Evidence for bias in C/N, $\delta^{13}$C and $\delta^{15}$N values of bulk organic matter, and on environmental interpretation, from a lake sedimentary sequence by pre-analysis acid treatment methods.


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Abstract

There is a known bias in C/N, $\delta^{13}$C and $\delta^{15}$N values of organic matter (OM) due to pre-analysis acid treatment methods. We report here, for the first time, the results of a pre-analysis acid treatment method comparison of measured C/N, $\delta^{13}$C and $\delta^{15}$N values in bulk OM from a sedimentary sequence of samples to illustrate this bias. Here we show that acid treatment significantly reduces the accuracy (between method biases) and precision (within method bias) of C/N, $\delta^{13}$C and $\delta^{15}$N values of OM, suggesting a differential response of sample OM between methods and sample horizons, and in some cases inefficient removal of inorganic carbon. We show that different methods can significantly influence environmental interpretation in some of our sample horizons (i.e. interpretation of aquatic vs. terrestrial OM source; C$_3$ vs. C$_4$ vegetation). Specifically, there are unpredictable and non-linear differences between methods for C/N values in the range of ~ 1 – 100; $\delta^{13}$C values in the range of 0.2 – 6.8 ‰ and; $\delta^{15}$N values in the range of 0.3 – 0.7 ‰. Importantly, these ranges are mostly much greater than the instrument precision (defined as the standard deviation of replicate analysis of standard reference materials; for this study, ± 0.5 for C/N values, ± 0.1 ‰ for $\delta^{13}$C values and; ± 0.1 ‰ for $\delta^{15}$N). The accuracy and precision of measured C/N, $\delta^{13}$C and $\delta^{15}$N values of bulk OM is not just dependent upon environmental variability, but on acid pre-treatment, residual inorganic carbon and organic matter state and composition. Collectively, this makes the correlation between samples prepared in different ways, including those from down core reconstructions, highly questionable.

Keywords: C/N ratios, $\delta^{13}$C, $\delta^{15}$N, organic matter; pre-analysis acid treatment methods, environmental interpretation, palaeoclimate.
1.1 Introduction

Bulk organic matter (OM) in lacustrine sediments is a heterogeneous composition of organic materials derived from aquatic (e.g. phytoplankton; macrophytes) and terrestrial origins (e.g. trees; shrubs; grasses; animals; see reviews in Meyers and Ishiwatari, 1993; Meyers, 1997; Sharpe, 2007). A number of factors contribute to the structure and isotopic composition of OM in lake sediment: the contribution of C and N from different source end-members; the state and availability of C and N in the environment; carbon fixation pathways; lake productivity; pre- and post-burial diagenetic processes (aerobic and anaerobic); dissolved CO$_2$ concentration, $p$CO$_2$, light, temperature, changes in palaeoenvironmental controls on OM C and N budget, and species composition (e.g. Stuiver, 1975; Meybeck, 1982; Hedges et al., 1986; Ehleringer and Monson, 1993; Hayes, 1993; Meyers and Ishiwatari, 1993; Meyers, 1994, 1997; Ehleringer et al., 1997; Krishnamurthy et al., 1999; Turney, 1999; Lehmann et al., 2003; Lucke et al., 2003; Perdue and Kopribnjak, 2007). Prima facie, these factors make the evaluation of the palaeoenvironmental and palaeoclimatic influence on sedimentary OM difficult. However, the investigation of lake sediment sequences with highly resolved age-depth models and high signal-to-noise ratios can still provide high amplitude palaeoenvironmental information (e.g. Lucke et al., 2003; Wei et al., 2010).

Despite the complexity of these processes on sediment OM, weight ratios of elemental carbon to nitrogen (C/N), and stable isotope ratios of C and N ($\delta^{13}$C and $\delta^{15}$N) from bulk OM, have been widely used to interpret OM provenance (e.g. aquatic versus terrestrial source) and vegetation type (e.g. C$_3$ versus C$_4$ plants; Meyers and Ishiwatari, 1993; Thornton and McManus, 1994; Meyers, 1997; Sampei and Matsumoto, 2001; Lamb et al., 2004, 2007; Street-Perrott et al., 2004; Wilson et al., 2005; Zong et al., 2006; Mackie et al., 2007). These proxies have subsequently underpinned palaeoenvironmental research and been used as a tool for understanding biogeochemical processes in a range of sedimentary sequences (Talbot and Johannessen, 1992; Street-Perrot et al., 1997; Holmes et al., 1997; Turney, 1999; Huang et al., 2001; Fuhrmann et al., 2003; Lucke et al., 2003; Baker et al., 2005; Lamb et al., 2007; Galy et al., 2008; Mampuku et al., 2008; Domingo et al., 2009; Langdon et al., 2010; Scholz et al., 2010; Yu et al., 2010; Wei et al., 2010).

In general, C/N ratios of OM tend to range from 3 – 9 (dominated by aquatic biomass; protein rich, lignin poor), 10 – 20 (admix of aquatics, including emergent aquatics, and terrestrial sources) and > 20 (dominated by terrestrial biomass; protein poor; lignin rich) (e.g. Meybeck,
The $\delta^{13}C$ of OM is broadly used as an indicator for carbon sources, productivity and photosynthetic pathways in plants. Values for land plants range from $\approx -6$ to $-35 \%$ (see overviews in Tyson, 1995; Meyers, 1997; Sharpe, 2007), and can differentiate between C$_3$ plants ($\delta^{13}C \approx -22$ to $-35 \%)$ and C$_4$ plants ($\delta^{13}C \approx -6$ to $-15 \%)$ in certain environments (e.g. estuaries, sea floors, lakes, soils; e.g. Smith and Epstein, 1971; O’Leary, 1988; Tyson, 1995; Meyers, 2003; Street-Perrot et al., 2004; Sharpe, 2007; Mampuku et al., 2008; Scholz et al., 2010). The $\delta^{13}C$ of OM has also been used for a range of other investigations including (1) assessment of carbon reservoir turnover times and soil C dynamics (Harris et al, 2001), (2) determination of trophic levels in environmental systems (Bunn et al., 1995; Pinnegar and Polunin, 1999; Kolasinski et al., 2008), (3) primary productivity reconstructions and estimation of carbon burial rates and, (4) to understand mineralisation processes (Midwood and Boutton, 1998; Freudenthal et al., 2001; Leng and Marshall, 2004). $\delta^{15}N$ has been used to understand trophic pathways in food webs (Bunn et al., 1995; Pinnegar and Polunin, 1999; Ng et al., 2007; Kolasinski et al., 2008); animal dietary tracers (e.g. Koch et al., 2007; Lee-Thorpe, 2008); OM provenance and degradation (Thornton and McManus, 1994; Meyers, 1997; Hu et al., 2006; Barros et al., 2010); denitrification in the water column (Altabet et al., 1995; Ganeshram et al., 2000); nitrate utilisation (Calvert et al., 1992; Teranes and Bernasconi, 2000); N$_2$-fixation (Haug et al., 1998) and; eutrophication (Owens, 1987; Vob et al., 2005). In addition, C/N values are used to support $\delta^{13}C$ and $\delta^{15}N$, for example through bi-plots providing a structure within which OM provenance and type can be broadly identified (e.g. Talbot and Johannessen, 1992; Thornton and McManus, 1994; Meyers, 1997; Meyers and Teranes, 2001; Krull et al., 2002; Lucke et al., 2003; Wilson et al., 2005; Lamb et al., 2006; Zong et al., 2006; Mackie et al., 2007; Sharpe, 2007; Yu et al., 2010). Investigators have subsequently deduced changes in environmental and/or climatic processes through interpretation of changing states of the system under investigation (e.g. terrestrial vs. aquatic biomass; C$_3$ vs. C$_4$ vegetation).

However, interpretations of C/N, $\delta^{13}C$ and $\delta^{15}N$ are predicated on the production of reliable proxy data, and the ability to disentangle the complex processes leading to OM preservation in the sedimentary record. This necessitates a complete understanding of the precision on the measured data, which, for C/N, $\delta^{13}C$ and $\delta^{15}N$ values from bulk sediment OM, are not widely discussed in the literature beyond instrument precision (reported as one standard deviation (1$\sigma$) of replicate runs of elemental and isotopic reference materials). Further, the instrument precision on C/N values is rarely (if at all) discussed.
The accurate determination of C/N and $\delta^{13}$C composition of OM requires the complete removal of any IC from the total carbon, commonly achieved through acid treatment. There is a variety of pre-analysis acid treatment methods that have been used in the published literature, from which it is clear there is no consensus on standard practice (see Brodie et al., 2011a for an overview). Research has shown significant non-linear bias on measured C/N, $\delta^{13}$C and $\delta^{15}$N values directly associated with these pre-analysis acid treatment methods, which can undermine an environmental interpretation of the data (Froelich, 1980; Yamamuro and Kayanne, 1995; Bunn et al., 1995; King et al., 1998; Lohse et al., 2000; Schubert and Nielsen, 2000; Ryba and Burgess et al., 2002; Kennedy et al., 2005; Schmidt and Gleixner, 2005; Galy et al., 2007; Fernandes and Krull, 2008; Brodie et al., 2011a). For example, Brodie et al. (2011a) noted a C/N value range of ~ 6 – 13, a $\delta^{13}$C range of −27.0 ‰ to −28.4 ‰ and a $\delta^{15}$N range of 0.8 ‰ to 1.8 ‰ for a terrestrial land plant (Broccoli) across pre-analysis acid treatment methods. These C/N values suggest OM derived largely from aquatic sources, or from an admixture of aquatic and terrestrial sources. More importantly, all of these offsets were shown to be non-linear and unpredictable within and between pre-analysis acid treatment methods (Brodie et al., 2011a; 2011b).

Differences in %C measured from modern and ancient, terrestrial and aquatic organic materials, as a function of acid treatment, have been reported at 5 – 78 % and for %N at 0 – 50%, either as a loss of C and N (Froelich, 1980; Yamamuro and Kayanne, 1995; Bunn et al., 1995; King et al., 1998; Lohse et al., 2000; Schubert and Nielsen, 2000; Ryba and Burgess, 2002; Schmidt and Gleixner, 2005) or as an artificial gain in C and N (Brodie et al., 2011a). Shifts in $\delta^{13}$C are variable, ranging from enrichment in $\delta^{13}$C of 0.2 – 8.0 ‰ (e.g. Schubert and Nielsen, 2000; Kolasinski et al., 2008; Brodie et al., 2011a), a depletion in $\delta^{13}$C of 0.1 – 1.9 ‰ (Kennedy et al., 2008; Komada et al., 2008; Brodie et al., 2011a) and no change (e.g. Midwood and Boutton, 1998; Kennedy et al., 2005). This is similar for $\delta^{15}$N, where results range from an enrichment of 0.1 – 3 ‰ (Bunn et al., 1995; Brodie et al., 2011b), to a depletion of 0.2 –1.8 ‰ (Bunn et al., 1995; Harris et al., 2001; Kennedy et al., 2005; Ng et al., 2007; Fernandes and Krull, 2008), and no significant change (Serrano et al., 2008). The bias on OM from acid treatment, alongside the complex processes that can influence OM prior to, and during, sedimentary preservation, suggests that reliance on the commonly reported instrument precision alone is unrealistic for robustly interpreting measured C/N, $\delta^{13}$C and $\delta^{15}$N values.
1.1.1 Unresolved Issues

Despite the considerable potential for acid treatment method to alter the bulk OM signal prior to C/N, δ\textsubscript{13}C and δ\textsubscript{15}N analysis, the potential bias in a sedimentary sequence of samples has hitherto never been investigated. In addition, the influence of inorganic carbon (IC) and inorganic nitrogen (IN; Hoefs, 1973; Sharpe, 2007); sample homogenisation (Baisden et al., 2002; Hilton et al., 2010) and sample size (Brodie et al., 2011a) can contribute additional inaccuracy and imprecision to measured data. There is an increase in the application of “dual-mode” isotope analysis (where C/N, δ\textsubscript{13}C and δ\textsubscript{15}N are measured simultaneously from the same pre-treated sample), implying an acidification of sample material prior to analysis. We note it is not common to acidify samples prior to δ\textsubscript{15}N analysis, but acidification is required for dual δ\textsubscript{13}C and δ\textsubscript{15}N analysis. It is clear, therefore, that the assumption that instrument precision alone accounts for the absolute imprecision on measured C/N, δ\textsubscript{13}C and δ\textsubscript{15}N values is questionable. Moreover, assumptions on the accuracy of the measured C/N, δ\textsubscript{13}C and δ\textsubscript{15}N are also questionable.

The aim of this study was to compare, for the first time, the effect of pre-analysis acid treatment methods on C/N, δ\textsubscript{13}C and δ\textsubscript{15}N of OM from a sedimentary sequence. We investigate an ancient lake cored sequence using the capsule and rinse methods alongside that of untreated materials (after Brodie et al., 2011a; 2011b). We test the null hypothesis that there is a significant difference between methods on the same sample horizon, implying that data precision exceeds the commonly discussed instrument precision. Specifically, the following research questions are addressed:

1. Are there significant differences between the results of pre-analysis acid treatment methods for C/N, δ\textsubscript{13}C and δ\textsubscript{15}N of bulk OM on a stratigraphical sequence of samples? (i.e. above instrument precision)

2. Can different pre-analysis acid treatment methods influence environmental interpretation of C/N, δ\textsubscript{13}C and δ\textsubscript{15}N of bulk OM?

1.2 Materials and Methods
1.2.1 Core material

A sedimentary sequence was extracted from Lake Tianyang (20°31’1.11” N, 110°18’43.02” E), south China, in January 2008, for multi-proxy palaeoenvironmental reconstruction, including C/N, δ\textsubscript{13}C and δ\textsubscript{15}N of OM. For this comparison, we sub-sampled 20 horizons in
the core from 7.00 – 10.24 m (16 cm resolution). This section of the core material was
selected due to the significant change in the lithology (Figure 1) from a brown clayey silt bed,
with few very fine sands and silts, and a low organic content (~0.3 – 1% OC; 10.24 m to 8.06
m), to an organic rich (amorphous) clay bed (~28 – 32% OC; 8.06 m to 7.09 m). In addition,
the $\delta^{13}$C of bulk OM derived from some pilot samples showed that this section produced an
overall $\delta^{13}$C range of ~15 % and, in particular, there is a ~12 % shift across the lithologic
boundary. Unfortunately, low levels of N precluded a full $\delta^{15}$N record across all of our
selected sample horizons so we only report $\delta^{15}$N values from 7.00 m to 7.46 m.

Figure 1: Lake Tianyang core lithology and description from 7.00 m to 10.20 m. The $^{14}$C age
is reported in $^{14}$C yrs BP (uncalibrated).

1.2.2 Cleaning Protocol
Prior to sample treatment, all sub-sampling equipment and glassware were thoroughly
washed in 1% nitric acid, rinsed in deionised water, followed by a wash in 2% soap solution
(neutracon®), a final deionised water rinse and then fired at 550°C for 3 hours. Ag capsules
were fired at 550°C for 3 hours prior to use and Sn capsules were submerged in methanol for
24 hours and then air dried. Cleaned capsules were then sealed in pre-cleaned containers and
stored until use.

1.2.3 Acidification methods
1.2.3.1 Rinse method
We compared the capsule and rinse methods using 5% w/w and 20% w/w HCl as the
acidifying reagents based on Brodie et al (2011a) who showed HCl tended to produce the
most coherent and reliable data. For the rinse method, approximately 250 mg of sample was
acidified in 50 ml of the chosen acid reagent for 24 h. Depending on the IC content additional
acid was added to maintain an acidic solution (checked with litmus paper) and left for a
further 24 h if necessary. After digestion, the sample material was sequentially rinsed 3 times
with deionised water, allowing 24 h between rinses to allow the sample to settle, using an
overall minimum of 1200 mls of deionised water. After the final decanting, the excess water
(50 – 100 mls) was allowed to evaporate off in a drying oven at ~50°C. Once dry, the sample
was loosened from the base of the beaker with a clean plastic spatula and transferred to an
agate pestle and mortar, ground, and a known quantity weighed into a Sn capsule (to provide
~500 μg C after acid treatment). Capsules were then crimped ready for elemental and δ^{13}C analysis.

1.2.3.2 Capsule Method
Sample material was weighed into open Ag capsules and recorded (to provide ~500 μg C after acid treatment). The capsules were then transferred on a metal tray to a cold hotplate and 10 μl of distilled water was added to moisten the samples, reducing the potential of an initial vigorous reaction from IC bearing materials. After moistening, 10 μl of the chosen acid reagent was added to the cold sample before the hotplate temperature was slowly increased to ~50°C. Additional acid was then added in steps of 10 μl, 20 μl, 30 μl, 50 μl and 100 μl without allowing the sample to dry out between additions. The samples were monitored for IC reaction by visual inspection but as the effervescence reduced, the reaction was checked using a binocular microscope at 50x magnification. The stepped addition of acid described here reduced problems associated with the ambiguous effervescence end-point, however, we also added a final 200 μl of acid to act as a “fail safe”. After the addition of the final aliquot of acid the capsules were left on the hotplate for c.1 hour to dry thoroughly. Once dry, the capsules were removed from the hotplate, left to cool before being crimped. All capsule method samples (traditionally analysed in Ag capsules only) were further wrapped in Sn capsules to ensure complete combustion (see Brodie et al., 2011b).

1.2.3.3 Untreated
Sample δ^{15}N is traditionally measured on untreated sample material (e.g. Muller, 1977; Altabet et al., 1995; Schubert and Calvert, 2001; Sampaei and Matsumoto, 2008), assuming a negligible influence from inorganic nitrogen (e.g. nitrates, ammonia; e.g. Sampaei and Matsumoto, 2008). Therefore, in addition to the rinse and capsule methods we also prepared untreated sample materials for C/N, δ^{13}C and δ^{15}N analysis, which involved directly weighing an untreated sample aliquot (500 μg for C/N and δ^{13}C and 15 μg for δ^{15}N) into a prepared Sn capsule, crimping and analysing.

1.2.4 Analytical Methods
The %C, %N and δ^{13}C values of sample OM were analysed using an online system comprising a Costech ECS4010 elemental analyser (EA), a VG TripleTrap, and a VG Optima mass spectrometer at the NERC Isotope Geosciences Laboratory (NIGL), with data reduction
carried out using DataApex Clarity ver 2.6.1 software package. Each analytical run contained three control materials: external standard SOILB (2 replicates), internal NIGL standard BROCC (10 replicates) and independent external standard SOILC (2 replicates). All standards returned values that were statistically indistinguishable from known sample values (p-value > 0.05) indicating the instrument measurements were accurate (in comparison to the long term values) and precise (within reported σ of known values), where C/N is ≤ 0.2 and δ¹³C ≤ 0.2 ‰. From knowledge of the laboratory standard’s δ¹³C value versus V-PDB (derived from regular comparison with international calibration and reference materials NBS-18 and NBS-19 and cross checked with NBS-22). ¹³C/¹²C ratios of the unknown samples were converted to δ values versus V-PDB as follows: δ = [(R_sample/R_standard) – 1] x 10³ ‰, where R = the measured ratio of the sample and standard respectively (for carbon and nitrogen).

Nitrogen isotope analyses were performed using a FlashEA 1112 elemental analyser linked to a Delta+XL isotope ratio mass spectrometer (EA-IRMS) via a Conflo III interface. Samples were combusted at 900°C with all samples acidified in the capsule method further wrapped in Sn capsules. Limits on analytical precision are mainly determined by conditions of combustion and chromatography in the elemental analyser. Within-run precision for δ¹⁵N is ≤ 0.13 ‰ (1σ for n = 13 samples).

Measurements of background C and N concentrations from capsules and acid reagents were below instrument detection limits suggesting contamination did not contribute to variability within our results (e.g. see Brodie et al., 2011a, 2011b for further details on acid methods and analytical methods).

### 1.2.5 Data Analysis

We compare our data using a one-way ANOVA, at the 95% confidence limit, to determine differences within (i.e. acid reagent) and between (i.e. untreated versus capsule method versus rinse method) the pre-analysis acid treatment methods, and take a p-value < 0.05 to indicate a significant difference. All data were tested for normality using an Anderson-Darling normality test, and tested for homogeneity of variances using a Bartlett’s test (which assumes data are normally distributed) and a Levene’s test (which assumes data are non-normally distributed). ANOVA comparisons for C/N and δ¹³C were carried out on data derived from acid treated samples but are not compared with untreated samples (untreated
measurements for C/N and δ^{13}C is not a common approach due to the potential for inorganic carbon contamination, hence the necessity for acid pre-treatment). For δ^{15}N, comparisons were made on data derived from acid treated samples and untreated samples as there is no consensus on the most appropriate method for N analysis (see Brodie et al, 2011b for an overview).

1.3 Results

The %C, %N, C/N and δ^{13}C data are presented in Figure 2 and ANOVA comparisons for C/N and δ^{13}C data from each pre-treatment method and reagent investigated are presented in Table 1.

1.3.1 %C and %N

From 7.00 to 7.52 m, the core material is characterised by high %C and %N values relative to the sample horizons below 7.52 m, where %C and %N are very low. For %C and %N, the rinse method samples above 7.52 m are consistently ~20% higher than capsule and untreated method samples. With the exception of the 5% HCl rinse method samples at 8.76 m for %C (probably a residual inorganic C signal), and 20% HCl capsule method sample from 7.80 – 8.60 m for %N, the data below 7.52 m are relatively coherent.

1.3.2 C/N and δ^{13}C

C/N values between methods are highly variable within specific sample horizons, especially within the capsule method samples (e.g. 7.48 m, 8.76 m, 9.24 m for 5 % HCl capsule method; 8.12 m, 8.60 m for 20 % HCl capsule), and between the capsule method samples and rinse method samples. An overall range of ~1 – 100 was evident between methods on some sample horizons. For example, at 8.76 m the capsule method samples returned C/N values of 81 to 122, the rinse method samples returned C/N values of ~34, and untreated values were ~174. At 9.08 m, samples in the capsule method return C/N values of ~19, and rinse method samples ~11. In general, data from the rinse method appear more coherent than data from the capsule method.

δ^{13}C data from samples between 7.00 m and 7.64 m across all methods tested are indistinguishable from one another (i.e. appear to be within instrument precision limits).

Between 9.00 m and 9.24 m, all measured values (i.e. within and between all acid treatment
methods) converge over a significant shift in the data, but are divergent above 9.00 m and below 9.24 m. However, our data also show sample horizons with incoherency between the methods (i.e. greater than instrument precision by a minimum of ~0.2 ‰), in particular from 7.64 m to 8.12 m and 8.60 m to 9.00 m. The greatest divergence in the data are from 7.64 m to 8.12 m and between the 20% HCl capsule method and 20 % HCl rinse method (~ 2.5 ‰ (7.64 m)), and from 8.60m to 9.00m which is caused by the 5 % HCl capsule method (~6 – 7 ‰ (8.60 m)). Between the remaining three methods at 8.60 m, the difference range is between 0.4 – 3.5 ‰. We note that the divergence in $\delta^{13}$C data, within and between methods, appear to become more evident in samples with relatively lower OC, but not in all instances (e.g. 9.00 m to 9.24 m). Given the evident differences between methods on any one sample horizon, a general trend in C/N and $\delta^{13}$C values between methods remains apparent, though the amplitude of the signal is variable.

**Figure 2:** Down-core plots of %C, %N, C/N and $\delta^{13}$C for data derived from the capsule method, rinse method and untreated samples. Instrument precision is not visible on these scales, but is 0.3% for %C, 0.3% for %N, 0.5 for C/N and 0.2 ‰ for $\delta^{13}$C. The embedded legend indicates the data for each method.

**Table 1:** ANOVA comparison results for C/N and $\delta^{13}$C for acid treated sample horizons only. P-values and r-squ values are based on comparisons of all measurements from a specific sample horizon after acid treatment, with a p-value < 0.05 deemed to represent a statistically significant difference. “nd” indicates no significant difference in measured values within and between acid treatment methods.

### 1.3.3 $\delta^{15}$N

$\delta^{15}$N values were only measureable between 7.09 m and 7.46 m due to extremely low %N. The $\delta^{15}$N data and ANOVA comparisons for each method and reagent are shown in Figure 3 and Table 2 respectively. Our results show that all acid treated samples produced lower $\delta^{15}$N in comparison to untreated samples, with the largest range in values between methods of ~0.8 ‰ (at 7.16 m). In general, capsule method samples produced lower values than rinse method samples, with the exception of 5 % HCl capsule samples at 7.09 m and 7.16 m (Figure 3). Overall, the rinse method samples produced more coherent results than the capsule method (< 0.2 ‰ overall range for all rinse method samples). ANOVA results
indicate statistically significant differences within the capsule method and between the capsule and rinse method data. In addition, all data derived from acid treated samples produced lower $\delta^{15}N$ in comparison to untreated values for all sample horizons, but highlighted no differences within the rinse method (i.e. no difference between samples acidified in 5% HCl or 20% HCl within the rinse method). We note a fractionation in $\delta^{15}N$ in all sample horizons, within and between methods, but no concomitant change in mass %N.

**Figure 3:** Down-core plots of $\delta^{15}N$ for data derived from the capsule method, rinse method and untreated samples. The scale bar in the plot represents instrument precision ($1\sigma = 0.13 \text{‰}$), and the embedded legend indicates the data for each method.

**Table 2:** ANOVA comparison results for $\delta^{15}N$ for all tested sample horizons. P-values and r-squ values are based on comparisons of all measurements from a specific sample horizon after acid treatment, and also in comparison to untreated samples.

### 1.4 Discussion

#### 1.4.1 Method differences

The pre-analysis acid treatment approach is underpinned by the assumption that the OM fraction is either unaltered during the process, or that any changes are at least systematic and proportional (i.e. predictable), and that all IC present is completely removed. This clearly suggests that, within instrument precision, results from any method followed should be indistinguishable from one another. Our results from the Lake Tianyang sedimentary sequence indicate an inconsistency in the application of any single pre-analysis acid treatment method in a down-core context for C/N, $\delta^{13}C$ and $\delta^{15}N$. There is evidence for significant differences in measured C/N, $\delta^{13}C$ and $\delta^{15}N$ values within and between pre-analysis acid treatment methods (Table 1 and 2). Differences between each acid treatment method in C/N, $\delta^{13}C$ and $\delta^{15}N$ values within and between sample horizons are highly variable, and not always in the same direction. For some sample horizons, differences between acid treatment methods for C/N values can be as high as ~90 (e.g. at 8.76 m) and as low as 0.2 (e.g. at 7.32 m; 8.44 m). Likewise, differences in $\delta^{13}C$ between acid treatment methods can be as high as 6.8 ‰ (e.g. 8.76 m) but for other horizons be within instrument precision (e.g. < 0.2 ‰; 7.09 m – 7.48 m). This may, in part, be a function of the overall %C and %N of the sample material, including organic and inorganic components. For example, our $\delta^{13}C$ data are generally in
good agreement with high %C. However, the imprecision on the data tends to increase within
and between methods as %C in the sample material becomes lower (e.g. 7.64 m to 9.00 m),
but this is not always the case (e.g. 9.00 m to 9.24 m). This suggests sample materials with
low %C may be more susceptible to acid method bias (and of greater magnitude), but this is
not a general rule (Brodie et al., 2011a, 2011b).

Where there is a high range of C/N values apparent between treatment methods, this can
fundamentally alter the support for $\delta^{13}C$ and $\delta^{15}N$ from cross-plots of these data. For example,
at 7.80 m, the 5% HCl capsule method points towards an environment dominated by
terrestrially sourced OM (C/N value ~ 32), whereas C/N values from all other methods
suggest an environment with a significant aquatic biomass contribution to total OM (C/N
value ~ 12). This contradictory position clearly indicates a serious discrepancy regarding the
interpretation of elemental and isotopic C and N proxies derived from bulk OM, both from
these cross-plots and in a down-core context. It also suggests that the assumptions
underpinning pre-analysis methods are invalid (Brodie et al., 2011a, 2011b). In addition,
there are known biases from IN contamination, which can lower C/N values below the true
organic C/N value (e.g. Muller, 1977; Schubert and Calvert, 2001; Sampei and Matsumoto,
2001; Meyers, 2003; Mampuku et al., 2008). For example, Muller (1977) reported C/N
values <4 from deep sea sediments as a consequence of inorganic ammonia. Furthermore, the
range of C/N values, as discussed in the context of marine versus terrestrial OM provenance,
is also more complex than the standard interpretation suggests; for example, C/N values
(weight ratio) of submerged aquatic macrophytes have documented ranges of 6 – 60 (e.g.
Atkinson and Smith, 1983) and macroalgae ranges from 16 – 68 (brown macroalgae; Fenchel
and Jørgensen, 1977). Brodie et al (2011a) also report a C/N range of ~ 6 – 13 for broccoli (a
terrestrial plant) which has a “typical” C3 $\delta^{13}C$ value of –27.4 ‰.

In addition, we also find that %C and %N are artificially concentrated (but not proportionally)
in samples from 7.00 m to 7.52 m analysed from the rinse method relative to untreated values
and capsule method samples. Brodie et al (2011a) suggested that this was likely a function of
the loss of fine grained inorganic material (e.g. clays) in the supernatant relative to the
amount of sample material treated with respect to that in other methods, despite the potential
losses of C and N through solubilisation (e.g. Schubert and Nielsen, 2000; Galy et al., 2007)
and absorption onto fine grained particles. We note that there is no concomitant shift in $\delta^{13}C$
values, though C/N values are disproportionally increased. Within the 20% HCl rinse method, and for %N only (from 8.12 m to 8.60 m), the %N values are substantially higher (Figure 3). Given the very low amounts of N within sample material, and the biasing effect of the acid treatment, the results are likely to be unreliable as %N is very close to instrument baseline conditions. Collectively, these factors point to a serious problem in the general theory on OM provenance as interpreted through C/N.

1.4.2 Residual inorganic carbon

In addition to the problems highlighted for C/N values, at 8.76 m, the 5 % HCl capsule method returned a $\delta^{13}$C value of $-12.5 \, \text{‰}$, ~ 6.8 ‰ more enriched than data from all other acidification methods (the overall $\delta^{13}$C range between treatment methods is $-21.3$ to $-12.5 \, \text{‰}$). Our measurements on untreated material from this sample horizon suggests the presence of a major IC component (~1.8% inorganic carbon by weight with $\delta^{13}$C = $-1.6 \, \text{‰}$), suggesting the 5% HCl capsule method is less efficient at IC removal in comparison to other methods for this sample, although it is widely assumed 5% HCl should efficiently remove calcite. Between all other methods at 8.76 m, the difference in $\delta^{13}$C value was ~ 2 – 3 ‰. However, in the context of the overall data trend (e.g. the ~12 ‰ shift from 7.00 m to 7.52 m; see Figure 2), the value returned for the 5 % HCl capsule method would not look out of place had this been the only method followed. This, subsequently, could have led to a misinterpretation of the core data in the context of sample OM: the 5 % HCl capsule data at 8.76 m suggest an environment dominated by C₄ type vegetation (both high C/N and $\delta^{13}$C values) and could be interpreted as being representative of a more arid environment, whereas the 3 other acid treatment methods tested produced more consistent results (though still potentially imprecise), suggesting a C₃ dominated environment, which could be interpreted as being representative of a more humid environment. We therefore do not recommend the use of 5 % HCl in the capsule method. These differences between the acid reagents and methods investigated here have three possible explanations: (i) the different effect of acid treatment on the IC component(s) within the sample material and (ii) non-linear and unpredictable offset on the OC component(s) within the sample material, or (iii) a combination of both. At 8.76 m, the offset in $\delta^{13}$C value in the 5% HCl capsule method is caused by inefficient removal of IC (see above), an offset not recorded in the other methods. This suggests that different methods and reagents (even at 5% HCl) have differential rates of removal of what is probably calcite (i.e. 5% HCl appeared to remove the IC in the rinse
method, likely due to the increased time of exposure of the sample to the acid in this method relative to the capsule method). Therefore, this problem is likely to be exacerbated where less soluble forms of IC exist in sample materials, such as dolomites and siderites, which can produce as large an offset to the $\delta^{13}C$ value as calcite. Moreover, an admixture of different IC components can further complicate the digestion process due to different rates of removal (i.e. stoichiometry of each IC component and combined stoichiometry, relative to dissolution reagent) and IC component grain size (Al-Aasm et al., 1990; Yui and Gong, 2003).

Where there is an IC contamination on $\delta^{13}C$ values, enrichment is usually expected in the $\delta^{13}C$ value due to the assumed relatively high $\delta^{13}C$ values of IC material; however, some freshwater, marine, authigenic, diagenetic and detrital carbonates can have very negative $\delta^{13}C$ values (Hoefs, 1973; Hangari et al., 1980; Mozley and Carothers, 1992; Mozley and Burns, 1993; Chow et al., 2000; Coniglio et al., 2000; El-ghali et al., 2006; Sharpe, 2007; Pierre et al., 2009). The $\delta^{13}C$ values of different forms of IC have been reported in the range of $+30 \%$ to $–51 \%$, a range which completely overlaps with the commonly cited $\delta^{13}C$ ranges for OM.

For example, Pierre et al (2009) reported values as low as $–51 \%$ for calcite/aragonite and $–38 \%$ for dolomite measured in marine authigenic carbonate, Chow et al (2000) reported a range of $–22 \%$ to $+8 \%$ for early diagenetic Mn-Fe carbonates, Hangari et al (1980) reported $\delta^{13}C$ values of between $–12 \%$ to $–30 \%$ for freshwater siderite, and Mosley and Burns (1993) provide an overview of $\delta^{13}C$ values of marine calcite, dolomite and siderite minerals illustrating the common nature of very depleted $\delta^{13}C$ values ($\leq –15 \%$).

If we take a hypothetical sample material, containing 3% OC with a $\delta^{13}C$ value of $–14 \%$, and 1% IC with a $\delta^{13}C$ value of $–30 \%$, then, by mass balance, the overall sample $\delta^{13}C$ value would be $–18 \%$ (i.e. a 4 % depletion in $\delta^{13}C$ due to IC contamination, not an enrichment that is commonly assumed), tending an interpretation towards C3 vegetation (e.g. more humid environment). Given the potential for more robust forms of IC to have very low $\delta^{13}C$ values, such as dolomite and siderite which are not readily digested by acid, then the potential for the depletion of measured $\delta^{13}C$ values as a result of residual IC is real, but largely unrecognised!

Our data illustrate a depletion of the $\delta^{13}C$ value, e.g. 7.64 m – 7.80 m, which suggests that the data may not only be affected by the inefficient (and disproportional) removal of IC from the sample (assuming an enrichment in $\delta^{13}C$ within this core from residual IC), but also by the
effect of acidification on the OC component (assuming the untreated values at these depths are not representative of IC contamination). We therefore suggest that the sample IC component should be identified and quantified to ensure no residual IC remains after treatment, or, where the IC exists as a more robust form (e.g. dolomite, siderite), the size of the offset can at least be partly accounted for and an investigation into the bias associated with the OC component can be undertaken. Sample OC must be understood in the context of IC within the same sample and alongside acid treatment biases: data presented without this explicit quantitative understanding are potentially unreliable. In addition, differences in interpretation of $\delta^{13}C$ from OM as an indicator for changes in C$_3$ and C$_4$ vegetation are also questionable, where the $\delta^{13}C$ of C$_3$ plant tissue has been reported in the range of $-13$ to $-29 \%$ (e.g. Hedges et al, 1986), and C$_4$ plants in the range of $-7$ to $-23 \%$ (e.g. Schilowski, 1987). This is counterintuitive relative to the widely used C$_3$ v C$_4$ interpretation, suggesting an additional environmental consideration, *inter alia*, in interpretation.

The structure and composition of C and N in OM from a down-core sedimentary sequence can vary substantially (e.g. relative proportions of lipids, lignins, proteins, amino acids, and cellulose; Fernandes and Krull, 2008), and may subsequently respond disproportionately under different acid treatment methods (i.e. differences in proportions of refractory and labile organic components). This suggests that C/N, $\delta^{13}C$ and $\delta^{15}N$ values are likely to be a relative proxy for the overall chemistry of the core material, but the degree with which it reflects the true OM value of the core, and thus a specific process, after acid treatment is highly variable and makes interpretation more difficult. In addition, where sample material is low in %C and %N, the effect of acidification on $\delta^{13}C$ and $\delta^{15}N$ could be significantly magnified (e.g. Brodie et al., 2011a, 2011b) which may be due to C and N isotopes becoming highly heterogeneous within the OM at these low levels. These factors add unpredictable, non-linear biasing to the dataset within sample horizons and with varying magnitude and proportions between sample horizons (i.e. suggesting the underlying trend of the data can be biased in a non-systematic fashion).

For $\delta^{15}N$, we note a fractionation between untreated and acid treated samples ($\sim 0.8 \%$), and between acid treated samples but with no concomitant loss in %N (no difference in %N values between treated and untreated data). The mechanisms for this are unclear; however, there seems to be a systematic shift across all acid treated samples towards lower $\delta^{15}N$ with
samples in the rinse method tending to produce the lowest $\delta^{15}$N. This shift towards more
depleted $\delta^{15}$N values may reduce the certainty on interpretations of water column
denitrification, for example, and biases of the order of ~0.8 ‰, or ~1.7 ‰ (Brodie et al.,
2011b), can account for between ~15 and 40 % of the variability in some records with an
overall range of ~5 ‰ (e.g. Altabet et al., 1995). Additionally, the isotopic signature of IN is
not significantly dissimilar to that of organic N, making the overall interpretation of the $\delta^{15}$N
of OM in the presence of IN difficult (e.g. Knies et al., 2007). This illustrates the importance
of fully understanding OM structure and composition, and the IC and IN components, within
the system under investigation where a bulk organic matter approach is adopted.

These findings have significant implications for the comparison of records that are (i) derived
in different laboratories following differing pre-treatment methods (or variations of the same
method), and (ii) derived from different environments where the amounts and relative
proportions of C and N in sample OM varies, and the amount, type and nature of OM, IC and
IN varies. The assumption that data are reliable (and the subsequent interpretation robust)
because of our ability to produce extremely high instrument accuracy and precision is a non
sequitur. Our data suggest the necessity to account for the acid treatment bias in full and
determine the size of the offset to ensure that the interpretation is more robust and
acknowledge the full range of “error” in the analysis (see section 1.6 for more detail). We
suggest that the biasing of the true OM signature during pre-analysis acid treatment is
inevitable, but unpredictable. The environmental interpretation of elemental and isotopic
values of OM is not necessarily dependent upon an environmental shift, but can be
significantly affected by both IC and IN, pre-analysis acid treatment method and the structure
and composition of OM across the land-sea gradient. It is imperative that the effect of pre-
analysis acid treatment methods on $\delta^{13}$C and $\delta^{15}$N values be pursued at the molecular level to
improve our understanding of the mechanisms controlling the bias evident in our data (and
most likely in other down-core records).

1.5 Implications for interpretation of C/N, $\delta^{13}$C and $\delta^{15}$N of bulk OM

Our findings have significant implications for the interpretation of measured C/N, $\delta^{13}$C and
$\delta^{15}$N values of bulk OM in the context of the established theory in the literature (e.g. OM
provenance and vegetation type), the estimation of organic and inorganic carbon burial and/or
accumulation rates (e.g. Twichell et al., 2002) and interpretation of carbon bi-plots (e.g.
Thornton and McManus, 1994; Meyers, 1997; Meyers, 2003; Lamb et al. 2006; Zong et al. 2006; Mackie et al., 2007; Yu et al., 2010). We show that the interpretation of C/N and $\delta^{13}$C data is not just dependant on an environmental shift, but can also be dependent on the bias due to pre-analysis acid treatment method. This is likely to be underpinned by the complexities in the structure and composition of OM within and between environments.

Specifically, it suggests that small changes in the down-core records (i.e. < 4 \%) may provide less reliable interpretations in comparison to much larger shifts (i.e. of the order of 10 \%, or greater). Interpretations of C/N, $\delta^{13}$C and $\delta^{15}$N values have been underpinned by the assumption that we can reliably determine C/N, $\delta^{13}$C and $\delta^{15}$N of sample OM. We have shown that this assumption is highly problematic, and that a detailed discussion and investigation on the potential source of bias, above that of the standard instrument precision, is essential for a robust interpretation of the data. It is clear that additional bias on C/N, $\delta^{13}$C and $\delta^{15}$N measurements in OM can derive from inorganic carbon (IC) and inorganic nitrogen (IN) content, pre-analysis acid treatment method followed and OM composition of the sample material.

However, our data also show sample horizons with no difference in results within and between methods, highlighting the inconsistency in any one method down-core. This suggests that the accuracy and precision with which C/N, $\delta^{13}$C and $\delta^{15}$N values from any one acid treatment method reflects sample OM is highly variable and unpredictable. Therefore, instrument precision should be interpreted as an absolute minimum precision on measured data (e.g. Brodie et al., 2011a, 2011b). The fact that pre-analysis treatment method can significantly influence the environmental interpretation of sedimentary OM is worrying, and cautions against the over interpretation of the minutiae of the data acquired. For example, we report differences within and between methods on our down-core record in the region of 2 – 3.5 \% for $\delta^{13}$C (excluding the excursion at 8.76 m which has a substantial IC contamination signal). A precision range of this magnitude can account for the overall range of some down-core studies (e.g. Turney et al., 1999; Zong et al., 2006; Mackie et al., 2007; Bertrand et al., 2010; Scholz et al., 2010; Yu et al., 2010). It is therefore critical the extent of bias due to acid treatment on elemental and isotopic measurements in OM is understood to ensure that any interpretation is grounded on a robust dataset reflecting sample OM, especially where inferences on climate variability and mechanisms are being proposed. In addition, these
findings suggest that the correlation of $C/N$ and $\delta^{13}C$ values of bulk OM derived from different sedimentary archives is highly problematic.

Given the current drive in the community to derive annual – centennial resolution from down-core records of past environmental change, and in the context of increasing use of data transformation techniques, such as spectral and wavelet transforms used to understand periodicities (e.g. Baker et al., 2005), it is imperative that the inaccuracy and imprecision of the data is fully understood and the subsequent limitations to interpretation acknowledged. For example, differences within and between methods of the order of $\sim 2 – 3.5 \, \%$ would significantly alter the amplitude and potentially change the frequency of a down-core record, which may be misinterpreted as being environmentally significant (i.e. the amplitude of environmental variability compared with the amplitude of variability in the data caused by inaccuracy and imprecision of the data). It may artificially cause high-frequency signals to manifest as significant periodicities in the core data during analysis, which may lead to incorrect interpretation. In addition, the bias due to acid treatment can also affect the underlying trend in the record, which can further undermine data analysis.

This suggests $C/N$ and $\delta^{13}C$ values from bulk OM are a less reliable tool for reconstructing environmental events with low amplitude variability. This is likely to have implications for the high resolution, high frequency reconstructions favoured in the recent literature. We did not carry out time-series analysis on our data – the analysis itself, in addition to the acid treatment bias, would have been undermined by the low resolution sampling and poor dating constraint across the data in the first instance, and made $a \ priori$ assumptions about the system and climatic processes responsible for the geochemical OM signature (e.g. Wunsch, 2010).

1.6 Implications for accuracy and precision

Based on our findings, we preliminarily assess the sources of inaccuracy and imprecision on $C/N (\Sigma_E)$, $\delta^{13}C (\Sigma_C)$ and $\delta^{15}N (\Sigma_N)$ values from sample bulk OM as follows:

\[
\Sigma_E \text{ (on individual } C/N \text{ values)} = c_d + c_{pic} + c_{pin} + c_{sh} + c_{ss} + c_{an}
\]

\[
\Sigma_C \text{ (on individual } \delta^{13}C \text{ values)} = c_d + c_{ic} + c_{sh} + c_{ss} + c_{an}
\]
Σ \( N \) (on individual \( \delta^{15}N \) values) = \( n_d + n_{in} + n_{sh} + n_{ss} + n_{an} \)

The inaccuracy and imprecision associated with each component of the above equations are summarised in Table 3. The diagenesis component (\( c_d; c_{ed}; n_d \)) can contribute a significant bias relative to the organic signal of the original source OM from pre- and post deposition processes, such as oxidation and microbial reworking, the extent of which will vary depending on the length of time OM takes to reach the sediment, the productivity in the water column and dissolved oxygen concentrations (Meyers, 1994; Krull et al., 2002; Lehmann et al., 2003). For example, Meyers and Ishiwatari (1993) noted that a diagenetic decrease in C/N values could occur in lake sediments of the order of ~ 26. A loss of non-lignin compounds from a C\( _4 \) marsh plant depleted \( \delta^{13}C \) by ~ 4 ‰ (Benner et al., 1987), though in sediments evidence for diagenetic bias is contradictory. Spiker and Hatcher (1984) noted a 4 ‰ depletion in lake sediments which they attributed to the loss of \( ^{13}C \)-rich carbohydrates, whereas Rea et al (1980) and Jasper and Gagosian (1989) noted no bias due to diagenesis.

For \( \delta^{15}N \), the effect of diagenetic processes on the primary sedimentary OM signal is also contradictory. Altabet and Francois (1994) reported a 5 ‰ enrichment in \( \delta^{15}N \) and Sigman et al (1999) noted an increase of ~ 4 ‰ in \( \delta^{15}N \) from Southern Ocean sediments. However, de Lange et al (1994) reported a decrease of ~1 ‰ in \( \delta^{15}N \), and Freudenthal et al (2001) noted a ~ 1 ‰ bias in eastern Atlantic Ocean sediments with no clear trend towards an increase or decrease in \( \delta^{15}N \). The degradation of organic compounds, which have distinctive isotopic signatures, appears to be non-discriminatory, implying that diagenetic processes must be accounted for on a system by system basis. For example, the loss of readily degradable amino acids and hydrocarbons, relative to terrestrially sourced compounds such as lignins and lipids, would deplete \( \delta^{15}N \) (loss of \( ^{15}N \) and \( ^{13}C \)). These contradictory results clearly imply that there is no emergent generalised affect on the \( \delta^{13}C \) and \( \delta^{15}N \) signature of bulk OM, and this can differ markedly between oxic and anoxic conditions (Tyson, 1995). However, a detailed molecular level investigation may allow the estimation of this bias on bulk OM.

The influence of IC and/or IN (\( e_{pic}; e_{pin}; c_{ic}; n_{in} \)) is dependent upon the efficiency of removal during acid treatment, and the structure and composition of the inorganic component (e.g. Al-Aasm et al., 1990; Yui and Gong, 2003; Knies et al., 2007). The bias on bulk \( \delta^{13}C \) in this study was shown to be an enrichment of ~ 6.8 ‰ at 8.76 m, though it was noted that residual IC could result in an overall depletion of the bulk \( \delta^{13}C \) value (e.g. Hoefs, 1973; Hangari et al.,
Additionally, and similar to $\delta^{13}$C, the extent of the bias on $\delta^{15}$N is less obvious owing to the similar values in both organic and inorganic N (Knies et al., 2007). The homogeneity of the sample material (csh; cah; nsh) can also contribute additional imprecision, likely increasing substantially on samples poorly homogenised or with significantly low amounts of C and N (e.g. Basiden et al., 2002). Sample size can also contribute significant bias, particularly those low in C and N before acid pre-treatment is undertaken (es; cs; ns). Where sample size becomes very small (especially in conjunction with an acid pretreatment), %C and %N can increase by over 50% (see Brodie et al., 2011a) and $\delta^{13}$C and $\delta^{15}$N values tend to become more positive, suggesting at least a ~1 ‰ deviation in the OM value (our analysis were carried out on sample sizes significantly above machine baseline conditions and therefore do not carry this additional inaccuracy).

Finally, the analytical term (ean; can; nan), comprising the bias from acid treatment and the instrument precision. Pre-analysis acid treatment has been shown here, and elsewhere, to substantially bias the elemental C and N values of sample OM through degradation of the OC fraction and/or inefficient removal of the IC fraction (and differential rates of removal linked to IC stoichiometry). For this core, we estimate this error to be in the region of ~ ± 2 – 3.5 ‰ associated with the OC fraction and ~ 6.8 ‰ (enrichment) associated with the IC fraction. The instrument precision, which is inherent to all measurements in this study, is of the order of ± 0.5 for C/N values, ± 0.1 ‰ for $\delta^{13}$C values and ± 0.1 ‰ for $\delta^{15}$N values.

We caution, however, that whilst these equations are more representative of the absolute inaccuracy and imprecision on measured C/N, $\delta^{13}$C and $\delta^{15}$N values than instrument precision alone, the terms are by and large inherently non-linear and unpredictable, implying that absolute inaccuracy and imprecision is unobtainable. An assumption of linearity of these terms would be seriously flawed. We conclude that the unpredictable, non-linear biasing to the data within sample horizons and with varying magnitude and proportions within and between sample horizons can undermine a robust interpretation of the data, with the size of bias varying considerably between different cores.

**Table 3**: Summary of inaccuracy and imprecision on C/N, $\delta^{13}$C and $\delta^{15}$N values measured from bulk OM. The size of bias is estimated from past literature (see section 1.6 for a discussion and evaluation) and biases due to pre-analysis acid treatment reported in this study.
1.7 Summary and Recommendations

1. This study has clearly demonstrated significant non-linear bias on bulk C/N, $\delta^{13}C$ and $\delta^{15}N$ values of OM associated with pre-analysis acid treatment method in a stratigraphical sequence of samples. We show that there is an inconsistency in the use of any one method within and between sample horizons and that where this bias is evident it is significantly above instrumental precision. The differences appear to be the result of (i) differential rates of removal of IC and (ii) disproportionate biasing to OC fraction of the sample material.

2. In light of our findings, we recommend that researchers do not interpret the minutiae of the bulk $\delta^{13}C$ and $\delta^{15}N$ of OM data, but restrict interpretations and discussions to those shifts significantly greater than a robust estimate of the inaccuracy and imprecision on the data (i.e. $\sim 4$ % on this down-core data). The estimation presented here is considerably greater than is normally assumed (i.e. standard instrument precision) and underlines the importance of determining the size of the bias on C/N, $\delta^{13}C$ and $\delta^{15}N$ data in a down-core record. Consequently, $\delta^{13}C$ data should be used as a first-order indication of potential changes in sample OM, which could be further investigated for environmental and climatic change at the molecular level.

3. The biases discussed here make the environmental interpretation of C/N values (e.g. terrestrial versus aquatic) and $\delta^{13}C$ values (e.g. C$_3$ versus C$_4$ vegetation) problematic. For example, at 7.80 m C/N values range from 12 – 32 between methods and at 8.60 m $\delta^{13}C$ values range from $-21.3$ to $-12.5$ %. In addition, it also makes the interpretation of C/N versus $\delta^{13}C$, C/N versus $\delta^{15}N$ and $\delta^{13}C$ versus $\delta^{15}N$ bi-plots questionable.

4. The rinse method can artificially elevate %C and %N values and significantly undermine the integrity of C/N values. We recommend including a centrifugation step in this method, but warn that this will not guarantee resolution of the problems associated with decanting.

5. The 5% HCl capsule method appears to be less efficient in the removal of IC leading to more enriched $\delta^{13}C$ values (e.g. 8.76 m), and so we therefore do not recommend the use of this reagent within the capsule method. However, we warn that the assumption that residual IC causes an overall enrichment of the measured bulk $\delta^{13}C$ value is invalid, where $\delta^{13}C$ values of IC can be very negative. This suggests a comprehensive understanding of sample IC alongside sample OC is required without which C/N and $\delta^{13}C$ values of OM may be unreliable.
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References


Black clayey amorphous organic material

Brown clayey silt with wood fragments grading to light grey clayey silt with small amounts of amorphous organic materials. Very fine to coarse sand grains throughout.

Lithology Key

- Wood Fragment
- Sand
- Amorphous Organic Material
- Clayey Silt
- Black amorphous organics

35148 ± 136
<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>(\delta^{13}C)</th>
<th>P-value (R-Squ)</th>
<th>Difference (%)</th>
<th>Cause</th>
<th>P-value (R-Squ)</th>
<th>Difference</th>
<th>Cause</th>
</tr>
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<td>7.09</td>
<td>0.197 (20.98)</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>0.00 (74.05)</td>
<td>3 – 4</td>
<td>5% and 20% HCl capsule higher than 5% rinse</td>
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<td>7.16</td>
<td>0.183 (22.52)</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>0.18 (23.18)</td>
<td>nd</td>
<td>-</td>
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<tr>
<td>7.32</td>
<td>0.04 (49.01)</td>
<td>0.2</td>
<td>5% HCl more enriched than 20% HCl in rinse method</td>
<td>0.29 (11.69)</td>
<td>nd</td>
<td>-</td>
<td></td>
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<td>7.48</td>
<td>0.55 (0.00)</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>0.143 (27.67)</td>
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<td>7.64</td>
<td>0.00 (94.69)</td>
<td>1.0</td>
<td>20% HCl capsule sample more enriched than 20% HCl rinse sample</td>
<td>0.97 (0.00)</td>
<td>nd</td>
<td>-</td>
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<td>7.80</td>
<td>0.00 (98.25)</td>
<td>0.6 – 1.5</td>
<td>All samples differ over 1.5 % range, with more enriched values in the capsule method.</td>
<td>0.00 (95.63)</td>
<td>22</td>
<td>5% HCl capsule higher than all other samples</td>
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<td>0.01 (64.38)</td>
<td>0.3 – 0.7</td>
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<td>0.94 (0.00)</td>
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<td>8.12</td>
<td>0.01 (60.68)</td>
<td>0.4</td>
<td>Capsule method samples more enriched than rinse method samples</td>
<td>0.02 (58.67)</td>
<td>4 – 6</td>
<td>5% and 20% capsule method different and 20% HCl capsule different from rinse samples</td>
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<td>nd</td>
<td>-</td>
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<td>nd</td>
<td>-</td>
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</tr>
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<td>HCl Concentration</td>
<td>Range</td>
<td>Results</td>
<td>Significance</td>
<td>Notes</td>
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<td>0.00 (98.83)</td>
<td>0.4</td>
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<td>0.00 (87.59)</td>
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<td>1.2 – 6.8</td>
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<td>5% HCl and 20% HCl capsule method higher than rinse method samples.</td>
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<td>0.00 (94.19)</td>
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<td>Capsule method samples higher than rinse method samples</td>
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<td>5% HCl capsule more enriched than all other samples</td>
<td>0.04 (47.69)</td>
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<td>5% HCl capsule higher than other samples</td>
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<tr>
<td>9.40</td>
<td>0.07 (40.58)</td>
<td>nd</td>
<td>-</td>
<td>0.03 (54.35)</td>
<td>10</td>
<td>5% and 20% HCl capsule method samples different</td>
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<tr>
<td>9.56</td>
<td>0.74 (0.00)</td>
<td>nd</td>
<td>-</td>
<td>0.31 (9.77)</td>
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<td>-</td>
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<tr>
<td>9.72</td>
<td>0.00 (88.05)</td>
<td>1.0 – 1.2</td>
<td>Rinse method samples more depleted than capsule method samples. 20% HCl capsule more enriched than all other samples</td>
<td>0.06 (41.74)</td>
<td>nd</td>
<td>-</td>
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<tr>
<td>9.88</td>
<td>0.09 (35.93)</td>
<td>nd</td>
<td>-</td>
<td>0.04 (49.52)</td>
<td>7</td>
<td>5% HCl capsule different from 20% HCl rinse</td>
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<td>10.02</td>
<td>0.00 (94.86)</td>
<td>1.0 – 1.8</td>
<td>Rinse samples more depleted than capsule samples. 5% HCl rinse</td>
<td>0.04 (49.95)</td>
<td>8</td>
<td>5% HCl different from both rinse samples</td>
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<td>Rinse method sample more depleted than capsule samples</td>
<td>0.00 (94.72)</td>
<td>0.3 – 0.6</td>
<td>0.00 (74.84)</td>
<td>6 – 8</td>
<td>5% HCl capsule higher than other samples</td>
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<td>Depth (m)</td>
<td>P-value (R-Squ)</td>
<td>Difference (‰)</td>
<td>Cause</td>
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<tr>
<td>7.09</td>
<td>0.00 (84.07)</td>
<td>0.46</td>
<td>Acid treated samples lower than untreated samples. 20% HCl rinse lowest.</td>
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<td>7.16</td>
<td>0.00 (89.14)</td>
<td>0.77</td>
<td>Acid treated samples lower than untreated samples.</td>
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<td>7.32</td>
<td>0.02 (51.33)</td>
<td>0.46</td>
<td>Acid treated samples lower than untreated samples. Capsule method samples lowest.</td>
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<td>7.48</td>
<td>0.00 (79.94)</td>
<td>0.42</td>
<td>Capsule method and 5% HCl rinse lower than untreated samples and 20% HCl rinse.</td>
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<td>Process</td>
<td>Bias variable</td>
<td>C/N Bias</td>
<td>δ¹³C Bias</td>
<td>δ¹⁵N Bias</td>
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<td>Diagenesis</td>
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<tr>
<td>Explanation</td>
<td>Bias associated with breakdown, oxidation and reworking of initial OM.</td>
<td>Bias associated with breakdown, oxidation and reworking of initial OM.</td>
<td>Bias associated with breakdown, oxidation and reworking of initial OM.</td>
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<td>Size of Bias</td>
<td>~5 – 26</td>
<td>~ 0.2 – 4 ‰</td>
<td>~ 0.1 – 5 ‰</td>
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<tr>
<td>Inorganic Carbon</td>
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<td>e_in</td>
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<td>Explanation</td>
<td>Bias associated with the structure, composition and quantity of sample IC.</td>
<td>Bias associated with the structure, composition and quantity of sample IC.</td>
<td>N/A</td>
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<td>Size of Bias</td>
<td>± 1 – 60</td>
<td>± 3.4 ‰</td>
<td>indefinable</td>
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<td>Inorganic Nitrogen</td>
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<td>Size of Bias</td>
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<td>± 3.4 ‰</td>
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<td>Analytical Bias</td>
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<td>n_an</td>
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<td>Size of Bias</td>
<td>± 0.5 – 15 (± 0.2)</td>
<td>± 4 ‰ (± 0.1 ‰)</td>
<td>± 0.7 ‰ (± 0.1 ‰)</td>
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<td>Sample Size</td>
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<td>n_sss</td>
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<tr>
<td>Explanation</td>
<td>Bias associated with the amount of C and N supplied for analysis.</td>
<td>Bias associated with the amount of C and N supplied for analysis.</td>
<td>Bias associated with the amount of C and N supplied for analysis.</td>
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<tr>
<td>Size of Bias</td>
<td>± 0.5 – 100</td>
<td>0.5 ‰ (or greater)</td>
<td>0.5 ‰ (or greater)</td>
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<td>Sample Homogenisation</td>
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<td>Explanation</td>
<td>Bias associated with homogenisation of sample material prior to treatment.</td>
<td>Bias associated with homogenisation of sample material prior to treatment.</td>
<td>Bias associated with homogenisation of sample material prior to treatment.</td>
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<tr>
<td>Size of Bias</td>
<td>± 0 – 0.2</td>
<td>± 0.0 – 0.1 ‰</td>
<td>± 0.0 – 0.1 ‰</td>
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