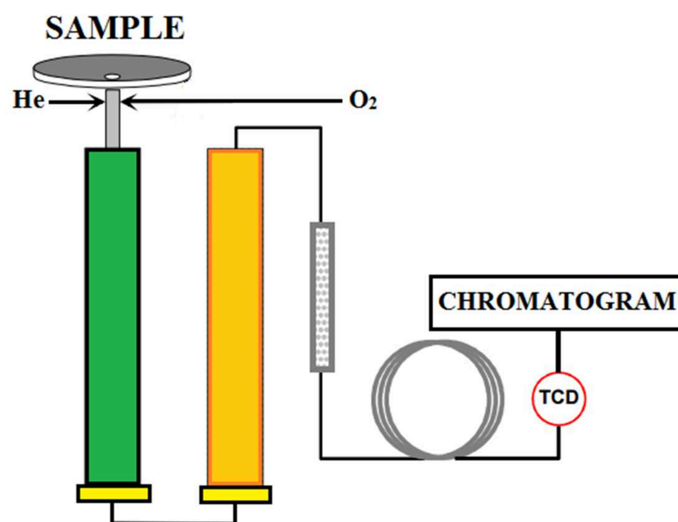


Fig. 5.2. Scheme representing a CHNS elemental analyzer. Modified from Costech ECS 4010 Reference Manual.

The working temperature of the instrument was set at 980°C for the left furnace and 70°C for the oven. The gas flows were 100 ml min^{-1} for both He and air, 30 ml min^{-1} for O_2. The time cycle parameters were set as follows: 12 (sample delay); 20 (sample stop); 40 (oxy stop); 70 (run time). The retention time (i.e. the time at which the C and N peaks appeared in the chromatograms) was daily checked and, in case, adjusted according to the last acquired standard samples. In the calibration settings, the curve type was set as linear for N and quadratic for C.

The possibility of leaks in the system was excluded by performing a "leak check" prior to any working session, according to the instruction provided by the manufactures.
The software EAS Clarity (DataApex Ltd., Prague, Czech Republic) was used for the acquisition and the analysis of the chromatograms. The amount of C and N in the samples was obtained by calculating the area of each peak in the chromatograms and interpolating the data from the standard curve.

5.2.6 Protein content

The protein content of *P. tricornutum* cells acclimated at the various culture conditions was measured spectrophotometrically by using the Peterson's (1977) method. Cells were collected by centrifugation (700·g for 7 minutes) and then washed twice with an ammonium formate (0.5 M). Algal pellets contained about 10^6 cells. Cell membranes in the algal pellets were lysed by adding a solution containing 1% SDS and 0.1 M NaOH (500 µl per pellet); pellets were vortexed for at least 1'. A solution ("reagent A") containing equal volumes of milliq water, 10% SDS, 0.8 M NaOH and CTC reagent (which consisted of 0.1 $CuSO_4$ $5H_2O$; 0.2% Na-K Tartrate; 10% Na_2CO_3, w/v) was then added to the samples (500 µl per pellet). The samples were immediately vortexed and then left 10' at room temperature before the addiction of the "reagent B" (250 µl per pellet), which contained milliq water and Folin-Ciocalteu reactive (Sigma F9252) in a 1:5 (v/v) ratio. Reagents "A" and "B" were freshly prepared prior to any measurement. For each sample, the absorbance at 750 nm was determined 30' after the addition of the reagent B. The blanks consisted of milliQ water. It was then possible to calculate the concentration of proteins in the samples by interpolating the measured absorbance values into a standard curve, produced by determining the absorbance of increasing amounts (0 to 150 µg) of bovine serum albumin (BSA; Sigma).
Absorbance was determined with a Beckman DU 640 spectrophotometer (Beckman Coulter, Bea, California, USA).

5.2.7 Fourier-transformed infrared (FTIR) spectroscopy

The principles of FTIR spectroscopy are described in the paragraph 3.2.3 of this thesis, which also provides a description of the modes of acquisition and analysis of the spectra. For *P. tricornutum*, the optimal cell number per window that gave the best possible signal to noise ratio was $5 \cdot 10^6$ cells. Peaks attribution to the various cell pools was performed as described in the paragraph 3.2.3. In addition, the peak at ~1074 cm^{-1} (Si-O of the siliceous frustules) was used as a proxy for silica.

Here, in addition to the ratios among the main organic pool in the cells, I also determined semiquantitatively the relative abundance of lipids and carbohydrates, according to Palmucci et al (2011) and using the absolute quantity of cell proteins as reference. This method is based on the Lambert–Beer's law, which describe the absorbance of a certain "x" pool in the sample (A_x) as follows:

$$A_x = C_x \cdot d_x \cdot \varepsilon_x$$

where C_x is the concentration of carbohydrates in the sample, d_x is the path length of the radiation, and ε_x is the molar absorptivity coefficient of the carbohydrate pool. The ratio between the FTIR absorbances of the "x" pool and the FTIR absorbance of proteins is thus described by the equation:

$$\frac{A_x}{A_{prot}} = \frac{C_x \cdot d_x \cdot \varepsilon_x}{C_{prot} \cdot d_{prot} \cdot \varepsilon_{prot}}$$

Assuming that the light path length is equal for both FTIR absorbances in the ratio, the concentration of the "x" pool will be equal to:

$$C_x = \frac{A_x}{A_{prot}} \cdot C_{prot} \cdot \frac{\varepsilon_{prot}}{\varepsilon_x}$$

Even if the value of the ratio of $\left(\frac{\varepsilon_{prot}}{\varepsilon_x}\right)$, named K, is not known (and thus absolute quantification of the pools of lipids and carbohydrate from FTIR data is not possible), it must positive and different from 0. Assuming that the K value for the same organic pool does not change significantly between different species, even when grown under different conditions (Palmuci et al 2011), it is possible to obtain a semiquantification that expresses the amount of the "x" pool relative to a reference concentration (Palmucci et al 2011).

5.2.8 Flow cytometry

The Guava EasyCyte flow cytometer (Merck Millipore, Darmstadt, Germany) was used in order to assess the proportion between the two algal populations (alternately represented by wild type and eYFP-tagged cells) at the beginning and at the end of the grazing experiments. The instrument allows the detection of five parameters: the forward scatter channel (FS), the side scatter channel (SS) and three fluorescence channels, which detect emissions at 525/30 nm, 583/26 nm and 690/50 nm (**Fig. 5.3**).

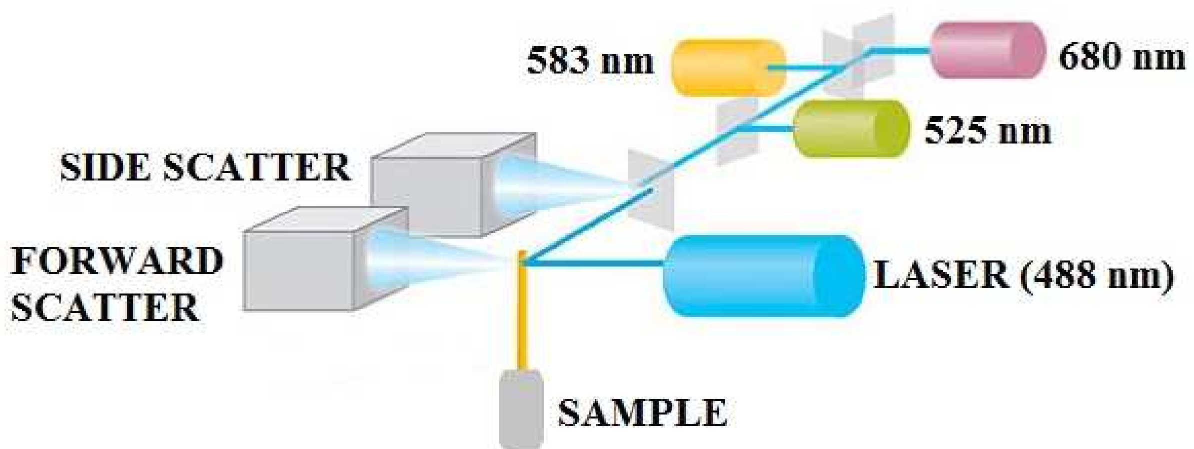


Fig. 5.3. Schematic representation of the flow cytometer configuration used for this study. Modified from Guava Easycyte flow cytometry systems data sheet.

The instrument is equipped with a single sample loader that communicates with a microcapillary flow cell; the latter removes the requirement for sheath fluid, i.e. the liquid that deliver the cells in the more traditional flow cytometers based on hydrodynamic focusing. In the sheath fluid systems, the sample is injected into a stream of sheath fluid, which is at lower pressure than that of sample; the sample particles are accelerated and restricted to the "core" of the cell, in which they align and interact with the laser beam (Suthanthiraraj and Graves 2013). The microcapillary system allows the direct aspiration of sample into the flow cell, where the cells pass one by one through a filament (**Fig. 5.4**). The most important advantages of this system are 1) substantial reduction of waste; 2) absolute counts of cells can be obtained without reference beads; 3) a large number of events can be acquired from small sample volumes.

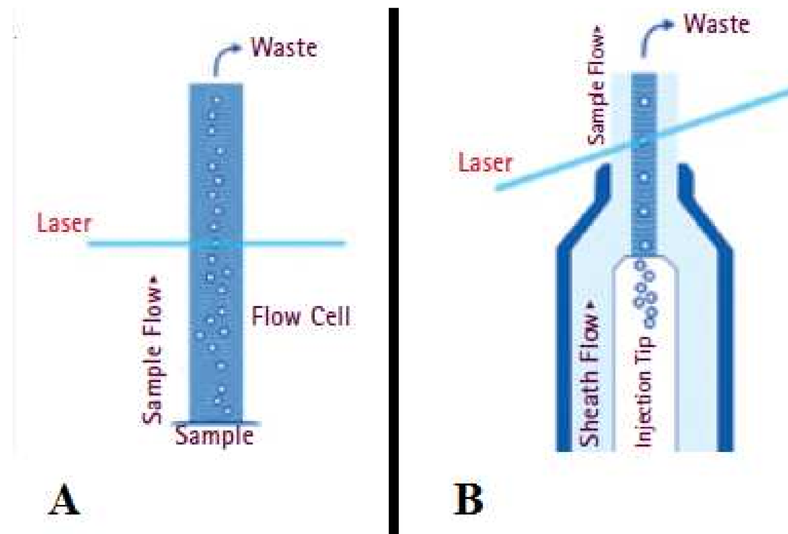


Fig. 5.4. Schematic representation of the microcapillary system mounted on the Guava Easycyte flow cytometer (**A**) compared to a traditional sheath fluid system (**B**). Modified from Guava Easycyte flow cytometry systems data sheet.

In my experiments, the discrimination between the two populations in each algal mixture was based on the fluorescence signal emitted by an enhanced yellow fluorescent protein (eYFP). This protein is a modification of the GFP (green fluorescent protein) from the jellyfish *Aequorea*. eYFP has an excitation peak at 514 nm and an emission peak at 527 nm (Day and Davidson 2009; **Fig. 5.5**); it is considered one of the brightest fluorescent proteins available nowadays (Miyawaki et al 2005).

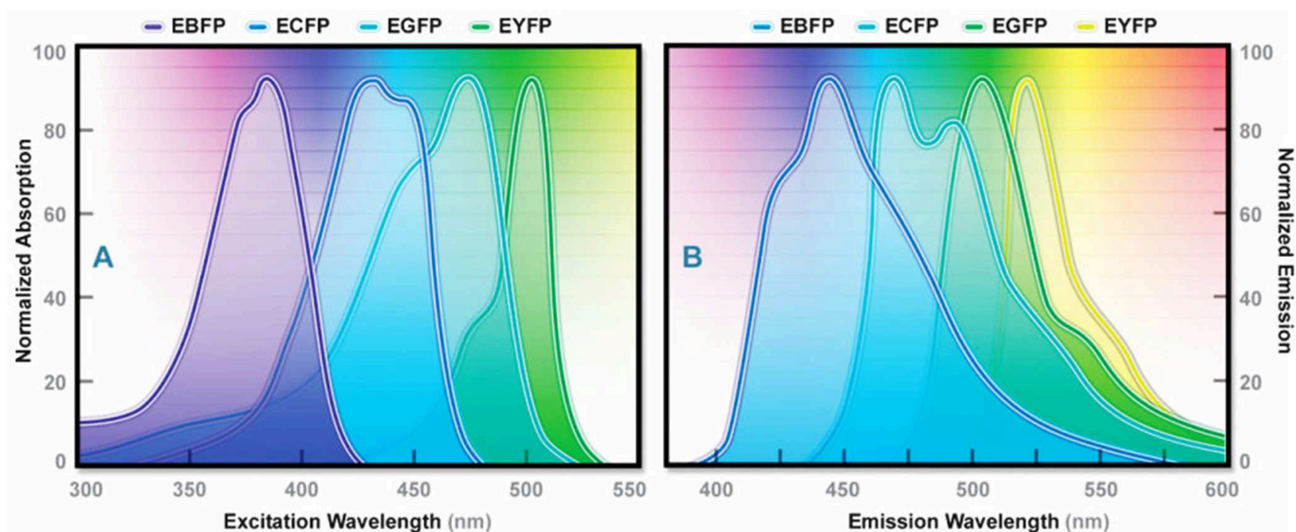


Fig. 5.5. Absorption and emission spectra of the Aequorea –derived fluorescent proteins that are most commonly employed as tools in cell biology and physiology. Modified from Day and Davidson 2009.

Algal suspensions were diluted immediately prior to be analyses, in order to obtain a concentration of 250,000 cells mL^{-1} in a total volume of 400 µL $sample^{-1}$. After excitation (provided by the laser at 488 nm) the fluorescence of eYFP was recorded at the GRN channel of the flow cytometer (525/30 nm). The following gain settings were set: FSC 189; SSC 53.8; G 53.8 Y 1; R 1; and a threshold of 100 on FSC. Fluorescence intensity was recorded on at least 5000 events per sample. The software InCyte 2.7 was used for the analysis of the data.

5.2.9 Statistics

All measurement were conducted on at least three biological replicates. The statistical significance of mean differences was verified by t-tests or 1-way ANOVA using GraphPad Prism 5 (San Diego, USA), setting the significance level at $p < 0.05$. The Principal Component Analysis (PCA) was performed with XLStat (Addinsoft, New York, NY).

5.3 RESULTS

5.3.1 Growth, cell volume and cell dry weight

The specific growth rate of *P. tricornutum* was affected by the culture conditions (**Fig. 5.6**). Low Light-grown cells grew significantly more slowly than all the other cells (0.71 d^{-1}); according to this, a growth rate of 0.70 d^{-1} was imposed by dilution to the semicontinuous cultures of *P. tricornutum* subjected to all culture treatments. Control cells, High CO_2-grown cells and 1 mM NH_4^+-grown cells also had a higher growth rate than 0.5 mM NH_4^+- and GeO_2-grown cells (**Fig. 5.6**).

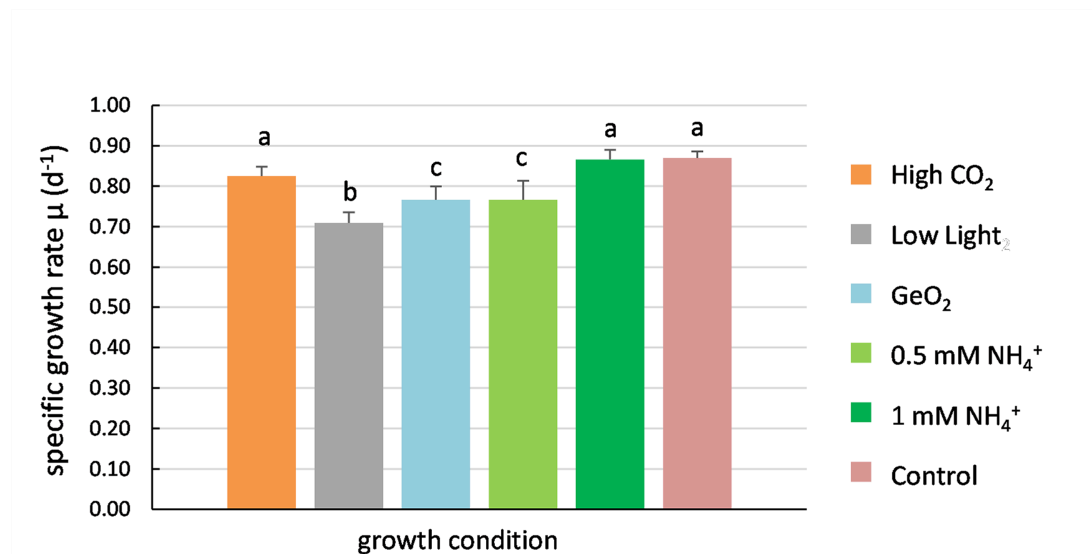


Fig. 5.6. Specific growth rate (μ) calculated from daily counts of *P. tricornutum* cells grown in batch cultures. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 5$).

The dry weight of *P. tricornutum* cells was equal for all growth conditions with the only exception of cells acclimated to the medium containing 1 mM NH_4^+; the latter were significantly heavier than the other cells (**Fig. 5.7**). The cell ash-free dry weight was affected in similar way (**Fig. 5.8**).

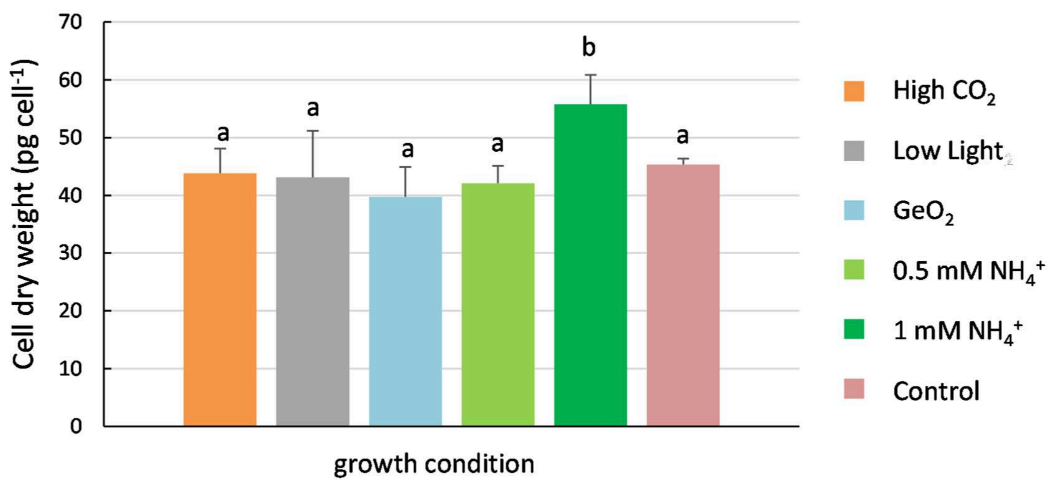


Fig. 5.7. Cell dry weight of *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

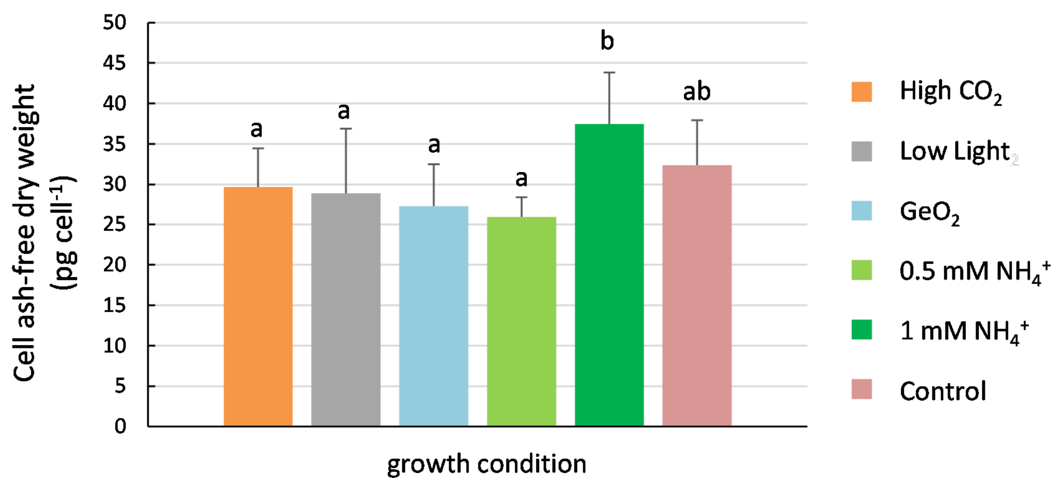


Fig. 5.8. Cell ash-free dry weight of *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

5.3.2 Cell elemental composition

Cells of *P. tricornutum* contained the same amount of C and N per unit of dry weight in all cultures conditions, with only one exception: the C content (% weight) of GeO_2-grown cells was significantly lower than that of cells acclimated to Low Light (**Fig. 5.9**; **Fig. 5.10**).

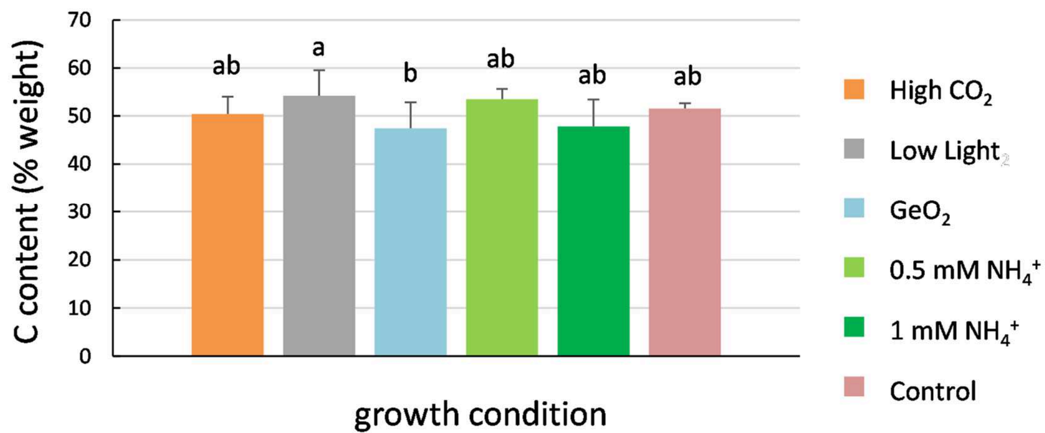


Fig. 5.9. Carbon content (expressed as percentage of the dry weight) in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

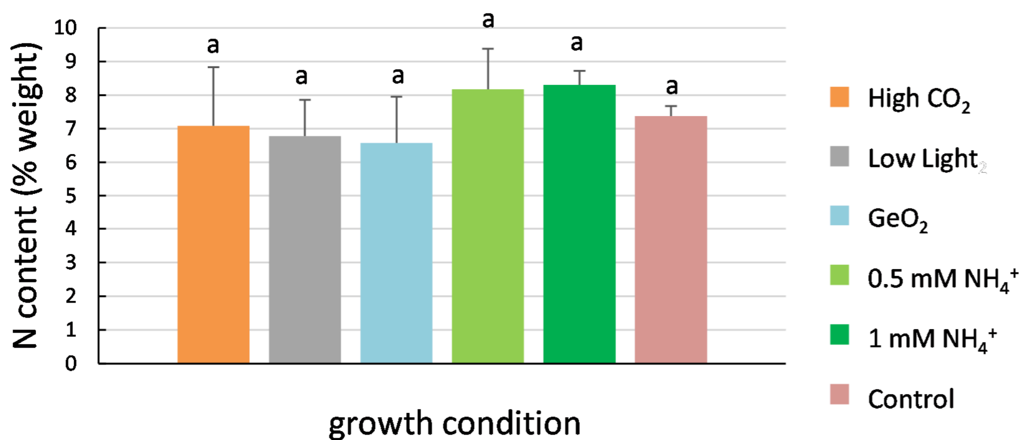


Fig. 5.10. Nitrogen content (expressed as percentage of the dry weight) in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

When expressed in picograms per cell, C content of GeO_2-grown *P. tricornutum* cells was significantly lower than those of both the Low Light-grown and 1 mM NH_4^+ -grown counterparts. The differences among the other treatments were not significant (**Fig. 5.11**).

When *P. tricornutum* was acclimated to the medium containing 1 mM NH_4^+, its N cell quota was significantly higher than that measured in all other culture conditions. No significant differences among the treatments were found (**Fig. 5.12**).

The comparison between algal cultures acclimated to 1 mM NH_4^+ and to Low Light showed that the latter had a higher C:N ratio than the former. When other treatments were compared, the C:N ratio resulted similar (**Fig. 5.13**).

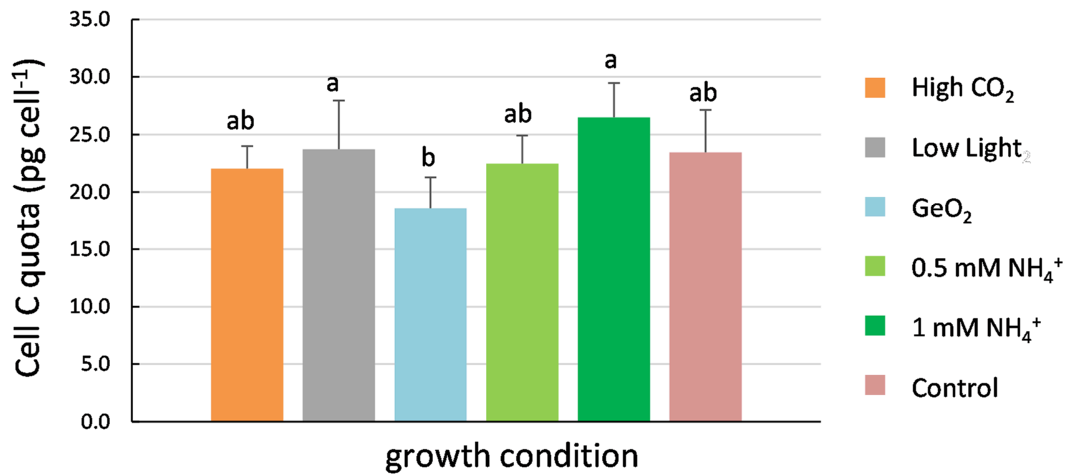


Fig. 5.11. Cell C quota (pg cell^{-1}) in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

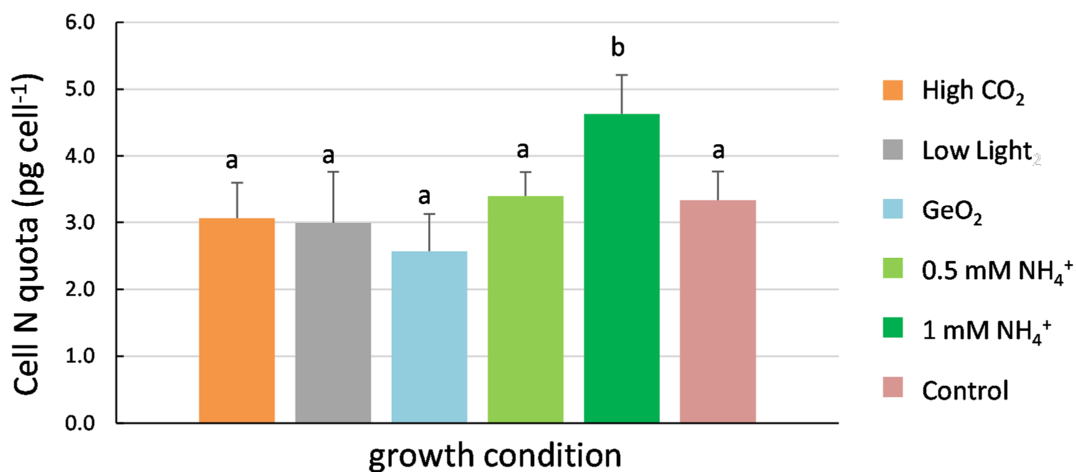


Fig. 5.12. Cell N quota (pg cell^{-1}) in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

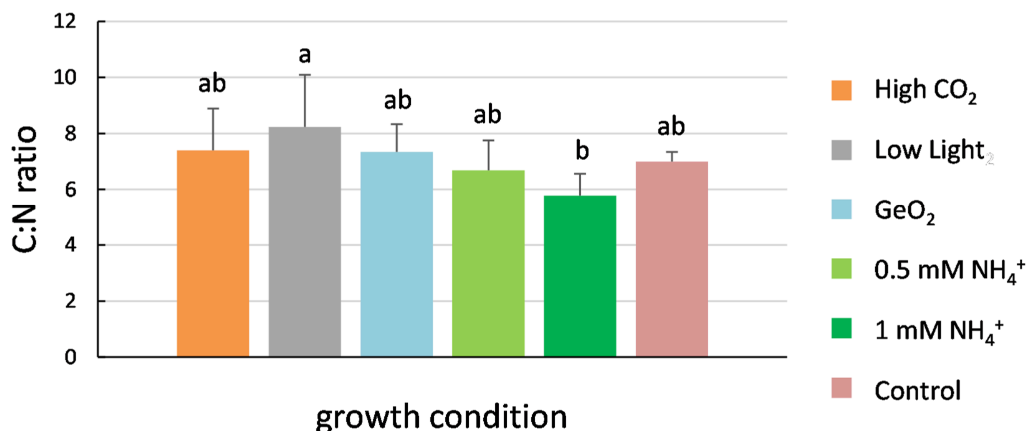


Fig. 5.13. The ratio between C and N in *P. tricornutum* cells grown in the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n \geq 6$).

5.3.3 Cell organic composition

5.3.3.1 FTIR absorbance ratios

The FTIR absorbance ratio between lipids and proteins was higher in GeO_2-grown cells than in the Low Light-grown counterparts, while no significant differences emerged by comparing the other culture conditions (**Fig. 5.14**).

P. tricornutum cells grown in the presence of 1 mM NH_4^+ had a higher carbohydrates to proteins ratio than the cells cultured in all other conditions, except in GeO_2. No other significant differences were found due to the treatments (**Fig. 5.15**).

Cells grown at 1 mM NH_4^+ also had a higher carbohydrates to lipids ratio than control cells, GeO_2-grown cells and 0.5 mM NH_4^+-grown cells (**Fig. 5.16**).

The silica to proteins FTIR absorbance ratio was significantly lower in cells acclimated to the medium containing GeO_2 than in the other cells. The silica to proteins ratio was higher in high CO_2- and 1 mM NH_4^+-grown cells than in the control (**Fig. 5.17**).

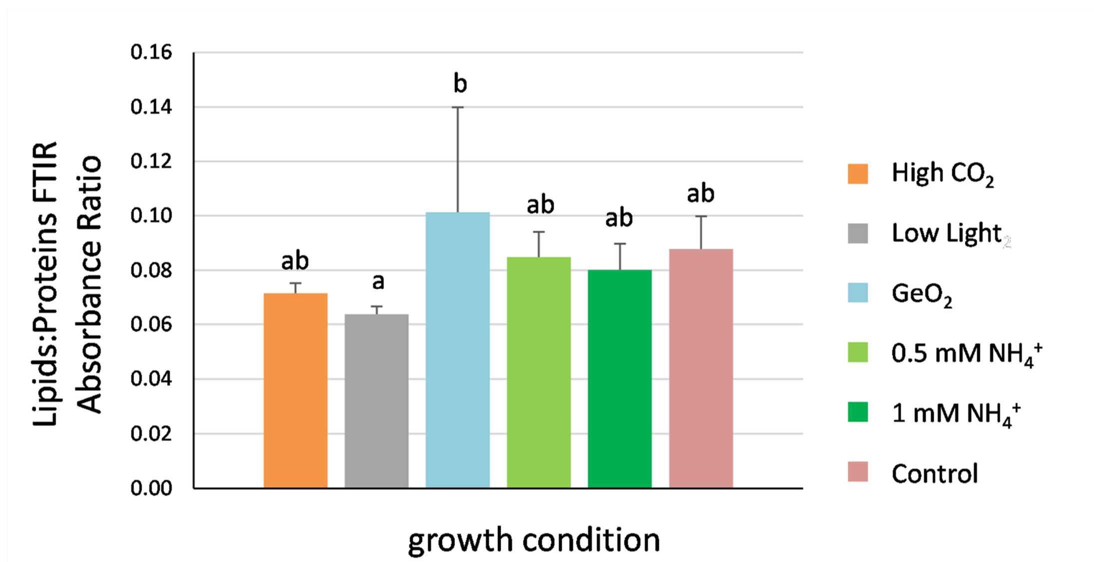


Fig. 5.14. FTIR absorbance ratio between lipids and proteins in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

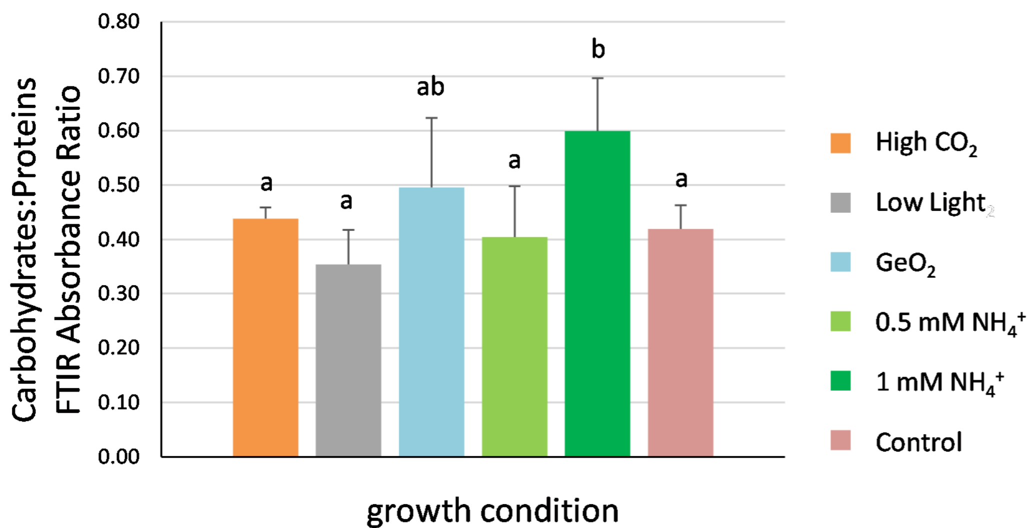


Fig. 5.15. FTIR absorbance ratio between carbohydrates and proteins in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

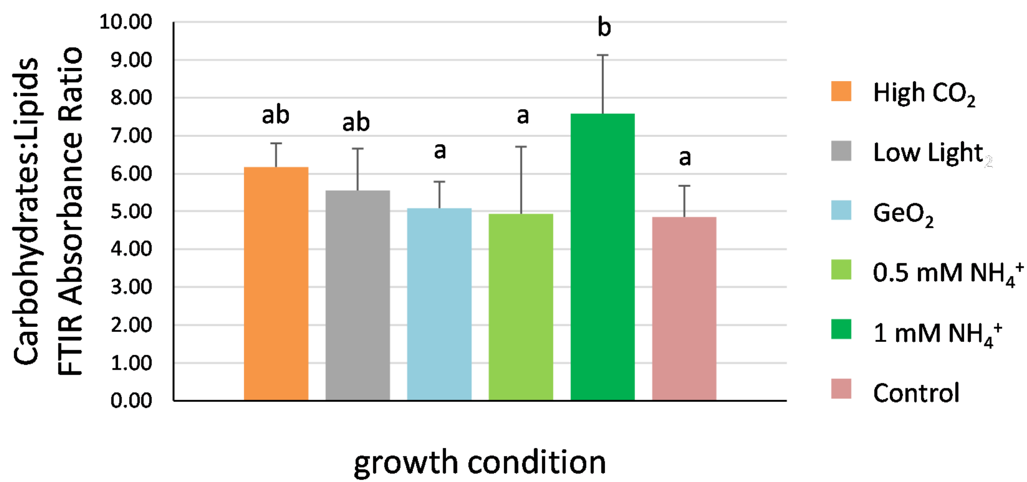


Fig. 5.16. FTIR absorbance ratio between carbohydrates and lipids in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

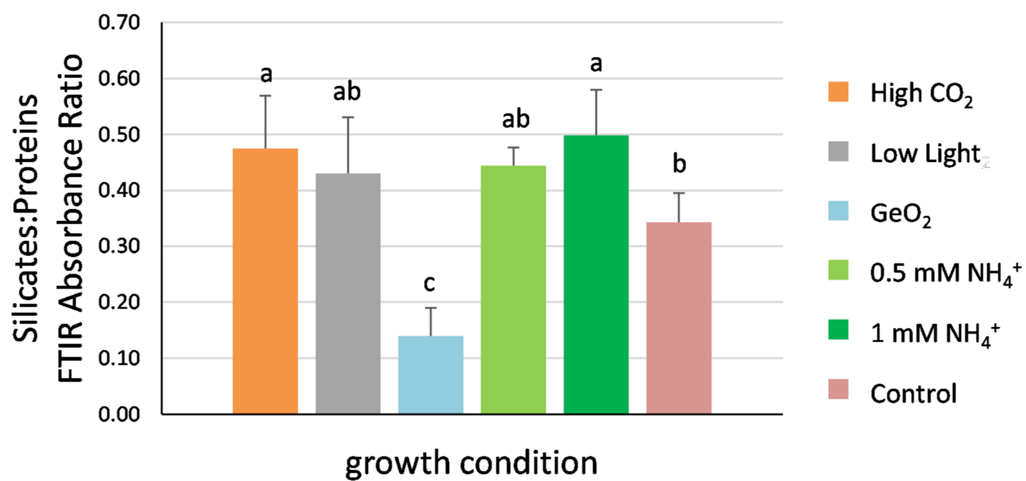


Fig. 5.17. FTIR absorbance ratio between silicates and proteins in *P. tricornutum* cells acclimated to the different culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

5.3.3.2 Protein, lipid, carbohydrate and silica content

The absolute amount of proteins was higher in *P. tricornutum* cells grown in the media containing GeO_2 and 1 mM NH_4^+ than in the LowLight-grown and in the control cells. Cells acclimated to Low Light also had fewer proteins than those acclimated to 0.5 mM NH_4^+ (**Fig. 5.18**).

The amount of lipids in Low Light- and GeO_2-grown cells was lower than in 0.5 mM NH_4^+-grown cells, 1 mM NH_4^+-grown cells and control cells. Cells grown in the presence of GeO_2 also had less lipids than cells grown at High CO_2 (**Fig. 5.19**).

P. tricornutum cells acclimated to 1 mM NH_4^+ had a significantly higher carbohydrate content than cells cultured in all others conditions. Differences were also detected in the relative amount of carbohydrates among the other conditions: control cells, 0.5 mM NH_4^+- and high CO_2-grown cells had more carbohydrates than GeO_2-grown cells (**Fig. 5.20**).

Cells grown at 0.5 mM NH_4^+, 1 mM $NH_4^+$and High CO_2 contained more silica than cells grown in the presence of GeO_2 and in control conditions. Low light-grown cells had less silica than 1 mM NH_4^+-grown cells, but more silica than GeO_2-grown cells (**Fig. 5.21**).

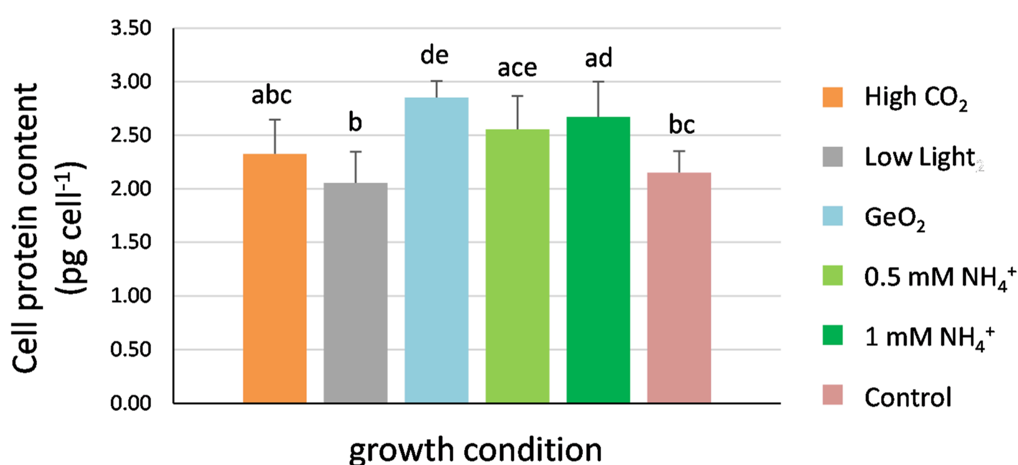


Fig. 5.18. Absolute protein content of *P. tricornutum* cells acclimated to the culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; n = 6).

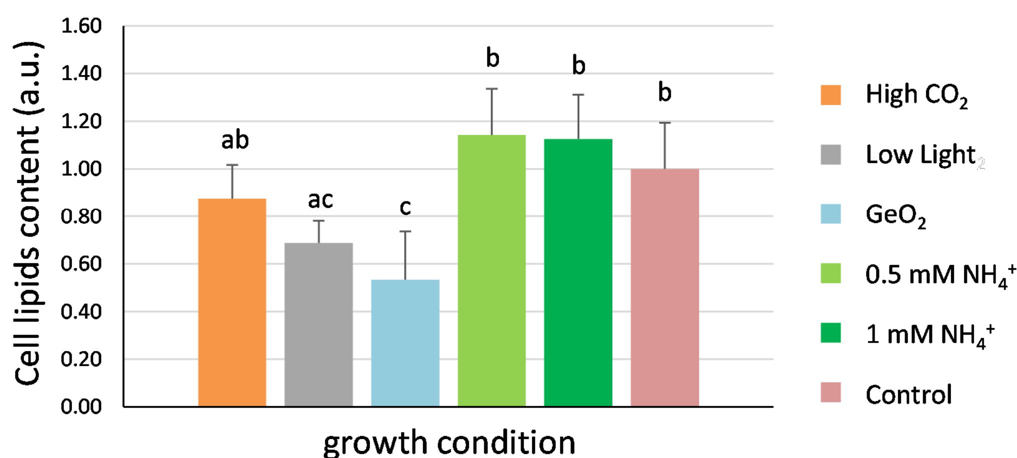


Fig. 5.19. Relative amount of lipids in *P. tricornutum* cells acclimated to the culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

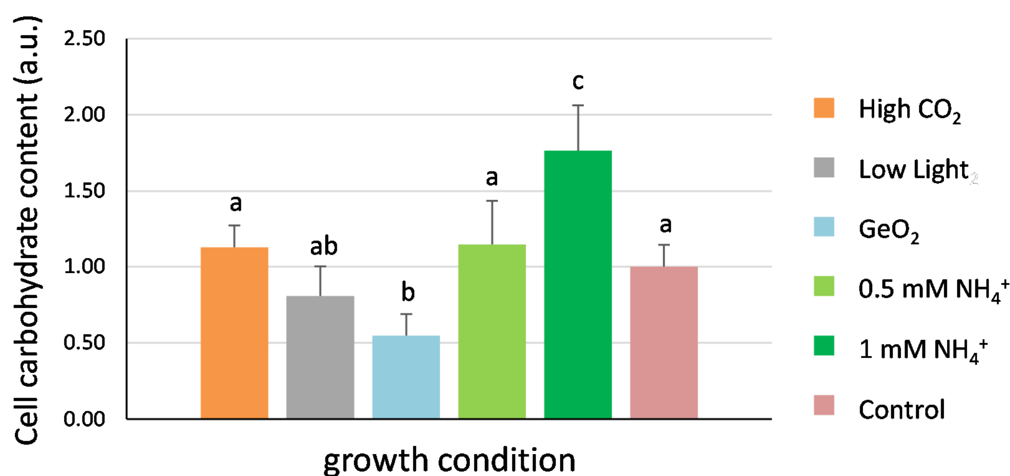


Fig. 5.20. Relative amount of carbohydrates in *P. tricornutum* cells acclimated to the culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

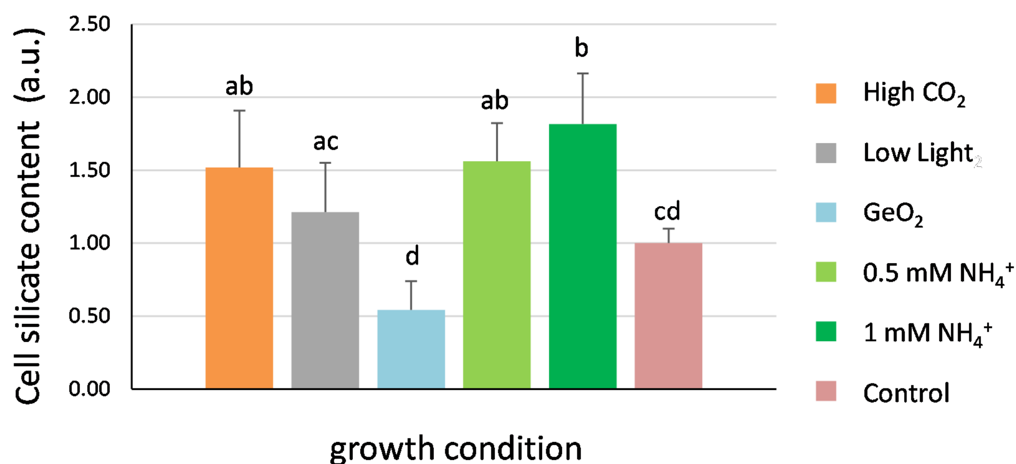


Fig. 5.21. Relative amount of silica in *P. tricornutum* cells acclimated to the culture conditions listed in the legend. Different letters above the bars indicate statistically different means ($p < 0.05$; $n = 6$).

Tab. 5.1 provides a summary of the differences between control *P. tricornutum* cells and cells acclimated to the different culture conditions.

The PCA in **Fig. 5.22** shows that cells of *P. tricornutum* grown in 1 mM NH_4^+ are quite different from cells having diverse nutritional histories, the latter grouping together on the left side of the score plot (see **Fig. 5.23** for the corresponding loadings plot).

Tab. 5.1. Summary of the differences in cell composition between cells of *P. tricornutum* grown in control conditions and cells grown at five experimental conditions. The green squares indicate that the parameter increased relative to the control cells; the red squares indicate that the parameter decreased relative to the control cells. The acronym "ns" stands for "not significant" and was used to identify the treatments that did not elicit significant changes in a certain parameter compared to the control cells. For each parameter, the units of measurement are specified by the superscript letters, according to the following legend: [a] pg cell^{-1}; [b] mass ratio; [c] FTIR absorbance ratio; [d] relative amount per cell.

Culture Conditions	Parameters												
	Dry Weight[a]	Ash-free dry weight[a]	Carbon[a]	Nitrogen[a]	$\frac{C}{N}$[b]	$\frac{Lipids}{Proteins}$[c]	$\frac{Carbohydrates}{Proteins}$[c]	$\frac{Carbohydrates}{Lipids}$[c]	$\frac{Silicates}{Proteins}$[c]	Proteins[a]	Lipids[d]	Carbohydrates[d]	Silicates[d]
High CO_2	ns	ns	ns	ns	ns	ns	ns	ns	■ (green)	ns	ns	ns	■ (green)
Low Light	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	■ (red)	ns	ns
GeO_2	ns	ns	ns	ns	ns	ns	ns	ns	■ (red)	■ (green)	■ (red)	■ (red)	ns
0.5 mM NH_4^+	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	■ (green)
1 mM NH_4^+	■ (green)	ns	ns	■ (green)	ns	ns	■ (green)	■ (green)	■ (green)	■ (green)	ns	■ (green)	■ (green)

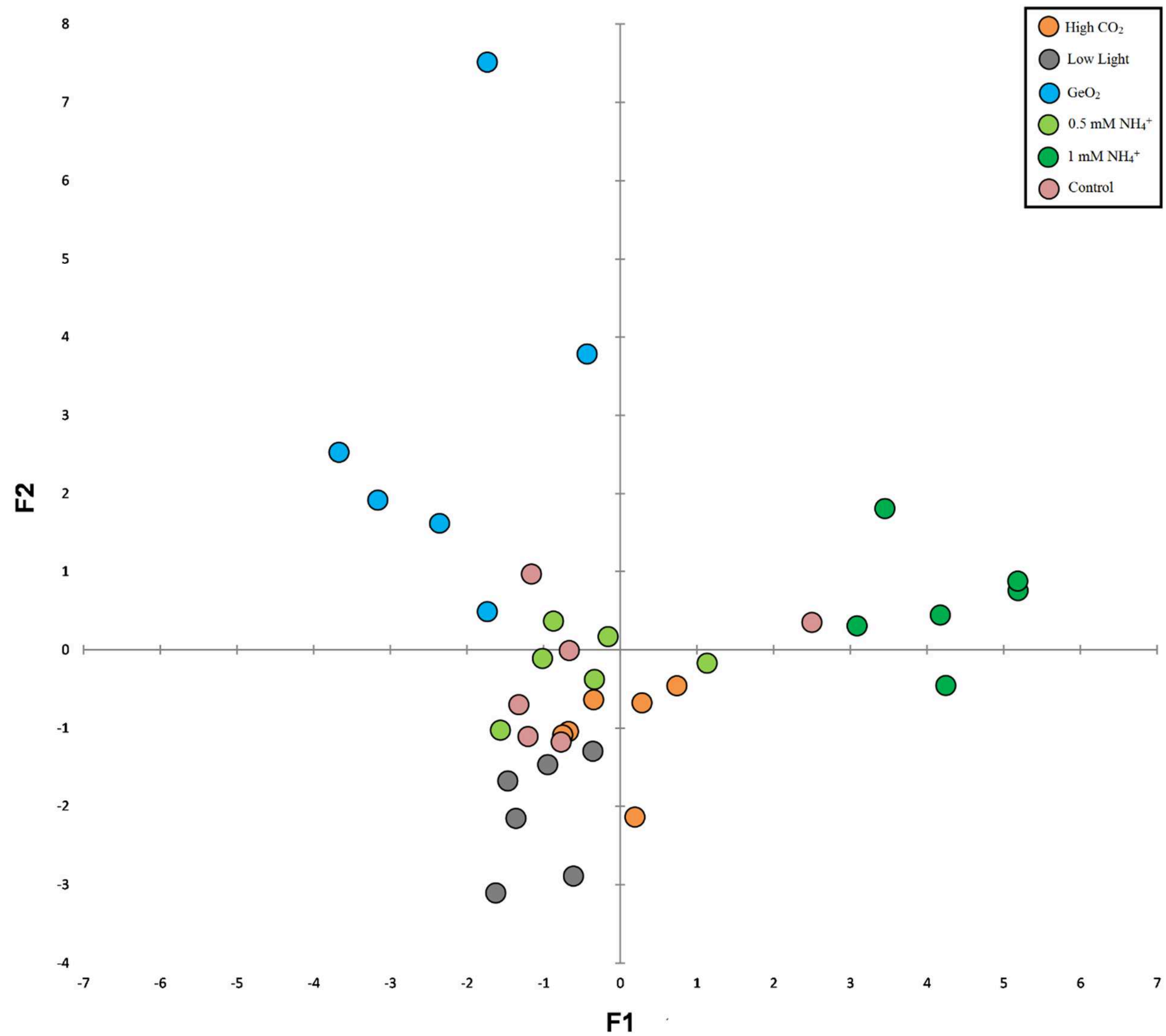


Fig. 5.22. Scatter chart of the score values of the Principal Component Analysis (PCA). Proteins, lipids, carbohydrates, silicates, carbon and nitrogen per cell and the lipid:protein, carbohydrates:protein, carbohydrates:lipids, silicates:protein and C:N were used as variables.

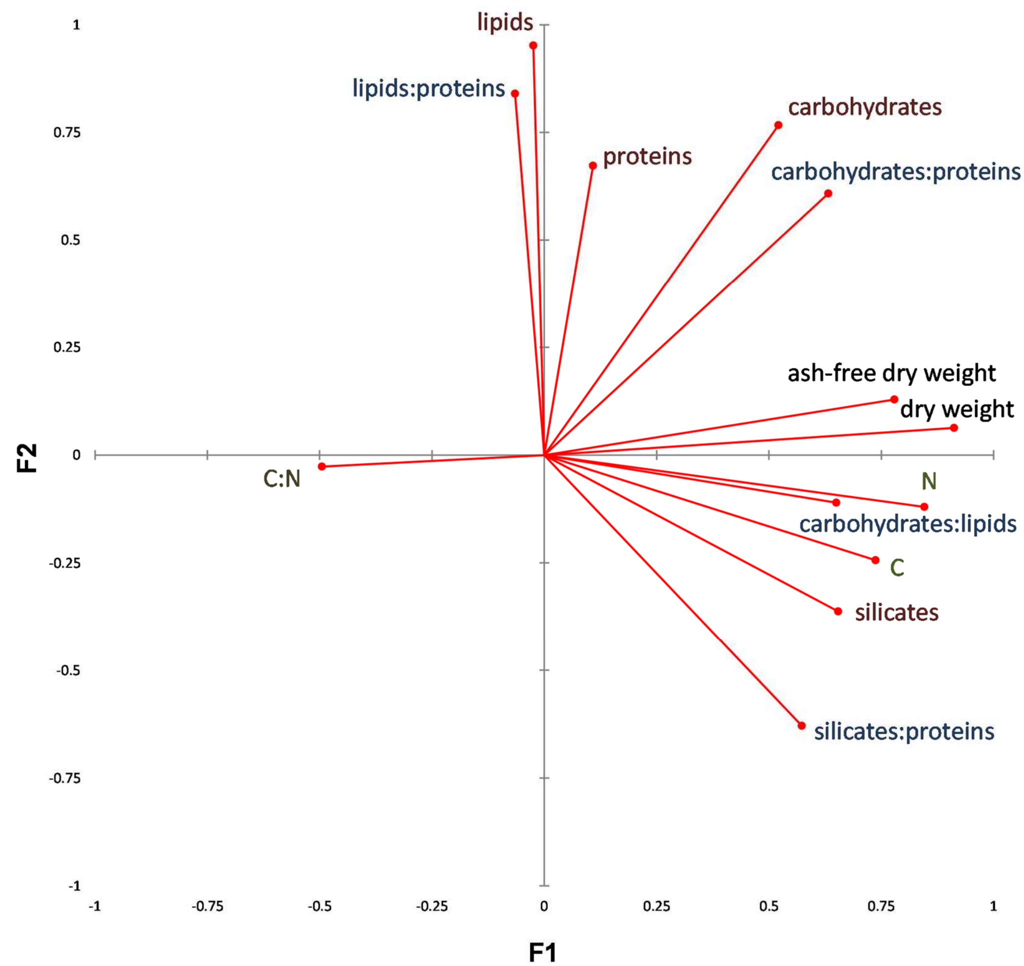


Fig. 5.23. Loading plot of the Principal Component Analysis (PCA). Proteins, lipids, carbohydrates, silicates, carbon and nitrogen per cell and the lipid:protein, carbohydrates:protein, carbohydrates:lipids, silicates:protein and C:N were used as variables.

5.3.4 Comparison between wild type and eYFP-tagged cultures

For each culture treatment, no significant differences were found between the growth rates of wild type and eYFP-tagged *P. tricornutum* cells grown in batch.

Wild type and transformants of *P. tricornutum* acclimated to the same culture medium did not differ in any of the factors examined. The results of the t-tests for growth, cell dry weight and elemental composition between wild type and fluorescent cells are shown in **Tab. 5.2**, those for cell organic composition are shown in **Tab. 5.3**.

Tab. 5.2 P-values resulting from the t-tests performed in order to assess whether wild type and eYFP-tagged cells of Phaeodactylum tricornutum were identical in terms of elemental composition and cell dry weight (n = 3).

Culture Condition	Parameter					
	Specific growth rate	Carbon	Nitrogen	C:N	Dry weight	Ash content
High CO_2	0.8200	0.5095	0.1581	0.2303	0.6987	0.7799
Low Light	0.2501	0.8829	0.8719	0.6645	0.7514	0.8565
GeO_2	0.2508	0.5251	0.7586	0.7589	0.5387	0.7580
0.5 mM NH_4^+	0.2869	0.7647	0.8932	0.9571	0.9261	0.8313
1 mM NH_4^+	0.7127	0.3819	0.3657	0.2493	0.6664	0.9635
Control	0.8575	0.9926	0.6629	0.7591	0.9070	0.7531

Tab. 5.3. P-values resulting from the t-tests performed in order to assess whether wild type and eYFP-tagged cells of Phaeodactylum tricornutum were identical in terms of organic composition (n = 3).

Culture condition	Parameter				
	Proteins	Lipids:Proteins FTIR abs ratio	Carbohydrates:Proteins FTIR abs ratio	Carbohydrates:Lipids FTIR abs ratio	Silicates:Proteins FTIR abs ratio
High CO_2	0.0507	0.3265	0.3260	0.3309	0.2584
Low Light	0.4583	0.5911	0.7916	0.6995	0.2321
GeO_2	0.7531	0.6950	0.6020	0.9760	0.4116
0.5 mM NH_4^+	0.1362	0.9296	0.6899	0.6951	0.6898
1 mM NH_4^+	0.941	0.0832	0.5386	0.6256	0.1624
Control	0.3932	0.5510	0.1007	0.0960	0.5800

In the flow cytometer, wild type and fluorescent cells produced the same signal in terms of red fluorescence (mostly due to the chlorophyll), forward and reverse scattering, indicating similar chlorophyll content, cell size and “granularity", respectively (Coulter Epics XL Flow Cytometer Reference Manual 1998). Mutant and wild type cells were identical for all the parameters I measured, and could be distinguished only based on the eYFP tag fluorescence (**Fig. 5.24**).

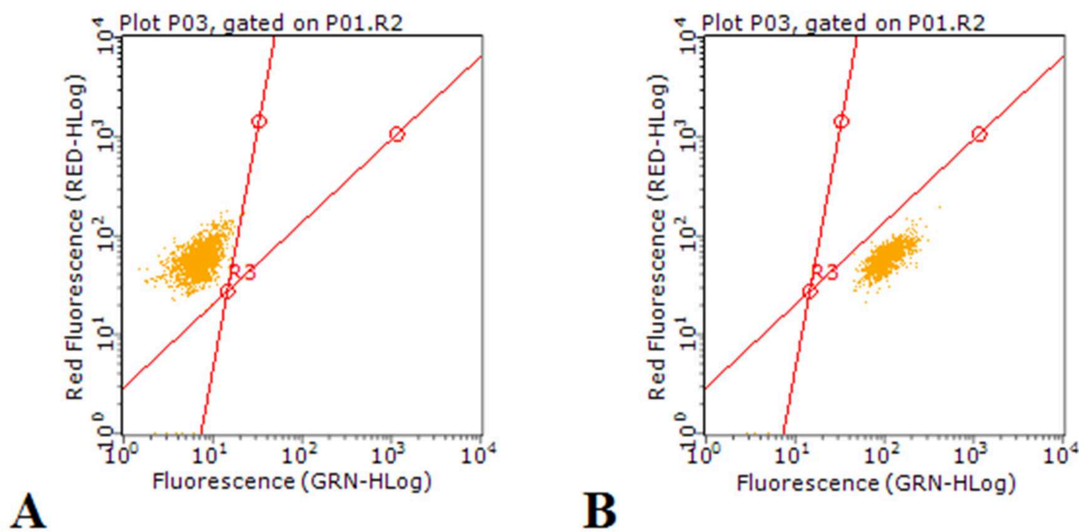


Fig. 5.24. Red fluorescence (detected at 690/50 nm) from wild type (**A**) and eYFP-tagged (**B**) cells of *P. tricornutum* was plotted against the fluorescence detected at 525/30 nm.

5.3.5 Growth of algae in the presence and in the absence of the grazers

The number of cells per unit of volume was calculated at the beginning and at the end of the predation experiments in each algal mixture constituted by two populations with different nutritional backgrounds and exposed or not to the predators. Based on this, the overall growth rates of the algal mixtures in the absence and in the presence of the grazers were calculated

Tab**. 5.4**; **Tab. 5.5**). I observed three types of output:

1) Algal mixtures that were not exposed to the predator had a significantly higher growth rate than those that were exposed.

2) Algal mixtures that were or were not exposed to the grazer grew at the same rate.

3) Algal mixtures that were not exposed to the predator had a significantly lower growth rate than algae that were exposed to the grazer.

The first result was the most common output of the experiments with the rotifers (7 out of 10 cases; **Fig. 5.25**). In the other three cases (Low Light + High CO_2; Low Light + 0.5 mM NH_4^+; 0.5 mM NH_4^+ + 1 mM NH_4^+) the difference between the growth rate of algal mixtures exposed or not exposed to the rotifers was not significant (**Fig. 5.25**).

In the experiments with the copepods, I did not observe any case in which algal mixtures that were exposed had a similar growth rate to those that were subjected to predation. In 8 cases, the growth rate of algae was lower in the presence than in the absence of the copepods (**Fig. 5.26**). In two cases only (Low Light + 1 mM NH_4^+ and High CO_2 + 1 mM NH_4^+) the growth rate of algal mixtures that were exposed to the copepods was higher than that of the mixtures that were incubated in the absence of copepods (**Fig. 5.26**).

Tab. 5.4. Algal cell concentrations (cells ml^{-1}) at the beginning and at the end of the grazing experiments with rotifers. For each row (i.e. for each couple of *P. tricornutum* populations) different letters indicate significantly different means ($n \geq 5$).

Algal mix	Algae - rotifers		Algae + rotifers	
	T0	T6	T0	T6
Low Light + High CO_2	$3.66E+06^{a}$	$4.26E+06^{a}$	$3.65E+06^{b}$	$4.10E+06^{b}$
	(1.33E+05)	(3.99E+04)	(1.70E+05)	(4.02E+04)
Low Light + 0.5 mM NH_4^+	$3.53E+06^{a}$	$4.23E+06^{a}$	$3.56E+06^{b}$	$4.13E+06^{b}$
	(1.22E+05)	(7.95E+04)	(9.09E+04)	(4.63E+04)
Low Light + 1 mM NH_4^+	$3.49E+06^{a}$	$4.21E+06^{a}$	$3.52E+06^{b}$	$4.10E+06^{c}$
	(2.51E+04)	(3.61E+04)	(4.69E+04)	(6.91E+04)
Low Light + GeO_2	$3.58E+06^{a}$	$4.24E+06^{a}$	$3.53E+06^{b}$	$4.03E+06^{c}$
	(9.69E+04)	(1.24E+05)	(9.03E+04)	(7.06E+04)
High CO_2 + GeO_2	$3.87E+06^{a}$	$4.55E+06^{ab}$	$3.94E+06^{c}$	$4.42E+06^{bc}$
	(3.01E+05)	(3.42E+05)	(3.36E+05)	(2.77E+05)
GeO_2 + 0.5 mM NH_4^+	$3.84E+06^{ab}$	$4.56E+06^{a}$	$3.67E+06^{c}$	$4.12E+06^{b}$
	(2.58E+05)	(2.39E+05)	(1.81E+05)	(1.19E+05)
0.5 mM NH_4^+ + 1 mM NH_4^+	$3.65E+06^{ab}$	$4.30E+06^{a}$	$3.53E+06^{c}$	$3.98E+06^{bc}$
	(2.67E+05)	(1.76E+05)	(2.01E+05)	(1.38E+05)
High CO_2 + 0.5 mM NH_4^+	$3.55E+06^{a}$	$4.23E+06^{ab}$	$3.96E+06^{b}$	$4.43E+06^{b}$
	(9.14E+04)	(4.64E+04)	(5.87E+05)	(4.92E+05)
GeO_2 + 1 mM NH_4^+	$3.59E+06^{a}$	$4.32E+06^{a}$	$3.52E+06^{b}$	$3.97E+06^{b}$
	(1.91E+05)	(1.00E+05)	(2.95E+05)	(2.69E+05)
High CO_2 + 1 mM NH_4^+	$3.63E+06^{a}$	$4.40E+06^{a}$	$3.65E+06^{b}$	$4.13E+06^{b}$
	(3.40E+05)	(3.11E+05)	(2.01E+05)	(1.74E+05)

Tab. 5.5. Algal cell concentrations (cells ml^{-1}) at the beginning and at the end of the grazing experiments with copepods. For each row (i.e. for each couple of *P. tricornutum* populations) different letters indicate significantly different means ($n \geq 5$).

Algal mix	Algae - copepods		Algae + copepods	
	T0	T6	T0	T6
Low Light + High CO_2	3.38E+06[a]	4.09E+06[a]	3.37E+06[b]	3.93E+06[b]
	(2.78E+05)	(3.83E+05)	(1.98E+05)	(1.92E+05)
Low Light + 0.5 mM NH_4^+	3.53E+06[a]	4.24E+06[a]	3.52E+06[b]	4.09E+06[b]
	(1.22E+05)	(1.38E+05)	(1.40E+05)	(9.68E+04)
Low Light + 1 mM NH_4^+	3.49E+06[a]	4.22E+06[b]	3.78E+06[c]	4.68E+06[d]
	(2.51E+04)	(5.47E+04)	(1.89E+05)	(2.09E+05)
Low Light + GeO_2	3.60E+06[a]	4.33E+06[a]	3.60E+06[b]	4.13E+06[b]
	(1.52E+05)	(1.91E+05)	(9.61E+04)	(9.01E+04)
High CO_2 + GeO_2	3.49E+06[a]	4.21E+06[a]	3.60E+06[b]	4.16E+06[b]
	(5.94E+04)	(1.09E+05)	(1.61E+05)	(2.09E+05)
GeO_2 + 0.5 mM NH_4^+	3.84E+06[ab]	4.62E+06[a]	3.58E+06[c]	4.12E+06[b]
	(2.58E+05)	(2.41E+05)	(1.12E+05)	(9.05E+04)
0.5 mM NH_4^+ + 1 mM NH_4^+	3.65E+06[a]	4.39E+06[a]	3.49E+06[b]	4.04E+06[c]
	(2.67E+05)	(2.32E+05)	(1.59E+05)	(1.51E+05)
High CO_2 + 0.5 mM NH_4^+	3.55E+06[a]	4.28E+06[a]	3.56E+06[b]	4.10E+06[c]
	(9.14E+04)	(4.93E+04)	(6.81E+04)	(6.25E+04)
GeO_2 + 1 mM NH_4^+	3.59E+06[a]	4.36E+06[a]	3.50E+06[b]	4.05E+06[c]
	(1.91E+05)	(1.60E+05)	(8.08E+04)	(4.26E+04)
High CO_2 + 1 mM NH_4^+	3.63E+06[a]	4.34E+06[a]	3.47E+06[b]	4.41E+06[b]
	(3.40E+05)	(3.73E+05)	(1.10E+05)	(1.27E+05)

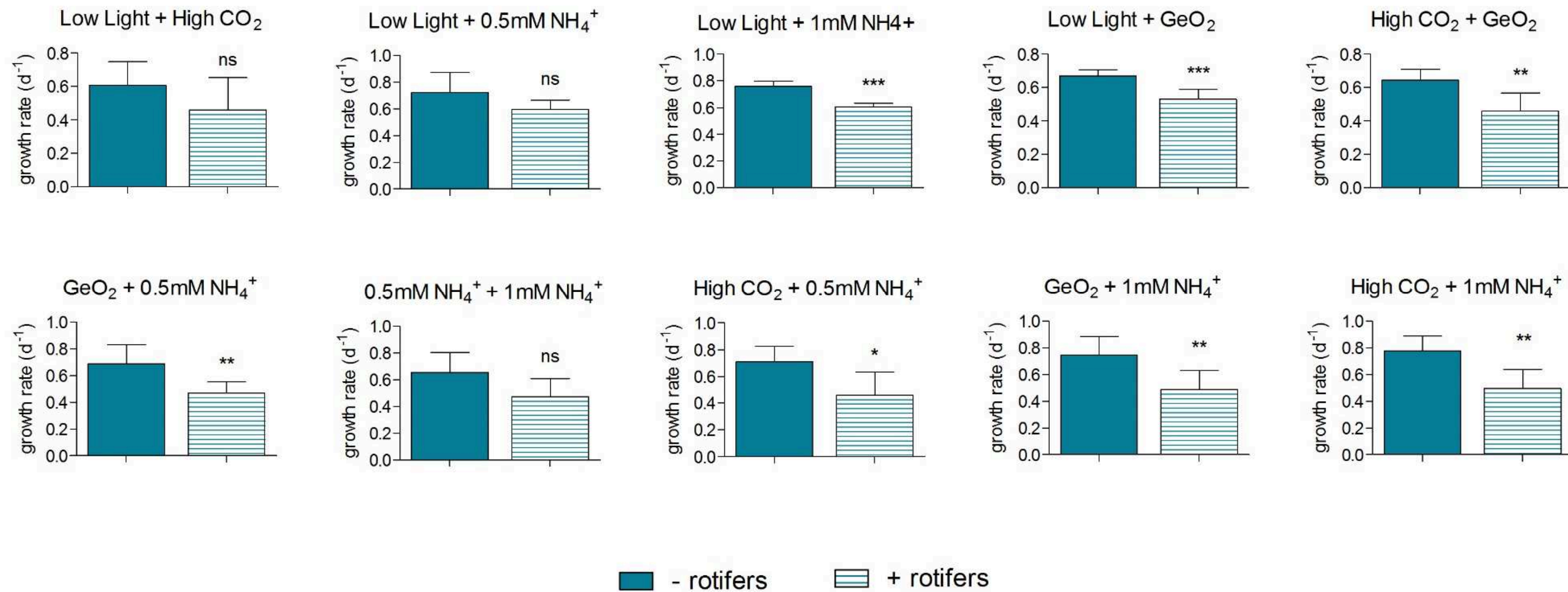


Fig. 5.25. Specific growth rates of algal mixtures constituted by couples of *P. tricornutum* populations with a different nutritional backgrounds, exposed (+ rotifers) or not (- rotifers) to the rotifers. Asterisks indicate that the differences between means in the absence and in the presence of the rotifers are significant ($n \geq 5$) (asterisks: p-value < 0.001; 2 asterisks: p-value from 0.001 to 0.01; 1 asterisk: p-value from 0.01 to 0.05).

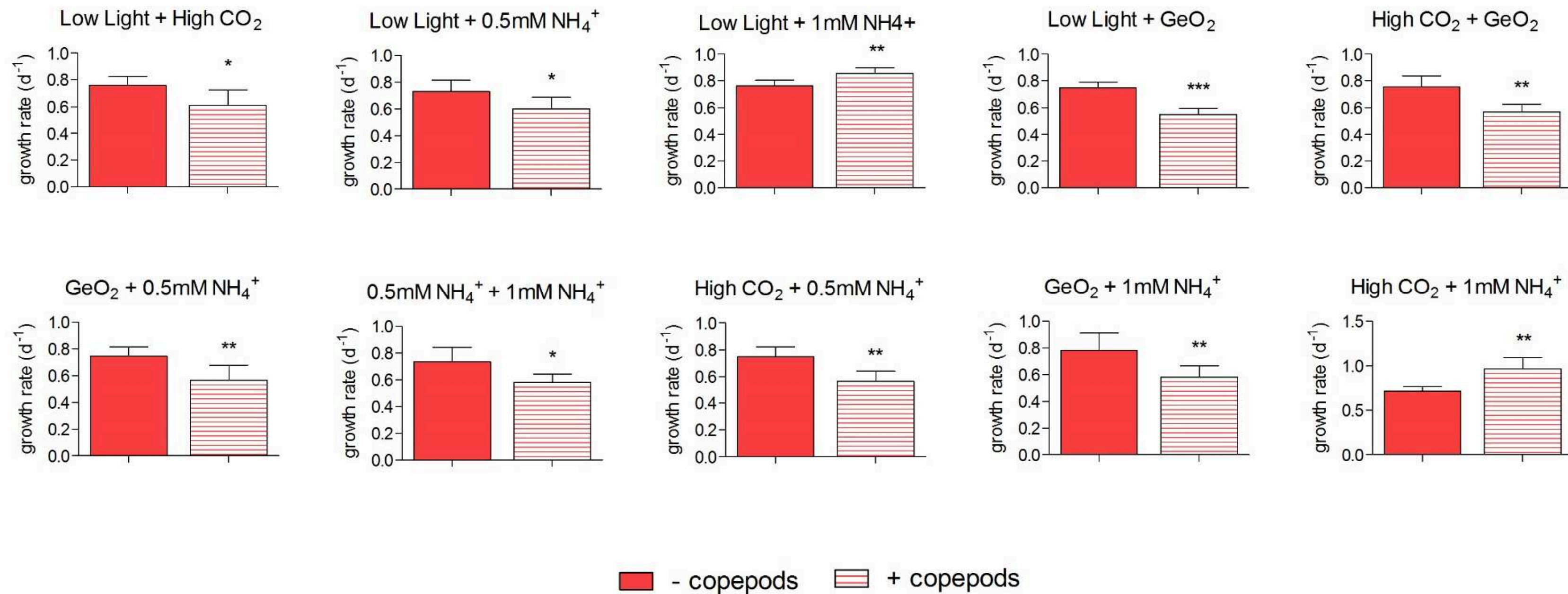


Fig. 5.26. Specific growth rates of algal mixtures constituted by couples of *P. tricornutum* populations with a different nutritional backgrounds, exposed (+ copepods) or not (- copepods) to the copepods. Asterisks indicate that the differences between means in the absence and in the presence of the copepods are significant ($n \geq 5$) (asterisks: p-value < 0.001; 2 asterisks: p-value from 0.001 to 0.01; 1 asterisk: p-value from 0.01 to 0.05).

5.3.6 Ratio between algal populations

Comparisons were made between:

- The population ratios at the end of the experiment in the absence and in the presence of the grazer (T6 – grazer vs T6 + grazer).
- The population ratios at the beginning and at the end of the experiment in the absence of the grazer (T0 – grazer vs T6 - grazer).
- The population ratios at the beginning and at the end of the experiment in the presence of the grazer (T0 + grazer vs T6 + grazer).

The ratios between the pairs of algal populations at the end of the grazing experiments were equal in the absence and in the presence of the rotifers (**Fig. 5.29**). The same was true when the copepods were the grazers, in 8 out of 10 cases (**Fig. 5.27**; **Fig. 5.30**). In two cases, instead, a significant difference was found between the population ratio in the presence and in the absence of the copepods. Specifically, this was observed when cells grown in 1 mM NH_4^+ were mixed with either Low Light or High CO_2-grown cells (**Fig. 5.28**; **Fig. 5.30**).

Tab. 5.6 and **Tab. 5.7** provide the results of the statistical analyses of the population ratios in these two cases.

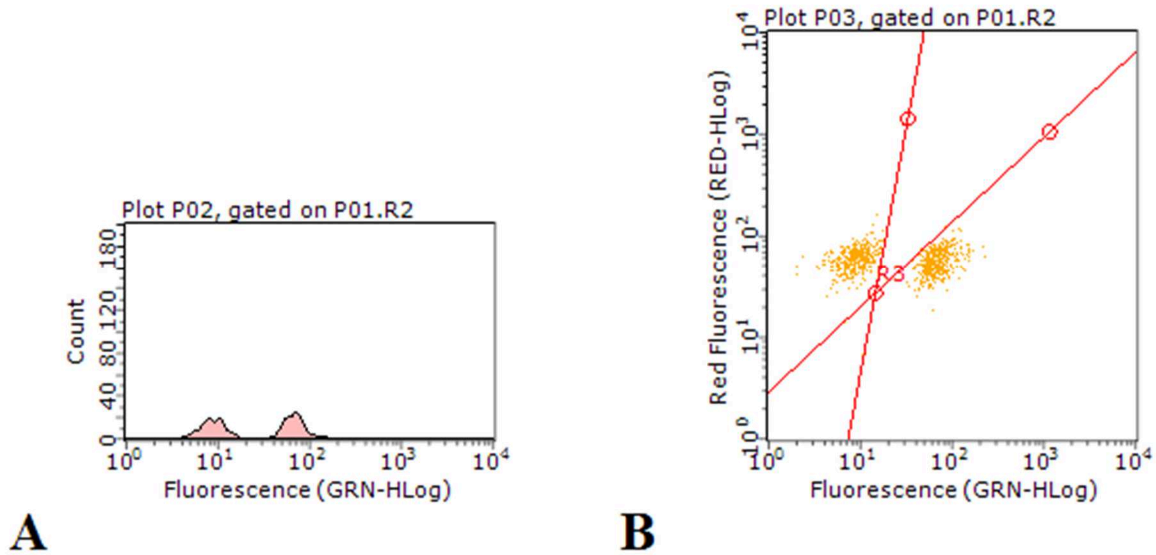


Fig. 5.27. Example of the output of experiments in which algal mixtures of two different populations were coupled in the presence of the grazers and then discriminated through flow cytometry. In this specific case, Low Light-grown cells were coupled with High CO_2-grown cells in the presence of copepods. **A.** Number of cells (“count”) vs fluorescence detected at the 525/30 nm channel of the flow cytometer. **B.** Cells are separated based on red fluorescence (y-axis; logarithmic) and on the fluorescence detected at the 525/30 nm channel of the flow cytometer (x-axis).

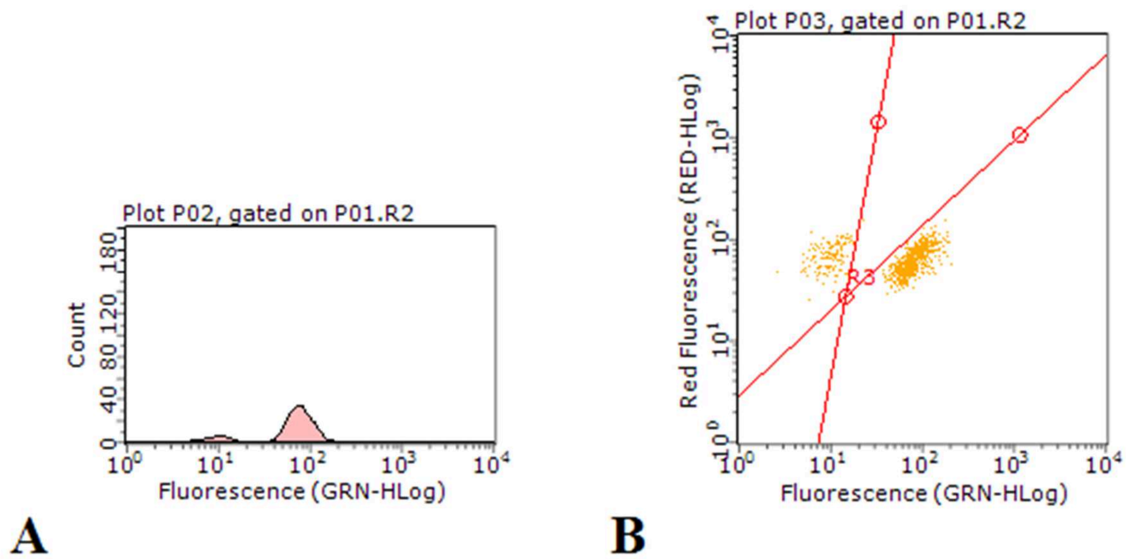


Fig. 5.28. Example of the output of experiments in which algal mixtures of two different populations were coupled in the presence of the grazers and then discriminated through flow cytometry. In this specific case, 1 mM NH_4^+-grown cells were paired with Low Light-grown cells in the presence of copepods. **A.** Number of cells ("count") vs fluorescence detected at the 525/30 nm channel of the flow cytometer. **B.** Cells are separated based on red fluorescence (y-axis; logarithmic) and on the fluorescence detected at the 525/30 nm channel of the flow cytometer (x-axis).

Tab. 5.6. Results of the statistical analysis of ratios between Low Light- and 1 mM NH_4^+-grown populations at different sampling time and in the presence or absence of the grazer. P-values < 0.05 are in bold font.

Comparison	p-value	
T0 – grazer vs T6 - grazer	0.2160	
	rotifers	copepods
T0 + grazer vs T6 + grazer	0.3818	**< 0.0001**
T6 – grazer vs T6 + grazer	0.6547	**< 0.0001**

Tab. 5.7. Results of the statistical analysis of ratios between High CO_2- and 1 mM NH_4^+-grown populations at different sampling time and in the presence or absence of the grazer. P-values < 0.05 are in bold font.

Comparison	p-value	
T0 – grazer vs T6 - grazer	0.5654	
	rotifers	copepods
T0 + grazer vs T6 + grazer	0.9568	**< 0.0001**
T6 – grazer vs T6 + grazer	0.4188	**< 0.0001**

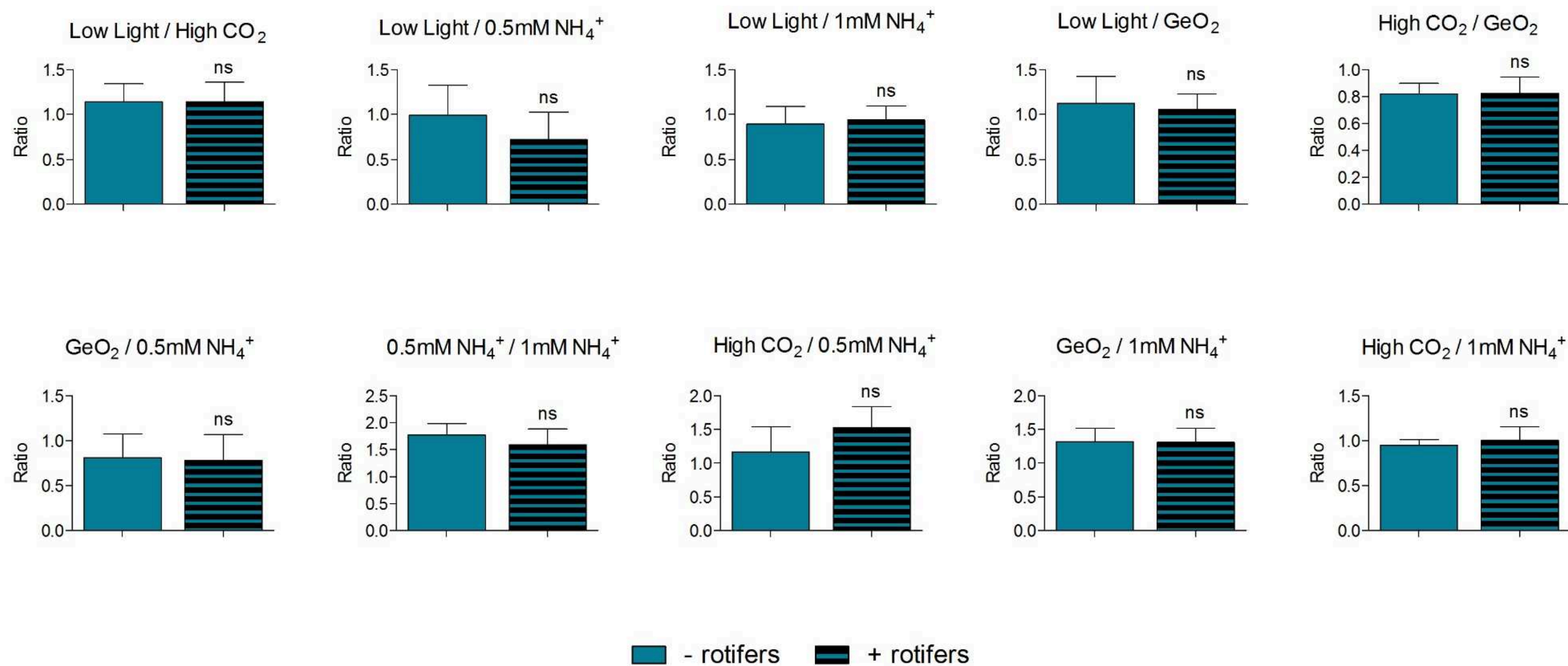


Fig. 5.29. Ratio between two algal populations after 6 hours in the presence (+ rotifers) or in the absence (- rotifers) of the rotifers. Asterisks indicate that the differences between means in the absence and in the presence of the rotifers are significant ($n \geq 5$) (asterisks: p-value < 0.001; 2 asterisks: p-value from 0.001 to 0.01; 1 asterisk: p-value from 0.01 to 0.05).

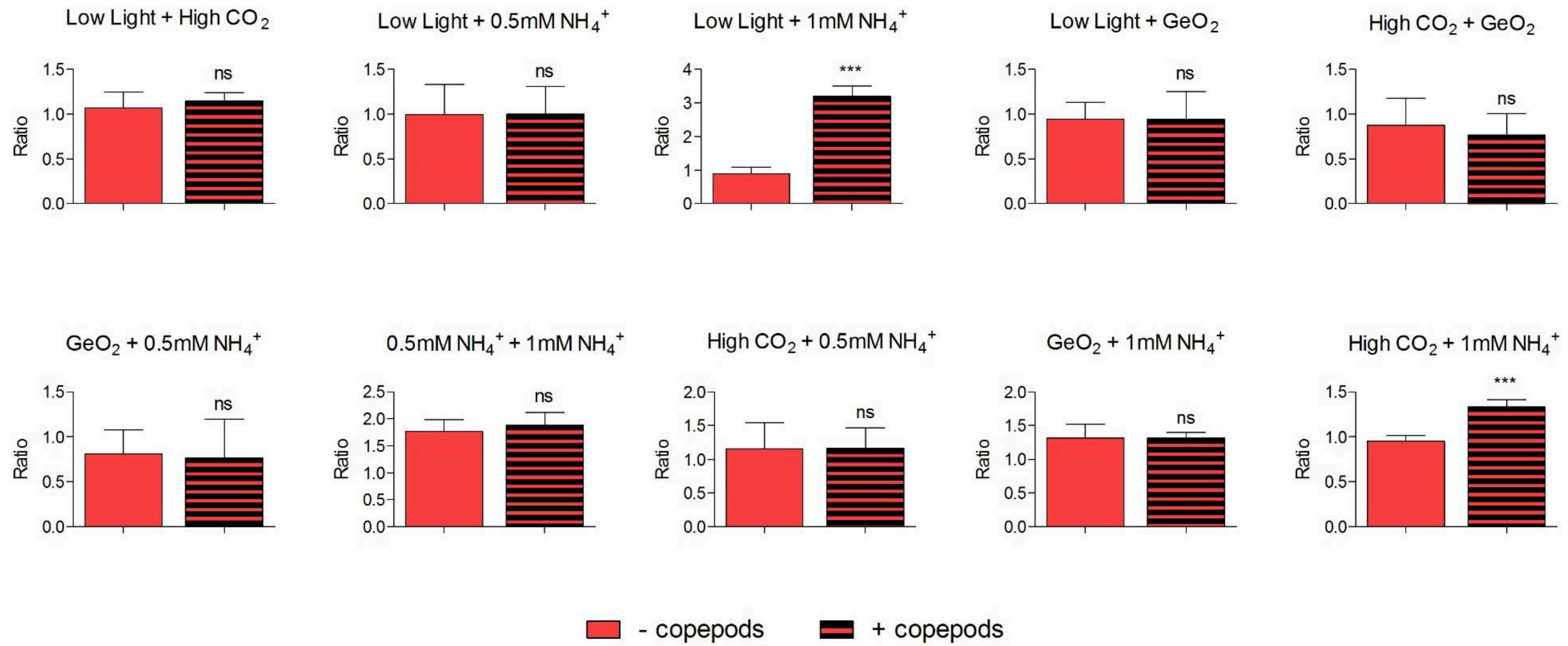


Fig. 5.30. Ratio between two algal populations after 6 hours in the presence (+ copepods) or in the absence (- copepods) of the copepods. Asterisks indicate that the differences between means in the absence and in the presence of the copepods are significant ($n \geq 5$) (asterisks: p-value < 0.001; 2 asterisks: p-value from 0.001 to 0.01; 1 asterisk: p-value from 0.01 to 0.05).

In two out of twenty experiments, the ratio between the algal populations changed over time in a similar way in the absence and in the presence of the grazers. This was observed when the 1 mM NH_4^+-grown population was mixed with the GeO_2-grown population (**Fig. 5.31**) or with the 0.5 mM NH_4^+-grown population (**Fig. 5.32**).

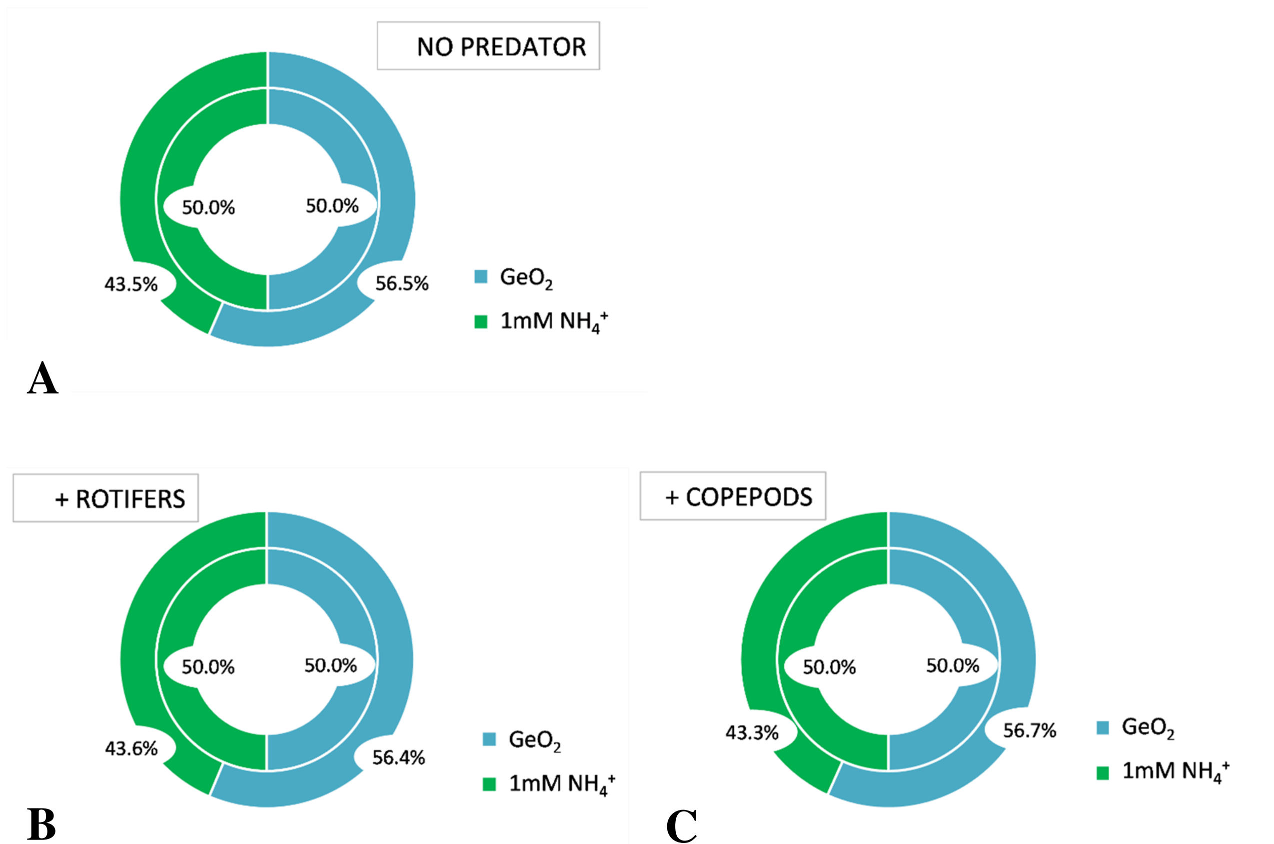


Fig. 5.31. Change over time of the ratio between GeO_2- and 1 mM NH_4^+-grown cells of P. tricornutu in the absence of predators (**A**), in the presence of rotifers (**B**) or in the presence of copepods (**C**). Tl inner circle represents the ratio at the beginning of the experiment; the outer circle represents the rat at the end of the experiment, after 6 hours. No significant differences were found between the rati at T6 in the absence and in the presence of either rotifers or copepods (n = 6).

Fig. 5.32. Change over time of the ratio between 0.5 mM NH_4^+- and 1 mM NH_4^+-grown cells of *P. tricornutum* in the absence of predators (**A**), in the presence of rotifers (**B**) or in the presence of copepods (**C**). The inner circle represents the ratio at the beginning of the experiment; the outer circle represents the ratio at the end of the experiment, after 6 hours. No significant differences were found between the ratios at T6 in the absence and in the presence of either rotifers or copepods (n = 6).

5.4 DISCUSSION

In this study, five culture treatments were selected and imposed on the pennate diatom *P. tricornutum*. Such treatments elicited changes of the cell composition of cells (**Tab. 5.1**) so that five *P. tricornutum* populations with a potentially different palatability for the grazers were generated.
A simple way to represent the interaction between different algal populations and the numerous analyzed parameters is a Principal Component Analysis (PCA). In the score plot of the PCA, observations from the same population - represented by dots of the same color – tended to cluster together and separate from those of the other populations (**Fig. 5.22**). Dots representing cells grown in 1 mM NH_4^+ are quite afar from the other dots. In the loading plot, we can observe that the position of the dots representing 1 mM NH_4^+ grown cells corresponds to the parameters related to the weight (dry weight and ash-free dry weight), to the cell N content and to the carbohydrates:lipids ratio (**Fig. 5.23**). This suggests that these features were the main determinants of the overall differences between 1 mM NH_4^+-grown cells and cells having a different nutritional history. The dots that represent the cells grown in the presence of GeO_2 are also very well distinct from the others, and gather on the top left quadrant of the PCA score plot, which is identified by the "lipid" and "lipid to protein ratio" parameters in the loading plot.
In the next paragraphs, I will examine the effect of the different culture treatments on *P. tricornutum* cultures in so far they affect algal cell composition and – in turn - the vulnerability of algae to the grazers. I will discuss the effects of the grazers on the algal growth and on the ratio between two coexisting algal population with different cell composition.

5.4.1 Effect of the grazers on the proportion between algal populations

Rotifers of the genus *Brachionus* were shown to select food based on size only (Rothhaupt 1990) and to be even capable of feeding on inert particles, providing that they had a suitable size (DeMott 1986). Copepods, instead, have been reported to discriminate particles taking advantage of the chemical information they convey (Poulet and Marsot 1978; Sjoqvist et al 2013). However, to the best of my knowledge, the incompetence of rotifers and the ability of copepods to feed selectively based on chemical information was never investigated before by using living algae identical in all but cell composition that coexisted in a communal medium. Instead, it has been mostly proved in laboratory experiments in which

inert materials were used to simulated food. Other investigations were conducted by employing different algal species representative of different cell compositions and thus did not allow a satisfying isolation of the factors that would possibly trigger selective feeding. In other works, the grazers were alternatively fed on cells of the same algal species having different nutritional backgrounds so that possible alterations in their growth and reproduction could be recorded (Rossol et al 2012; Schoo et al 2013). Rossol and co-authors showed that High CO_2- and low CO_2-grown *Thalassiosira pseudonana* cells had a different fatty acids profile; when the copepod *Acartia tonsa* was fed on the High CO_2-grown cells, its growth and egg production were compromised (Rossol et al 2012). Schoo and co-authors observed that larval development of *Acartia tonsa* was compromised when fed on cells of *Rhodomonas salina* cultivated at elevated CO_2 (Schoo et al 2013). Other studies have found no indirect (i.e. mediated by the food quality) effects of high CO_2 on feeding, egg production and hatching of copepods: it is the case of Isari and collaborators, who found that the reproduction of cultures of the calanoid copepod *Acartia grani* fed on *Heterocapsa* cells grown at different CO_2 concentrations did not vary significantly (Isari et al 2015). Similar experimental setups, however, cannot directly demonstrate the occurrence of an actual choice by the grazer, because different algae were not concomitantly exposed to the predator.

Copepods have very precise nutritional requirements to complete their life cycle. Since it is known that the poor nutritional value of ingested food can negatively affect growth, development and egg production of these predators, a selective pressure may have promoted the evolution of systems that allowed the copepods to recognize cells that are worth consuming.

What makes an algal cell more or less palatable to copepods and other selective feeders, however, is still matter of debate. Various factors have been proposed as critical, among which:

- Ratio between C and other nutrient. High ratios between C and other nutrients – not only N - are often indicated as suggestive of a low biomass quality for herbivores (Sterner and Elser 2002; Malzahn et al 2007; Boersma et al 2008). Some works consider the C:N ratio as a major determinant for algal biomass quality (Kiørboe 1989; Jones et al 2002).
- Phosphorus and fatty acid (especially polyunsaturated fatty acids, PUFA) are also thought to be important determinants of the quality of algal biomass as a food for zooplankton (Sterner 1994; Muller-Navarra 1995). The occurrence of a direct correlation between P and PUFA content in algae, which may affect zooplankton selection of food, has been postulated but not strongly confirmed, as well as the direction and extent of such link (Gulati and Demott 1997). Diatoms and dinoflagellates would be preferred over cyanobacteria because of their higher fatty acid content (Ahlgren et al 1990). In nature, cyanobacteria would be neglected by some zooplankters because often poor in P and not

(or not only) because of size-or toxicity-related constraints (Bernardi and Giussani 1990; Gulati and Demott 1997).

It is likely that the answer to the question "what makes algae more or less attractive to grazers?" depends both on the kind of grazer we consider and on its life stage and physiological state. My results appear to confirm that rotifers and copepods have a different propensity to select algae as food on the basis of chemical information. The rotifer *Brachionus plicatis* did not exhibit preferences among *P. tricornutum* cells with different cell composition. The copepod *Acartia tonsa*, instead, exhibited a preference for *P. tricornutum* cells that were previously acclimated to a culture medium containing 1mM NH_4^+ (**Fig. 5.30**). Such preference, however, was effective or emerged only when these cells were paired with those grown at Low Light and at High CO_2, while it was not noticed in other combinations of algal populations.

Low Light- and High CO_2-grown cells had a lower dry weight (**Fig. 5.7**) and ash-free dry weight (**Fig. 5.8**) than the 1 mM NH_4^+-grown counterparts; they also contained less N (**Fig. 5.12**) and fewer carbohydrates (**Fig. 5.20**) per cell, and their carbohydrate:protein ratio was lower (**Fig. 5.15**), than cells grown at 1 mM NH_4^+. However, such differences were also observed in algae having different nutritional backgrounds (specifically, in cells of all the other populations, for dry weight, cell N and carbohydrate content; in all cells but the control cells, for the ash-free dry weight; in all cells but GeO_2-grown cells for the carbohydrate:protein ratio) (**Tab. 5.8**).

Some of the features that made Low Light-grown cells different from 1mM NH_4^+-grown cells were not shared with High CO_2-grown cells (i.e. the higher C:N ratio and the lower protein, lipid and silicate contents). The lower C:N ratio in 1mM NH_4^+-grown than in Low Light-grown cells, due to a higher quota of N and not to a lower quota of C (**Fig. 5.11**, **Fig. 5.12**, **Fig. 5.13**), was likely associated to the need of these cells to assimilate NH_4^+ by directing C allocation toward N-rich compounds. Differences in the C:N ratio within algal cells can determine different patterns of allocation of resources into C and N organic pools (Rivas-Ubach et al 2012); the higher protein content in 1mM NH_4^+-grown than in Low Light-grown cells is probably linked to their lower C:N ratio. Since differences in the C:N ratio were only found between 1mM NH_4^+-grown and Low Light-grown cells, the differences among other algal populations in the organic cell composition were not driven by stoichiometric (i.e. C:N) unbalances.

All information above suggests that one of the two following scenarios may have occurred:

1) Selective feeding by *A. tonsa* was primarily ascribable to one of the factors that determined the differences both between Low Light- and 1 mM NH_4^+-grown cells and between High CO_2- and

1mM NH_4^+-grown cells. In the case of Low Light-grown cells, this factor may be the C/N ratio, whose value was lower than in 1 mM NH_4^+-grown cells. In the case of High CO_2- grown cells, this factor is not included between those that were examined in this study, since no differences between High CO_2- and 1mM NH_4^+-grown cells was exclusive of this couple of algal population.

2) The preference of *A. tonsa* for certain *P. tricornutum* cells is due to a (unique or not) combination of factors, which however I could not detect in my experiments. Surely, algae that possess lower dry weight, ash-free dry weight, N content, carbohydrates content ratio and carbohydrates:proteins ratio are not necessarily less predated by copepods than co-occurring algae having different characteristics. This combination of parameters, in fact, distinguished the 1 mM NH_4^+-grown cells not only from High CO_2- and Low Light-grown cells, but also from other algal populations (e.g. that grown at 0.5mM NH_4^+), for which however the grazers did not exhibit any preference.

Tab. 5.8. Summary of the differences in cell composition between NH_4^+ 1mM-grown cells of *P. tricornutum* and cells grown in all other culture conditions. The green squares indicate that the parameter increased relative to the NH_4^+ 1mM-grown cells; the red squares indicate that the parameter decreased relative to the NH_4^+ 1mM-grown cells. The acronym "ns" stands for "not significant" and was used to identify the treatments that did not elicit significant changes compared to the NH_4^+ 1mM-grown cells. For each parameter, the units of measurement are specified by the superscript letters, according to the following legend: [a] pg $cell^{-1}$; [b] mass ratio; [c] FTIR absorbance ratio; [d] relative amount per cell.

Culture Conditions	Parameters												
	Dry Weight[a]	Ash-free dry weight[a]	Carbon[a]	Nitrogen[a]	$\frac{C}{N}$[b]	$\frac{Lipids}{Proteins}$[c]	$\frac{Carbohydrates}{Proteins}$[c]	$\frac{Carbohydrates}{Lipids}$[c]	$\frac{Silicates}{Proteins}$[c]	Proteins[a]	Lipids[d]	Carbohydrates[d]	Silicates[d]
High CO_2	■ (red)	■ (red)	ns	■ (red)	ns	ns	■ (red)	ns	ns	ns	ns	■ (red)	ns
Low Light	■ (red)	■ (red)	ns	■ (red)	■ (green)	ns	■ (red)	ns	ns	■ (red)	■ (red)	■ (red)	■ (red)
GeO_2	■ (red)	■ (red)	■ (red)	■ (red)	ns	ns	ns	■ (red)	■ (red)	ns	■ (red)	■ (red)	■ (red)
0.5 mM NH_4^+	■ (red)	■ (red)	ns	■ (red)	ns	ns	■ (red)	■ (red)	ns	ns	ns	■ (red)	ns
Control	■ (red)	ns	ns	■ (red)	ns	ns	■ (red)	■ (red)	■ (red)	■ (red)	ns	■ (red)	■ (red)

In some cases, factors related to cell composition may co-determine selective feeding together with factors not related to cell compositions; for instance, algae with different elemental stoichiometry or absolute abundance of elements may be able to develop thicker/thinner cell walls, so becoming less/more attractive for the grazers. The possibility that a similar scenario occurs was investigated in the present study by comparing cells whose different cell composition was expected to determine differences in mechanical properties of cells. *P. tricornutum* cells grown in the presence of GeO_2, specifically, were expected – and actually had – a lower silica content (**Fig. 5.21**). However, this did not translate into a higher grazing pressure, either because it did not significantly weight on the cell wall properties or because a less-silicified cell wall was not important for the grazers' choice. Anyhow, it is possible that other combinations of algal populations, involving cells with a much wider difference in terms of silicate content, will produce a different outcome.

The comprehension of which algal features are important for selective feeding is expected to: 1) clarify the prey-predator dynamics in the modern oceans and 2) cast light on the evolution of algae. In fact, cell composition may be targeted by natural selection, if it influences the survival of algae to grazing; this has previously been proposed for other algal features, such as size, morphology and strength of the cell wall. Algal cell composition can be tightly linked to environmental conditions, and especially to the absolute and relative amount of nutrients (Sterner and Elser 2002). Algae whose internal composition is extremely dependent on the external chemical environment can be disadvantaged if the environmental conditions lead them to become more palatable to the grazer; in such conditions, algae that are able to maintain compositional homeostasis would be favored. Thus, the homeostatic/acclimatory capabilities of algae, having the potential to affect the palatability of algae to grazers, may also be targeted by selection.

5.4.2 Effect of the grazer on the number of cells in the algal mixtures

At the end of the grazing experiments, in 2 out of 20 cases, the number of cells per unit of volume of algal mixtures was significantly higher in the presence of the predator than in its absence (**Tab. 5.4**; **Tab. 5.5**). This occurred when High CO_2–grown and Low Light-grown cells were paired and exposed to *A. tonsa*. Interestingly, these are the same combinations in which the ratio between the two algal populations was affected by the grazer.

It should be pointed out that the algal cell concentration at the end of the grazing experiments (and thus the growth rates of the mixtures of algal populations; **Fig. 5.25**; **Fig. 5.26**) results from a

combination of factors: the depletion of cells from the medium by grazing; possible changes in the growth of one algal population; possible changes in the growth of both algal populations (not necessarily in the same direction).

Many instances in literature indicate that the presence of the predators could stimulate algal growth and/or photosynthesis. Already in 1978, a positive correlation between zooplankton pressure and algal growth was reported (Lampert 1978). Later on, Redfield noticed that the presence of zooplankters could enhance the algal productivity:biomass ratio; this increase was positively correlated with the abundance of zooplankters and was proposed to be a consequence of nutrient regeneration (Redfield 1980). Sterner found that both growth and reproduction of N-limited natural algal communities were positively correlated with grazer density (but they saturated at low grazer density; Sterner 1986); according to the author, the stimulatory effect on algal growth was ascribable to the N regeneration operated by *Daphnia pulex* (Sterner 1986). More recently, Trommer and coauthors showed that phytoplankton assemblages dominated by diatoms and dinoflagellates increased in biomass when grown in the presence of *Acartia tonsa* and *Brachionus plicatilis* (Trommer et al 2012). Ratti and co-workers observed an increase of the growth rate of *Tetraselmis suecica* and *Thalassiosira weissflogii* in the presence of two kind of grazers (the copepod *Acartia tonsa* and the protist *Euplotes* sp.) and speculated this was due to the animal metabolism, responsible for an increase in the nutrients turnover (Ratti et al 2013).

The hypothesis that the predators operate a “fertilization” of the environment, stimulating the growth of nutrient-limited algae, has been postulated and, in some cases, verified experimentally (Axler et al 1981; Sterner 1986). Grazers are expected to influence – through the supply of regenerated nutrients - only the growth of algae that experience a shortage of those nutrients (mostly P and N; Redfield 1980). Phytoplankton is limited by N availability in much of the surface low-latitude ocean (Moore et al 2013). A noteworthy amount of the N that is ingested by copepods (> 50% in some cases) is then released in the external medium (Gardner and Paffenhofer 1982). Although urea and amino acids are also released in lower amounts, the N is prevalently released in the form of ammonium (Corner and Newell 1967; Corner et al 1976). This ammonium has an important role for the primary producers that are N-limited (McCarthy and Goldman 1979). Some authors suggest that the release of N by copepods is probably a minor flux, if compared, for instance, to microbial N regeneration (Miller and Glibert 1998). However, the regeneration of nitrogen via zooplankton excretion was reported as capable, by itself, to satisfy a big fraction of the phytoplankton N total requirement (Axler et al 1981). It should be pointed out that the availability of regenerated N of zooplankton origin to N-limited phytoplankton is not always stimulatory for algae; in cells in which the assimilation of C and N

compete for ATP and reducing power, for instance, the availability of regenerated ammonium can ultimately reduce algal photosynthesis (Falkowski and Stone 1975).
The influence of herbivores on an overall algal community, therefore, may result from the interplay of various factors, among which direct consumption and regeneration of nutrients. The sum of these factors may be null or may lead to either stimulation or suppression of algal growth (Porter 1973). In the cases examined here, 1 mM NH_4^+-grown cells, for which copepods showed a predilection compared to High CO_2-grown and Low Light-grown cells, had a higher dry weight, more N and carbohydrates per cell than *P. tricornutum* cells with different nutritional backgrounds. By eating them, therefore, the copepods gained access to a higher amount of N than they would have obtained by eating an equal number of cells grown in other culture media. It can be speculated that the supply of regenerated ammonium by zooplankton had stimulated the growth of *P. tricornutum* algae that were not eaten, providing them more N *pro capite*. This requires the important assumption that the growth of algal populations in the mix was either N-limited (and thus stimulated by N supply) or energy limited. In fact, the use of nitrate is generally more expensive than that of ammonium, so that cells that have been cultivated in nitrate (among which High CO_2-grown and Low Light-grown cells), whose growth is energy-limited, are expected to especially benefit from the regenerated ammonium released by zooplankton that have been eating “richer” cells.
The stimulatory effect on the growth of nitrate-grown cells, anyhow, only emerged when a higher number of N-rich cells than of other cells was consumed by *A. tonsa*, possibly because the greater amount of ingested algal N resulted in a greater amount of regenerated N.
Even if 1 mM NH_4^+-grown cells also had more silicate than the other cells, it is unlikely that the availability of a greater amount of Si per cell due to the consumption of Si-rich grazed cells had stimulated the growth of the other cells. It should be considered, indeed, that recycling efficiency varies among the elements; specifically, Si is recycled by grazer with much less efficiency than, for instance, P and N, because it is regenerated in a chemical form that is unsuitable for uptake by algae (Sterner 1990).

5.4.3 Changes of the ratio between algal populations that do not depend on the presence of the grazer

When the *P. tricornutum* cells grown at 1 mM NH_4^+ were incubated together with either those grown in the presence of GeO_2 or those grown at 0.5 mM NH_4^+, the ratio between the populations turned out to be altered independently of the presence of either kind of grazers. In both cases, after 6 hours

of coexistence, cells previously grown at 1 mM NH_4^+ were significantly less abundant than the other cells.

When acclimated to 1 mM NH_4^+, *P. tricornutum* cells had a higher dry weight and contained more N, proteins, carbohydrates and silicates than cells acclimated to the control medium (**Tab. 5.1**). This suggests that the control medium was somehow less favorable to *P. tricornutum*. It can be postulated that the transfer of 1 mM NH_4^+ cells to the control medium had challenged their ability to produce new equally rich cells, being the new environment less favorable. Why this would only emerge when they are paired with cells grown with GeO_2 and at 0.5 mM NH_4^+, however, is hard to understand by using the available information.

The possibility that GeO_2- and 0.5 mM NH_4^+-grown cells prevailed over 1 mM NH_4^+-grown cells by acquiring nutrients more efficiently appears unlikely. In fact, such a process has been only demonstrated in the case of cells whose nutritional history comprised a limitation for a nutrient; these cells would be much more capable to uptake the same nutrient than their counterparts grown in replete conditions (Goldman and Gilbert 1982).

Furthermore, in the absence of direct determination of infochemicals in the medium, I cannot exclude that the observed outcome is the result of chemical communication between conspecific populations. In nature, conspecific cells with a different nutritional background (i.e. acclimated to different environmental conditions) may get in touch, for example, after the breakage of a temporary physical barrier. Similar scenarios are examined in more detail in the chapter 6.

5.5 CONCLUSIONS

My data suggest that algal cell composition is of utmost importance for the interaction with their natural predators. The outcome of this work shows that the ability of feeding selectively based on chemical information is not a prerogative of all grazers. While the rotifer *Brachionus plicatis* was unable to discriminate among *P. tricornutum* cells having six different nutritional backgrounds, the copepod *Acartia tonsa* exhibited feeding preferences between co-occurring algae that differed for cell composition.

Since cell composition and environment are strictly related, the nutritional history of algae emerges as a major determinant of the relationships between algae and grazers. The food preferences of the predators may have had a role in the evolution of algae, for instance by driving the affirmation of physiological processes that allow cell composition to vary in an opposite direction compared to the grazers' preferences.

If algal cell composition – as it emerges from my results – has great importance in the relationships with the grazers, then also the ability to modulate cell composition is expected to affect the possibility of algae to survive (to grazing). This has important implications, suggesting that cell composition and homeostatic/acclimatory capabilities of algae are natural candidate as targets for natural selection.

Changes in the ratio between two algal populations were also observed independently from the presence of the grazers. This suggests the occurrence of competition and/or communication mechanisms among algal cells that are genetically identical but have distinct environmental (i.e. nutritional) histories.

The present study would also like to stimulate further investigations. For instance, cultures of *P. tricornutum* acclimated to identical culture conditions than those described in this work can be examined for numerous characteristics that have not been investigated here (e.g. the content of polyunsaturated fatty acids, which has been proposed as a main regulator of zooplankton choice). This would clarify in more detail the mechanisms at the basis of copepods' preferences.

A further interesting issue that is worth to address in the future is whether algal cell composition is affected by the presence of the copepods. Some evidences of this (i.e. algae that increased the silicification of the frustule in the presence of the copepods; Pondaven et al 2007; Ratti et al 2013) have been reported already. Whether this occurs (and, in case, whether at similar extent and in similar way) in the presence of competitors is a further stimulating issue.

The method I have developed, which entails the use of tagged cells having different nutritional background, is a valuable candidate for investigating this possibility. Cells with different composition

may be paired and exposed to the grazers over a longer period of time than in this work (which was intended to maintain the cell organic composition constant). Then, the application of a cell sorting system downstream of flow cytometry and the study of the biomass of cells originally belonging to different populations would reveal the differential ability of algae to “tune” their own cell composition in order to be, for instance, less attractive to the grazers.

Algal cells have been shown to be able to modulate size and shape in the presence of the grazers (e.g. Pondaven et al 2007). If cell composition, as suggested by my results, is a selectable factor affecting the probability of algae to survive, just like size and morphology do, we can expect algae to possess the capability of modulating their cell composition in the presence of the grazers.

5.6 APPENDIX

5.6.1 Consideration about the growth of *P. tricornutum* cultures

In the semicontinuous cultures that served as inocula for the grazing experiments, all cells grew at the same rate because a communal growth rate was imposed by dilution. From the comparison of the growth rates of the batch cultures, however, it can be deduced that differences existed in the way treatments influenced growth.

The specific growth rate (μ) calculated in this study from daily count of the control batch cultures was 0.87 d^{-1}. We do not know whether the "control" conditions used for this study allowed *P. tricornutum* to reach its "maximum growth rate", uniquely dependent on the genetic properties of the species. Substantial disparity exists among the results present in literature, likely due to even slight different culture conditions or to the use of different strains of *P. tricornutum.* For instance, the strain CCAP 1052/1A of *P. tricornutum*, cultivated between 15.5 and 17.5 °C at 150 μmol photons m^{-2} s^{-1}, grew at rates comprised from 1.25 to 1.37 (little variations depended on the pH; Bartual et al 2008). The strain Pt 1 8.6 (the same I have used for my experiments) was shown to grow at rates comprised between 0.34 and 0.49 d^{-1} in a temperature range from 15 to 20°C (De Martino et al 2011).

Our data show that *P. tricornutum* cells acclimated to 1% CO_2 had the same specific growth rate (μ) of the control cells, which were grown at 0.04% CO_2 (**Fig. 5.6**). This suggests that in our culture conditions, inorganic carbon availability does not limit the growth of *P. tricornutum*, as it occurs in nature at atmospheric CO_2 (see the paragraph 0), despite of course noteworthy differences exist between our culture conditions (nutrient-replete, continuously illuminated…) and natural conditions.

For what concerns the growth of *P. tricornutum* in NH_4^+, it was already known that *P. tricornutum* is able to grow on different N sources (Regan 1988). A higher biomass production was reported for *P. tricornutum* grown on urea or nitrate rather than ammonium (Yongmanitchai and Ward 1991). Other works showed that the growth rate of *P. tricornutum* was not affected by the N source (Lourenço et al 2002; Liang et al 2006). The divergent results obtained by different authors may reflect the use of diverse *P. tricornutum* strains (which in some cases is not specified) or of different culture conditions.

My results show that batch cultures grown at the lower concentration of NH_4^+ had a lower growth rate than cultures grown at 1 mM NH_4^+ (**Fig. 5.6**). This seems to confirm the results described by Giordano (2013), according to which the growth of *P. tricornutum* is not inhibited by very high concentrations (10 mM NH_4^+); furthermore, it was reported to be even stimulated when the NH_4^+ concentration raised from 1 mM to 10 mM NH_4^+ (Giordano 2013). The growth rate of 0.5 mM NH_4^+-

grown cultures, however, was also lower than that of control cultures. While NO_3^- can be stored in vacuoles within the cells (Geider and La Roche 2002), NH_4^+ cannot be accumulated and can be actively released from the cytosol (Britto et al 2001) or incorporated anaplerotically into organic compounds (Norici and Giordano 2002). I hypothesize that the presence of 0.5 mM NH_4^+ in the medium may have not been sufficient for the activation of the systems that allow a tight control of intracellular NH_4^+ and that this may have elicited differences in the growth rates between the 0.5 mM NH_4^+-grown cultures and both the 1 mM NH_4^+-grown and control cultures.

My results show that *P. tricornutum* cells grown in the presence of GeO_2 had a slower growth rate than cells grown in control conditions, at High CO_2 and at 1 mM NH_4^+ (**Fig. 5.6**). This suggests that GeO_2 is able to constrain the growth of *P. tricornutum* in spite of the fact that this species is the only diatom that does not have an absolute requirement of Si for growth (De Martino et al 2007). Nevertheless, this alga takes up Si from the environment (Nelson et al 1984). The half-saturation constant for Si uptake in *P. tricornutum* is estimated to be 97.4 µM, which is a much higher value than those reported for diatoms having a recognized Si requirement for growth (1-6 µM) (Kilham et al 1977; Nelson et al 1984). The saturation of the uptake systems in *P. tricornutum* occurs when the concentration of dissolved Si is above 200 µM (Nelson et a 1984), which is far above the ranges reported in nature (see above).

When Lewin monitored the growth of several monospecific diatom cultures in the presence of 2 mg SiO_2 L^{-1} and at different concentrations of GeO_2, he found that it was significantly decreased in most species. The most responsive species-treatment combination was *Cylindrotheca fusiformis* cultivated at 10 mg GeO_2 L^{-1} (growth rate was inhibited by 92%); in *P. tricornutum* the growth rate declined by 4-38% in the presence of 1 mg GeO_2 L^{-1} and by 65 to 89% with 10 mg GeO_2 L^{-1} (Lewin 1966). The fact that *P. tricornutum* was the least sensitive diatom to GeO_2 was ascribed to the very small SiO_2 amount in this species (Lewin and Guillard 1963; Lewin 1966).

The connection between growth and Si metabolism in this unusual diatom is controversial. Fusiform *P. tricornutum* cells grown in the absence of silicic acid were shown to grow at the same rate as cells grown in the presence of silicic acid (53 µM; De Martino et al 2007). Zhao and collaborators, instead, showed that Si depletion was responsible for a reduction of the growth of *P. tricornutum* only in certain culture conditions, i.e. when green light at 491-574 nm was provided instead of cool white light and when cells were cultivated at 10°C instead of 20°C (Zhao et al 2014).

The *P. tricornutum* batch cultures acclimated to Low Light (i.e. 50 µmol photons m^2 s^{-1}, ¼ of the photon flux rate provided to control cultures) grew slower than all the other cultures. This indicates that growth of *P. tricornutum* at 50 µmol photons m^2 s^{-1}, at least in our culture conditions, did not saturate growth, in agreement with previously published papers (Terry et al 1983).

6. INTRASPECIFIC CHEMICAL COMMUNICATION IN MICROALGAE

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Running head: Chemical communication between conspecific microalgae

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6.1 ABSTRACT

The relevance of infochemicals in the relationships between organisms is emerging as a fundamental aspect of aquatic ecology. Exchanges of chemical cues are likely to occur not only between organisms of different species, but also between conspecific individuals. Especially intriguing is the investigation of chemical communication in microalgae, due to the relevance of these organisms for global primary production and their key role in trophic webs. Intraspecific communication between algae has been investigated mostly in relation to sexuality and mating. The literature also contains information on other types of intraspecific chemical communication that has not always been explicitly tagged as ways to communicate to conspecifics. On the other hand, the proposed role of certain compounds as intraspecific infochemicals appears questionable. In this paper, we make use of this plethora of information in describing the various instances of intraspecific chemical communication between conspecific microalgae and to identify the common traits and ecological significance of intraspecific communication. We also discuss the evolutionary implications of intraspecific chemical communication and the mechanisms by which it can be inherited. I paid special attention to the genetic diversity among conspecific algae, including the possibility that genetic diversity is an absolute requirement for intraspecific chemical communication.

Keywords: infochemicals, intra-specific communication, signalling, evolution, species concept

6.2 INTRODUCTION

Any communication system is essentially made of three components: an "emitter", a "receiver" and a "field" in which the exchange of information occurs. Chemical communication among organisms requires biological machinery, including genes, RNA, proteins and other chemical messengers; the interaction among these components allows the production, the release and/or the detection of chemical cues. Equally important for a successful communication are the constraints imposed by the environment in which the infochemical (**Tab. 6.1**) is released (Legrand *et al,* 2003). For instance, medium viscosity can limit the diffusion of substances; electromagnetic radiation can affect the activity of the infochemicals (Sterr and Sommaruga, 2008); chemical agents, such as compounds identical or similar to the cue or that interact with it modifying its signalling function, can act as infodisruptors and interfere with the transmission of information (Lürling and Scheffer, 2007).

Within the present contribution, we recognize three main subsets of modes of chemical communication, depending on identies of the emitter and the receiver:

a) Chemical communication between different microalgal species. The most obvious example is allelopathy, which occurs when a species produces chemicals that have a detrimental effect on a competitor for space, nutrients or light. The outcome of this type of communication can be death, paralysis (for motile cells) or inhibition of growth, of the receiver (Legrand *et al,* 2003).

b) Signalling between microalgae and other organisms. Microalgae can release compounds harmful to their grazers (Ianora and Miralto, 2010; Ratti *et al,* 2013) or to non-algal competitors for resources such as bacteria and fungi (Burkholder *et al,* 1960; Kellam *et al,* 1988; Hagmann and Jüttner, 1996; Issa, 1999). We cannot exclude the possibility that microalgae are involved in chemical interactions with multiple actors (i.e. "multitrophic" interactions), for instance by producing compounds beneficial for individuals whose presence is disadvantageous for grazers, as observed for other organisms (Vet and Dicke, 1992; De Vos *et al,* 2006; Stout *et al,* 2006; Nevitt 2008). Certainly, microalgae can be part of multitrophic interactions in which they act as neither the donors nor the receivers: this was for instance observed in the case of waterborne cues released by seastars of the genus *Leptasterias* that deter the snail *Tegula funebralis* from feeding on tide pool microalgae (Gravem *et al,* 2016).

c) Intra-specific chemical communication. Despite the paucity of investigation in this field, several processes are likely to implicate transfer of infochemicals among conspecifics. A population of algae of a given species can be genetically homogeneous or heterogeneous; the occurrence of chemical communication in these two cases has never been explicitly addressed.

In this review, we collected and discussed such information (**Tab. 6.2**) in order to assess how common

and important chemical communication is among conspecific algae. The description of the instances available in the literature is directed at the identification of common traits and differences between intraspecific infochemicals and serves as a context for discussing the ecological and evolutionary implication of intraspecific chemical communication.

6.3 WHAT DOES “INTRASPECIFIC” MEAN IN THE CASE OF MICROALGAE?

No definition of “species”, and thus of “intraspecific”, exists that can be generically applied to microalgae (Hartl and Clark, 1989; Templeton, 1989; Hey, 2001). For example, the morphological species concept (i.e. conspecific individuals look the same and differ morphologically from individuals of other species) is not easily applicable to organisms with different life stages (as it is common among algae) that do not share the same appearance while being conspecifics. Other species definitions have proved to be more inclusive. The concept of “genetic species” (or “biological species”, *sensu* Mayr, 1963) refers to groups of natural interfertile populations that are reproductively isolated from other populations. This definition cannot be applied to asexual organisms. The “evolutionary species” concept identifies lineages (i.e. ancestral-descendant sequences of populations) that evolved separately from other evolutionary units and “have their own unitary evolutionary roles and tendencies” (Simpson, 1961). According to Simpson, the “evolutionary species” concept does not exclude asexual organisms; other authors have further explained the theoretical context in which it can be used (see Wiley, 1978 for a thorough reflection on the applicability of the evolutionary species concept to asexual organisms). Furthermore, the recognition of the “evolutionary tendency”, as Simpson himself admitted, is totally arbitrary in some cases (e.g. in a fossil sample) (Mayr, 2000).

The difficulty to identify species, especially for asexual organisms, translated into the use of different approaches for the delimitation of microalgal species. Morphology has been the historically prevalent criterion for the identification of microalgal species (Hey, 2001); after the application of molecular techniques in taxonomic studies, some of these species attributions became questionable (e.g. Medlin *et al,* 1995; Montresor *et al,* 2003, Ellegaard *et al,* 2008). The difficulty in the definition of species for microalgae occurs particularly in cyanobacteria, for which the application of the bacterial nomenclature has been proposed and strain codes increasingly replace linnean binomial nomenclature (Castenholz, 1992; Pinevich, 2015).

In the ensuing text, we shall identify species in accordance with the current literature on each organism used as example. We acknowledge that this brings in some degree of subjectivity in the definition of “intraspecific”, but the unresolved debate on this matter leaves us with no alternative. In some cases (paragraphs 6.5 and 6.7.4), the discussion required a further discrimination between conspecific algae that have complete genetic identity and conspecific algae that share lower degrees of genetic relatedness.

6.4 MODULATION OF INTRASPECIFIC CHEMICAL COMMUNICATION

The synthesis or the reception of chemicals conveying information can be either constitutive or modulated through inducible mechanisms (Agrawal and Karban, 1999; Wolfe, 2000). Induction may be controlled by either environmental factors or endogenous factors (i.e. the passing of set points, such as a certain cell size). An instance of constitutive production of a chemical that transmits information to conspecific algae is given by the inhibition of heterocyst formation by existing heterocysts (see paragraph 6.7.1 for details). Notice that the constitutive nature of infochemical production does not necessarily correspond to a constant presence of the signal in the environment. Pheromones, for instance, are often ephemeral (Watson, 2003) so that their persistence in water depends on the persistance of the cell type that constitutively produces them. Various examples of inducible communication mechanisms in microalgae exist in the literature, although they are not always explicitly labeled as such (Vardi *et al,* 2007; see paragraph 6.7.3). In the diatom *Pseudostaurosira trainorii*, the production of a pheromone (**Tab. 6.1**) by female cells that have achieved a certain cell size was also inferred to be "constitutive"; this signal induces the production of male gametes, initiating a further cascade of chemical signals (Sato *et al,* 2011; see paragraph 6.7.2 for further details). However, we believe that the "constitutive" nature of such signalling is highly questionable, since the passing of a minimum cell size represents, in our opinion, an example of (endogenous) induction of communication.

Consistent with the topic of this review, the examples above concern intraspecific communication; however, we can also recognize instances of both constitutive and inducible communication mechanisms between organisms of different species. For instance, *Alexandrium minutum*, a bloom-forming dinoflagellate, activates a defense mechanism against copepod grazers through the production of a toxin induced by waterborne cues from the grazers themselves (Selander *et al,* 2006). Other dinoflagellates constitutively produce chemical compounds that are toxic for potential grazers (e.g. Huntley *et al,* 1986; Hansen *et al,* 1992).

Inducibility is obviously linked to the management of resources; in the case of infochemicals, this means confining the production of the signalling molecules and its energetic and other metabolic expenditure, to the time of requirement (Haukioja and Hakala, 1975). The production of infochemicals may bear consequences for basal metabolism: an example of this is provided by Pohnert (2000, 2005), who suggested that diatoms' polyunsaturated aldehydes have their precursors in fatty acids such as the C20 eicosapentenoic acid. The impact of infochemical production on metabolism in terms of diversion of resources (energy, chemicals) may also be of relevance, although no study, to the best of our knowledge, has yet directly addressed this aspect.

In some cases (e.g. in genetically identical organisms), the differential expression of the systems devoted to production and reception of infochemicals may be necessary to prevent self-communication (i.e. the same cell both produces and receives the signal) that may occur after induction. In the bacterial pheromone-induced conjugation (**Tab. 6.1**), cells potentially capable of both emitting and receiving the signal activate only one of the two abilities, preventing self-communication (Dunny *et al,* 1995). The inducibility of the production and the reception mechanisms are not necessarily mutually exclusive and self-signalling may occur. For instance, it has been proposed that the wound-induced production of polyunsaturated aldehydes (PUAs) in diatoms not only does affect other algae, bacteria and microinvertebrate (see Ianora and Miralto, 2010 for a review on the effect of the PUAs on various organisms and Dutz *et al,* 2008 for contrasting results), but also the emitters themselves, although, allegedly, the intoxication of neighbouring diatoms is minimized by the fact that grazers ingest most of the PUAs (Casotti et al 2005; Wichard et al*,* 2007). If PUAs are passively released from dead cells rather than being actively produced by wounded cells, this questions the existence of chemical communication (as discussed in paragraph 6.7.3).

Tab. 6.1. Glossary of terms

Allelochemical	Compound involved in interspecific interactions, which is produced by a donor organism and elicits a response in a receiver organisms belonging to a different species (Whittaker, 1970a,b).
Allomone	Allelochemical that triggers a response in the receiver that is favorable to the donor organism only (Nordlund and Lewis, 1976).
Apoptosis	Process that leads to specific morphological and biochemical changes during the genetically controlled death of cells. These modifications include reduction of cell volume, condensation of chromatin, fragmentation of nucleus, little or no ultrastructural changes of cytoplasmic organelles and blebbing of plasma membrane in the final stage (Kerr et al 1972; Kroemer et al 2009).
Colonial (organism)	Aggregation of individuals with identical genotype and phenotype (Kirk, 2000). The genera *Microcystis*, *Skeletonema* and *Gonium* include examples of colonial microalgae.
Conjugation (bacterial)	Transmission of genetic material from one bacterial cell to another through direct contact between cells (Clark and Adelberg, 1962).
Diazotrophy	Metabolic ability to reduce atmospheric N_2 to forms that can be assimilated into the organic matter (e.g. Postgate, 1998).
Diplontic Life Cycle	Life cycle in which mitotic cell divisions and development occur uniquely in the diploid phase. Gametes, originating from meiosis, fuse and restore the diploid phase (Mable and Otto, 1998).
Founder Effect	Mechanism by which a few individuals from a parent population give rise to a new population with reduced genetic variability, outside of the spatial range of the parent population (Mayr, 1942, Provine, 2004).
Gene Flow	Movement of genes from one population to another (Slatkin, 1985).
Hormone	The original definition comes from the medical field and describes hormones as chemical messengers produced by an organ in response to the "physiological needs of the organism" and intended to reach and affect another organ (Starling, 1905). Hormones of multicellular organisms have also been found in unicellular organisms, in which they exhibit signalling function; nevertheless, there is no consensus on the use of the term "hormone" for single-celled organisms (Lenard, 1992).

Haplodiplontic Life Cycle	Life cycle in which development occurs in both a haploid vegetative phase, interposed between meiosis and sexual fusion, and a diploid vegetative phase (Mable and Otto, 1998).
Haplontic Life Cycle	Life cycle in which mitotic cell divisions and development occur solely in the haploid phase. Gametes fuse and form a zygote, which undergo meiosis (Mable and Otto, 1998).
Infochemical	A chemical that conveys information from an emitter to a receiver and elicits a behavioral or physiological response in the receiver (Dicke and Sabelis, 1988). Infochemicals include pheromones and allelochemicals. Infochemical nomenclature is context-specific and not compound-specific, e.g. a substance acting as a pheromone in one species may be used as a kairomone in the interaction with another species (Dicke and Sabelis, 1988; Nordlund and Lewis, 1976).
Kairomone	Allelochemical that triggers a response in the receiver that is favorable to the receiver organism only (Nordlung and Lewis, 1976).
Multicellular (organism)	Aggregation of individuals with identical genotype but different phenotype (i.e. functionally and/or morphologically different) (Kirk, 2000). Examples of multicellular microalgae are *Anabaena* spp. and *Pleodorina* spp.
Pheromone	Substance secreted to the outside by an individual and received by a second individual of the same species, in which it triggers a specific response (Karlson and Lüscher, 1959). This response may be adaptively favorable to the donor and/or the receiver organism (Vet and Dicke, 1992).
Random Genetic Drift	Process that causes a change in a population allele frequency due to the random sampling of genes from generation to generation (Slatkin, 1985; Masel, 2011).
Semiochemical	Chemical involved in the interaction between organisms (Nordlund and Lewis, 1976). Semiochemicals include toxins and nutrients (which are beneficial or detrimental to the interacting organisms *per se*) and infochemicals (which are beneficial or detrimental to the organisms through the response they evoke in the reveiver) (Dicke and Sabelis, 1988).

Synomone	Allelochemical that triggers a response in the receiver that is favorable to both the donor and the receiver organism (Nordlund and Lewis, 1976).

6.5 GENETICAL AND PHENOTYPICAL BASES FOR INTRASPECIFIC COMMUNICATION

We see no theoretical impediment to the possibility that intraspecific communication occurs both between genetically identical and between genetically distinct individuals. The information in the literature suggests that within-species variability is fertile ground for the development of communication, because it allows intraspecific diversification in the mechanism and extent of signal emission and reception (Wood and Leatham, 1992; Eigemann *et al,* 2013). However, signals can also be exchanged within a genetically uniform population, providing a feedforward mechanism that allows the response to external stimuli to propagate across the population. Clearly, interacting clonal cells are capable of acting as both emitter and receiver; the same is not necessarely true when the communicating cells are not genetically identical.

6.5.1 Origin of genetic diversity in microalgal species

The generation of intra-populational genetic variability is mainly due to sexual reproduction. The probability of generation of variability among different populations, in the case of microalgae, is often considered to be low, because these organisms often reproduce asexually and have a high dispersal capacity (de Meester *et al,* 2002). However, large genetic differences have been detected within algal populations. This is not only true for algal populations that are spatially distant, but also occurs within the same population; substantial genetic variability was even observed in phytoplankton blooms (Evans *et al,* 2005; Rynearson and Ambrust, 2005; Rynearson *et al,* 2006; Shilova et al 2016).
Over time, microalgae have adapted to changes in environmental conditions, so that the gene complement of an extant algal species is likely to be different from that of the same species in the past. This may have led to speciation, or genetic heterogeneity within the same species. Obviously, organisms that do not coexist in time, whether conspecific or not, are unable to exchange chemical information. This may not be true for conspecific populations that differentiate *across space* due to adaptive responses to local selective forces and/or stochastic events such as founder effects and genetic drift (**Tab. 6.1**; Hartl and Clark, 1989; for a recent review on phytoplankton biogeography, see Padisák *et al,* 2016). In this review, we shall therefore focus on the case of spatial differentiation. The occurrence of genetically distinct populations of microalgae may result from the interruption of gene flow (**Tab. 6.1**) due to the development of geographical barriers (Kim *et al,* 2004; but also see Koester *et al,* 2012 and Scholin *et al,* 1994 for cases in which genetic distance and geographic

separation seem not to be correlated). On the other hand, the absence of geographical barriers does not prevent the development of distinct populations of the same species (metapopulations *sensu* Levins, 1969). The dispersal of individuals over a large region may be followed by recombination, genetic drift, and/or genotype selection based on local conditions that lead to a differentiation from the original population(s) (Vanormelingen *et al,* 2009). This may be similar to what was observed by Koester and co-workers (2012), who reported positive selection of several genes in different strains of *T. pseudonana*, living in connected habitats in which different selective forces prevailed (e.g. temperature and UV radiations). Furthermore, biological interactions, such as predation, competition and allelopathy, can operate as "environmental filters" that may impede or favor the successful establishment of a population in a new environment, and selectively act upon the genotypes it contains (Naselli-Flores and Padisák, 2016).

6.5.2 Communication between genetically distinct conspecific individuals: premises and paradigms

The generation of genetic diversity becomes relevant with respect to intraspecific communication when individuals coexist. Coexistence is a condition that can either preceed the generation of genetic diversity (e.g. via sexual reproduction within a single algal population) or follow it. The latter case can occur by the breakdown of a geographic barrier that allows the populations from the two sides to converge into a single population or in the case of chemical or physical barriers that constitute a boundary with intermediate properties. The actual occurrence of the breakdown of geographical barriers is questioned by those who deny the existence of such barriers for phytoplankton (see Hedlund and Staley, 2004 for a review on this debate). According to these authors, the geographic isolation of algal populations is hampered by the fact that these organisms are numerous, small-sized, have a great dispersal ability and most of them can produce resting stages (Finlay, 2002; Cermeño and Falkowski, 2009). This calls to mind the famous assertion of the Dutch microbiologist Martinus Wilhelm Beijerinck (1851-1931): "everything is everywhere, the environment selects" (reported by Baas-Becking, 1934). This paradigm, however, has been challenged, for instance, by Kim and coauthors (2004), who reported evidences for geographically isolated populations of the dinoflagellate *Peridinium limbatum* that diverged genetically because of a limited gene flow. A restriction of the gene flow between distant populations of the diatom *Pseudo-nitzschia pungens* was also reported (Casteleyn *et al,* 2010).

The existence of boundary zones between different marine habitats (e.g. benthic vs planktonic, deep chlorophyll maximum vs surface mixed zone; areas separated by well defined thermoclines or chemoclines, etc.) that are not separated by geographical barriers is more widely accepted (see for instance Ribalet *et al,* 2010).

6.5.3 Communication between genetically identical conspecific individuals

Environmental variations may trigger differential gene expression in genetically identical individuals (Choi and Kim, 2007; Roberfroid et al 2015). Such differential expression may result in the activation of intercellular communication, i.e. inducible communication between identical cells can be based on the generation of a phenotypic difference. If the environmental variation affects only a portion of the area occupied by the population, the activation of communication may concern a fraction of the population, which may then propagate the signal to the cells that have not yet encountered the environmental perturbation (**Fig. 6.1**). In nature this is only possible in specific conditions, implying short diffusional distances among cells that share information. This network of communication can be beneficial for the receiver or not (e.g. leading to population control processes). From an evolutionary point of view, the case of an altruistic network is more easily comprehended, for a clonal population (kin selection; see 6.7.4). The case we just described is based on a cause-effect process determined by environmental inducers (nutrients, toxic agents, presence of grazers etc.). Other scenarios may be represented by the activation of communication pathways in randomly selected individuals, because of stochastic gene expression (Roberfroid *et al,* 2015). Another possibility is that interacting conspecific cells represent different stages in the life cycle of a non-synchronous population, which have proved to exhibit, in some cases, very different phenotypes originated by drastic differentiation in gene expression (e.g. diploid and haploid phase in *Emiliana huxleyi*; Frada *et al,* 2008; Von Dassow *et al,* 2009).

Other examples of communication between genetically identical cells are provided by the exchange of information between cells in filaments resulting from mitotic division from a single progenitor. These cases will be discussed in detail in 6.7.1.

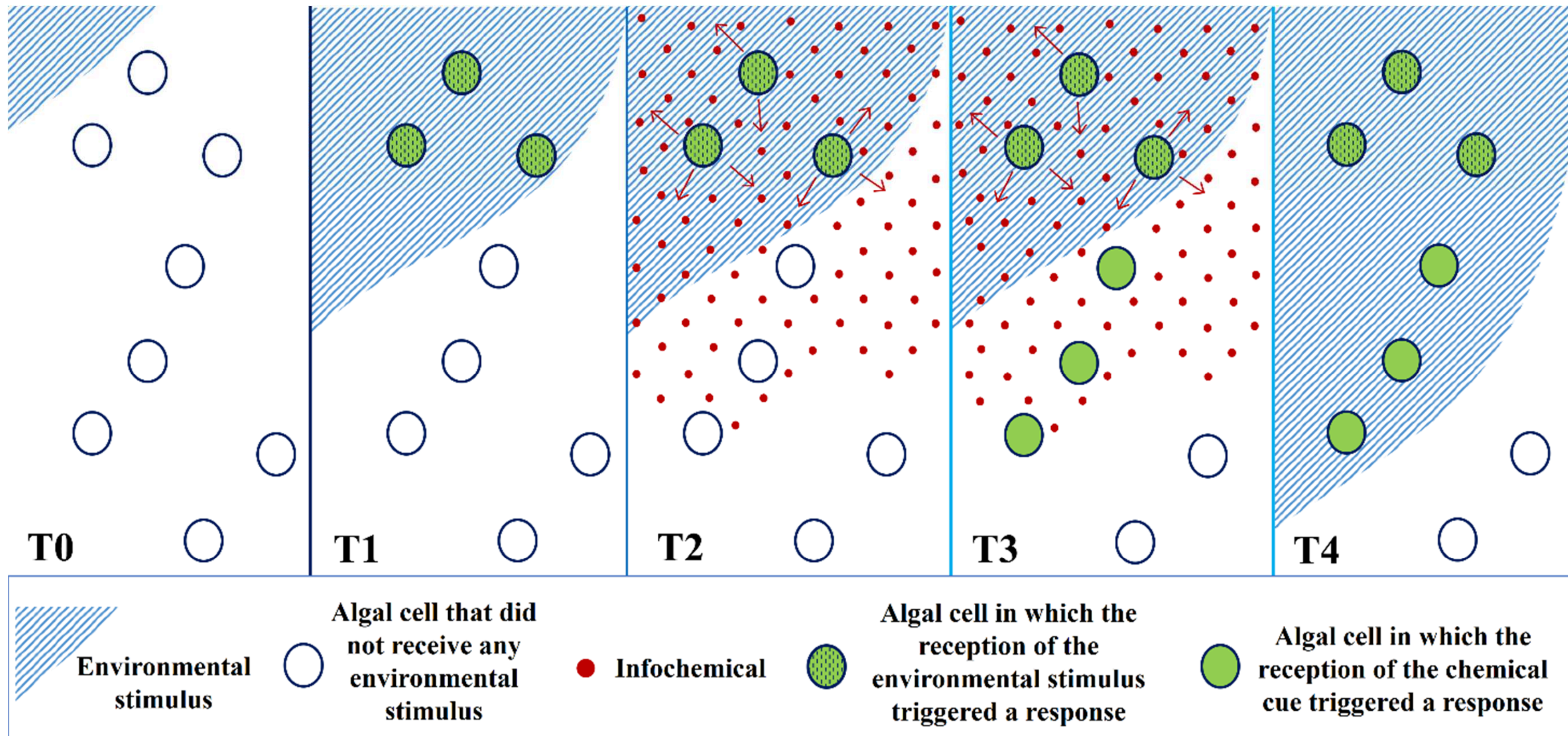

Fig. 6.1. Gradual interaction of a clonal algal population with an environmental perturbation. T0: The perturbation has not reached the populations. T1: the front of the perturbation has reached the cells at the borders of the population, triggering a phenotypic response. T2: The cells that responded to the perturbation in T1 produced and released a chemical cue, which conveys information on the environmental change. T3: Cells that were not directly affected by the perturbation received the cue and responded to it. T4: The perturbation front moved forward and reached other cells in the population; these cells were already «prepared» to the environmental change. Cells represented in this model are close enough to ensure that the diffusion of the chemical cue toward "not-affected" cells is faster than the propagation of the environmental perturbation.

6.5.4 Phenotypic differences that are relevant for communication between conspecific organisms

Among the phenotypic differences that have been observed between conspecific individuals, the ability to produce toxins (Carrillo, 2003), the vulnerability to toxic compounds (e.g. Behra et al 1999; Lopez-Rodas et al 2011) and the sensitivity to allelochemicals (**Tab. 6.1**; Eigemann et al 2013) are of special relevance to this review.

When the individuals of a population are distributed along a physical and/or chemical gradient, they can perceive the same stimulus with different sensitivity (Maan et al 2006; Yewers et al 2015). This different sensitivity often results from the presence of distinct types or number of receptors (Schoonhoven, 1982) or from differences in other cell components involved in signal processing (e.g. enzymes involved in the modification of the chemical that is sensed; Van Straalen, 1994). Differences in the sensitivity to chemicals between conspecific algae have been reported (Fisher, 1977; Murphy and Belastock, 1980). For instance, estuarine clones of *Thalassiosira pseudonana* and *Skeletonema costatum* are less susceptible to chemicals from industrial waste than conspecific strains that originated in other environments (Murphy and Belastock, 1980). Different strains of *Scenedesmus obliquus* exhibited different responses to chemical substances released by *Daphnia*: cells of three strains responded by forming 4 and 8-celled coenobia; cells of another strain responded by irregularly aggregating in colonies having more than 8 cells; cells of a fifth strain did not show obvious responses (Lürling, 1999).

In animals, sensory abilities are an important component of the reproductive behavior and can result in reproductive isolation and speciation, through natural selection based on sensory performance ("sensory drive hypothesis"; Schluter and Price, 1993; Seehausen et al 2008). In the light of the obvious role of signal sensing in mating, it is likely that also in microalgae sensory capabilities of individuals drive behavioural and genetic differentiation (i.e. changes to the nucleotide sequence in the genome) through selection of distinct signaling modalities.

6.6 POSSIBLE EVOLUTIONARY TRAJECTORIES OF INTRASPECIFIC CHEMICAL COMMUNICATION

Infochemicals probably derive from chemicals that had different original functions or from products released by the cells as waste (Wyatt, 2003). More specifically, it can be hypothesized that intraspecific chemical communication derives from the conversion of a molecule capable of conveying signals within a cell into an infochemical that operates on different cells of the same species (**Fig. 6.2**). In other words, a hormone-like (**Tab. 6.1**) molecule capable of modulating metabolic processes within a cell may be able to interact with cells that share similar metabolic pathways and intracellular signalling cascades. The more the metabolic pathways and intracellular signalling cascades between the cell of origin and the target cells are similar, the more likely is the acquisition of an infochemical function for a hormone-like molecule.

A similar evolutionary process has been proposed for metazoans by Sorensen and Stacey (1999). These authors proposed that fish pheromones evolved in three steps: in the first phase, some individuals of a species produce a hormone that (by definition; Starling, 1905) functions as internal chemical messenger (in the context of unicellular organisms, this would be equivalent to intracrine or autocrine signalling; Csaba, 1996). In the second phase, some other individuals evolve the ability to detect, and respond to, the hormone accidentally released by conspecifics, for instance after algal cell breakage; these receiver organisms obtain a benefit from this acquired ability (notice that at this stage, only the receiver develop a new ability). Finally, in the third phase, which does not necessarily occur, the receivers responds to the signal. If this response, which does not inevitably involve the generation of chemical cues and may consist, for instance, in the sexualization of the receivers, elicits a positive response in the producers, the establishment of a two-way communication can occur (Sorensen and Stacey, 1999). Although similar models have been mostly proposed for Metazoa, the underlying mechanisms do not seem to belong to any group in particular, and are likely to have occurred in all organisms with intraspecific chemical communication.

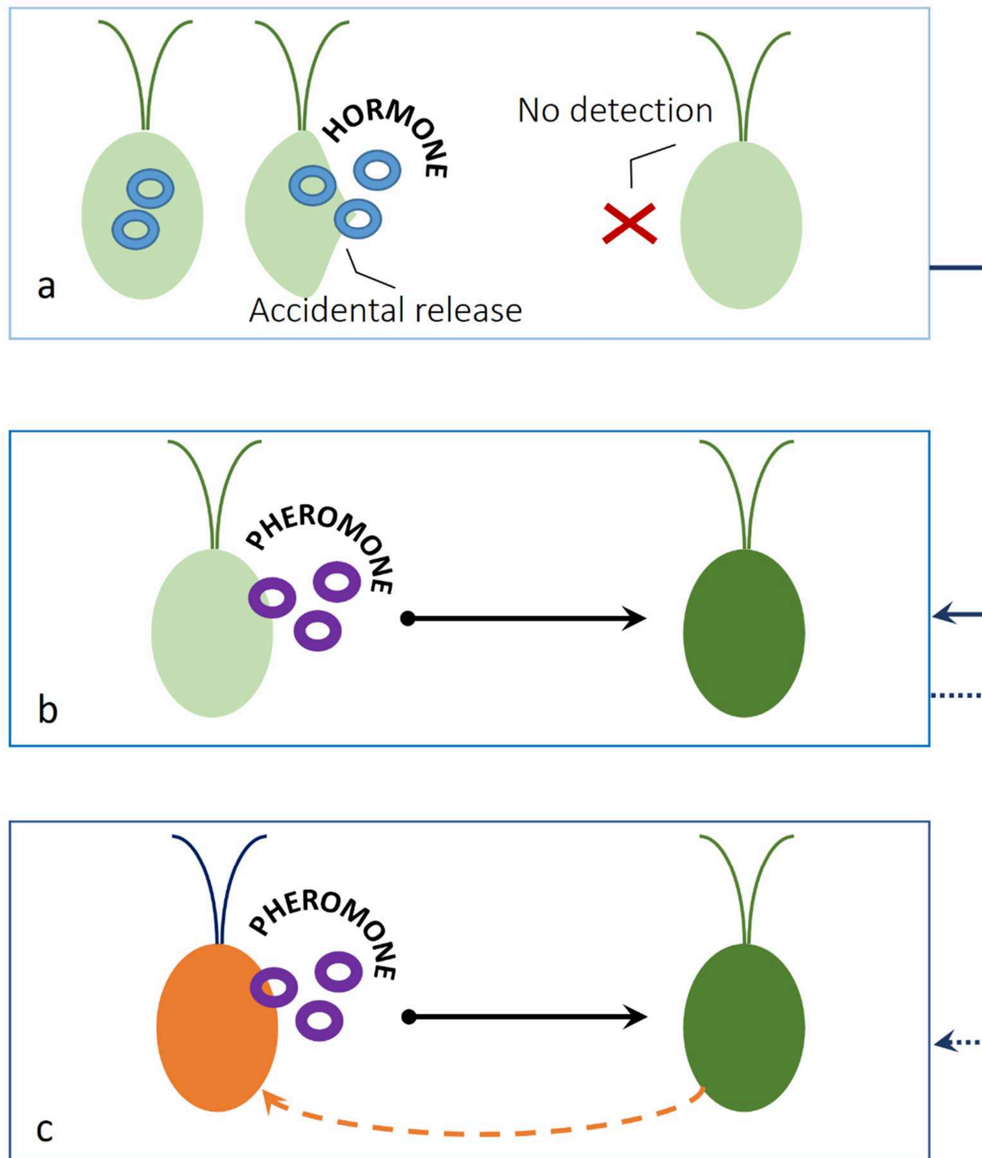

Fig. 6.2. Evolution of pheromones from pre-existing hormones. A) A hormone is accidentally released into the medium and conspecifics are unable to detect it. B) The receiver specializes to detect the hormone and respond to it. C) The response of the receiver exerts a selective pressure for the specialization of the producers. Changes in the color of the cells (receiver in b; receiver and donor in c) indicate specialization. The dashed line indicates that the last step may occur or not. Redrawn from Sorensen and Stacey (1999).

6.7 PROCESSES ENTAILING INTRASPECIFIC COMMUNICATION

6.7.1 Cell-cell communication in colonial and multicellular microalgae

Intraspecific communication, in most cases, serves the scope of coordinating functions among individual unicellular microalgae in a population. It is not known if this coordination is an evolutionarily precursor to the tighter interaction among individuals in colonial (no inter-cellular differentitation) and multicellular (with intercellular differentiation) organisms (Beardall et al 2008; **Tab. 6.1**). Nevertheless, it has been pointed out that several features that were considered a hallmark of multicellular organization (among which: programmed cell death; cell-cell adhesion and communication) probably evolved in ancestral unicellular organisms (Ameisen, 2002; Grosberg and Strathmann, 2007).

The signalling machinery of multicellular organisms was inherited, in several cases, from their unicellular ancestors (Kawabe et al 2015 for amoebae; also see König and Nedelcu, 2016 for a discussion on the evolution of multicellularity in volvocalean algae). It is also worthwhile noticing that cell differentiation in multicellular organisms is commonly recognized as one of the main selective factors in favor of the evolution of this habit (Stanley, 1973). Multicellularity is attained from sequential divisions of a single cell without separation of the progeny (Bell and Mooers, 1997). Since cells share the same genetic profile and no major differential effect can be exerted on them by the environment, so the production of distinct cell types presumably results from communication between the descendents of the first cell. Another possible scenario is the production of different cell types (somatic vs reproductive) in *Volvox*, which is determined through a series of asymmetric divisions (Kirk 2003), in a size-related process (Kirk et al 1993; 1995): the smaller cells produce a transcriptional repressor of reproduction-related genes (thus they become the somatic cells).

It is interesting to notice that the transition from single cells to colonies has been shown to respond to chemical cues from herbivores or epiphytes (Lürling, 2003; Leflaive et al 2008; Verschoor et al 2009). The link between the formation of colonies and communication with other species certainly endorses the idea that coordination among cells can result from exchange of infochemicals.

In filamentous cyanobacteria, cell-cell communication allows genetically identical cells within the same filament, originated by mitotic division, to carry out distinct tasks and thus become multicellular (Beardall et al 2008). Diazotrophy (**Tab. 6.1**) in *Anabaena*, for instance, requires the differentiation of specialized cells, the heterocysts, in which nitrogen fixation occurs and oxygen diffusion is

hampered (Wolk et al 1994). Such differentiation takes place in approximately one in every ten cells (Wolk et al 1994) and is controlled by a set of genes that are expressed in a precise sequence (Golden and Yoon, 2003). According to the "activator-inhibitor model" (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 1974), the development of heterocysts and the maintenance of their spacing depend on the presence of a protein activator (HetR) acting over a short spatial range and of an inhibitor (the peptide PatS) acting over a longer spatial distance. The transcription of the *het*R gene is triggered by low external combined nitrogen; when nitrogen is abundant, the gene hetR is transcribed at basal levels in all cells. The process is self-promoting because HetR stimulates its own synthesis. Increased levels of HetR also stimulate the transcription of *pat*S, whose product (PatS) is responsible for the inhibition of *het*R transcription and thus heterocyst formation (Black and Wolk, 1994; Gerdtzen et al 2009). The inhibition only operates in the cells surrounding the heterocyst, which itself is insensitive to PatS (Haselkorn, 1998; Yoon and Golden, 1998). This mechanism bears similarities to the plasmid-mediated system that prevents bacterial emitter cells from self-signalling during pheromone-induced conjugation (Dunny et al 1995).

6.7.2 Role of infochemicals in mating and sexual differentiation

Sexual reproduction requires that two sexually competent cells meet and recognize reciprocally. These cells can originate from meiosis of diploid cells or by activation of haploid cells, respectively in algae with diplontic, or diplohaplontic and haplontic life cycle (**Tab. 6.1**; Frenkel et al 2014). Sex reshuffles the deck of genes in the population, but can also be seen as a strategy to overcome hostile environmental conditions, for instance through the production of resistant zygotes. Environmental stressors such as high temperature or limited nutrient supply have been shown to trigger sexuality (Starr et al 1995; Chepurnov et al 2004). Nedelcu and coauthors (2004) proposed that sex and death are the outcomes of different extents of environmental stress. Pheromones, which can be involved in different phases of sexual reproduction and whose synthesis and activity are often subject to environmental influence, may play a pivotal role in the signalling pathway linking sex to the environment (Fukumoto et al 1998; Gillard et al 2013).

In many diatoms, mostly centric, asexual cell division causes a gradual decrease in cell size; the original cell size is restored by vegetative enlargement or, more commonly, by sexual reproduction (Round et al 1990). The latter does not occur in cells whose size is above the "sexual size threshold" (SST; Drebes, 1977). Below this limit, external factors such as light, photoperiod and temperature can trigger sexualization in some of of the cells in a population (Werner, 1971; Furnas, 1985;

Armbrust et al 1990); sexualized cells can then initiate the sexualization of other cells mediated by chemical signals.

The multicellular alga *Volvox carteri*, for instance, which lives in temporary ponds that dry out in the summer, reproduce asexually most of the time (Hallmann *et al,* 1998); when the temperature is critical, this alga switches to sexual reproduction and forms dormant zygotes that survive the drought (Sumper and Hallmann, 1998). Heat shock, specifically, induces somatic cells to produce a glycoprotein infochemical that triggers gametogenesis in the gonidia (Starr and Jaenicke, 1974; Kirk and Kirk, 1986; Sumper *et al,* 1993). The same signal, later on, is released at lower concentrations by the male gametes to induce differentiation and agglutination in the surrounding cells (Gilles *et al,* 1984; Hallmann *et al,* 1998; Sumper and Hallmann, 1998).

In the pennate diatom *Pseudostaurosira trainorii*, some vegetative cells in the “sexual size range” (hereinafter named “A”) produce a signal of not specified chemical nature (ph-1); this signal induces meiosis in other vegetative cells (“B”) that subsequently release two motile (amoeboid) male gametes (it is worth mentioning that amoeboid motion can only occur on a solid surface, so the “A” and “B” cells must be close to each other). The “B” cells and/or the “male” gametes themselves release another chemical message (ph-2; also of unknown identity); ph-2 triggers sexualization of the “A” cells that undergo meiosis and produce female gametes. The “A” cells, then, are thought to produce a third pheromone (ph-3), which stimulates the amoeboid movement of the “male” gametes toward the “female” gametes (Sato et al, 2011). In *Seminavis robusta*, a heterothallic pennate diatom, meiosis occurs only after the pheromone-mediated interaction between two types of compatible cells (mating types) has taken place (Gillard et al, 2013).

The contact between sexually competent motile cells can happen by chance, as observed in *Chlamydomonas reinhardtii* and *Volvox* (Starr *et al,* 1995; Coleman, 2012), or can be guided by pheromone-induced chemotaxis (Tsubo, 1961; Ichimura, 1971; Hill *et al,* 1989; Al-Hasani and Jaenicke, 1992; Maier, 1993; Starr *et al,* 1995; Mori and Takanashi, 1996). The greater the resource investment for the synthesis of attracting signals, the greater the benefits obtained by the alga in terms of increased encounter rates of gametes (Frenkel et al 2014). The trade-offs associated with the cost of producing the attracting signals, however, are not known. This is not the only aspect of chemically regulated sexuality in algae that still needs to be clarified. Whether or not sexual determination in heterothallic species is only genetically pre-determined, as recently observed for the diatom *Seminavis robusta* (Vanstechelman et al 2013), or environmental factors can concur to the modulation of such phenomena , as happens in macroalgae such as *Laminaria religiosa*, *Lessonia nigrescens* and *Lessonia variegata* (Funano, 1983; Nelson, 2005; Oppliger et al 2011), is also not clear. It is interesting that some of the compounds operating in brown algae, were also found in diatoms,

although their infochemical role in microalgae has not been demonstrated (Pohnert and Boland 1996; Hombeck and Boland 1998). Sex determination may also be determined epigenetically by the interaction with conspecifics, possibly through the mediation of chemical cues (Godwin et al 2003).

Tab. 6.2. Compounds involved in intra-specific chemical communication in microalgae. In the column "Donor species, strain or cell type", different colors indicate organisms belonging to different algal groups (grey: cyanobacteria; green: green algae; orange: diatoms; blue: dinoflagellates; yellow: haptophytes). The numbers in parenthesis refer to the references (see footnote).

Infochemical name and chemical nature	**Biosynthetic precursor**	**Donor species, strain or cell type**	**Molecular mass (kDa)**	**Role as infochemical**	**Active concentration**	**Conditions for activity**	**Effect on other algae**
HetR (protein)	-	*Anabaena* sp. (1)	-	Control of heterocysts development (1)	HetR in heterocysts is 20-fold more than in other cells of the filament (2)	Transcription of hetR gene is stimulated by low external combined nitrogen (2)	-
PatS (peptide)	-	*Anabaena* sp. (3)	-	Inhibition of heterocysts development (3)	-	-	-
(Unsaturated fatty acids)	-	Lysed cells of *Phormidium tenue* (4)	-	Autotoxin (4)	-	-	-
L-Glutamic acid (amino acid)	-	*Volvox capensis* K27 strain (5)	0.15	Induction of development of sexual spheroids (5)	$< 7 \cdot 10^{-8}$ M (5)	Developing spheroids must be exposed not later than at 64-cell stage (5)	No sexual induction in *V. carteri* (5)

(Glycoprotein)	-	Male spheroids of *Volvox carteri* f. *nagariensis* (6)	30 (7)	Induction of development of sexual spheroids (8; 9)	$6\cdot10^{-17}$ M (7) probable signal amplification (9)	6 h of exposure to trigger the activity (7)	-
(Glycoprotein)	-	*Volvox carteri* f. *weismannia* strain 65-30 (10)	27 (10)	Induction of development of sexual spheroids (8)	10^{-12} M (10)	-	Sexual induction in *V. carteri* f. *nagariensis* at 10^{5}-fold higher concentration (10)
(Glycoprotein)	-	*Volvox carteri* f. weismannia strain 1b (10)	28.5 (10)	Induction of development of sexual spheroids (10)	10^{-13} M (10)	-	Sexual induction in *V. carteri* f. *nagariensis* at 10^{5}-fold higher concentration (10)
Sexual cell division inducing pheromone SCD-IP (glycoprotein)	-	Mt- cells of *Closterium ehrenbergii* (11)	18-20 (11)	Induction of sexual cell division of mt+ vegetative cells, (11)	-	Light-dependent release	-

Lurlene (pentosylated hydroquinone)	Plastoquinone-4 (12) Plastoquinone-6 (13)	Female gametes of *Chlamydomonas allensworthii* (13; 14)	532[a] (13)	Attraction of the motile male gamete (13)	< 10^{-12} M (14) Synthetic analogue: 10^{-13} M (12)	-	-
di-L-prolyl diketopiperazine (“diproline”) (2,5-diketopiperazine)	-	Mt- cells of *Seminavis robusta* (15)	0.11	Attraction of mt+ cells (15)	-	Production is light-dependent (15) and induced by SIP+ (16)	-
2-*trans*, 4-*trans*-decadienal (polyunsaturated aldehyde)	-	*Thalassiosira weissflogii* (17)	0.15	Triggering of degenerative processes that culminate in the death of cells (17)	-	-	-
Ph-1	-	Female vegetative cells of *Pseudostaurosira trainorii* in the sexual size range (18)	-	Sexualization of male cells (18)	-	-	-

Ph-2	-	Male vegetative cells of *Pseudostaurosira trainorii* in the sexual size range (18)	-	Sexualization of female cells (18)	-	-	-
Sex Inducing Pheromone SIP+	-	Mt+ cells of *Seminavis robusta* (16)	-	Induction of cell cycle arrest in G1 and synthesis and secretion of diproline in mt- cells (16)	Sip+ is secreted at much lower concentration than diproline (16)	-	-
15-hydroxyeicosa-pentaenoic acid	-	Dying and dead cells of *Skeletonema constatum* (19)	0.32	Autotoxin (19; 20)	-	-	Also toxic for other diatoms but not 2 tested dinoflagellates (19)
(Protease)	-	Senescing cells of *Peridinium gatunense* (21)	-	Sensitizes younger cells to oxidative stress (21)	-	-	-

Unknown	-	Cells of *Alexandrium ostenfeldii* that were infected by the flagellate *Parvilucifera infectans* (22)	-	Stimulates motile cells of *A. ostenfeldii* to form temporary cysts (22)	-	-	-
1-[hydroxyl-diethyl malonate]-isopropyl dodecenoic acid	-	*Isochrysis galbana* (23)	414[a] (23)	Autotoxin (23)	-	-	Also toxic for 8 other species of microalgae (23)
Unknown	-	*Diacronema lutheri* (24)	-	Autotoxin (24)	-	-	-

[a] m/z

References: (1) Buikema and Haselkorn, 1991. (2) Zhou et al 1998. (3) Yoon and Golden, 1998. (4) Yamada et al 1994. (5) Starr et al 1980. (6) Starr and Jaenicke, 1974. (7) Gilles et al 1984. (8) Starr, 1970. (9) Sumper et al 1993. (10) Al-Hasani and Jaenicke, 1992. (11) Fukumoto et al 2002. (12) Mori and Takanashi, 1996. (13) Jaenicke and Marner, 1995. (14) Starr et al 1995. (15) Gillard et al 2013. (16) Moeys et al 2016. (17) Casotti et al 2005. (18) Sato et al 2011. (19) Imada et al 1991. (20) Imada et al 1992. (21) Vardi et al 2007. (22) Toth et al 2004. (23) Yingying et al 2008. (24) Yamasaki et al 2015.

6.7.3 Role of infochemicals as inducer of death of conspecific individuals

Senescent or dead cells in microalgal populations have been shown to release chemical compounds that determine the death of cells of the same species (Imada *et al,* 1991, 1992; Yingying *et al,* 2008; Yamasaki *et al,* 2015). The signalling nature of these so-called autotoxins or autoinhibitors is, however, not always clear. If they are passively released and interact with the receiver cell only after the death of the producer cell, this eliminates one of the elements characterizing cell-cell communication: the existence of an emitter (Dicke and Sabelis, 1988). The existence of cells with a reduced viability, in which photosynthesis still occurs but membranes are compromised and thus allow the efflux of metabolites (Veldhuis et al 2001) makes the recognition of an actual donor even more complicated.

It should also be noted that most of the work on autotoxins has been conducted on laboratory cultures. Yamada and co-workers showed that a mixture of fatty acids from lysed cells of *Phormidium tenue* exerted an autotoxic activity on axenic cultures of the same species (Yamada et al 1994). The algal population was completely annihilated in the absence of non photosynthetic bacteria, while it continued to grow when bacteria of the genera *Flavobacterium* and *Micrococcus* were present in the culture: according to these authors, bacteria, which are typically present in algal blooms in large numbers (Mayali and Azzam, 2004), were capable of degrading the toxins (Yamada et al 1994).

Programmed Cell Death (PCD) is defined as the genetically regulated self-destruction of a cell. It requires a highly coordinated interaction among receptors, nuclear factors, signal kinases and other cellular components (Leist and Nicotera, 1997; Aravind et al 1999). PCD is essential for development and tissue turnover in multicellular organisms (Leist and Nicotera, 1997; Pennell and Lamb, 1997; Fukuda, 1997). In several filamentous cyanobacteria, the PCD of “sacrificial” cells known as necridia is involved in the separation of hormogonia (i.e. short motile filaments that act as dispersal units) from a trichome (Lamont, 1969; Reichenbach and Golecki, 1975). PCD has also been described in non-photosynthetic bacteria (Yarmolinsky, 1995; Lewis, 2000), yeasts (Fröhlich and Madeo, 2000), protozoa (Lee et al 2002) and algae (Berges and Falkowski, 1998; Vardi et al 1999; Veldhuis et al 2001; Ning et al 2002; Segovia et al 2003; Berman-Frank et al 2004; Casotti et al 2005), although for these organisms the molecular hallmarks of the cell death machinery are not unequivocal and some are still controversial (Franklin et al 2006; Deponte, 2008; Jiménez et al 2009). Several rationalizations have been proposed for PCD in unicellular microalgae (e.g. removal of mutated/damaged cells, limitation of viral infection), which are critically reviewed by Nedelcu *et al* (2010), together with the possibility that programmed cell death is the side-effect of the mechanistic

basis of an adaptive trait (Nedelcu et al 2010). Environmental changes such as nutrient and light limitation (Berges and Falkowski, 1998), high doses of UV (Moharikar et al 2006), increase of salinity (Ning et al 2002) or oxidative stress (Vardi et al 1999) are alleged determinants of PCD in microalgae. In the case of oxidative stress, Nedelcu and co-workers (2004) propose that the effect is dose-dependent, with low levels of oxidative stress inducing sexuality and high levels triggering PCD. Some chemical compounds have been reported to induce PCD in microalgae. Casotti and co-authors (2005) showed that the application of synthetic polyunsaturated aldehydes (PUAs) to diatom cultures leads to degenerative processes that culminate in apoptosis-like death of the cells (**Tab. 6.1**; Casotti et al 2005). Similarly, Vardi and co-workers (2006) demonstrated that the application of the PUA (2*E*,4*E/Z*)-decadienal to *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* cultures triggers a cascade of events which culminates in the death of diatom cells (Vardi *et al,* 2006). These PUAs or other chemical compounds produced by microalgae may play a role as PCD-inducers in conspecifics (Casotti *et al,* 2005; Vardi et al, 2006;Vidoudez and Pohnert, 2008), although a conclusive proof of this is still to be produced.

A proteolytic activity present in the spent medium from ageing cultures of the dinoflagellate *Peridinium gatunense* increases the sensitivity of younger conspecific cultures to oxidative stress, wich in turn triggers PCD (Vardi *et al,* 2007). In this case, the viability of donor cells has been confirmed and the observations are suggestive of an active secretion of the active compound (Vardi *et al,* 2007).

In bacteria, the compounds that cause intra-specific induction of death have been proposed to derive from substances that kill other species, in bacteria (Ameisen, 2002). This is a plausible scenario for microalgae as well. The final phase of an algal bloom is often characterized by increasing levels of cell death due to viral (Bratbak et al 1993; Jacquet et al 2002; Frada et al 2008) and bacterial (Mayali and Azzam, 2004) attack, nutrient limitation (Van Boekel et al 1992) or the interaction among these factors (Pal et al 2006). Franklin and co-authors (2006) suggested that PCD may explain why dinoflagellates blooms can vanish in times that are too short to be justified by sedimentation or grazing (Franklin et al 2006; also see Raven and Waite 2004 for non-PCD means of removing infected cells from the population). The triggering of PCD by intraspecific communication appears especially suited for the control of populations density in an unfavorable environment (Vardi et al 2007), similarly to what happens in bacterial biofilms in which PCD is propagated via quorum sensing (Oleskin et al 2000).

6.7.4 Chemical communication in processes that benefit conspecifics

Chemical signalling may be beneficial to conspecifics that receive the signal in many ways; for instance, the infochemicals can warn of the presence of predators and/or induce defensive behavioral responses. Toth and co-authors (2004) showed that temporary cysts of the dinoflagellate *Alexandrium ostenfeldii* are more resistant to the parasitic flagellate *Parvilucifera infectans* than motile cells; when *A. ostenfeldii* motile cells were exposed to cell-free spent media from a culture of conspecifics that were infected by the parasite, they formed temporary cysts (Toth et al 2004). These authors proposed that this is a response to either a chemical that leaks from infected cells or an unknown infochemical actively released by infected cells, forewarning other members of the population of the presence of the parasite (Toth et al 2004). In organisms with a biphasic life cycle, changes in ploidy have also been related to different degrees of sensitivity to infections (e.g. shift from diploid to haploid phase, the latter being more resistant to viral infection, in *Emiliana huxleyi*, Frada et al 2008). Whether these changes in ploidy are mediated by communication among conspecifics, however, is not known.

Some authors showed that the death of unicellular algae can generate an immediate benefit to the neighboring cells through the mediation of chemicals (but see above for the actual infochemical role of substances released by dead cells). Durand and co-authors (2011) reported that *Chlamydomonas reinhardtii* cells that undergo PCD release unidentified compounds that stimulate growth of conspecific cells, while chemicals coming from cells that died by causes different from PCD inhibit growth in cells of the same strain (Durand et al 2011). The effect determined by compounds deriving from cells that died by PCD stimulates growth only in *C. reinhardtii*, but inhibits growth of *C. moewusii* and *C. debaryana* (Durand et al 2014).

Vardi et al (1999), in the above mentioned work on PCD in *P. gatunense*, propose that, under unfavorable environmental conditions, part of the population sacrifices itself via PCD so that other cells can encyst and re-establish the population when favorable environmental conditions resume. Whether a cell is destined to die or to encyst is determined through processes that involve cysteine protease(s), since the application of a commercial cysteine protease inhibitor (E-64) prevents PCD and also stimulates cyst formation; the link between these two pathways has been proposed as the result of high selective pressure for the formation of cysts from non-stressed cells only, while the other cells are purged out from the population (Vardi et al 1999). It is not clear whether the "other cells" are genotypically distinct from those that encyst and, in this case, how their genotype is preserved so that the same mechanism can occur again.

The existence of an "altruistic" chemical signalling that favors conspecific individuals raises many doubts. Group selection, i.e. the selectability of the group (Wynne-Edwards 1986; Wilson 1997) as a

counterpart for the generally accepted selection of genes (Dawkins 1989) and individuals, is much more difficult to maintain through natural selection. Furthermore, a "cheating" strategy (i.e. the ability of cells to take advantage of shared resources without giving any contribution in return) could rapidly spread through a non clonal population by virtue of the higher fitness it confers (Thornton, 2002). Kin selection, involving the sacrifice of individuals in permitting the survival of the same genotype in other members of a clonal population (Hamilton 1963; 1964) has much more support (Nedelcu et al 2010; Durand et al 2011; Pepper et al 2013; Durand et al 2014).

As pointed out by Franklin and co-authors (2006), if "altruism" is restricted to clonal populations, blooms may not provide suitable conditions for kin selection to occur (Thornton, 2002), since they seem to be more genetically heterogeneous than it was previously believed (Medlin et al 2000; Rynearson and Armbrust, 2005). However, this would depend on the degree of relatedness of the cells in the bloom.

6.8 CONCLUSIONS

1. The latest findings on chemical communication in algae suggest that the exchange of signals between conspecifics is more common than generally believed. Intraspecific chemical communication appears to be present in all major algal groups (**Tab. 6.2**) and is involved in several processes, such as sexual reproduction, cell differentiation in algal colonies, induction of death and protection of conspecifics from grazers, viruses and parasites.

2. Intraspecific communication operates on both clonal and non-clonal populations. In the former case, the potential of each cell to act as both a producer and a receiver of the signal can be modulated, resulting in either a producer- or a receiver-phenotype (e.g. through differential gene expression); this appears particularly useful for the rapid propagation of a response to an environmental stimulus through the whole population, because it allows the cells that firstly come in contact with the stimulus to 'inform' other cells of the population of the external change. In the case of interacting, genetically different cells, the diversification of communication mechanisms can also have genetic bases. Such diversification allows communication whenever the cells come in contact; this does not exclude cells that owe their genetic distinctness to a temporary geographic segregation.

3. The evolution of intraspecific communication was presumably based on the recruitment of pre-existing mechanisms and compounds that operated within the cell with other functions. It can be envisaged that communication between similar cells originated via the perception of hormone-like molecules occasionally released by related neighbors.

7. GENERAL CONCLUSIONS

My research has given a contribution to the comprehension of the complex interactions between algae and environment. Furthermore, the possible modes through which such interactions modify the relationships between algae and other organisms have been investigated.

In the first part of my study, I have elucidated the mechanisms through which obligate photoautotrophs may try to deal with a prolonged exposure to darkness and discovered that different algae, in this situation, consume their internal organic pools in different ways. I have also found that compositional homeostasis in algae is a much more common response to environmental stimuli than previously believed. The costly process of acclimation is chosen less frequently, at least in response to changes in the environmental CO_2 concentration and in the N source.

In the second part of my work, I have analyzed the responses of *Chromera velia*, a photosynthetic relative to the parasite Apicomplexa, to elevated CO_2. The outcome of this investigation revealed that this organism is perfectly able to withstand very high CO_2, which even stimulates the overall C production and increases the nutrient use efficiency by *C. velia*.

In the third part of my study, I dealt with the relationships between algae and grazers, which I found to be strongly affected by the nutritional background (and thus by the cell composition) of algae. Differences among grazers have been confirmed: copepods were able to feed selectively on diatom cells with various cell composition, while rotifers were not.

At last, my study has also given a technical contribution, through the development of new protocols and methods that will hopefully be employed in the future for further investigations.

In conclusion, my research has given a contribution towards the understanding of the modes through which microalgae cope with a changing environment and of the possible ecological implications of such strategies.

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