Evidence for bias in measured δ^{15}N values of terrestrial and aquatic organic materials due to pre-analysis acid treatment methods.

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Abstract

We investigate the effect of acid treatment methods on δ^{15}N from a range of environmental organic materials in the context of the increased application of "dual-mode" isotope analysis (the simultaneous measurement of δ^{13}C and δ^{15}N from the same acid treated sample). Three common methods are compared; (i) untreated samples; (ii) acidification followed by sequential water rinse (rinse method); (iii) acidification in silver capsules (capsule method). The influence of capsule type (silver and tin) on δ^{15}N is also independently assessed (as the capsule and rinse methods combust samples in different capsule; silver and tin respectively). We find significant differences in δ^{15}N values between methods and the precision of any one method varies significantly between sample materials and above instrument precision (> 0.3 ‰). δ^{15}N of untreated samples did not produce the most consistent data on all sample materials. In addition, the capsule type appears to influence the measured δ^{15}N value of some materials, particularly those combusted only in silver capsules. We also compare the new δ^{15}N data with previously published δ^{13}C on the same materials. The response of δ^{13}C and δ^{15}N within and between methods and sample materials to acidification appears to be relatively disproportionate, which can influence environmental interpretation of the measured data. In addition, statistical methods used to estimate inorganic nitrogen are shown to be seriously flawed.

Introduction

The analysis of stable isotope ratios of nitrogen ($\delta^{15}N$) provides valuable information on the complex processes within the global nitrogen cycle. $\delta^{15}N$ has been used to understand trophic pathways in food webs [e.g. 1, 2, 3, 4], organic matter (OM) provenance and degradation [e.g. 5, 6, 7, 8], denitrification in the water column and nitrate utilisation in ocean sedimentary records [e.g. 9], and eutrophication [10, 11]. C/N ratios (weight ratios of the elements) have been widely used to support $\delta^{15}N$ (and $\delta^{13}C$) by broadly distinguishing between terrestrial and aquatic sourced OM. The N composition of aquatic derived OM (protein and lipid rich) is considerably greater than for that of terrestrial OM (cellulose and lignin rich) dependent upon the availability of N in the environment. In addition, $\delta^{13}C$ of OM has been employed to identify carbon sources and photosynthetic pathways in plants [e.g. C3 and C4 plant differentiation; 12, 13], assess C dynamics in soil systems and study trophic levels in environmental systems [1, 2, 4, 14], and understand C mineralisation processes [e.g. 15, 16, 17].

These interpretations assume that C/N ratios, $\delta^{13}C$ and $\delta^{15}N$ of OM can be reliably determined. For C/N ratios and $\delta^{13}C$, this relies upon the complete removal of inorganic carbon (IC) from the sample total carbon pool, typically achieved through acid pre-treatment, without alteration of sample OM [e.g. 18]. In contrast, measurements of %N and $\delta^{15}N$ are commonly made on untreated sample material, on the assumption that inorganic nitrogen (IN) does not contribute to the total sample N (though this may not be valid in all environments [e.g. 19, 20, 21]). However, it is becoming increasingly common to measure C/N ratios, $\delta^{13}C$ and $\delta^{15}N$ as part of a single, "dual-mode" analysis [e.g. 4, 22, 23, 34]. In this case, if IC must be removed by acid treatment it is necessary to establish that this treatment does not affect the %N or $\delta^{15}N$ of OM.

The effect of pre-analysis acid preparation methods on %N and $\delta^{15}N$ can be significant [e.g. 1, 3]. Losses in %N have been reported in the range of $0 - 50\%$ [e.g. 18, 25, 26, 27] alongside artificial gains of ~ 20 % [18]. In addition, and like known variabilities in $\delta^{13}C$ previously reported [18], results for shifts in $\delta^{15}N$ are variable indicating an increase of $0.1 - 3$ ‰ [1], a

decrease of 0.2 –1.8 ‰ [1, 3, 14, 22, 28], and no significant change [29, 31], with no apparent trend in the size of offset related to the type of material (i.e. modern or ancient, terrestrial or aquatic). In addition, disproportionate and non-systematic offsets in C and N of OM due to acid treatment contribute to this variability [e.g. 18, 30, 31], and could preclude interpretation of sedimentary $\delta^{15}N$ records with a range of ~ 1 – 4 ‰ [e.g. 20, 32, 33, 34]. An investigation into the effects of pre-analysis acid preparation methods on $\delta^{15}N$ of OM is therefore justified.

We aim to investigate the effect of pre-analysis acid preparation methods on measured $\delta^{15}N$ values of OM. We test the hypothesis that the measured $\delta^{15}N$ values are not different between pre-analysis acid preparation methods. We compare untreated material, and material acid treated in the two most common methods; i) the capsule method and; ii) the rinse method; and use HCl as the acidifying reagent. We also independently investigate the effect of capsule type. These methods all use different capsules; the rinse method and untreated method use tin (Sn) capsules, whereas the capsule method uses silver (Ag) capsules, thus an investigation into the influence of different capsules is necessary [see 18]. Specifically, the following research questions are addressed:

1. Are there significant differences in measured $\delta^{15}N$ values of OM between methods?
2. Does capsule type effect measured $\delta^{15}N$ values?
3. Does acid treatment method influence environmental interpretation of measured $\delta^{15}N$ values?

Sample materials and Preparation Methods

Sample materials

A recent comprehensive study into the effects of acid treatment methods on OC in OM of different sample materials showed significant differences in C/N and $\delta^{13}C$ of sample materials that were prepared in different ways [18]. As a follow on from this study, we selected 3 sample materials for this study from those previously analysed which represent different environments and which showed considerably different treatment effects for C/N and $\delta^{13}C$ values between them. These samples are BROC, SOILB and TYC (a plant, a modern soil and an ancient lake sediment, see Table 1). As these sample materials represent three very different environments, and hence different amounts and composition of OM (e.g. terrestrial, freshwater, marine, plant or animal; modern or ancient), and show differences in C/N and $\delta^{13}C$ between acid treatment methods [e.g. 18], $\delta^{15}N$ might also be expected to be biased by acid treatment. All sample materials were freeze dried and freeze milled to a flour

(grain size $\leq 63\mu m$) prior to analysis. None of these sample materials contain an IC component (so we were able to compare untreated with treated sample aliquots).

Table 1: Description of sample materials. [1]International organic soil standard from LECO corporation (part no. 502 – 308). [2]Internal NIGL laboratory standard. [3]Measured on untreated sample material. Values are calibrated to V-PDB against international standards NBS-18 and NBS-19, and crossed checked with NBS-22.

Sample Preparation Methods

Prior to sample preparation, all sub-sampling equipment and glassware were thoroughly washed in 1% nitric acid, rinsed in deionised water, followed by a wash in 2% neutracon® solution (DECON Laboratories Ltd., UK), 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. HCl was the only reagent selected for removing IC, because it produced more coherent $\delta^{13}C$ and C/N data within and between methods than 6% H_2SO_3 and 6% H_3PO_4 [see 18]. Two strengths of HCl were tested, 5% w/w and 20% w/w, based on findings in [18]. Sample materials were prepared as follows:

Untreated samples: Samples were weighed directly into Sn capsules.

Capsule method: Samples were weighed into open Ag capsules, transferred to a metal tray on a hotplate and 10 μL of distilled water was added to moisten the samples. 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, followed by a final 200 μL, without allowing the sample to dry out between additions.

Rinse method: Sample material was mixed with excess acid in a beaker and allowed to stand for 24 hours. The beaker was topped up to 500 mls with deionised water and the sample material allowed to settle for a further 24 hours. Once settled, the supernatant was decanted, ensuring minimal disturbance of the remaining material, and the beaker then topped up again to 500 mls with deionised water. Dilution was repeated 3 times in total with an overall minimum of 1200 mls of deionised water used. After the final decanting, the excess water (50 – 100 mls) was allowed to evaporate off in a drying oven at ~50°C, and the sample transferred to a Sn capsule.

The capsule method uses Ag capsules, because they are resistant to acid attack whereas Sn capsules disintegrate under acidification, especially with HCl. Unlike Sn, however, Ag does not oxidise exothermally in the elemental analyser, so that sample combustion temperatures in Ag are lower than in Sn. We therefore also analysed samples with their Ag capsules further wrapped in tin (Ag+Sn) to test whether this significantly affected $\delta^{15}N$ values. The rinse method traditionally uses Sn capsules, and these were replicated by wrapping in silver (Sn+Ag). All treatments were performed in triplicate.

Analytical Methods

Nitrogen isotope analyses were performed using an elemental analyser linked to an isotope ratio mass spectrometer (EA-IRMS). Samples were loaded into an autosampler (Costech Zero Blank, Costech Analytical Technologies, Valencia, USA) and dropped into a 1.6 mL sec^{-1} stream of helium in a FlashEA 1112 elemental analyser (Thermo Fisher Scientific, Bremen, Germany). The combustion column contained copper oxide and silvered cobaltous oxide at $900^{o}C$, and combustion products were cleaned of oxygen and nitrogen oxides by passage through copper at $680\,^{o}C$, and of water and carbon dioxide by passage through magnesium perchlorate and carbosorb before passage through the GC column (chemicals supplied by Elemental Microanalysis, Okehampton, England and Pelican Scientific, Stockport, England). The helium stream with sample N_2 was led via a Conflo III interface to a Delta+XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) for determination of the $^{15}N/^{14}N$ ratio. In order that all treatments of a particular sample material could be analysed in a single run, correction of the measured sample $^{15}N/^{14}N$ ratios to $\delta^{15}N$ values was not based on comparison with internal standard samples (the normal procedure) but rather on comparison with a reference gas whose $\delta^{15}N$ value versus atmospheric N_2 had been determined in separate runs. The quoted $\delta^{15}N$ values are therefore correct relative to one another for the same sample material, but only approximately correct in absolute terms versus atmospheric N_2. Limits on analytical precision are mainly determined by conditions of combustion and chromatography in the elemental analyser. For organic materials containing a few %N (e.g. BROC) within-run precision for $\delta^{15}N$ is better than 0.3‰ (1 SD for n = 10 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 [18].

Data Analysis

All data are plotted as mean ± standard deviation (1σ). Our data were analysed by One-Way ANOVA using Minitab 15.0 (MINITAB Inc. 2007). Data were tested for normality using an Anderson-Darling normality test (all p-values > 0.05) and homogeneity of variances using a Bartlett's Test (assumes normality within each factor level) and Levene's Test (does not assume normality within each factor level) [50, 51]. For our data, the p-value for the Bartlett and Levene tests were all above 0.05 indicating the variances are the same for each factor. These tests validate the use of ANOVA on our data. We test the null hypothesis that there is no significant difference in measured $\delta^{15}N$ values between treatment methods, capsule type and acid reagent strength (i.e. within method variability). The results for C/N and $\delta^{13}C$ have been previously reported [18] and were measured on different sample aliquots than those for $\delta^{15}N$.

Results

Results for $\delta^{15}N$ for SOILB, BROC and TYC are presented in Figures 1A-C and are reported as mean ± standard deviation (Table 2), alongside previously reported C/N and $\delta^{13}C$ [see 18]. Results from the one-way ANOVA are presented in Table 3. There is a statistically significant difference between pre-analysis acid treatment methods, and within and between sample materials (Table 3) that is significantly greater than instrument precision (~0.3 ‰). In SOILB and TYC, measured values of $\delta^{15}N$ in acid treated are lower than for untreated samples (Figures 1A and 1C; Table 3). For SOILB, the precision of $\delta^{15}N$ determinations was better for untreated and rinse method samples than for capsule method samples (value represented graphically by horizontal grey bars on Figure 1A). In TYC precision was marginally better for acid treated samples than for untreated samples (untreated sample in the Ag capsule significantly influenced this; see Figure 1C). We notice a slight depletion in $\delta^{13}C$ and lower C/N in SOILB, with a relatively depleted $\delta^{15}N$ (to untreated samples). In TYC, the rinse method show enriched $\delta^{13}C$ and higher C/N values, concomitant with a relatively depleted $\delta^{15}N$.

In BROC, the measured $\delta^{15}N$ values were generally better for acid treated samples than for untreated samples (value represented graphically by horizontal grey bars on Figure 1B), though the rinse method samples produced the lowest $\delta^{15}N$ values of all methods for this material. This poor sample precision contributed to no statistical differences being detected between untreated and acid treated sample means. However a significant difference between

data in the capsule and rinse methods of ~ 1.2 ‰ exists (Figure 1B), with the rinse method data showing relatively higher $\delta^{15}N$, concomitant with depleted $\delta^{13}C$ and higher C/N relative to known values [18].

Comparisons of results within the same method but combusted in different capsules showed significant differences (Table 3). In particular, untreated samples analysed only in Ag capsules showed significantly lower results in all materials (see unfilled circles in Figures 1A – 1C). We note, in the capsule method, there was no common trend in data for samples further wrapped with a Sn capsule, though for TYC $\delta^{15}N$ were depleted by ~0.5 ‰ relative to other samples within the capsule method. For the rinse method, across all sample materials, samples further wrapped in an Ag capsules showed higher $\delta^{15}N$ values of between 0.4 – 0.8 ‰.

Figure 1a: SOILB C/N, $\delta^{13}C$ and $\delta^{15}N$ values for each method and acid. Horizontal solid grey lines indicate mean values for each method, and perforated grey lines 1σ. Vertical perforated lines split the untreated, capsule and rinse methods. The transparent grey bar on the C/N and $\delta^{13}C$ plots represents known values. Error bars are calculated as standard deviation (1σ) of replicate measurements. Unfilled circles represent samples analysed in Ag capsules only.

Figure 1b: BROC C/N, $\delta^{13}C$ and $\delta^{15}N$ values for each method and acid. Horizontal solid grey lines indicate mean values for each method, and perforated grey lines 1σ. Vertical perforated lines split the untreated, capsule and rinse methods. The transparent grey bar on the C/N and $\delta^{13}C$ plots represents known values. Error bars are calculated as standard deviation (1σ) of replicate measurements. Unfilled circles represent samples analysed in Ag capsules only.

Figure 1c: TYC C/N, $\delta^{13}C$ and $\delta^{15}N$ values for each method and acid. Horizontal solid grey lines indicate mean values for each method, and perforated grey lines 1σ. Vertical perforated lines split the untreated, capsule and rinse methods. Error bars are calculated as standard deviation (1σ) of replicate measurements. Unfilled circles represent samples analysed in Ag capsules only.

Table 2: Mean and standard deviation (1σ) for $\delta^{15}N$ values in SOILB, BROC and TYC.

Table 3: Results of One-Way ANOVA analysis for each sample material for untreated, capsule and rinse methods. Data were tested at the 0.05 significance level.

Discussion

Methodological differences in $\delta^{15}N$

As reported for C/N and $\delta^{13}C$ data [18], the measured $\delta^{15}N$ values show evidence of significant within (affect of acid reagent strength and capsule type) and between (untreated versus capsule method versus rinse method) method differences. Across all sample materials, the differences in sample data between methods (up to ~1.5 ‰) are significantly greater than the instrument precision (~ 0.3 ‰). The untreated method is the most common approach in the literature [e.g. 4, 22], though simultaneous $\delta^{13}C$ and $\delta^{15}N$ measurements ("dual-mode" analysis), which require acid removal of carbonate, are increasingly common [e.g. 4, 23, 24, 34]. Our results show significant differences in measured $\delta^{15}N$ value between acid treatment methods *and* within untreated samples (Figures 1A-C). For example, data for SOILB and TYC from acid pre-treated samples were significantly different from untreated by up to ~1.5 ‰, with the overall range in the untreated method ~0.5 ‰ (excluding the samples combusted only in Ag capsules). In the rinse method, $\delta^{15}N$ may be biased by the loss of soluble organic and/or inorganic N species [e.g. 35, 36], or very fine particles [e.g. 14, 18; 36, 37, 38, 39]. For example, the loss of ^{14}N rich species (e.g. protein; ammonium) would increase the $\delta^{15}N$ value [e.g. 35]. This suggests problems of reliability on $\delta^{15}N$ measurements, particularly from acid treated samples. This has implications for the applicability of any one method in a short or long core, where type, amount and nature of OM can vary significantly, and on modern organic materials.

These findings partially agree with Kennedy [22] and Fernandes and Krull [28], who find that the precision of acid treated samples was equal to or less than that of untreated samples. Interestingly, Kennedy et al [22] find no significant difference between results, and suggest that measurements of $\delta^{15}N$ on acid treated samples as an appropriate methodology whereas Fernandes and Krull [28] report differences of up to 2 ‰ (offsets due to volatilisation (capsule method) and solubilisation (rinse method)), and suggest measurement on untreated material. Such variability suggests the different organic chemical composition of sample materials from different environments can influence the reliability of isotopic C and N values obtained using these acid treatment procedures necessitating an understanding of the complexities of sample OM within and between environments [18, 28]. This has significant

interpretative implications for $\delta^{15}N$ in sedimentary records. Published studies have reported $\delta^{15}N$ data ranges in the order of ~1 – 5 ‰, on surface samples, short cores [e.g. 7, 20, 23, 40, 41], and long cores [e.g. 16, 24, 32, 33, 42, 43]. The differences reported here for BROC (~ 0.1 – 1.2 ‰), SOILB (~ 0.2 – 1.3 ‰) and TYC (~ 0.8 – 1.6 ‰) are only a function of pre-analysis acid treatment (i.e. an artefact), and therefore have the potential to significantly influence environmental interpretation of the data. Where different sample preparations (e.g. untreated versus acid treated) or analytical methods (e.g. "single" versus "dual" mode isotope analysis) are followed, this will preclude the comparison of $\delta^{15}N$ from different down-core records.

Effect of Capsule type

It has been shown that there is a systematic depletion in $\delta^{13}C$ in aquatic material within the capsule method for samples further wrapped in Sn (Ag+Sn)[18]. We therefore test whether capsule type influenced measured $\delta^{15}N$ results. For all sample materials, untreated sample aliquots wrapped only in an Ag capsule showed significantly lower $\delta^{15}N$ values in comparison to all other untreated sample aliquots (unfilled circles on Figures 1A – C). The absence of Sn in the combustion process has an influence on the overall combustion temperature as there is no additional exothermal heat supply through the Sn oxidative reaction. This, combined with the 900°C combustion temperature, which is lower than traditionally used in C/N and $\delta^{13}C$ analysis of OM (~ ≥ 1000°C), likely has a direct influence on the ease with which labile and recalcitrant components of the sample materials react. The presence of Sn appeared to have less marked influence on results for acid treated samples, but this might be due to the acidification process obscuring differences between labile and recalcitrant components [18, 28].

Alongside the potential for offsets in elemental and isotopic C and N due to the acid reagent and/or method followed, it appears that the capsule material in which the sample is combusted, may also influence data distribution. This has implications for the comparison of data between methods, and between laboratories (because the rinse method uses Sn capsules and the capsule method Ag capsules) in addition to the likelihood for offsets linked to the type, amount and nature of OM [28].

Coupled offsets in $\delta^{13}C$ and $\delta^{15}N$

A comparison of $\delta^{13}C$ and $\delta^{15}N$ within and between methods clearly indicates that method induced alterations of the sample OM can influence measured $\delta^{13}C$ and $\delta^{15}N$ values in a disproportionate and non-linear fashion. $\delta^{13}C$ and $\delta^{15}N$ shifts are not consistent or in the same direction across the methods in any one material analysed (Figures 1A-C). For BROC, relative to untreated samples, the capsule method showed a slight enrichment in $\delta^{13}C$ of ~0.2 ‰ (and slight increase in C/N value) coupled with a depletion in $\delta^{15}N$ of ~0.6 ‰ relative to untreated. This is in contrast to the results in the rinse method, which show a depletion in $\delta^{13}C$ of ~1.2 ‰ (C/N value increased by ~3 – 6), and an enrichment in $\delta^{15}N$ of ~0.7 ‰ relative to untreated. For the capsule method, the results suggest a loss of ^{15}N enriched compounds and no significant change in $\delta^{13}C$ but for the rinse method, a loss of ^{13}C enriched compounds, but no differential loss of ^{15}N compounds (which is not too dissimilar to untreated values).

The differential loss of ^{15}N rich compounds has previously been reported from pre-analysis acid treatment methods [e.g. 1, 2, 3, 4], through solubilisation or volatilisation [e.g. 18, 26, 27, 28, 44]. It has been proposed that losses or alterations to proteins, nucleic and amino-acids are the primary cause [e.g. 26, 44]. These compounds also tend to be relatively deficient in ^{13}C, suggesting the trends noted in BROC in the rinse method are from these nitrogenous compounds with losses greater than for the capsule method. Fernandes and Krull [28] also reported greater losses in the rinse method by at least double. The capsule method showed a depleted $\delta^{15}N$ signature, but no significant change in $\delta^{13}C$ from known values. This clearly indicates that both $\delta^{13}C$ and $\delta^{15}N$ can be significantly affected by acid preparation method, but more importantly can also vary independently of one another (i.e. in significantly different proportions). The different types of N (and C) within a sample (organic and inorganic, and there relative proportions) can respond variably suggesting that the magnitude of the method affect will vary considerably between sample materials (i.e. different environments representing different OM provenance and composition). We therefore warn of the dangers of using 'dual-mode' analysis without a robust understanding of sample OM in the context of pre-analysis acid treatment effects on N (and C). Additionally, where sample size of C and N of OM is small, acid treatment induced alterations of OM can promote less reliable data which further undermines a "dual-mode" analysis approach [e.g. 18]. Sample mineralogy may also influence the precision of C and N data [e.g. 28].

$\delta^{13}C$ and $\delta^{15}N$ cross-plots

Cross plots of $\delta^{13}C$ and $\delta^{15}N$ have been used to indicate OM provenance [e.g. 7, 45] in a similar fashion to C/N and $\delta^{13}C$ [e.g. 46, 47, 48], though the extent to which acid treatment method could influence the distribution of data on these plots has not been discussed. Brodie et al [18] reported this method bias on C/N and $\delta^{13}C$ cross-plots, illustrating the method and acid used could dictate the spread of data and subsequent environmental interpretation. This provides justification for examining $\delta^{13}C$ against $\delta^{15}N$. Our $\delta^{13}C$ and $\delta^{15}N$ cross-plots for each material similarly illustrate this bias (Figure 2). This may consequently preclude a robust interpretation, especially in environments where the amount, type and nature of OM varies and, there is more than one OM end member (i.e. rivers, estuaries, lakes, marine environments).

Figure 2: Bi-plots of $\delta^{13}C$ and $\delta^{15}N$ values of SOILB (A), BROC (B) and TYC (C) data. The filled circle represents untreated samples; the unfilled circle represents rinse method samples and; the filled triangle capsule method samples. Error bars represent the standard deviation (1σ) of triplicate treatments for $\delta^{13}C$ and $\delta^{15}N$. As discussed, samples analysed in Ag capsules only have been discarded from further comparison and are not represented in these plots.

Inorganic Nitrogen

An important assumption underpinning the interpretation of %N, C/N and $\delta^{15}N$ values of OM is that all sample N is OM bound, and that any IN component is insignificant in terms of error on data and subsequent interpretation [e.g. 21]. However, it is not common place to quantify IN of sample materials, despite the potential influence on organic %N and C/N values especially in samples containing low OC [19, 20, 21, 31, 32, 49]. For example, Schubert and Calvert [20] reported C/N values of Arctic Ocean surface sediments from total N ranging from 4 – 8, and from organic N from 8 – 15. This represents a clear shift in interpretation from OM dominated by aquatic biomass to OM with an increasing terrestrially sourced component. Despite this, measurements for %N and $\delta^{15}N$ are commonly reported, whether on treated or untreated samples.

In lieu of IN quantification, it has been proposed that the IN content can be estimated through a regression of %C and %N values assuming a perfect linear relationship and using the calculated r^2 value. The estimation of IN content as a percentage deviation from that perfect linear relationship is calculated from $(1 - r^2)$ x 100 [e.g. 7, 19, 45]. The approach of Muller [19], Meyers [45] and Hu et al [7] is the regression of a number of samples within a large system (e.g. lacustrine and estuarine surface samples), whereas our approach is sample specific (i.e. one sample rather than numerous spatial distributed samples). We follow this IN estimation method for our analysed sample materials by linearly regressing data from all methods for each material (providing one r^2 statistic for the data – the "best fit") and then bootstrapping the data (i.e. computing 2000 iterations of the linear regression model on the data to assess the distribution of the r^2 statistic across the data set; this allows an assessment of the influence of acid treatment method on the spread of the data relative to the "best fit"). If acid treatment method has little to no influence on the spread of the %C and %N data, there should be little deviation from the "best fit" scenario, and hence little to no change in the estimation of IN.

Based on our bootstrapped r^2 estimates, and subsequent calculation of percentage IN, ~60 – 86% of BROC, ~27 – 49% of SOILB and ~ 55 – 75% of TYC may be attributed to IN (i.e. variation in %N explained by the regression model). The spread of data shown in Figures 3A-C represents i) method bias; and ii) the true C and N distribution, and indicates a limitation to the interpretation of C/N values. These estimation ranges for IN content point toward an emergent linearity within our sample data, suggesting that the data be non-normally distributed either by nature, or by bias due to acid treatment method. This violates the key assumption of normality in the linear regression model and suggests an analysis of numerous spatially distributed samples [e.g. 7, 19] will propagate significant (but unrecognised) bias onto the interpretation of %C, %N and C/N values.

The overall statistical approach is misleading, and assumes i) a dependence of %N on %C in sample OM within and between environments; ii) a systematic and proportional co-variability of both pools within sample OM during sample pre-treatment and; iii) the offsets from this are related to intrinsically non-linear IN processes. In the context of different formation, transportation and diagenesis processes, across significantly different environments, an emergent linearity from a complex C and N system is unlikely to be real, particularly across diverse systems where the OM composition and structure varies. There is no apparent

mechanism promoting linearity of C and N in a specific sample or indeed in a system, suggesting that the emergent linearity reported here can be substantially dictated by method. In addition, the disproportionate and non-systematic variability in elemental C and N concentrations previously reported [18], suggests that this procedure of estimation is further undermined, even if a non-linear relationship is assumed (which would likely lead to significantly greater bias). The idea that the emergent linearity can be used to derive an understanding of the non-linear IN pool based on the deviation from the presumed perfect linear relationship is incorrect, especially in the context of a down-core profile. Further, it does not reliably improve the understanding of the sample OM. Even if the prescribed linear fit was correct, the assumptions made are seriously flawed. We conclude that the application of this statistical technique for IN estimation, in the context of method bias and the complexity of C and N in the environment, is an aberration and should be abhorred. We recommend that IN be quantified following an organic N digestion process [e.g. KOBr-KOH: 20, 21].

Figure3a: Bi-plot of %C and %N values of SOILB data for untreated (diamond); capsule (square) and; rinse methods (circle).

Figure 3b: Bi-plot of %C and %N values of BROC data for untreated (diamond); capsule (square) and; rinse methods (circle).

Figure 3c: Bi-plot of %C and %N values of TYC data for capsule (square) and rinse methods (circle).

Summary and Recommendations
1. Our results show clear evidence for significant differences in measured δ^{15}N values between pre-analysis acid treatment methods and between untreated samples. The bias is of the order of ~ 1.5 ‰.
2. Differences between δ^{13}C and δ^{15}N on acid treated samples are non-linear and disproportionate. This implies that the type, amount and nature of OM, and its potential alteration during pre-analysis acid treatment methods, is an important factor underpinning the reliable determination of C/N, δ^{13}C and δ^{15}N of sample OM. Importantly, these offsets can significantly undermine environmental interpretation of δ^{15}N values.
3. Capsule type can have a significant influence on the reliability of δ^{15}N in sample OM. Untreated samples combusted only in Ag capsules particularly produce aberrant results

(exclusive of bias linked to acidification). We therefore recommend further wrapping capsule method samples (which traditionally only use Ag capsules) in Sn capsules after acid treatment as this increases the combustion temperature in the reactor column. This is recommended with the codicil that the affect of acidification could obscure the affect of the capsule.

4. We do not recommend "dual-mode analysis" of sample materials due to these unpredictable, non-linear differences, which suggest its inapplicability. Samples should be processed and analysed for C and N on separate aliquots, in the knowledge of the potential for acid induced offsets. It should be noted that measurement of N on untreated samples does not guarantee reliable results.

5. The estimation procedure used for IN, namely linear regression, is seriously flawed, and provides no sensible or reliable information on the influence of IN on measured %N (and hence influence on C/N values) or $\delta^{15}N$ values of OM. IN should therefore be quantified on separate sample aliquots, and corrected for [e.g. 20, 21].

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SOILB
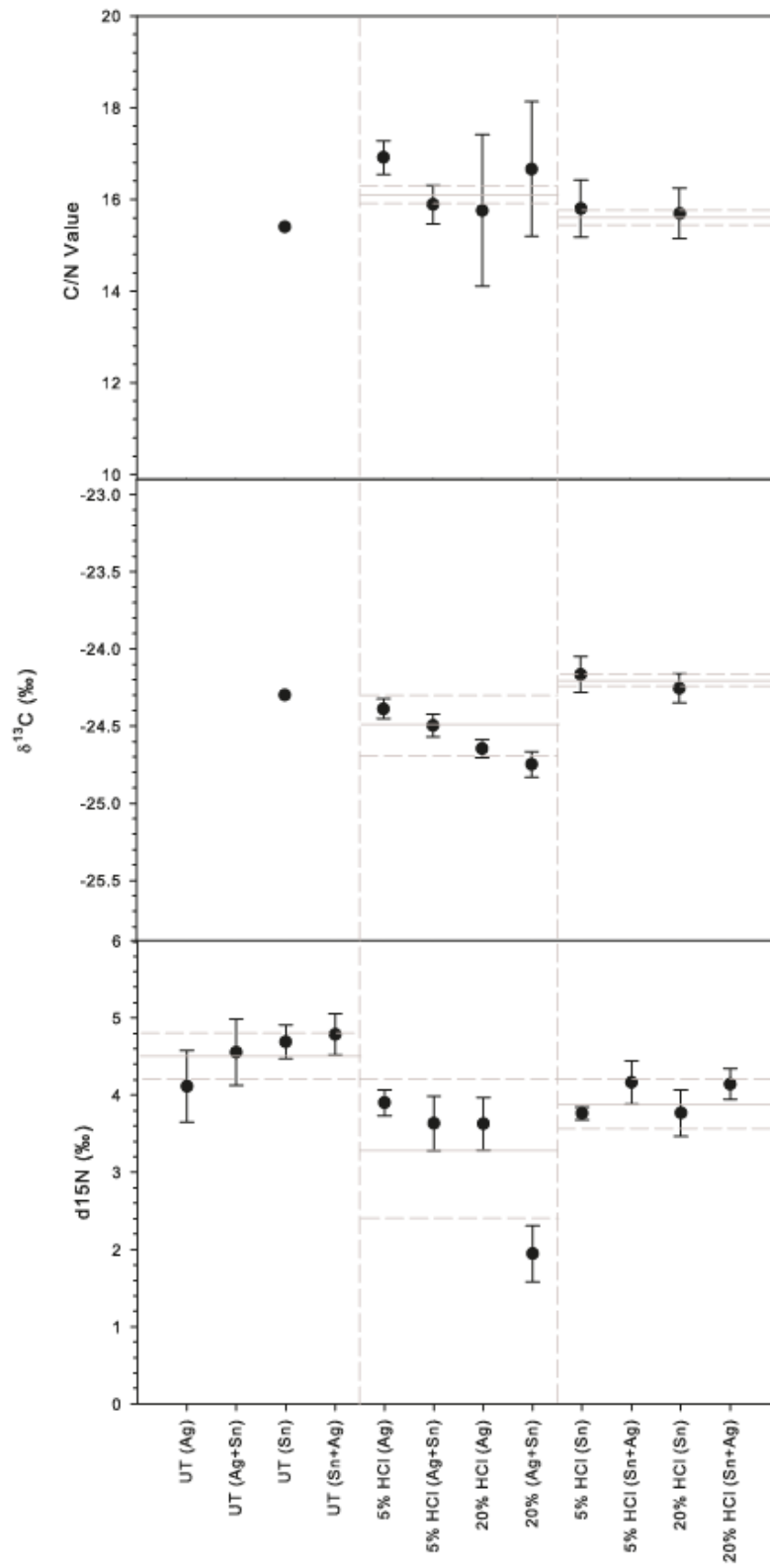
BROC
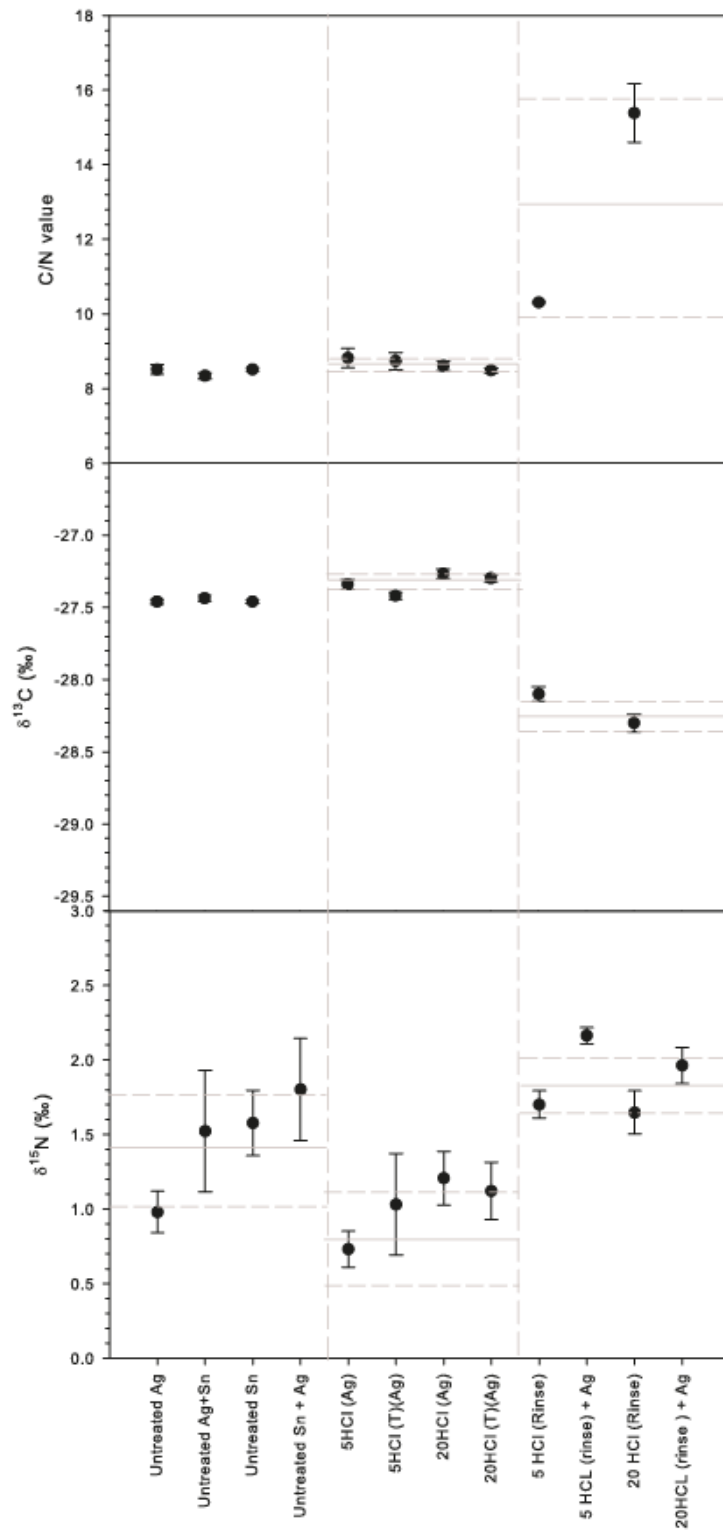
TYC
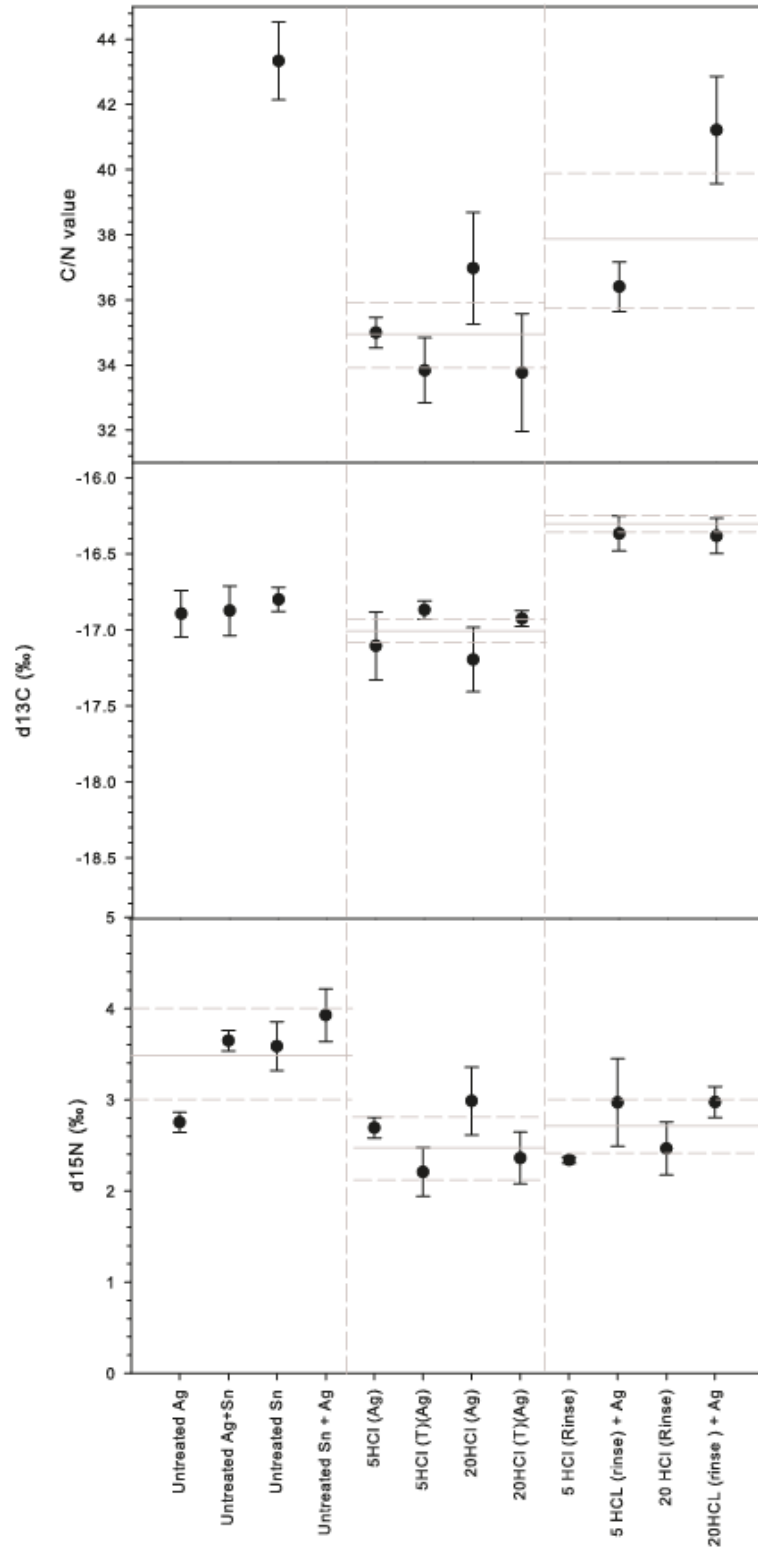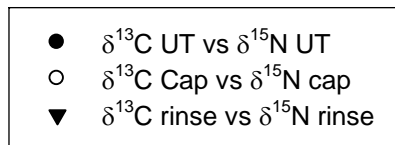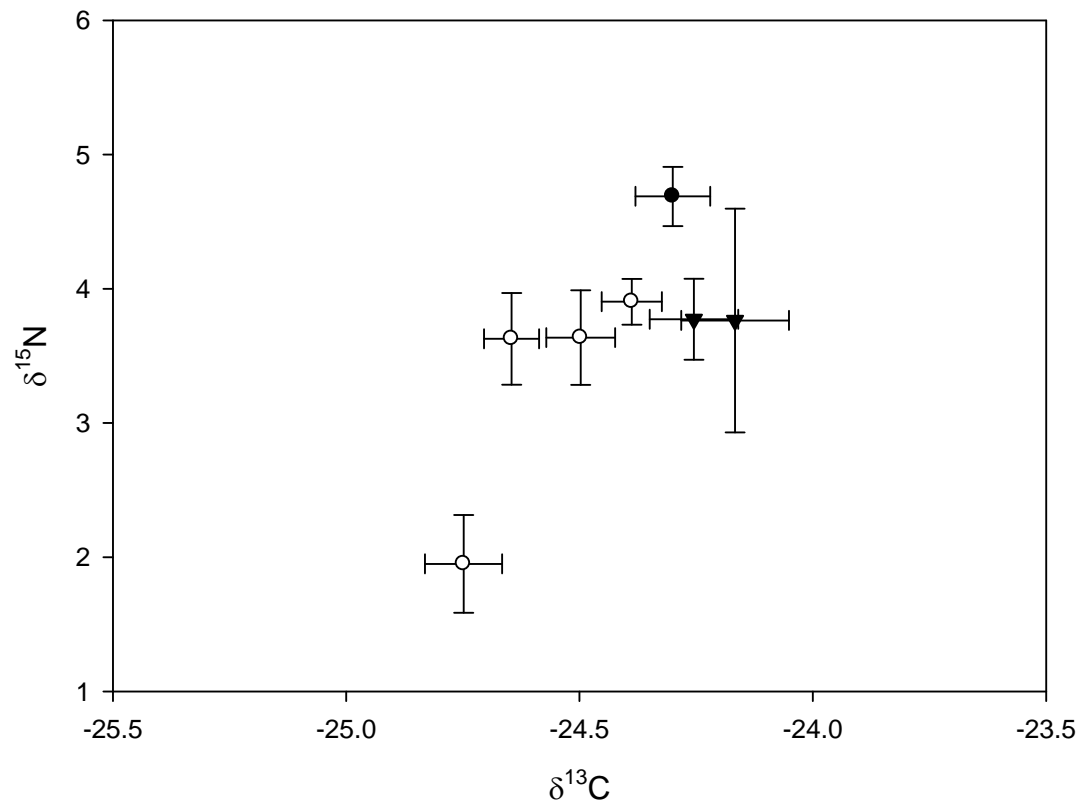
Table 2: Description of sample materials, with values for %C, %N, C/N and $\delta^{13}C$ measured on untreated sample material. %C, %N and C/N were calibrated against ???; and $\delta^{13}C$ values versus VPDB against standards NBS-18 and NBS-19. (Adapted from Brodie et al (In review))

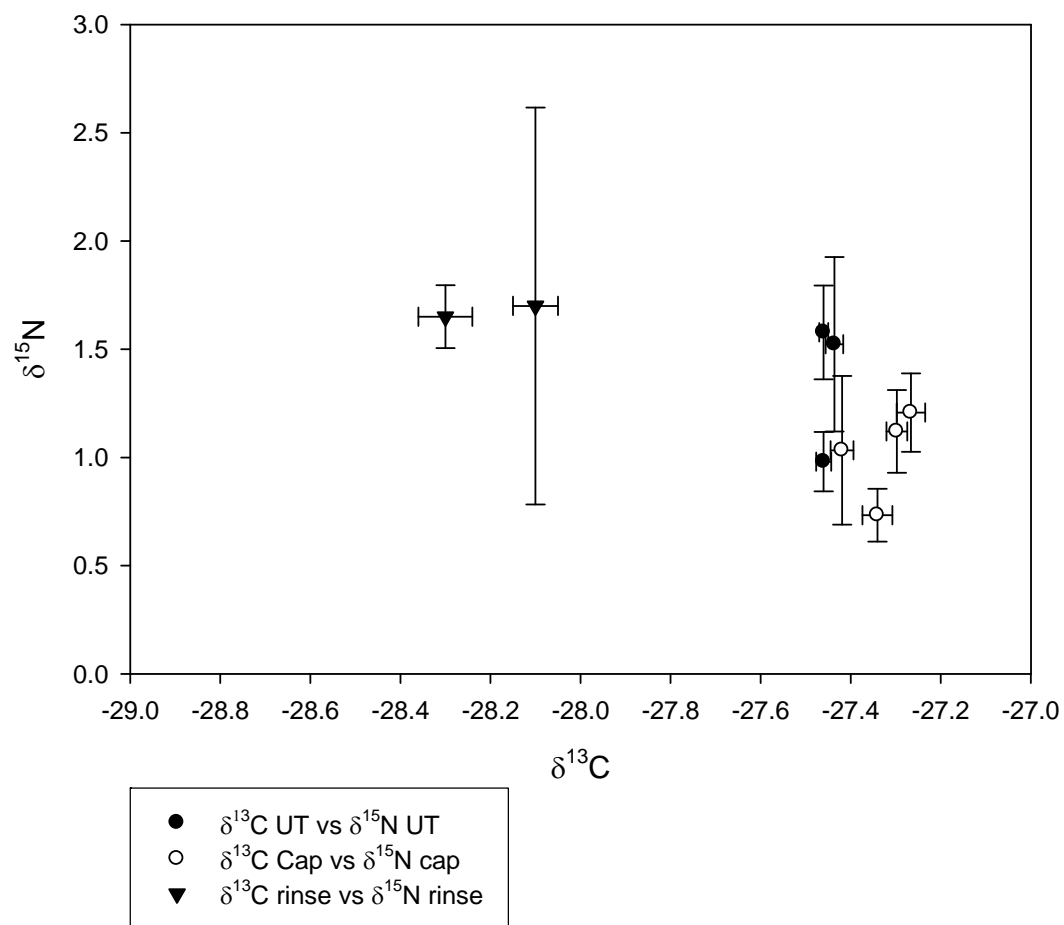| Sample Identifier	Description	%C	%N	C/N	$\delta^{13}C$ (‰)
SOILB	International soil standard from LECO corporation	3.00±0.05	0.20±0.01	15.4	−24.3±0.1
BROC	*Brassica oleracea* (broccoli) florets, used as an NIGL laboratory standard.	41.8±0.05	4.4±0.01	9.5	−27.4±0.1
TYC	Down-core lake sediment sample from maar Lake Tianyang, south China (6.62m depth).	5.82±0.05	0.14±0.01	41.6	−16.8±0.2

Table 3: T-test results of δ^{15}N values

	BROC			SOILB			TYC		
	T-Statistic	P-Value	Significant?	T-Statistic	P-Value	Significant?	T-Statistic	P-Value	Significant?
UT v cap and UT v rinse									
UT (Ag) V 5% HCl	2.46	0.06	NS	0.74	0.50	NS	0.67	0.55	NS
UT (Ag) V 20% HCl	-1.89	0.12	NS	1.46	0.22	NS	-1.05	0.41	NS
UT (Sn) V 5% HCl (rinse)	-0.91	0.40	NS	6.81	0.00	99%	10.21	0.00	99%
UT (Sn) V 20% HCl (rinse)	-0.49	0.64	NS	5.02	0.00	99%	5.44	0.01	99%
UT (Ag+Sn) v 5% HCl (T)	1.68	0.15	NS	2.87	0.05	95%	8.59	0.01	99%
UT (Ag+Sn) v 20% HCl (T)	1.56	0.18	NS	8.00	0.00	99%	7.31	0.02	99%
UT (Sn+Ag) v 5% HCl (rinse+Ag)	0.49	0.64	NS	3.14	0.02	95%	3.12	0.09	NS
UT (Sn+Ag) v 20% HCl (rinse+Ag)	-0.76	0.48	NS	3.54	0.01	95%	5.87	0.00	99%
Capsule V rinse									
5% HCl (Ag) v 5% HCl (Sn)	-11.06	0.00	99%	1.29	0.27	NS	5.21	0.04	99%
20% HCl (Ag) V 20% HCl (Sn)	-3.29	0.30	95%	-0.54	0.62	NS	1.92	0.15	NS
5% HCl (Ag) v 20% HCl (Sn)	-8.39	0.00	99%	0.67	0.54	NS	1.27	0.33	NS
5% HCl (Sn) v 20% HCl (Ag)	-4.22	0.05	95%	-0.67	0.54	NS	3.01	0.10	NS
5% HCl (Ag+Sn) v 5% HCl (Sn+Ag)	-5.66	0.01	99%	-2.04	0.11	NS	-2.41	0.10	NS
20% HCl (Ag+Sn) v 20% HCl (Sn+Ag)	-6.43	0.00	99%	-9.14	0.00	99%	-3.22	0.05	95%
Capsule method comp.									
5% HCl (Ag) v 20% HCl (Ag)	-3.81	0.02	95%	1.25	0.28	NS	-1.31	0.32	NS
5% HCl (Ag) v 5% HCl (Ag+Sn)	-1.44	0.22	NS	1.20	0.30	NS	2.89	0.10	NS
20% HCl (Ag) v 20% HCl (Ag+Sn)	0.57	0.60	NS	5.81	0.00	99%	2.32	0.10	NS
5% HCl (Sn+Ag) v 20% HCl (Sn+Ag)	-0.39	0.72	NS	5.75	0.01	99%	-0.68	0.55	NS
Rinse method comp.									
5%HCl (Sn) v 20% HCl (Sn)	0.53	0.632	NS	-0.04	0.97	NS	-0.74	0.54	NS
5% HCl (Sn) v 5% HCl (Sn+Ag)	-7.45	0.01	99%	-2.39	0.08	NS	-2.28	0.15	NS
20% HCl (Sn) v 20% HCl (Sn+Ag)	-2.88	0.06	NS	-1.79	0.15	NS	-2.63	0.08	NS
5% HCl (Sn+Ag) v 20% HCl (Sn+Ag)	2.61	0.12	NS	0.09	0.93	NS	-0.01	0.99	NS
Untreated comparisons									
UT (Ag) v UT (Sn)	-4.96	0.00	99%	-2.45	0.05	95%	-6.15	0.00	99%
UT(Ag+Sn) v UT (Sn+Ag)	-1.11	0.32	NS	-0.96	0.38	NS	-1.92	0.11	NS
UT (Ag) v UT (Ag+Sn)	-2.52	0.9	NS	-1.21	0.29	NS	-9.89	0.00	99%
UT(Sn) v UT(Sn+Ag)	-1.24	0.26	NS	-0.63	0.55	NS	-1.93	0.10	NS
UT (Ag) V UT (Sn + Ag)	-4.88	0.01	99%	-2.67	0.04	99%	-8.18	0.00	99%
UT (Sn) v UT (Ag+Sn)	0.25	0.82	NS	0.60	0.57	NS	-0.46	0.67	NS

SOILB

BROC

Legend:
- ● $\delta^{13}C$ UT vs $\delta^{15}N$ UT
- ○ $\delta^{13}C$ Cap vs $\delta^{15}N$ cap
- ▼ $\delta^{13}C$ rinse vs $\delta^{15}N$ rinse

TYC

BROC

$y = 0.067x + 1.659$

$r^2 = 0.40$

%N

%C

1
2
3
4
5
6
7

SOILB

$y = 0.05x + 0.04$
$r^2 = 0.73$

%N

%C

8
9
10
11
12
13
14

TYC

$y = 0.025x + 0.015$
$r^2 = 0.45$

15
16
17
18
19
20
21