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Dead in the Water: The Vicious Cycle of Blanks During Natural Level ^{14}C Manipulation of Marine Algal Cultures

Stephanie Kusch[1,2†], Albert Benthien[1], Klaus-Uwe Richter[1], Björn Rost[1,3] and Gesine Mollenhauer[1,2]*

[1] Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany, [2] Department of Geosciences, University of Bremen, Bremen, Germany, [3] Faculty of Biology/Chemistry, University of Bremen, Bremen, Germany

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***Correspondence:**
Stephanie Kusch
stephanie.kusch@uni-koeln.de

†Present address:
Stephanie Kusch,
CologneAMS, University of Cologne, Cologne, Germany

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Authentic biomarker standards were obtained from algal cultures in an attempt to accurately determine blank C added during sample processing for compound-specific radiocarbon analysis. *Emiliania huxleyi* and *Thalassiosira pseudonana* were grown under manipulated $\Delta^{14}C$ dissolved inorganic carbon (DIC) levels and chlorophyll *a* and either alkenones (*E. huxleyi*) or low molecular weight (LMW) alkanoic acids (*T. pseudonana*) were isolated from the respective biomass using preparative liquid chromatography (LC), wet chemical techniques or preparative gas chromatography, respectively. DI^{14}C in the seawater medium was determined pre- and post-growth. Biomarker $\Delta^{14}C$ values mostly agree within 1σ or 2σ analytical uncertainties. In those cases where biomarker $\Delta^{14}C$ values differ significantly, chlorophyll *a* is up to 104‰ more ^{14}C-depleted than alkenones or LMW alkanoic acids, consistent with a larger LC blank compared to the other purification methods. However, in the majority of experimental setups pre- and post-growth DIC $\Delta^{14}C$ values seem to be compromised by an unknown and variable blank C contribution. DIC $\Delta^{14}C$ values deviate strongly from the anticipated $\Delta^{14}C$ values (by up to ca. 560‰), pre- and post-growth $\Delta^{14}C$ values differ significantly (by up to ca. 460‰), and changes are not unidirectional. Accordingly, since the substrate $\Delta^{14}C$ value cannot unequivocally be constrained, blank C contributions for the different biomarker purification methods cannot be accurately calculated. This study illustrates the challenges and problems of producing authentic standards that are not readily commercially available and exemplifies how a laborious and time-consuming culturing approach may enter a vicious cycle of blank C contamination hampering accurate blank C determination.

Keywords: compound-specific radiocarbon analysis, authentic standards, blank, chlorophyll *a*, alkenones, alkanoic acids, algal cultures, natural level ^{14}C manipulation

INTRODUCTION

Compound-specific radiocarbon analysis (CSRA) has revolutionized our understanding of carbon cycling in the ocean including both sedimentary and metabolic processes in the water column and sediments (e.g., Pearson et al., 2005; Ingalls et al., 2006; Mollenhauer and Eglinton, 2007; Mollenhauer et al., 2007), as well as land to ocean carbon transfer (e.g., Drenzek et al., 2009;

Kusch et al., 2010b; Feng et al., 2013). Accordingly, there are efforts to extend CSRA to an ever-increasing number of compounds and compound classes. Nonetheless, CSRA is quite laborious and compound isolation requires various wet-chemical techniques as well as analytically more advanced steps such as preparative gas chromatography (GC) or preparative liquid chromatography (LC). In order to achieve meaningful results, it is a pivotal necessity to accurately determine the blank carbon contribution associated with compound isolation (sample processing), i.e., both the amount as well as ^{14}C isotopic composition of this blank C (e.g., Shah and Pearson, 2007). Two approaches can be taken for this purpose (i) obtaining "true" blank runs void of any sample, e.g., collection of the LC effluent volume, or (ii) processing authentic standards with known Δ^{14}C values. "True" blank runs require the pooling of many consecutive runs in order to retrieve sufficient C amounts for ^{14}C analysis, which may not always be successful (Shah and Pearson, 2007), and yield the combined blank C isotopic composition. In contrast, processing of authentic standards is likely quicker and allows the characterization of blank C contributions from both fossil ("^{14}C-dead") and modern (atmospheric ^{14}C) sources if these standards have modern and fossil Δ^{14}C values, respectively.

However, for a range of compounds or even compound classes typically found in marine particulate organic matter, authentic standards are not readily available. Either, they are not produced commercially or are manufactured solely from either plant biomass or petroleum sources limiting our ability to characterize either the fossil or modern blank sources, respectively. Under these circumstances, scientists are required to use surrogate standards (e.g., Shah and Pearson, 2007; Birkholz et al., 2013) or to obtain their own authentic standards for example via culturing approaches (Mollenhauer et al., 2005). Surrogate standards only provide the best approximation of the actual blank C, since they do not have chemical properties identical to the target compounds, e.g., they will show different chromatographic behavior and, thus, entrain slightly different column "bleed" from stationary phases during chromatography. Authentic standards can be obtained when culturing marine algae in the laboratory under controlled dissolved inorganic carbon (DIC) Δ^{14}C levels (Mollenhauer et al., 2005). While this approach is laborious and time-consuming, it allows for the determination of blank C contribution associated with the isolation of target compounds from different compound classes and with various Δ^{14}C endmembers. However, it is crucial that the Δ^{14}C isotopic composition of C sources during culturing remains constrained and the culturing experiments themselves are not associated with significant blank C contributions.

Here, we illustrate the challenging nature of natural level ^{14}C manipulation in batch cultures of *Emiliania huxleyi* and *Thalassiosira pseudonana* grown under three different Δ^{14}C DIC levels (40‰, –480‰, and –1000‰). We show that blank C of unknown origin contributed significantly to the DIC Δ^{14}C isotopic composition during the experiments, impairing a valuable assessment of blank C associated with the purification of chlorophyll *a* as well as alkenones (*E. huxleyi*) and low molecular weight (LMW) alkanoic acids (*T. pseudonana*).

MATERIALS AND METHODS

Algal Culture Conditions

North Sea seawater was enriched with nutrients including nitrate, phosphate, silicate (for *T. pseudonana*), metals, and vitamins at concentrations equaling f/2 medium (Guillard and Ryther, 1962). The seawater was filtered through sterile 0.2 µm PTFE filters and collected in sterile 2.4 l borosilicate glass bottles. The seawater was percolated with CO_2-free air for 36 h at 25°C to expel DIC species (**Figure 1**). A similar setup is routinely used to achieve DIC-free medium, which has been verified by direct measurements using Membrane-Inlet MS (Rost et al., 2007). Subsequently, CO_2 of known ^{14}C isotopic composition was percolated through the seawater at 380 ppm for 48 h at 25°C each. CO_2 endmembers represent "Modern" (40‰; ambient air in AD2010/AD2011; Levin et al., 2013) and "Fossil" (–1000‰; Air Liquide N45) Δ^{14}C values as well as a 1:1 "Intermediate" mixture (–480‰; gas volume v:v using a custom-made gas flow controller). The seawater was cooled to 15°C and the *E. huxleyi* strain RCC 1238 (Roscoff Culture Collection) or the *T. pseudonana* strain CCMP 1335 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton) were inoculated at a cell density of approximately 200 cells ml^{-1}. Both strains were checked for their vitality under a light microscope prior to inoculation. Culture bottles were topped with seawater to reduce headspace volume and closed with sterile PTFE-lined caps (to limit diffusion of atmospheric ^{14}C). Bottles were stored in a RUMED 1200 light-thermostat at 15°C with a photon flux density of 100 µmol photons m^{-2} s^{-1} to stimulate pigment production (Nielsen, 1997). Starter cultures were grown in 16 h: 8 h light: dark cycles until they reached stationary phase (based on cell densities determined from 1 ml aliquots). Cell densities were determined using a Beckman Multisizer 3 Coulter Counter (*E. huxleyi*) or visual counting under a microscope (*T. pseudonana*). Afterward

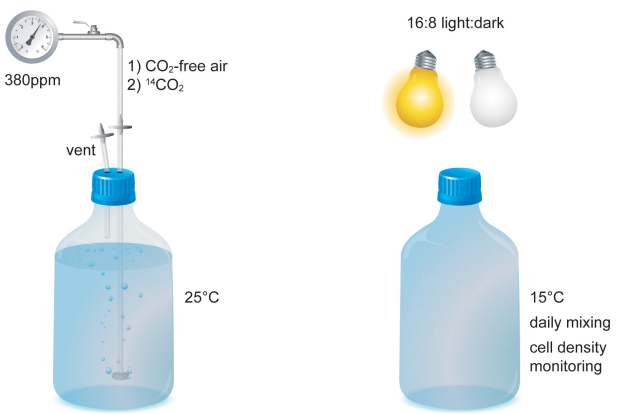

FIGURE 1 | Schematic of the experimental setup during **(A)** DIC ^{14}C manipulation and **(B)** batch culture growth of the *E. huxleyi* and *T. pseudonana* cultures.

an aliquot of the starter cultures was taken and approximately 200 cells ml^{-1} of the isotopically equilibrated starter culture were then inoculated into triplicates of new medium (sterile 2.4 l borosilicate glass bottles) and main cultures were grown in 16 h: 8 h light: dark cycles. After reaching stationary phase (9–13 days), an aliquot of the main culture was taken for final cell density determination and cultures were harvested (mid-day) by filtration onto pre-combusted GF/F filters (–200 m bar; triplicates of each batch were combined) and stored frozen at –20°C in the dark until analysis.

Pigment and Lipid Extraction and Purification

For pigment extraction, GF/F filters were transferred into pre-combusted glass vials and 20 ml dehydrated acetone was added, samples were extracted for 15 min in an ultrasonic ice bath, centrifuged at 1200 rpm for 3 min, and the acetone was recovered. This extraction step was repeated twice. Subsequently, the combined acetone extracts were concentrated under N_2 and transferred into a 1:3 hexane: Seralpure® mixture for liquid-liquid extraction. Liquid-liquid extraction was performed using hexane (pigments) and dichloromethane (residual lipids), each extraction was repeated until the solvent layer was colorless. The hexane fraction was concentrated under N_2, rinsed through pre-combusted sodium sulfate to remove Seralpure® residues, dehydrated dimethylformamide was added, the sample homogenized, and stored at –20°C overnight. Chlorophyll *a* was purified with an Agilent 1200 Series HPLC/DAD system using the method described in Kusch et al. (2010a). After initial ultrasonic extraction of pigments, the GF/F filters were additionally extracted with 9:1 dichloromethane: methanol using Soxhlet (48 h; cellulose thimbles pre-extracted 24 h) in order to ensure maximum lipid recovery. The total lipid extract (TLE) was combined with the dichloromethane fraction recovered during liquid-liquid extraction of the acetone-Seralpure® mixture. The TLE was saponified in 0.5 M potassium hydroxide in methanol for 3 h at 85°C to separate alkanoic acids and neutral lipids. Alkenones were purified wet-chemically according to Ohkouchi et al. (2005). LMW alkanoic acids were methylated and purified using an Agilent HP6890N GC connected to a Gerstel preparative fraction collector following Kusch et al. (2010b).

Isotope Measurements

For radiocarbon measurements, chlorophyll *a*, alkenones, and LMW alkanoic acids methyl esters (FAMEs) were converted into CO_2. The samples were transferred into pre-combusted quartz tubes (900°C, 4 h), 150 μg pre-combusted copper oxide were added as oxygen source, and samples were evacuated and flame-sealed *in vacuo*. Afterward, the samples were combusted to CO_2 at 900°C for 8 h. The resulting CO_2 gas was stripped of water *in vacuo* and quantified manometrically. AMS measurements of the $\Delta^{14}C$ of chlorophyll *a*, alkenones, and FAME isolates containing >1 μmol CO_2 were performed at the National Ocean Sciences Accelerator Mass Spectrometry Facility (NOSAMS) at Woods Hole Oceanographic Institution, United States following the protocol for small samples (Pearson et al., 1998). Aliquots of the CO_2 were measured on a VG Optima to obtain $\delta^{13}C$ values. ^{14}C analyses of biomarker isolates <1 μmol CO_2 were performed at the Ion Beam Physics Department at ETH, Switzerland using the MICADAS gas ion source system (Ruff et al., 2007). Seawater sampled before and after batch culturing was transferred into 500 ml pre-combusted borosilicate glass bottles, 100 μl saturated mercuric chloride solution was added, and bottles were filled without headspace. Seawater DIC was analyzed at NOSAMS following standard protocols (McNichol et al., 1994).

Radiocarbon data are reported as $\Delta^{14}C$ in ‰ according to Stuiver and Polach (1977), $\delta^{13}C$ data are reported in ‰ relative to Vienna Pee Dee Belemnite (VPDB). FAME $\Delta^{14}C$ and $\delta^{13}C$ values were corrected for the addition of one methyl group during derivatization.

RESULTS

"Modern" Cultures

In the "Modern" *E. huxleyi* culture, pre- and post-growth DIC $\Delta^{14}C$ values are 60.0 ± 3.4‰ and 83.0 ± 3.5‰, respectively (**Figure 2** and **Supplementary Table S1**). The stable carbon isotopic composition ($\delta^{13}C$) of the seawater DIC was –1.0 ± 0.1‰ prior to culture growth and 4.0 ± 0.1‰ after *E. huxleyi* growth. The alkenone $\Delta^{14}C$ value of 77.0 ± 3.9‰ falls within the pre- and post-growth DIC $\Delta^{14}C$ values, while the chlorophyll *a* $\Delta^{14}C$ value (24.5 ± 10.6‰) is significantly (>2σ analytical uncertainty) more depleted. Corresponding $\delta^{13}C$ values are –20.2 ± 0.1‰ for alkenones and –15.4 ± 0.1‰ for chlorophyll *a*.

Pre- and post-growth DIC $\Delta^{14}C$ values for the "Modern" *T. pseudonana* culture show the highest deviation of the entire data set (∼460‰) with a pre-growth $\Delta^{14}C$ value of –43.9 ± 2.9‰ and a post-growth $\Delta^{14}C$ value of –505.2 ± 2.5‰. Analogous to the "Modern" *E. huxleyi* culture, seawater DIC became ^{13}C-enriched during the course of the experiment increasing from a pre-growth $\delta^{13}C$ value of –14.8 ± 0.1‰ to a $\delta^{13}C$ value of –11.0 ± 0.1‰ post-growth. Both, *T. pseudonana* derived $C_{14:0}$ alkanoic acid (–348.8 ± 3.1‰) and chlorophyll *a* (−452.3 ± 1.9‰) $\Delta^{14}C$ values fall within the range of the pre- and post-growth DIC $\Delta^{14}C$ values but deviate significantly (>2σ analytical uncertainty) from one another. The $\delta^{13}C$ values of the $C_{14:0}$ alkanoic acid and chlorophyll *a* are –30.2 ± 0.1‰ and –27.8 ± 0.1‰, respectively.

"Intermediate" Cultures

The seawater DIC $\Delta^{14}C$ value in the "Intermediate" *E. huxleyi* culture was –380.1 ± 2.4‰ before culture growth and −437.1 ± 2.0‰ after growth (**Figure 2** and **Supplementary Table S1**) with corresponding pre- and post-growth $\delta^{13}C$ values of –14.5 ± 1.0‰ and –12.1 ± 0.1‰, respectively. Both, alkenones and chlorophyll *a* from *E. huxleyi* are more ^{14}C-depleted than DIC with $\Delta^{14}C$ values of –451.3 ± 2.1‰ and −463.9 ± 2.0‰, respectively, and $\delta^{13}C$ values of –35.0 ± 0.1‰ and –27.6 ± 0.1‰, respectively.

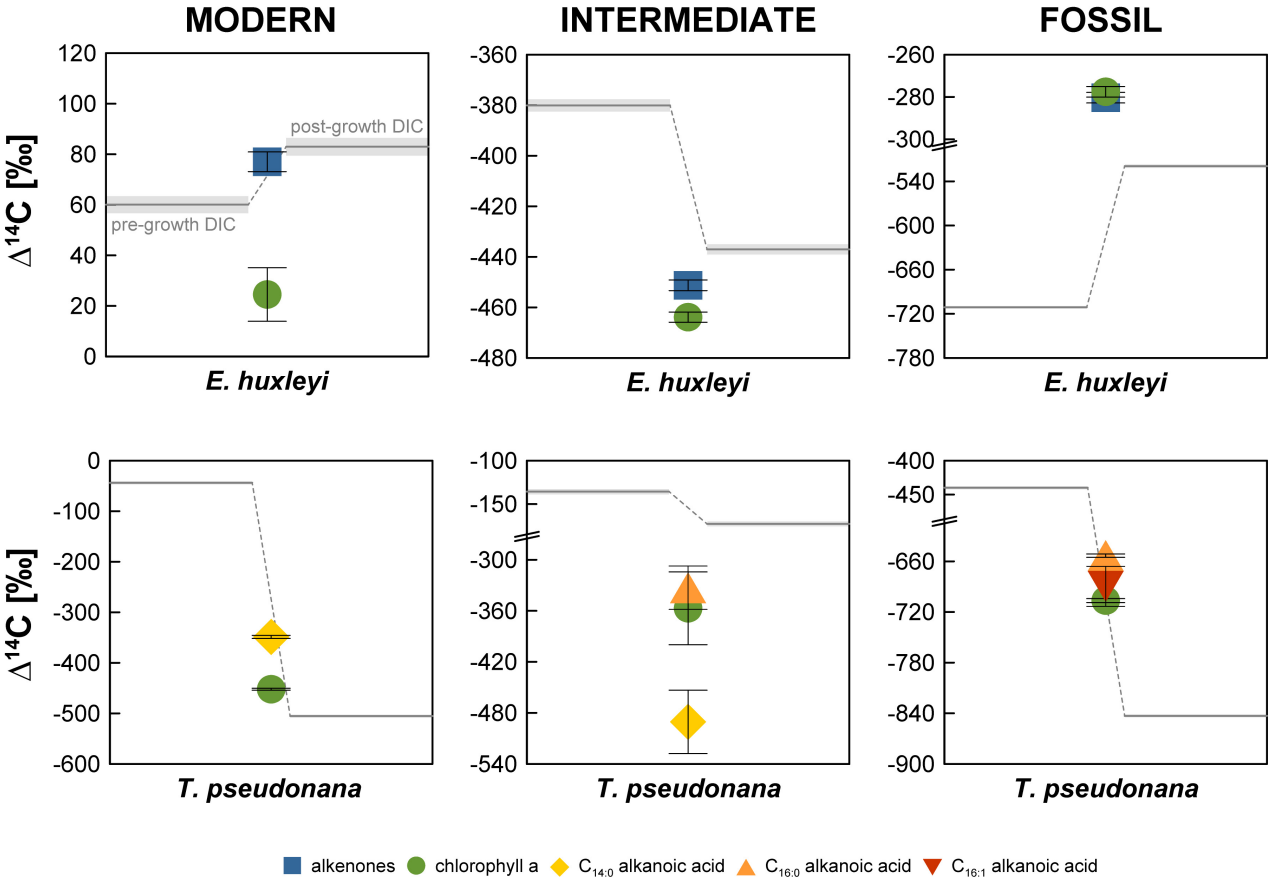

FIGURE 2 | Radiocarbon isotopic composition (Δ^{14}C in ‰) of the *Emiliania huxleyi* and *Thalassiosira pseudonana* cultures. Errors bars (shaded area for DIC) represent 1σ analytical uncertainty.

In the "Intermediate" *T. pseudonana* culture both the pre-growth DIC (–135.7 ± 3.0‰) and the post-growth DIC (–172.8 ± 2.6‰) Δ^{14}C values are considerably ^{14}C-enriched in comparison to the anticipated Δ^{14}C value (ca. –480‰) that should have resulted from a 1:1 CO_2 mixture of ambient air and Air Liquide N45. Pre- and post-growth seawater δ^{13}C values are –8.8 ± 0.1‰ and –2.4 ± 0.1‰, respectively. The biomarker Δ^{14}C values were measured on ultra-small samples sizes (<1 μmol) and are, thus, associated with large errors. While $C_{14:0}$ alkanoic acid (–490.5 ± 37.2‰) and $C_{16:0}$ alkanoic acid (–332.9 ± 25.5‰) deviate significantly, $C_{14:0}$ alkanoic acid and chlorophyll *a* (–357.0 ± 42.8‰) agree within 2σ analytical uncertainty and $C_{16:0}$ alkanoic acid and chlorophyll *a* agree within 1σ analytical uncertainty. Biomarker δ^{13}C values could not be measured independently on splits of the sample CO_2 due to size restrictions.

"Fossil" Cultures

In the "Fossil" *E. huxleyi* culture, pre- and post-growth seawater DIC Δ^{14}C values (–711.1 ± 1.6‰ and –519.4 ± 2.1‰, respectively) differ significantly with a ~200‰ enrichment in ^{14}C during culture growth (**Figure 2** and **Supplementary Table S1**). A concurrent strong isotopic enrichment is also evident for the seawater DIC δ^{13}C. The pre-growth value was –22.1 ± 0.1‰ whereas the post-growth value increased to –13.1 ± 0.1‰. Both alkenones (–280.3 ± 2.5‰) and chlorophyll *a* (–277.6 ± 2.5‰) are even more enriched in ^{14}C than the post-growth DIC but agree within 1σ analytical uncertainty with each other. Alkenones have a δ^{13}C value of –30.2 ± 0.1‰. Chlorophyll *a* has a δ^{13}C value of –22.5 ± 0.1‰.

The pre-growth seawater DIC Δ^{14}C value (–439.8 ± 2.2‰) in the "Fossil" *T. pseudonana* culture is significantly ^{14}C-enriched in comparison to the post-growth Δ^{14}C value (−843.1 ± 1.3‰). The δ^{13}C values of the pre- and post-growth DIC show the exceptional pattern of a ^{13}C-depletion during the culture growth. While the DIC δ^{13}C value equaled –16.5 ± 0.1‰ before growth of *T. pseudonana*, it decreased to –23.6 ± 0.1‰ after culture growth representing the most depleted DIC δ^{13}C value of the entire data set. All biomarker Δ^{14}C values are bracketed by the DIC Δ^{14}C values. The $C_{16:0}$ alkanoic acid (−653.3 ± 2.0‰) and the $C_{16:1}$ alkanoic acid (–689.6 ± 23.7‰) agree within 2σ analytical uncertainty. Chlorophyll *a* is more ^{14}C-depleted (–706.5 ± 2.3‰) than both concurrent alkanoic acids but agrees within 1σ analytical uncertainty with the $C_{16:1}$ alkanoic acid. The δ^{13}C value of the $C_{16:0}$ alkanoic acid (–42.6 ± 1.0‰) is the most ^{13}C-depleted value of the

entire data set. Likewise, chlorophyll *a* is substantially more ^{13}C-depleted (–34.7 ± 1.0‰) than chlorophyll *a* in any of the other cultures.

DISCUSSION

All *E. huxleyi* and *T. pseudonana* main cultures reached stationary phase after 9–13 days. During this time interval, DIC utilization by algae ranged from ∼0.8–1.2 mmol/kg in the *E. huxleyi* cultures (in good agreement with final biomass; r^2 = 0.99) and ∼0.4–0.8 mmol/kg in the *T. pseudonana* cultures (**Supplementary Table S2**). While pre-growth DIC concentrations of 1.8–2.2 mmol/kg were in the range typically observed in the global ocean (Feely et al., 2001), it is obvious that the pre-growth DIC Δ^{14}C values differ from the theoretical values of the CO_2 used to manipulate the original seawater DIC, i.e., 40‰ (ambient air in AD2010/AD2011; Levin et al., 2013), –480‰, and –1000‰ for the "Modern," "Intermediate," and "Fossil" cultures, respectively. Only the pre-growth DIC Δ^{14}C value in the "Modern" *E. huxleyi* culture (60.0 ± 3.4‰) is close to the anticipated Δ^{14}C value. With offsets of approximately –85‰ and –100‰, the pre-growth DIC Δ^{14}C values in the "Modern" *T. pseudonana* culture and the "Intermediate" *E. huxleyi* cultures (**Figure 2** and **Supplementary Table S1**), respectively, are moderately close to the anticipated DIC Δ^{14}C values. In contrast, the pre-growth DIC Δ^{14}C values in the "Intermediate" *T. pseudonana* culture and both "Fossil" cultures are significantly more Δ^{14}C-enriched (∼290–560‰). This ^{14}C-enrichment is significantly higher than the ^{14}C-enrichment observed by Mollenhauer et al. (2005) in their "dead air" *Isochrysis* sp. culture (∼120–170‰). These authors attributed the ^{14}C-enrichment to exchange with the ambient air during the experiment (equaling ca. 10% of the total DIC species). Assuming diffusion of ambient air (Δ^{14}C = 40‰) was the cause in our experiments as well, it would account for 66.2, 27.8, and 53.9% of the DIC species in the "Intermediate" *T. pseudonana* culture, the "Fossil" *E. huxleyi* culture, and the 'Fossil" *T. pseudonana* culture, respectively. However, our post-growth DIC Δ^{14}C values challenge this explanation for our experiments. Relative to pre-growth DIC, the post-growth DIC Δ^{14}C values are only ^{14}C-enriched in the "Modern" and "Fossil" *E. huxleyi* cultures and are in fact more ^{14}C-depleted in the "Intermediate" *E. huxleyi* and all *T. pseudonana* cultures (**Figure 2**), which cannot be explained by diffusion of atmospheric ^{14}C. This observation requires the introduction of a ^{14}C-depleted carbon source to the DIC pool, which, however, is not observed in the post-growth DIC pool of the "Modern" and "Fossil" *E. huxleyi* cultures. The ^{14}C-depletion cannot be explained by kinetic or equilibrium isotope fractionation either. Equilibrium fractionation of carbon isotopes occurs during exchange of gaseous CO_2 and the different seawater DIC species, but equilibrium effects should result in Δ^{14}C-enrichment of bicarbonate and carbonate (the primary DIC species at pH ∼8) relative to $CO_{2(aq)}$/carbonic acid analogous to δ^{13}C values (Mook, 1986; Zhang et al., 1995). For example, an equilibrium effect of ∼8–9‰ in δ^{13}C for total DIC at pH ∼8.2 and 15°C (Mook, 1986; Zhang et al., 1995) would result in an approximately 16–18‰ enrichment in Δ^{14}C. Likewise, algal DIC utilization causes ^{13}C and ^{14}C enrichment of the substrate, as evident for the δ^{13}C values in our data set, which show enrichment ranging from 2.4 to 9.0‰ (**Figure 3** and **Supplementary Table S1**). Accordingly, kinetic effects would account for roughly 5 to 18‰ Δ^{14}C-enrichment irrespective of which DIC species was utilized. However, Δ^{14}C values are corrected for such fractionation effects by normalizing to a δ^{13}C value of –25‰ (Stuiver and Polach, 1977). Accordingly, the ^{14}C-depletion in the "Intermediate" *E. huxleyi* and all *T. pseudonana* cultures have to be explained by other factors.

The mismatch between the anticipated DIC Δ^{14}C values and the measured Δ^{14}C values may indicate problems during initial DIC manipulation. In comparison to Mollenhauer et al. (2005), we did not acidify the seawater to expel DIC, but rather expelled DIC by sparging with CO_2-free air directly. This is a common approach in phytoplankton physiology to achieve DIC-free media, e.g., for testing the DIC-dependence of photosynthesis (Badger et al., 1994; Rost et al., 2003). Even though acidification accelerates the procedure, our setup has been routinely used to achieve DIC-free medium without acidification (typically within 24 h), which has been confirmed by direct measurements using Membrane-Inlet MS (Rost et al., 2007). In general, the measured DIC δ^{13}C and Δ^{14}C values attest to isotopic exchange of the DIC species since they would otherwise mirror natural North Sea surface seawater values, i.e., δ^{13}C values between 0‰ and 1‰ (Burt et al., 2016) and Δ^{14}C values close to atmospheric values in AD2010. We did not obtain Δ^{14}C values for untreated North Sea seawater DIC and (to our knowledge) no direct observational data are available for the most recent decades, but post-bomb surface water Δ^{14}C values until AD1989 inferred from *A. islandica* indicate that Δ^{14}C values should be slightly more enriched than atmospheric Δ^{14}C values (Scourse et al., 2012). Mass balance using pre-growth DIC Δ^{14}C values for the "Fossil" *E. huxleyi* and *T. pseudonana* cultures reveals that 72 and 46% of the total DIC was exchanged, respectively (endmembers –1000‰ and 40‰). We cannot exclude problems during DIC manipulation such as potential contamination with other CO_2 sources in the custom-made gas flow control unit or deviations from 1:1 v:v gas mixtures, which may explain the deviation of the pre-growth DIC Δ^{14}C values from the anticipated values. However, such problems cannot explain the observed isotopic shift to more depleted post-growth DIC Δ^{14}C values in the "Intermediate" *E. huxleyi* and all *T. pseudonana* cultures (**Figure 2**). In the absence of other processes explaining this ^{14}C-depletion, we conclude that contribution of blank carbon is the most likely explanation for the observed isotopic shift between pre- and post-growth DIC Δ^{14}C values.

Final DIC-derived C amounts ranged from 66.7 to 140.6 μmol, i.e., 0.8–1.7 mg C (**Table 1**). Accordingly, we consider combustion and graphitization blanks, combined typically in the range of ∼1 μg C (e.g., Pearson et al., 1998; Shah and Pearson, 2007; Santos et al., 2010), negligible. Hence, it is more likely that the shift in both pre- and post-growth DIC Δ^{14}C values has occurred due to the contribution of some unknown blank source during the experiment or during sample storage until ^{14}C analysis. A significant sample storage effect has been

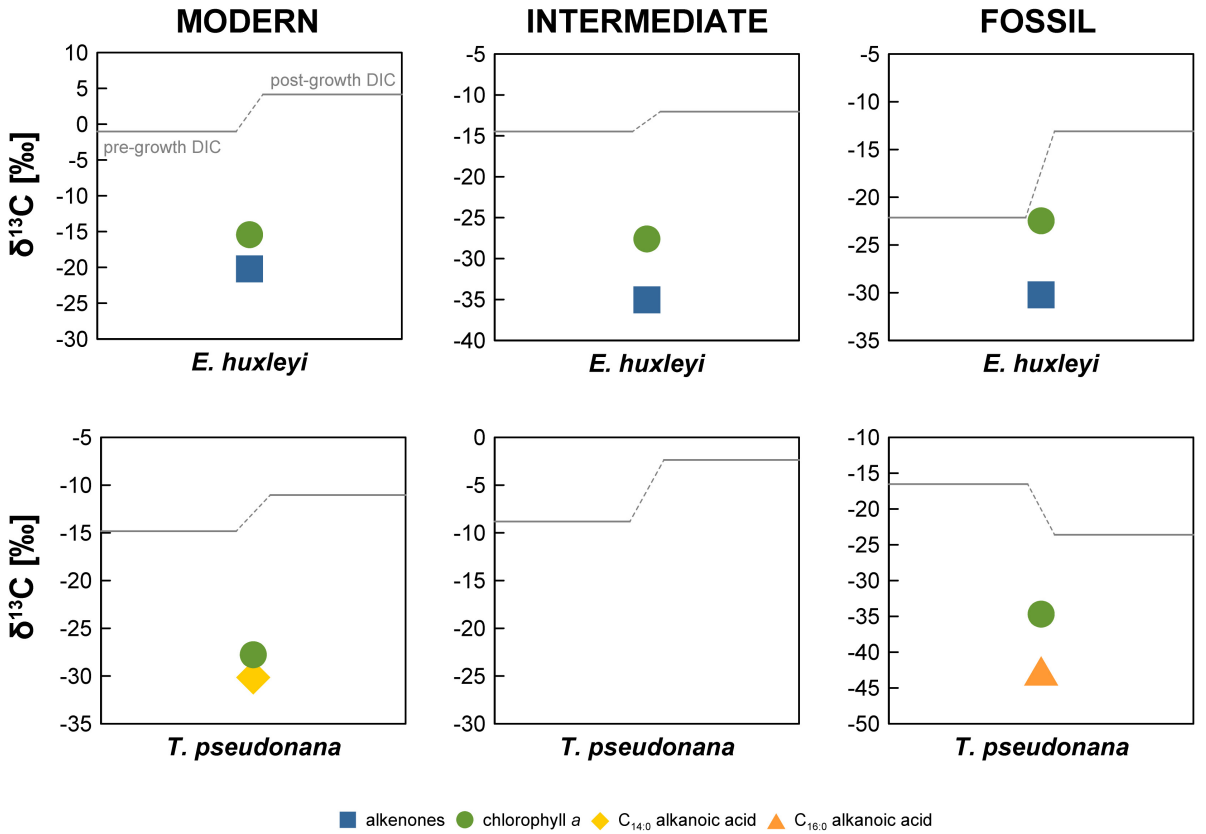

FIGURE 3 | Stable carbon isotopic composition (δ^{13}C in ‰) of the *E. huxleyi* and *T. pseudonana* cultures. Errors bars are ±0.1‰, not shown due to scale.

TABLE 1 | Sample amounts of DIC and purified compounds.

	Modern		Intermediate		Fossil	
Sample	**Graphite (μmol C)**	**GC (μg compound)**	**Graphite (μmol C)**	**GC (μg compound)**	**Graphite (μmol C)**	**GC (μg compound)**
E. huxleyi						
DIC pre-growth	186.2	n.d.	179.3	n.d.	164.0	n.d.
DIC post-growth	66.7	n.d.	115.6	n.d.	73.2	n.d.
chlorophyll *a*	3.9	n.d.	6.7	n.d.	6.1	n.d.
alkenones	75.2	410.0	57.0	147.9	49.2	134.9
T. pseudonana						
DIC pre-growth	209.2	n.d.	217.2	n.d.	232.3	n.d.
DIC post-growth	136.0	n.d.	140.6	n.d.	183.4	n.d.
chlorophyll *a*	6.4	n.d.	n.g.	n.d.	20.0	n.d.
$C_{14:0}$ alkanoic acid	16.5	283.7	n.g.	4.2	–	–
$C_{16:0}$ alkanoic acid	–	–	n.g.	6.4	3.0	23.0
$C_{16:1}$ alkanoic acid	–	–	–	–	n.g.	18.0

n.d., not determine; n.g., not graphitized. Sample analyzed as CO_2.

observed for DIC δ^{13}C values due to gas exchange through the septum even in the absence of an atmospheric headspace (Olack et al., 2018). As discussed above, an atmospheric blank C source cannot explain ^{14}C-depletion of DIC during the "Intermediate" *E. huxleyi* and all *T. pseudonana* experiments or subsequent sample storage, but likely has to derive from material produced from petroleum sources. However, the only such material used during the experiments were the septa, which are made of chemically highly resistant PTFE. It is also puzzling that the observed shifts are not unidirectional in each of the *E. huxleyi* and *T. pseudonana* cultures grown under the same manipulated DIC ^{14}C levels, e.g., ^{14}C-enrichment in the "Fossil" cultures and

^{14}C-depletion in the "Modern" cultures if the actual blank C had some intermediate Δ^{14}C value. The non-unidirectional nature of the shift between pre- and post-growth DIC Δ^{14}C makes it virtually impossible to determine a common blank source in the samples. Moreover, although the pre- and post-growth DIC Δ^{14}C value in the "Modern" *E. huxleyi* culture is close to the anticipated value of 40‰, it is obvious that a ^{14}C-enriched blank C source was added during this particular experiment, resulting in pre- and post-growth DIC Δ^{14}C values of 60.0 ± 3.4‰ and 83.0 ± 3.5‰, respectively. In this particular DIC sample, the δ^{13}C values are ^{13}C-enriched (−1.0 ± 0.1‰ and 4.2 ± 0.1‰, **Figure 3**) in comparison to atmospheric δ^{13}C values, which globally were around −8.3‰ in AD2010/AD2011 (Graven et al., 2017), but in agreement with equilibrium fractionation effects (∼9.0‰ for total DIC at pH ∼8.2 and 15°C; Mook, 1986) in the pre-growth DIC sample. With the exception of the "Fossil" *T. pseudonana* culture, δ^{13}C values in the post-growth DIC are enriched compared to pre-growth DIC as can be expected due to kinetic isotope fractionation during DIC assimilation in algal biomass (Degens et al., 1968). Irrespective of a kinetic effect, however, it is evident that the pre-growth DIC δ^{13}C values differ significantly (5.6–13.8‰) between the *E. huxleyi* and *T. pseudonana* cultures of each ^{14}C manipulation experiment further emphasizing significant blank C contribution of unknown origin during the experiments (based on the ^{14}C-depleted DIC signature of the "Modern" *T. pseudonana culture)*. Overall, the large scatter of DIC Δ^{14}C (and δ^{13}C) values and the non-unidirectionality of the pre- and post-growth DIC Δ^{14}C shifts (**Figure 2**) does not allow us to constrain a common blank C source.

The somewhat random pattern of pre- and post-growth DIC Δ^{14}C values also impairs our ability to accurately quantify the blank C added during biomarker purification. If the DIC Δ^{14}C values were properly constrained (or at least the pre-growth DIC Δ^{14}C value), deviations of biomarker Δ^{14}C values from the DIC Δ^{14}C values could be used to calculate the amount and isotopic composition of the blank C using propagated isotope mass balance (e.g., Shah and Pearson, 2007). In the absence of uncompromised DIC Δ^{14}C values, biomarker Δ^{14}C values can only be compared relative to one another, which may allow blank C calculations. The comparison shows that LC-purified chlorophyll *a* is significantly more ^{14}C-depleted in the "Modern" and "Intermediate" *E. huxleyi* and the "Modern" *T. pseudonana* cultures compared to wet-chemically purified alkenones and the GC-purified $C_{14:0}$ alkanoic acid (**Figure 2**). In contrast, chlorophyll *a* and alkenones as well as chlorophyll *a* and LMW alkanoic acids agree within 2σ analytical uncertainty in the "Fossil" *E. huxleyi* culture and the "Intermediate" and "Fossil" *T. pseudonana* cultures. So far, the wet-chemical alkenone isolation procedure and preparative GC methods have primarily been shown not to add significant blank C to samples, i.e., Δ^{14}C values of pure and processed standards agree within 2σ analytical uncertainty (Mollenhauer et al., 2005; Zencak et al., 2007; Mollenhauer and Rethemeyer, 2009; Wakeham and McNichol, 2014). Theoretically, alkenone and LMW *n*-alkanoic acid Δ^{14}C values should, thus, mirror DIC Δ^{14}C values. In comparison, LC isolation methods have been shown to add blank C with an Δ^{14}C isotopic composition of approximately −400‰ to −600‰, the amount of which depends on the effluent volume (Shah and Pearson, 2007; Birkholz et al., 2013). This would be consistent with a ^{14}C-depletion of chlorophyll *a* in comparison to alkenones and LMW alkanoic acids in the "Modern" cultures. However, closer inspection of the actual Δ^{14}C values of compound pairs in our cultures shows that this is not a consistent pattern. For example, the LMW alkanoic acids in the "Modern" and "Intermediate" *T. pseudonana* cultures agree within 2σ analytical uncertainty, but chlorophyll *a* is significantly more ^{14}C-depleted in the "Modern" culture while it agrees with LMW alkanoic

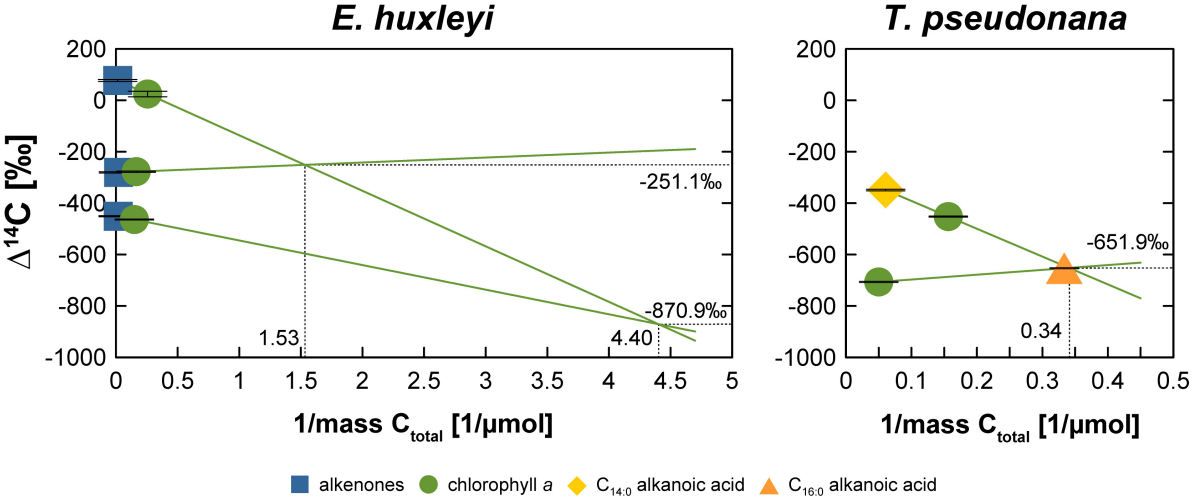

FIGURE 4 | Blank C characterization for the chlorophyll *a* isolation procedure assuming alkenones (*E. huxleyi*) or alkanoic acids (*T. pseudonana*) represent the "true" Δ^{14}C value for the different ^{14}C manipulation experiments of each algal culture. The intercepts of the respective regression lines represent the Δ^{14}C value and mass (1/µmol) of the respective blank C. C_{total} is defined as the C concentration of the sample plus blank.

acids in the "Intermediate" culture (**Figure 2**). Accordingly, if we use the alkenone or alkanoic acid $\Delta^{14}C$ values to calculate the blank for the LC-based chlorophyll *a* isolation method (**Figure 4**), a non-unidirectionality of the blank C contribution to the different chlorophyll *a* samples becomes obvious as well as the resulting large range of possible blank amount and isotopic composition. Assuming alkenones or alkanoic acids were unaffected by extraneous carbon (**Supplementary Figures S1–S11**) and represent the $\Delta^{14}C$ (DIC) value of each culture, extrapolation of the $\Delta^{14}C$ and 1/mass values of the respective compound pairs in each culture reveals that the blank C contribution (based on the intercepts) falls somewhere between 0.23 and 0.65 μmol (2.7 to 7.8 μg) with a $\Delta^{14}C$ value of –870.9‰ to –251.1‰, respectively, for the *E. huxleyi* culture and 2.93 μmol (35.3 μg) with a $\Delta^{14}C$ value of –651.9‰ in the *T. pseudonana* culture. Further, if 1σ analytical uncertainties are included, the chlorophyll *a* isolation blank in the *E. huxleyi* culture for example ranges from 0.15 μmol with a $\Delta^{14}C$ value of –1000.0‰ to 0.89 μmol with a $\Delta^{14}C$ value of –223.4‰. Such large ranges are obviously of little help for blank corrections. Whether this simply results from the relative blank C amount in the respective chlorophyll *a* sample or whether alkanoic acids may in fact also be affected by blank C cannot unequivocally be determined in the absence of reliable DIC $\Delta^{14}C$ constraints. The respective kinetic ^{13}C fractionation factors also do not aid in distinguishing these effects since they are similar for the alkenones (–19.2‰ and –20.5‰) or chlorophyll *a* (–14.4‰ and –13.1‰) in both the "Modern" and "Intermediate" cultures, respectively (**Figure 3**). Furthermore, in case of the "Fossil" *E. huxleyi* culture, the good agreement of chlorophyll *a* and alkenone $\Delta^{14}C$ values and their positive offset to pre-growth DIC $\Delta^{14}C$ values would suggest that both purification methods are affected by similar ^{14}C-enriched blank C contributions. However, the $\delta^{13}C$ value of chlorophyll *a* in this sample basically mirrors the pre-growth DIC $\delta^{13}C$ value, which would imply the absence of any kinetic isotope fractionation during pigment synthesis in this batch. This could result from ^{13}C-enrichment of DIC during batch growth, but DIC uptake was even higher in the "Modern" *E. huxleyi* culture (**Supplementary Table S2**) and chlorophyll *a* is ^{13}C-depleted relative to pre-growth DIC in that culture (and all other cultures). This strongly suggests that the chlorophyll *a* $\Delta^{14}C$ value is affected by some modern blank C. Mass balance calculations to obtain blank C amounts and isotopic compositions based on relative biomarker $\Delta^{14}C$ differences, thus, need to be corroborated by reliable DIC $\Delta^{14}C$ values. Alternatively, bulk biomass $\Delta^{14}C$ values could aid at determining blank C contributions, unfortunately, however, sample size restrictions did not permit bulk analyses in our study.

Our study illustrates the difficulties associated with natural level ^{14}C manipulation of algal cultures in an attempt to obtain authentic "isotopically labeled" biomarker standards, which are not commercially available. Our data show that we entered a vicious cycle of blank C contamination hampering our initial goal to accurately determine the blank C associated with different compound purification methods for CSRA. Considering that culturing is a laborious and time-consuming approach to obtain authentic standards, researchers should be certain that natural level ^{14}C manipulation of DIC has yielded the anticipated $\Delta^{14}C$ values prior to inoculation of algae and that these $\Delta^{14}C$ values can be maintained during the course of the experiment (e.g., via continuous sparging with CO_2). This, however, is only possible if access to AMS facilities and very quick turnaround is assured to avoid blank C addition during sample storage until inoculation. This possibly restricts the ^{14}C-manipulation culturing approach to laboratories housing both culturing and AMS facilities. In any case, potential blank C addition during sample storage should be tested in future studies and we would also recommend obtaining total biomass $\Delta^{14}C$ values if samples sizes permit these analyses.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

SK, GM, and BR designed the study. SK, AB, and K-UR performed the analyses. SK wrote the manuscript with input from all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2019.00780/full#supplementary-material

REFERENCES

Badger, M. R., Palmqvist, K., and Yu, J.-W. (1994). Measurement of CO_2 and HCO_3^- fluxes in cyanobacteria and microalgae during steady-state photosynthesis. *Physiol. Plant.* 90, 529–536. doi: 10.1034/j.1399-3054.1994.900314.x

Birkholz, A., Smittenberg, R. H., Hajdas, I., Wacker, L., and Bernasconi, S. M. (2013). Isolation and compound specific radiocarbon dating of terrigenous branched glycerol dialkyl glycerol tetraethers (brGDGTs). *Organ. Geochem.* 60, 9–19. doi: 10.1016/j.orggeochem.2013.04.008

Burt, W. J., Thomas, H., Hagens, M., Pätsch, J., Clargo, N. M., Salt, L. A., et al. (2016). Carbon sources in the North Sea evaluated by means of radium and stable carbon isotope tracers. *Limnol. Oceangr.* 61, 666–683. doi: 10.1002/lno.10243

Degens, E. T., Guillard, R. R. L., Sackett, W. M., and Hellebust, J. A. (1968). Metabolic fractionation of carbon isotopes in marine plankton—I. Temperature and respiration experiments. *Deep Sea Res. Oceanogr. Abstr.* 15, 1–9. doi: 10.1016/0011-7471(68)90024-7

Drenzek, N. J., Hughen, K. A., Montlucon, D. B., Southon, J. R., Santos dos, G. M., Druffel, E. R. M., et al. (2009). A new look at old carbon in active margin sediments. *Geology* 37, 239–242. doi: 10.1130/g25351a.1

Feely, R. A., Sabine, C. L., Takahashi, T., and Wanninkhof, R. (2001). Uptake and storage of carbon dioxide in the ocean: the global CO_2 survey. *Oceanography* 14, 18–32. doi: 10.5670/oceanog.2001.03

Feng, X., Vonk, J., van Dongen, B., Gustafsson, Ö, Semiletov, I. P., Dudarev, O. V., et al. (2013). Differential mobilization of terrestrial carbon pools in Eurasian Arctic river basins. *Proc. Natl. Acad. Sci. U.S.A.* 110, 14168–14173. doi: 10.1073/pnas.1307031110

Graven, H., Allison, C. E., Etheridge, D. M., Hammer, S., Keeling, R. F., Levin, I., et al. (2017). Compiled records of carbon isotopes in atmospheric CO_2 for historical simulations in CMIP6. *Geosci. Model Dev.* 10, 4405–4417. doi: 10.5194/gmd-10-4405-2017

Guillard, R. R. L., and Ryther, J. H. (1962). Studies of marine planktonic diatoms: I. *Cyctotella nana* Hustedt, and *Detonula confervacea* (Cleve) gran. *Can. J. Microbiol.* 8, 229–239. doi: 10.1139/m62-029

Ingalls, A. E., Shah, S. R., Hansman, R. L., Aluwihare, L. I., Santos, G., Druffel, E. R. M., et al. (2006). Quantifying archaeal community autotrophy in the mesopelagic ocean using natural radiocarbon. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6442–6447. doi: 10.1073/pnas.0510157103

Kusch, S., Kashiyama, Y., Ogawa, N. O., Altabet, M., Butzin, M., Friedrich, J., et al. (2010a). Implications for chloro-and pheopigment synthesis and preservation from combined compound-specific $\delta^{13}C$, $\delta^{15}N$, and $\Delta^{14}C$ analysis. *Biogeosciences* 7, 4105–4118. doi: 10.5194/bg-7-4105-2010

Kusch, S., Rethemeyer, J., Schefuß, E., and Mollenhauer, G. (2010b). Controls on the age of vascular plant biomarkers in Black Sea sediments. *Geochim. Cosmochim. Acta* 74, 7031–7047. doi: 10.1016/j.gca.2010.09.005

Levin, I., Kromer, B., and Hammer, S. (2013). Atmospheric $\Delta^{14}CO_2$ trend in Western European background air from 2000 to 2012. *Tellus B* 65, 57–57.

McNichol, A. P., Osborne, E. A., Gagnon, A. R., Fry, B., and Jones, G. A. (1994). TIC, TOC, DIC, DOC, PIC, POC—unique aspects in the preparation of oceanographic samples for ^{14}C-AMS. *Nuclear Instr. Methods Phys. Res. B* 92, 162–165. doi: 10.1016/0168-583x(94)95998-6

Mollenhauer, G., and Eglinton, T. I. (2007). Diagenetic and sedimentological controls on the composition of organic matter preserved in California Borderland Basin sediments. *Limnol. Oceangr.* 52, 558–576. doi: 10.4319/lo.2007.52.2.0558

Mollenhauer, G., Inthorn, M., Vogt, T., Zabel, M., Sinninghe Damsté, J. S., and Eglinton, T. I. (2007). Aging of marine organic matter during cross-shelf lateral transport in the Benguela upwelling system revealed by compound-specific radiocarbon dating. *Geochem. Geophys. Geosyst.* 8:Q09004. doi: 10.1029/2007GC001603

Mollenhauer, G., Montlucon, D. B., and Eglinton, T. I. (2005). Radiocarbon dating of alkenones from marine sediments: II. Assessment of carbon process blanks. *Radiocarbon* 47, 413–424. doi: 10.1017/s0033822200035190

Mollenhauer, G., and Rethemeyer, J. (2009). Compound-specific radiocarbon analysis – Analytical challenges and applications. *IOP Confer. Ser. Earth Environ. Sci.* 5, 1–9.

Mook, W. G. (1986). ^{13}C in atmospheric CO_2. *Netherlands J. Sea Res.* 20, 211–223. doi: 10.1016/0077-7579(86)90043-8

Nielsen, M. V. (1997). Growth, dark respiration and photosynthetic parameters of the coccolithophorid *Emiliania huxleyi* (prymnesiophyceae) acclimated to different day length-irradiance combinations. *J. Phycol.* 33, 818–822. doi: 10.1111/j.0022-3646.1997.00818.x

Ohkouchi, N., Xu, L., Reddy, C. M., Montlucon, D., and Eglinton, T. I. (2005). Radiocarbon dating of alkenones from marine sediments: I. Isolation protocol. *Radiocarbon* 47, 401–412. doi: 10.1017/s0033822200035189

Olack, G. A., Colman, A. S., Pfister, C. A., and Wootton, J. T. (2018). Seawater DIC analysis: the effects of blanks and long-term storage on measurements of concentration and stable isotope composition. *Limnol. Oceanogr. Methods* 16, 160–179. doi: 10.1002/lom3.10235

Pearson, A., McNichol, A. P., Schneider, R. J., von, R. F., and Zheng, Y. (1998). Microscale AMS ^{14}C measurement at NOSAMS. *Radiocarbon* 40, 61–75. doi: 10.1017/s0033822200017902

Pearson, A., Seewald, J. S., and Eglinton, T. I. (2005). Bacterial incorporation of relict carbon in the hydrothermal environment of Guaymas Basin. *Geochim. Cosmochim. Acta* 69, 5477–5486. doi: 10.1016/j.gca.2005.07.007

Rost, B., Kranz, S. A., Richter, K.-U., and Tortell, P. D. (2007). Isotope disequilibrium and mass spectrometric studies of inorganic carbon acquisition by phytoplankton. *Limnol. Oceanogr. Meth.* 5, 328–337. doi: 10.4319/lom.2007.5.328

Rost, B., Riebesell, U., Burkhardt, S., and Sültemeyer, D. (2003). Carbon acquisition of bloom-forming marine phytoplankton. *Limnol. Oceanogr.* 48, 55–67. doi: 10.4319/lo.2003.48.1.0055

Ruff, M., Wacker, L., Gäggeler, H. W., Suter, M., Synal, H.-A., and Szidat, S. (2007). A gas ion source for radiocarbon measurements at 200kV. *Radiocarbon* 49, 307–314. doi: 10.1017/s0033822200042235

Santos, G. M., Southon, J. R., Drenzek, N. J., Ziolkowski, L. A., Druffel, E., Xu, X., et al. (2010). Blank assessment for ultra-small radiocarbon samples: chemical extraction and separation versus AMS. *Radiocarbon* 52, 1322–1335. doi: 10.1017/s0033822200046415

Scourse, J. D., Wanamaker, A. D., Weidman, C., Heinemeier, J., Reimer, P. J., Butler, P. G., et al. (2012). The marine radiocarbon bomb pulse across the temperate North Atlantic: a compilation of Δ^{14}C time histories from *Arctica islandica* growth increments. *Radiocarbon* 54, 165–186. doi: 10.2458/azu_js_rc.v54i2.16026

Shah, S. R., and Pearson, A. (2007). Ultra-microscale (5–25 μg C) analysis of individual lipids by ^{14}C AMS: assessment and correction for sample processing blanks. *Radiocarbon* 49, 69–82. doi: 10.1017/s0033822200041904

Stuiver, M., and Polach, H. A. (1977). Discussion: reporting of ^{14}C Data. *Radiocarbon* 19, 355–363. doi: 10.1017/s0033822200003672

Wakeham, S. G., and McNichol, A. P. (2014). Transfer of organic carbon through marine water columns to sediments - insights from stable and radiocarbon isotopes of lipid biomarkers. *Biogeosciences* 11, 6895–6914. doi: 10.5194/bg-11-6895-2014

Zencak, Z., Reddy, C. M., Teuten, E. L., Xu, L., McNichol, A. P., and Gustafsson, Ö (2007). Evaluation of gas chromatographic isotope fractionation and process contamination by carbon in compound-specific radiocarbon analysis. *Anal. Chem.* 79, 2042–2049. doi: 10.1021/ac061821a

Zhang, J., Quay, P. D., and Wilbur, D. O. (1995). Carbon-isotope fractionation during gas-water exchange and dissolution of CO_2. *Geochim. Cosmochim. Acta* 59, 107–114. doi: 10.1016/0016-7037(95)91550-d

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