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Group-specific primary production based on stable-isotope labeling of phospholipid-derived fatty acids

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Abstract

Stable-isotope labeling of phospholipid-derived fatty acids (PLFAs) is a potentially powerful technique to study group-specific primary production of phytoplankton, as many algal groups possess a specific PLFA composition, and it is relatively simple to measure the isotopic composition of a large number of PLFAs. Experiments with cultured algae showed that differences exist in labeling among PLFAs and between PLFAs and particulate organic carbon (POC), most likely owing to differences in biosynthesis pathways causing some components of the cell with short pathways to be labeled faster. These differences were constant, however, during the first few hours of incubation, and correction factors were used to convert PLFA labeling to total carbon uptake. In algal cultures, growth rates based on ^{13}C-PLFA labeling agreed well with those based on biomass increases in terms of PLFA concentrations and cell numbers. At two contrasting sites in the Scheldt estuary, PLFA synthesis rates were calculated using 2-h incubations. Group-specific primary production was estimated from PLFA synthesis rates and PLFA compositional spectra of samples and algal taxa using the matrix factorization program Chemtax. When accompanied with studies or information on system-relevant phytoplankton, this sensitive method will be applicable to a wide range of pelagic ecosystems and also to benthic systems such as algal mats.

Introduction

Since its introduction, the ^{14}C tracer technique (Steeman-Nielsen 1952) has provided a wealth of information on primary production in freshwater, brackish, and marine ecosystems and revealed the importance of aquatic photosynthesis in the global carbon cycle (Falkowski and Raven 1997, Field et al. 1998). These measurements provide information on total carbon fixation. A great variety of eukaryotic algae and cyanobacteria can contribute to primary production, however, and large differences in growth and ecological characteristics are known among species. The standing stock of phytoplankton at any given point and time is the result of the combined effects of gain and loss processes, many of which are species or group specific. Differences between species such as maximum growth rates, nutrient acquisition efficiency, or minimum required light levels are intrinsic properties of species (Furnas 1990, Raven and Kubler 2002, Sunda and Huntsman 1997), but extrinsic factors such as viral attacks (Brussaard 2004) or predation by herbivores (Tillmann 2004, Verity et al. 2002) can also be species or group specific. Therefore, primary production is not necessarily proportional to standing-stock group abundance, and refining bulk photosynthesis into narrower taxonomic entities can help greatly in deepening our understanding of aquatic ecosystem production and functioning (e.g., Barber and Hiscock 2006).

A number of methods have been developed and applied to measure group-specific rates of primary production. Many studies have used size fractionation in combination with the ^{14}C tracer technique (e.g., Lignell et al. 2003), as size is a determining factor in many growth-related processes such as nutrient uptake and intrinsic growth rates (Raven and Kubler 2002). Ecological differences between the taxonomic groups (Chisholm 1992, Latasa et al. 2005), as well as species-specific differences (e.g., Tang 1996), appear to override the influence of size on growth potential, however, and the method is difficult to apply

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in sediment or in turbid systems such as estuaries, where algae may be attached to large particles or where colony formation occurs. The dilution method in combination with high-performance liquid chromatography (HPLC)-based pigment analysis (Landry 1993) can provide group-specific values for both algal growth and grazing rates. Samples are diluted with predator-free water and several dilutions are compared, based on the idea that algal growth rates remain the same while mortality decreases owing to fewer encounters with potential grazers. Recent technical developments have allowed fluorescence-based measurements of photosynthetic performance of single cells (Dijkman and Kromkamp 2006b, Gorbunov et al. 1999). Quantifying carbon fixation of individual cells is hampered, however, because it remains difficult to measure the light absorption of single cells. In addition, the origin of the differences commonly observed between fluorescence-derived electron transport rates and carbon and oxygen measurements is still not fully solved (e.g., Geel et al. 1997).

Other methods make use of compound-specific labeling with either stable or radioactive isotopes. The chlorophyll labeling method was initially introduced by Redalje and Laws (1981) to calculate the growth rate of the phytoplankton community. The method was later refined to measure group-specific algal growth rates by including the isotopic labeling of carotenoids (Goericke and Welschmeyer 1993a). Applying a similar procedure, Popp et al. (2006) used stable-isotope labeling of alkenones to calculate the growth rates of alkenone-producing haptophytes, and Pel et al. (2004) combined flow-cytometric cell sorting with stable-isotope labeling of fatty acids to measure growth of cyanobacteria and eukaryotic algae.

In this article, we explore the use of stable-isotope labeling of phospholipid-derived fatty acids (PLFAs) to study group-specific primary production. There were several reasons for turning to PLFAs as biomarkers. Although pigments are the most obvious biomarkers for phytoplankton, many algal taxa have a characteristic PLFA composition as well (Dijkman and Kromkamp 2006a, Viso and Marty 1993, Volkman et al. 1989, Zhukova and Kharlamenko 1999), and depending on the taxonomic composition, PLFAs may provide a more detailed image of the phytoplankton composition than pigments (Dijkman and Kromkamp 2006a). An advantage is that information on non-algal taxa, such as bacteria, can be derived as well because PLFAs are important constituents of cellular membranes in eukaryotes and bacteria. This makes it possible to follow transfer of labeled material to other trophic levels applying the same methodology (Boschker 2004, Middelburg et al. 2000). Furthermore, the isotopic composition of PLFAs can be measured relatively simply using gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS) (Boschker 2004). In contrast, measurement of the isotopic composition of pigments, which are most commonly used as biomarkers for phytoplankton, is not straightforward and requires collection of fractions after separation of the pigments by thin-layer chromatography or HPLC (Goericke and Welschmeyer 1993a, 1993b) or with in-line scintillation counting in combination with radioactive isotopes (Ornolfsdottir et al. 2004; Pinckney et al. 2001, 1996). We used phospholipid-derived fatty acids rather than total lipid-derived fatty acids, because phospholipids are rapidly degraded upon cell death and are therefore representative of viable biomass (Pinkart et al. 2002). Furthermore, phospholipids are not used as storage material in contrast to neutral lipids, which include variable amounts of storage molecules in the form of triacylglycerols. Phospholipids are therefore a more stable fraction of cellular biomass total lipids. PLFA synthesis indicates synthesis of cellular membranes and thus growth. The rate of PLFA synthesis should give a good proxy for growth rate calculations based on isotopic labeling.

We measured the rate of incorporation of ^{13}C in PLFAs in both cultured material and field samples during incubations in the presence of ^{13}C-labeled bicarbonate (^{13}C-HCO_3^-). In algal cultures, growth rates were calculated from labeling data and then validated with those based on increases in cell numbers and PLFA concentrations. In field samples, PLFA synthesis rates were calculated from PLFA concentrations and isotopic data and the taxonomic composition and group contribution to primary production were estimated using full PLFA spectra.

Materials and procedures

^{13}C-labeling of cultivated strains—Species were chosen to represent the phytoplankton in the Scheldt estuary, a turbid, tidal system (Belgium, the Netherlands). Algal cultures were grown in Erlenmeyer flasks containing the appropriate medium (Table 1) at an irradiance of 40 µmol m^{-2} s^{-1} with a light:dark cycle of 14:10 and a temperature of 16°C. The cultures were kept in the logarithmic growth phase for at least 2 weeks before measurements by regular dilution with fresh medium. Incubations were carried out in closed bottles. The cultures were enriched with ^{13}C-$NaHCO_3$ (99% ^{13}C; Cambridge Isotope Laboratories, Inc.) to approximately 4% of the ambient dissolved inorganic carbon (DIC) concentration.

The green alga *Ankistrodesmus angustus* and the diatom *Thalassiosira pseudonana* were incubated at the growth irradiance for a full 24-h period. Cell numbers and concentration and isotopic composition of PLFAs and of particulate organic carbon (POC) were followed in time. The carbon stable-isotope ratio of total DIC was determined according to Miyajima et al. (1995) using a Fisons NA-2500 Elemental Analyzer (column: Haysep Q, flow rate 80 mL min^{-1}) coupled online with a Finnigan Delta S isotope ratio mass spectrometer. A larger number of species was incubated for 2 h at a higher irradiance of 210 µmol m^{-2} s^{-1}. The exact enrichment of DIC in these incubations was calculated after DIC concentration was determined by potentiometric titration as described in detail in Gazeau et al. (2005). Cell numbers were determined using a cell counter (Coulter Multisizer II). Cells for PLFA and POC analysis were harvested on glass fiber filters (GF/F) (Whatman) using low

Table 1. ^{13}C labeling (δ^E) relative to 16:0 after 2-h incubation.

Species	Strain code	Medium	δ^E-DIC	14:0	16:0	16:1 (n-7)	16:2 (n-4)	16:3 (n-4)	16:3 (n-3)	16:4 (n-3)	18:3 (n-3)	18:4 (n-3)	18:5 (n-3)	18:5 (n-3,6,9,12,16)[a]	20:5 (n-3)	22:6 (n-3)
Thalassiosira pseudonana (Bacillariophyceae)	CCMP1013	F/2	1253	0.31	1	0.77	0.42	0.05				0.38			0.18	0.08
Chaetoceros calcitrans (Bacillariophyceae)	PCC537	F/2	546	0.49	1	0.84	0.27	0.09				1.59			0.25	0.11
Cylindrotheca closterium (Bacillariophyceae)	CCY9601	F/2	716	0.70	1	0.55	0.61	0.39				1.13			0.16	0.05
Ankistrodemus angustus (Chlorophyceae)	CCY9713	BG11	2728	0.19	1	0.70			1.54	0.24	0.24	0.04				
Chlorella pyrenoidosa (Trebouxiophyceae)	CCY9618	BG11	4892	0.35	1	0.33			0.16		0.24					
Rhodomonas maculata (Cryptophyceae)	CCY0234	F/2-Si	680	0.64	1	0.21	0.13				0.93	0.52			0.07	0.03
Emiliania huxleyi (Haptophyceae)	CCY0319	F/2-Si	9745	0.96	1	0.75	0.26				0.37	0.19	0.05	0.06	0.31	0.27

Only PLFAs that are discriminative between phytoplankton groups are indicated. CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; F/2, artificial seawater enriched with F/2 nutrients (Guillard and Ryther 1962); PCC, Plymouth Culture Collection; CCY, Culture Collection Yerseke; BG11, blue-green freshwater medium (Stanier et al. 1971); F/2-Si, identical to F/2 but Si omitted.

[a]In a previous publication (Dijkman and Kromkamp 2006a), this PLFA was erroneously assumed to have a length of 20 carbon atoms and was indicated as 20:x(n-x). Mass spectrometry on pycolinyl esters (see "Materials and procedures") has since identified this PLFA as 18:5(n-3,6,9,12,16). Because the double bonds in this PLFA are not regularly interrupted by methylene groups, the position of each double bond is given.

pressure filtration (–0.1 to –0.2 atmospheric pressure). The filters for PLFA analysis were frozen immediately in liquid nitrogen and stored at –80°C until analysis. Filters for particulate organic carbon and nitrogen analysis were stored at –20°C until analysis.

Field experiments—Incubations for ^{13}C-labeling of natural phytoplankton communities were performed at salinities of 0 and 28 in the Scheldt estuary. In April 2003, water samples were taken from 1 m depth with a Niskin water sampler and incubated on board the research vessel *Luctor*. The samples were enriched with ^{13}C-$NaHCO_3$ (99% ^{13}C; Cambridge Isotope Laboratories, Inc.) to 2% to 4% of the ambient DIC concentration. The absolute ^{13}C enrichment was calculated after DIC was determined by potentiometric titration. The samples were incubated for 2 h in closed 1-L bottles at an irradiance of 250 µmol m^{-2} s^{-1} in an incubator at the in situ temperature. Four bottles were incubated at each station. Two bottles were pooled before taking subsamples for $\delta^{13}C$-POC and $\delta^{13}C$-PLFA to end up with duplicate samples. Incubations were ended by filtration over GF/F filters using low-pressure filtration. PLFA extraction was started immediately by placing the filters in the solvent mixture (see "PLFA extraction and analysis") and stored at –20°C until continuation of the extraction procedure in the laboratory. Filters for analysis of particulate organic carbon and nitrogen were stored at –20°C until analysis.

PLFA extraction and analysis—PLFAs were extracted and analyzed as in Boschker (2004). In short, lipids were extracted in a mixture of chloroform, methanol, and water (1:2:0.8 vol/vol/vol), using a modification of the method of Bligh and Dyer (1959). Phase separation was induced by the addition of chloroform and water to a final composition of chloroform:methanol:water of 1:1:0.9 (vol/vol/vol). The chloroform layer containing the total lipid fraction was collected. The total lipid extract was fractionated into different polarity classes on silica columns (0.5 g Kieselgel 60; Merck) eluted sequentially with chloroform (7.5 mL), acetone (7.5 mL), and methanol (15 mL). The methanol fraction, containing mainly phospholipids, was derivatized using mild alkaline methanolysis (1 mL of 0.2 M sodium methanolate, 15 min at 37°C) to yield fatty acid methyl esters (FAMEs), which were recovered by hexane extraction. Separate glassware was used for enriched and non-enriched samples to avoid contamination. FAMEs were separated on a very polar analytical column (BPX-70, 50 m length, 0.32 mm diameter, 0.25 µm film; Scientific Glass Engineering). FAME concentrations were determined by gas chromatography–flame ionization detection (GC-FID) (Interscience HRGC MEGA 2 series). The isotopic composition of individual FAMEs was determined using GC-C-IRMS; a HP G1530 GC (Hewlett Packard) was connected to a Deltaplus IRMS via a type-III combustion interface from

Thermo Finnigan (Bremen). The combustion oven of the interface was kept at 950°C, and no reduction oven was used. The GCs were equipped with a split/splitless injector that was used in the splitless mode. The injector temperature was 240°C, the column flow was kept at 2 mL min^{-1}, and the following temperature program was applied: initial 60°C for 2 min, then to 110°C with +25°C/min, to 230°C with +3°C/min, to 250°C with +25°C/min, and hold 15 min. The splitless period was 1.5 min and the total run time about 55 min. Non-enriched samples were measured before enriched samples. Blanks and a standard mixture were measured regularly in between samples to check for system stability and possible contamination, with regard to both concentration and isotopic composition. FAME identification was based on comparison of retention times with known reference standards. The identity of FAMEs not present in the reference standards was determined from mass spectrometry (Finnigan Voyager) on pycolinyl esters prepared from samples as described in Dubois et al. (2006). This allows identification of the molecular mass and the number and position of double bonds in FAMEs.

Stable carbon isotope ratios for individual FAMEs were corrected for the one carbon atom in the methyl group that was added during derivatization to yield PLFA ratios using the following formula:

$$\delta^{13}C_{PLFA} = [(n + 1) \times \delta^{13}C_{FAME} - 1 \times \delta^{13}C_{methanol}]/n,$$

where n is the number of carbon atoms in the PLFA. The isotopic composition of the methanol that was used for derivatization was determined separately by GC-C-IRMS (–43‰).

Chemical purity was checked by measuring several samples on a nonpolar column (HP-5MS; Agilent, see Boschker 2004 for additional information) in addition to the normal measurements on the very polar BPX70 column. These columns separated the compounds by different principles. Both retention time and the elution order of saturated versus unsaturated fatty acids differ. A comparison of concentrations and isotopic data showed that some PLFAs overlapped, mainly on the nonpolar HP5 column. The reported data are for non-overlapping PLFAs on the polar BPX70 column.

The fatty acid notation consists of the number of carbon atoms followed by a colon and the number of double bonds present in the molecule. The position of the first double bond relative to the aliphatic end of the molecule is given between parentheses [e.g., 16:1(n-7)]. If relevant, "c" indicates the *cis*-orientation of the double bonds. The other possible double bonds are methylene interrupted. Prefixes "i" (iso) and "ai" (anteiso) represent the location of a methyl branch one or two carbons, respectively, from the aliphatic end (e.g., i15:0).

Particulate organic carbon—POC was analyzed using a Fisons NA-2500 Elemental Analyzer following an in situ acidification procedure as described in Nieuwenhuize et al. (1994). The carbon isotopic composition was determined using a Fisons NA-2500 elemental analyzer coupled online with a Finnigan MAT Delta S mass spectrometer.

Data analysis—Stable isotope data are expressed in the delta notation ($\delta^{13}C$) relative to Vienna Pee Dee Belemnite (VPDB) calculated from the stable isotope ratio (R): $\delta^{13}C = (R/R_{std}^{-1}) \times 1000$, where R is the $^{13}C/^{12}C$ ratio measured in the sample and in the standard ($R_{std} = R_{VPDB} = 0.0111797$). Following Maddi et al (2006), we use the enrichment (δ^E notation) as a measure of label enrichment; $\delta^E = [(\delta^{13}C_s + 1000)/(\delta^{13}C_b + 1000) - 1] \times 1000 = (R_s/R_b - 1) \times 1000$, where R_b is the isotope ratio in the background and R_s in the sample. This enrichment notation is exact and thus accurate for high levels of enrichment. The production per PLFA was calculated from the difference in the fraction of ^{13}C at the start and the end of the incubation, multiplied by the concentration of this PLFA at the start of the incubation. The fraction of ^{13}C is calculated as $^{13}C/(^{13}C + ^{12}C) = R/(R + 1)$. R is the isotope ratio and is derived from $\delta^{13}C$ values as $R = (\delta^{13}C/1000 + 1) \times R_{VPDB}$. The data were corrected for the % ^{13}C enrichment and the incubation time to calculate PLFA production rates (nmol L^{-1} h^{-1}).

Growth rates (μ) were calculated from several parameters for experiments with algal cultures; μ (day^{-1}) was calculated from cell numbers and the concentration of PLFA as:

$$\mu = \frac{1}{t} \ln\left(\frac{N_t}{N_0}\right) \quad (1)$$

where N_0 and N_t are cell numbers or PLFA concentration at the start of the experiment and the time of sampling, respectively, and t is time (days). Growth rate (μ) ($days^{-1}$) was also calculated from the isotopic data of PLFAs or POC in combination with DIC analogous to the chlorophyll-based measurements of growth rates as reported in Welschmeyer and Lorenzen (1984):

$$\mu = -\frac{1}{t} \ln\left(1 - \frac{\delta^E C_x}{\delta^E C_{DIC}}\right) \quad (2)$$

where $\delta^E C_X$ is the enrichment in ^{13}C of PLFAs or POC and $\delta^E C_{DIC}$ is the enrichment of DIC.

The group composition of the standing stock of biomass and of primary production was estimated for field experiments using the matrix factorization program Chemtax (Mackey et al. 1996). The program requires a data file containing sample data and an input ratio file containing the biomarker composition of the groups present in the samples. Chemtax uses an iterative process to find optimal PLFA ratios per group and estimates the fraction of the total biomarker pool belonging to each group. The program was originally developed to analyze pigment data, in which case the results are expressed as the fraction belonging to chlorophyll *a*. Using PLFAs, we express the results as the fraction belonging to the PLFA 16:0, since this PLFA is present in all groups relevant to our samples.

The taxonomic composition for the standing stock of biomass was estimated based on the PLFA composition as described in Dijkman and Kromkamp (2006a) and checked against microscopy counts (data not shown). The field measurements reported here are two of five stations analyzed for

standing stocks in that publication. Based on PLFA data, six groups were distinguished: bacteria, two groups of green algae (Chlorophyceae and Trebouxiophyceae), diatoms, cryptophytes, and a combined group of dinoflagellates and haptophytes. The latter group contains haptophytes such as *Phaeocystis* sp. and *Emiliania huxleyi*, but some other haptophytes (e.g. *Isochrysis* sp. and *Pavlova* sp.) are more similar to diatoms and are not represented by the combined dinoflagellate–haptophyte group. The input ratio matrix for the analysis of the standing stock of phytoplankton was based on the PLFA composition measured in cultured algae and literature data (for the functional group "bacteria") (Table 2).

The input ratio matrix is adapted by the CHEMTAX program during the analysis to best fit the data. The adapted ratio files from the analysis of the standing stock were used as the starting point (input ratio file) for the analysis of phytoplankton groups contributing to primary production. The data were grouped per station, and therefore separate ratio files were used for the freshwater and marine station. Moreover, differences occur in the degree of labeling of individual PLFAs from a single group during short-term incubations (see "Assessment," Fig. 1, and Table 1), and the adapted ratio file resulting from the analysis of the standing stock was therefore multiplied by the corresponding correction factors presented in Table 1. Three diatom species are included in Table 1, and the group "diatoms" was corrected with the average values of three tested species. Growth of bacteria was not detected during the incubations, and bacteria—and PLFAs found only in bacteria—were removed from the input ratio file. We also removed PLFAs with deviating labeling kinetics as described in "Assessment" (Table 3). The ratio file was again adapted during the fitting procedure (Table 4).

Assessment

Culture experiments: Use of ^{13}C labeling of PLFAs to determine growth rates—The labeling kinetics of individual PLFAs and the calculation of growth rates from labeling data were investigated using batch cultures. The time-course ^{13}C-labeling of individual PLFAs and POC was followed during a 24-h period in the green alga *Ankistrodesmus angustus* and the diatom *Thalassiosira pseudonana*. Enrichment of DIC (δ^E-DIC) was 2725 and 1244 for *A. angustus* and *T. pseudonana*, respectively. δ^E increased in time for all PLFAs (Fig. 1); however, the rate of increase was PLFA specific. In both algal species, δ^E initially increased faster and was higher for 18:1(n-9) and 18:2(n-6) than for the other PLFAs. In *A. angustus*, 16:3(n-3) followed a similar deviating labeling pattern. δ^E of the remaining PLFA initially increased linearly with time, at least for the first 8 h of incubation. Coefficients of determination (r^2) for linear regression of δ^E versus time until 8 h were higher than 0.95 for all PLFAs in both species. As a general pattern, δ^E increased faster in short PLFAs than in long PLFAs, although there were exceptions to this pattern. For example, 16:4(n-3) was labeled more slowly than several longer PLFAs in *A. angustus*. Similarly, labeling was slower for 14:0 and 16:3(n-4) than for several longer PLFAs in *T. pseudonana*. δ^E of POC increased at an

Table 2. Chemtax ratio files for the station with salinity 0: Input ratio file for the analysis of the standing stock.

	Chlorophyceae	Trebouxiophyceae	Diatoms	Cryptophytes	Dinoflagellates–haptophytes	Bacteria
i14:0						0.10
14:0	0.05	0.01	0.81	0.33	0.74	0.33
i15:0						0.33
ai15:0						0.49
15:0			0.04	0.02	0.11	0.11
16:0	1	1	1	1	1	1
16:1(n-7c)	0.40	0.54	1.68	0.20	0.19	0.67
16:2(n-4)			0.45		0.09	
16:3(n-4)			0.74			
16:3(n-3)	0.20	1.549				
16:4(n-3)	1.26					
16:4(n-1)			0.68			
18:1(n-9c)	0.58	0.20	0.05	0.09	0.52	0.14
18:1(n-7c)	0.03	0.07	0.45	0.69	0.37	0.38
18:2(n-6)	0.46	0.56	0.08	0.08	0.10	0.09
18:3(n-3)	3.34	3.18		1.63	0.45	
18:4(n-3)	0.42		0.52	3.78	1.21	
18:5(n-3)					0.39	
18:5[a]					1.31	
20:5(n-3)			2.27	2.28	0.36	
22:6(n-3)			0.48	1.31	1.60	

[a](n-3,6,9,12,16).

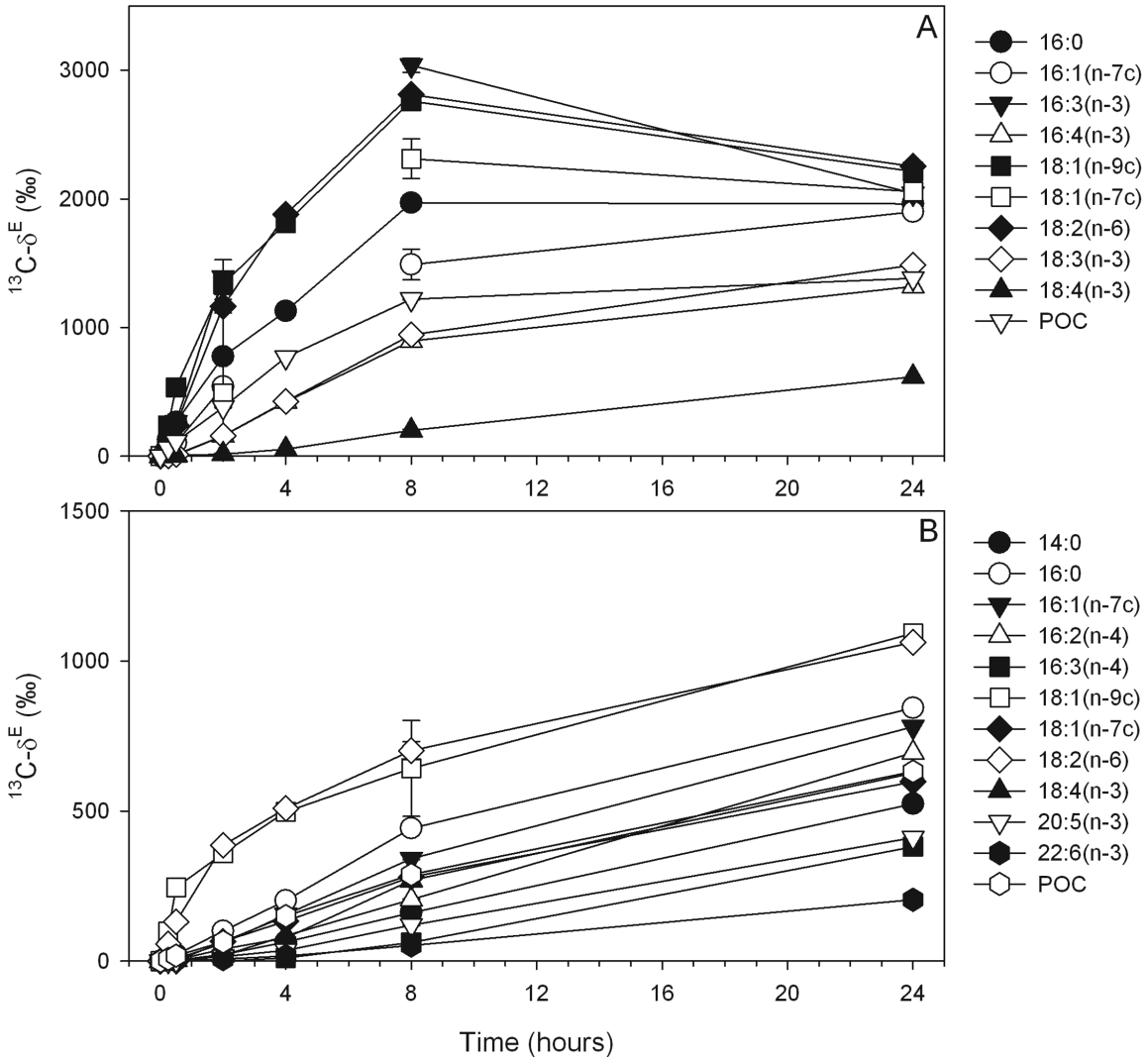

Fig. 1. Development of δ^E (specific labeling) with time in the green alga *Ankistrodesmus angustus* (A) and the diatom *Thalassiosira pseudonana* (B) during 24-h incubation. Error bars (where larger than marker size) present differences between duplicate incubations.

intermediate rate in both species. In *A. angustus*, the difference in δ^E values decreased after 24 h; in *T. pseudonana*, convergence was not observed during 24 h of incubation.

The total incubation time of 24 h was sufficiently long for a measurable increase in the concentration of individual PLFAs. The increase in PLFA concentration was balanced, and the relative PLFA composition did not change during the incubation (data not shown). Differences among PLFAs as observed for their δ^E were not found for increases in concentration.

As the relative differences in labeling were more or less constant during the first few hours of the incubation, a larger number of species was incubated for 2 h only. The labeling pattern of individual PLFAs was reproducible among species, although the relative amplitude varied. Similar to the results for *A. angustus* and *T. pseudonana*, after 2 h δ^E was always highest in 18:1(n-9) and 18:2(n-6) and was generally higher in short PLFAs than in long PLFAs (Fig. 1). This difference in labeling between individual PLFAs and POC is probably due to differences in biosynthesis routes, where fast-labeled compounds are first intermediates in pathways and other compounds get labeled more slowly, explaining the lower labeling in total POC (see "Discussion"). Table 1 lists the ratio of PLFA δ^E relative to δ^E of 16:0 for several PLFAs that can potentially be used as biomarkers for the calculation of group-specific production.

Growth irradiance for the algal cultures and the incubation irradiance for the 24-h incubations was chosen to represent average irradiance in the turbid estuary where we did our field measurements. We switched to a higher irradiance for the 2-h incubations to approximate the irradiance used in the field incubations, which was chosen to represent near-surface irradiance. The change in incubation irradiance did not affect the labeling pattern (Table 1).

Table 3. Chemtax ratio files for the station with salinity 0: Input ratio file for the analysis of primary production (adapted ratio file from the analysis of standing stock, multiplied by the correction factors presented in Table 1).

	Chlorophyceae	Trebouxiophyceae	Diatoms	Cryptophytes	Dinoflagellates–haptophytes
14:0	0.01	0.00	0.19	0.21	0.71
16:0	1	1	1	1	1
16:1(n-7c)	0.28	0.18	1.21	0.04	0.15
16:2(n-4)			0.20		0.02
16:3(n-4)			0.15		
16:3(n-3)	0.30	0.25			
16:4(n-3)	0.30				
18:3(n-3)	0.80	0.76		1.52	0.17
18:4(n-3)	0.02		0.24	1.96	0.25
18:5(n-3)					0.02
18:5[a]					0.08
20:5(n-3)			0.45	0.16	0.11

[a](n-3,6,9,12,16).

Table 4. Chemtax ratio files for the station with salinity 0: Adapted ratio file after the analysis of primary production.

	Chlorophyceae	Trebouxiophyceae	Diatoms	Cryptophytes	Dinoflagellates–haptophytes
14:0	0.01	0.01	0.16	0.21	1.18
16:0	1	1	1	1	1
16:1(n-7c)	0.28	0.18	1.58	0.04	0.15
16:2(n-4)			0.37		0.02
16:3(n-4)			0.27		
16:3(n-3)	0.30	0.25			
16:4(n-3)	0.30				
18:3(n-3)	0.80	0.61		0.50	0.17
18:4(n-3)	0.02		0.27	1.96	0.23
18:5(n-3)					0.02
18:5[a]					0.02
20:5(n-3)			0.52	0.16	0.11

[a](n-3,6,9,12,16).

For all species and at all sampling times, δ^E for POC was lower than for 16:0. δ^E of POC was 61% of that of 16:0 as determined from linear regression ($r^2 = 0.95$) (Fig. 2). This was independent of the species and irradiance, indicating that the rate of cellular carbon labeling relative to that of carbon in 16:0 was well constrained among species during the first few hours of incubation.

Growth rates for *A. angustus* and *T. pseudonana* could be calculated from several independent parameters, based on changes in either biomass (μ_N and μ_{PLFA}) or isotopic labeling ($\mu_{16:0}$ and μ_{POC}). μ_{PLFA} was the average of the growth rate calculated from the change in concentration for each PLFA individually. $\mu_{16:0}$ was multiplied by 0.61 to compensate for the difference in labeling between 16:0 and POC as described in the preceding paragraph. $\mu_{16:0}$ and μ_{POC} were the average of the values calculated for each sampling time. For both species, the different methods led to comparable growth rates (Fig. 3). Differences among these proxies for algal growth rate were significant for *A. angustus* (ANOVA, $P < 0.001$) but not for *T. pseudonana* (ANOVA, $P > 0.05$). For *A. angustus*, the difference between the highest and the lowest growth rate was 30% of the maximum value. For *T. pseudonana*, this difference was maximally 20%. Despite these differences being significant for *A. angustus*, these variations are well within the range reported for growth rates calculated from isotope labeling of other cellular compounds such as pigments (Riemann et al. 1993), alkenones (Popp et al. 2006), and total fatty acids (Pel et al. 2004).

Field measurements: Group contribution to primary production—Stable-isotope labeling of PLFAs allows the calculation of the contribution of algal groups to total primary production. This approach was tested at two sites in the Scheldt estuary differing in salinity: one site with salinity 0 in the freshwater tidal reaches of the estuary and another with salinity 28 at the mouth of the estuary.

A large number of different PLFAs were identified in the samples (Fig. 4A). The differences in PLFA concentration and

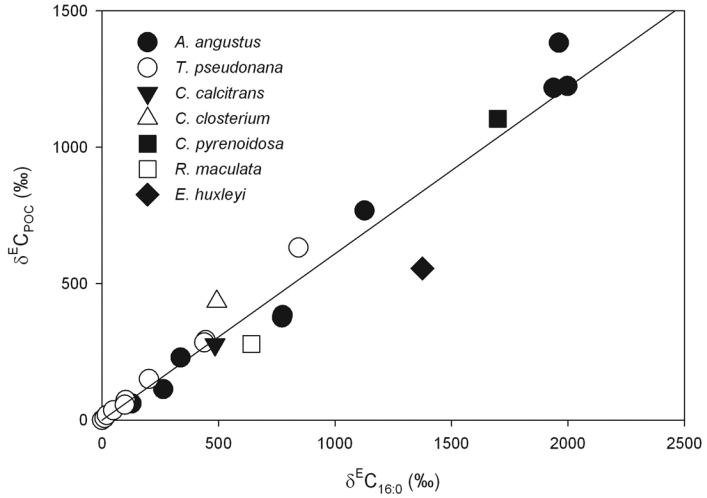

Fig. 2. δ^E_{POC} versus $\delta^E_{16:0}$ for all measured species and all sampling times. The line represents linear regression ($\delta^E_{POC} = 0.61 \times \delta^E_{16:0}$, $r^2 = 0.95$, $P < 0.0001$).

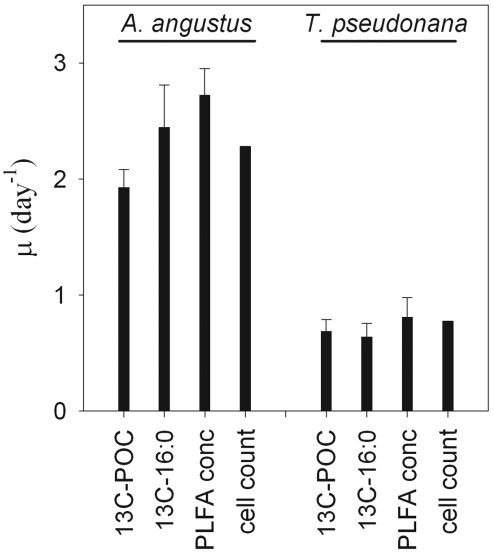

Fig. 3. Growth rates for the green alga *Ankistrodesmus angustus* and the diatom *Thalassiosira pseudonana* calculated from labeling of POC ($^{13}C_{POC}$), labeling of 16:0 ($^{13}C_{16:0}$), changes in PLFA concentration (PLFA conc), and changes in cell numbers. Error bars present differences between duplicate incubations, except for cell counts that were not replicated.

their relative abundance between stations are due to variations in the standing stock of bacteria and phytoplankton. Some of the PLFAs are found in most groups present in the Scheldt estuary [e.g., 14:0, 16:0, 16:1(n-7), 18:1(n-7), 18:1(n-9)]; other PLFAs are restricted to specific groups. Branched fatty acids such as i14:0, i15:0, and ai15:0 are exclusively found in bacteria (Kaneda 1991, Ratledge and Wilkinson 1988); 16:2(n-4), 16:3(n-4), 20:5(n-3), and 22:6(n-3) are typically important in diatoms; 16:3(n-3), 16:4(n-3), and 18:3(n-3) in green algae; and 18:5(n-3) and 18:5(n-3,6,9,12,16) are characteristic for dinoflagellates and some haptophytes (e.g., *Phaeocystis* sp.) (Dijkman and Kromkamp 2006a). At the freshwater station, the relatively high concentrations of 16:3ω4, 20:5ω3, and 22:6ω3 indicate a diatom-dominated phytoplankton community. The most obvious difference between the two stations is the presence of 18:5(n-3) and 18:5(n-3,6,9,12,16) at the high-salinity station, because these PLFAs were absent in the low-salinity station. This difference was caused by a bloom of the haptophyte *Phaeocystis* sp. in the mouth of the estuary.

DIC was enriched with 1.4% (δ^E-DIC = 1289) and 3.2% ^{13}C (δ^E-DIC = 2902) at stations 0 and 28, respectively. δ^E was different for nearly every PLFA (Fig. 4B). Differences in δ^E are the result of not only variations in labeling of PLFAs within a single organism (Fig. 1), but also activity and biomass of taxonomic groups contributing to a particular PLFA. Some of the patterns observed in the culture experiments, such as high labeling of 18:1(n-9) and 18:2(n-6), were obvious in the field experiments as well.

The production rates of individual PLFAs were calculated from concentration and labeling data (Fig. 4C). Only PLFAs typical for phytoplankton became labeled. The production rate of branched fatty acids, which are unique to bacteria, was close to zero, indicating that bacteria did not acquire substantial amounts of ^{13}C during incubation. Growth of green algae, diatoms, and dinoflagellates–haptophytes was revealed by synthesis of PLFAs typical for these groups. Cryptophytes contain no biomarkers that are not also present in the other groups, and growth of this group can therefore not be readily determined from PLFA production.

Analyzing the data with a compositional estimator program such as Chemtax provides an estimation of the relative abundance of the groups and their contribution to primary production. The contribution of groups without unique biomarkers, such as the cryptophytes, can be detected as well, since the analysis is based on the complete PLFA pattern and not solely on unique biomarkers. In Fig. 5, group composition of standing stock and primary production are compared. Data for standing stock were analyzed including bacteria as a group, but only the relative abundance of algal groups is shown in Fig. 5 to facilitate a comparison with the data for group-specific primary production. At both stations, the same groups were detected in the standing stock and the primary production. The most obvious difference between standing stock and primary production was found at station 28, where dinoflagellates–haptophytes contributed more to primary production than to standing stock.

The outcome of the Chemtax analysis depends at least to some degree on the data provided by the user in the input ratio matrix, as this matrix contains the groups to be considered and their PLFA abundance ratios (e.g., Latasa 2007). We tried several fitting options analyzing the standing stock (adding green algae as a single combined group instead of two

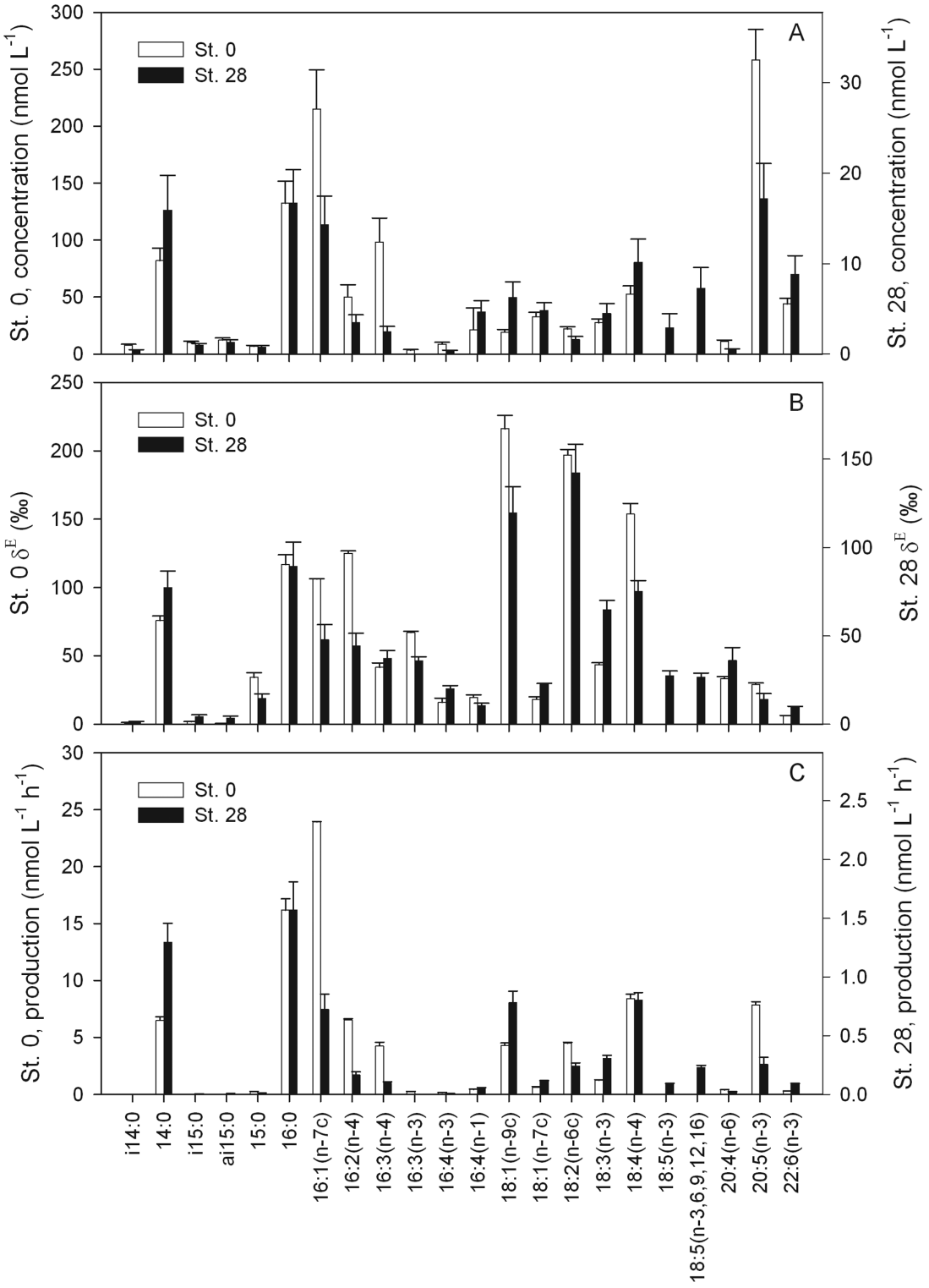

Fig. 4. Concentration (A), enrichment (δ^E) (B), and production (C) of PLFAs in the Scheldt estuary in April 2003 at a salinity of 0 (white bars) and 28 (black bars). The data are not corrected for the difference in labeling between PLFAs. Error bars present differences between duplicate incubations.

separate groups, varying PLFA ratios for bacteria, limiting the changes allowed in the ratio matrix), but these did not lead to significantly different results in terms of algal group abundance. Furthermore, the derived group composition is in good

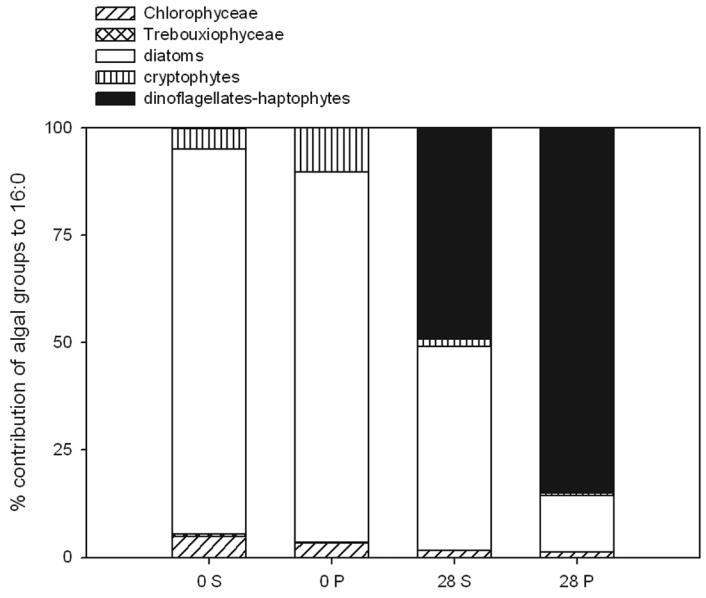

Fig. 5. Group composition, estimated with Chemtax, of the standing stock (S) and primary production (P) in the Scheldt estuary in April 2003 at a salinity of 0 and 28.

agreement with pigment data within the limitations of both approaches as discussed in Dijkman and Kromkamp (2006a).

The adapted ratio files from the analysis of standing stock are in principle the best starting point for Chemtax analysis of primary production, as these PLFA ratios have been optimized for the samples. Not all species represented by a group necessarily contribute to primary production, however, and correction for differences in labeling of PLFAs within a certain group introduces uncertainty. Therefore, we tried several fitting options in addition to the methodology outlined in "Materials and procedures." Options tested were (1) allowing no change in the PLFA input matrix; (2) combining both green algal groups (Chlorophyceae and Trebouxiophyceae) into a single group, as these share many PLFAs, allowing changes in the input matrix; and (3) same as (2) but allowing no changes in the input ratio matrix. The largest variations observed (relative to the results as presented in Fig. 5) were 8.2% of 16:0 for diatoms [station (st) 0, option (1)], 1.1% for green algae [st 28, option (3)], 7.5% for the cryptophytes [st 0, option (3)], and 5.1% for dinoflagellates–haptophytes [st 28, option (1)]. These changes are minor considering the relative abundance of these groups, except for the cryptophytes, as 7.5% is a large difference for a group contributing at most 10% of 16:0. A fourth option was not correcting for the differences in labeling. This increased the contribution of cryptophytes to 16:0 to unrealistically high values, up to 22% higher at st 28 compared to results shown in Fig. 5. The relative differences between the other groups remained the same, however. Finally, rather than using the full spectra of PLFAs with Chemtax, single biomarkers to 16:0 ratios were used: 16:4(n-3) for green algae, 20:5(n-3) for diatoms, and 18:5(n-3) for dinoflagellates–haptophytes. This again returned the same general pattern for the relative contribution of these three groups to primary production. A disadvantage of this last method is that it cannot detect groups that contain no unique biomarkers, such as cryptophytes. Thus, although some uncertainty is inevitably associated with estimations of group abundance, the pattern of group abundance was well constrained and not overly dependent on PLFA abundance input ratio matrices and correction factors for isotopic labeling.

Precision of measurements—The typical precision of $\delta^{13}C$ determination of PLFAs by GC-C-IRMS is 0.3‰. After 2 h of incubation, δ^E values for algal PLFAs were higher than 10‰, indicating that analytical reproducibility is better than 3%. Most uncertainty in group-specific primary production estimates is associated with differences in labeling of individual PLFAs, as discussed above, and variations between replicate samples. The good analytical reproducibility also implies that very low incorporation of label can be detected. An enrichment of DIC with ^{13}C-HCO_3^- of 1.4% to 3.2%, as used in the field experiments, led to a well-measurable increase in PLFA labeling after 2 h, leaving room for either using even lower enrichment or studying less productive systems. The actual detection limit depends on ^{13}C enrichment of DIC, incubation time, phytoplankton biomass, and analytical precision.

Discussion

Several requirements have to be met if biomarker labeling is used for calculating group-specific growth rates: (1) growth must be balanced, i.e., physiological acclimation should not take place during the incubation (Goericke and Welschmeyer 1993a); (2) the biomarker must be a stable end product that does not turn over (Goericke and Welschmeyer 1993a); and (3) specific activity of biomarker and total carbon must be equivalent after a given time period (Redalje and Laws 1981, Welschmeyer and Lorenzen 1984).

Acclimation to incubation conditions can cause growth to be severely unbalanced. Well known are the changes in cellular pigment composition and content due to photoacclimation (Goericke and Welschmeyer 1993a, 1993b; McManus 1995). Lipids are primarily a constitutive component and make up a rather constant fraction of biomass (Wainman et al. 1999). Although studied less than for pigments, variations in PLFA content and composition in response to irradiance seem to be much more limited in comparison to variations in pigment composition (Brown et al. 1993, 1996; Dijkman and Kromkamp, unpublished data). PLFA composition is known to respond to shifts in temperature, as length and degree of saturation are adapted to maintain membrane fluidity (Sushchik et al. 2003). Because we incubated at in situ temperatures, however, we can neglect this possible source of error.

Pigments are stable end products that are not respired and have negligible turnover (Goericke and Welschmeyer 1993a). PLFAs meet this requirement only partially, since some PLFAs are intermediates in the synthesis toward other PLFAs. De

novo synthesis of fatty acids occurs in chloroplast as acyl-carrier protein (ACP) thioesterss which are not included in the polar lipid fraction. 16:0-ACP and 18:1-ACP are the major products of plastid fatty acid synthesis (Browse and Somerville 1991). Further elongation and desaturation, leading to the synthesis of longer and/or more unsaturated fatty acids, can proceed along various pathways, partly via phosphatidylcholine (PC)- and phosphatidylethanolamine (PE)-linked fatty acids (Arao and Yamada 1994, Domergue et al. 2002, Khozin-Goldberg et al. 2002, Sushchik et al. 2003). Either synthesized fatty acids are released as free fatty acids and subsequently bound to other lipids or the diacylglycerol moieties are released from the phospholipids and incorporated into other lipids (Khozin-Goldberg et al. 2002).

The above biosynthesis route of fatty acids explains several observations in our experiments. The differences in δ^E reflect the biosynthesis route of fatty acids, with high labeling of PLFAs early in the biosynthesis route and lower labeling in PLFAs that are further down the biosynthesis route. The two PLFAs with a deviating labeling pattern in all species, 18:1(n-9) and 18:2(n-6), are the first intermediates toward all other long-chain polyunsaturated fatty acids (Arao and Yamada 1994, Domergue et al. 2002, Khozin et al. 1997, Sushchik et al. 2003). The only other PLFA with a deviating labeling pattern, 16:3(n-3) in *Ankistrodesmus angustus*, was of minor importance in terms of biomass and was probably mainly acting as an intermediate toward 16:4(n-3). Some loss of label was observed for 18:1(n-9) and 18:2(n-6) in *Ankistrodemus angustus* after 24 h; the synthesized fatty acids can also be incorporated into neutral lipids or glycolipids and, hence, can be lost from the phospholipid fraction. 18:1(n-9) and 18:2(n-6) are nonspecific PLFAs that are found as a minor fraction in all eukaryote algae (Dijkman and Kromkamp 2006a). Because of their deviating labeling pattern and nonspecific biomarker properties, they are not useful to derive group-specific rates. Labeling of most other PLFAs was linear with time, at least for the first few of hours of an incubation. Therefore, fatty acid synthesis partly taking place attached to phospholipids does not pose a problem.

Apart from minor differences caused by discrimination against the heavier isotope ^{13}C in certain enzymatic reactions, the isotopic composition of cellular compounds will eventually become equal. As our results indicate, however, it may take a considerable period before PLFAs and POC become equally labeled. To calculate growth rates, either incubations should be long enough to reach equal labeling or the data have to be corrected for the difference in labeling, in which case the incubation period should be short enough to ensure that the difference in labeling relative to 16:0 is constant. Differences in specific activity of marker molecules have been observed for pigments (Goericke and Welschmeyer 1993a) and alkenones (Popp et al. 2006) as well, and are thought to be caused by the presence of an unlabeled precursor pool at the start of the experiment and isotopic disequilibrium of the internal carbon pool, precursor pools, and marker molecules.

Our field measurements were done in a highly eutrophic and highly productive system that allowed the use of intermediately high labeling. The aforementioned isotopic discrimination against the heavier isotope was not corrected for. With some precautions, the method can equally well be applied in oligotrophic or less productive systems. When labeling is low, either because lower DIC enrichment is used or because of lower productivity of the studied system, isotopic discrimination should be taken into account as was done in Welschmeyer and Lorenzen (1984) for pigments. Another issue that becomes important when labeling is low is that fatty acids are usually somewhat depleted ^{13}C relative to total biomass (1‰–8‰) and that a few ‰ difference can exist in natural abundance of ^{13}C in individual PLFAs from a single organism due to fractionation at different steps in the biosynthesis route (Schouten et al. 1998, Dijkman and Kromkamp, unpublished data).

As pointed out in the "Introduction," an important reason for using PLFAs is the relative ease with which the isotopic composition can be measured. As biomarkers, PLFAs have both advantages and disadvantages. For example, the green algae can be split into two classes based on PLFAs, and *Phaeocystis* sp., of which the local strain lacks the biomarker pigment 19′-hexanoyloxyfucoxanthin, is easily recognized by the presence of 18:5(n-3) and 18:5(n-3,6,9,12,16) (Dijkman and Kromkamp 2006b). The division of haptophytes in two groups, however, is not consistent with the major clades in these algae based on phylogenetic studies (e.g., Edvardsen et al. 2000). It should be pointed out here that evolutionary pathways of pigments and PLFAs are not necessarily the same. Phospholipids are membrane lipids, most of which originated from the host cell, whereas pigments are restricted to the chloroplast and thus originated from the endosymbiont. Algae acquired their chloroplasts by multiple endosymbiotic events (Jeffrey and Wright 2006), and thus the evolutionary pathways of PLFAs and pigments may well be different. As with any compound used as biomarker, a careful examination of the sample is necessary to determine which organisms are present and to ascertain a correct interpretation of the data.

Pel et al. (2004) used total fatty acids (of which PLFAs are a subfraction) to measure algal growth rates. They first separated lacustrine phytoplankton samples by flow cytometry and analyzed the resulting fractions for fatty acids. The cell sorting resulted in well-defined fractions of phytoplankton, which facilitated assigning fatty acids to specific taxa. This method worked well to separate cyanobacteria from eukaryote algae. Because the fractions collected from the flow cytometer were very small, however, pyrolytic methylation was required to prepare the fatty acids. Unfortunately, this resulted in selective loss of long-chain (C20 and C22) fatty acids, which prevented a proper differentiation between eukaryotic algal taxa. Furthermore, in turbid environments such as rivers and estuaries, flow-cytometric cell sorting is often not possible since many species are attached to other algae or to detritus particles. For

example, microscopic observations of phytoplankton at station 28 showed that nearly every *Phaeocystis* sp. colony had chains of the diatom *Thalassionema nitzschioides* attached to it.

We calculated algal growth rates based on labeling of the PLFA 16:0, but growth rates can be based on any of the other PLFAs, provided that data are corrected for the difference in labeling relative to 16:0. If applied in field measurements, contribution of bacteria and eukaryotes other than algae will have to be considered, as these contain PLFAs as well. In water samples collected by filtering, bacteria and algae are the main contributors to biomass. In our samples, bacteria contributed approximately 20% of 16:0 (Dijkman and Kromkamp 2006a). Bacteria do not synthesize long-chain, polyunsaturated fatty acids such as the PLFAs 18:3(n-3) and 20:5(n-3), and these PLFAs can be used for calculating growth rates.

Whether growth rates can be calculated in field samples depends on the experimental setup. Proper algal growth rates require 24-h incubations, as algal growth continues during the dark period on internally stored carbon. Furthermore, it requires incubating under the correct irradiance conditions, with regard to both light intensity and spectral composition, and should therefore preferably be carried out in situ (Goericke and Welschmeyer 1993a). Short-term incubation also limits the effect of grazing. In the Scheldt estuary with its strong tidal currents and heavy shipping traffic, in situ incubations are not feasible. Therefore, we used short incubations and estimated the group composition of the primary production from the PLFA synthesized. Calculation of production from biomarker labeling and initial biomass estimates is based on the assumption that biomass remains constant during incubation (Latasa et al. 2005). This was one of the reasons for using short incubations. At the freshwater station, all groups that were present in the standing stock of phytoplankton contributed to primary production more or less proportionally to abundance (Fig. 5). In contrast, at the marine station, the dinoflagellate–haptophyte group contributed much more to the production than expected based on its abundance, indicating that they were growing relatively fast and that a spring bloom of this group was developing. Similar measurements in other systems showed that even larger differences can occur. For example, in coastal microbial mats, diatoms were found to grow much faster than the cyanobacteria that were the dominant component of the mats (Dijkman and Kromkamp, unpublished data).

Comments and recommendations

A number of compounds (pigments, alkenones, PLFAs) have been used in compound-specific labeling studies to investigate group-specific primary production. The choice of method and compound will depend on the system to be studied, the taxonomic composition of such a system, the research question, and the analytical tools at hand. We have shown here that it is possible to dig into the black box of bulk production by using PLFA labeling. The activity of the individual algal groups and their contribution to the overall primary productivity can be estimated, and by doing so, a more accurate picture on biodiversity function relationships can be obtained. The main advantage of using PLFAs is that information on a large number of PLFAs can be obtained from a single sample so that a detailed image of primary production can be acquired. With some additional studies on system-relevant phytoplankton, this sensitive method will be applicable to a wide range of pelagic ecosystems and also to benthic systems such as algal mats.

In the Scheldt estuary, three of five identified phytoplankton taxa contained specific biomarkers. For two other taxa, however, only the relative importance of different PLFAs was indicative. One then has to take the entire spectrum of analyzed PLFAs into account by using a compositional estimator. Our approach is based on the most commonly used and well-known technique Chemtax (Mackey et al. 1996), which has identifiability problems and requires iterative ad hoc modifications to generate acceptable results (Latasa 2007). Knowledge of phytoplankton groups present in the system under investigation is essential to produce the right input ratio matrix. However, our results were rather robust and not very sensitive to combining groups or limited changes in the ratio matrix. Recently, van den Meersche et al. (2008) introduced a novel compositional estimator for microbial taxonomy based on Bayesian interference. Their approach allows for better and more accurate inclusion of variance in input ratios among taxa and environments. Combination of this Bayesian compositional estimator and stable-isotope labeling of PLFAs will likely resolve some of the uncertainty related to variability of input ratio files. Another improvement circumventing the lack of biomarker PLFAs would be in the use of more specific types of biomarkers such as ribosomal RNA (e.g., Miyatake et al. 2009).

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