Vol. 223: 157–165, 2001 | MARINE ECOLOGY PROGRESS SERIES Mar Ecol Prog Ser | Published November 28

# Measurement of seagrass production using the $^{13}C$ stable isotope compared with classical $O_2$ and $^{14}C$ methods

**Miguel A. Mateo*, Pere Renom, Marten A. Hemminga, Jan Peene**

**Netherlands Institute of Ecology, Centre for Estuarine and Coastal Ecology, Korringaweg 7, 4401 NT Yerseke, The Netherlands**

ABSTRACT: The methods currently employed for seagrass production estimates include biomass, marking and metabolic techniques, the latter including the $O_2$ and $^{14}C$ methods. Both are currently in general use and have generated a great deal of seagrass-production data. In this work, we measured carbon incorporation rates in *Zostera marina* L. using the stable carbon isotope $^{13}C$ as a metabolic tracer. We tried this method in an attempt to overcome the 2 major methodological problems of the $O_2$ and $^{14}C$ methods, i.e. the limitations when measuring low production rates and the hazardous and laborious handling of radioactive isotopes, respectively. To validate the $^{13}C$ method we compared it with the classical $O_2$ and $^{14}C$ methods in microcosm experiments. The 2 carbon-tracer techniques were in overall good agreement ($^{13}C = 0.12 + 1.03 \times {}^{14}C$; $R^2 = 0.964$, $p < 0.001$, $n = 10$). Production rates derived from the $^{13}C$ method were on average $1.34 \pm 0.03$ ($n = 42$) times higher than those obtained with the $O_2$ method ($^{13}C = 0.99 + 1.15 \times O_2$; $R^2 = 0.788$, $p < 0.001$, $n = 42$), suggesting that for short incubation lengths (in this study between 0.5 and 4.0 h) the $^{13}C$ method provides estimates very close to gross production. The pros and cons of all 3 methods are critically discussed.

KEY WORDS: Seagrass production · *Zostera marina* · Stable carbon isotopes · $O_2$ method · $^{14}C$ method · $^{13}C$ method

*Resale or republication not permitted without written consent of the publisher*

## INTRODUCTION

The direct methods currently in use to estimate seagrass productivity are (1) measurement of biomass differences at certain time intervals, (2) marking of different parts of a plant followed by determination of growth increments and (3) metabolic techniques (e.g. Phillips & McRoy 1980, Larkum et al. 1989). Indirect methods include lepidochronology (Pergent et al. 1989) and the plastochrone method (the measurement of internode numbers: Duarte et al. 1994). Biomass techniques provide useful productivity estimates only if the species under study have a marked annual life cycle, and if the losses of plant matter as a consequence of herbivory, mechanical breaking or biomass turnover between observation times can be neglected (Kirkman & Cook 1987). This is by no means the case for many seagrass species, resulting in the underestimation of productivity when such methods are used. Despite these limitations, Methods 1 and 2 give valuable information when a time-integrated production estimate is required.

The marking technique (Zieman 1974) is the most generally accepted method of obtaining direct net seagrass production estimates. Unfortunately, certain leaf morphologies and sizes (e.g. those of *Halodule* sp., *Syringodium* sp., *Zostera noltii* or some forms of *Z. marina*) do not allow adequate marking (see Bittaker & Iverson 1976 and references therein). Practical disadvantages, furthermore, are the amount of underwater (marking and relocation of marked samples) and laboratory work required and the long time period necessary to measure appreciable growth (at least 4 d, Zieman 1975).

*Present address: Departament d'Ecologia, Universitat de Barcelona. Diagonal 645, 08028 Barcelona, Spain.
E-mail: mateo@porthos.bio.ub.es

© Inter-Research 2001 · www.int-res.com

Metabolic techniques that follow the photosynthetic evolution of oxygen or uptake of carbon dioxide/bicarbonate suffer from various technical and interpretational difficulties. The main problems concern the unknown extent of internal recycling or storage of gases within the extensive lacunal system of seagrasses and, in the case of field measurements, the difficulties in discriminating the gas flux actually due to seagrass metabolism from that of faunal and bacterial respiration (Hillman et al. 1990 and references therein).

The isotopic technique based on $^{14}C$ uptake developed by Steeman Nielsen (1952) to measure phytoplankton productivity was adapted by Wetzel (1964, 1965) to aquatic vascular plants. It has since been applied to several seagrasses (e.g. McRoy 1974, Bittaker & Iverson 1976, Lindeboom & de Bree 1982, Moncreiff et al. 1992) and to many freshwater submersed vascular plants (e.g. Lipkin et al. 1986). The $^{14}C$ technique has proved to be very comparable to the marking techniques when appropiate corrections are applied (Bittaker & Iverson 1976), and has the advantages of higher sensitivity and much shorter incubation times (e.g. McRoy 1973). In addition, it can be universally applied to all autotrophic components of the marine ecosystem (phytoplankton, benthic microflora, macroalgae, epiphytes, and seagrasses).

Understandably, the use of $^{14}C$ in the natural environment has been virtually abandoned in recent years, because of the associated radioactive hazards. Moreover, radioactivity handling requires that the operators have special training and permits. In the field of phytoplankton research, such limitations have led to attempts to replace the radioactive carbon isotope ($^{14}C$) with the stable carbon isotope ($^{13}C$). Slawyk et al. (1977) were the first to demonstrate a good agreement between $^{14}C$ and $^{13}C$ techniques in experiments with marine phytoplankton. This agreement has since been confirmed by other authors (Sakamoto et al. 1984, Slawyk et al. 1984, Mousseau et al. 1995). One interesting feature of the $^{13}C$ method is that it can be easily coupled (i.e. in the same incubation bottles or incubation bells) with uptake measurements of various $^{15}N$-labelled compounds (Dugdale & Goering 1967), permitting insight into the interactions between photosynthesis and inorganic nitrogen assimilation (Slawyk et al. 1977).

In this paper we explore the possibilities of using the stable isotope $^{13}C$ to measure carbon uptake rates in the seagrass *Zostera marina*. First, changes in carbon uptake rates effected by (1) the amount of isotopic addition and (2) the duration of the incubation have been assessed. Second, the $^{13}C$ method has been compared to the classical $O_2$ and the $^{14}C$ methods in microcosm experiments. The pros and cons of all 3 methods are critically discussed.

## MATERIALS AND METHODS

Seedling eelgrass *Zostera marina* L. were harvested in an intertidal meadow in Zandkreek Bay, Oosterschelde estuary, SW Netherlands, in the summer of 1996. The shoots were transplanted the same day to a plastic flow-through tank (75 × 50 × 40 cm) in a climatized room, rooted in their own sediment. Light (60 µE $m^{-2}$ $s^{-1}$), aeration and Oosterschelde seawater (temperature 16°C, renewal rate 2 times $d^{-1}$) were supplied to the tank. The seedlings were allowed to acclimate to mesocosm conditions for 2 wk before experimental use.

**Preliminary experiments.** Three different experiments were performed to assess the adequacy of the experimental setup before attempting comparisons between methods.

In the first 2 experiments, 7 to 8 pieces of ca 7 cm of epiphyte-free green leaves (total dry weight ranging from 35 to 85 mg) were cut off the shoots and incubated in Winkler bottles (ca 294 ml) under magnetic stirring (stirring speed set just below turbulent flux). Incubations were performed in filtered (Millipore 0.2 µm) Oosterschelde water from a single large container (dissolved inorganic carbon [DIC] = 30 ± 0.6 mg C $l^{-1}$, n = 6, aliquots; $\delta^{13}C$ = –0.86 ± 0.12‰, n = 3 aliquots; means ± SE error; see 'Analysis of stable carbon isotopes and carbon fractions'). Incubations were performed in a climatized chamber at 16°C and 174 to 265 µE $m^{-2}$ $s^{-1}$ (spatial variability within the chamber). This range is at the top end of the light intensity required for saturating photosynthesis in *Zostera marina* (e.g. Marsh et al. 1986, Goodman et al. 1995, Zimmerman et al. 1995). Samples were pre-incubated for 1 h to minimize stress effects that could affect photosynthetic performance (e.g. wounds after cutting the leaves into fragments or sudden changes in temperature and irradiance).

In the first experiment, the water was amended with 2.5, 5, 10 or 20 mg $l^{-1}$ $NaH^{13}CO_3$ (99%, Sigma) to study the effect of carbon addition on carbon incorporation rates. Three replicates of each treatment, including control bottles (samples incubated in light bottles without isotopic addition) and dark bottles (samples incubated in opaque bottles with isotopic addition), were incubated for 3 h. In the second experiment, the samples were incubated for 0.5, 2, 4, or 6 h to check for a possible effect of incubation length on carbon incorporation rates. Six replicates (except for the control and dark incubations, where n = 3) per incubation time were incubated in seawater amended with 10 mg $l^{-1}$ $NaH^{13}CO_3$.

To ascertain the linearity of photosynthetic production under constant incubation conditions, a time-course experiment using the $O_2$ method was performed with a single Winkler bottle (ca 500 ml) adapted to allow air-tight fitting with an oxygen electrode. A 6 h

incubation was carried out, during which the $O_2$ concentration was measured every 10 or 15 min using an oxygen meter (Eijkelkamp Z621) fitted with a polarographic oxygen electrode (Eijkelkamp 9009/2). Light, temperature and stirring conditions were as above.

At the end of the incubations, plant material was immediately recovered from the bottles, washed with seawater, and briefly rinsed with deionized water to flush any isotopic label off the leaf surfaces. The samples were subsequently oven-dried at 60°C until constant weight, and ground to powder. Prior to each experiment, 6 groups of leaves from the same batch were kept apart for isotopic analysis. Because the plant material used in the incubations was essentially epiphyte-free, samples were not acidified to eliminate carbonates prior to isotopic analysis.

**$O_2$ method versus $^{13}C$ method.** An experiment consisting of 5 series of incubations, on different days, at different temperatures (11.4 to 20.5°C), and using samples from different *Zostera marina* batches, compared the $O_2$ and $^{13}C$ methods. The general experimental conditions were the same as those for the preliminary experiments. Carbon incorporation rates were measured by both methods in each incubation bottle. Isotopic addition was 10 mg $l^{-1}$ $NaH^{13}CO_3$ and incubation time 3 h. The characteristics of the water for incubation were the same as described above.

After the incubation period, the plant material was removed and the $O_2$ concentration in the medium immediately measured. Leaf samples were treated as above.

**$^{13}C$ method versus $^{14}C$ method.** To compare the $^{13}C$ method with the homologous metabolic technique of the $^{14}C$ method, a fifth experiment was designed. Whereas the $O_2$ and $^{13}C$ techniques could be compared in the same incubation bottles, the $^{13}C$ and $^{14}C$ techniques necessitated the use of separate bottles. (Simultaneous employment might contaminate the mass spectrometer when measuring the $^{13}C/^{12}C$ ratio of the $^{14}C$-containing samples.) The incubations for this experiment were therefore performed in an incubator fitted with 10 rotating discs placed at regular, increasing distances from a source of light and immersed in a temperature-controlled water bath. Each disc was provided with 10 bottle holders (for details of the incubator see Kromkamp & Peene 1995). About 5 pieces of *Zostera marina* leaf blades ca 3 cm in length (7 to 12 mg dry weight) were incubated in 50 ml glass bottles at 16°C for 3 h at different light intensities ranging from 13 to 515 µE $m^{-2}$ $s^{-1}$. Sample weight was reduced to maintain the water volume to leaf sample ratio of the previous experiments. For all 10 light treatments, 3 replicates were incubated in filtered Oosterschelde water (Millipore 0.2 µm; DIC = 36 ± 0.8 mg C $l^{-1}$, n = 6, aliquots) amended with 10 µCi $l^{-1}$ $NaH^{14}CO_3$ (Amersham), and 3 other replicates with 10 mg $l^{-1}$ $NaH^{13}CO_3$ (Sigma); 3 bottles without addition served as blanks. Non-photosynthetic incorporation (dark uptake, passive incorporation or adsorption of the isotopic label) was checked by including 3 dark bottles with labelled medium either amended with $^{13}C$ or $^{14}C$. Directly after incubation, 4 ml of a buffered formaline 4% solution were added to all $^{14}C$-amended bottles to stop photosynthetic activity (Moncreiff et al. 1992). The material incubated in the $^{13}C$-enriched medium was treated as described in the previous subsection. The plant material incubated in the radioactive medium was ground in a porcelain mortar before being taken up in scintillation solvent (0.5% w/v, PPO 2,5-diphenyloxazol: Merk; in technical-grade toluene: Baker). Radioactivity was counted in a Beckmann LSC (LS5000TD). Quenching was corrected by automatic external standardization. No correction was applied for self-quenching of undissolved material.

**Analysis of stable carbon isotopes and carbon fractions.** Water samples for DIC $\delta^{13}C$ determination were collected in 200 ml dark glass bottles. Water was sterilized by adding 10 drops (5 drops per 100 ml sample) of an $I_2$-KI solution (1.5 g $I_2$ and 3 g KI in 100 ml). Bottles were completely filled, airtight-sealed, and stored in the dark at 5°C until analysis. DIC was isolated from the water samples by acidification (85% $H_3PO_4$) and stripping under vacuum following Mook (1968, 1970). The $CO_2$ obtained was then dried prior to the determination of the carbon isotopic composition using an isotope-ratio mass spectrometer Micromass SIRA-9. The mass spectrometer internal error was 0.05‰, and the total analytical error (i.e. analytical precision) 0.1‰. The $\delta^{13}C$ of the plant material was determined with a Finnigan Delta S isotope-ratio mass spectrometer (IRMS) equipped with a Conflo II interface (internal error 0.05‰) with an overall analytical error of 0.13‰. The above-described IRMS equipment requires a minimum amount of organic carbon in the sample (ca 10 µg). Because the carbon content of *Zostera marina* is ca 35%, the minimum amount of plant material required for isotopic analysis is ca 29 µg. In this study, ca 1 mg of powdered dried sample was combusted (at 1000°C in the presence of oxygen and $V_2O_5$) for each determination. $\delta^{13}C$ values are given in common delta notation:

$$\delta^{13}C(‰) = \frac{R_{sa} - R_{std}}{R_{std}} \times 1000 \quad (1)$$

where $R$ is the isotopic ratio $^{13}C/^{12}C$ of the sample (sa) or of the standard (std) VPDB.

DIC concentration was determined by potentiometric titration. The total carbon concentration in the plant material was analyzed using an elemental analyzer (Fisons NA1500).

**Numerical procedures.** Uptake rates ($P$) determined with the $^{13}C$ method were calculated using an equation derived from Hama et al. (1983):

$$P\left(\text{mg C g}^{-1}\text{ dry wt h}^{-1}\right) = \frac{C\cdot\left(a_{is}-a_{ns}\right)}{t\cdot\left(a_{ic}-a_{ns}\right)}\cdot\frac{V}{\text{DW}} \qquad (2)$$

where $a_{is}$ is the at.% of $^{13}C$ in the incubated sample, $a_{ns}$ the at.% of $^{13}C$ in the natural sample, $a_{ic}$ the at.% of $^{13}C$ in the total inorganic carbon (incubation medium), $C$ the particulate organic carbon (POC, mg C) in the incubated sample, $t$ the incubation time (h), $V$ the incubation volume (litres), and DW the dry weight (mg) of the incubated sample. The variable $a_{ic}$ was calculated as the $^{13}C$ at.% of the DIC of the seawater (measured) plus the amount of tracer added (calculated). No correction factor for isotopic discrimination against $^{13}C$ has been applied to the calculations.

Uptake using the $^{14}C$ technique was calculated following Penhale (1977):

$$P\left(\text{mg C g}^{-1}\text{ dry wt h}^{-1}\right) = \frac{\text{dpm}_{is}\cdot\text{DIC}}{\text{DW}\cdot\text{dpm}_{added}\cdot t} \qquad (3)$$

where $\text{dpm}_{is}$ and $\text{dpm}_{added}$ are the disintegrations per minute of the incubated sample and those of the tracer added to the incubation medium, respectively, DIC is the dissolved inorganic carbon (mg) in the water (before isotopic addition), DW the dry weight (mg) of the sample, and $t$ the incubation time (h).

The significance of differences between treatments in the various experiments was tested using Student's $t$-tests. A standard linear regression model was used for comparison of methods (Model II in Sokal & Rohlf 1981). Oxygen data were converted to carbon equivalents by assuming a photosynthetic quotient (PQ), $O_2$/C, of 1.29 (Sepers 1981, Lindeboon & de Bree 1982, Oviatt et al. 1986; see also discussions on PQs in Williams et al. 1979 and in Laws 1991).

## RESULTS AND DISCUSSION

### Preliminary experiments

#### Control and dark incubations

A small but significant increase in $\delta^{13}C$ ($\Delta\delta^{13}C$ = 1.41 ± 0.12‰, $p < 0.01$, n = 3) was observed in the *Zostera marina* samples from the dark bottles (incubated for 3 h in a medium amended with 10 mg $NaH^{13}CO_3$ $l^{-1}$). Radioactivity was also significantly higher in the samples from the dark $^{14}C$ incubations than in the corresponding control samples ($\Delta$dpm = 292.4 ± 8.1 dpm, $p < 0.01$, n = 3). Both results evidence the involvement of light-independent phenomena that lead to carbon enrichment of the samples. The increase in the final production estimates derived from this enrichment ranged from 0.014 to 0.047 mg C $g^{-1}$ dry wt $h^{-1}$. This represented 0.1 to 11.8% of the total carbon incorporation, being <1% in 80% of the measurements.

With carbon isotopic techniques, dark bottles integrate all processes leading to label incorporation by the samples in the absence of light. The exact processes responsible for such incorporation are still a matter of controversy, and whether the observed incorporation rates should be subtracted or ignored remains unclear (Peterson 1980, Legendre et al. 1983). An increase in $^{13}C$ in a sample during dark incubation could be due to both metabolic and non-metabolic processes. Experimental evidence suggests that fixation in phytoplankton can proceed in the darkness, with a certain dependence upon the fixation rate prior to light deprivation, and that enzymatically driven reactions may be involved (Syrett 1962, Lean & Burnison 1979, Legendre et al. 1983). A similar pattern has been described for marine macrophytes, including some seagrass species, by Cabello-Pasini & Alberte (1997), who reported a maximum light-independent carbon fixation rate always lower than 5% of $P_{max}$. This observation is consistent with our results. Only a very small portion of the label measured in the dark-incubated samples can be attributed to non-metabolic $^{13}C$ incorporation (i.e. adsorption), since the anion-exchange capacity of plant and algal cell walls is very low. Label accumulation in the external surface of the plant samples is also unlikely, because the leaf fragments were thoroughly rinsed with unlabelled seawater and then briefly rinsed with deionized water prior to isotopic determination. A brief exposure of samples to acid fumes prior to isotopic analysis would help minimize eventual label adsorption to carbonated particles. Because in the present experiments dark uptake was usually very low (<1% of total carbon incorporation), it has been ignored in the calculations of carbon incorporation rates. The fact that the standard error calculated for the production estimates (± 0.7 mg C $g^{-1}$ dry wt $h^{-1}$) was in most cases much higher than the dark uptake values (0.04 mg C $g^{-1}$ dry wt $h^{-1}$) supports this decision.

The $\delta^{13}C$ values of the control samples (light bottle incubations without $^{13}C$ addition) did not significantly differ from the initial $\delta^{13}C$ values of the samples except in 1 case, in which a significant increase was observed (0.8‰: Table 1). The almost complete lack of change in the control samples indicates that any change observed in the $\delta^{13}C$ values of the $^{13}C$-incubated samples was due to the uptake of $^{13}C$ from the incubation medium and that no other phenomena leading to changes in the $^{13}C/^{12}C$ ratio of the plant were involved.

Table 1. *Zostera marina*. Differences in carbon isotope composition $\Delta\delta^{13}C$ (‰) of samples (n = 6) in the control incubations during the various incubation periods. *Significant difference between initial and final $\delta^{13}C$ values

| Incubation time (h) | $\delta^{13}C$ (‰) Initial | Final | $\Delta\delta^{13}C$ (‰) | p |
|---|---|---|---|---|
| 0.5 | –11.69 | –11.44 | 0.25 | 0.186 |
| 0.5 | –12.99 | –12.63 | 0.36 | 0.494 |
| 1.0 | –13.08 | –13.09 | –0.01 | 0.758 |
| 1.0 | –12.24 | –12.50 | –0.26 | 0.479 |
| 2.0 | –11.07 | –11.08 | –0.01 | 0.814 |
| 3.0 | –11.86 | –11.05 | 0.81 | 0.003* |
| 4.0 | –11.74 | –11.61 | 0.13 | 0.417 |
| 6.0 | –11.06 | –10.78 | 0.28 | 0.085 |

## Effect of carbon addition on uptake rates

An increase in productivity can potentially be expected as the consequence of a fertilization effect resulting from the addition of carbon to the incubation medium (Beer & Waisel 1979, Durako 1993, Beer & Koch 1996, Björk et al. 1997). Our results, however, showed no significant effect of additions in the range 2.5 to 20 mg $NaH^{13}CO_3$ $l^{-1}$, which represents 1.3 to 10.2% of the natural DIC concentration in the incubation medium ($p = 0.87$, n = 3; Fig. 1). This suggests that the widely accepted rule of a $^{13}C$ addition of ca 10% of the ambient DIC was also appropriate for our experimental conditions. It is notable that even in the incubations with the smallest label addition (2.5 mg $l^{-1}$ $NaH^{13}CO_3$, i.e. 0.4 mg $^{13}C$ $l^{-1}$) the $\delta^{13}C$ content of the incubated leaf samples increased conspicuously by about 70‰. As the overall analytical procedures used for $\delta^{13}C$ determinations in this work allow the detection of changes as small as 0.13‰, it follows that even lower tracer additions could be expected to yield adequate $^{13}C$ sample enrichment for isotopic analysis.

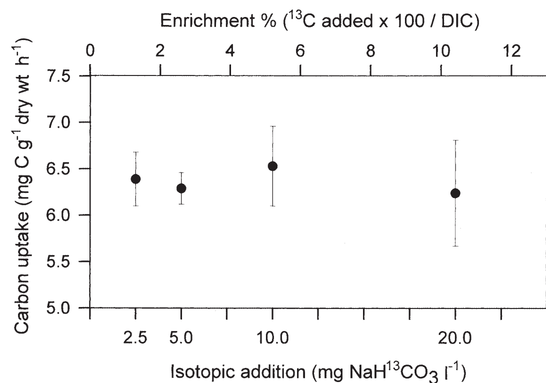


Fig. 1. *Zostera marina*. Response of carbon incorporation rates (means ± SE, n = 3) to increasing $NaH^{13}CO_3$ additions. Incubations were performed in a climatized chamber at 16°C and 174 to 265 µE $m^{-2}$ $s^{-1}$ (range of light variability within chamber) over 3 h. Percent carbon enrichment represented by the $^{13}C$ addition with respect to the natural DIC

## Effect of incubation time on uptake rates

Any method measuring the productivity of confined samples whereby the incubation medium is not renewed may result in underestimation of the uptake rates as a consequence of nutrient exhaustion and/or deterioration of sample vitality. The latter may be especially relevant when leaf fragments instead of entire plants are used for the incubations. The second preliminary experiment ($^{13}C$ uptake vs incubation time, see 'Materials and methods') showed a linear response of carbon uptake between 1 and 4 h incu-bation (Fig. 2: top graph). The uptake rate after 6 h incubation was not linear (lower) and showed the lowest replicability. The time-course experiment ($O_2$ evolution vs incubation time in a single incubation bottle, see 'Materials and methods'), however, showed an excellent linear response for 10 min to 6 h incubation times ($R^2 = 0.994$, $p < 0.001$, n = 24; Fig. 2: bottom graph). This last result indicates that no nutrient exhaustion or plant vitality decrease was significantly affecting the photosynthetic

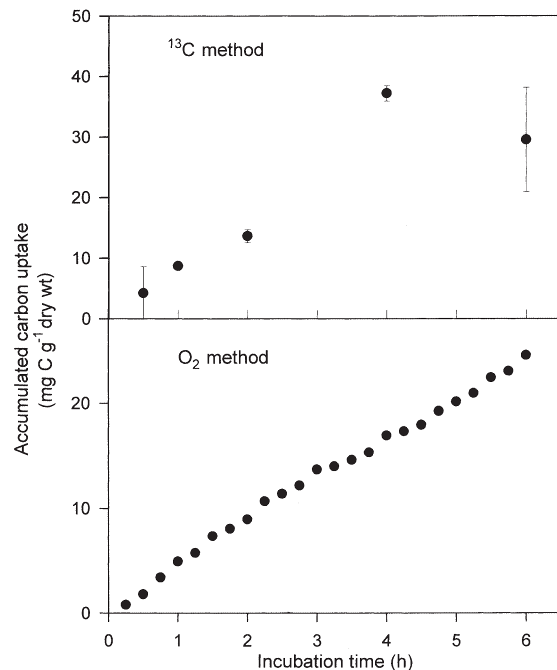


Fig. 2. *Zostera marina*. Top graph: response of carbon incorporation (means ± SE, n = 3) to increasing incubation time estimated by $^{13}C$ method (incubation water was amended with 10 mg $NaH^{13}CO_3$ $l^{-1}$); bottom graph: time-course of $O_2$ evolution; oxygen concentration was measured every 10 to 15 min for 6 h in a single incubation bottle. Oxygen data converted to carbon equivalents

performance of the incubated samples for incubation periods up to 6 h. The significant loss of linearity in the 6 h incubation ($^{13}C$ uptake experiment) is discussed further below.

## Methods comparison

### $O_2$ method versus $^{13}C$ method

In each of the bottles from the 5 series of incubations performed for comparing the 2 methods, carbon incorporation rates were estimated by measuring the increase of $O_2$ dissolved in the water and of $^{13}C$ in the plant tissues. Both methods were significantly correlated ($^{13}C = 0.99 + 1.15 \times O_2$; $R^2 = 0.788$, $p < 0.001$, $n = 42$; Fig. 3: top graph) but production rates derived from the $^{13}C$ method were on average 1.34 ± 0.03 (n = 42) times higher than those obtained with the $O_2$ method. This difference supports what seems to be one of the few points of agreement between marine biologists who have dealt with the problem of interpreting $^{14}C$-derived carbon uptake values, i.e. that the $^{14}C$ method measures something between net and gross productivity (e.g. Peterson 1980, Lindeboom & de Bree 1982 and references therein). It is also generally accepted that the $O_2$ method approximates net productivity.

Although the $^{14}C$ method is one of the most widely used, both in freshwater and marine studies, we are not aware of any authoritative statement in the literature concerning its overall accuracy. This uncertainty is mainly attributed to the unknown degree of carbon refixation that occurs during photosynthesis and that leads to unknown degrees of underestimates of carbon uptake rates (Lean & Burnison 1979, Peterson 1980, Legendre et al. 1983, Larkum et al. 1989). We are not aware of any publication comparing the $^{13}C$ method with the $O_2$ method. Those studies which have compared the $O_2$ method with the $^{14}C$ method have reported different degrees of agreement between them, usually finding that the $^{14}C$ method substantially underestimates carbon incorporation rates relative to the $O_2$ method (Bittaker & Iverson 1976, Williams et al. 1979, Peterson 1980, Lindeboom & de Bree 1982). Owing to the homology of the $^{13}C$ and $^{14}C$ methods, the aforementioned arguments can also apply to the comparison between the $O_2$ and $^{13}C$ methods.

Our data suggest that underestimation of uptake in the C-tracer methods due to carbon refixation is not sufficiently important to override the increased production rates (relative to the $O_2$ method) resulting from the fact that these methods give rates closer to gross than to net productivity. Lindeboom & de Bree (1982) measured the gross and net productivity of eelgrass in

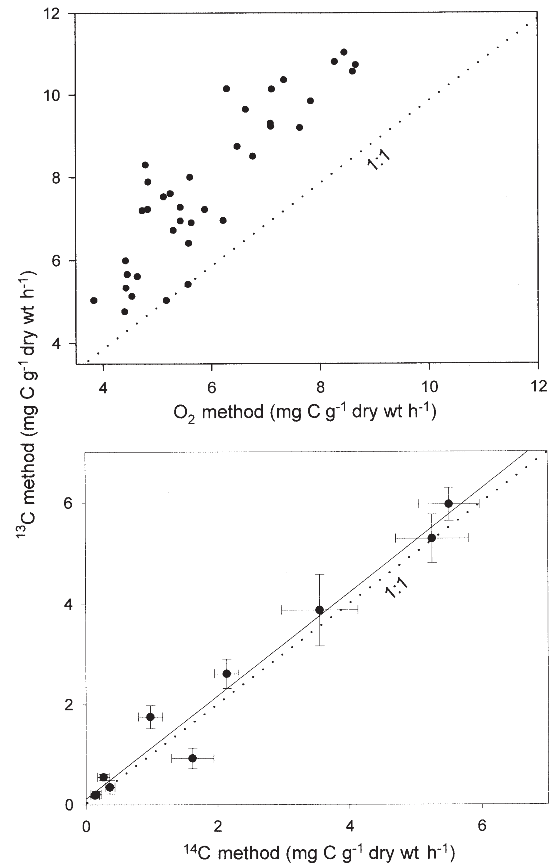


Fig. 3. *Zostera marina*. Top graph: comparison between carbon incorporation rates obtained when $^{13}C$ and $O_2$ methods were applied simultaneously in each incubated bottle ($^{13}C = 0.99 + 1.15 \times O_2$; $R^2 = 0.788$, $p < 0.001$, $n = 42$); bottom graph: comparison between carbon incorporation rates obtained with $^{13}C$ and $^{14}C$ methods ($^{13}C = 0.12 + 1.03 \times {}^{14}C$; $R^2 = 0.964$, $p < 0.001$, $n = 10$). Dotted lines represent 1:1 proportions

Lake Grevelingen (SW Netherlands) using the $O_2$ method. From results in their Table III, we have inferred that gross productivity was an average of 1.41 times higher than net productivity, a factor very close to that obtained in this study when comparing the $O_2$ and $^{13}C$ methods (1.34; Fig. 3: top graph). This strongly suggests that in this work the estimates achieved by the $^{13}C$ method were very close to gross productivity, and indicates that in the 3 h incubation experiments all isotopically labelled carbon was incorporated by the plants, with no respiration or refixation processes substantially affecting the final production rates. This contrasts with the results of other studies which reported serious underestimations of productivity attributable to $O_2$ accumulation in macrophyte lacunal storage systems (McRoy & McMillan 1977, Zieman & Wetzel 1980). It also contrasts with observations that $O_2$ production and consumption rates in eelgrass showed an immediate and linear response over several hours in

response to provision and withdrawal of light, respectively, suggesting that the influence of the lacunal storage system was negligible (Lindeboom & de Bree 1982).

In the context of the above, another complicating factor may be incubation length, upon which the relationship between the $O_2$ and $^{13}C$ methods is likely to depend. A short-term incubation (e.g. of 3 h) is likely to reflect only the influx of $^{13}C$. Respiratory losses of $^{13}C$ are probably negligible because of the low $^{13}C$ content of plant material. The production rate determined by the $^{13}C$ method may reflect net productivity only when isotopic equilibrium between inorganic carbon and plant material is established, a process requiring longer incubation times. In summary, the higher estimates obtained using the $^{13}C$ method compared to the $O_2$ method in this study may reflect the fact that isotopic equilibrium had not been achieved, and therefore the estimates were closer to gross than to net production. The decrease in accumulated carbon incorporation after 6 h incubation (Fig. 2: top graph) may reflect an evolution towards isotopic equilibrium, i.e. towards net production.

### $^{13}C$ method versus $^{14}C$ method

The techniques based on isotopically labelled carbon showed an overall good statistical agreement ($^{13}C = 0.12 + 1.03 \times {}^{14}C$; $R^2 = 0.964$, $p < 0.001$, $n = 10$; Fig. 3: bottom graph) as would be expected from a comparison of homologous methods.

### Method sensitivity

A major disadvantage of the $O_2$ method lies in its inability to measure low production rates. The resolution of a typical research oxygen-meter is 0.1 mg $O_2$ $l^{-1}$ (as was the case for that used in this study). In our experiments, a variation of 0.1 mg $O_2$ $l^{-1}$ represented a variation of around 0.3 mg C $g^{-1}$ dry wt $h^{-1}$. The resolution of the $^{13}C$ method is about 2 orders of magnitude higher, potentially allowing the detection of variations as small as 0.004 mg C $g^{-1}$ dry wt $h^{-1}$ (i.e. a detectable variation in $^{13}C$ at.% excess enrichment of ≈0.001%).

### Other considerations

An important potential source of error in $O_2$ measurements is the formation of gas bubbles (largely $O_2$). This problem becomes particularly relevant at high irradiance levels (= high production rates) or when $O_2$ measurements are performed at the room temperature of the laboratory, with the latter much higher than the experimental incubation temperature, which can result in $O_2$ oversaturation of the incubation medium. To solve this problem in this study, we submerged the incubation bottles in a cold-water bath at the experimental temperature while measuring the oxygen concentration. Deterioration of the electrode membrane and the need for regular calibration and temperature compensation are other significant sources of error associated with the $O_2$ method (errors up to 10%: see discussion in Hootsmans & Vermaat 1994). Regular calibration with the Winkler method is recommended to minimize these types of error.

An important criticism of the $^{13}C$ method is its requirement for more laborious post-incubation procedures. However, an important simplification can be made without introducing any substantial error into the estimates. Determination of the $\delta^{13}C$ in the DIC of the *in situ* seawater (used as incubation medium) can be omitted if the added $^{13}C$ greatly exceeds the natural $^{13}C$ (Mousseau et al. 1995): a value typical for $\delta^{13}C$ in the DIC of the incubation water can be used instead. The sensitivity of the calculations with respect to such omission has been checked. To achieve a 1% change in carbon uptake rates, a change of about 34‰ would be required in the $\delta^{13}C$ of the natural *in situ* water. The total DIC in oceanic water has a $\delta^{13}C$ value of around 0‰ (e.g. Anderson & Arthur 1983, Galimov 1985, present study: –0.86‰). Substantial deviations from this average value occur only in extreme cases. An increase in $\delta^{13}C$ in marine DIC has been reported as a consequence of photosynthetic discrimination against $^{13}C$ (+3‰: Anderson & Arthur 1983), while $\delta^{13}C$ values lower than 0‰ may occur in marine coastal areas with significant riverine input. Because the lower limit of $\delta^{13}C$ in the DIC of river waters is around –15‰ (Fry & Sherr 1984), it follows that even in a marine area strongly influenced by riverine efflux the error introduced into productivity rates by assuming an oceanic $\delta^{13}C$ value for DIC of ca 0‰ will be always substantially lower than 1%.

## Final remarks

The $^{13}C$ method for estimating carbon uptake rates in *Zostera marina* in microcosm conditions was in overall good agreement with an homologous method using $^{14}C$. For short incubation periods (in this study, between 0.5 and 4.0 h), the $^{13}C$ method provides estimates very close to gross production. Our results provide strong evidence supporting the comparability of the C and $O_2$ methods.

The situation in the literature, however, is of a longstanding controversy, with an abundance of contradic-

tory results for the 3 methodological approaches. We therefore recommend caution in comparing production rates derived from the $^{13}C$ and $^{14}C$ methodological approaches, especially when combining them in ecosystem carbon-mass balances.

*Acknowledgements.* The authors are grateful to Dr Yves Collos for advice on numerical procedures, and to Joop Nieuwenhuize and Jos van Soelen for technical assistance. This work has benefited from the helpful comments of 6 anonymous referees. The work was funded by the European Union (Fellowship MAS2-CT-94-5022) and the Nederlands Instituut voor Oecologisch Onderzoek, Centrum voor Estuariene en Mariene Oecologie (NIOO-CEMO). Publication 2869 N100 Centre for Estuarine and Coastal Ecology, Yerseke, The Netherlands.

LITERATURE CITED

Anderson TF, Arthur RA (1983) Stable isotopes of oxygen and carbon and their application to sedimentologic and paleoenvironmental problems. In: Arthur MA, Anderson TF, Kaplan IR, Veizer J, Land LS (eds) Stable isotopes in sedimentary geology. Society of Economic Paleontologists and Mineralogists, Tulsa, OK

Beer S, Koch E (1996) Photosynthesis of marine macroalgae and seagrasses in globally changing $CO_2$ environments. Mar Ecol Prog Ser 14:199–204

Beer S, Waisel Y (1979) Some photosynthetic carbon fixation properties of seagrasses. Aquat Bot 7:129–138

Bittaker HF, Iverson RL (1976) *Thalassia testudinum* productivity: a field comparison of measurement methods. Mar Biol 37:39–46

Björk M, Weil A, Semesi S, Beer S (1997) Photosynthetic utilisation of inorganic carbon by seagrasses from Zanzibar, East Africa. Mar Biol 129:363–366

Cabello-Pasini A, Alberte RS (1997) Seasonal patterns of photosynthesis and light-independent carbon fixation in marine macrophytes. J Phycol 33:321–329

Duarte CM, Marbà N, Agawin N, Cebrián J, Enríques S, Fortes MD and 6 others (1994) Reconstruction of seagrass dynamics: age determinations and associated tools for the seagrass ecologist. Mar Ecol Prog Ser 107:195–209

Dugdale RC, Goering JJ (1967) Uptake of new and regenerated forms of nitrogen in primary productivity. Limnol Oceanogr 12:196–206

Durako MJ (1993) Photosynthetic utilization of $CO_{2(aq)}$ and $HCO_3^-$ in *Thalassia testudinum* (Hydrocharitaceae). Mar Biol 115:373–380

Fry B, Sherr EB (1984) Delta 13C measurements as indicators of carbon flow in marine and freshwater ecosystems. Contrib Mar Sci 27:13–47

Galimov EM (1985) The biological fractionation of isotopes. Academic Press, New York

Goodman JL, Moore KA, Dennison WC (1995) Photosynthetic responses of eelgrass (*Zostera marina* L.) to light and sediment sulfide in a shallow barrier island lagoon. Aquat Bot 50:37–47

Hama T, Miyazaki T, Ogawa Y, Iwakuma T, Takahashi M, Otsuki A, Ichimura S (1983) Measurement of photosynthetic production of a marine phytoplankton population using a stable $^{13}C$ isotope. Mar Biol 73:31–36

Hillman K, Walker DI, Larkum AWD, McComb AJ (1990) Productivity and nutrient limitation. In: Larkum AWD, McComb EAJ, Shepherd SA (eds) Biology of seagrasses. Elsevier, Amsterdam, p 635–685

Hootsmans MJM, Vermaat JE (1994) Light-response curves of *Potamogeton pectinatus* L. as a function of age and irradiance level during growth. In: Van Vierssen W, Hoostmans MJM, Vermaat JA (eds) Lake Veluwe, a macrophyte-dominated system under eutrophication stress. Kluwer Academic Publishers, Dordrecht, p 57–130

Kirkman H, Cook IH (1987) Distribution and leaf growth of *Thalassodendron pachyrhizum* den Hartog in southern Western Australia. Aquat Bot 27:257–266

Kromkamp J, Peene J (1995) Possibility of net phytoplankton primary production in the turbid Schelde estuary. Mar Ecol Prog Ser 121:249–259

Larkum AWD, Roberts G, Kuo J, Strother S (1989) Gaseous movement in seagrasses. In: Larkum AWD, McComb AJ, Shepherd SA (eds) Biology of seagrasses. Elsevier, Amsterdam, p 686–722

Laws EA (1991) Photosynthetic quotients, new production and net community production in the open ocean. Deep-Sea Res 38:143–167

Lean DR, Burnison BK (1979) An evaluation of errors in the $^{14}C$ method of primary production measurement. Limnol Oceanogr 24:917–928

Legendre L, Demers S, Yentsch CM, Yentsch CS (1983) The $^{14}C$ method: patterns of dark $CO_2$ fixation and DCMU correction to replace the dark bottle. Limnol Oceanogr 28: 996–1003

Lindeboom HJ, de Bree BHH (1982) Daily production and consumption in an eelgrass (*Zostera marina*) community in saline lake Grevelingen: discrepancies between the $O_2$ and $^{14}C$ method. Neth J Sea Res 16:362–379

Lipkin Y, Beer S, Best EPH, Kairesalo T, Salonen K (1986) Primary production of macrophytes: terminology, approaches and a comparison of methods. Aquat Bot 26:129–142

Marsh JAJ, Dennison WC, Alberte RS (1986) Effects of temperature on photosynthesis and respiration in eelgrass (*Zostera marina* L.). J Exp Mar Biol Ecol 101:257–267

McRoy CP (1973) Seagrass ecosystems: recommendations for research programmes. International Seagrass Workshop, Leiden.

McRoy CP (1974) Seagrass productivity: carbon uptake experiments in eelgrass, *Zostera marina*. Aquaculture 4: 131–137

McRoy CP, McMillan C (1977) Production ecology and physiology of seagrasses. In: McRoy CP, Helfferich C (eds) Seagrass ecosystems: a scientific perspective. Marcel Dekker, New York, p 53–87

Moncreiff CA, Sullivan MJ, Daehnick AE (1992) Primary production dynamics in seagrass beds of Mississippi Sound: the contributions of seagrass, epiphytic algae, sand microflora, and phytoplankton. Mar Ecol Prog Ser 87:161–171

Mook WG (1968) Geochemistry of the stable carbon and oxygen isotopes of natural waters in the Netherlands. PhD thesis. Groningen University.

Mook WG (1970) Stable carbon and oxygen isotopes of natural waters in the Netherlands. In: Proceedings of the International Atomic Agency Conference on Isotope Hydrology. IAEA, Vienna, p 163–190

Mousseau L, Dauchez S, Legendre L, Fortier L (1995) Photosynthetic carbon uptake by marine phytoplankton: comparison of stable ($^{13}C$) and radioactive ($^{14}C$) isotope methods. J Plankton Res 17:1449–1460

Oviatt CA, Rudnick DT, Keller AA, Sampow PA, Almquist GT (1986) A comparison of system ($O_2$ and $CO_2$) and $^{14}C$ measurements of metabolism in estuarine mesocosms. Mar Ecol Prog Ser 28:57–67

Penhale PA (1977) Macrophyte-epiphyte biomass and productivity in an eelgrasss (*Zostera marina* L.) community. J Exp Mar Biol Ecol 26:211–224
Pergent G, Boudouresque CF, Crouzet A, Meinesz A (1989) Cyclic changes along *Posidonia oceanica* rhizomes (lepidochronology): present state and perspectives. PSZN I: Mar Ecol 10:221–230
Peterson PJ (1980) Aquatic primary productivity and the $^{14}C$-$CO_2$ method: a history of the productivity problem. Annu Rev Ecol Syst 11:359–385
Phillips RC, McRoy CP (1980) Handbook of seagrass biology: an ecology system perspective. Garland STPM Press, New York
Sakamoto M, Tilzer MM, Gachter R, Rai H, Collos Y, Tschumi P, Berner P, Zbaren D, Zbaren J, Dokulil M, Bossard P, Uehlinger U, Nusch EA (1984) Joint field experiments for comparisons of measuring methods of photosynthetic production. J Plankton Res 6:365–383
Sepers ABJ (1981) The aerobic mineralization of amino acids in the saline Lake Grevelingen and the freshwater Haringvliet basin (The Netherlands). Arch Hydrobiol 92:114–129
Slawyk G, Collos Y, Christian J (1977) The use of the $^{13}C$ and $^{15}N$ isotopes for the simultaneous measurement of carbon and nitrogen turnover rates in marine phytoplankton. Limnol Oceanogr 22:925–932
Slawyk G, Minas M, Collos Y, Legendre L, Roy S (1984) Comparison of radioactive and stable isotope tracer techniques for measuring photosynthesis: $^{13}C$ and $^{14}C$ uptake by marine phytoplankton. J Plankton Res 6:249–257
Sokal RR, Rohlf FJ (1981) Biometry. The principles and practice of statistics in biological research, 2nd edn. WH Freeman & Co, New York
Steeman Nielsen E (1952) The use of radioactive carbon ($C^{14}$) for measuring organic production in the sea. J Cons Perm Int Explor Mer 18:117–140
Syrett PJ (1962) Nitrogen assimilation. In: Lewin RA (ed) Physiology and biochemistry of algae. Academic Press, New York, p 171–188
Wetzel RG (1964) A comparative study of the primary productivity of higher aquatic plants, periphyton and phytoplankton in a large, shallow lake. Int Rev Ges Hydrobiol 49:1–61
Wetzel RG (1965) Techniques and problems of primary productivity measurements in higher aquatic plants and periphyton. Mem Ist Ital Idrobiol Dott Marco Marchi 18(Suppl):249–267
Williams PJl, Raine RCT, Bryan JR (1979) Agreement between $^{14}C$ and oxygen methods of measuring phytoplankton production: reassessment of the photosynthetic quotient. Oceanol Acta 2:411–416
Zieman JC (1974) Methods for the study of the growth and production of turtle grass, *Thalassia testudinum* König. Aquaculture 4:139–143
Zieman JC (1975) Seasonal variation of turtle grass, *Thalassia testudinum* König, with reference to temperature and salinity effects. Aquat Bot 1:107–123
Zieman JC, Wetzel RG (1980) Productivity in seagrasses: methods and rates. In: Phillips RC, McRoy CP (eds) Handbook of seagrass biology: an ecosystem perspective. Garland STPM Press, New York, p 87–117
Zimmerman RC, Reguzzoni JL, Alberte RS (1995) eelgrass (*Zostera marina* L.) transplants in San Francisco Bay: role of light availability on metabolism, growth and survival. Aquat Bot 51:67–86

*Editorial responsibility: Otto Kinne (Editor), Oldendorf/Luhe, Germany*

*Submitted: May 10, 1999; Accepted: February 6, 2001*
*Proofs received from author(s): November 1, 2001*