European Journal of Phycology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713725516

Biochemical responses and photosynthetic performance of **Gracilaria** sp. (Rhodophyta) from Cádiz, Spain, cultured under different inorganic carbon and nitrogen levels

Jesus R. Andria; Juan J. Vergara; J. Lucas Perez-Llorens

To cite this Article: Andria, Jesus R., Vergara, Juan J. and Perez-Llorens, J. Lucas , 'Biochemical responses and photosynthetic performance of **Gracilaria** sp. (Rhodophyta) from Cádiz, Spain, cultured under different inorganic carbon and nitrogen levels', European Journal of Phycology, 34:5, 497 - 504
To link to this article: DOI: 10.1080/09541449910001718851
URL: http://dx.doi.org/10.1080/09541449910001718851

Biochemical responses and photosynthetic performance of *Gracilaria* sp. (Rhodophyta) from Cádiz, Spain, cultured under different inorganic carbon and nitrogen levels

JESUS R. ANDRIA, JUAN J. VERGARA AND J. LUCAS PEREZ-LLORENS

Departamento de Ecología, Facultad de Ciencias del Mar, Universidad de Cádiz, E-11510 Puerto Real, Cádiz, Spain

(*Received 2 March 1999; accepted 24 June 1999*)

Photosynthetic acclimation and the interactions between carbon (C) and nitrogen (N) metabolism have been studied in the red macroalga *Gracilaria* sp. from Cádiz, Spain, cultured under different inorganic C and N levels. The use of chemostats and buffered medium allowed continuous restoration of the alkaline reserve and constancy of pH during the experiments. The N:C ratios and phycobiliprotein, chlorophyll *a* and soluble protein contents decreased when *Gracilaria* sp. was grown at low N levels. Algae grown in a high inorganic C concentration (5% CO_2) displayed a higher soluble carbohydrate concentration and maximum photosynthesis rates but a lower photosynthesic affinity for inorganic C, and lower phycobiliprotein and Rubisco contents, than those cultured at low inorganic C levels (air CO_2). The inorganic C enrichment also affected the N uptake and assimilation in *Gracilaria* sp., causing a decrease in the N uptake rate even under conditions of N sufficiency. These results reflect the significant influence of the inorganic C growth regime on N assimilation in *Gracilaria* sp.

Key words: carbon, chemostat, *Gracilaria*, nitrogen, photosynthesis, phycobiliproteins, Rubisco.

Introduction

Recently much interest has been focused on the photosynthetic acclimation of aquatic autotrophs to a CO_2-enriched environment because of the increase in CO_2 during the last century (Johnston & Raven, 1990; Bowes, 1991, 1993; Riebesell *et al.*, 1993; Beer & Koch, 1996). Human activities in coastal areas have also greatly increased the inputs of inorganic nutrients into many estuaries and salt-marshes, promoting changes in the specific abundances of macrophytes and alterations at the population and ecosystem levels (Valiela *et al.*, 1997). However, little information exists on the influence of concomitant carbon (C) and nitrogen (N) enrichment on the biochemical composition and photosynthetic performance of marine macroalgae.

The metabolic pathways of C and N are highly coordinated and coupled, since CO_2 fixation and N assimilation compete for assimilatory power and C skeletons (Lara *et al.*, 1987; Turpin *et al.*, 1988; Huppe & Turpin, 1994). However, the effect of N availability (Hanisak, 1983; Turpin, 1991) and inorganic C concentration (Johnston & Raven, 1990; Raven, 1991) on photosynthetic acclimation have often been regarded as unrelated topics. Among the few studies that consider both aspects (Rivers & Peckol, 1995; Giordano & Bowes, 1997; Gordillo, 1998) none has been carried out in chemostats. This system has been widely applied to algal and bacterial cultures (Monod, 1950; Droop, 1966) to avoid the gradual changes in the properties of the culture medium associated with batch systems. Chemostats are very appropriate for determining the effect of any variable on the physiology of macroalgae in culture since they can be controlled and manipulated easily (Vergara *et al.*, 1993). In contrast to batch cultures, the use of continuous systems avoids the gradual depletion of alkaline reserve of the medium in those experiments in which the effect of different dissolved inorganic carbon (DIC) levels on the algal physiology is tested.

The aim of this work was to study the combined effect of different DIC and N levels on the biochemical composition and photosynthetic performance of the red macroalga *Gracilaria* sp. cultured in chemostats.

Correspondence to: J. R. Andría. Fax: +34 956 016019. e-mail: jesus.andria@uca.es

Materials and methods

Plant material

Gracilaria sp. was collected in the tidal channels of Los Toruños salt-marsh (El Puerto de Santa María, Southern Spain) during June 1997. Individuals harvested from these populations were identified as *Gracilaria gaditana* nom. prov. (M. Steentoft, personal communication). Plants were cleaned of epiphytes and maintained for 1 week in the laboratory in aerated 20 l aquaria with filtered (Whatman GF/C) natural sea water (NSW, 35‰). The algae were

preincubated in artificial sea water (ASW) (modified MCM, 'Marine Culture Medium', Woelkerling *et al.*, 1983; by omitting boric acid) for 2 weeks prior to the experiments. Both NSW and ASW were enriched with 40 μM NO_3^- and 2 μM H_2PO_4 (final concentrations). Temperature was maintained at 19 ± 1 °C, and the photon fluence density (PFD) at 85 μmol photons m^{-2} s^{-1} (Sylvania GRO-LUX, F-36W/GRO-T8, Erlangen, Germany) in a 12:12 h light:dark cycle. The PFD was quantified with a quantum spherical PAR sensor (LiCor Li-193SA) connected to a data logger (LiCor Li-1000).

Experimental design

Gracilaria sp. was cultured in chemostats (Vergara *et al.*, 1993). This continuous system of culture operates by controlling the rate of nutrient supply, resulting in nearly constant culture medium conditions and a population with steady-state growth (Miller-Way & Twilley, 1996). A peristaltic pump (Watson-Marlow 503S) provided a continuous and controlled flow of culture medium (ASW buffered with 25 mM Tris at pH 8·0). The use of a synthetic medium (ASW) instead of natural sea water (NSW) allowed a finer control of the nutrient availability (Vergara *et al.*, 1993). Biomass density was kept approximately constant at 0·8 g fresh weight (FW) per litre throughout the cultures to avoid limitation of growth (Vergara *et al.*, 1993). The volume of culture medium was 5 l in a reactor of 6 l total capacity, and the renewal rate of the medium was 0·5 d^{-1}. Cultures were agitated by a gentle bubbling near the bottom of the reactor. Different levels of dissolved inorganic carbon (DIC) and N were applied for 12 days: high DIC and high N (DIC + N +), high DIC and low N (DIC + N −), low DIC and high N (DIC − N +) and low DIC and low N (DIC − N −). In the low DIC treatments, ASW was aerated with air from outside (air CO_2; 2·2 mM DIC), while in the high DIC treatments, medium was bubbled with 5% CO_2 enriched air (flow rate of 1·5 ml s^{-1} of 99% CO_2; final concentration *c.* 3·7 mM DIC). In the high N treatments, the culture medium contained 75 μM NO_3^- and 2 μM $H_2PO_4^-$, and 15 μM NO_3^- and 2 μM $H_2PO_4^-$ in the cultures with low N level. Light and temperature conditions were maintained as described above.

Growth estimation

Biomass was monitored throughout the experiment to estimate growth and production. The mean relative growth rate (μ), expressed as % d^{-1}, was calculated according to the exponential model: $\mu = [\ln(w_2/w_1)/(t_2 - t_1)] \cdot 100$, where w_2 and w_1 are FW at times t_2 and t_1.

Oxygen evolution rates

Net photosynthesis rates (NPS) were measured, at 19 ± 1 °C, with a Hansatech polarographic O_2 electrode (Hansatech, Norfolk, UK) lit with a high-intensity light source (LS2, Hansatech) and connected to a chart recorder (Linseis L6512). Photosynthesis–DIC curves (P–C curves) were performed in DIC-free artificial sea water (ASW-DIC) buffered at pH 8·2 with 25 mM Tris. The sea water was further bubbled with air which was previously passed through a freshly prepared 6 N KOH solution. The range of DIC concentrations (0–2500 μM) was achieved by injecting different volumes of a 20 mM $NaHCO_3$ solution into the incubation chamber once a zero net O_2 exchange rate was achieved. For each treatment, duplicate measurements were carried out at a saturating PFD of 160 μmol photons m^{-2} s^{-1} (I_k is about 100 μmol photons m^{-2} s^{-1}) (cosine collector, LiCor LI-192SB). Data were fitted to a biphasic curve according to Andría *et al.* (1999). The photosynthetic response of *Gracilaria* sp. to different DIC concentrations does not follow a simple saturation curve but a biphasic pattern (Andría *et al.*, 1999). *Gracilaria* sp. is able to use DIC by two complementary mechanisms (external carbonic anhydrase-mediated mechanism and direct transport of HCO_3^-) which operate simultaneously. P–C curves therefore display two phases: a high-affinity phase which operates at low DIC concentrations with a lower maximal capacity, and a low-affinity phase at higher DIC levels.

Analytical methods

The analytical measurements were performed on thallus fragments harvested during the experiment. The plant material was frozen in liquid nitrogen and maintained at −80 °C until analysed. Duplicate samples (40–60 mg FW) were ground in phosphate buffer 0·1 M pH 6·5 at 4 °C, extracted overnight and centrifuged at 19000 *g* for 25 min (Sigma Laborzentrifugen 2K15, Germany). The content of the phycobiliproteins (PBP) r-phycoerythrin (RPE) and r-phycocyanin (RPC), and the soluble protein concentration, were determined spectrophotometrically (UNICAM UV/Vis Spectrometer UV2) from the supernatant fraction. PBP were determined using the chromatic equations of Beer & Eshel (1985) and soluble protein according to Bradford (1976). The pellet fraction was processed in 5% (w:v) trichloroacetic acid for 3 h at 80–90 °C (Bird *et al.*, 1982), and centrifuged at 19000 *g* for 15 min to determine insoluble carbohydrate content according to the phenol–sulphuric acid method (Kochert, 1978). Soluble carbohydrate content was also quantified from the previous supernatant fraction using this method.

Duplicate samples were also taken to determine the liposoluble pigment content by grinding in 90% (v:v) acetone, overnight extraction at 4 °C and filtration. Chlorophyll *a* (chl *a*) concentration was determined spectrophotometrically according to Talling & Driver (1963).

Parallel duplicate samples were dried in an oven at 60 °C for 48 h to determine total C and N content (Perkin-Elmer 240-C elemental autoanalyser). The FW:DW relationship was also determined to normalize the results on a dry weight (DW) basis (g DW/g FW = 0·103).

For Rubisco determination, two samples (0·1 g FW) per treatment were powdered in liquid nitrogen and sonicated for two cycles of 10–20 s (Microson Ultrasonic Cell Disruptor) in 1 ml of ice-cold TCA-acetone (10% w:v). Proteins were precipitated for 1 h at −20 °C, and centrifuged at 20000 *g* for 5 min. Pellets were washed in 0·3 ml of ice-cold 100% acetone and air dried. Protein content was measured according to the bicinchoninic acid method (BCA; Smith *et al*., 1985). Samples were prepared following Greene *et al*. (1991), subjected to SDS-PAGE (14% resolving gel; 6% stacking gel) according to Laemmli (1970), and loaded on an equal protein basis. All reagents and molecular weight markers were from Sigma. Different dilutions of partially purified Rubisco from spinach (R-8000; Sigma) were used as standards during the electrophoretic measurements. Gel staining and destaining were carried out according to Rintamaki *et al*. (1988). Gels were scanned (Agfa Studio Star Desktop Colour Scanner), and the area and mean intensity of the bands, identified as Rubisco large subunits, were quantified by an image analysis program (NIH Image 1·55).

Nutrients

Nitrate and nitrite concentrations were determined in samples from the inflow and outflow of the chemostats (Bran & Luebbe Technicon Traacs 800) according to Wood *et al*. (1967) and Shinn (1941), respectively. The mean nitrate uptake rate for a given interval of time was calculated according to Carmona *et al*. (1996). The mean nitrogen requirement was estimated using the equation:

$$[\mu\text{mol N (g FW)}^{-1}\ \text{d}^{-1}] = (\text{N}_{\text{int}}).(\text{DW:FW}).(\mu)/\text{AW}_{\text{N}}$$

where N_{int} is the internal N content expressed as (μg N (g DW)$^{-1}$), AW_{N} is the N atomic weight, DW:FW is the ratio between dry and fresh weight, and μ is the mean relative growth rate expressed as d^{-1}.

Statistics

Statistical analyses (two-way ANOVA) were applied to test the significance of the results ($p < 0{\cdot}05$).

Results

Growth and production

The biomass increased with a doubling time of 12 days. The growth rate was significantly higher ($p < 0{\cdot}05$; Table 1) in the low N treatments (N−) than in high N (N+), regardless of the DIC level in the culture medium (Table 2).

Internal N and C content

The N content was significantly affected by the N concentration in the medium ($p < 0{\cdot}01$; Table 1), irrespective of the DIC levels (Fig. 1*A*), with higher values corresponding to N+ treatments. The internal C content increased slightly during the experiment, but did not show a clear picture (Fig. 1*B*). The C:N ratio was the mirror image of N values, since variability in this ratio was influenced markedly by N content (Fig. 1*C*).

Table 1. *F* values of two-way ANOVA with replication ($n = 2$) of growth rates, biochemical variables and photosynthetic parameters after exposure to different dissolved inorganic carbon (DIC) and N levels for 12 days

	Source of variation		
	DIC	N	Interaction
Growth rates	NS	10·26*	NS
Biochemical variables			
Internal C	NS	NS	NS
Internal N	NS	44·08**	NS
RPE	NS	27·21**	NS
RPC	NS	18·90*	NS
Chl *a*	NS	15·35*	NS
SP	24·93**	12·50*	NS
Rub (prot)	14·95*	NS	NS
Rub (g FW)	48·11**	24·39**	NS
SCH	17·41*	NS	NS
ICH	NS	NS	NS
Photosynthetic parameters			
NPS_{max} (HA)	15·73*	NS	NS
NPS_{max} (LA)	NS	NS	NS
K_{m} (HA)	100·49***	NS	NS
K_{m} (LA)	NS	NS	13·25*

Biochemical variables: internal N, internal C, r-phycoerythrin (RPE), r-phycocyanin (RPC), soluble protein (SP), relative units of Rubisco per protein [Rub (prot)] and per gram fresh weight [Rub (g FW)], soluble carbohydrate (SCH) and insoluble carbohydrate (ICH). Photosynthetic parameters: maximum photosynthesis rates (NPS_{max}) and half-saturation constant (K_{m}) of the high-affinity (HA) and low-affinity (LA) phases. Significance levels: *** $p < 0{\cdot}001$; ** $p < 0{\cdot}01$; * $p < 0{\cdot}05$; NS, no significant difference ($p > 0{\cdot}05$).

Table 2. Mean relative growth rates (μ) for *Gracilaria* sp. cultured at different dissolved inorganic carbon (DIC) and N concentrations

Treatment	μ (% d^{-1})
DIC+N+	5·6±0·5
DIC+N−	7·1±0·4
DIC−N+	5·5±0·5
DIC−N−	6·9±0·4

Data are presented as the value±SD.

Inorganic N

The time course of the NO_3^- concentration in the culture medium is shown in Fig. 2. For the N+ treatments, the NO_3^- concentration remained close to 40 μM (DIC−) or

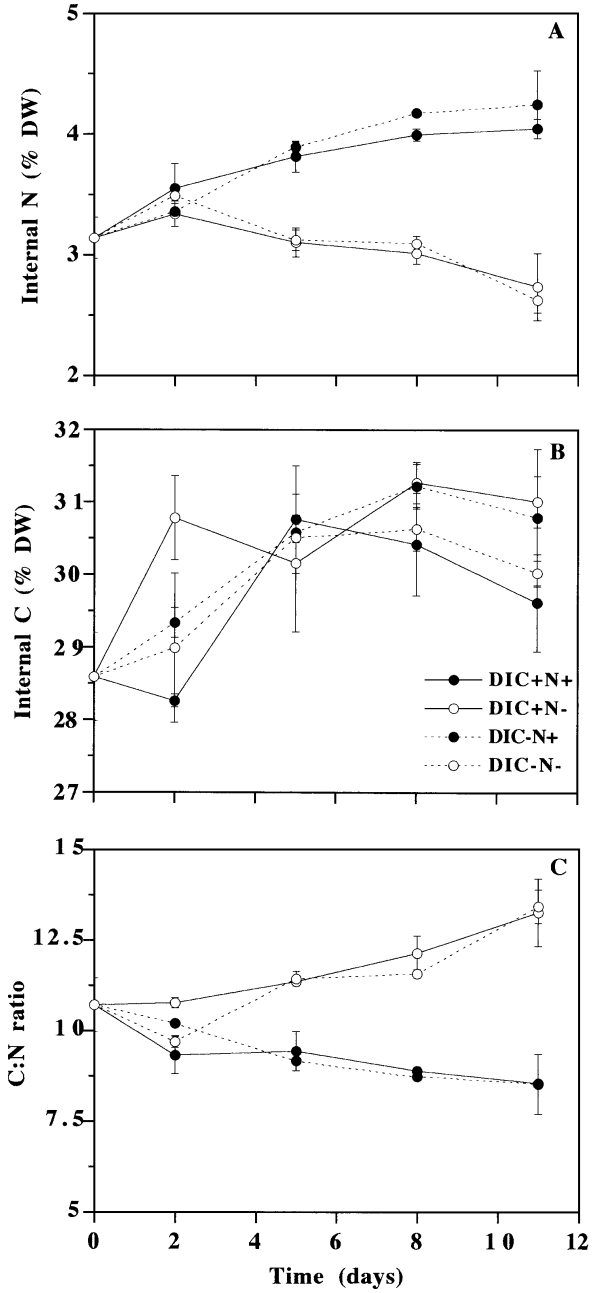

Fig. 1. Time course of (*A*) internal N, (*B*) C content and (*C*) C:N ratio in *Gracilaria* sp. grown in chemostats. Different levels of dissolved inorganic carbon (DIC) and N were applied for 12 days: high DIC and high N (DIC+N+), high DIC and low N (DIC+N−), low DIC and high N (DIC−N+) and low DIC and low N (DIC−N−). Temperature was maintained at 19±1 °C, and the photon fluence density (PFD) at 85 μmol photons m^{-2} s^{-1} in a 12:12 h light:dark cycle. Data are presented as mean±SD ($n = 2$).

60 μM (DIC+), after 4 days of experiment. In the N− treatments, the NO_3^- concentration decreased down to a constant value of 2 μM, after 2 days. The NO_2^- concentration was less than 0·5 μM in all treatments (data not shown). The highest NO_3^- uptake rates were recorded for N+ treatments, especially for DIC−N+, allowing a N supply in excess of the algal requirement (Table 3). The DIC enrichment decreased the NO_3^- uptake rate under N sufficiency conditions. The NO_3^- uptake rates for N− treatments only supported 50% of the algal N requirements (Table 3).

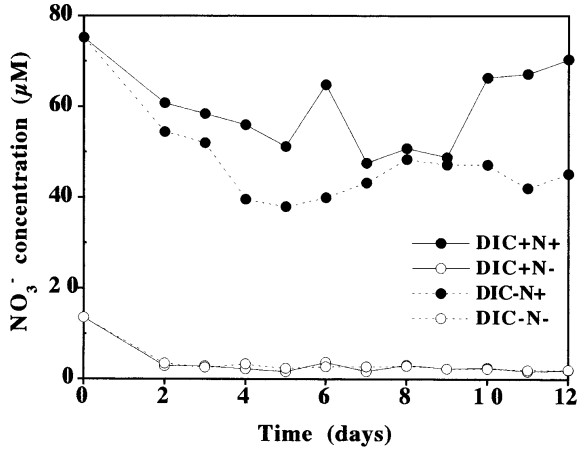

Fig. 2. Time course of the NO_3^- concentration in the chemostats where *Gracilaria* sp. was grown under different dissolved inorganic carbon (DIC) and N conditions. Treatments were as for Fig. 1.

Photosynthetic pigments

The pigment contents (RPE, RPC and Chl *a*) were significantly higher in N+ treatments (Table 1; Fig. 3). The DIC enrichment promoted a slight decrease in pigment contents for treatments with the same N level.

Proteins

The DIC and N availability affected the soluble protein content significantly (Table 1, Fig. 4). The highest values of soluble protein content were obtained in the DIC−N+ treatment, while the lowest concentrations were recorded under DIC+N− conditions. Algae grown in DIC+N+ showed similar values to those cultured in DIC−N− conditions. The highest values of the enzyme Rubisco were also obtained in algae grown under low DIC (air CO_2) conditions, especially in the DIC−N+ treatment (Table 4). Relative units of Rubisco per unit protein were affected by the DIC level in the medium, with significantly lower values under high DIC conditions ($p < 0{\cdot}05$; Tables 1, 4). Significant differences were obtained with respect to DIC and N level when data were expressed on a fresh weight basis (Tables 1, 4), the lower values also being found at high DIC concentrations.

Carbohydrates

The insoluble carbohydrate content increased during the first few days of the experiment for all treatments, specially for the N− treatments (Fig. 5*A*). At the end of the experiments, no significant differences were obtained among the treatments (Table 1). Soluble carbohydrates accounted for a much lower proportion of cellular C in *Gracilaria* sp. The differences among the soluble carbo-

Table 3. Mean nitrate uptake rate and mean nitrogen requirement in *Gracilaria* sp. grown at different dissolved inorganic carbon (DIC) and N levels

Treatment	Mean NO_3^- uptake rate (μmol NO_3^- (g FW)$^{-1}$ d^{-1})	Mean N requirement (μmol N (g FW)$^{-1}$ d^{-1})	Ratio (uptake:requirement)
DIC+N+	12·0	15·0	0·80
DIC+N−	7·9	15·6	0·51
DIC−N+	21·8	15·0	1·46
DIC−N−	7·5	15·3	0·49

Mean nitrate uptake rate was calculated from the NO_3^- concentration in the chemostat, biomass, volume and renewal rate of the medium according to Carmona *et al.* (1996). Mean nitrogen requirement was calculated from internal N content (expressed as mass of N per unit DW basis), DW:FW ratio, the atomic weight of N and the mean relative growth rate (μ).

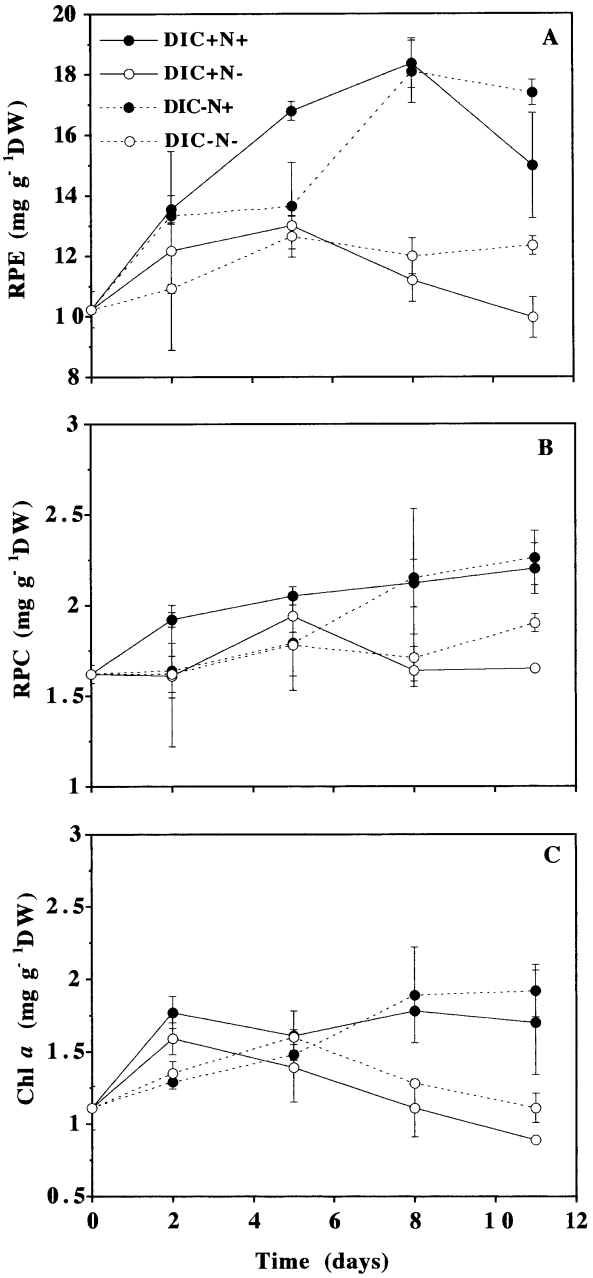

Fig. 3. Time course of (*A*) r-phycoerythrin (RPE), (*B*) r-phycocyanin (RPC) and (*C*) chlorophyll *a* (Chl *a*) concentrations in *Gracilaria* sp. cultured in chemostats at different dissolved inorganic carbon (DIC) and N levels. Data are presented as means ± SD ($n = 2$). Treatments were as for Fig. 1.

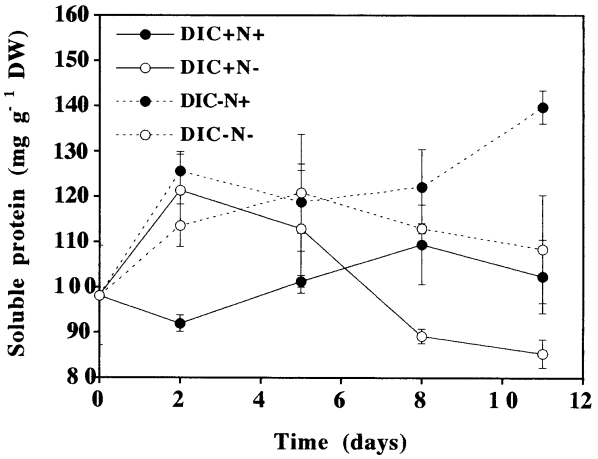

Fig. 4. Time course of the total soluble protein content in *Gracilaria* sp. cultured in chemostats at different dissolved inorganic carbon (DIC) and N concentrations. Data are presented as mean ± SD ($n = 2$). Treatments were as for Fig. 1.

Table 4. Relative units of Rubisco per protein and on a fresh weight (FW) basis for *Gracilaria* sp. cultured at different dissolved inorganic carbon (DIC) and N concentrations

Treatment	Relative units of Rubisco per protein	Relative units of Rubisco per gram fresh weight
DIC+N+	99·3 ± 3·6	130·3 ± 15·0
DIC+N−	88·2 ± 1·1	96·2 ± 4·8
DIC−N+	137·0 ± 12·8	245·2 ± 29·2
DIC−N−	111·4 ± 8·4	153·1 ± 5·4

Data were calculated as a percentage of the values on the initial day of the experiment. Data are presented as mean ± SD ($n = 2$).

hydrate concentrations were significant with respect to DIC level in the medium ($p < 0·05$; Table 1), the final concentration being higher under DIC-enriched conditions (Fig. 5 *B*).

P–C curves

The P–C curves were affected by DIC and N levels. Curves displayed a biphasic pattern regardless of the

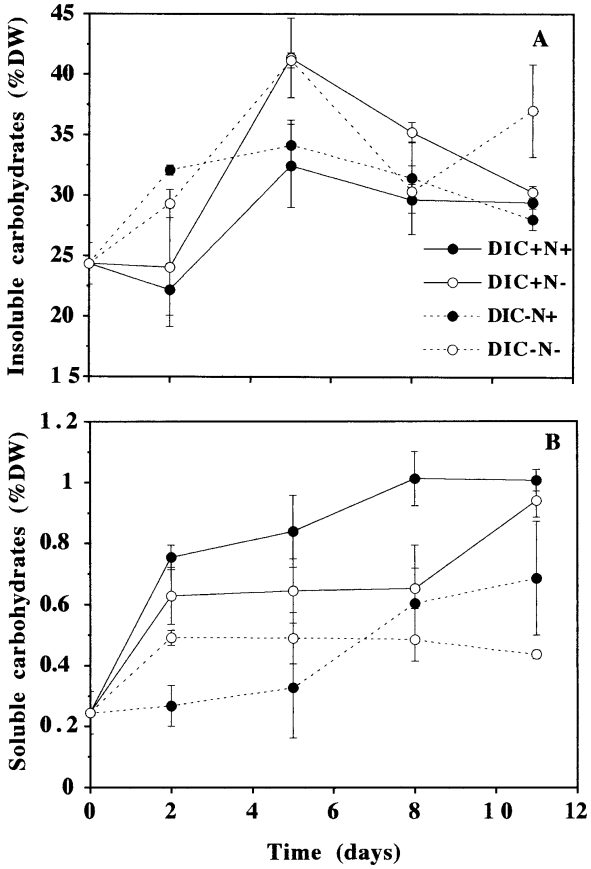

Fig. 5. Time course of (*A*) insoluble and (*B*) soluble carbohydrate concentrations in *Gracilaria* sp. grown in chemostats at different dissolved inorganic carbon (DIC) and N levels. Data are presented as mean ± SD ($n = 2$). Treatments were as for Fig. 1.

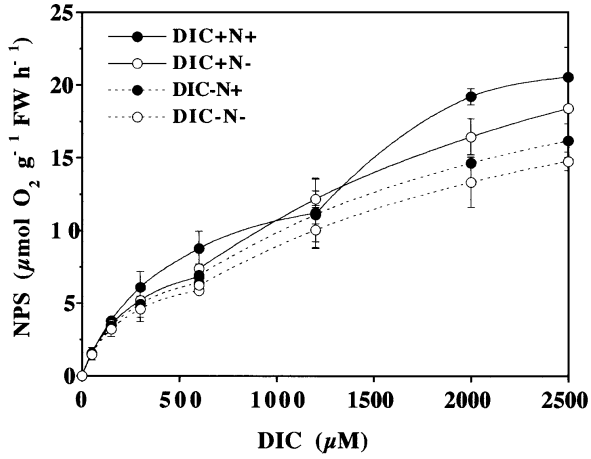

Fig. 6. Net photosynthesis rates (NPS) as a function of the dissolved inorganic carbon (DIC) concentration, in *Gracilaria* sp. cultured at different DIC and N concentrations. Data are presented as mean ± SD ($n = 2$).

treatment (Fig. 6). In the high-affinity phase, the NPS_{max} and K_m were significantly affected by DIC treatments (Tables 1, 5), the higher values being at high DIC level. In contrast, for the low-affinity phase, these parameters showed no significant differences with respect to DIC or N levels in the culture medium (Tables 1, 5).

Discussion

Populations of *Gracilaria* sp. occur in tidal shallow creeks in Los Toruños salt-marsh (Cádiz Bay). In this fluctuating environment, macroalgae are subjected daily to emersion cycles and continuous and abrupt changes in temperature, pH, alkalinity, irradiance and nutrient levels, specially at low tide (unpublished data), so it is important to analyse the combined effects of DIC and N levels in this species.

Limitation of algal growth by N availability results in a decrease in N:C ratios, photosynthetic pigments and protein content (Lapointe & Duke, 1984; Turpin, 1991; Vergara *et al.*, 1993). In our study these variables displayed a decline in the low N treatments (DIC+N− and DIC−N−), where N supply only supported 50% of the algal N requirements. N limitation in red macroalgae redirects the flow of N metabolites towards the synthesis of non-pigmented proteins and away from PBP (Vergara & Niell, 1993). This result was confirmed by the parallel decline in RPE and internal N content in *Gracilaria* sp. grown in low N cultures. It reflected the role of these nitrogenous compounds as a N reserve under N limitation (Lapointe & Duke, 1984). The DIC enrichment further decreased the photosynthetic pigments and soluble protein content regardless of the N level in the cultures. These results were in accord with those obtained by García-Sánchez *et al.* (1994), who reported a decrease in pigment content, soluble protein and Rubisco when *Gracilaria tenuistipitata* was cultured at 5% CO_2 compared with control (air CO_2) conditions.

The photosynthetic acclimation to high DIC concentration resembles the algal response to high irradiance, resulting in a decrease in pigment content which can be explained by some kind of control of DIC level on the photosynthetic pigments (García-Sánchez *et al.*, 1994). The increase in soluble carbohydrate content represses the transcription of genes related to photosynthetic pathways (Sheen, 1994). In fact, acclimatory losses of Rubisco and carboxylation capacity under an elevated CO_2 growth regime have been linked to an increase in carbohydrate content (Webber *et al.*, 1994; Drake *et al.*, 1997). However, some authors have recently concluded that the changes in carbohydrate content when vascular plants are exposed to a CO_2-enriched environment cannot be the sole explanation of the rapid alterations in Rubisco gene expression detected in their experiments (Gesch *et al.*, 1998). In our study, lower PBP, soluble protein and Rubisco contents were obtained under a DIC-enriched growth regime (5% CO_2), while internal N content was not significantly affected by DIC level. Thus, the exposure and acclimation to high CO_2 would involve the reallocation of resources, like N, away from Rubisco and towards other limiting components (electron transport, carbohydrate synthesis and non-photosynthetic processes) (Bowes, 1991).

The maximum rate of photosynthesis (NPS_{max}) is frequently related to Rubisco activity (Lapointe & Duke, 1984). The Rubisco content per cell decreases under N

Table 5. Kinetic parameters from the photosynthesis–dissolved inorganic carbon (DIC) curves, maximum photosynthesis rate (NPS_{max}) and half-saturation constant (K_m) for *Gracilaria* sp. after culturing at different DIC and N levels for 12 days

	High-affinity phase			Low-affinity phase		
Treatment	NPS_{max} (μmol O_2 (g FW)$^{-1}$ h^{-1})	K_m (μM C_i)	r	NPS_{max} (μmol O_2 (g FW)$^{-1}$ h^{-1})	K_m (μM C_i)	r
DIC+N+	20·6 ± 0·8	618 ± 51	0·82	35·1 ± 2·8	2627 ± 295	0·93
DIC+N−	14·6 ± 4·4	592 ± 49	0·99	32·2 ± 5·6	1815 ± 97	0·99
DIC−N+	8·9 ± 1·2	203 ± 21	0·99	26·5 ± 1·9	1732 ± 41	0·99
DIC−N−	6·6 ± 1·8	156 ± 42	0·94	29·4 ± 4·3	2098 ± 81	0·99

Data are presented taking into consideration the two phases of the dual kinetic pattern of the photosynthesis–DIC curves (high- and low-affinity phases). Data were calculated by fitting the Michaelis–Menten equation applying an iterative procedure. Data are presented as mean ± SD ($n = 2$).

limitation, resulting in a significant decline in the cellular photosynthetic capacity (Turpin, 1991). However, in *Gracilaria* sp. the Rubisco content was maintained practically constant under low N conditions after 12 days of experiment. This indicates that *Gracilaria* sp. was not subjected to a strong N limitation in the low N treatments, since a slight increase in growth rate was obtained. The DIC enrichment caused a significant decrease in the Rubisco content but higher NPS_{max} values than at low DIC level, even under low N conditions. It could denote the existence of factors other than Rubisco levels controlling photosynthesis.

The biphasic pattern of the P–C curves in *Gracilaria* sp. can be supported by the occurrence of a C-concentrating mechanism that involves two different DIC acquisition pathways: an indirect use by an external carbonic anhydrase activity (CA) and a direct use mediated by an anion exchange protein (Andría *et al.*, 1999). The potential contribution of the two DIC acquisition mechanisms decreases when *Gracilaria* sp. is cultured under low pH conditions (similar to high CO_2 levels), resulting in a reduction of the photosynthetic DIC affinity (Andría *et al.*, 1999). A slight decline in CA activity was also reported in *Gracilaria tenuistipitata* cultured under high CO_2 concentrations in the medium (Haglund & Pedersen, 1992). In our study, K_m was affected by DIC level for the high-affinity phase, being higher in a DIC-enriched culture medium. These results agree with those obtained by Giordano & Bowes (1997), who reported a lower affinity for DIC in *Dunaliella salina* cells grown at high CO_2 concentration. In addition, Matsuda & Colman (1996) reported K_m values significantly higher in high CO_2-grown *Chlorella ellipsoidea* than in air-grown cultures, the external CA activity being related to recognition mechanisms for DIC concentration in the medium.

With respect to NPS_{max}, in the high-affinity phase the highest values were obtained at a high DIC level under N sufficiency conditions. Similar results were obtained by Rivers & Peckol (1995), who found that under conditions of N sufficiency, DIC enrichment promoted an increase in NPS_{max} in *Cladophora vagabunda* and *Gracilaria tikvahiae*. Gordillo (1998) also reported an increase in NPS_{max} in the microalgae *Dunaliella viridis* and *Phaeodactylum tricornutum* under high CO_2 and N concentrations, while a decrease in NPS_{max} as a consequence of the high CO_2 concentration in the medium was found for the macroalgae *Ulva rigida* and *Porphyra leucosticta*. Furthermore, *Gracilaria tenuistipitata* cultured under 5% CO_2 displayed lower NPS_{max} values than under air CO_2 conditions (García-Sánchez *et al.*, 1994). These results reflect the heterogeneity of the algal photosynthetic response to different CO_2 regimes.

In conclusion, when *Gracilaria* sp. was cultured under different DIC and N conditions in a flow-through system, there were significant variations in the photosynthetic response and biochemical composition such as pigment content, soluble protein, Rubisco, and insoluble and soluble carbohydrates, indicating interactive effects between C and N metabolism. DIC enrichment affected N assimilation in *Gracilaria* sp., promoting a decrease in N uptake and a change in the allocation of the internal N compounds.

Acknowledgements

This work was financed by the projects AMB 96-0782 from the Ministerio Español de Educación y Cultura and 12/REGII/6/96 from INTERREG II. J.R.A. is supported by a grant from Consejería de Educación y Ciencia de la Junta de Andalucía. The authors thank Sebastián Tirado for his help in the arrangement of the experimental set-up, and Manuel Fernández-Prada for assistance with the nutrient analytical measurements.

References

Andría, J.R., Pérez-Lloréns, J.L. & Vergara, J.J. (1999). Mechanisms of inorganic carbon acquisition in *Gracilaria gaditana* nom. prov. (Rhodophyta). *Planta*, **208**: 564–573.

Beer, S. & Eshel, A. (1985). Determining phycoerythrin and phycocyanin concentrations in aqueous crude extracts of red algae. *Aust. J. Mar. Freshwater Res.*, **36**: 785–792.

Beer, S. & Koch, E. (1996). Photosynthesis of marine macroalgae and seagrasses in globally changing CO_2 environments. *Mar. Ecol. Prog. Ser.*, **141**: 199–204.

Bird, K.T., Habig, C. & De Busk, T. (1982). Nitrogen allocation and storage patterns in *Gracilaria tikvahiae* (Rhodophyta). *J. Phycol.*, **18**: 344–348.

Bowes, G. (1991). Growth at elevated CO_2: photosynthetic responses mediated through Rubisco. *Plant, Cell Environ.*, **14**: 795–806.

Bowes, G. (1993). Facing the inevitable: plants and increasing atmospheric CO_2. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **44**: 309–332.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of micrograms quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248–254.

Carmona, R., Vergara, J.J., Pérez-Lloréns, J.L., Figueroa, F.L. & Niell, F.X. (1996). Photosynthetic acclimation and biochemical responses of *Gelidium sesquipedale* cultured in chemostats under different qualities of light. *Mar. Biol.*, **127**: 25–34.

Drake, B.J., González-Meler, M.A. & Long, S.P. (1997). More efficient plants: a consequence of rising atmospheric CO_2? *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **48**: 609–639.

Droop, M.R. (1966). Vitamin B_{12} and marine ecology. II. An experiment with a chemostat. *J. Mar. Biol. Assoc. U.K.* **46**: 659–667.

García-Sánchez, M.J., Fernández, J.A. & Niell, F.X. (1994). Effect of inorganic carbon supply on the photosynthetic physiology of *Gracilaria tenuistipitata*. *Planta*, **194**: 55–61.

Gesch, R.W., Boote, K.J., Vu, J.C.V., Allen, H. & Bowes, G. (1998). Changes in growth CO_2 result in rapid adjustments of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene expression in expanding and mature leaves of rice. *Plant Physiol.*, **118**: 521–529.

Giordano, M. & Bowes, G. (1997). Gas exchange and C allocation in *Dunaliella salina* cells in response to the N source and CO_2 concentration used for growth. *Plant Physiol.*, **115**: 1049–1056.

Gordillo, F.J. (1998). Efecto del incremendo de CO_2 sobre el metabolismo del carbono y del nitrógeno en algas. PhD thesis, Universidad of Málaga.

Greene, R.M., Geider, R.J. & Falkowski, P.G. (1991). Effect of iron limitation on photosynthesis in a marine diatom. *Limnol. Oceanogr.*, **36**: 1772–1782.

Haglund, K. & Pedersen, M. (1992). Growth of the red alga *Gracilaria tenuistipitata* at high pH: influence of some environmental factors and correlation to an increased carbonic-anhydrase activity. *Bot. Mar.*, **35**: 579–587.

Hanisak, M.D. (1983). The nitrogen relationships of marine macroalgae. In *Nitrogen in the Marine Environment* (Carpenter, E.J. & Capone, D.G., editors), 699–730. New York: Academic Press.

Huppe, H.C. & Turpin, D.H. (1994). Integration of carbon and nitrogen metabolism in plant and algal cells. *Annu. Rev. Plant. Physiol. Plant Mol. Biol.*, **45**: 577–607.

Johnston, A.M. & Raven, J.A. (1990). Effects of culture in high CO_2 on the photosynthetic physiology of *Fucus serratus*. *Br. Phycol. J.*, **25**: 75–82.

Kochert, G. (1978). Carbohydrate determination by the phenol sulfuric acid method. In *Handbook of Phycological Methods: Physiological and Biochemical Methods* (Hellebust, J.A. & Craigie, J.S., editors), 95–97. Cambridge: Cambridge University Press.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **277**: 680–685.

Lapointe, B.E. & Duke, C.S. (1984). Biochemical strategies for growth of *Gracilaria tikvahiae* (Rhodophyta) in relation to light intensity and nitrogen availability. *J. Phycol.*, **20**: 488–495.

Lara, C., Romero, J.M., Coronil, T. & Guerrero, M.G. (1987). Interactions between photosynthetic nitrate assimilation and CO_2 fixation in cyanobacteria. In *Inorganic Nitrogen Metabolism* (Ullrich, W.R., Aparicio, P.J., Syrett, P.J. & Castillo, F., editors), 45–52. Berlin: Springer.

Matsuda, Y. & Colman, B. (1996). Active uptake of inorganic carbon by *Chlorella saccharophyla* is not repressed by growth in high CO_2. *J. Exp. Bot.*, **47**: 1951–1956.

Miller-Way, T. & Twilley, R.R. (1996). Theory and operation of continuous flow systems for the study of benthic–pelagic coupling. *Mar. Ecol. Prog. Ser.*, **140**: 257–269.

Monod, J. (1950). La technique de culture continué: théorie et applications. *Ann. Inst. Pasteur Paris*, **79**: 390–410.

Raven, J.A. (1991). Physiology of inorganic C acquisition and implications for resource use efficiency by marine phytoplankton: relation to increased CO_2 and temperature. *Plant Cell Environ.*, **14**: 779–794.

Riebesell, U., Wolf-Gladrow, D.A. & Smetacek, V. (1993). Carbon dioxide limitation of marine phytoplankton growth rates. *Nature*, **361**: 249–251.

Rintamaki, E.A., Keys, A.J. & Parry, M.A. (1988). Comparison of the specific activity of ribulose-1,5-bisphosphate carboxylase/oxygenase from some C_3 and C_4 plants. *Physiol. Plant.*, **74**: 326–331.

Rivers, J.S. & Peckol, P. (1995). Interactive effects of nitrogen and dissolved inorganic carbon on photosynthesis, growth, and ammonium uptake of the macroalgae *Cladophora vagabunda* and *Gracilaria tikvahiae*. *Mar. Biol.*, **121**: 747–753.

Sheen, J. (1994). Feedback control of gene expression. *Photosynth. Res.*, **39**: 427–338.

Shinn, M.B. (1941). A colorimetric method for the determination of nitrite. *Ind. Eng. Chem. Anal. Edn.*, **13**: 33–35.

Smith, P.K., Krohn, R.I., Hermanson, T.I., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenck, D.C. (1985). Measurements of protein using bicinchoninic acid. *Anal. Biochem.*, **150**: 76–85.

Talling, J.F. & Driver, D. (1963). Some problems in the estimation of chlorophyll *a* in phytoplankton. Proceedings of a Conference on Primary Productivity Measurement, Marine and Freshwater, Hawaii, USA. *Atomic Energy*, **7633**: 142–146.

Turpin, D.H. (1991). Effects of inorganic N availability on algal photosynthesis and carbon metabolism. *J. Phycol.*, **27**: 14–20.

Turpin, D.H., Elrifi, I.R., Birch, D.G., Weger, H.G. & Holmes, J.J. (1988). Interactions between photosynthesis, respiration and nitrogen assimilation in microalgae. *Can. J. Bot.* **66**: 2083–1087.

Valiela, I., McClelland, J., Hauxwell, J., Behr, P.J., Hersh, D. & Foreman, K. (1997). Macroalgal blooms in shalllow estuaries: controls and ecophysiological and ecosystem consequences. *Limnol. Oceanogr.*, **42**: 1105–1118.

Vergara, J.J. & Niell, F.X. (1993). Effects of nitrate availability and irradiance on internal nitrogen constituents in *Corallina elongata* (Rhodophyta). *J. Phycol.*, **29**: 285–293.

Vergara, J.J., Niell, F.X. & Torres, M. (1993). Culture of *Gelidium sesquipedale* (Clem.) Born. *et* Thur. in a chemostat system: biomass production and metabolic responses affected by N flow. *J. Appl. Phycol.*, **5**: 405–415.

Webber, A.N., Nie, G.Y. & Long, S.P. (1994). Acclimation of photosynthetic proteins to rising atmospheric CO_2. *Photosynth. Res.*, **39**: 413–425.

Woelkerling, W.J., Spencer, K.G. & West, J.A. (1983). Studies on selected Corallinaceae (Rhodophyta) and other algae in a defined marine culture medium. *J. Exp. Mar. Biol. Ecol.*, **67**: 61–77.

Wood, E.D., Armstrong, F.A.J. & Richards, F.A. (1967). Determination of nitrate in seawater by cadmium–copper reduction to nitrite. *J. Mar. Biol. Assoc. U.K.*, **47**: 23–31.