# 14C labelling of algal pigments to estimate the contribution of different taxa to primary production in natural seawater

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# $^{14}C$ labelling of algal pigments to estimate the contribution of different taxa to primary production in natural seawater samples

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Several attempts have been made in the past to measure taxon-specific growth rates in natural phytoplankton populations in order to evaluate the conditions leading to success of individual taxa, to estimate the specific role of the various taxonomic components of algae in the food web and in nutrient cycling, and to explain succession. The method of Redalje and Laws, who studied the pattern of $^{14}C$ labelling of Chl *a* (the pigment common to all microalgae), has now been adapted by following $^{14}C$ incorporation into the carotenoids that are tags of taxonomic groups. The results obtained so far indicate that even in oligotrophic regions different species co-occurring in one sample usually incorporate $^{14}C$ at a very different rate, which suggests that it is not only eutrophic pelagic plankton communities that are subject to rapid shifts in growth conditions. The pigment labelling method is still open to challenge from several directions. More research must be done to transform this approach into a technique that is undeniably accurate and reliable for the assessment of growth rates of algal taxa – or to show when and under what circumstances it cannot be used. Special care should be taken to avoid radioactive contamination of pigments separated by HPLC, and isotope dilution during experiments. The methods that are available now can also be applied directly to studies of the turnover and eventual fate of the various pigments synthesized by algae, be they photosynthetically active or photoprotective, e.g., in studies of photoadaptation, for example the response of algae to increased ultraviolet radiation. $^{14}C$ labelling of both chlorophylls and carotenoids may even become a tool in studies of large-scale carbon cycling because a considerable part of phytoplankton carbon biomass in the sea and in sediments is associated with pigments or their degradation products.

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## Introduction

In mixed natural phytoplankton populations each taxonomic group that is present usually has its own response to biotic and abiotic environmental conditions and so has a specific balance between production and loss. In order to predict the success of an individual group and understand succession in the phytoplankton it is necessary not only to know taxon-specific loss rates by grazing and sinking, but also the primary production potential of each single entity in mixed algal populations.

Attempts to distinguish between growth rates of different components in microbial communities have often been made, most recently by Carpenter and Chang (1988), Newell and Fallon (1991), and Conley (1992), who all used methods based on species- or taxon-specific cell cycle and growth characteristics. Size fractionation, often used to distinguish picoplankton from nano- and microplankton primary production, is a simpler method, but unfortunately the most important taxonomic groups are all in the picoplankton size range in most oceanic regions. Since the late 1970s a new technique of phytoplankton analysis has become popular: HPLC (high-performance liquid chromatography), called at that time "the new king of analytical chemistry" (Robinson, 1979). It soon became obvious that all taxonomic groups

in seawater samples could be detected quite well by a characteristic set of pigments, often even by one specific pigment (for a recent review see Wright *et al.*, 1991), the most characteristic ones being found among both the carotenoids and the chlorophylls. It seemed therefore logical to follow in the footsteps of Redalje and Laws (1981) and extend their method of $^{14}C$ labelling of Chl *a* (the pigment common to all algal classes) to labelling of all pigments.

Our first attempts with labelling carotenoids were made in the laboratory in the early 1980s (Buma, 1984) and a more systematic study was undertaken in Indonesian waters in 1984 during the "Snellius expedition" (Gieskes and Kraay, 1989).

In the Redalje and Laws version (Redalje and Laws, 1981; Laws, 1984) the carbon-specific $^{14}C$ activity of Chl *a* provides an estimate of the specific activity of phytoplankton carbon (cf. Smith and Geider, 1985). It is thereby possible to determine carbon biomass and the growth rate of phytoplankton without complications of $^{14}C$ possibly associated with detritus or consumers (Welschmeyer and Lorenzen, 1984). The condition is that after a sufficiently long incubation the specific activity of Chl *a* carbon is identical to the specific activity of the total phytoplankton carbon. Until recently, $^{14}C$ labelling of pigments as a means to determine biomass and carbon-specific growth rates of phytoplankton was only used by a few others: Goericke, who evaluated the chlorophyll labelling methodology in a dissertation (Goericke, 1990; see also Goericke and Welschmeyer, 1992a, b, c; Goericke, 1992), and two Scandinavian groups that tested the method with laboratory cultures of some marine and freshwater species of algae, with Riemann *et al.* (in press) arguing in favour of the approach of Redalje and Laws, and Jespersen *et al.* (1992) against application.

## Pigment labelling for biomass and production estimates

When a phytoplankton sample is incubated with $^{14}CO_2$, all algal cell components that contain carbon will eventually be labelled more or less, including the pigments. In the past, the time course of labelling of cellular components has been followed mostly to monitor production and breakdown processes or the conversion of one substance from another, such as the daytime production of carbohydrates that provide the energy for night-time protein synthesis in algal cells (e.g., Li and Harrison, 1982; Smith and Geider, 1985).

It can be argued that after a sufficiently long incubation all cell components are labelled with $^{14}C$ to a similar degree, including pigments such as Chl *a*, so

$$R^*_{CF} = R^*_{Chl\,a}$$

i.e., the specific activity $R^*$ of the carbon of the whole cell ($C_F$ is phytoplankton carbon) equals the specific activity of the carbon in the Chl *a* molecules (the carbon content of a molecule is 0.7399). The specific activity is normally expressed as dpm/mgC. In other words: activity of the whole cell/$C_F$ = activity of Chl *a*/Chl *a* − C or $C_F$ ($\mu$g/l) = activity of whole cell at end incubation/$R^*_{chl\,a}$. The growth rate can be calculated from $C_F = C_0 \cdot e^{\mu t}$ (t = h incub.). Since $P_t = C_t - C_0 = C_F (1 - e^{-\mu t})$, $\mu = -t^{-1} \ln (1 - P_t \cdot C_F^{-1})$; $P_t$ = productivity as measured in the normal way with $^{14}C$. Both the phytoplankton carbon biomass and the growth rate can thus be calculated readily (for details see Redalje and Laws, 1981; Laws, 1984; Welschmeyer and Lorenzen, 1984; Gieskes and Kraay, 1989; modified labelling equations were designed by Goericke, 1990; Goericke and Welschmeyer, 1992a, b, c; see also Riemann *et al.*, 1993).

Up to now the method has been applied with most success for estimates of carbon-specific growth rates ($\mu$) and algal carbon biomass ($C_F$) in tropical waters. For example, Gieskes and Kraay (1986) found in the central Atlantic along 20°N that $\mu$ was as high as expected, in agreement with the high rate of recycling in the microbial food web (Goldman, 1984): 0.16 per hour during daytime near the surface; 0.017–0.040 just above the deep chlorophyll maximum layer at 75–100 m depth. The carbon:Chl *a* ratio found with the Redalje–Laws method was around 107 near the surface and much lower (67) at the depth of the chlorophyll maximum. These figures are not unexpected: near the surface of tropical ocean waters picoplanktonic cyanobacteria (*Synechococcus*) tend to dominate (Gieskes *et al.*, 1988), with a mean C:Chl *a* ratio of 82, while at depth picoplanktonic Prochlorophytes contribute most to the phytoplankton (Chisholm *et al.*, 1988), with a much lower C:Chl *a* ratio (Veldhuis and Kraay, 1990), presumably as an adaptation to low light conditions.

## Testing the Redalje–Laws method

The weakest point in the series of assumptions that has to be made when the Redalje and Laws (1981) method is to be applied is when the turnover of one cellular component (*in casu* a pigment) is different from the rest; the $^{14}C$ labelling of Chl *a*, for example, can then be quite out of phase with the labelling of the total carbon pool. In fact, as long as 15 years ago it was suggested that Chl *a* turns over much more rapidly, in a few hours, than other cell constituents (Riper *et al.*, 1979). However, the precursor pool from which Chl *a* is labelled may have the same specific radioactivity as the rest of the cellular carbon. This is probably the reason why the specific

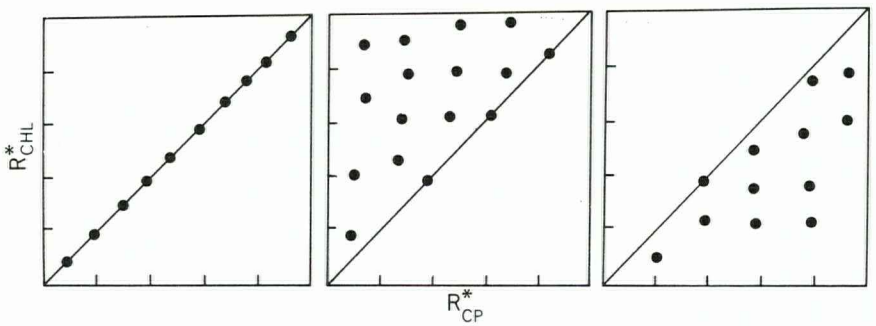


Figure 1. Relationship between the specific activities of chlorophyll *a* carbon and of phytoplankton carbon in cultures according to the three conceivable models $R^*_{chl} = R^*_{cp}$(a), $R^*_{chl} \geq R^*_{cp}$ (b), and $R^*_{chl} \leq R^*_{cp}$ (c).

activity of Chl *a* carbon has been found by most investigators to be closely related to that of the whole cell carbon (Fig. 1a; a compilation of data presented by Redalje and Laws, 1981; Laws, 1984; Redalje, 1983; Welschmeyer and Lorenzen, 1984; Riemann *et al.*, in press). Taking this as given (see Fig. 1a) it is just one further step to assuming that also other pigments in a cell can become labelled in a similar way as Chl *a*. Pigment synthesis and carbon fixation must of course be balanced if one wants to use $^{14}C$ labelling of pigments and relate this to algal growth.

Work on cultures by Jespersen *et al.* (1992) suggests that complications may arise. They found that Chl *a* carbon sometimes seems to be labelled much more rapidly than the rest of the cell (Fig. 1b), so the most basic assumption of Redalje and Laws is not fulfilled. The fact that POC (particulate organic carbon) specific activity in a culture is less than the specific activity of Chl *a* carbon may imply that the pool of recently assimilated carbon is respired much more rapidly than the subset of recently assimilated Chl *a* carbon. In other words, the POC is losing activity due to respiration much faster than the Chl *a* carbon pool. However, another explanation for the high specific activity of chlorophyll compared to the rest of the cell may be radioactive contamination of pigments by coeluting compounds, according to Goericke (1992) (see HPLC methods section for a description of methods to avoid contamination). Finally, in dense laboratory cultures of algae the specific activity of dissolved inorganic carbon (DIC) may decline when the amount of POC that is produced during incubation exceeds the DIC concentration. Growth rate calculations must then be corrected for isotope dilution (Riemann *et al.*, in press).

Alternatively (Fig. 1c), the specific Chl *a* carbon activity was found to be consistently *below* POC activity by Buma (1984) in laboratory cultures – again not in accord with Redalje and Laws' assumption. This was ascribed to suboptimal culture conditions and the resulting bad physiological condition of the population. However, the phenomenon may also have been caused by photoadaptation: an algal cell can respond with increased growth when moved to higher light intensities and with reduced pigment synthesis, so the pigment pool can become rapidly diluted during growth (Post *et al.*, 1984, 1985). Thus, photoadaptation may lead to different, counteracting rates of chlorophyll synthesis. Time-course labelling of intracellular pools has often shown that the carbon-specific activity of the various compartments can differ widely. In their model, Smith and Platt (1984) assume that the phytoplankton carbon pool includes an exchange pool and a synthetic pool. The former exchanges carbon with the exogenous inorganic C pool ($^{14}C$ and $^{12}C$), and can be from 0 to 100% of the total carbon pool. The synthetic pool does not exchange with exogenous $^{14}CO_2$ but accumulates carbon from the exchanging pool. Therefore, the specific activity may vary from 0 to uniform labelling in a few hours. Taking all this into consideration one would expect that the specific activity of Chl *a* will often provide a completely biased estimate of specific activity of the total phytoplankton carbon. However, as we have seen, most of the data have shown that this is not the case (see Fig. 1a). The most recent results of Riemann *et al.* (in press) also support the assumption that is the basis of the Redalje and Laws' method: they found that in a large number of cultures of different species of microalgae growth rates based on specific labelling of chlorophyll carbon averaged 112% (81–118%) of growth rates estimated from the rate of increase of particulate organic carbon (mostly phytoplankton carbon, not bacterial).

## Testing the carotenoid labelling method in cultures

### HPLC methods

Our methods differ slightly from those recently published by Goericke and Welschmeyer (1992a, b, c),

Goericke (1992), and Welschmeyer *et al.* (1991). The solvents used for the pigment separations in our culture experiments with *Isochrysis* and *Rhodomonas* were A (51.8 ml 0.5 M $KH_2PO_4$, brought to a pH of 7 with 0.5 M NaOH, diluted with methanol to 30%) and B (20% ethylacetate in methanol), pumped through a Lichrosorb C 18 HPLC column in a linear gradient from t = 0 100% A to t = 10 or 20 min. 100% B. Pigments were detected at absorption wavelengths of 436 and 658 nm. To assess the specific activity of pigments, contaminant $^{14}C$ associated with other cell consituents must be removed carefully (see also above under Testing the Redalje–Laws method). Goericke (1990, 1992) used a phaeophytinization procedure to separate activity of labelled colourless compounds from the activity of chlorophyll. The isolated $^{14}C$-labelled Chl *a* is converted to phaeophytin by weak acidification and this is rechromatographed for the collection and determination of specific activity of phaeophytin *a*.

Our method is different, but as effective as Goericke's. Samples extracted in 90% acetone were washed in 10% NaCl and diethyl ether, which is the normal procedure for transfer of pigments to ether. The ether extract was brought onto a Sep-pak silica cartridge after this was rinsed with 10 ml petroleum diethylether 100%. Next, rinsing took place in six steps: first with 10 ml petroleum-ether, followed by 5% chloroform in petr.-ether, then by 10, 20, and 50% chloroform in diethyl ether, and finally with 100% chloroform. The pigments were then removed from the cartridge with ether, the ether was evaporated, and the pigments were redissolved in 90% acetone and this contaminant-free extract injected into the HPLC system. Peaks of the various pigments were collected in Packard's Instagel 50% (in methanol) for liquid scintillation counting. As a check, blanks were measured close to the peaks. The specific activity of a pigment is:

dpm/amount of pigment collected × carbon contents of pigment.

The C contents of the pigments is known (Chl *a*: 0.7399; fucoxanthins: 0.7553; diadinoxanthin: 0.8164; alloxanthin: 0.8500; etc.). For calculation of the maximum specific activity ($R^*_{max}$; cf. Welschmeyer and Lorenzen, 1984) one needs to know the concentration of DIC (dissolved inorganic carbon) in the sample, the amount of $^{14}C$ activity that is added, and the concentration of POC (particulate organic carbon) in the culture: $R^*_{max}$ (dpm/$\mu$g C) = activity added/DIC + POC.

## Results and comments

The striking results presented in Figures 2 and 3 are certainly not as expected when compared with the results obtained by the originators of the Redalje–Laws method (Redalje and Laws, 1981; Laws, 1984; Redalje, 1983; Welschmeyer and Lorenzen, 1984).

For *Isochrysis*, the various components did not show parallel labelling in these time course experiments (Fig. 2). Diadinoxanthin was the only compound that reached $R^*_{max}$ (max. specific activity), as it assimilated the label much faster than the other carotenoid, fucoxanthin. Is diadinoxanthin a precursor of fucoxanthin? Both carotenoids increased their specific activity at night. For this nocturnal increase in turnover they probably drew carbon from a precursor pool that was synthesized (and labelled) during the previous light period. The specific

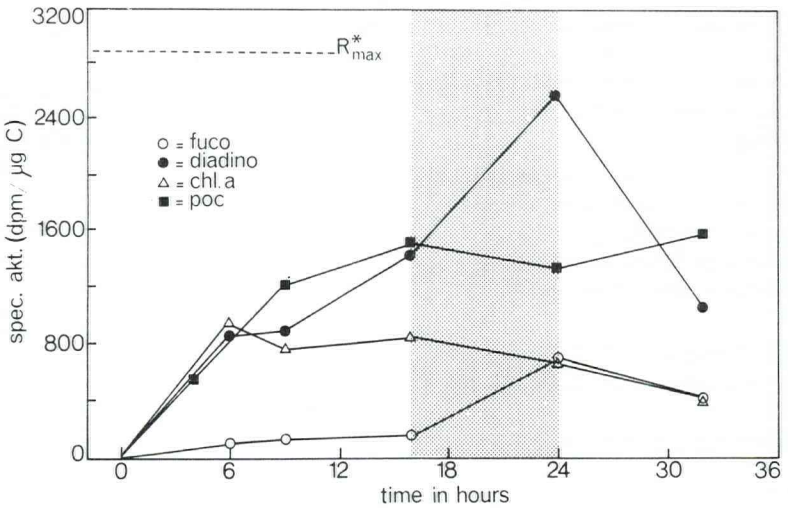


Figure 2. Development of specific activity in particulate organic carbon (POC), Chl *a*, diadinoxanthin and fucoxanthin in cultures of *Isochrysis* sp. Maximum specific activity (see text) is indicated with a dotted line.

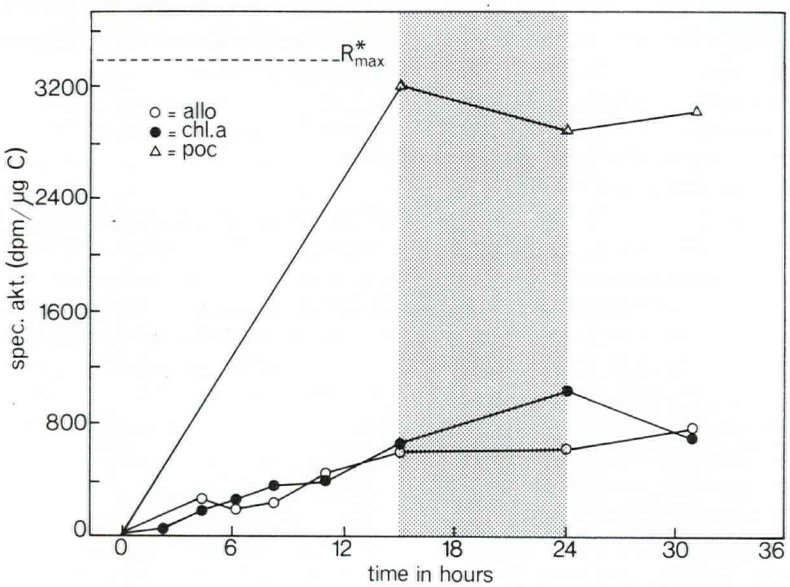


Figure 3. Development of specific activity in particulate organic carbon (POC), Chl *a* and alloxanthin in cultures of *Rhodomonas* sp. Maximum specific activity (see text) is indicated with a dotted line.

activity of POC showed a night-time decrease which may be ascribed to respiratory activity of the algal cells during which the labelled part was broken down preferentially; respiration apparently went on also during the light period, since the POC increase was less than $^{14}C$ fixation. The night-time decrease in specific activity of Chl *a* also suggests that the labelled part of the pool was broken down preferentially, rather than the unlabelled part – or chlorophyll was synthesized in the dark from unlabelled carbon, derived either from $\beta$ fixation or from unlabelled carbon mobilized from storage pools. With respect to *Rhodomonas* sp. (Fig. 3), the specific activity of the whole cell ($R^*_{POC}$) was highest while labelling of the pigments alloxanthin and Chl *a* was parallel (Fig. 3). The maximum specific activity was only attained by POC (see Fig. 3).

We also estimated the specific activity development of newly synthesized pigment, calculated from the increase in pigment concentration during the first and second light periods, the carbon content of the pigments, and the measured specific activity after 12 and 24 h:

Sp. act. new pigment (dpm/$\mu$gC) =
= $R^*_{pigm}$ (dpm/$\mu$gC) × [pigment] × C contents of pigment/
/amount of newly synthesized pigment × C contents of pigment

The expectation was that new material should be 100% labelled, while old existing material would not become labelled at all. It was found that the specific activity of new pigment was always much higher than $R^*_{max}$ (Table 1), so also the non-newly synthesized, "old" pigment turned over. If this was not the case, then $R^*_{new}$ would have been equal to $R^*_{max}$. Collectively, these results are hard to interpret in the light of the assumptions of Redalje and Laws (1981). It is obvious that much research is required to learn more about: (1) the proportion of "old" and "new" material that is either degraded or synthesized, (2) the pigment precursor turnover rates, (3) the effect of the flux rate of precursor into the pigment proper. Labelling kinetics of pigments are certainly determined by their own turnover and the turnover rate of their precursors, (4) the effect of culture conditions: the influence of nutrients, light, and temperature, and of the physiological condition of cells at different stages of growth and at different phases of the cell cycle.

Table 1. Specific acitivity (dpm/C) of newly synthesized pigment compared with maximum specific activity, measured after 12 h and 24 h of $^{14}C$ exposure in cultures. See text for details on calculation.

| Experiment | $R^*_{new}$, 12 h | $R^*_{new}$, 24 h | $R^*_{max}$ |
|---|---|---|---|
| 1 | 5004 | 5593 | 2850 |
| 2 | 3047 | 3239 | 2792 |
| 3 | 4739 | 5215 | 3658 |
| 4 | 4148 | 3464 | 3224 |
| 5 | 2683 | 4111 | 2320 |

## The carotenoid labelling in natural waters: a success story

The slow labelling of pigments described in the foregoing section may partly be explained by slow growth in cultures with cells in a bad physiological condition. In natural tropical and subtropical waters and in the subarctic Pacific the Redalje–Laws method has been much more successful since growth and increase in phytocarbon were usually neatly correlated with pigment labelling (Redalje, 1983; Welschmeyer and Lorenzen, 1984; Gieskes and Kraay, 1989; Goericke, 1990; Welschmeyer *et al.*, 1991). We have collected strong evidence that the rates of taxon-specific pigment synthesis equal the rates of growth of the various algal classes in Indonesian waters during incubations of no more than 9 h. The carbon-specific growth rate calculated by following Chl *a*-carbon labelling (the Redalje and Laws, 1981, method) provided us with estimates of the "bulk" phytoplankton production (Chl *a* is the pigment common to all algal classes) at a number of stations throughout the Banda Sea (eastern Indonesia). Taxon-specific growth rates of the various algal classes, based on measurements of labelling of the carbon of taxon-specific pigments, revealed that the various classes were often growing at very different rates (Table 2). Interestingly, and as expected, in samples where diatoms dominated the phytoplankton, "$\mu$-fucoxanthin" was close to "$\mu$-Chl *a*" (resp. 0.73 and 0.63); where Prymnesiophyceae dominated, "$\mu$-hexanoyloxyfucoxanthin" was close to "$\mu$-Chl *a*" (resp. 0.85 and 0.65), and where cyanobacteria dominated "$\mu$-zeaxanthin" was close to "$\mu$-Chl *a*" (resp. 0.62 and 0.67). For all stations, $\mu$ (calculated by the labelling of carotenoid carbon) varied between 0.15 and 0.87 (for 9 h experiments).

Apparently, there is not an inherent difference in the turnover of pigments. It is also remarkable that the growth rate of the whole phytoplankton community (calculated by Chl *a* labelling) had at most stations a value that was roughly the mean of values calculated for the growth rates of the various taxonomic constituents; a result that was expected. However, at stations 17 and 65 (Table 2) a rapidly growing component was apparently missed. Note that the carbon-specific growth rate of an entire mixed phytoplankton population should lie *somewhere between* the extreme carbon-specific growth rates of the population components, being closer to the minimum if phytoplankton carbon is dominated by the slowest-growing component and closer to the maximum if phytoplankton carbon is dominated by the fastest-growing component.

Welschmeyer *et al.* (1991) recently reported on carotenoid labelling experiments in the subarctic Pacific; diatoms had the highest specific growth rates of all taxa in this region, yet they were present at lowest cell numbers and biomass. They were apparently kept in check by grazing of large herbivores – clearly not by iron limitation.

## Future research

The carotenoid-labelling extension of the method of Redalje and Laws (1981) allows distinction of the contribution of different taxonomic groups to total phytoplankton growth by following the $^{14}C$ incorporation into taxon-specific pigments, without the necessity to correct for losses due to grazing. Taxon-specific responses to changing environmental conditions can readily be assessed, allowing prediction of the future success of any representative with a good marker pigment combination in a mixed phytoplankton population. Moreover, pigment synthesis and breakdown can be monitored by time-course sampling from an incubated population, e.g., in studies of photoadaptation and in relation to

Table 2. Carbon-specific growth rates measured during 9 h incubations (at 30% surface irradiance) of surface samples taken throughout the Banda Sea (Indonesia) in August 1984. Total phytoplankton $\mu$ calculated on the basis of $^{14}C$ labelling of chlorophyll *a*; major algal taxonomic group growth rates on the basis of labelling of flucoxanthin (diatoms), zeaxanthin (Cyanobacteria) and 19'-hexanoyloxyflucoxanthin (Prymnesiophyceae). f = failed; n.m. = not measurable.

| Station | $\mu$-Chl *a* | $\mu$-Zeax | $\mu$-Fuco | $\mu$-hex. fuco |
|---|---|---|---|---|
| 11 | 0.27 | 0.40 | 0.25 | 0.15 |
| 17 | 0.37 | 0.20 | 0.16 | 0.06 |
| 23 | 0.56 | 0.87 | 0.43 | 0.27 |
| 41 | 0.58 | 0.80 | 0.25 | 0.20 |
| 47 | 0.44 | 0.34 | n.m. | n.m. |
| 53 | 0.63 | n.m. | 0.73 | n.m. |
| 59 | 0.36 | 0.75 | 0.19 | f |
| 65 | 0.67 | 0.62 | 0.30 | 0.16 |
| C | 0.65 | 0.81 | 0.18 | 0.85 |
| A | f | 0.62 | 0.08 | f |

ultraviolet light effects on photo-oxidation. The flux of carbon through pigments should receive more attention in large-scale carbon flux studies such as those undertaken in JGOFS: a considerable part of plant carbon is associated with pigments and their precursors and derivatives.

Future work should include a comparison of growth rates based on pigment labelling with independent measurements (POC and protein increase). The effect of temperature, nutrient, and light conditions and the physiological condition of cells should be established because these factors seem to affect the turnover rate of pigments and pigment precursors and the flux of $^{14}C$ from precursors to pigments.

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