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Application of infrared absorption spectrometry for measuring the photosynthetic production of phytoplankton by the stable ^{13}C isotope method*

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Abstract: The application of infrared absorption spectrometry was examined for measuring the photosyntetic rate of phytoplankton by the stable ^{13}C isotope method. In laboratory experiments using the cultured marine diatom, *Skeletonema costatum*, the photosynthetic rates obtained by the present method showed good agreement with those by the ^{14}C method, indicating the usefulness of infrared absorption spectrometry. The advantage of the present method is its ease of use and effectiveness of saving labour and time for the analysis of ^{13}C abundance in samples, compared with those by mass spectrometry or by nuclear magnetic resonance. Some technical problems in the application of stable ^{13}C isotope for measuring the photosynthetic rate of phytoplankton were also examined.

1. Introduction

Since the epoch-making work of STEEMANN NIELSEN (1952), the ^{14}C method has long been applied for measuring the photosynthetic rate of natural phytoplankton in the aquatic environments. The high sensitivity of the ^{14}C method extended the possibility of measuring the phytoplankton production even in waters of low productivity, and our global knowledge of primary productivity has been constructed (e.g. KOBLENTZ-MISHKE, 1965; ARUGA, 1973).

Despite of its ease of use and its sensitivity, the use of ^{14}C radioisotope in the natural environment has been severely restricted, particularly in Japan, mainly due to its radioactivity hazards. Thus, it is better to use stable isotopes, which have no hazardous radioactive problems, instead of radioisotopes. The stable ^{13}C isotope for the determination of the uptake rate of carbon in photosynthesis was first introduced by SLAWYK *et al.* (1977) using cultured phytoplankton. Recently, HAMA *et al.* (1983) demonstrated the usefulness of application of stable ^{13}C isotope for measuring the photosynthetic rate of natural phytoplankton. Although it has gradually been adopted (e.g. MIYAZAKI *et al.*, 1985a, b), the ^{13}C method has been used not so widely yet for determining the photosynthetic activity in natural waters. One of the main reasons is the difficulty and complexity of working with a mass spectrometer (MS) or with nuclear magnetic resonance (NMR). Technical skill is needed for operation and maintenance of these instruments. The incidental and most practical problem of handling these instruments is that a considerable length of time is required for analyzing a sample.

To overcome these problems, infrared absorption spectrometry, which was first proposed by MCDOWELL (1970), has recently been developed for measuring the ^{13}C abundance in plant materials or in exhaled breath using the JASCO EX-130 ^{13}C analyzer (Japan Spectroscopic Co., Ltd., Tokyo) by several investigators (KOKUBUN and SASAKI, 1979; KOKUBUN and YANAGISAWA, 1982; YANAGISAWA and KUMAZAWA, 1982). The instrument is easy to use and enables the researcher to save labour and time in the analysis.

In this paper we describe the results of experiments conducted to show the usefulness of infrared absorption spectrometry for measuring the ^{13}C abundance in phytoplankton for the calculation of photosynthetic activity.

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2. Material and methods

The concentrations of organic carbon and the isotopic ratio of ^{12}C and ^{13}C of the phytoplankton samples were determined by infrared absorption spectrometry using the JASCO EX-130 ^{13}C analyzer (Japan Spectroscopic Co., Ltd., Tokyo). Details of this system were described by KOKUBUN and SASAKI (1979), KOKUBUN and YANAGISAWA (1982) and/or YANAGISAWA and KUMAZAWA (1982). The phytoplankton samples collected on glass fiber filters (Whatman GF/C) were automatically dropped into the combustion furnace where the samples were immediately oxidized to carbon dioxide at 900°C with O_2 gas inflowing continuously (100 ml/min). The carbon dioxide evolved was introduced into the absorption cell in the system with a carrier gas and the absorption intensity of infrared radiation by $^{12}CO_2$ or $^{13}CO_2$ was measured in two different infrared regions. Time required for the analysis of one sample was only about 3 minutes. The calibration curves for the determination of ^{13}C content were made according to the method described by OKANO *et al.* (1983), and the atom percent of ^{13}C was calculated using the following equation:

$$^{13}\text{C atom } \% = \frac{^{13}\text{C}}{^{13}\text{C}+^{12}\text{C}} \times 100.$$

The photosynthetic rate of phytoplankton was calculated with the following equation in the same way as HAMA *et al.* (1983):

$$P(\text{mgC/m}^3\text{/hr}) = \frac{POC \times (a_{is} - a_{ns})}{T \times (a_{ic} - a_{ns})} \times f,$$

where a_{is} is the atom % of ^{13}C in incubated sample, a_{ns} the atom % of ^{13}C in natural sample (natural abundance), a_{ic} the atom % of ^{13}C in total inorganic carbon, POC the particulate organic carbon in incubated sample (mgC/m^3), T the duration (hours) of incubation, and f the discrimination factor of ^{13}C ($f=1.025$).

The marine diatom *Skeletonema costatum* (GREVIELLE) CLEVE was cultured in the medium (SW-II) of IWASAKI (1961) under illumination of 100 $\mu E/m^2/sec$ (L:D cycle of 14:10 hr) and 20°C. The cells in their logarithmic growth phase were harvested and diluted with the filtered seawater, and used for the experiments.

First experiment: The time course of $^{13}CO_2$ uptake by the alga was examined under the same conditions as in culture. Samples were transferred into the 300 ml BOD bottle, added with $Na_2{}^{13}CO_3$ (6.78 % of a_{ic}; Prochem, UK) and incubated for 1, 2, 3 and 4 hours. After incubation, the samples were filtered immediately through precombusted glass fiber filters (Whatman GF/C) and stored at −20°C until analysis. The samples were completely dried at 60°C and the concentrations of organic carbon (^{12}C and ^{13}C) were measured by the infrared absorption spectrometry mentioned above.

Second experiment: To examine the enrichment effect of inorganic carbon on the estimation of photosynthetic rate, samples were added with different concentrations of ^{13}C, 5.51 and 12.6 % of a_{ic}, and incubated under the same conditions as in the first experiment. After incubation, the samples were treated with the same procedures as described above.

Third experiment: The effects of washing with filtered seawater or exposure to HCl fumes for removing inorganic carbonate from the samples retained on glass fiber filters were examined. The loss of organic ^{13}C by the fixation with chemicals (0.04 % $HgCl_2$) was also examined.

All these experiments were followed by experiments using the ^{14}C method. The samples were filtered through Millipore RA filters (pore size, 1.2 μm), solubilized with acetone, and then added with Dimilume-30 (Packard, USA) as liquid scintillation cocktail with chemiluminescence inhibitor. Their radioactivities were counted with a Mark-III liquid scintillation counter (Seale Analytic Inc., USA).

The concentrations of chlorophyll *a* in the samples were determined according to the method in SCOR-UNESCO (1966).

3. Results and discussion

Table 1 shows the natural abundance (atom %) of ^{13}C in cultured *Skeletonema costatum* determined by infrared absorption spectrometry (IR). Even though the carbon content in the samples varied considerably from 26 to 602 μg, the natural abundance of ^{13}C was almost constant with the mean value of 1.098 % (S.D. 0.006, C.V. 0.55 %). In samples of lower carbon content than 25 μg, the natural abundance obtained was

Table 1. Natural abundance (atom %) of ^{13}C in cultured diatom *Skeletonema costatum*.

Sample No.	^{12}C (μg)	^{13}C (μg)	atom % of ^{13}C
1	26.07	0.290	1.100
2	84.89	0.953	1.110
3	89.79	0.986	1.087
4	151.78	1.695	1.104
5	152.03	1.686	1.097
6	240.60	2.683	1.103
7	365.10	4.027	1.091
8	548.22	6.061	1.094
9	557.74	6.169	1.099
10	588.40	6.534	1.098
11	595.46	6.620	1.100
Mean			1.098
S.D.			0.006
C.V. (%)			0.55

fluctuated. Although the natural abundance of ^{13}C is fluctuated within several percents in natural samples depending on the species and environmental growth conditions (FONTUGNE and DUPLESSY, 1981; OKANO *et al.*, 1983), the natural abundance of ^{13}C is generally about 1.10 %. Thus, the natural abundance of ^{13}C obtained in the present measurements is considered to be reasonable, indicating that the present IR method is reliable enough to determine the ^{13}C abundance in phytoplankton samples for the calculation of their photosynthetic rates. In addition, it is noted that the sensitivity of IR must be maintained for obtaining the accurate value of ^{13}C abundance. It is also advisable to prepare the samples containing at least more than 25 μg of carbon.

A linear relationship was obtained between the ^{13}C abundance in *Skeletonema costatum* and the incubation time when *S. costatum* samples were incubated with an a_{ic} of 6.78 % (Fig. 1). The regression obtained under the light was

$$Y=0.12X+1.06 \quad (r=0.964,\ n=12),$$

where Y is the ^{13}C abundance in samples and X is the incubation time. The mean photosynthetic rate calculated from this equation was 2.03 mgC/mg. chl. *a*/hr. The rate coincided well with that measured by the ^{14}C method in the same samples. This result indicates that the sufficient increase of ^{13}C abundance in the samples for measuring their photosynthetic rate can be

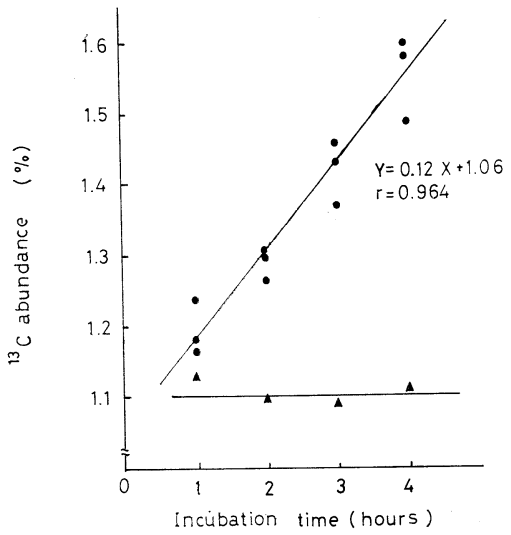

Fig. 1. Relationship between the incubation time and the ^{13}C abundance in *Skeletonema costatum* (a_{ic}=6.78 %). Solid circle, a_{is} of the light bottle. Solid triangle, a_{is} of the dark bottle.

Table 2. Effects of different concentrations of a_{ic} and chlorophyll *a* on the determination of photosynthetic rate.

a_{ic} (%)	Chl. *a* (mg/m^3)	Photosynthetic rate (mgC/mg. chl. *a*/hr)
5.51 (n=3)	0.4	2.01±0.17* (8.5)**
5.51 (n=3)	4.0	2.34±0.22* (9.4)**
12.6 (n=3)	4.0	2.08±0.11* (5.3)**

* S.D., ** C.V. (%)

obtained within the incubation time of 2 to 4 hours.

In the ^{13}C method, it is required to add more isotope-containing carbon than in the ^{14}C method because of its lower sensitivity. This enrichment might accelerate or reduce the activity of carbon uptake. Table 2 shows the results of enrichment experiments for the carbon uptake under different concentrations of a_{ic} and chlorophyll *a*. The photosynthetic rates obtained at two concentrations of a_{ic}, 5.51 and 12.6 %, were 2.34 mgC/mg. chl. *a*/hr (S.D. 0.22, C.V. 9.4 %) and 2.08 mgC/mg. chl. *a*/hr (S.D. 0.11, C.V. 5.3 %), respectively. No marked difference was observed in the photosynthetic rate obtained in the sample of reduced chlorophyll *a* concentration (0.4 mg/m^3). Thus, it is concluded that the enrichment of ^{13}C-bicarbonate in such an amount can be

Table 3. Effects of washing the filter with filtered seawater (F.S.W.) or exposure of it to HCl fumes for removing inorganic carbonate.

	Washing with F.S.W. (mgC/mg. chl. *a*/hr)	Exposure to HCl (mgC/mg.chl.*a*/hr)
^{13}C ($n=4$)	2.69±0.77* (29)**	2.36±0.18*(7.8)**
^{14}C ($n=4$)	2.63±0.25* (9.4)**	2.36±0.12*(5.2)**

* S.D., ** C.V. (%)

considered to have no significant effect on the carbon uptake of natural phytoplankton. Similar results were demonstrated by HAMA *et al.* (1983) with the marine phytoplankton and also by MIYAZAKI *et al.* (1985a) in the lake.

It is absolutely necessary in the ^{13}C method to remove completely unused ^{13}C-bicarbonate because a large enrichment of ^{13}C is needed. In the ^{14}C method, unused ^{14}C-bicarbonate can be removed generally by "fuming" the filter over HCl (STRICKLAND and PARSONS, 1972). Similarly, unused ^{14}C-bicarbonate can be removed by "washing" the samples with filtered seawater (MCMAHON, 1973). To check these problems, the effect of "washing" with filtered seawater or "fuming" over HCl was examined. Samples of cultured *Skeletonema costatum* (chl. *a* 2.0 mg/m^3) were incubated with ^{13}C or ^{14}C for 3 hours under the same conditions, and used for the different treatments after filtration. The value obtained by both treatments was determined with 6.4 % of the coefficient of variation (Table 3). The results indicate that unused bicarbonate can be removed adequately by "washing" or "fuming".

There exists another unavoidable problem accompanying in the ^{13}C and ^{14}C methods. Several researchers have argued over the problems of "losses" of fixed carbons from the cells; the loss during chemical fixation (SILVER and DAVOLL, 1978; SHIMURA *et al.*, 1978) and the loss through cellular damage during vacuum filtration (ARTHUR and RIGLER, 1967). The most serious cause of loss can be considered to be chemical fixation at the end of incubation. To check this problem, losses of ^{13}C and ^{14}C activity after chemical fixation of the algal cells were examined. The cells which took up ^{13}C or ^{14}C tracer were fixed with mercuric chloride (0.04 %), and filtered through Whatman GF/C

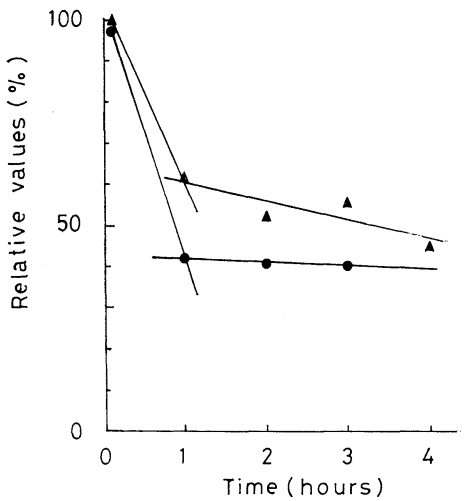

Fig. 2. Decrease of the retained ^{13}C (solid triangles) and ^{14}C (solid circles) in *Skeletonema costatum* cells due to the loss caused by fixation with $HgCl_2$ (0.04 %).

filters (for ^{13}C) or Millipore RA filters (for ^{14}C) at intervals of one hour. The amount of ^{13}C or ^{14}C retained in the fixed cells was expressed relative to the levels found in the unfixed cells (Fig. 2). In the cells filtered immediately after fixation, the amount of both ^{13}C and ^{14}C retained was almost 100 %. However, it decreased to 64 % and 41 %, respectively, after one hour of fixation. Thereafter, only a slight decrease was observed relative to the time of fixation until the final level of about 46 % in ^{13}C and about 40 % in ^{14}C was reached (Fig. 2). A little difference observed between the results in ^{13}C and ^{14}C might be attributed partly to the difference of filters used to filtration. Losses in the samples treated with such a strong fixative as mercuric chloride were two times higher than those of the unfixed samples when filtered immediately after incubation. The extracellular release in *Trichodesmium thiebautii* treated with a similar fixative was reported to be 3 to 6 times higher than that in the unfixed samples (SHIMURA *et al.*, 1978). One of the reasons for this difference could be attributed to the fact that *Skeletonema costatum* cells used in the present study form their skeleton tightly. In any way, ^{13}C or ^{14}C incorporated in the cells can be partly lost by chemical fixation. Hence, it is emphasized that the best way to perform the experiments

is without chemical fixation. In addition, it is best to conduct a filtration immediately after incubation and wash the cells sufficiently with filtered seawater or expose them to HCl fumes.

In conclusion, the results of the present study suggest the effectiveness of infrared absorption spectrometry to determine the ^{13}C abundance in phytoplankton samples for calculating the rate of photosynthetic carbon uptake. The simplicity and briefness of this method will save labour and time in analysis.

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安定同位体 ^{13}C を用いた赤外分光法による植物プランクトンの光合成生産の測定

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要旨: 植物プランクトンの光合成を測定するために，安定同位体 ^{13}C を用いた赤外分光法を検討した。培養した海産珪藻 *Skeletonema costatum* を用いて比較した結果，本法による光合成速度は ^{14}C 法による光合成速度とよく一致した。本法は質量分析法や核磁気共鳴法と比較して簡便であり，迅速な分析が可能である。また，本法を用いた場合の無機炭酸除去および植物プランクトンの固定についても技術的な検討を行った。