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The effect of acidification on the determination of organic carbon, total nitrogen and their stable isotopic composition in algae and marine sediment

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We investigated the effects of sample acidification on the stable carbon and nitrogen isotopic composition ($\delta^{13}C$ and $\delta^{15}N$), as well as the organic carbon (OC) and total nitrogen (TN) composition, of an algal culture and a marine sediment. Replicate measurements of untreated and acid-treated samples were made using 1 M, 2 M and 6 M HCl, 6% H_2SO_3 and 1 M H_3PO_4. For all treatments the precision of the analysis for the acid-treated sample was equal to or less than that in the non-acidified sample. For the algae, analysis of variance (ANOVA) indicated no significant differences in the mean OC and TN concentration, or $\delta^{13}C$ and $\delta^{15}N$ composition, between any acid treatment and non-acidified samples. For the sediment sample a comparison could only be made between the different acid treatments because the untreated contained significant amounts (~30%) of carbonate carbon. ANOVA indicated that the mean OC determined in sediment samples after the 1 M HCl treatment and the mean $\delta^{13}C$ values after the 6% H_2SO_3 and 1 M H_3PO_4 treatments were significantly different ($p < 0.013$ and <0.05, respectively) from all other treatments. Mass balance calculations indicate that in some instances $\delta^{13}C$ values were biased due to a contribution from unreacted carbonate carbon. There were no significant differences in the mean TN between any acid-treated and non-acidified samples. The mean $\delta^{15}N$ values after 6 M HCl, 6% H_2SO_3 and 1 M H_3PO_4 treatments were significantly different from the untreated sediment sample ($p < 0.044$). Based on the significant bias observed for the $\delta^{15}N$ and $\delta^{13}C$ values, a weak (1–2 M) HCl solution is confirmed as the most appropriate acid for the removal of inorganic carbon from natural materials requiring elemental and isotopic analysis. Copyright © 2005 John Wiley & Sons, Ltd.

In any organism, the biochemical processes that are intimately associated with the assimilation and incorporation of elements into their tissues leave a geochemical signature that can be related to an element's source and mode of incorporation. Because the chemical and isotopic compositions of food and water depend on environment, then the elemental and stable isotope ratios of carbon and nitrogen incorporated into plant and animal tissues can provide information on their physiology, environmental conditions and/or diet. Organic matter synthesis and calcium carbonate precipitation both provide suitable repositories that can be interrogated to yield current and/or palaeo-information of ecological importance.

While the determination of total nitrogen (TN) and its isotopic composition ($\delta^{15}N$) requires no sample pre-treatment, analysis of organic carbon (OC) and its isotopic composition ($\delta^{13}C$) usually calls for the prior removal of inorganic carbon, calcium carbonate ($CaCO_3$), as they have very different $\delta^{13}C$ signatures. Two procedures are commonly employed to prepare samples for stable isotope analysis of organic material. First, $CaCO_3$ can be removed using acid and the remaining material rinsed and dried, or alternatively decalcification can be undertaken *in situ*, within sample cups, and the sample re-dried.

The rinsing procedure has been shown to significantly alter elemental OC and TN concentrations[7,17] and their isotopic composition.[1,8,19,20] The causes of the shifts in elemental and stable isotopic composition are most likely due to the leaching, acid solubilisation and removal of a fraction of the original organic matter. The fraction lost and the isotopic composition of this material are variable and possibly depend on the nature and lability of the organic matter being treated. It has been found that treatment with phosphoric acid, hydrochloric acid and acetic acid significantly alters the C and N contents of labile material like seagrass tissues.[17] Even in materials that are likely to contain a large proportion of refractory organic matter it has been reported that 5–45% of the original organic carbon can be solubilised and lost during rinsing of marine sediments.[7]

In studies where the isotopic composition has been monitored, the results after rinsing have been contradictory.

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No difference was found in $\delta^{15}N$ or $\delta^{13}C$ between acid-treated or untreated samples of marine biota.[5] For labile organic matter such as plant material rinsing has been reported to cause little change in $\delta^{13}C$ ($\leq$0.03‰) but a larger change for $\delta^{15}N$ ($\leq$0.3‰).[1] In more refractory material, such as sediments, acidification and washing can cause small differences in $\delta^{13}C$ (mean 0.3‰, max 0.7‰) and $\delta^{15}N$ (max deviation 0.35‰) between rinsed and unrinsed samples.[20,23] Because of these artefacts, many authors now suggest that separate analyses of samples should be made, with no acid treatment for $\delta^{15}N$ analyses and acidification and rinsing for $\delta^{13}C$ analyses.[2,12]

To avoid any artefacts due to rinsing, the addition of acid directly to a sample cup has been encouraged.[17,18,22] Even with these precautions, variability in elemental data has suggested that the use of some acids (H_2SO_3, H_3PO_4) can result in organic matter loss from the sample.[11,13] Surprisingly there has been no systematic study of variability in stable isotopic composition using a range of different acids, although it has been reported[3,14] that no significant change in $\delta^{13}C$ or $\delta^{15}N$ occurred in suspended organic matter treated to acid fuming (12 M HCl) or in animal tissues between treated (1 M HCl) and untreated samples.

The use of dual isotope analysis in stable isotope applications is increasing and often mass balance equations are used in the interpretation of the data, requiring concurrent analysis of elemental C and N concentrations. However, most studies of the integrity of sample composition during preparation have focused on either elemental or isotopic changes and often only relate to carbon or nitrogen rather than to both elements. Because of the known problems associated with acidification, our aim was to undertake a systematic study that could determine the veracity of the *in situ* acidification technique for both the elemental (OC, TN) and the stable isotopic ($\delta^{13}C_{OC}$ and $\delta^{15}N_{TN}$) composition of samples. For a rigorous test we chose samples that represented both fresh, labile, $CaCO_3$-free (algal culture) and refractory, $CaCO_3$-rich (marine sediment) organic matter. We used a range of commonly used acids and, where possible, made direct comparisons between acidified and non-acidified samples.

EXPERIMENTAL

Samples

For an algal sample a naked dinoflagellate was used to obtain $CaCO_3$-free labile organic matter. The material was aggregated by centrifugation from an actively growing culture of *Rhinomonas reticulata*, was dried at 40°C and homogenised by grinding. The marine sediment was obtained from a box core collected from 15°S 77°W at a depth of 2530 m. A 2 cm section (~30% $CaCO_3$) was dried (40°C) and finely ground.

Sample preparation and analysis

For elemental analysis the method of carbonate removal was similar to previously published protocols.[18] Approximately 5 mg of material from the *R. reticulata* culture and 100 mg from the homogenised marine sediment were accurately weighed in pre-combusted (500°C, 3 h) silver pans. The use of silver pans provides two benefits; first, silver is more resistant to acid than tin or aluminium,[18] and, secondly, the silver is available to react with halogens present in the sample (especially marine samples) or added as part of the treatment. Both types of sample were treated in an identical manner. Five replicates of the culture and the sediment were analysed without any pre-treatment to serve as a control, while another five replicates of each sample type were treated with 1 M, 2 M and 6 M HCl, 6% H_2SO_3 and 1 M H_3PO_4. Acid treatment consisted of repeated additions of small aliquots of acid (up to 530 μL), with the samples being left to dry in a warming oven at 40°C between each acid addition. This procedure was repeated until there was no visual evidence of effervescence. The marine sediment was calcareous, and the different acid treatments required variable additions of acid (530 μL of 1 M HCl, 350 μL of 2 M HCl, 180 μL of 6 M HCl, 400 μL of 6% H_2SO_3 and 250 μL of 1 M H_3PO_4). It has been reported that for some acid treatments increasing aliquots of added acid resulted in a concomitant increased loss of organic nitrogen.[13] To remove bias of this kind, the volume of acid added to the algal culture was the same as was added to the marine sediment (even though this was well in excess of what was necessary to remove any inorganic carbon present in the algal culture). Treatment of the two sample types with the different acids was carried out consecutively to avoid the possibility of acid fumes affecting any other batch of samples while in the warming oven. The C and N elemental composition was determined using a EUROPA Roboprep CN elemental analyser.

For stable isotopic analyses, 100 mg of each material were used and treated with acid in the same manner as that used for elemental analysis and run alongside a no-acid control batch. Upon completion of the acid treatment and oven-drying, the samples were placed in pre-combusted (910°C, 3 h) quartz tubes with copper and copper oxide.[10] The stable C and N isotopic composition was determined on CO_2 and N_2 generated by vacuum combustion and is reported in the δ notation as the ratio of the heavy to the light stable isotope in the material, R_{sample}, relative to that of a standard, R_{std}, with the standards Vienna Pee Dee Belemnite (VPDB) and air, for carbon and nitrogen, respectively, i.e., $\delta_{sample} = 1000\left(\frac{R_{sample}}{R_{std}} - 1\right)$. The gases were separated and collected by vacuum distillation from the same sample, and were analysed on a EUROPA-PDZ GEO 20/20 isotope ratio mass spectrometer ($\delta^{13}C$) and a VG SIRA II dual inlet isotope ratio mass spectrometer ($\delta^{15}N$).

The precision of the determinations was based on the standard deviation from the mean value and is reported as 95% confidence interval, while outliers were assessed using Dixon's test.[4] Acid treatments were compared with one another and with untreated samples where applicable by one way analysis of variance (ANOVA). Means with an ANOVA F-value significant at or less than 5% probability level ($p \leq 0.05$) were considered significantly different.

RESULTS AND DISCUSSION

Elemental analysis

The mean OC value obtained from the non-acidified algal material was 16.9 ± 0.44 mmol C g^{-1} (Fig. 1). The mean of the acidified samples varied from 15.2 ± 1.58 mmol C g^{-1}

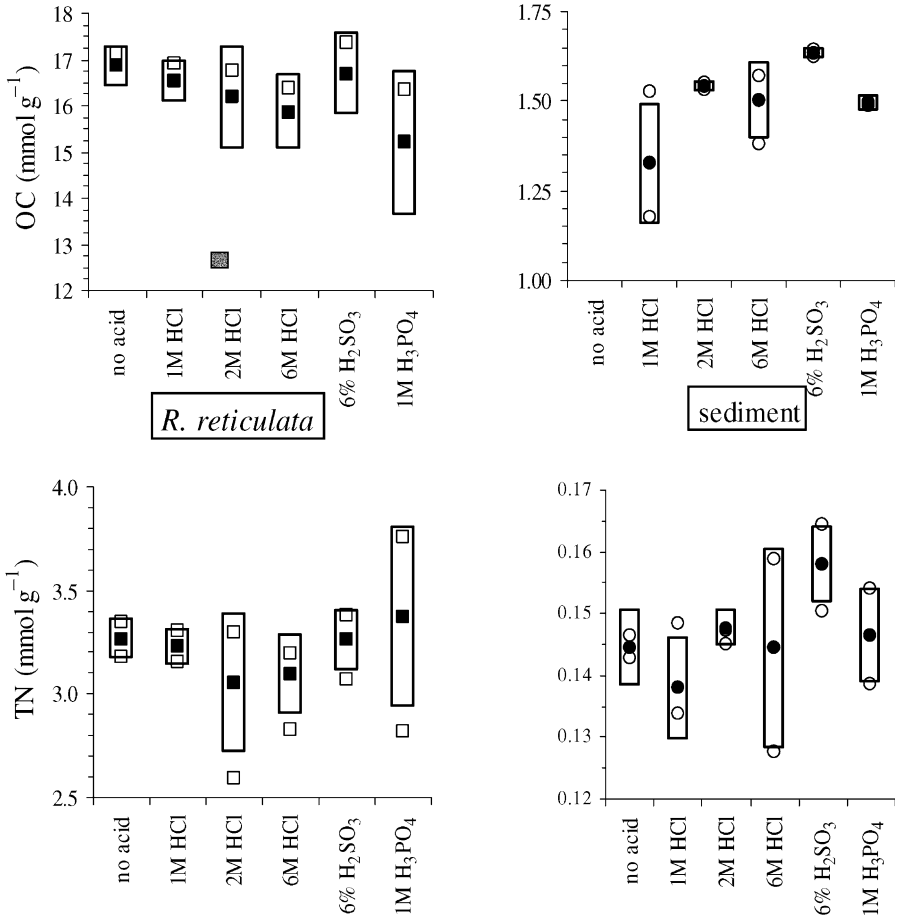

Figure 1. Organic carbon (POC) and nitrogen (PON) content in *R. reticulata* (□, ■) and, POC and total nitrogen (TN) content in marine sediment (○, ●) vs. acid treatment. Enlarged semitransparent symbols indicate outliers (Dixon's test), while open symbols linked with solid line indicate the mean value of replicate determinations per acid treatment (n = 5). The open columns indicate the ±95% confidence interval based on the standard deviation from the mean value.

(1 M H_3PO_4) to 16.7 ± 0.88 58 mmol C g^{-1} (6% H_2SO_3). These losses are equivalent to 1–10% of the OC in the non-acidified algal material and were presumably due to loss of labile (i.e. easily oxidised) organic carbon. The precision of OC determinations on acidified samples of the algal material (±0.44 to ±1.58 mmol C g^{-1}) was generally poorer than in the non-acidified material (±0.44 mmol C g^{-1}). The mean TN value obtained from the non-acidified algal material was 3.3 ± 0.1 mmol N g^{-1}. The mean of the acidified samples varied from 3.1 ± 0.34 mmol N g^{-1} (2 M HCl) to 3.4 ± 0.44 mmol N g^{-1} (1 M H_3PO_4), giving a loss ranging from 6% to greater than 10%. A similar trend of reduced precision after acid treatment (±0.1 to ±0.44 mmol N g^{-1}) was generally observed for TN determinations on the algal material relative to determinations on non-acidified (±0.1 mmol N g^{-1}) samples. Despite these trends, ANOVA indicated no significant differences ($p > 0.100$) in the mean OC and TN content between acid treatments and between any acid-treated and untreated samples.

The mean OC value obtained from the acidified sediment could not be tested relative to non-acidified material due to the presence of significant amounts of carbonate carbon in the latter (equivalent to ca. 30 wt% $CaCO_3$). The means of the acid-treated sediment varied from 1.3 ± 0.17 mmol C g^{-1} (1 M HCl) to 1.6 ± 0.02 mmol C g^{-1} (6% H_2SO_3). The precision of the OC analysis in the sediment samples was better (±0.02 to ±0.17 mmol C g^{-1}) than that obtained with the algal material. ANOVA between the different acid treatments indicated that the mean of the 1 M HCl treatment was significantly different from all other treatments ($p < 0.013$), and the difference between the means of the 6% H_2SO_3 and 1 M H_3PO_4 treatments was also significant ($p = 0.050$).

The nitrogen value reported for the sediment sample, total nitrogen (TN), represented both ON and ammonium (NH_4^+) that are adsorbed onto the clay.[2] The mean TN value obtained from the non-acidified sediment sample was 0.144 ± 0.006 mmol N g^{-1}. The mean TN after acidification varied from 0.137 ± 0.008 mmol N g^{-1} (1 M HCl) to 0.158 ± 0.006 mmol N g^{-1} (6% H_2SO_3) (Fig. 1) and, in a similar manner to the algal material, represented both net gains and losses of TN. The precision of the TN determinations on acidified samples of the sediment samples (±0.003 to ±0.016 mmol N g^{-1}) in all but one case was poorer than in the non-acidified material (±0.006 mmol N g^{-1}). ANOVA indicated no significant differences in the mean TN concentration between acid-treated and untreated samples ($p \gg 0.050$).

In previous studies where *in situ* acidification of the sample has been employed, sulphurous acid has been shown to volatilise ON resulting in losses ranging from 0–50%, even when relatively refractory organic matter has been analysed.[11,13,16] Additionally, substantial losses of OC have been reported after acidification of algal material with phosphoric acid.[11] In our study, although there was a tendency for TN loss with some acid treatments, none of these differences were significant. In fact the results for the elemental analyses show that there were no significant differences between OC and TN in non-acidified and acidified samples of algal material, or between and TN in non-acidified and acidified samples of sediment. A similar comparison for OC of the sediment could not be made due to the presence of calcium carbonate. The difference between the previously reported elemental nitrogen losses and our study may be due to the temperature at which the samples were dried. While we do not have the evidence to show that our drying temperature of 40°C would cause a significantly smaller effect on the loss of N than drying at 60°C, as used in other studies, this could be a plausible explanation of our results.

Isotopic analysis

The stable carbon and nitrogen isotopic compositions of the non-acidified algal material, $\delta^{13}C$ and $\delta^{15}N$, were -34.2 ± 0.04‰ and -1.2 ± 0.02‰, respectively. After acid treatment the mean $\delta^{13}C$ ranged from -34.1 ± 0.06‰ (6% H_2SO_3) to -34.2 ± 0.14‰ (1 M HCl) and the mean $\delta^{15}N$ ranged from -1.1 ± 0.18‰ (1 M HCl) to -1.3 ± 0.23‰ (1 M H_3PO_4). The precision of the non-acidified algal material was equal to or better than that of the acidified samples ($\delta^{13}C$: 0.06–0.24‰; $\delta^{15}N$: 0.02–0.18‰). ANOVA indicated that all products of acid treatments were indistinguishable from each other and from the untreated material (Fig. 2).

A comparison could not be made between the mean $\delta^{13}C$ values obtained from the acidified and non-acidified sediment due to the presence of significant amounts of carbonate carbon in the latter. The means of the acid-treated sediment varied from -20.0 ± 0.06‰ (6% H_2SO_3) to -21.2 ± 0.01‰ (6 M HCl). The precision of the $\delta^{13}C$ analysis in the acidified sediment samples was very poor for H_3PO_4 (0.69‰), but for all other samples was better (±0.01 to ±0.12‰) than that obtained with the algal material (0.06–0.24‰). ANOVA between the different acid treatments indicated that the means of the HCl treatments were not statistically discernible from one another ($p > 0.08$). The 6% H_2SO_3 and 1 M H_3PO_4 treatments had isotopically heavier mean values than those after HCl treatment by +1.2 to +1.3‰ and +0.6 to +0.65‰, respectively. This positive isotopic shift in the mean $\delta^{13}C$ value after the 6% H_2SO_3 and 1 M H_3PO_4 acid treatments is considerable and statistically significant ($p < 0.05$).

The mean value for the $\delta^{15}N$ of the non-acidified sediment was 7.9 ± 0.12‰. After acid treatment the $\delta^{15}N$ ranged from 7.7 ± 0.03‰ (6% H_2SO_3) to 8.0 ± 0.03‰ (2 M HCl). Analysis of the non-acidified sediment was less precise than that of the acidified samples (±0.01 to ±0.04‰). ANOVA indicated that

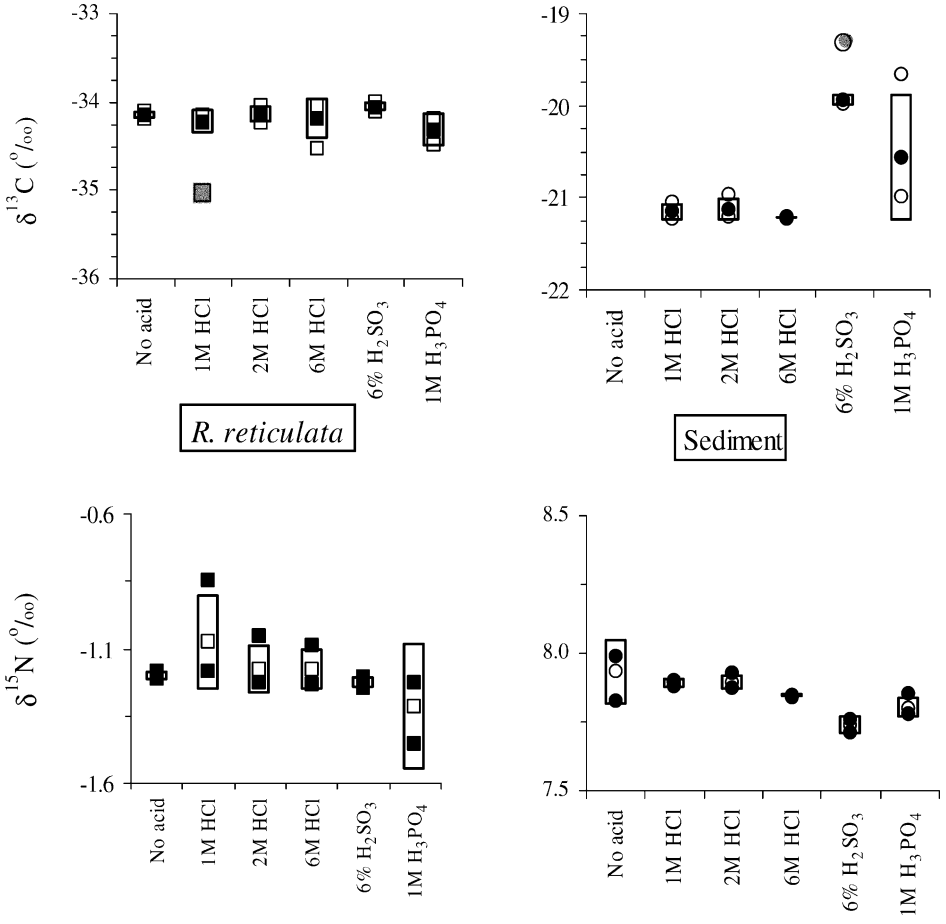

Figure 2. Stable isotopic composition of organic carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in *R. reticulata* (□, ■) and marine sediment (○, ●) vs. acid treatment. Symbols are as in Fig. 1.

the means of the 1 M HCl and 2 M HCl treatments were both indistinguishable from the mean $\delta^{15}N$ value of the untreated marine sediment ($p > 0.340$) and from each other ($p = 0.664$). The means of the 6 M HCl (7.9 ± 0.01‰), 6% H_2SO_3 (7.8 ± 0.03‰) and 1 M H_3PO_4 (7.8 ± 0.004‰) treatments were significantly different from (i.e. isotopically lighter than) the untreated sediment sample ($p < 0.044$), and from one another ($p = 0.007$).

The positive isotopic shift in $\delta^{13}C$ in marine sediment acidified with 6% H_2SO_3 and 1 M H_3PO_4 could have resulted from incomplete removal of carbonate carbon, which is typically more enriched in the heavy carbon isotope than in organic carbon in marine sediments. An estimate of the isotopic composition of carbonate carbon can be made based on mean elemental and isotopic carbon determinations on non-acidified marine sediment sample (i.e. total bulk sediment carbon) and using the mean of the determinations on samples treated with HCl as the best possible estimate of the organic carbon content and its stable isotopic composition in the marine sediment sample. Specifically, the mean total carbon content, C_T, and its $\delta^{13}C$ in the non-acidified marine sediment sample were 4.8 ± 0.06 mmol C g^{-1} and -6.8 ± 0.01‰, respectively, while the overall means obtained after HCl treatment were 1.5 ± 0.07 mmol C g^{-1} and -21.2 ± 0.04‰. By mass balance and propagation of errors,[4] it can be calculated that the stable isotopic composition of bulk carbonate carbon in the marine sediment sample was -0.58 ± 0.11‰. Similarly, using mass balance calculations, the positive isotopic shift observed after the 6% H_2SO_3 and 1 M H_3PO_4 acid treatments could have resulted from the presence of unreacted residual carbonate carbon contributing ca. 6% and 3%, respectively, to the mean carbon determined after these treatments. This contribution of unreacted residual carbonate carbon (0.04–0.10 mmol C g^{-1}) to the OC estimate in the marine sediment sample is small compared with the precision of elemental analysis after any one acid treatment (Fig. 1). Even so, the contribution becomes clearly manifest in the statistically significantly higher elemental yield for 6% H_2SO_3 as discussed earlier (Fig. 1) and in the isotopic composition determined after the 6% H_2SO_3 and 1 M H_3PO_4 treatments (Fig. 2). The source of the unreacted carbonate in the sediment samples may be dolomite as it has been shown that its complete dissolution requires heating to 70°C.[9]

The negative isotopic shift relative to the mean $\delta^{15}N$ of the untreated marine sediment sample, most pronounced after the 6% H_2SO_3 and 1 M H_3PO_4 treatments, was small by comparison with the positive shift observed in the $\delta^{13}C$ of the same material (Fig. 2) but still cannot be explained by the processes that have previously been reported to bias TN and $\delta^{15}N$ analyses. The nitrogen content and isotopic composition determined in marine sediments are influenced by the amount of NH_4^+ adsorbed on, as well as fixed into, the clay mineral matrix.[21] This contribution to the true PON content of marine sediment samples, from the inorganic N pool associated with the mineral matrix, should be the same for all subsamples. In addition, the final, high-temperature combustion step of both elemental and isotopic analyses (~1000°C) should liberate all inorganic N available from the mineral matrix and so its presence should not produce any bias in the analysis. Volatilisation of oxidised nitrogenous organic compounds as NH_3 during acid treatment can reduce the mean TN content[13] and would be expected to generate a positive isotopic shift in the mean $\delta^{15}N$ determined due to the strong positive fractionation, $\varepsilon_{NH_3}^{NH_4^+}$, between the NH_4^+, in solution and the volatilised NH_3 ($\varepsilon_{NH_3}^{NH_4^+} = +19$ to $+21$‰).[6] This scenario contrasts with the slight negative isotopic shift observed and is not supported by the elemental TN analysis on the marine sediment samples. The data could be explained if a small amount of a volatile component is lost with a very positive isotopic composition relative to the total nitrogen fraction. Adsorbed proteinaceous compounds can form a significant component of TN[13] and amino acids can have widely differing (~30‰) $\delta^{15}N$ values.[15] However, the combined effects of the isotopic composition and the susceptibility of N-containing compounds to volatilisation cannot be addressed with the data available.

CONCLUSIONS

For labile organic matter such as fresh algal material there were no significant changes to the elemental or isotopic composition of the organic matter during the acid treatment. The same results were not observed for more refractory material, as represented by the marine sediment. In this case the 6 M HCl, 6% H_2SO_3 and 1 M H_3PO_4 acid treatments resulted in higher OC concentrations and heavier $\delta^{13}C$ values than with the other acid treatments. These results suggest incomplete removal of carbonate, possibly present as dolomite, which requires higher temperatures for full removal. The 6% H_2SO_3 and 1 M H_3PO_4 acid treatments also resulted in significantly more negative values for $\delta^{15}N$ relative to the untreated sediment. The reasons for these changes could not be determined during this study as the isotopic changes occurred without significant loss or gain of TN.

Overall, the results suggest that the use of a weak (2 M) HCl solution yields the most accurate and consistent results and that, with this strength acid, pre-treatment of samples reduces the precision of the analysis, but does not significantly affect the elemental or isotopic composition of the organic matter.

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