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ULTRA-MICROSCALE (5–25 µg C) ANALYSIS OF INDIVIDUAL LIPIDS BY ^{14}C AMS: ASSESSMENT AND CORRECTION FOR SAMPLE PROCESSING BLANKS

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ABSTRACT. Measurements of the natural abundance of radiocarbon in biomarker molecules can be used to elucidate the biogeochemical roles of marine bacteria and archaea in the oceanic water column. However, the relatively low concentration of biomass, especially below the euphotic zone, inevitably results in small sample sizes for compound-specific analyses. In ultra-microscale $\Delta^{14}C$ measurements, which we define as measurements on samples smaller than 25 µg C, the process of isolating pure compounds and preparing them for measurement adds significant background carbon. This additional blank carbon can contribute up to 40% of the total sample mass; therefore, it is necessary to quantify all components of the processing blank in order to make appropriate corrections. Complete propagation of error is critical in order to report the correct analytical uncertainty. The carbon blank is composed of at least 3 different sources: i) those that scale in proportion to the mass of the sample; ii) sources that contribute a constant mass of blank, e.g. closed-tube combustion; and iii) contaminants from vacuum lines and/or other aspects of sample handling that are difficult to quantify. We approached the problem of correcting for the total sample processing blank by deriving a 4-part isotopic mass balance based on separating the 3 exogenous components from the sample. Subsequently, we derived the appropriate equations for the full propagation of error associated with these corrections. Equations for these terms are presented. Full treatment of a set of raw data is demonstrated using compound-specific $\Delta^{14}C$ data from the North Central Pacific water column.

INTRODUCTION

Biogeochemical cycles in the marine water column are mediated largely by the activity of the complex prokaryotic community. The roles of different groups of prokaryotes in these cycles—especially those species that have few or no representatives in culture—are not yet completely understood. Examples include the recent discovery that anaerobic ammonia oxidation (anammox) by Planctomycetes is a significant sink for fixed nitrogen (Dalsgaard et al. 2003; Kuypers et al. 2003); evidence that marine Crenarchaeota are responsible for widespread oxidation of ammonia (Francis et al. 2005; Herndl et al. 2005); and evidence that marine archaea fix a significant quantity of inorganic carbon in the dark ocean (Ingalls et al. 2006). One of many potential tools that can be used to link prokaryotes with particular processes is measurement of the radiocarbon content of specific biomarkers taken from the water column. ^{14}C measurements can trace the sources of carbon to organisms since the bulk pools of dissolved inorganic carbon (DIC), sinking and suspended particulate organic carbon (POC), and dissolved organic carbon (DOC) have distinct ^{14}C contents below the mixed layer (Druffel et al. 1992). Individual-compound ^{14}C analyses of biomarker lipids have the potential to distinguish carbon transformation processes at a level more specific than gross transfers between these bulk pools of carbon. The utility of compound-specific radiocarbon analysis (CSRA) has been demonstrated for marine sediments (e.g. Eglinton et al. 1997; Pearson et al. 2001; Ohkouchi et al. 2002), but analytical constraints long prevented application of CSRA to the pelagic environment. Only recently has it become possible to take this approach with lipids from the water column. Several recent studies have focused on $\Delta^{14}C$ analysis of lipid fractions extracted from water-column particulate matter (Wang et al. 1998; Hwang and Druffel 2003; Loh et al. 2004), but it was not possible to measure individual compounds in these studies, due to the small sample sizes recovered. CSRA in the water column has great scientific potential, but it has been difficult to achieve because of the challenges associated with collecting sufficient biomass and with making $\Delta^{14}C$ measurements at the lower limit of sample size.

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The concentration of suspended POC below 100 m depth in the water column of the North Central Pacific is approximately 0.3–0.7 µM (Hernes and Benner 2002). A rough estimate for the quantity of seawater required to obtain 0.5–1.0 g of biomass can be made assuming that there are 10^4–10^5 prokaryotic cells/mL (Francis et al. 2005; Herndl et al. 2005) below the photic zone in the oligotrophic ocean, and that they have approximately 20–40 fg C/cell (Herndl et al. 2005). Such a low concentration of biomass in the water column requires sampling of >10^5 L of seawater to obtain adequate quantities of biomarkers for ^{14}C-AMS measurement of single compounds (Ingalls et al. 2006). We recently completed a water-column CSRA project (Ingalls et al. 2006) in which we filtered approximately 208,000 L of 670-m seawater onto 0.2-µm Pall Supor® filters from the continuously flowing pipeline available at the Natural Energy Laboratory of Hawaii Authority (NELHA). Even after filtering and extracting such a large volume of water, the amount of carbon obtained for ^{14}C-AMS analysis per individual lipid compound was very small: 5–40 µg C (Ingalls et al. 2006). In order to report *accurate* Δ^{14}C values in Ingalls et al. (2006), it was necessary to perform a detailed assessment of and careful correction for all contributions of exogenous carbon that the samples accumulated before being submitted to the AMS facility. Once these blanks were assessed, a full mathematical propagation of uncertainty was performed in order to report correctly the analytical *precision* for the Δ^{14}C values. Here, we report the data and method used to make these corrections.

Previous approaches to the analysis of small samples by ^{14}C AMS have focused on analytical issues relevant to samples of size >25 µg C (Pearson et al. 1998; von Reden et al. 1998). However, the recent development of an accurate and precise AMS source that can measure samples at sizes <25 µg C (Santos et al. 2004, forthcoming; Southon et al. 2004) now permits analysis of samples to the 5-µg C threshold. We define the "ultra-micro" AMS range as this sample size class: between 5–25 µg C. Here, we show that AMS facility-reported error generally limits the analytical precision for samples >25 µg C, with errors typically in the range of ±10‰ for samples of modern ^{14}C content. In contrast, the overall precision in Δ^{14}C values for samples smaller than 25 µg C depends critically on the precision with which the laboratory processing blanks can be assessed. These blanks typically contribute more to the reported errors (> ±10‰) than does the facility contribution. To develop these arguments, the following sections are arranged topically:

- Isolation and purification methods for CSRA of lipid samples by high-performance liquid chromatography (HPLC).
- Assessment of the sizes and isotopic compositions of the combustion and HPLC-derived blanks.
- The correction of Δ^{14}C values of environmental samples for all components of the processing blank by isotopic mass-balance.
- Calculation of the total analytical uncertainties for corrected Δ^{14}C measurements.
- Comparison of the reported AMS facility errors with our values for total propagated error.

ISOLATION AND PURIFICATION OF WATER-COLUMN LIPID SAMPLES FOR CSRA

The CSRA data presented by Ingalls et al. (2006) include 12 Δ^{14}C values of individual lipids extracted from water-column POC and purified by high-performance liquid chromatography (HPLC). Accurate determination of Δ^{14}C values of individual lipids requires that raw Δ^{14}C measurements be corrected for unavoidable process blanks. These include the blanks associated with purification of the individual compounds by HPLC and with combustion of the sample to CO_2 for AMS measurement. We assume that all contaminants associated with sample filtration, extraction of total lipids, and other procedures are removed or minimized during chromatography; individual compounds are separated from this background material during preparative HPLC. Therefore, the last HPLC step (purification) represents the initial source of background contamination that must be

assessed. It includes potential contributions from column bleed, solvent, co-elution of undesired compounds, and carry-over of chromatographic impurities from the total lipid sample.

The complete methods for separation of individual lipids by HPLC were described in Ingalls et al. (2006). Briefly, tetra-ether lipids of marine archaea were separated by mass and retention time using normal-phase HPLC and atmospheric-pressure chemical ionization mass spectrometry (APCI-MS) for detection. Individual lipids were collected in 1-min fractions and the content of each fraction was confirmed by flow-injection analysis (FIA). This normal-phase separation and collection of individual lipids required many repeated HPLC injections. The lipid collected from each injection was pooled into crude total samples for each lipid. Such pooling of repeated injections effectively combines the contributions of HPLC contamination from each injection, and the total contamination is proportional to the number of injections and the volume of effluent. For this reason, a final purification step was developed to remove these contaminants and to standardize the amount of background carbon that should be present in each sample. The crude samples of individual lipids were purified using a reverse-phase HPLC program with an Agilent ZORBAX Eclipse XDB-C_8 column at 30.0 °C (4.6 × 150 mm, 5 µm): 100% solvent A (80% acetonitrile, 20% water) to 90% A and 10% ethyl acetate (EtOAc) over 4 min, to 65% A over 10 min, to 31% A over 6 min, to 100% EtOAc over 7 min (1 mL/min). Burdick & Jackson HPLC-grade acetonitrile and Burdick & Jackson GC^2-grade EtOAc were used. The individual archaeal lipids eluted in 100% EtOAc and were separated by >10 min of retention time from all other detectable components in the initial fractions. The final samples were colorless; when solvent was removed, they dried to fine, white crystalline powder. Most importantly, each final sample of lipid was recovered in only 2 mL or 4 mL of total HPLC effluent.

ASSESSMENT OF THE COMBUSTION AND HPLC-DERIVED BLANKS

Mass of Combustion and HPLC Blanks

HPLC effluent blanks representing a range of volumes (6, 12, 18, 36, 72, and 108 mL) were collected from the 100% EtOAc section of the reverse-phase HPLC program. These blanks were dried under ultra-high purity N_2(g) in precombusted (850 °C for 5 hr, no more than 36 hr before use) 9-mm quartz tubes, amended with ~0.1 g of precombusted cupric oxide, evacuated to 10^{-5} torr, and flame-sealed. The tubes were combusted for 5 hr at 850 °C, and the CO_2 gas was trapped by cracking the quartz tubes onto a vacuum line, passing the gases through a –70°C water trap, and trapping the CO_2 over liquid N_2. The CO_2 yield was quantified manometrically in a known volume and flame-sealed inside 6-mm precombusted Pyrex® tubes. The CO_2 was sent to the Keck Carbon Cycle AMS facility at the University of California, Irvine, where it was converted to C (*gr*) for measurement by ^{14}C AMS.

The masses of the combusted HPLC effluent samples (Table 1) show a strong linear relationship with the volume of effluent collected (Figure 1). Extrapolating the linear regression line ($R^2 = 0.97$) to zero effluent volume indicates that the mass of the combustion blank (m_{CB}) is about 1.0 µg C, and the mass of the blank derived from running the sample through the HPLC (m_{LC}) is approximately 0.03 µg C/mL of HPLC effluent. At 2–4 mL of HPLC effluent per sample of environmental lipid, the magnitude of m_{LC} is 0.06–0.12 µg C. We expect our estimate of the HPLC blank to represent the introduction of exogenous carbon by similar HPLC methods, when HPLC is performed with stable columns and high-purity, low-volatility solvents. Previous assessment of the HPLC blank made using a different column and solvent conditions and for different analytical purposes yielded very similar results (Ingalls et al. 2004). However, it is the uncertainty in each of these numbers that is important for determining the overall precision of reported $\Delta^{14}C$ values.

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Table 1 Mass and $\Delta^{14}C$ values of blanks and standards discussed in the text.

Sample	HPLC effluent (mL)	Mass (µg C)	m_{CB_i}	m_{LC_i}	UCIAMS-reported $\Delta^{14}C$ (‰)	Δ_{LC_i}	Δ_{CB_i}
LC Blank	6	1.24	1.05	0.043			
LC Blank	12	1.08	0.71	0.009	n.p.[a]		
LC Blank	18	1.70	1.14	0.040			
LC Blank	36	2.01	0.88	0.029	s.l.[b]		
LC Blank	72	3.56	1.30	0.036			
LC Blank	108	4.18	0.79	0.030	–611 ± 26		
Stigmasterol	2	8.52			117 ± 10	–774	123
Stigmasterol	4	10.53			109 ± 15	–751	49
Stigmasterol	4	15.03			133 ± 12	–842	346
Stigmasterol	2	8.98			100 ± 13	–737	6
Stigmasterol	4	15.49			95 ± 12	–664	–232
Stigmasterol[c]	n.a.	1020			126 ± 5		

[a]n.p. = non-performing sample.
[b]s.l. = sample lost.
[c]Large sample not purified through HPLC and assumed to be true $\Delta^{14}C$ value of stigmasterol.

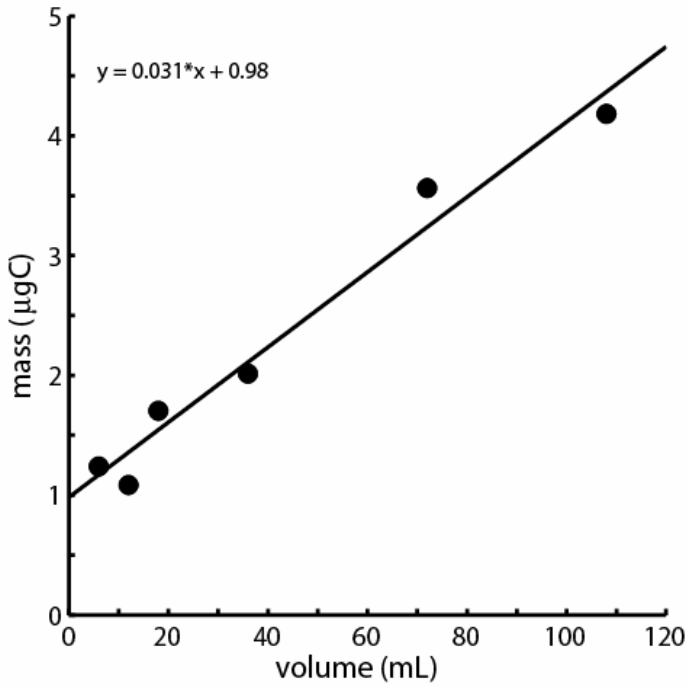

Figure 1 Plot of volume of mass vs. HPLC effluent volume for combusted HPLC effluent samples. Linear regression was used to determine the mass of combustion and HPLC blanks. R^2 for linear regression: 0.97.

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The uncertainty in the mass of the combustion blank ($\sigma_{m_{CB}}$) is determined using the combustion blank for each individual sample. It is calculated by first subtracting out the regression-derived HPLC blank:

$$m_{CB_i} = m_{T_i} - v m_{LC} \tag{1}$$

where m_{T_i} is the mass of the total blank sample and v is the volume of HPLC effluent. The uncertainty ($\sigma_{m_{CB}}$) is determined from the root mean square (rms) of the difference between the regression-derived combustion blank and each individually calculated blank. This results in a m_{CB} value of 1.0 ± 0.2 µg C (Table 2). Similarly, uncertainty in the mass of the HPLC blank ($\sigma_{m_{LC}}$) is determined by the rms of the difference between the regression-derived m_{LC} and the HPLC blanks calculated for each individual sample. The values of m_{LC_i} are calculated by subtracting the regression-derived combustion blank and normalizing by the volume of effluent in each blank:

$$m_{T_i} = \frac{m_{S_i} - m_{CB}}{v} \tag{2}$$

The resulting size and uncertainty for m_{LC} is 0.03 ± 0.01 µg C/mL (Table 2).

Table 2 Parameters used to correct the data for process blanks and for complete propagation of error; see equations in the text.

Parameter name	Parameter description	Value	How determined
m_{CB}	mass of combustion blank	1.0 µg C	measurements
$\sigma_{m_{CB}}$	error in mass of combustion blank	0.2 µg C	measurements
Δ_{CB}	$\Delta^{14}C$ of combustion blank	59 ‰	calculated from blank and stigmasterol measurements
$\sigma_{\Delta_{CB}}$	error in $\Delta^{14}C$ of combustion blank	208 ‰	calculated from blank and stigmasterol measurements
m_{LC}	mass of HPLC blank	0.03 µg C/mL	measurements
$\sigma_{m_{LC}}$	error in mass of HPLC blank	0.01 µg C/mL	measurements
Δ_{LC}	$\Delta^{14}C$ of HPLC blank	–734 ‰	calculated from blank and stigmasterol measurements
$\sigma_{\Delta_{LC}}$	error in $\Delta^{14}C$ of HPLC blank	64 ‰	calculated from blank and stigmasterol measurements
m_{XC}	mass of contaminant blank	0.07 µg C	lipid measurements
$\sigma_{m_{XC}}$	error in mass of contaminant blank	0.05 µg C	lipid measurements
Δ_{XC}	$\Delta^{14}C$ of contaminant blank	–1000 ‰	assumed
$\sigma_{\Delta_{XC}}$	error in $\Delta^{14}C$ of contaminant blank	0 ‰	assumed
m_S	mass of stigmasterol	—	variable
σ_{m_S}	error in mass of stigmasterol	[a]	vacuum line uncertainty
Δ_S	$\Delta^{14}C$ of stigmasterol	126 ‰	measured
σ_{Δ_S}	error in $\Delta^{14}C$ of stigmasterol	5 ‰	measured

[a]Uncertainty in known volume of vacuum line determined experimentally in Pearson Lab (0.44 cm^3): mass (µg C) = $P \times 0.3$. Although all masses of natural samples were determined at UCIAMS, the uncertainty is assumed to be the same as that calculated from the Pearson Lab vacuum line, and agreement in values among samples processed on both lines is ±5%.

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Δ^{14}C Values of Combustion and HPLC Blanks

^{14}C-AMS measurements were made on the 108-mL sample of HPLC effluent; the combined 6-, 12-, and 18-mL samples; and the combined 36- and 72-mL samples (Table 1) at the Keck Carbon Cycle AMS facility, UC Irvine (UCIAMS). Only the Δ^{14}C measurement of the 108-mL sample was suitable for further analysis, due to analytical difficulties with the smaller samples. The mass of the combined 6-, 12-, 18-mL sample was very small, resulting in a highly uncertain measurement, and the combined 36- and 72-mL sample was lost (Table 1). The Δ^{14}C measurement of a sample of combusted HPLC effluent includes both HPLC blank and combustion blank:

$$\Delta_T = \frac{m_{CB}\Delta_{CB} + vm_{LC}\Delta_{LC}}{m_T} \quad (3)$$

where m_T refers to the total mass measured and Δ_T refers to the Δ^{14}C measurement of the total sample. m_{CB} and m_{LC} are defined above, v is the volume of HPLC effluent, and Δ_{CB} and Δ_{LC} are the unknown values for the combustion blank and the HPLC blank, respectively. Because there are 2 unknowns, Δ_{CB} and Δ_{LC}, it is not possible to accurately deconvolve the combustion and HPLC contributions to the total value of Δ_T.

To resolve these unknowns, Δ^{14}C measurements also were made for a lipid standard, stigmasterol (Sigma Lot #47H5033; Table 1). Stigmasterol was used rather than ^{14}C-dead bituminous coal because stigmasterol is expected to behave similarly to our archaeal lipid samples through the purification and combustion steps. We also anticipated that the combustion and HPLC blanks were likely to be ^{14}C-depleted; a low ^{14}C-content blank would be detected more easily using a modern compound as a standard. The Δ^{14}C value of a 1-mg sample of stigmasterol, combusted to CO_2 as described above (with the exception that about 2 g of cupric oxide was used rather than 0.1 g) was taken to be the authentic Δ^{14}C value of the standard (Δ_S) (Tables 1, 2). This is a reasonable assumption, as the combustion blank would contribute only 0.1% of the total sample mass. Five additional "small" samples of stigmasterol in the size range of our environmental lipid samples (5–15 µg C) were purified with the HPLC program described above, collected in 2–4 mL of effluent and combusted to CO_2 (Table 1). The total mass of carbon in these latter samples ($m_{T'}$) includes contributions from the stigmasterol standard, from the combustion blank, and from the HPLC blank:

$$\Delta_{T'} = \frac{m_S\Delta_S + m_{CB}\Delta_{CB} + v'm_{LC}\Delta_{LC}}{m_{T'}} \quad (4)$$

where $m_{T'}$ represents the total mass and $\Delta_{T'}$ is the Δ^{14}C value of the total sample. The volume of HPLC effluent that the stigmasterol was collected in is v'. The unknown values are Δ_{CB} and Δ_{LC}, which are the same 2 unknowns described above. Rearranging (3) and (4) using the relation:

$$m_s = m_{T'} - m_{CB} - v'm_{LC} \quad (5)$$

results in a set of 2 equations with 2 unknowns, which can be solved using the values in Table 2:

$$\Delta_{LC} = \frac{m_{T'}\Delta_{T'} - m_T\Delta_T - m_S\Delta_S}{m_{LC}(v' - v)} \quad (6)$$

and

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$$\Delta_{CB} = \frac{m_T\Delta_T - vm_{LC}\Delta_{LC}}{m_{CB}} \quad (7)$$

This results in 5 pairs of calculated values for Δ_{LC} and Δ_{CB}, one for each of the 5 "small" measurements of stigmasterol. The uncertainty in these calculated values ($\sigma_{\Delta_{CB}}$ and $\sigma_{\Delta_{LC}}$) is the sample standard deviation of the set of 5 solutions. The final values of the HPLC and combustion blanks, including uncertainties, are Δ_{LC} = –754 ± 64‰ and Δ_{CB} = 59 ± 208‰ (Table 2). The uncertainty in the $\Delta^{14}C$ value of the combustion blank is significantly larger than the uncertainty of the HPLC blank and becomes the limiting factor for precise determination of $\Delta^{14}C$ values of small samples (discussed below).

CORRECTING INDIVIDUAL $\Delta^{14}C$ MEASUREMENTS FOR COMBUSTION AND HPLC BLANKS

Natural samples of archaeal membrane lipids and sterols were obtained from the water column of the North Central Pacific as described in Ingalls et al. (2006). Here, we use the data from these 5 compounds (representing 8 $\Delta^{14}C$ measurements) as examples of the application of this blank-correction method (Table 3). The raw $\Delta^{14}C$ measurements represent ^{14}C content in the total mass of each sample (m_T), which includes contributions from the lipid, combustion blank, and HPLC blank, as well as potential contribution from residual laboratory contamination that has not been adequately quantified as part of one of the above components. Isotopic mass balance corrections can be applied. In this case, it is the $\Delta^{14}C$ value of the lipid that is the unknown:

$$\Delta_{lipid'} = \frac{m_T\Delta_T - m_{CB}\Delta_{CB} - vm_{LC}\Delta_{LC}}{m_{lipid'}} \quad (8)$$

where Δ_T refers to the raw measurement of the total sample and $m_{lipid'} = m_T - m_{CB} - vm_{LC}$. The resulting $\Delta_{lipid'}$ is the corrected value from which the contributions of the combustion and HPLC blank have been removed (Table 3, column 5).

Table 3 $\Delta^{14}C$ measurements of natural samples measured at UCIAMS, corrected according to Equation 11 and total propagated error calculated according to Equation 18.

Sample description	HPLC effluent (mL)	Mass (µg C)	UCIAMS-reported $\Delta^{14}C$ (‰)	$\Delta^{14}C$ corrected for m_{CB} and m_{LC} (‰)	$\Delta^{14}C$ corrected for m_{CB}, m_{LC}, and m_{XC} (‰)	Total propagated error (‰)
670m GDGT I	2	5.4	–179 ± 11	–226	–68	±64
670m GDGT I replicate	3	30.0	–128 ± 7	–133	–110	±11
670m GDGT III	2	6.1	–161 ± 10	–198	–60	±53
670m GDGT IV	4	7.6	–197 ± 9	–226	–127	±40
670m GDGT IV replicate	4	9.8	–130 ± 13	–143	–64	±32
670m GDGT II	4	9.6	–138 ± 12	–152	–72	±32
670m GDGT II replicate	2	24.0	–89 ± 8	–94	–64	±13
670m GDGT VI	2	2.8	–223 ± 36	–367	98	±254
21m GDGTs I,III,IV,V	4	27.8	42 ± 9	45	77	±13
21m GDGTs II, VI	5	32.2	51 ± 8	51	84	±12
21m C27 sterol	6	41.7	34 ± 8	37	56	±9
21m C29 sterol	6	46.3	49 ± 8	52	69	±9

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ASSESSING AND CORRECTING FOR RESIDUAL CONTAMINANTS

All values of $\Delta_{lipid'}$ should agree within propagated errors, as they represent 8 measurements of 5 compounds, all of which have the same biosynthetic source: pelagic archaea living at 670 m. The values of $\Delta_{lipid'}$ were not expected to show any correlation with the mass of the sample, unless there remained an additional contribution from a contaminant that had not yet been subtracted. The initial clue that this was indeed the case was apparent when examining the data for individual samples that had been measured in duplicate (Table 3). Samples GDGT-I and GDGT-I-replicate yielded $\Delta^{14}C$ values of –226‰ and –133‰ after corrections using Equation 8, respectively. Similar cases were observed for GDGT-II and GDGT-II-replicate (–152‰, –94‰) and GDGT-IV and GDGT-IV-replicate (–226‰, –143‰). These differences are too large to be explained by AMS machine error (statistical counting error) and are unexpected. These measurements each represent 2 authentic replicates of the same initial sample. As Figure 2 illustrates, the values of $\Delta_{lipid'}$ continue to show a strong and significant negative correlation with mass^{-1} ($R^2 = 0.93$). This result is consistent with the presence of an additional contaminant of constant mass with an isotopically-negative $\Delta^{14}C$ signature in each sample. This unidentified component affects samples of smaller mass to a greater extent than it biases samples of larger mass. Potential sources of contamination during preparation of compound-specific ^{14}C samples include blanks associated with flame-sealing of quartz and Pyrex tubes, blanks associated with the use of vacuum lines, contamination on interface devices (tube crackers and fittings), and blanks associated with the portions of the outsides of the tubes that must be exposed to the interior of the vacuum environment. Each of these possible sources of additional carbon could contribute a constant—but small—mass per sample. An underestimate of the uncertainty in or absolute value of the Δ_{LC} component of Equation 8 could also be responsible. Since the stigmasterol used earlier in determining the isotopic composition of the liquid chromatography background does not elute identically to the lipids, the uncertainty in Δ_{LC} may be underestimated.

Thus, the values of $\Delta_{lipid'}$ contain contributions from the archaeal lipid and also from the additional contaminant:

$$m_{lipid'}\Delta_{lipid'} = m_{lipid}\Delta_{lipid} + m_{XC}\Delta_{XC} \tag{9}$$

where m_{lipid} (and Δ_{lipid}) refer to the pure archaeal lipid, m_{XC} is the mass of the extra contaminant carbon, and Δ_{XC} represents its ^{14}C content. This equation can be rearranged to the equation of a line using $m_{lipid} = m_{lipid'} - m_{XC}$:

$$\Delta_{lipid'} = (m_{XC}(\Delta_{XC} - \Delta_{lipid}))\frac{1}{m_{lipid'}} + \Delta_{lipid} \tag{10}$$

Plotting the $(m_{lipid'})^{-1}$ against $\Delta_{lipid'}$ (Figure 2) allows the mass m_{XC} to be calculated from the slope. Using the assumption that these unmeasurable contaminants are most likely to be ^{14}C-dead (Δ_{XC} = –1000‰), the mass of the contaminant carbon is 0.7 µg C (Table 2). Therefore, the complete isotopic mass balance correction that must be applied to each raw CSRA measurement includes three components of exogenous carbon contamination, m_{LC}, m_{CB}, and m_{XB}. Values for the true isotopic composition of the individual lipids, Δ_{lipid}, are shown in Table 3, column 6:

$$\Delta_{lipid} = \frac{m_T\Delta_T - m_{CB}\Delta_{CB} - vm_{LC}\Delta_{LC} - m_{XC}\Delta_{XC}}{m_{lipid}} \tag{11}$$

The uncertainty in m_{XC} is determined by considering the definition of the R^2 value. The R^2 value of the regression line (0.93) implies that $m_{lipid'}$ predicts the value of $\Delta_{lipid'}$ with 93% certainty. Since

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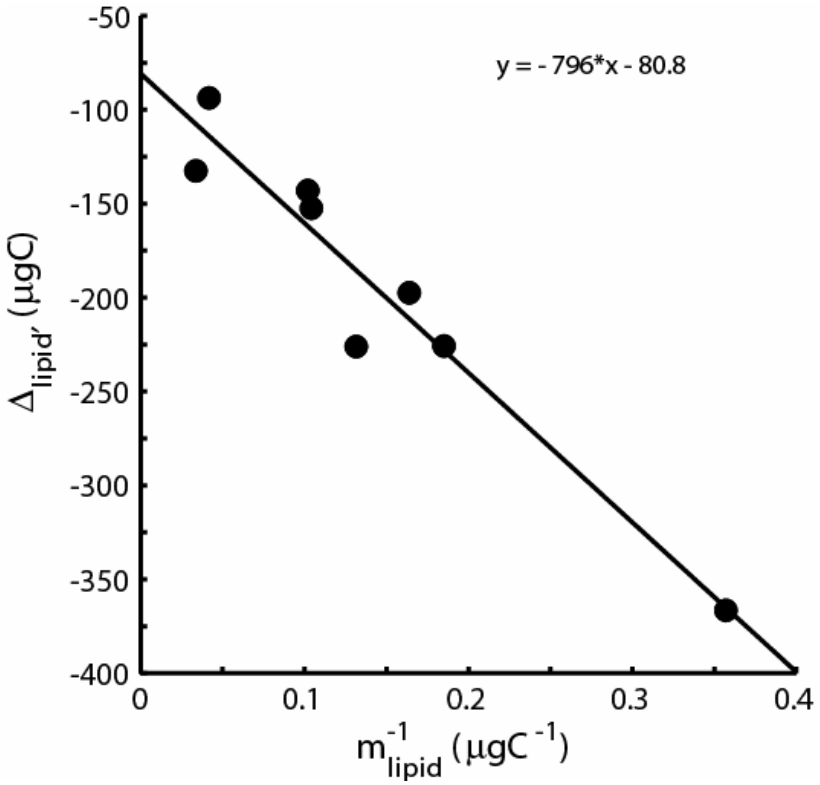

Figure 2 Plot of $\Delta^{14}C$ measurements of lipid samples corrected for combustion blank and HPLC blank ($\Delta_{lipid'}$) vs. $1/m_{lipid'}$ ($m_{lipid'} = m_T - m_{CB} - m_{LC}$). Equation of regression line corresponds to Equation 10. $R^2 = 0.93$.

we have calculated m_{XC} with this relation, the uncertainty in m_{XC} would be $m_{XC} \times (1–0.93)$, or 0.05 µg C, although this could be an underestimate (Table 2).

Each of the sources of exogenous carbon, particularly the unidentified contaminant carbon, represents an integration of multiple individual sources that may vary independently in time. Repeated assessment of these blanks on a periodic basis would have to be performed to ensure that the values in Table 2 remained representative of current conditions. Such self-monitoring is critical to obtain both accurate and precise calculation of corrected $\Delta^{14}C$ values for ultra-microscale samples.

The additional $\Delta^{14}C$ measurements reported in Ingalls et al. (2006) are 2 samples of sterols and 2 samples of archaeal lipids extracted from 21 m in the North Central Pacific. Both of these categories of samples contain only 2 data points per compound class, not enough to independently confirm our assessment of the unmeasurable contaminant. However, as they both have been purified by HPLC and combusted to CO_2 in the same way as the archaeal lipids from 670 m, we believe they are likely to contain identical blanks. Therefore, they were corrected for the combustion blank, HPLC blank, and contaminant carbon using the same values for all of the components of the blank (Table 2). Results for these data also are summarized in Table 3.

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TOTAL ANALYTICAL UNCERTAINTY FOR BLANK-CORRECTED $\Delta^{14}C$ MEASUREMENTS

Calculating the total uncertainty for lipids purified and analyzed by the HPLC-CSRA methods described here requires propagating the errors associated with each of the individual contributions from the sample and blanks. Each value of Δ_T reported by an AMS facility represents the average of many individual measurements, i.e. a finite number of intervals of time over which individual ^{14}C ions were counted as ratios to the numbers of ^{13}C or ^{12}C counts. As such, the AMS facility-reported error can be represented as a statistical function dependent on N measurements, or counts. Each count of the measurement, Δ_{T_n}, also is a function of m_{lipid_n}, Δ_{lipid_n}, m_{CB_n}, Δ_{CB_n}, m_{LC_n}, Δ_{LC_n}, m_{XC_n}, Δ_{XC_n}. Therefore, each final corrected value of Δ_{lipid} also represents an average of many individual measurements, n:

$$\overline{\Delta_{lipid}} = \frac{1}{N}\sum_{1}^{N}\Delta_{lipid_n} \tag{12}$$

The variance in these individual measurements is:

$$\sigma^2_{\Delta_{lipid}} = \frac{1}{N}\sum_{1}^{N}(\Delta_{lipid_n} - \overline{\Delta_{lipid}})^2 \tag{13}$$

A more explicit representation of the difference between each individual measurement and the average measurement is:

$$\Delta_{lipid_n} - \overline{\Delta_{lipid}} = f(m_{T_n}, \Delta_{T_n}, m_{CB_n}, \Delta_{CB_n}, m_{LC_n}, \Delta_{LC_n}, m_{XC_n}, \Delta_{XC_n}) -$$

$$f(\overline{m_T}, \overline{\Delta_T}, \overline{m_{CB}}, \overline{\Delta_{CB}}, \overline{m_{LC}}, \overline{\Delta_{LC}}, \overline{m_{XC}}, \overline{\Delta_{XC}}) =$$

$$f[(m_{T_n} - \overline{m_T}), (\Delta_{T_n} - \overline{\Delta_T}), (m_{CB_n} - m_{CB}), (\Delta_{CB_n} - \overline{\Delta_{CB}}), (m_{LC_n} - \overline{m_{LC}}), (\Delta_{LC_n} - \overline{\Delta_{LC}}),$$

$$(m_{XC_n} - \overline{m_{XC}}), (\Delta_{XC_n} - \overline{\Delta_{XC}})] \tag{14}$$

Assuming the deviations in Δ_{lipid_n} from $\overline{\Delta_{lipid}}$ are caused by small random deviations of m_{T_n} from $\overline{m_T}$, of Δ_{T_n} from $\overline{\Delta_T}$, and of similar deviations for m_{CB}, Δ_{CB}, m_{LC}, Δ_{LC}, m_{XC}, and Δ_{XC}, a Taylor series expansion (keeping only the first-order terms) results in:

$$\Delta_{lipid_n} - \overline{\Delta_{lipid}} = (m_{T_n} - \overline{m_T})\frac{\partial\Delta_{lipid}}{\partial m_T} + (\Delta_{T_n} - \overline{\Delta_T})\frac{\partial\Delta_{lipid}}{\partial\Delta_T} + (m_{CB_n} - \overline{m_{CB}})\frac{\partial\Delta_{lipid}}{\partial m_{CB}} +$$

$$(\Delta_{CB_n} - \overline{\Delta_{CB}})\frac{\partial\Delta_{lipid}}{\partial\Delta_{CB}} + (m_{LC_n} - \overline{m_{LC}})\frac{\partial\Delta_{lipid}}{\partial m_{LC}} + (\Delta_{LC_n} - \overline{\Delta_{LC}})\frac{\partial\Delta_{lipid}}{\partial\Delta_{LC}} +$$

$$(m_{XC_n} - \overline{m_{XC}})\frac{\partial\Delta_{lipid}}{\partial m_{XC}} + (\Delta_{XC_n} - \overline{\Delta_{XC}})\frac{\partial\Delta_{lipid}}{\partial\Delta_{XC}} \tag{15}$$

Substituting this definition in Equation 13 to determine the variance results in:

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$$\sigma^2_{\Delta_{lipid}} = \frac{1}{N}\sum_{1}^{N}\left[\begin{array}{c}(m_{T_n}-\overline{m_T})\frac{\partial\Delta_{lipid}}{\partial m_T}+(\Delta_{T_n}-\overline{\Delta_T})\frac{\partial\Delta_{lipid}}{\partial\Delta_T}+(m_{CB_n}-\overline{m_{CB}})\frac{\partial\Delta_{lipid}}{\partial m_{CB}}+\\ (\Delta_{CB_n}-\overline{\Delta_{CB}})\frac{\partial\Delta_{lipid}}{\partial\Delta_{CB}}+(m_{LC_n}-\overline{m_{LC}})\frac{\partial\Delta_{lipid}}{\partial m_{LC}}+(\Delta_{LC_n}-\overline{\Delta_{LC}})\frac{\partial\Delta_{lipid}}{\partial\Delta_{LC}}+\\ (m_{XC_n}-\overline{m_{XC}})\frac{\partial\Delta_{lipid}}{\partial m_{XC}}+(\Delta_{XC_n}-\overline{\Delta_{XC}})\frac{\partial\Delta_{lipid}}{\partial\Delta_{XC}}\end{array}\right]^2 \quad (16)$$

Multiplying out the summed part of this equation results in 36 terms, 24 of which are covariance terms. For simplicity, we assume the mass and $\Delta^{14}C$ values of the lipid samples and of all of the process blanks are independent of each other, so their covariance is zero. The resulting 8 terms give the formula for total propagation of uncertainty associated with our final reported values of Δ_{lipid}:

$$\sigma^2_{\Delta_{lipid}} = \left(\frac{\partial\Delta_{lipid}}{\partial m_T}\right)^2\sigma^2_{m_T}+\left(\frac{\partial\Delta_{lipid}}{\partial\Delta_T}\right)^2\sigma^2_{\Delta_T}+\left(\frac{\partial\Delta_{lipid}}{\partial m_{CB}}\right)^2\sigma^2_{m_{CB}}+\left(\frac{\partial\Delta_{lipid}}{\partial\Delta_{CB}}\right)^2\sigma^2_{\Delta_{CB}}+$$

$$\left(\frac{\partial\Delta_{lipid}}{\partial m_{LC}}\right)^2\sigma^2_{m_{LC}}+\left(\frac{\partial\Delta_{lipid}}{\partial\Delta_{LC}}\right)^2\sigma^2_{\Delta_{LC}}+\left(\frac{\partial\Delta_{lipid}}{\partial m_{XC}}\right)^2\sigma^2_{m_{XC}}+\left(\frac{\partial\Delta_{lipid}}{\partial\Delta_{XC}}\right)^2\sigma^2_{\Delta_{XC}} \quad (17)$$

Expanding the derivative terms results in:

$$\sigma^2_{\Delta_{lipid}} = \left(\frac{m_{CB}(\Delta_{CB}-\Delta_T)+m_{LC}(\Delta_{LC}-\Delta_T)+m_{XC}(\Delta_{XC}-\Delta_T)}{(m_T-m_{CB}-m_{LC}-m_{XC})^2}\right)^2\sigma^2_{m_T}$$

$$+\left(\frac{m_T}{m_T-m_{CB}-m_{LC}-m_{XC}}\right)^2\sigma^2_{\Delta_T}+\left(\frac{m_T(\Delta_T-\Delta_{CB})+m_{LC}(\Delta_{CB}-\Delta_{LC})+m_{XC}(\Delta_{CB}-\Delta_{XC})}{(m_T-m_{CB}-m_{LC}-m_{XC})^2}\right)^2\sigma^2_{m_{CB}}$$

$$+\left(\frac{-m_{CB}}{m_T-m_{CB}-m_{LC}-m_{XC}}\right)^2\sigma^2_{\Delta_{CB}}+\left(\frac{m_T(\Delta_T-\Delta_{LC})+m_{CB}(\Delta_{LC}-\Delta_{CB})+m_{XC}(\Delta_{LC}-\Delta_{XC})}{(m_T-m_{CB}-m_{LC}-m_{XC})^2}\right)^2\sigma^2_{m_{LC}}$$

$$+\left(\frac{-m_{LC}}{m_T-m_{CB}-m_{LC}-m_{XC}}\right)^2\sigma^2_{\Delta_{LC}}+\left(\frac{m_T(\Delta_T-\Delta_{XC})+m_{CB}(\Delta_{XC}-\Delta_{CB})+m_{LC}(\Delta_{XC}-\Delta_{LC})}{(m_T-m_{CB}-m_{LC}-m_{XC})^2}\right)^2\sigma^2_{m_{XC}}$$

$$+\left(\frac{-m_{XC}}{m_T-m_{CB}-m_{LC}-m_{XC}}\right)^2\sigma^2_{\Delta_{XC}} \quad (18)$$

The final data, as reported in columns 6 and 7 of Table 3 (and in Ingalls et al. 2006) show that all of the $\Delta^{14}C$ values for replicate samples agree after correction for all 3 components of the process blank: m_{CB}, m_{LC}, and m_{XC}. Although m_{CB} and m_{LC} were determined on the vacuum line in our lab, and m_{XC} and m_T from measurements made at UCIAMS, the small relative differences in volume calibration (<5% error in mass determinations) between the 2 facilities introduce much less error than the total relative uncertainty in m_{CB} and m_{LC} (10–30%). Thus, there are no complications introduced by working with multiple vacuum lines (in this case). More significantly, the total propagated uncertainty associated with our reported values is much larger than the uncertainty associated with AMS facility processes (AMS facility-reported error; Table 3, column 4).

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The uncertainties reported by AMS facilities include not only counting statistics, as described above, but also contributions from sample handling and graphitization (detailed description of this process and the associated data corrections can be found in Santos et al., forthcoming). The data obtained from AMS facilities, including the data reported here, typically already include corrections for graphitization, handling, and AMS stability. Uncertainties in these variables are reported as part of the total AMS facility error, σ_{Δ_T}. However, our analysis of laboratory blanks associated with CSRA shows that σ_{Δ_T} always is significantly smaller than the complete propagated error ($\sigma_{\Delta_{lipid}}$) when samples are smaller than 25 µg C (Table 3). The difference between AMS error and the total sample error ($\sigma_{\Delta_{lipid}}$) is shown in Figure 3. These data highlight the rapid increase in total measurement uncertainty at very small sample sizes. The figure also highlights the importance of reporting the complete propagated error when dealing with extremely small samples, since blank carbon—most significantly the combustion blank—contributes up to 40% of the total sample mass, and the uncertainty in both the mass and isotopic composition of this blank is very large.

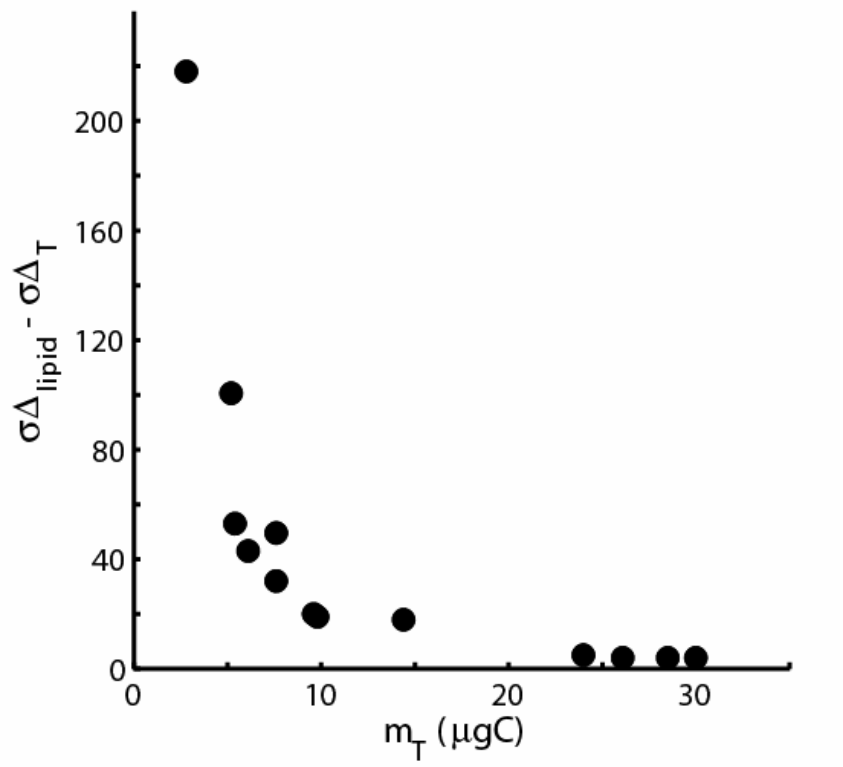

Figure 3 Difference between AMS facility-reported uncertainty (σ_{Δ_T}) and total propagated uncertainty ($\sigma_{\Delta_{lipid}}$) plotted against total sample mass (m_T). The data include 3 unpublished measurements of bacterial fatty acids not included in Table 3.

CONCLUSIONS

We have defined a new category of AMS measurement: the ultra-microscale measurement, which refers to samples that contain 5 to 25 µg C. Unlike larger samples—for which the uncertainty in the AMS measurement is determined primarily by factors associated with sample graphitization, counting statistics, and tuning and stability parameters inherent to the AMS machine—the uncertainty in

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ultra-microscale measurements derives mostly from unavoidable sample processing blanks. Therefore, these ultra-small samples require meticulous assessment of all contributions of exogenous carbon, followed by careful propagation of associated uncertainties. These corrections are needed to report the true $\Delta^{14}C$ values with appropriate accuracy and precision.

The approaches to error analysis explained here also can be generalized to other applications. Our goal in this paper was to describe the process by which ^{14}C measurements of small samples of individual compounds are corrected for background carbon. In the case of non-CSRA samples, the quantified blanks would have to be replaced with more general and/or integrative blanks that are specific to the type of sample collection and processing that is used.

We find that the biggest impediment is the uncertainty associated with the combustion blank. The mass contributed by closed-tube combustion is about 1 μg C, and as such it represents 4–20% of the total mass of carbon analyzed. Although this quantity can be subtracted by mass balance, the substantial uncertainty associated with its mass (±0.2 μg C) and its $\Delta^{14}C$ value (±208‰) is enough to dwarf all other contributions to the total uncertainty. Additional strategies to minimize the size of the combustion blank are possible (including the use of Vycor® rather than quartz tubes), but this in turn would complicate the accurate determination of both the magnitude and especially the variability of Δ_{CB}.

We find that it is impractical to attempt to measure samples smaller than 5 μg C using the customary techniques that include closed-tube combustion. The magnitude of the total propagated uncertainty becomes unreasonable for nearly all geochemical applications below 5 μg C, as witnessed by our single measurement of a sample containing 2.8 μg C (measured to only ±254‰, Figure 3). For studies requiring measurement uncertainty to be limited to ≤±30‰, samples smaller than 10 μg C also are too small to yield useful results (Figure 3). New approaches to sample combustion or oxidation are needed to minimize the magnitude and uncertainty of the combustion blank. Meanwhile, the practical limit for ultra-microscale ^{14}C AMS measurements, and therefore applications to problems requiring CSRA, will be limited to samples larger than 5 μg C. Despite these analytical constraints, ultra-microscale AMS measurements combined with compound-specific approaches promise to provide insights into the biogeochemical roles of prokaryotes when samples can be obtained at the ≥5-μg C threshold.

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