LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 3, 2005, 511–519

Measuring carbon isotope ratios of microphytobenthos using compound-specific stable isotope analysis of phytol

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Abstract

Carbon stable isotope analysis is one of the most common methods for validating theories about the role microphytobenthos (MPB) plays in estuarine food webs. However, difficulties extracting MPB from sediments to determine a pure isotope signature have hampered such studies. We have developed compound-specific isotope analysis (CSIA) of phytol $\delta^{13}C$ ($\delta^{13}C_{phytol}$) as an accurate proxy for $\delta^{13}C$ of bulk MPB ($\delta^{13}C_{bulk}$). In most circumstances MPB is the dominant source of phytol in estuarine sediments, particularly in shallow water environments, so our method circumvents the need to extract MPB. We have demonstrated in the laboratory the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$, and could predict $\delta^{13}C_{bulk}$ within 1.8‰ (95% CI) of observed values at moderate replication ($n = 5$). If water temperature is included, $\delta^{13}C_{phytol}$ predicts $\delta^{13}C_{bulk}$ within 1.3‰. With greater replication ($n = 10$), predictions are extremely good (within 1.0‰). A two-source mixing model assessed the usefulness of each method for estimating MPB contribution to consumer nutrition. At moderate replication ($n = 5$), for a gap between MPB and another producer of 6.0‰, estimates were more precise (i.e., 95% CIs were smaller) when both $\delta^{13}C_{phytol}$ and temperature were used (95% CI 0.40) rather than only $\delta^{13}C_{phytol}$ (0.46). The greatest difference in precision was for a gap of 6.0‰, close to the average gap in the literature (5.5‰). Given the difficulty of extracting MPB from sediment, carbon isotope studies of food webs could benefit by using CSIA of phytol, especially in conjunction with simple measurements of water temperature. The advance in methodology will allow a reevaluation of the trophic importance of MPB.

Increasing anthropogenic pressure on coastal areas has led to an emphasis on the conservation of estuarine habitats, especially those of nutritional importance for fisheries species. Although macrophytes such as seagrasses and mangroves are relatively well protected, little consideration is given to the abundant cyanobacteria and diatoms (known collectively as microphytobenthos [MPB]) in superficial sediments. Although inconspicuous, MPB can be highly productive (up to 10 Kcal m^{-2} y^{-1}; Kennish 1995) and may be an important source of nutrition for many estuarine consumers.

To determine the contribution of MPB to consumer diets, recent studies have applied stable isotope analysis (SIA). Unlike more traditional methods (e.g., gut content analysis), SIA distinguishes between food which is merely ingested by an organism and that which is assimilated, and is able to trace the ultimate source of nutrition for a consumer (Peterson and Fry 1987). This is particularly useful in the case of MPB, which, although often ingested directly by benthic invertebrates, may be used via microbe intermediaries (e.g., Middelburg et al. 2000).

Carbon stable isotope analysis uses the ratio of the rare, heavy isotope (^{13}C) to the common, lighter isotope (^{12}C) of producers and consumers. Producer signatures differ depending on the source of nutrients and fractionation (discrimination between heavy and light isotopes) during uptake and assimilation. Because the signature of a heterotrophic consumer reflects that of its food (Peterson and Fry 1987), comparison of the consumer signature with those of potential autotrophic sources can indicate one or more ultimate sources of organic matter even after multiple trophic transfers (Peterson and Fry 1987).

Studies using stable isotope analysis have supported the assimilation of MPB by both lower-level consumers (e.g., nematodes, Montagna et al. 1995, Riera and Hubas 2003) and higher-order species (e.g., oysters, Riera and Richard 1996; commercially harvested fish, Melville and Connolly 2003). However, accurate

Acknowledgments

We thank Ian Jameson for assistance with microalgae growth techniques, Gustaaf Hallegraeff for species identifications, and Frances D'Souza, John Volkman, Rhys Leeming, and two anonymous reviewers for valuable comments on the manuscript. This work was partly funded by a Queensland Government 'Growing the Smart State' grant and the Commonwealth Scientific and Industrial Research Organisation (CSIRO).

Table 1. Methods for obtaining MPB $\delta^{13}C$ in selected studies, highlighting potential for contamination with bacteria, detritus, and meiofauna (P = possible, U = unlikely/not possible) and expected discrimination against components of the MPB community.

General method	Specifics	Potential contamination			MPB discriminated against	Reference
		Bacteria	Detritus	Meiofauna		
Scraping of sediment surface	Bulk sediment	P	P	P	None	Loneragan et al. (1997) Paterson & Whitfield (1997) Dittel et al. (2000) Waldron et al. (2001)
	MPB colonies/mats	P	P	P	Noncolonial	Sullivan & Moncreiff (1990) Kwak & Zedler (1997) Stribling & Cornwell (1997)
	Gut contents of fish known to consume MPB	P	P	P	Various	Newell et al. (1995)
Physical separation of MPB from sediment	Migration into Nitex nylon mesh/silica powder/lens tissue	P	U	P	Nonmotile	Couch (1989) Currin et al. (1995) Page (1997) Lee (2000) Wainright et al. (2000) Cook et al. (2004)
	Centrifugation in colloidal silica	U	P	P	Larger or smaller	Créach et al. (1999) Melville & Connolly (2003) Guest et al. (2004) Hamilton et al. (2005)
Use of biomarker	CSIA of phytol	U	P	U	None	This study

use of stable isotope analysis relies on clear definition of the isotopic signatures of potential sources, and the signatures assumed for MPB in previous studies are questionable (Table 1).

In studies to date, estimation of $\delta^{13}C$ of bulk MPB (i.e., whole algal cells) has been through either analysis of material scraped from the sediment surface (sediment or MPB mats) or attempts to physically separate MPB from sediments before analysis. Where isotopic signatures of bulk sediment are used as a proxy for MPB $\delta^{13}C$ (e.g., Dittel et al. 2000), signatures are contaminated by detritus and meiofauna within the sediment. Samples may also be contaminated with bacteria, although relative biomass is very low. Allowing MPB to migrate onto Nitex nylon mesh (e.g., Couch 1989), lens tissue (e.g., Cook et al. 2004), or silica powder (e.g., Riera and Richard 1996) may give very clean samples, but discrimination against non- or less-motile MPB may give biased isotopic signatures (Cook et al. 2004). Centrifuging the sediment-MPB mixture in colloidal silica (Hamilton et al. 2005) avoids discrimination against nonmotile species but sometimes fails to isolate larger algal cells when sediment requires prior sieving (Oakes pers. observation unref.). Contamination can be low, especially in sandy sediments with minimal organic matter, but where MPB standing biomass is relatively low, detritus and meiofauna may still be significant in the sample (S. Y. Lee, unpublished observations). Such uncertainty regarding MPB $\delta^{13}C$ may have resulted in misinterpretation of trophic linkages. There remains, therefore, a need for a reliable method to determine MPB $\delta^{13}C$ in studies of marine food webs.

The isotopic signature required for comparison to that of consumers is $\delta^{13}C$ of the whole algal cell (MPB $\delta^{13}C_{bulk}$). Microalgae contain many different compounds, however, and MPB $\delta^{13}C_{bulk}$ is simply the weighted average of the signatures of each of these. The determination of the isotopic abundances of individual compounds such as chlorophyll-*a* (chl-*a*; Sachs et al. 1999), phytol (Riebesell et al. 2000), and fatty acids (Uhle et al. 1997) has been made possible by the recent development of compound-specific isotope analysis (CSIA). If the isotopic signature of a compound uniquely associated with MPB (i.e., a biomarker) is predictably related to that of the whole algal cell, then extraction and analysis of this compound may be used to estimate MPB $\delta^{13}C_{bulk}$.

Compound-specific isotope analysis has been used in only a few studies of trophodynamics to date (e.g., Middelburg et al. [2000] used CSIA of fatty acids to trace ^{13}C uptake by bacteria). No trophic studies have used CSIA of MPB. The current study describes the development and validation of a method to predict MPB $\delta^{13}C_{bulk}$ using CSIA of phytol to effectively isolate the living autotrophic component of sediment (i.e., MPB). This negates the need for physical isolation of algal cells from the sediment matrix.

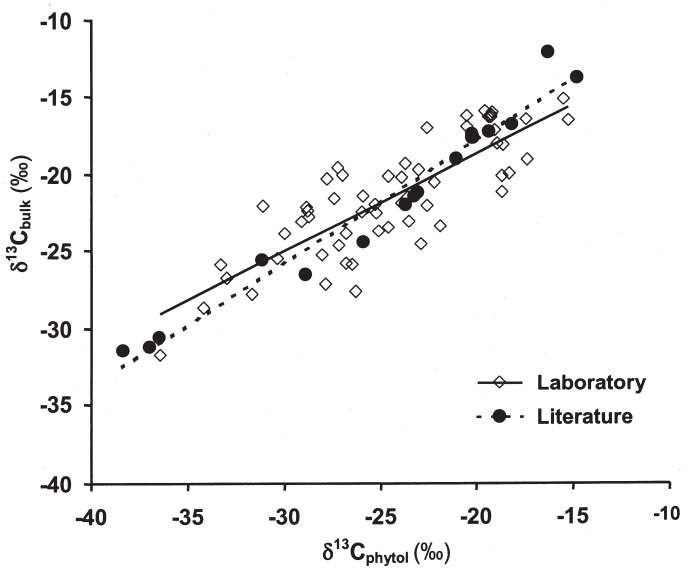

Fig. 1. Relationship between $\delta^{13}C$ of bulk plant material and $\delta^{13}C$ of phytol for literature data (terrestrial plants and marine macroalgae, van Dongen et al. 2002; marine phytoplankton, Sakata et al. 1997, Riebesell et al. 2000) ($R^2 = 0.96$, $F_{1,15} = 392$, $P < 0.001$) and for MPB grown in the laboratory ($R^2 = 0.68$, $F_{1,56} = 117$, $P < 0.001$).

Studies indicate that chl-*a* $\delta^{13}C$ is an effective proxy for $\delta^{13}C_{bulk}$ of phytoplankton (e.g., Laws et al. 1995); however, extraction of chl-*a* from sediments is relatively complex (see Sachs and Repeta 2000). In comparison, phytol can be extracted using fewer steps (this article), reducing the possibility of fractionation. Phytol is the side chain of chlorophyll-*a*, and its $\delta^{13}C$ is therefore expected to be predictably related to MPB $\delta^{13}C_{bulk}$. Although phytol is found within all green plants, rapid degradation of phytol in the water column and senescent cells of detritus (Rontani and Volkman 2003) suggests that phytol within sediments should be of relatively recent origin and most likely produced predominately in situ by MPB, particularly in shallow water environments.

A relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ exists for terrestrial plants (van Dongen et al. 2002) and marine phytoplankton (Sakata et al. 1997; Riebesell et al. 2000) and macroalgae (van Dongen et al. 2002; Fig. 1) but has not been shown for MPB. Sources, uptake, and assimilation of carbon may differ for benthic autotrophs (i.e., MPB); therefore the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ may also differ from that described for other producers. Temperature (Hinga et al. 1994), taxon-specific biosynthetic pathways, and the size, membrane permeability (Laws et al. 1995), and geometry (Popp et al. 1998) of cells may affect the uptake and assimilation of carbon in MPB. The composition of MPB communities and the temperatures to which they are exposed are likely to be both temporally and spatially variable (Currin et al. 1995). Therefore, the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ may be both species- and temperature-dependent.

This study aimed to:

(a) determine if a predictive relationship exists between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ for MPB and then compare this to the literature regression for terrestrial plants and marine phytoplankton and macroalgae,

(b) investigate potential influences of growth temperature and species on the phytol-bulk relationship, and

(c) demonstrate the precision and effectiveness of measuring $\delta^{13}C_{bulk}$ using $\delta^{13}C_{phytol}$ through determining the expected difference between predicted and observed $\delta^{13}C_{bulk}$ and, using a two-source mixing model, assess the change in 95% confidence intervals for estimations of MPB contribution to consumer nutrition.

Materials and procedures

Algae culture—To maximize chances of identifying species differences, different taxa and various sizes and shapes of MPB were selected, namely: the cyanobacterium, *Oscillatoria* sp., the diatoms *Nitzschia closterium* and *Nitzschia frustulum*, two naviculoid species (sp. 1 and sp. 2), *Pleurosigma* sp., *Gyrosigma* sp., and *Bacillaria paxillifer*. All species were isolated from sandy sediment from Moreton Bay, southeast Queensland, Australia, with the exception of *N. frustulum* (CSIRO Collection of Living Microalgae; Strain CS-258/1 http://www.marine.csiro.au/microalgae/collection.html), which was isolated from Beaver Cay, north Queensland, Australia, in 1987.

MPB species were grown in flasks containing 300 mL sterile F_2 culture medium (Guillard and Ryther 1962) on a light:dark cycle of 12 h:12 h. As MPB $\delta^{13}C_{bulk}$ typically varies with growth rate, which increases with temperature (Fielding et al. 1998), diatoms were cultured at a range of temperatures to deliberately extend the applicable range of the relationship. Each species was cultured at average temperatures for each flask of 10, 15, 20, 25, 30, or 35° C (one flask for each species per temperature). To mimic field conditions, temperatures for each of these treatments varied within 2° C, increasing slightly during the light period and decreasing during the dark period. These conditions were essentially stable throughout the culture period and are termed "constant" from here on. To determine if temperature fluctuations between days would affect the $\delta^{13}C_{phytol}$-$\delta^{13}C_{bulk}$ relationship, the temperatures for another 10 flasks of each of *Nitzschia closterium*, *Gyrosigma* sp., and *Oscillatoria* sp. were varied by as much as 10° C from day to day so that the growth temperature was not constant for the culture period. Cells were harvested when most cultures appeared to have attained sufficient biomass for analysis: 16 d for *Nitzschia frustulum*, 24 d for other MPB species grown at constant temperature, and 14 d for MPB grown with variable temperature.

Algae were collected by filtration onto precombusted (450° C, 12 h) 25-mm glass fibre filters (GF/Fs). Degradation of phytol is minimal in frozen samples (Sun et al. 1998), so filters were stored frozen in aluminum foil until processed (up to 7 d). Approximately 25% of the filtered material was retained on filter papers for bulk analysis. The remainder was extracted for phytol.

Extraction of phytol—The neutral fraction, within which phytol is found, was extracted and isolated using an adaptation of the method of Bligh and Dyer (1959). Shredded filters were sonicated for 10 min and centrifuged at 2000 rpm for 3 min three times, in 30, 40, and 30 mL 3:6:1 dichloromethane: methanol:H_2O. The extracts were combined in a separating funnel. Dichloromethane (DCM; 30 mL) and milli-Q H_2O (30 mL) were added, and the phases were allowed to separate. The bottom layer was drawn into a round-bottom flask, and the process was repeated using 15 mL DCM. Total extracts were evaporated to near dryness at 40° C on a rotary evaporator and transferred to test tubes. The extract was blown to dryness with N_2 and saponified with 5% KOH in methanol:H_2O (80:20; 3 mL) to give a pH of 12-14. Samples were flushed with ultrapure N_2 and heated (80° C, 2 h). After cooling, 3 mL milli-Q H_2O and 3 mL 4:1 hexane:DCM were added, and samples were shaken and centrifuged at 2000 rpm for 2 min. The top solvent layer was transferred to a test tube. This process was repeated twice more with 2 mL hexane:DCM. The volume was reduced with N_2, and the extract was transferred to a vial. In preparation for gas chromatography, samples were blown to dryness with N_2 and derivatized (60° C, 2 h) with 100 µL pure BSTFA.

Gas chromatography and gas chromatography-mass spectrometry—Each extract was analyzed by gas chromatography to ensure that there was sufficient phytol for analysis and that there were no co-eluting compounds that could interfere with analysis. Gas chromatography was initially performed using a Varian CP 3800 equipped with a split/splitless injector and flame ionization detector (FID), interfaced with Galaxy chromatography software. For the analysis of the neutral fraction, the gas chromatograph was fitted with a 50 m × 0.32 mm inner diameter (i.d.) cross-linked 5% phenyl-methyl silicone (HP5, Hewlett Packard) fused-silica capillary column; hydrogen was the carrier gas. The initial oven temperature was 45° C held for 1 min, with a 30° C min^{-1} ramp rate to 140° C and then a 3° C min^{-1} ramp rate to 310° C, which was held for 5 min. 5′(H)-cholestan-24-ol was used as an internal standard. Initial peak identifications were based on retention times relative to authentic and laboratory standards and subsequent gas chromatography-mass spectrometry (GC-MS) analysis.

Verification of the identity of individual compounds by GC-MS analysis was performed on a Thermoquest/Finnigan GCQ-Plus benchtop mass spectrometer fitted with a direct capillary inlet and an automated on-column injector. Data were acquired in scan acquisition or selective ion monitoring and processed using Xcalibur software supplied with the instrument. The nonpolar column (HP5) and operating conditions were the same as those described above for GC-FID analyses, except that helium was used as the carrier gas.

Stable isotope analysis—**Bulk analysis.** Algal samples on glass-fiber filters were dried at 60° C for several hours before being punched and packed into tin cups (Elemental Microanalysis Ltd., Okehampton, UK) for analysis. Before the cups were closed, a few drops of sulfurous acid (6% wt/wt min; Australian Chemical Reagents, Qld) were added to remove any carbonates present; this was done within cups to prevent loss of acid-soluble organic carbon (Verardo et al. 1990). Samples were redried and analyzed for $\delta^{13}C$ using a Carlo Erba NA1500 CNS analyser interfaced via a Conflo II to a Finnigan Mat Delta S isotope ratio mass spectrometer operating in the continuous flow mode. Combustion and oxidation were achieved at 1090° C and reduction at 650° C. Samples were analyzed at least in duplicate. Results are presented in standard δ notation:

$$\delta^{13}C(‰) = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \times 100\%$$

where $R = {}^{13}C/{}^{12}C$. The standard for carbon is Vienna Pee Dee Belemnite (VPDB). The reproducibility of the stable isotope measurements was ~0.2‰.

Compound-specific isotope analysis. Not all species of MPB grew to sufficient biomass for CSIA of phytol at all temperatures. Only those samples where a sufficiently strong, pure phytol peak was obtained were analyzed. Compound-specific isotope ratio mass spectrometry was performed using a Hewlett Packard 5890 series II gas chromatograph, which was coupled via a Finnigan MAT GC combustion interface to the isotope ratio mass spectrometer described above. The gas chromatograph was equipped with a 60 m J&W DB-1, 0.32 mm i.d. column with He as the carrier gas. Samples were injected on-column via a cold on-column injector ("Duck Bill," Hewlett Packard). The initial oven temperature of 40° C was maintained for 1 min followed by a 30° C min^{-1} ramp rate up to 120° C, then a 4° C min^{-1} ramp rate to 315° C, which was held for 15 min. Samples were coinjected twice with C_{16} and C_{24} deuterated n-alkanes of known isotopic composition; the average of the 2 injections is reported here. To obtain the ratio for phytol, the carbon isotope ratios of the derivatized samples were corrected for the number of carbon atoms that had been added during derivatization. Results are presented in standard δ notation as described above.

Assessment

Establishing a predictive $\delta^{13}C_{phytol}$-$\delta^{13}C_{bulk}$ relationship—Both phytol and bulk $\delta^{13}C$ values were obtained for a total of 58 samples. Although MPB in this study was not within sediment, the extraction procedure was suitable for use on field-collected samples with a complex sediment matrix. For all species pooled, and regardless of whether algae were grown at constant or fluctuating temperature, $\delta^{13}C_{phytol}$ was linearly correlated with $\delta^{13}C_{bulk}$ (Fig. 1):

$$\delta^{13}C_{bulk} = 0.638 \times \delta^{13}C_{phytol} - 6.135 \quad (1)$$

This regression (Eq. 1) was not significantly different from data in the literature (ANCOVA: test for heterogeneity of slopes, $F_{1,71} = 3.72$, $P = .057$; test for differences in elevations, $F_{1,72} = 0.03$, $P = .853$) (Fig. 1). The similarity of the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ for such diverse producers as

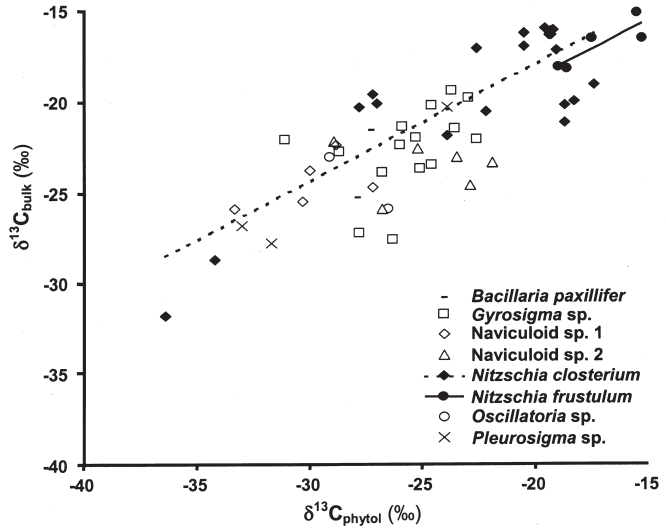

Fig. 2. Relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ for different MPB species. Regression lines are shown for *Nitzschia closterium* (R^2 = 0.68, $F_{1,18}$ = 39.2, P < 0.001) and *Nitzschia frustulum* (R^2 = 0.74, $F_{1,3}$ = 8.58, P = .061).

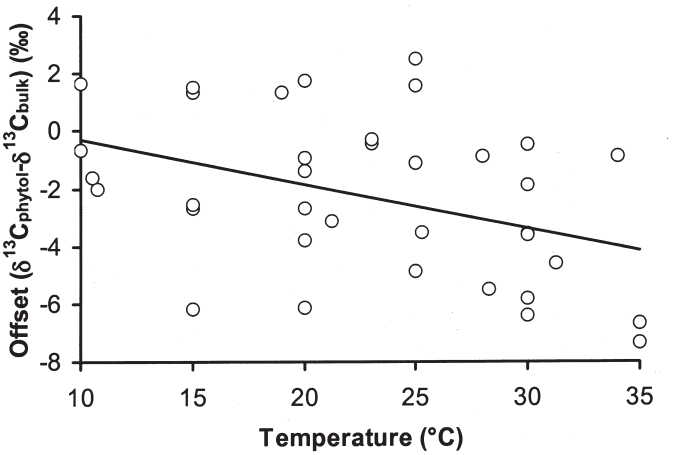

Fig. 3. Relationship between growth temperature and the offset between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ (R^2 = 0.17, $F_{1,35}$ = 7.12, P = .011).

MPB, terrestrial plants, marine phytoplankton, and marine macroalgae suggests that the observed relationship is widely applicable. There was more variation in the relationship for MPB than for these other plant groups from the literature. The relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ for MPB may be inherently more variable due to MPB taxonomic and physiological diversity. Alternatively, the source of this variation may be the fluctuating temperatures at which MPB was cultured in the laboratory. There may be even greater variability in environmental samples, where MPB are subject to extremes of temperature, pH, and salinity beyond the range simulated in the laboratory.

Influence of species effect on the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$—Of the MPB species studied, three (*Oscillatoria* sp., *Pleurosigma* sp., and *Bacillaria paxillifer*) had insufficient data for regression analysis (n < 5). Regression analyses showed the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ to be significant for *Nitzschia closterium* (Fig. 2) and close to significant for *Nitzschia frustulum* (Fig. 2). Regressions for the remaining species were not significant. The relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ did not significantly differ among species (ANCOVA: slopes, $F_{4,4}$ = 5.80, P = .058; elevations, $F_{1,8}$ = 3.14, P = .115) (Fig. 2).

The similarity of the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ among MPB species indicates that the biosynthetic pathways responsible for partitioning carbon into phytol within cells is similar for all species studied.

Influence of culture temperature on the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$—For the 37 cultures grown at constant temperature, $\delta^{13}C_{phytol}$ was generally depleted relative to $\delta^{13}C_{bulk}$. The offset between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ was smaller at higher culture temperatures (Fig. 3). The relationship between offset and culture temperature was similar regardless of whether the measure of temperature used was the average temperature or the temperature extremes during either the light or the dark periods. Sachs et al. (1999) reported that the offset between $\delta^{13}C_{bulk}$ and chl-*a* $\delta^{13}C$ of marine phytoplankton was influenced by growth rate, with use of more enriched carbon sources in chl-*a* synthesis at faster growth rates. However, growth rate is often positively correlated with temperature (Fielding et al. 1998), which was not considered separately in the study by Sachs et al. (1999). Hinga et al. (1994) found growth rate to have no effect on carbon isotope fractionation during uptake by marine phytoplankton, but fractionation increased with temperature. The relationship observed by Sachs et al. (1999) may therefore be attributable to differences in temperature rather than growth rate. Although Sachs et al. (1999) studied marine phytoplankton, chl-*a* is also found in MPB. Assuming that the enrichment of chl-*a* they reported is in fact due to increased temperature, this may account for the change in offset between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ observed in the current study at increased temperatures. As phytol is a side chain of chl-*a*, enrichment of chl-*a* at higher temperatures would also result in phytol becoming less depleted relative to bulk MPB. Nevertheless, the current study demonstrates that growth conditions can affect cell components independent of the whole cell.

Both $\delta^{13}C_{phytol}$ and temperature are considered in a multiple regression equation:

$$\delta^{13}C_{bulk} = 0.671 \times \delta^{13}C_{phytol} + 0.136 \times \text{temperature} - 8.799 \qquad (2)$$

This equation explained more of the variation in $\delta^{13}C_{bulk}$ values (80%; multiple regression [R^2] = 0.80, $F_{2,34}$ = 69.81, P < 0.001) than when $\delta^{13}C_{phytol}$ alone was used (Eq. 1; 68%). There was no indication of collinearity between $\delta^{13}C_{phytol}$ and temperature.

Predictive ability of the $\delta^{13}C_{phytol}$-$\delta^{13}C_{bulk}$ relationship—Although there is a significant regression between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ (Eq. 1), and between temperature, $\delta^{13}C_{phytol}$, and $\delta^{13}C_{bulk}$ (Eq. 2),

Table 2. Precision of $\delta^{13}C_{bulk}$ estimates relative to observed $\delta^{13}C_{bulk}$ values when replication is small (n = 3), average (n = 5), or large (n = 10) using Eq. 1 or Eq. 2.

	Mean		95% CI	
n	Eq. 1	Eq. 2	Eq. 1	Eq. 2
3	0.9	0.8	2.1	1.9
5	0.8	0.6	1.8	1.3
10	0.5	0.4	1.3	1.0

Mean difference between observed and predicted $\delta^{13}C_{bulk}$ (‰) and 95% confidence intervals of predictions for each scenario.

the ability to predict $\delta^{13}C_{bulk}$ using these equations needs to be further assessed to ensure usefulness in food web applications.

Difference between observed and predicted $\delta^{13}C_{bulk}$—To determine the average difference expected between observed and predicted $\delta^{13}C_{bulk}$ with different numbers of samples, a small (3), average (5), or large (10) number of replicate $\delta^{13}C_{phytol}$ values were randomly selected from those measured for the algae cultured in the current study. For each level of replication, this was repeated 100 times to form 100 groups. Within these groups, each $\delta^{13}C_{phytol}$ value was entered into Eq. 1 or Eq. 2 to predict $\delta^{13}C_{bulk}$. The difference between the $\delta^{13}C_{bulk}$ value predicted and that measured for the sample (observed $\delta^{13}C_{bulk}$) was calculated. The mean difference for each group was determined, and these values were used to calculate the overall mean difference between observed and predicted $\delta^{13}C_{bulk}$ for the 100 groups for each level of replication (Table 2). The difference between observed and predicted $\delta^{13}C_{bulk}$ expected in 95% of cases was also determined (Table 2).

In every scenario, when temperature and $\delta^{13}C_{phytol}$ are both used as predictors for $\delta^{13}C_{bulk}$ (Eq. 2), the difference between observed and predicted $\delta^{13}C_{bulk}$ is smaller than when $\delta^{13}C_{phytol}$ is the sole predictor (Eq. 1). For example, when 5 samples are used, $\delta^{13}C_{bulk}$ predicted from Eq. 1 will typically lie within 0.8‰ of observed values, and will be within 1.8‰ 95% of the time. Predicted $\delta^{13}C_{bulk}$ from Eq. 2 will typically be within 0.6‰ of observed values and within 1.3‰ 95% of the time. To resolve food webs where the gap between $\delta^{13}C$ of MPB and a second producer is small, greater precision of MPB signatures will be required, and Eq. 2 should therefore be applied.

Resolution of MPB signatures can also be improved by increasing replication. In all cases, the expected mean difference between observed and predicted $\delta^{13}C_{bulk}$ was greater when replication was decreased.

Influence of gap distance—A two-source mixing model (Phillips and Gregg 2001) was used to assess the usefulness of each of the equations in estimating source contribution to consumer nutrition. This model incorporates variation in consumer and producer $\delta^{13}C$, sample size, and gap distance (the differences between mean $\delta^{13}C$ of sources) and calculates 95% confidence intervals for estimates of source contribution. Where the precision of these estimates is greater, 95% CIs are smaller.

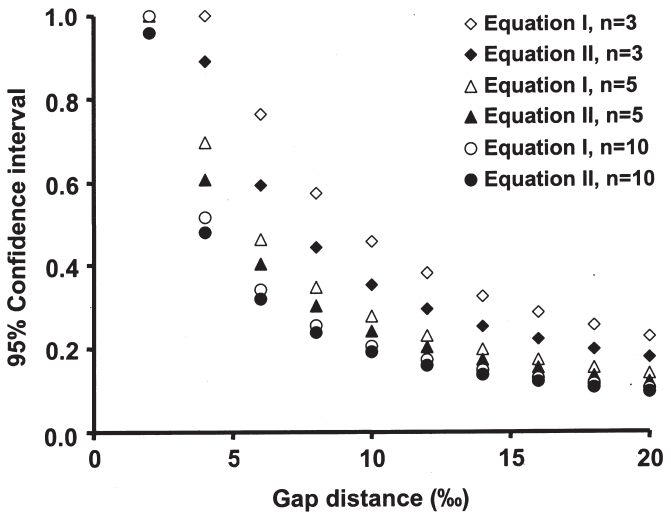

Fig. 4. Improvement in 95% confidence intervals for predictions of MPB contribution to consumer nutrition with increased sample size and gap size for Eq. 1 and Eq. 2.

Modeling was simulated assuming 3, 5, or 10 replicates, where the variation was calculated for $\delta^{13}C_{bulk}$ predictions using Eq. 1 or Eq. 2 and gap distances between MPB and a second producer varied from 2 to 20‰. The sample sizes (10 for both) and standard deviations (1.00 and 1.09, respectively) specified for the consumer and the second producer were typical values for Moreton Bay, from which MPB were isolated.

For all gap distances greater than 2‰, the smallest (i.e., best) 95% CIs were obtained for a sample size of 10 where Eq. 2 was used for predictions (Fig. 4). The greatest benefit gained from the addition of temperature as a predictor for $\delta^{13}C_{bulk}$ was at a sample size of 3. When the sample size was 10, there was only a slight improvement in CIs at any gap distance when Eq. 2 was applied in preference to Eq. 1. The greatest difference between CIs among the methods was when gap distance was 6.0‰. As the average offset between producers is 5.5‰ (calculated from available literature), great benefit is expected from taking extra samples and/or measuring temperature for inclusion in Eq. 2. However, the method selected will depend on the needs of the researcher, availability of resources, and gap distances between $\delta^{13}C$ of MPB and other potential producers.

Discussion

We have demonstrated that MPB $\delta^{13}C_{bulk}$ can be determined from measurements of $\delta^{13}C_{phytol}$ (Eq. 1) or, with greater confidence, through measurement of both $\delta^{13}C_{phytol}$ and temperature (Eq. 2). When 10 $\delta^{13}C_{phytol}$ measurements are made and temperature is included as a further predictor, $\delta^{13}C_{bulk}$ can be estimated within 1.0‰ of observed values 95% of the time (Table 2). Estimation of MPB $\delta^{13}C$ from $\delta^{13}C_{phytol}$ therefore potentially allows adequate distinction from other producers with a signature within 1.0‰ of the true MPB signature.

Regardless of the equation used, the confidence of $\delta^{13}C_{bulk}$ predictions is greater when replication is increased.

Values of MPB $\delta^{13}C_{bulk}$ determined using the $\delta^{13}C_{phytol}$ method may be more reliable than those measured using alternative methods, for which the influence of contamination on MPB $\delta^{13}C$ is unknown. Use of values from the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ in trophic studies may thus lead to a reevaluation of the importance of MPB as a source of nutrition for consumers in estuarine systems.

Comments and recommendations

Existing techniques for physical separation of MPB before MPB $\delta^{13}C_{bulk}$ determination rely on immediate and/or field-based processing of samples, without which live MPB may alter their $\delta^{13}C_{bulk}$. The ability to freeze and store samples before applying the $\delta^{13}C_{phytol}$ method for MPB $\delta^{13}C_{bulk}$ circumvents this problem. A further advantage of the $\delta^{13}C_{phytol}$ method is the reliability of the MPB $\delta^{13}C_{bulk}$ values obtained. There has been little effort to quantify the extent to which MPB $\delta^{13}C$ estimated using existing methods is compromised by contamination with detritus and meiofauna, and/or discrimination against some components of the MPB community. However, the $\delta^{13}C_{phytol}$ method for MPB $\delta^{13}C_{bulk}$ determination incorporates all components of the MPB community, and use of phytol as a biomarker removes the possibility of contamination by meiofauna. Although most phytol in detritus degrades before it enters sediments (Rontani 2001), residual phytol has the potential to interfere with predictions, especially where detrital fragments are large (Cuny et al. 1999). This should be minimal in areas with only shallow water, but the contribution of phytol from alternative sources to the pool in the sediment needs to be established. To minimize the potential for contamination with non-MPB phytol, it is recommended that sediment be sieved through appropriate mesh sizes to remove larger detritus particles (e.g., >1 mm).

The temperatures to which diatoms are subjected, and the composition of MPB communities in the field, vary considerably both spatially and temporally (Currin et al. 1995). In the current study, variation in bulk signatures in the range of –15‰ to –28‰ was achieved by manipulation of temperature. Therefore, variation in temperature potentially causes considerable spatial and temporal variation in MPB $\delta^{13}C_{bulk}$ in estuaries. For example, at lower temperatures in shaded areas of intertidal zones, or during winter, MPB signatures may be relatively depleted in ^{13}C. Overall, MPB $\delta^{13}C$ may also become more depleted if a community becomes dominated by a species with more depleted $\delta^{13}C$ values. This emphasizes the need to estimate MPB signatures on a case-by-case basis using the equations described in this article.

In applying Eq. 2 to food web studies, it should be noted that although we found including temperature to improve the ability to predict $\delta^{13}C_{bulk}$ using $\delta^{13}C_{phytol}$, presumably as a result of an overall increase in growth rate with temperature, the same may not be the case in the field. At any given temperature in the field, some of the species within a diverse MPB community may be at or above their optimal temperature for growth, beyond which growth rate will not increase. Thus temperature and growth rate will not always be clearly correlated across the entire range of possible temperatures. The preferred measure of temperature is that of the superficial sediments (e.g., upper 5 mm) when the majority of the algal biomass to be sampled was synthesized. Fluctuations in temperature do not affect the predictive ability of Eq. 1, as the described relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ incorporates this variation; however, the $\delta^{13}C$ of cell components is influenced by the temperature at which they are synthesized (Hinga et al. 1994). If the temperature used in Eq. 2 differs markedly from when biomass was created, errors may result in estimations made using Eq. 2. Further investigation is required to determine the extent to which this occurs and, if necessary, improve methods for incorporating the effect of temperature when predicting $\delta^{13}C_{bulk}$ using $\delta^{13}C_{phytol}$.

The precision of MPB $\delta^{13}C_{bulk}$ estimates made using the equations described in this article is sufficient to discriminate MPB from other potential sources of nutrition in food web studies. It would be beneficial to assess the variation in phytol content of different MPB taxa, as this may affect the contribution of components of a community to the overall $\delta^{13}C_{bulk}$. The variability of the relationship between $\delta^{13}C_{phytol}$ and $\delta^{13}C_{bulk}$ for a wider variety of MPB taxa should also be considered. However, given the similarity of the relationship between $\delta^{13}C_{bulk}$ and $\delta^{13}C_{phytol}$ for terrestrial plants and marine macroalgae and phytoplankton (Fig. 1), and the relationships for each MPB species in the current study across a range of growth conditions, the proposed method is expected to have wide applicability in predicting MPB $\delta^{13}C$ in food web studies in estuarine and marine environments. The method may also be useful in predicting $\delta^{13}C_{bulk}$ of other producers.

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Submitted 27 February 2005
Revised 1 September 2005
Accepted 11 October 2005