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Chemistry and Ecology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713455114>

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To cite this Article Collos, Y. , Descolas-Gros, C. and Mornet, F.(2005) 'Grazing affects carbon fixation pathways by phytoplankton in coastal marine ecosystems', *Chemistry and Ecology*, 21: 6, 503 – 509

To link to this Article: DOI: 10.1080/02757540500439217

URL: <http://dx.doi.org/10.1080/02757540500439217>

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Grazing affects carbon fixation pathways by phytoplankton in coastal marine ecosystems

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(Received 15 April 2005, accepted 15 September 2005)

During parallel sampling of seawater samples in bottles and in free water (1000–2000 m³ clay ponds), we have measured phytoplankton biomass, Ribulose biphosphate carboxylase (Rubisco), phosphoenol pyruvate carboxylase, and phosphoenol pyruvate carboxykinase activities and major nutrients (ammonium, nitrate, phosphate, silicate). This was done in two ecosystems: one with high grazing pressure due to the presence of oysters and another with low grazing pressure (no oysters). In the ecosystem subjected to high grazing pressure, anaplerotic carbon fixation by phytoplankton in free water was higher in the light period and could represent 25% of total carbon fixation. Incubating samples in bottles led to a major increase in Rubisco activity (80% in 3 h) relative to values measured in free water, a decrease in β -carboxylases activity (70% in 24 h) due to ammonium exhaustion, as well as disappearance of its diel periodicity. This indicates *a contrario* that grazers, which are excluded from incubation bottles, drive ecosystems toward heterotrophy *in situ* by favoring the β -carboxylation pathway through excretion products such as ammonium. Therefore, incubations in high grazing environments (characterized by a grazing rate near 2 day⁻¹) change the way carbon is fixed by unicellular algae within 3 h through a change in the form of nitrogen taken up.

Keywords: Ammonium; Anaplerotic; Carbon assimilation; Carboxylases; Grazing

1. Introduction

Two major pathways of carbon assimilation are responsible for phytoplankton growth in aquatic systems. One is the ribulose biphosphate carboxylase (Rubisco) pathway (Calvin–Benson cycle) and the other one is the β -carboxylase (anaplerotic) pathway. The latter one is generally considered to be quantitatively of minor importance [1], excepted in transient situations of nitrogen supply [2] which are often considered to dominate in aquatic ecosystems [3–5] and in heterotrophic nutrition [6–8]. Rubisco uses light as an energy source, whereas

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the β -carboxylases use internal or external metabolites [8]. β -carboxylases activities are also known to depend on ammonium in the medium of cultures of unicellular algae [9, 10]. This anaplerotic carbon fixation allows the synthesis of molecules that are essential for growth and cannot be provided by the Calvin–Benson cycle [11]. Here we examine the possibility that enclosing water samples has an effect on the activities of those enzymes and the re-routing of carbon relative to the situation *in situ*, where macrograzers are present [12, 13]. Traditional measurements of primary production in aquatic ecosystems are generally carried out by adding isotopic carbon tracers to water samples and incubating those in containers of various sizes and materials [14, 15]. While this approach has been criticized in many respects, it still remains the main way by which global fluxes of carbon are estimated in the oceans [16, 17]. One difficulty of validating the widely used incubation method comes from the movement of water masses *in situ* which makes comparisons between free water and incubated water impossible to carry out. Here we report such comparisons by using enzymatic measurements which do not require incubations and mesocosms which are large enough to support several trophic levels [18] but are still manageable in terms of homogeneity and size to allow large scale extrapolations from point sampling.

2. Materials and methods

To test the effect of incubation on carboxylating enzymes, we compared changes in bulk nutrients, phytoplankton biomass and carboxylase activities with changes in the same variables measured on samples enclosed in bottles and incubated in the same water body. Sampling took place from two mesocosms which had been filled with fresh seawater during the previous high tide [19]. These were 1000–2000 m³ clay ponds on the Atlantic coast of France (CREMA experimental site). The main difference between the two mesocosms was the presence of oysters and sediment filter feeders in one (high grazing system), which made it similar to a continuous culture, with relatively low steady-state chlorophyll *a* levels (4–10 $\mu\text{g chl } a/l$) but nevertheless high phytoplankton growth rates [20]. The other one (low grazing system) acted more like a batch culture, with high phytoplankton biomass levels (20–150 $\mu\text{g chl } a/l$) reached within a few days after filling and isolation from the feed channel. Preliminary tests showed the water column of the mesocosms to be homogenous in chemical and biological properties [19]. Measurements took place in the summer (July) and lasted about 40 h, with a sampling interval ranging from 2 to 12 h.

Samples were taken directly from the mesocosms (referred to as *in situ* in the text) and from mesocosm water enclosed in bottles. Large volume (4–8 l) transparent polycarbonate bottles were filled at time zero with surface seawater, sealed and incubated at the surface of the mesocosms. Aliquots were taken for nutrients (ammonium, nitrate, soluble reactive phosphorus (SRP), silicate), particulate matter and enzymatic analysis. Chlorophyll *a* (collected on Whatman GF/F filters) was extracted in 90% acetone [21] within 30 min of collection. Bacteria were estimated by epifluorescence direct counting [22]. Nutrients (ammonium, nitrate, phosphate, silicate) were measured on a continuous flow analyzer [23].

For carboxylating enzyme assays, samples were collected by filtering 250–750 ml of sea water through GF/F filters which were placed immediately in cryotubes and stored in liquid nitrogen. This storage did not affect the enzyme activities [24]. Activities were determined by measuring the incorporation of radioactive bicarbonate at 25°C into stable products. Ribulose biphosphate carboxylase (Rubisco), phosphoenol pyruvate carboxylase, and phosphoenol pyruvate carboxylase activities were measured in the same extract [8, 24].

We did not have replicate bottles, but we used several bottles in series because of the large volumes required for all analyses. Those bottles were all monitored by *in vivo* fluorescence to have an idea of the reproducibility, at least at the level of chlorophyll *a* fluorescence. From this variability, we could compute a coefficient of variation of about 8% at the beginning of incubation to about 3% at the highest values observed.

3. Results

Table 1 shows the range of variables during the experiment. In the low grazing system, the large phytoplankton was dominated by *Prorocentrum minimum*, *Scrippsiella trochoidea* and *Gymnodinium* spp. In the high grazing system, the diatom *Surirella* and *Diploneis* genera dominated. Water temperature ranged between 21.8 and 24.5 °C. SRP and silicate concentrations remained high and were not limiting phytoplankton growth.

In the low grazing mesocosm, the incubation did not change the phytoplankton biomass or the activities of either Rubisco or β -carboxylases relative to the free water situation, at least on time scales of 1–2 days used here (figure 1). In contrast, in the mesocosm under high grazing pressure, the first obvious effect of incubation was a large (fivefold) increase in phytoplankton biomass (figure 1) which was due to release of macrograzing pressure. In the free water, the biomass remained low (4–8 $\mu\text{g/l}$) and exhibited a diel periodicity with lower values at night than during the day.

At the level of carbon fixation enzymes, Rubisco/chl *a* activity in the bottles increased by about 80% relative to free water activity within 3 h (figure 2), overriding the strong diel periodicity observed in the free water and related to the day–night cycle. Over the longer term (days), this activity decreased because of nutrient depletion (dissolved inorganic nitrogen decreased from about 8 to 1 $\mu\text{mol N l}^{-1}$). In parallel, β -carboxylase activities decreased by about 70% in incubation bottles relative to their activities in the free water on the second day (figure 3). We have expressed β -carboxylase activity on the basis of chl *a* because bacterial biomass decreased over the experiment [25], so that the trends shown are mostly due to phytoplankton. The higher β -carboxylase activity during the day also confirms that anaplerotic carbon fixation is higher in the light than in the dark [26]. In the low grazing system, β -carboxylase activity remained low and similar in bottles and in free water over the duration of the experiment (figure 3).

Table 1. Ranges of chemical and biological variables during the experiment.

	Low grazing	High grazing
Ammonium ($\mu\text{mol N l}^{-1}$)	1.1–7.1	0.6–5.7
Nitrate	0.1–4.0	0.5–2.2
Phosphate (SRP)	4.1–9.0	1.3–2.6
Silicate	14.9–36.0	13–25.4
Chl <i>a</i> ($\mu\text{g l}^{-1}$)	16–27	4–22
μ_{max} (div day $^{-1}$)	3.8	2.6
Phytoplankton		
Large	Dinoflagellates	Diatoms
Small 3–5 μm (10^6 cells l^{-1})	150	1.5
Bacteria (10^7 cells ml^{-1})	2.5–3.3	8.3–9.7

Notes: SRP, soluble reactive phosphorus; μ_{max} , maximum net growth rate from increases in chlorophyll *a*.

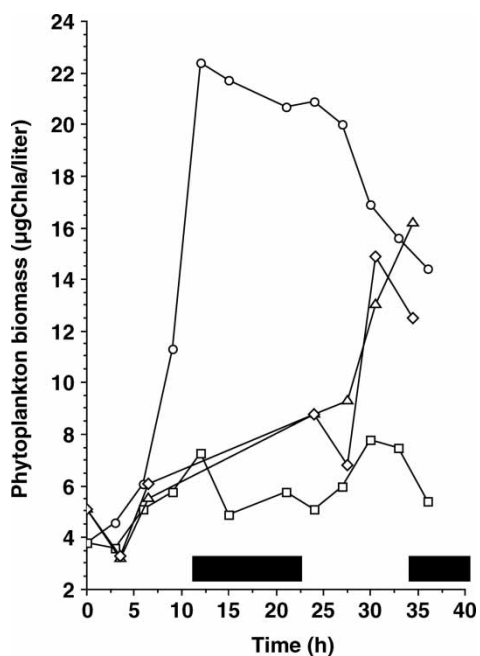


Figure 1. Changes in phytoplankton biomass with time in the high grazing mesocosm *in situ* (squares) and incubated (circles) samples and in the low grazing mesocosm *in situ* (diamonds) and incubated (triangles) samples. Dark bars represent night time.

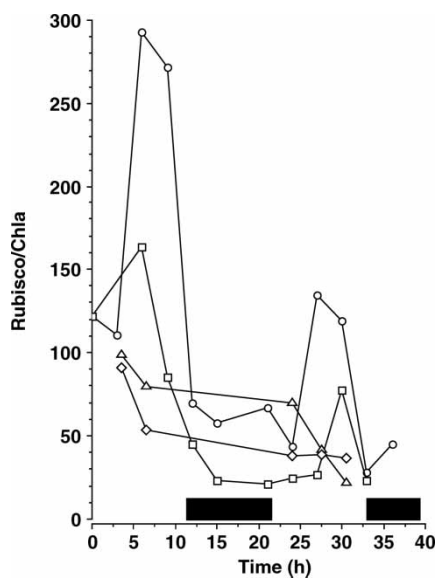


Figure 2. Changes in ribulose biphosphate carboxylase (Rubisco) activity per unit chlorophyll *a* with time in the high grazing mesocosm *in situ* (squares) and incubated (circles) samples and in the low grazing mesocosm *in situ* (diamonds) and incubated (triangles) samples. Units in $\text{nmol CO}_2 \mu\text{g Chl } a^{-1} \text{ h}^{-1}$. Dark bars represent night time.

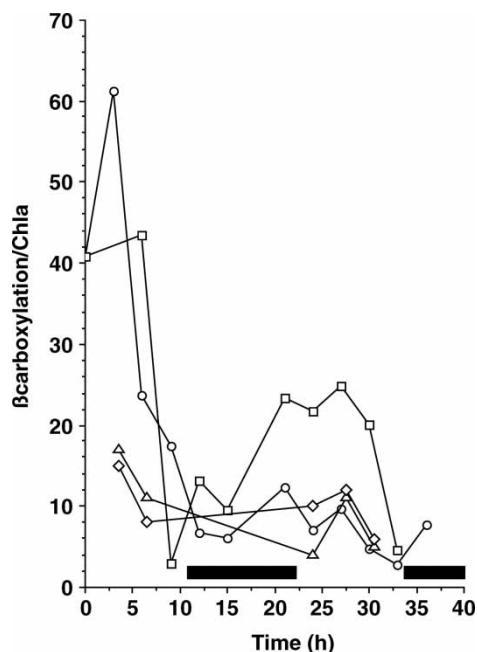


Figure 3. Changes in β -carboxylase activity per unit chlorophyll *a* with time in the high grazing mesocosm *in situ* (squares) and incubated (circles) samples and in the low grazing mesocosm *in situ* (diamonds) and incubated (triangles) samples. Units in $\text{nmol CO}_2 \mu\text{g Chl } a^{-1} \text{ h}^{-1}$. Dark bars represent night time.

The activity of β -carboxylases was closely related to ammonium concentrations in the high grazing system ($r = 0.887$, $p = 0.0001$), whereas much more loosely in the low grazing system ($r = 0.617$, $p = 0.0326$). As ammonium decreased with time both in incubation bottles and in free water, β -carboxylase activity also decreased with time, but in a faster way in the incubation bottles because the ammonium supply from macrograzers was missing and ammonium concentration decreased in a faster way than in free water.

In fact, the relationship between ammonium in free water and in bottles was much more variable in the high grazing system ($r = 0.704$, $p = 0.0131$) than in the low grazing system ($r = 0.975$, $p = 0.0001$), which is expected because the ammonium excreted by the grazers in free water is not present in incubation bottles where the major grazers (oysters and sediment filter feeders) are excluded with 100% efficiency. In addition, the slope of the relationship was not different from unity in the low grazing system, while ammonium decreased more rapidly (by about 30%) in bottles than in free water in the high grazing system.

4. Discussion

The high phytoplankton growth rate (2.6 divisions per day or 1.8 day^{-1}) estimated for the high grazing environment was obtained from net increases in chl *a* concentrations in bottles. The lack of a similar increase in phytoplankton biomass *in situ* (figure 1) implies that grazing is in equilibrium with growth and is also equal to 1.8 day^{-1} . This value is in the high range of grazing rates recorded in aquatic environments [27] and is close to the value of 2.1 day^{-1} observed by others at an oyster farming site [28]. The discrepancy in phytoplankton growth estimates between incubation and in free water methods is likely to be a function of the grazing intensity of grazers that are excluded from bottles. Our high grazing mesocosm is therefore

probably a worst case scenario exemplified by the wide divergence among the evolutions of biomass (figure 1), Rubisco (figure 2) and β -carboxylases (figure 3). Concerning rates of carbon fixation, figures 2 and 3 show that such rates begin to diverge widely beyond 3 h of incubation. This duration would then be the upper limit for obtaining representative estimates of rates when using incubation bottles in such environments. The effect of grazing of microzooplankton in incubation bottles can be accounted for by using the so called dilution method [29]. However, this does not account for grazers outside incubation bottles. In a recent review of zooplankton grazing [30], it was estimated that the latter consumed about 40% of phytoplankton daily growth in coastal environments.

Concerning the ecological implications of the use of bottle incubations in relation with the ammonium/heterotrophy link, the diverging trends in Rubisco activity between incubated and free water in the high grazing system (figure 2) indicate that the enclosed phytoplankton is less dependent on ammonium or dissolved organic matter excreted by the excluded grazers (less heterotrophy) due to the confinement in bottles and relies more on inorganic carbon (and nitrate) for its nutrition. In addition, the rapid decrease in ammonium concentrations in bottles in the high grazing system leads to a decrease in β -carboxylase activity. This results in a greater reliance on other nitrogen forms such as nitrate and a switch from regenerated production (on ammonium) to new production (on nitrate). Therefore, incubations in high grazing environments change the way carbon is fixed by unicellular algae through a change in the form of nitrogen taken up.

A strong feedback thus exists between macrograzers and carbon assimilation pathways in phytoplankton, through excreted nitrogenous nutrients, here represented by ammonium. As β -carboxylase activity is generally associated with heterotrophy [6–8], the above relationship can also be considered as representing heterotrophy on the nitrogen level. The traditional ‘incubation cum tracer’ method thus severs the regeneration link and the ammonium supply from macrograzers. This further leads to underestimate heterotrophy whose importance has been shown by other approaches in both marine [31] and freshwater [32] ecosystems.

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