

# Chemical, isotopic and enzymatic monitoring of free and enclosed seawater: implications for primary production estimates in incubation bottles

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**ABSTRACT:** Enclosing seawater samples in incubation bottles had 3 main effects on the phytoplankton: first, a large (5-fold in 12 h) increase of phytoplankton biomass, due to the release of grazing pressure by herbivores. Second, an increase (2-fold in 6 h) in ribulose biphosphate carboxylase specific activity (per unit chlorophyll *a*), which could be due to a smaller dependence on regenerated production (less heterotrophy on excreted compounds). This was also supported by a tight relationship between phosphoenol pyruvate carboxykinase activity and dissolved ammonium. Finally, the large changes in the carbon isotopic composition of the incubated particulate matter, which were highly dependent on the dissolved inorganic nitrogen concentration, were due mainly to physiological processes such as biochemical fractionation rather than physical processes such as CO<sub>2</sub> limitation. For environments with high phytoplankton growth rate and high grazing pressure, a duration of incubation greater than 3 h may significantly change the physiology of the organisms and lead to different estimates of carbon assimilation by autotrophs than bulk water estimates.

## INTRODUCTION

Present estimates of primary production in aquatic environments rely mainly on data obtained by enclosing water samples in incubation bottles for various amounts of time. However, recent comparisons with changes in bulk water properties have revealed wide discrepancies in estimates of primary production (Jenkins 1982, Reid & Shulenberger 1986, Chavez & Barber 1987, Pace et al. 1987), calling into question the traditional incubation method. We have tried to assess the validity of incubating seawater samples by comparing chemical, isotopic and enzymatic estimates of

primary production in bottles to direct *in situ* sampling during a 40 h time series in an homogenous, isolated water mass. Large differences in phytoplankton biomass changes between bulk water and incubated samples had already been documented under those conditions (Sornin et al. 1990). As the use of <sup>14</sup>C was precluded in the open study site, we used stable isotope and enzymatic estimates of carbon assimilation.

## MATERIALS AND METHODS

Sampling took place from an oyster pond (No. 4 of the CREMA experimental site, L'Houmeau, Charente Maritime, France) which had been filled with fresh seawater. The pond surface was about 1500 m<sup>2</sup>, with an average depth of 0.5 m, i.e. a total volume of 750 m<sup>3</sup>. For a detailed description of the study site, see Collos

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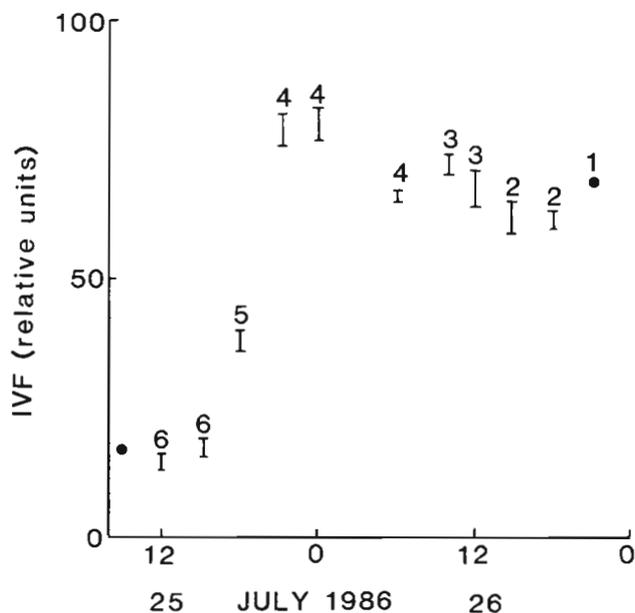


Fig. 1 Range of values of chlorophyll *in vivo* fluorescence (IVF, in relative units) between samples taken from different incubation bottles as a function of time. Numbers above vertical bars: no. of bottles remaining; (●) at left: initial sample

et al. (1988). Incubation bottles (4 l volume, polycarbonate) were cleaned by soaking in 10 % HCl and rinsed with deionized water (0.06  $\mu\text{S}$  conductance). Several of those bottles were directly filled with surface seawater and incubated at the pond's surface during about 40 h. Aliquots were taken for chemical, enzymatic and isotopic analyses. Several bottles were used in succession. Fig. 1 shows the spread in values of *in vivo* chlorophyll fluorescence (IVF) between the different bottles, indicating that the trends were essentially the same in all bottles. In addition, simultaneous samples were taken directly from the pond's surface. Previous results showed the pond to be homogenous in chemical and biological properties (Collos et al. 1988). The sampling interval varied from 2 to 6 h.

Surface irradiance was measured and recorded every 20 min with an Aandera pyranometer (Model 2770). The total energy values (in  $\text{W m}^{-2}$ ) were converted to the visible part of the spectrum (400 to 700 nm) by multiplying by 0.42 (Jitts et al. 1976) and converted to total quanta by multiplying by  $2.5 \times 10^{18}$  (Morel & Smith 1974). The final values were expressed in  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Within 30 min of collection, chlorophyll *a* (chl *a*) was extracted in 90 % acetone (Holm-Hansen et al. 1965) following the recommendations of Holm-Hansen & Riemann (1978) concerning the acidification step, and measured on a fluorometer fitted with an F4T5B lamp and a R136 photomultiplier. Nutrients were analyzed immediately during daytime on a continuous flow analyzer (Skalar Analytical,

Breda, Netherlands) for nitrate, nitrite, ammonium, phosphate and silicate (Grasshoff et al. 1983). Night samples were frozen and analyzed the next day.

Samples for cell counts were preserved in 4 % (final concentration) formalin and enumeration was carried out in 5 ml chambers according to the technique of Utermöhl (1958), using a Diaphot inverted microscope (Nikon) with phase contrast equipment. The whole bottom chamber was examined at  $100\times$  magnification for larger cells (mainly diatoms). At  $400\times$  magnification, one or more diameter transects were counted, for smaller cells and flagellates, according to their abundance. Identification to species level was attempted whenever possible. Bacterial numbers were estimated using the epifluorescence direct counting technique (AODC) of Hobbie et al. (1977), from 18 ml samples of seawater fixed with phosphate buffered formaldehyde (2 % v/v).

Carboxylating enzyme assays were performed according to Descolas-Gros & Fontugne (1985, 1988) and Descolas-Gros & Oriol (1992). Phytoplankton cells were collected by filtering 250 to 750 ml seawater through GF/C glass fiber filters which were placed immediately in cryotubes (Nunc R) and stored frozen at  $-196^\circ\text{C}$  in liquid nitrogen. Activities were determined by measuring the incorporation of radioactive bicarbonate into stable products and expressed in  $\text{nmol CO}_2$  fixed per liter seawater and per hour ( $\text{nmol CO}_2 \text{ l}^{-1} \text{ h}^{-1}$ ). Ribulose biphosphate carboxylase (Rubisco), phosphoenol pyruvate carboxylase (PEPC), and phosphoenol pyruvate carboxykinase (PEPCK) activities were measured in the same extract.

For stable carbon isotope ratio measurements, phytoplankton was collected by filtering 350 ml seawater through pre-cleaned GF/C glass fiber filters (Chesselet et al. 1981). The filters were decarbonated with 0.1 N HCl, dried at  $60^\circ\text{C}$  and stored in the dark at  $4^\circ\text{C}$  before combustion in oxygen (Fontugne & Duplessy 1978, 1981). The  $\text{CO}_2$  gas obtained was analyzed using a VG Micromass 602D mass spectrometer. The reproducibility on the carbon isotope ratio is 0.1 ‰, and results are expressed relative to the PDB standard in the usual notation:

$$\delta^{13}\text{C} (\text{‰}) = \left[ \frac{^{13}\text{C}/^{12}\text{C} \text{ sample}}{^{13}\text{C}/^{12}\text{C} \text{ standard}} - 1 \right] \times 1000$$

## RESULTS

The pond contained a population of phytoplankton (Table 1), consisting mostly of diatoms. Bacterial numbers decreased from  $9.65 \times 10^6$  cells  $\text{ml}^{-1}$  on the first day to  $8.32 \times 10^6$  on the second day, an overall decrease of about 14 % during the experiment.

Table 1. Phytoplankton populations during the study period. Values are cell counts on 26 July 1986 at 6:00 h

Genus or size class	Cells l <sup>-1</sup>
<i>Surirella</i>	$4.5 \times 10^3$
<i>Diploneis</i>	$11.5 \times 10^3$
8 to 10 $\mu\text{m}$ cells	$400 \times 10^3$
3 to 5 $\mu\text{m}$ cells	$1.5 \times 10^6$

Fig. 2 shows the changes in dissolved inorganic nitrogen (DIN) and chl *a* in particulate matter as a function of time, for samples taken directly from the pond and for those incubated in the bottles. In both cases, all 3 nitrogen (N) forms (nitrate, nitrite, ammonium) were taken up simultaneously. The trends were similar *in situ* and in bottles, excepted for ammonium, which was higher *in situ* on the morning of the second day. Nitrite (not shown) ranged between 0.6 and 0.1  $\mu\text{M}$  (not shown). Concerning phytoplankton biomass estimated as chl *a*, the trends were very different *in situ* and in the bottles. The chl *a* levels *in situ* remained more or less constant, with possibly a slight diurnal variation showing an increase in the afternoon and a decrease at night. In the bottles, chl *a* increased from an initial value of 3.8  $\mu\text{g l}^{-1}$  to a maximum of 22.4  $\mu\text{g l}^{-1}$  in about 12 h. From these values, a maximum phytoplankton growth rate can be estimated to be about 2.6 doublings per 12 h day. The chl *a* values then decreased to 14  $\mu\text{g l}^{-1}$  over the next 24 h.

The same difference was observed for ribulose biphosphate carboxylase (Rubisco) activity per unit of chl *a* (Fig. 3). Six hours after the beginning of incubation (15:00 h local time), this activity had increased by a factor of 2 relative to *in situ* samples. This difference remained at night and during the second day. Beta-

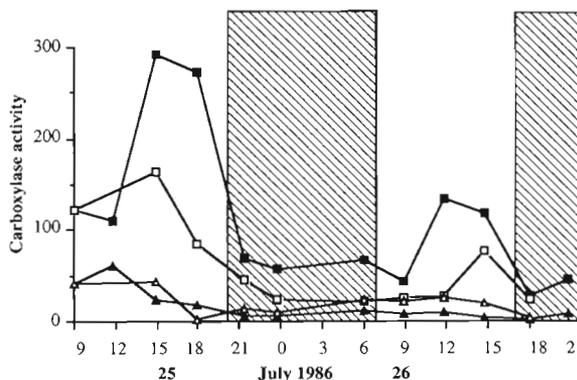


Fig. 3. Changes in Rubisco and  $\beta$ -carboxylase activities ( $\text{nmol CO}_2 \mu\text{g}^{-1} \text{chl } a \text{ h}^{-1}$ ) in *in situ* samples ( $\circ$  and  $\square$  respectively) and incubated samples ( $\blacksquare$  and  $\blacktriangle$  respectively). Hatching: hours of darkness

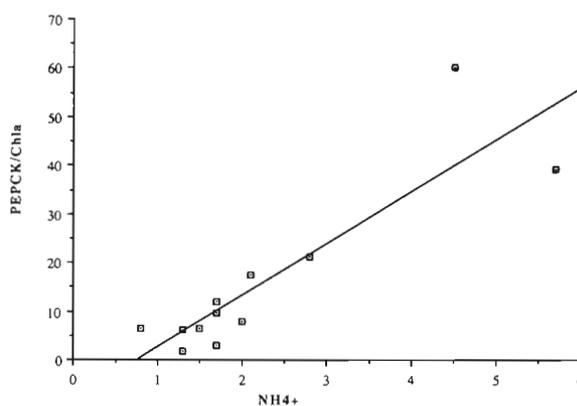


Fig. 4. Phosphoenol pyruvate carboxykinase (PEPCK) specific activity ( $\text{nmol CO}_2 \mu\text{g}^{-1} \text{chl } a \text{ h}^{-1}$ ) for incubated samples as a function of ammonium concentration ( $\mu\text{g-at. N l}^{-1}$ )

carboxylase activities were mainly due to PEPCK, as PEPC activity remained low over the sampling period. The same increase in specific activity was noted in bottles for those enzymes. In addition, the specific activity of PEPCK became highly correlated with ammonium (Fig. 4).

Over the duration of the experiment, the  $\delta^{13}\text{C}$  of the particulate matter remained rather constant *in situ* (mean value  $\pm 95\%$  confidence intervals  $-15.26 \pm 1.44$ ), but became more negative in bottles (Fig. 5), where  $\delta^{13}\text{C}$  values decreased markedly as biomass (chl *a*) increased ( $r = -0.67$ ;  $p < 0.01$ ,  $n = 12$ ). The greatest changes in  $\delta^{13}\text{C}$  occurred during the beginning of the experiment, and corresponded to the greatest changes in DIN per unit biomass and unit time. The values of  $\delta^{13}\text{C}$  and DIN were inversely correlated in a highly significant way (Fig. 6). The lines join the data points for the first 24 h.

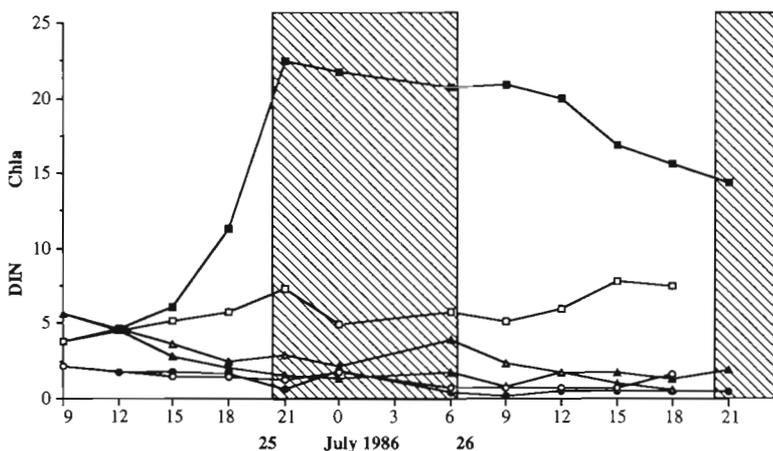


Fig. 2. Changes in ammonium, nitrate and chlorophyll *a* *in situ* ( $\circ$ ,  $\square$ , and  $\square$  respectively) and in bottles ( $\blacktriangle$ ,  $\bullet$ , and  $\blacksquare$  respectively) incubated at the pond's surface as a function of time. DIN: dissolved inorganic nitrogen ( $\mu\text{g-at. N l}^{-1}$ ); Chla: chlorophyll *a* (in  $\mu\text{g l}^{-1}$ ). Hatching: hours of darkness

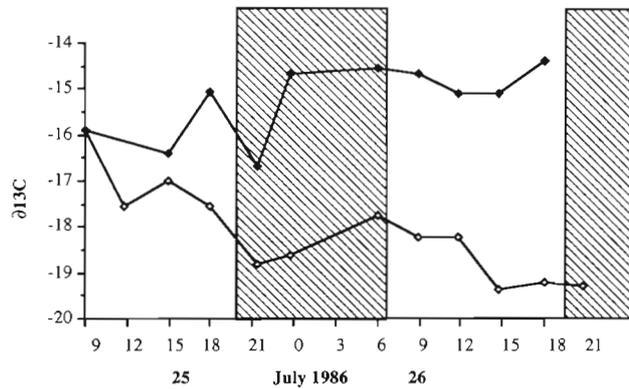


Fig. 5. Changes in  $\delta^{13}\text{C}$  in the particulate matter (‰) in *in situ* samples ( $\blacklozenge$ ) and incubated samples ( $\circ$ ) as a function of time. Hatching: hours of darkness

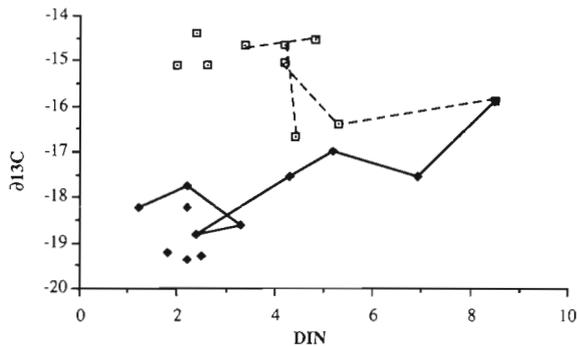


Fig. 6.  $\delta^{13}\text{C}$  (‰) in particulate matter as a function of dissolved inorganic nitrogen (DIN,  $\mu\text{g-at. N l}^{-1}$ ). ( $\blacklozenge$ ) incubated samples (correlation coefficient:  $r = -0.81$ ,  $p < 0.01$ ); ( $\square$ ) samples taken directly *in situ*. Time zero is to right of graph. Lines join data points for first 24 h of sampling

Samples taken directly from the pond indicated a significant negative correlation between Rubisco and  $\delta^{13}\text{C}$  values ( $r = -0.63$ ;  $p < 0.05$ ,  $n = 10$ ), while there was absolutely no correlation ( $r = 0.02$ ) between those 2 variables in bottles. However, a cross correlation study showed that, in bottles, the correlations between Rubisco, chl *a* and  $\delta^{13}\text{C}$  became significant when values of enzyme activities at  $T+1$  were considered. For example, the coefficient of correlation between Rubisco and chl *a* became 0.53 ( $p < 0.05$ ) and between Rubisco and  $\delta^{13}\text{C}$  became  $-0.50$  ( $p < 0.05$ ) with this transformation of variables. The same pattern was found between the ratio of  $\beta$ -carboxylase/Rubisco activity ( $\beta\text{-C/R}$ ) at  $T+1$  and  $\delta^{13}\text{C}$  ( $r = 0.72$ ;  $p < 0.01$ ). But for  $\delta^{13}\text{C}$  and PEPCK activity, the correlation was highest for both values at time  $T$  ( $r = 0.52$ ;  $p < 0.05$ ).

## DISCUSSION

There was no sign of a detrimental effect of enclosing the water samples in bottles as sometimes reported

(Venrick et al. 1977, Marra et al. 1988). On the contrary, this led to an increase in the phytoplankton biomass relative to the bulk water, as also reported in the open ocean (Saijo et al. 1969, Gieskes & Kraay 1982, Cullen et al. 1992). The material used here for incubation (polycarbonate) is generally preferred to glass because it does not disturb the ionic balance of seawater (Robertson 1968, Fitzwater et al. 1982). However, it has also been found that polycarbonate (PC) can be inferior to glass containers for diatom growth (Metaxas 1989). Comparisons between changes in chl *a* in samples enclosed in PC bottles and changes in bulk water in a pond without grazers failed to show any significant differences (Collos 1987), so that at least for our samples, it seems that the incubation material had no effect on the phytoplankton. We therefore assume that the observed changes are due to physiological reasons.

The most striking results from this study concern the large increases in both phytoplankton biomass and Rubisco as well as the large decrease in the  $\delta^{13}\text{C}$  and its dependence on the DIN upon isolation of the water sample from bulk water. The rise in chl *a* is similar to that previously reported in the same pond (Sornin et al. 1990) and indicates that it is most probably due to removal of grazing pressure by the oysters and other filter feeding organisms present in the sediment. Such an interpretation is consistent with other reports on grazing control of primary production in the open ocean (Cullen et al. 1992).

The increase in Rubisco specific (per unit of chl *a*) activity is more difficult to explain. One possibility is that the enclosed phytoplankton is less dependent on organic matter excreted by oysters (less heterotrophy) due to the confinement in bottles and relies more on inorganic carbon (and nitrogen) for its nutrition.

Oyster pond microalgae are known to be able to grow on organic substrates (Robert et al. 1982, Maestrini & Robert 1987) but such a capacity is by no means restricted to this kind of environment (see reviews by Bonin & Maestrini 1981, Flynn & Butler 1986, Amblard 1991). It is therefore possible that the algae sampled here were partly heterotrophic due to their use of organic compounds excreted by the grazers.

This greater reliance on autotrophy during incubation is supported by the greater dependence of chl *a* on DIN in bottles than *in situ* ( $r = 0.876$  vs  $0.717$  *in situ*). The same can be said of  $\delta^{13}\text{C}$  ( $r = 0.811$  vs  $0.471$  *in situ*). In addition, the highest correlation coefficient was found between PEPCK specific activity and ammonium concentration in bottles. Such a dependence of carboxylases on ammonium concentration or assimilation rate has been described in cultures of diatoms (Rosslenbroich & Döhler 1982) and green algae (Vanlerberghe et al. 1990).

The reduced dependence of carbon fluxes on regenerated compounds due to incubation conditions could also explain the unexpected lagged correlations involving Rubisco since the changes in enzymatic activities are generally faster than those in isotopic composition of the cells.

*In situ*, the increase of the  $\beta\text{C}/\text{R}$  ratio which was accompanied by a decrease in chl *a* could be due to grazing. Such predation appears to modify the metabolism of algae (Amouroux et al. 1989), but this effect is species dependent, stimulating the growth of some and inhibiting the growth of others.

Finally, the large changes of the carbon isotope composition of the particulate matter described here confirm previous results (Collos et al. 1992), but are in the opposite direction as those reported by Fry & Wainright (1991) for diatom blooms. One explanation for the difference could be the incubation duration which was up to 8 d in the latter study. In a closed medium like an incubation bottle, the photosynthetic activity yields particulate matter with a low  $^{13}\text{C}$  content due to isotopic fractionation by Rubisco, as exemplified in the present study and elsewhere (Descolas-Gros & Fontugne 1990 and references therein). But, for very long incubations such as those used by Fry & Wainright (1991), as the total inorganic carbon pool is small, the enrichment in  $^{12}\text{C}$  of the particulate matter induces an enrichment in  $^{13}\text{C}$  of the inorganic carbon pool. This enrichment increases with time during the bloom and must be carried over to the synthesized particulate matter.

In our short-term study,  $\text{CO}_2$  limitation does not seem to be the dominant factor, inasmuch as we observed a decrease in  $\delta^{13}\text{C}$  in the bottles (Fig. 5), which could be related to the increase in Rubisco activity. This suggests that changes in carbon fluxes in the phytoplankton compartment upon enclosing water samples in bottles are due more to physiological (biochemical fractionation) than physical ( $\text{CO}_2$  limitation) processes.

In the pond, the relatively constant phytoplankton biomass indicates an approximate steady-state between microalgal growth and grazing processes, which both appear to occur at high rates. Our results show that, even for small time scales (hours), phytoplankton switch towards greater autotrophy during incubation. Those changes indicate that, apart from possible methodological artefacts such as sampler or bottle toxicity (Robertson 1968, Fitzwater et al. 1982, Metaxas 1989), and for incubation longer than 3 h, bulk water estimates of primary production can be different from those obtained from incubation bottles due to the physical separation of phytoplankton from the grazers.

The features of the system we examined make it somewhat extreme with respect to the difficulties fac-

ing incubation studies. These include relatively high phytoplankton biomass, high specific growth rates, and high grazing pressure from organisms which are excluded from incubation bottles with 100 % efficiency (oysters and sediment filter feeders).

However, the following generalizations can be made from our study: Firstly, there is some upper limit to the length of useful incubation experiments performed on seawater. Secondly, this upper limit probably decreases as autotrophic biomass, growth rate and the likelihood of excluding the main grazers from the incubation bottles increases. Thirdly, in the worst cases (such as that examined here), incubations longer than 3 h should not be interpreted as good estimators of *in situ* rates.

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