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TECHNICAL NOTE

# Technical note: Examining the use of ethylenediaminetetraacetic acid for humic extraction of ancient bone

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## Abstract

We examined the efficacy of ethylenediaminetetraacetic acid (EDTA) for removing humic contaminants from collagen extracted from ancient bone. Humic contaminants must be removed to obtain reliable stable isotope values from ancient bone collagen, given that humic acids have consistently lower $\delta^{13}C$ values than collagen. The purpose of our research was to examine if EDTA treatment could effectively remove humic contaminants from bone collagen and thus serve as an alternative to the commonly implemented sodium hydroxide (NaOH) treatment, which may be associated with large collagen losses in poorly preserved samples. We compared the isotopic and elemental composition of ancient samples treated with EDTA alone, samples demineralized in hydrochloric acid (HCl) and rinsed in EDTA, samples treated with HCl alone, and samples demineralized in HCl and rinsed with NaOH. The samples used in the analyses were selected because they presented evidence of substantial humic contamination. We found that NaOH was the most effective agent for reducing humic contaminants as evidenced by the samples treated with this agent having higher $\delta^{13}C$ values and lower C:N ratios relative to other treatments. The results from samples treated with EDTA suggest that this chemical cannot effectively remove humic contaminants given that these samples had significantly higher C:N ratios and lower $\delta^{13}C$ and $\delta^{15}N$ values relative to the HCl/NaOH treatment. Our results demonstrate that when performing stable isotope analysis of ancient bone collagen suspected to be contaminated with humic acids, NaOH is the most effective chemical agent to remove humic contaminants, while EDTA cannot perform this task.

**KEYWORDS**
bone collagen, EDTA, humic extraction, sodium hydroxide, stable isotope analysis

## 1 | INTRODUCTION

### 1.1 | Humic contaminants in ancient bone collagen

Humic acids are common contaminants in ancient bone samples collected from a variety of different burial environments. These contaminants are closely associated with the collagen helices that are often the analytical substrate of choice for stable isotope analysis (van Klinken & Hedges, 1995). Humic acids are the product of decaying organic material, most of which is derived from primary producers (Hiradate et al., 2004; Sutton & Sposito, 2005). Humic acids in $C_3$ environments are characterized by $\delta^{13}C$ values that are substantially lower than the $\delta^{13}C$ values of bone collagen from terrestrial and marine animals in that environment (Hiradate et al., 2004; Szpak Krippner & Richards, 2017). Additionally, humic acids have a much higher atomic C:N ratio than collagen due primarily to the small

  

proportion of nitrogen and secondarily due to the increased proportion of carbon in humic acids (wt% C of 50%–60% for humics) (Christl et al., 2000; Lobartini et al., 1997). The presence of humic acids is often evident in bone samples because of the impact that the dark coloration of the humics has on the appearance of the bone sample (Schnitzer & Khan, 1975). Ancient collagen samples having higher than expected C:N ratios (DeNiro, 1985; Guiry & Szpak, 2021) can also indicate that these contaminants may be present. Although the exact chemical bonding relationship between collagen amino acids and humic acids is unknown (Tan et al., 2008), it is thought that humic contaminants can be co-extracted with the collagen during typical pretreatment processes, such as demineralization and solubilization, especially when a chemical humic extraction is not conducted (van Klinken & Hedges, 1995). It is, however, critically important to remove these contaminants in order to generate reliable isotopic data. Given the discrepancy in the range of observed $\delta^{13}$C values between bone collagen and humic acids, the presence of humic contaminants will skew the $\delta^{13}$C measurements of the bone collagen samples lower than the endogenous signal in almost all cases (Guiry & Szpak, 2021). The $\delta^{15}$N values of bone collagen can also be affected by humic contamination in a similar manner, with humic acids having lower $\delta^{15}$N values as compared with most collagen samples (Guiry & Szpak, 2021; Szpak Krippner & Richards, 2017). The difference in $\delta^{15}$N values between these two compounds is, however, not believed to be a consequential issue (Guiry & Szpak, 2021) because of the small amount of nitrogen present in the humic compounds compared with the amount found in bone protein (Christl et al., 2000; Lobartini et al., 1997). This is, however, dependent on the magnitude of the difference in the $\delta^{15}$N values between the humic contaminants and the endogenous bone protein. If the $\delta^{15}$N values are extremely diverged, then even a small amount of contamination can have an impact on the stable isotope composition of the bone.

## 1.2 | Methods for the removal of humic contaminants

To decrease the impact of humic contaminants on the stable isotope composition of ancient bone collagen, different extraction methods have been developed to isolate the contaminants from the collagenous proteins. Rinsing with sodium hydroxide (NaOH) post-demineralization is a commonly implemented procedure to remove these contaminants (Brock et al., 2010), which are soluble in basic solution (Kipton et al., 1992). Although generally effective at mostly removing these contaminants (Deviese et al., 2018; van Klinken & Hedges, 1995), treatment with NaOH can be associated with a significant loss of sample material, especially in poorly preserved samples (Chisholm et al., 1983; Szpak Krippner & Richards, 2017). There is also the potential for the generation of salts if samples are not rinsed thoroughly enough after NaOH treatment and prior to solubilization in weak acid, increasing the probability that collagen samples will unnecessarily fail quality control criteria due to lower than expected amounts of carbon and nitrogen (Ambrose, 1990).

As a result of the potential drawbacks associated with NaOH treatment, other extraction methods (both mechanical and chemical) have been explored as potential alternatives. Ultrafiltration, which uses size-based filters to retain the fraction of material greater than a specified molecular weight (often 30 kDa), have been shown to be ineffective at removing these contaminants (Szpak Krippner & Richards, 2017). In fact, humic contaminants may actually be selectively retained alongside the higher molecular weight collagen (Szpak Krippner & Richards, 2017). Treatment with EDTA is an alternative chemical method that has been suggested as a means to remove humic contaminants from bone samples without excessive reductions in collagen yield (Tuross, 2012; Tuross et al., 1988). Nevertheless, there has yet to be any published research supporting this assertion, nor has there been any rigorous methodological studies comparing the effectiveness of EDTA against other treatment methods related to the removal of humic contaminants from bone collagen.

EDTA has been used to remove humic acids from materials such as soils and sludges (Liu & Fang, 2002; Tsai & Olson, 1992). Additionally, it is used as a bone demineralization agent, although there is comparatively little data surrounding EDTA in contrast to more common agents such as HCl (Collins & Galley, 1998; Tuross, 2012; Tuross et al., 1988). EDTA demineralization has been hypothesized to be particularly useful for poorly preserved samples that cannot withstand a treatment in strong acid (Tuross et al., 1988). While there has been a collection of studies comparing different demineralization techniques with EDTA as a treatment, there have been no studies that have sought to test the ability of EDTA to remove humic contaminants from bone collagen. If EDTA could indeed remove humic contaminants, then the demineralization and humic extraction pretreatments could be combined into a single chemical treatment. As a result, the sample loss associated with demineralization and humic extraction could be reduced, making this potential method extremely useful for poorly preserved samples or those from heavily humic-contaminated environments. It is important, however, that EDTA be able to effectively extract the contaminants in the same conditions as is required for demineralization (i.e., a neutral solution of EDTA, at a concentration of ~0.5 M, with treatment occurring for prolonged periods of time). If this is not the case, then these two pretreatments would need to be conducted separately in different EDTA solutions, thus diminishing the benefits of this method and its potential to reduce sample loss.

## 1.3 | Experimental approach

Our research examines the impact of EDTA treatment on the removal of humic contaminants from bone collagen by comparing the elemental and isotopic composition of humic-contaminated collagen samples treated with EDTA against untreated samples. This allows us to identify if treatment with EDTA has a significant impact on the $\delta^{13}$C values and atomic C:N ratios of bone collagen and whether these data suggest that EDTA can or cannot remove these contaminants. We also compare the relative effectiveness of EDTA treatment for humic

removal against the traditional NaOH method, to determine whether the EDTA method provides a substantial benefit in terms of higher collagen yields and improved quality control criteria.

## 2 | METHODS

### 2.1 | Sample selection and preparation

Ancient bone samples collected from different sites occupied between 1000 and 1918 AD from a variety of different species were used in the analyses for a total sample size of 41 (Table S1). The sample set included bones from 35 large marine mammals (*Callorhinus ursinus*, $n = 19$, *Odobenus rosmarus* $n = 7$, *Pusa hispida* $n = 6$, Unknown $n = 3$), 4 large terrestrial mammals (*Rangifer tarandus* $n = 1$, Unknown $n = 3$), and 2 birds (*Alle alle* $n = 2$). The samples chosen for analysis were all well-preserved and produced high collagen yields. Sample selection was biased toward samples that appeared to be humic-contaminated based on a dark coloration of the bone (Figure 1). Sixteen samples for which data had previously been obtained demonstrating a higher-than-expected atomic C:N ratio indicative of humic contaminants were also included in the analyses.

Each bone was cleaned and then cut into small chunks using an Ultimate XL-D micromotor with a diamond-tipped cutting wheel (NSK-Nakanishi International). Bone chunks were then crushed into smaller fractions using a Plattner mortar and pestle. The samples were then sieved to isolate a 1–2 mm size fraction which was used for all subsequent treatments. The material between 1–2 mm was homogenized to minimize any intrabone isotopic variation and then separated into four different culture tubes and weighed.

### 2.2 | Sample pretreatment

For the HCl/0 treatment, 9 ml of 0.5 M HCl was added to each culture tube and the samples were placed on an orbital shaker to increase the rate of demineralization and ensure that the demineralization occurred evenly across sample chunks. The samples were removed from acid when they felt soft when prodded with a glass pipette and/or could be cut through smoothly using a razor blade. The samples were then rinsed in 10 ml of Type I water (resistivity >18.2 MΩ·cm) until the pH of the solution was neutral. Following rinsing, the samples were solubilized in 3.5 ml of 0.01 M HCl for 36 h at 75°C. Post-solubilization, the samples were centrifuged and the liquid fraction containing the soluble collagen was transferred to glass vials. Samples were then frozen for at least 24 h and lyophilized.

The HCl/EDTA-treated samples underwent the same demineralization protocol that was used for HCl/0 samples with an additional step post-demineralization to attempt humic removal. After demineralization and rinsing, 8 ml of 0.5 M EDTA at a pH of 7.4 was added to each sample (Simpson et al., 2016). Samples were placed on an orbital shaker for 30 min and each sample was assessed at the end to determine if a color change in the solution had occurred. If the solution darkened in color during treatment, the EDTA was pipetted away, and an additional 8 ml of new EDTA solution was added. The process of treating the samples in 8 ml of EDTA was repeated for all samples until no color change was visible after 30 min, which took between 2 and 6 treatments depending on the coloration of the sample. Each sample was then rinsed 15 times in 10 ml of Type I water to ensure that any residual EDTA was removed (Tuross et al., 1988). Samples were then solubilized and lyophilized in the same manner as described in the HCl/0 treatment (Figure 2).

The demineralization procedure for HCl/NaOH-treated samples proceeded according to the same protocol as the above two methods (Figure 2). Post-demineralization and rinsing, the samples were placed in 8 ml of 0.1 M NaOH for humic extraction (Beaumont et al., 2010). The samples were placed in the NaOH solution for 30 min on an orbital shaker. If the NaOH solution changed color after 30 min, the solution was replaced and another rinse for 30 min was performed (van Klinken & Hedges, 1995). After treatment with NaOH, the samples were rinsed to neutrality with multiple aliquots of 10 ml of Type I water, then solubilized and lyophilized.

The final treatment (EDTA/0) utilized a different demineralization procedure to attempt to simultaneously remove humic contaminants and demineralize the sample. Each sample was placed in 5 ml of 0.5 M EDTA at a pH of 7.4. Samples were agitated on an orbital shaker throughout the treatment and the solution was changed every 3 days. Once samples appeared to be demineralized

**FIGURE 1** Photographs of two very darkly colored samples used in the analyses. The samples come from marine mammals, although the species are unknown

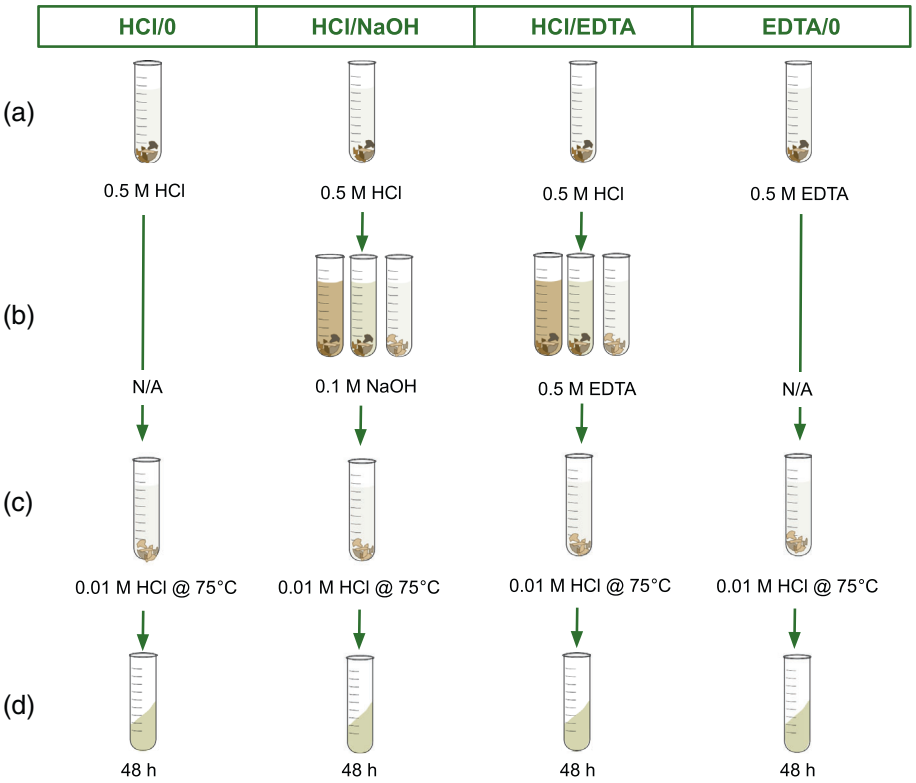


**FIGURE 2** Outline of the major pretreatment steps for each of the different methods used. (a) Demineralization treatment, (b) humic extraction treatment, (c) solubilization, and (d) lyophilization

(according to the criteria described in the first treatment), they were rinsed 15 times in Type I water (Tuross et al., 1988). Solubilization and lyophilization followed the same protocol described in the previous three treatments.

## 2.3 | Stable isotope and elemental analysis

Between 0.5 and 0.6 mg of dried collagen from each sample was weighed into a tin capsule for isotopic and elemental analysis. The analyses were performed in the Trent University Water Quality Center (Peterborough) using a Nu Horizon continuous flow isotope ratio mass spectrometer coupled to a Euro Vector EA 300 elemental analyzer. Results were calibrated relative to atmospheric nitrogen (AIR) for $\delta^{15}$N and Vienna Pee Dee Belemnite (VPDB) for $\delta^{13}$C using USGS40 and USGS66 or USGS63 (Schimmelmann et al., 2016) (Table S2). Analytical precision and accuracy were monitored using the following in-house laboratory reference materials: SRM-1 (caribou bone collagen), SRM-2 (walrus bone collagen), and SRM-14 (polar bear bone collagen) (Table S3). Duplicate samples were also included throughout the analyses (10% duplication rate) to assess the homogeneity of the samples relative to known pure substances such as standards (glycine, caffeine, etc.). The average difference in the $\delta^{13}$C values of duplicates was 0.04‰, while the average difference was 0.06‰ for $\delta^{15}$N values. The analytical uncertainty for the analyses was ± 0.10‰ for $\delta^{13}$C and ± 0.23‰ for $\delta^{15}$N (Szpak Metcalfe & Macdonald, 2017). The stable isotope composition, elemental composition, and collagen yield for each of the samples can be found in Data S1 (Table S1).

## 2.4 | Statistical analyses

Given that our sample set included many different species with a wide variety of feeding ecologies, Wilcoxon tests for paired sample isotopic composition were used because there is a considerable amount of inter-sample variation beyond what may be caused by varying experimental treatment. Mann–Whitney *U* tests were used to compare the elemental composition of samples treated using different chemical agents. A one-way analysis of variance (ANOVA) test was performed to compare the atomic C:N ratios of all samples, given that these should be approximately the same regardless of species and therefore we expect the variation within treatments to be small (Guiry & Szpak, 2020). A post hoc ANOVA test was not performed to compare the differences in atomic C:N ratio among treatments because Mann–Whitney *U* tests provide equivalent information. Collagen yield was calculated by dividing the mass of the final dried product by the initial mass of bone and differences in yield between treatments were also assessed using Mann–Whitney *U* tests. All statistical tests were performed using PAST version 4.05 (Hammer et al., 2001).

# 3 | RESULTS

## 3.1 | Isotopic compositions

### 3.1.1 | $\delta^{13}$C values

Samples treated with NaOH had consistently and significantly higher $\delta^{13}$C values relative to all other treatments (Table 1, Figure 3). The $\delta^{13}$C values of HCl/NaOH samples were on average 0.29‰ higher than the $\delta^{13}$C values of EDTA/0-treated samples (Figure 4, Figure 3b, HCl/NaOH

vs. EDTA/0 comparison). Samples that were not exposed to the NaOH treatment had $\delta^{13}$C values that were lower on average than the NaOH-treated samples. The HCl/0 samples had $\delta^{13}$C values that were not significantly different from the EDTA/0 treatment (average difference of 0.03‰) (Figure 4). The EDTA/0 samples had $\delta^{13}$C values that were not significantly different than the HCl/EDTA samples (Table 1).

### 3.1.2 | $\delta^{15}$N values

The $\delta^{15}$N values were significantly different when comparing the HCl/NaOH samples to any of the other treatment groups (Table 2). On average the $\delta^{15}$N values of HCl/NaOH-treated samples were 0.13‰ higher than samples treated only with HCl (Figure 5, HCl/NaOH vs. HCl/0 comparison). Samples treated solely with HCl had significantly higher $\delta^{15}$N values as compared with EDTA/0 samples (Table 2). The differences in $\delta^{15}$N values were not significant when comparing HCl/0 samples to HCl/EDTA-treated samples (Table 2). The average difference in $\delta^{15}$N values between the HCl/0 and HCl/EDTA samples (0.03‰) was below the range we would expect to observe for normal measurement uncertainty (Figure 5). The EDTA/0 and HCl/EDTA samples were associated with a significant difference in $\delta^{15}$N values, with samples treated only with EDTA having lower $\delta^{15}$N values on average (Figure 5).

**TABLE 1** *p*-values from Wilcoxon tests comparing $\delta^{13}$C values between treatments

| *df* | Treatment | HCl/0 | HCl/NaOH | EDTA/0 |
|---|---|---|---|---|
| 40 | HCl/NaOH | **<0.001** | — | — |
| | EDTA/0 | 0.71 | **<0.001** | — |
| | HCl/EDTA | 0.02 | **0.001** | 0.05 |

*Note*: Bolded values represent *p*-values below the alpha threshold ($\alpha = 0.05$).

## 3.2 | Elemental composition

The atomic C:N ratios were significantly different for all comparisons between HCl/0-treated samples and any of the other pretreatments (Table 3). Results from a one-way analysis of variance indicate that sample treatment does have a significant

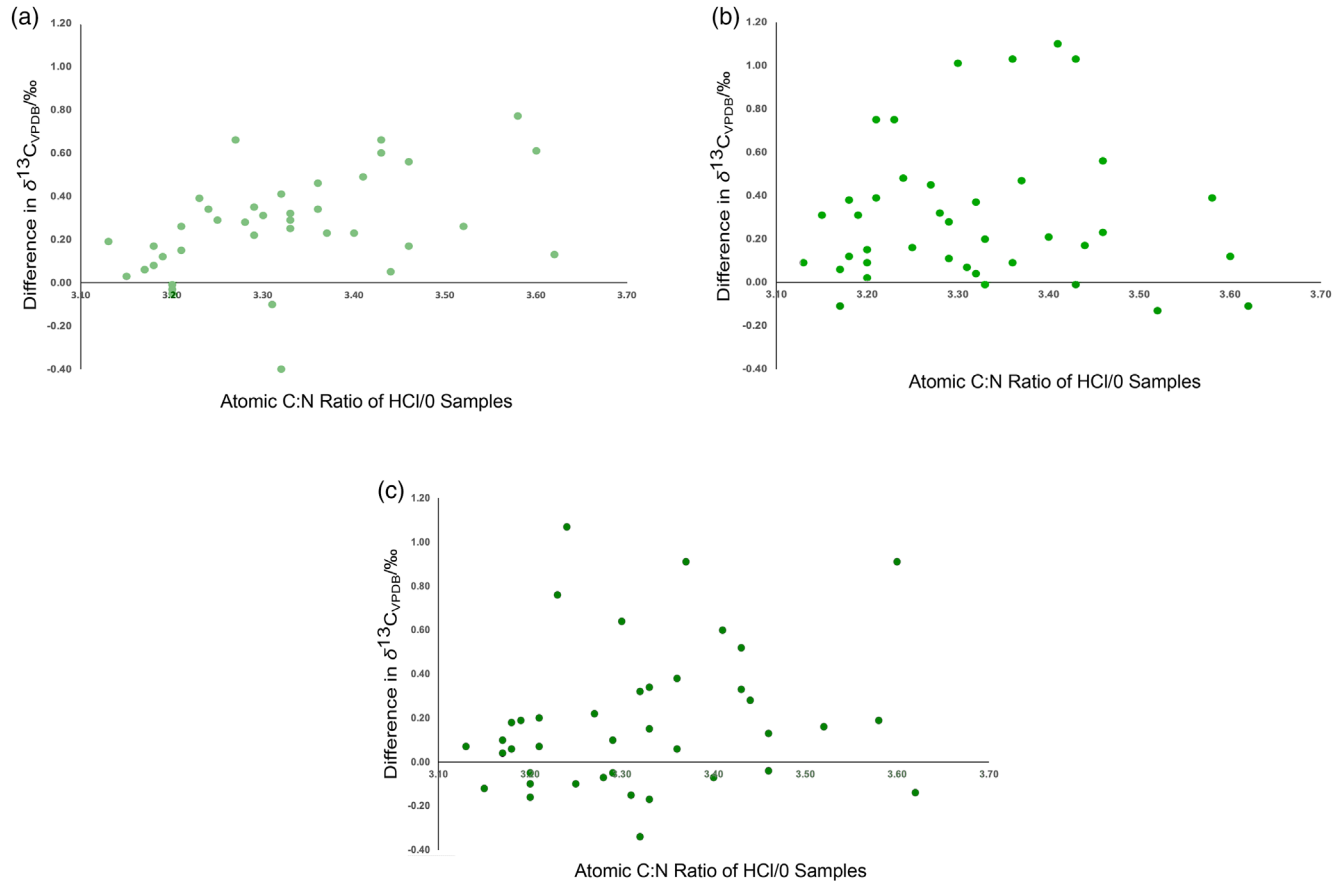


**FIGURE 3** Plot of atomic C:N ratios of HCl/0 ("untreated") samples against the corresponding difference in $\delta^{13}$C values between two treatments. A large difference in $\delta^{13}$C values between treatments is indicative that one treatment is more effective at removing humic contaminants than the other. We expect to observe larger differences in $\delta^{13}$C values between treatments for HCl/0 samples that have higher C:N ratios a priori and therefore likely contain more humic acids. (a) HCl/NaOH-HCl/0. (b) HCl/NaOH-EDTA/0. (c) HCl/NaOH-HCl/EDTA

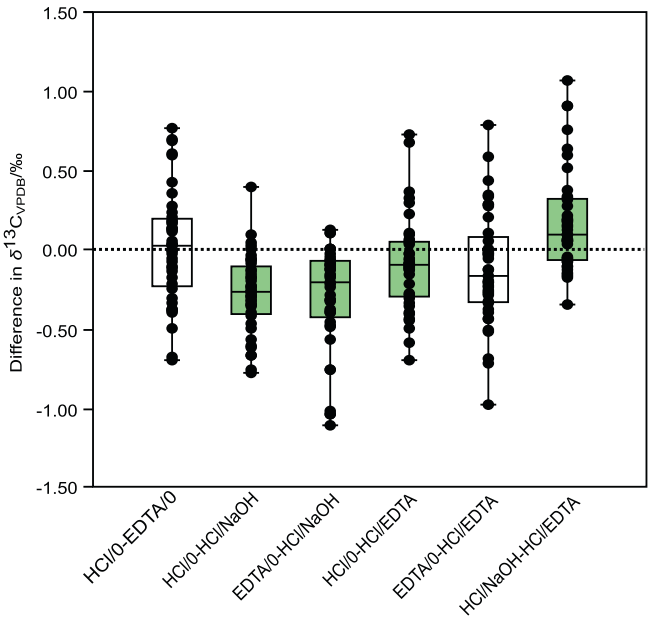


**FIGURE 4** Plot of the average differences in $\delta^{13}$C values between sample treatments. The dotted line denotes an average difference of zero. Note that if a point plots above the line, it indicates that the first treatment has a higher $\delta^{13}$C (less negative) value than the second treatment and vice versa, if the point plots below the line. For example, the $\delta^{13}$C values in the EDTA/0 treatment were lower in nearly all cases than the $\delta^{13}$C values in the HCl/NaOH treatment (third box from the left)

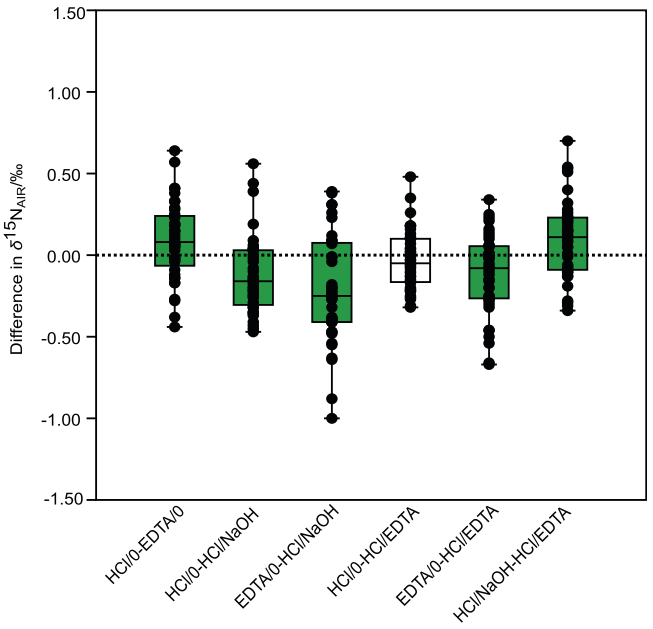


**FIGURE 5** Plot of the average difference in $\delta^{15}$N values between sample treatments. The dotted line denotes an average difference of zero. Note that if a point plots above the line, it indicates that the first treatment has a higher $\delta^{15}$N value than the second treatment and vice versa if the point plots below the line

**TABLE 2** *p*-values from Wilcoxon tests comparing $\delta^{15}$N values between treatments

| *df* | Treatment | HCl/0 | HCl/NaOH | EDTA/0 |
|---|---|---|---|---|
| 40 | HCl/NaOH | **0.001** | — | — |
| | EDTA/0 | **0.02** | **<0.001** | — |
| | HCl/EDTA | 0.21 | **0.02** | **0.007** |

*Note*: Bolded values represent *p*-values below the alpha threshold ($\alpha = 0.05$).

**TABLE 3** *p*-values from Mann–Whitney *U* tests comparing atomic C:N ratios between treatments

| *df* | Treatment | HCl/0 | HCl/NaOH | EDTA/0 |
|---|---|---|---|---|
| 40 | HCl/NaOH | **<0.001** | — | — |
| | EDTA/0 | **0.006** | **0.04** | — |
| | HCl/EDTA | **0.04** | **0.006** | 0.35 |

*Note*: Bolded values represent *p*-values below the alpha threshold ($\alpha = 0.05$).

impact on the atomic C:N ratios of the samples ($F = 8.08$, $p = <0.001$, $df = 163$). On average, the samples treated with NaOH had lower atomic C:N ratios than samples not treated with this agent (Figure 6, Table 4). The samples treated only with EDTA had the next lowest average atomic C:N ratio, while the samples treated only with HCl had the highest atomic C:N ratios on average (Figure 6, Table 4). The direction of the difference remained very uniform for all comparisons, with one treatment having a consistently higher or lower atomic C:N ratio, however, the magnitude of the differences were quite small (Tables 3, 4). Samples treated with EDTA/0 did not have significantly different atomic C:N ratios than HCl/EDTA-treated samples (average difference of 0.04) (Table 3). The wt% C and wt% N were quite variable among the different treatments, particularly between the HCl/NaOH treatment and the other treatments (Tables 4, S4, S5).

## 3.3 | Collagen yield

Collagen yield was not significantly different for HCl/NaOH and HCl/EDTA treated samples (Table S6, Figure 7). Moreover, the additional NaOH humic extraction step did not reduce sample yield significantly relative to samples treated only with HCl (Table S6). Samples treated only with EDTA had significantly higher collagen yields than HCl/EDTA-treated samples. The EDTA/0 samples also had significantly different yields relative to HCl/0 and HCl/NaOH samples. It should be noted, however, that the average collagen yield was very similar across treatments (20%–22% for all treatments), despite the statistically significant difference identified for some of the comparisons. All samples produced adequate collagen yields regardless of treatment, with the collagen yield ranging between 6% and 47% (Table S6). The atypically high collagen yields (i.e., >30%) were limited to samples from northwestern Greenland that had a very low density, suggesting a loss of some of the mineral component in the burial environment (Nielsen et al., 2018).

### 3.4 | Sample coloration

Many of the samples chosen for analysis were selected because they had a very dark coloration (to increase the chances of humic contamination) and changes in the color of the demineralization and humic extraction solutions were commonly observed. During the demineralization process, most samples caused the solution to discolor over time, turning the clear solution from a light yellow to a dark brown depending on the sample. During treatment with EDTA or NaOH post-demineralization, the color of the solution continued to change (darkening the color of the clear solution to a dark brown) even after many rinses (Figure S1). Often four or more treatments with NaOH or EDTA were conducted to ensure that a color change in the solution was not occurring after the 30-min treatment period. Approximately the same number of rinses were required for the post-demineralization EDTA and NaOH treatments for a given sample. Despite the thorough treatment of samples, and significant rinsing post-treatment, the solubilized collagen solution continued to appear dark yellow after refluxing.

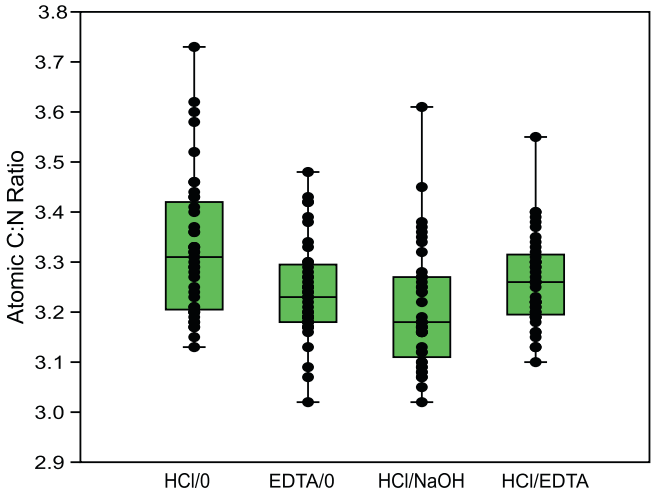


**FIGURE 6** Boxplot of the atomic C:N ratios of all samples according to treatment type

## 4 | DISCUSSION

### 4.1 | Effect of EDTA treatment on the isotopic and elemental composition of humic contaminated bone collagen

EDTA is not an effective chemical agent to perform the removal of humic acids in ancient bone collagen samples under the experimental conditions tested in this research. The elemental and isotopic data from EDTA-treated samples support the conclusion that under demineralization conditions (neutral pH of 7.4 and concentration of 0.5 M),

**TABLE 4** Average elemental compositions for all samples by treatment type

| Treatment | HCl/0 | EDTA/0 | HCl/NaOH | HCl/EDTA |
|---|---|---|---|---|
| C:N Ratio | 3.33 ± 0.14 | 3.24 ± 0.10 | 3.21 ± 0.12 | 3.26 ± 0.09 |
| wt% C | 40.6 ± 2.8 | 43.3 ± 2.1 | 40.3 ± 2.2 | 41.2 ± 2.1 |
| wt% N | 14.2 ± 0.8 | 15.6 ± 0.8 | 14.7 ± 0.6 | 14.8 ± 0.8 |

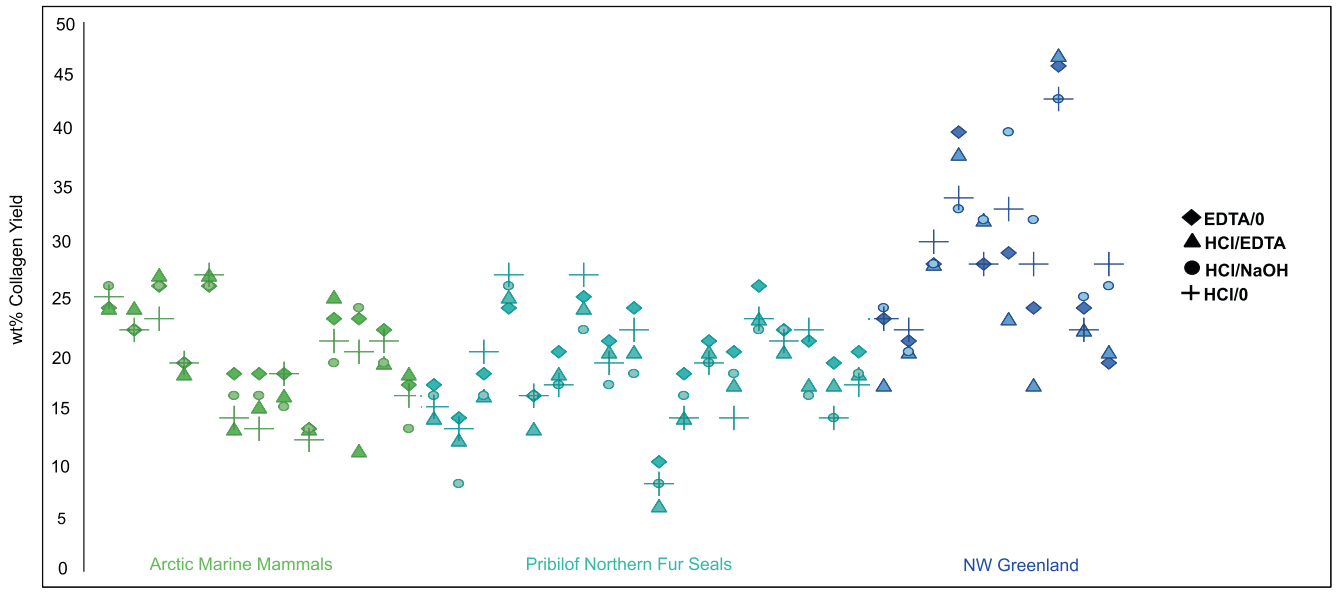


**FIGURE 7** Plot of the wt% collagen yield of all samples examined. The different shapes represent the different treatment types. The light green section of the graph plots collagen yields from various marine mammals. The light blue section of the graph plots fur seals from the same burial environment. The dark blue section corresponds to samples originating from Greenland from the same burial environment which promotes bone demineralization

EDTA does not remove humic contaminants. The samples treated with EDTA, either as a demineralization agent, or as a post-demineralization rinse to attempt humic removal, had consistently lower $\delta^{13}C$ and $\delta^{15}N$ values as compared with NaOH-treated samples (Figures 3b and 4). Moreover, the $\delta^{13}C$ values of the HCl/0 and EDTA/0 treatment were, on average, nearly indistinguishable (Figure 4, HCl/0—EDTA/0 comparison). These results suggest that the presence of humic acids, which generally have lower $\delta^{13}C$ and $\delta^{15}N$ values relative to bone collagen, have skewed the isotopic composition of EDTA-treated samples. The EDTA-treated samples also had $\delta^{13}C$ values that were very similar to samples treated only with HCl. Given that humic acids are insoluble in HCl, we do not expect the HCl/0 treatment to remove the humics to any extent. Similar results for EDTA treatments (relative to the HCl/0 treatment) suggest that EDTA is similarly ineffective at removing humics. Despite implementing two different EDTA-based methods for humic removal, neither seemed to effectively remove humic contaminants. Adding an EDTA rinsing step after demineralization via HCl had no impact on the $\delta^{13}C$ or $\delta^{15}N$ values of the samples (Figure 4, HCl/0 vs. HCl/EDTA comparison). In summary, the isotopic evidence supports the conclusion that EDTA treatment has no effect on the presence of humic contaminants in ancient bone collagen. The elemental data associated with EDTA-treated samples also supports the conclusion that this chemical agent cannot effectively remove humic contaminants. Samples treated with EDTA (EDTA/0 or HCl/EDTA treatments) had higher atomic C:N ratios as compared with NaOH-treated samples (Figure 6). These results indicate that humic acids, which have a higher atomic C:N ratio than collagen, are more abundant in the samples treated with EDTA, relative to those treated with NaOH.

Contrary to the pattern observed with the isotopic data, the EDTA-treated samples did not have elemental compositions that were in agreement with HCl-treated samples. On average the EDTA-treated samples had lower atomic C:N ratios than HCl/0 samples, which at first glance makes it appear as though EDTA may have removed more humic acids than the HCl/0 samples. Bone collagen samples treated with EDTA, however, have lower atomic C:N ratios than equivalent samples treated with HCl, without any appreciable or consistent difference in stable isotope composition (Tuross, 2012; Wilson & Szpak, 2022). Therefore, the lower atomic C:N ratio associated with EDTA-treated samples observed in this study relative to HCl treated samples may be the product of factors unrelated to the ability of this chemical agent to remove humic contaminants, such as the cleavage of amide groups from collagen chains by HCl during demineralization (Simpson et al., 2016). Another consideration is that EDTA-treated samples had higher wt% C and wt% N compositions compared with the other treatments, which may imply a lower salt concentration in these samples relative to the samples treated with other methods. It should be noted that the strict rinsing protocol following EDTA treatment (15 rinses) may be the cause of this difference in wt% C and wt% N composition, and it is possible that if a similarly strict rinsing protocol were used for HCl treated samples to remove salts, the elemental compositions may be more comparable. We can still conclude, however, based on the elemental compositions of the samples, that EDTA cannot effectively remove humic contaminants given that the atomic C:N ratios of these samples are consistently and significantly higher than NaOH-treated samples.

### 4.2 | Effect of sodium hydroxide treatment on the isotopic and elemental composition of humic contaminated bone collagen

Our results suggest that chemical treatment with NaOH can remove humic contaminants to some extent. Given that humic acids have relatively low $\delta^{13}C$ and $\delta^{15}N$ values and high atomic C:N ratios as compared with the collagen samples, we would predict that the sample pretreatment that removes these contaminants most effectively would produce samples with relatively high $\delta^{13}C$ and $\delta^{15}N$ values and low atomic C:N ratios compared with equivalent samples left untreated or treated with other reagents. Our results demonstrate that samples treated with NaOH consistently follow these trends, having on average lower atomic C:N ratios and higher $\delta^{13}C$ and $\delta^{15}N$ values than the three other treatments (Figures 3, 4, 5, 6). The enhanced removal of humic acids from NaOH treated samples is expected to be the cause of the difference in isotopic composition amongst treatments, because using different demineralization methods on non-humic contaminated samples does not affect collagen stable isotope composition (Tuross, 2012; Wilson & Szpak, 2022). Therefore, we can conclude based on the elemental and isotopic data that NaOH performs humic removal more thoroughly than any of the other methods investigated. These results are consistent with previous studies that suggest NaOH is effective for the removal of humics within the context of stable isotope analysis of bone collagen (Ambrose, 1990; Sealy et al., 2014; Szpak Krippner & Richards, 2017).

Although NaOH performs humic removal more effectively than the other methods examined, it is unlikely that complete humic removal can be performed without destroying the protein in the process (van Klinken & Hedges, 1995). If NaOH treatment were able to completely remove humic contaminants, we would expect to see the largest differences in $\delta^{13}C$ values between NaOH treated and untreated samples when the untreated samples have elevated atomic C:N ratios and thus are expected to be highly humic-contaminated. This trend, however, is not observed in these data (Figure 3a). These data suggest that in highly humic-contaminated samples, complete humic removal is not possible regardless of treatment, although NaOH treatment remains the most effective option for pretreatment. Furthermore, although previous studies have found that NaOH treatment significantly reduces sample yield (Chisholm et al., 1983; Szpak Krippner & Richards, 2017), this effect was not observed in our samples, which were, however, predominantly well-preserved. Additional studies using more aggressive NaOH treatments (i.e., longer treatment times, more treatments, more concentrated NaOH, or some combination of these) to remove humics from well-preserved bones with high C:N ratios would be useful to determine the point at which the benefit of humic extraction is overwhelmed by the detrimental effects of collagen loss.

### 4.3 | Informing future pretreatment decisions

Sodium hydroxide treatment is a widely-used process that has been shown to remove humic contaminants (Ambrose, 1990; Szpak Krippner & Richards, 2017). Our results do not suggest that EDTA could serve as a substitute for NaOH for this purpose. In fact, one of the main concerns associated with NaOH treatment is that it can significantly reduce collagen yield. But, contrary to our expectations, we observed that the NaOH treatment did not consistently reduce the collagen yield for well-preserved samples (Figure 7). This observation may not extend to poorly preserved samples, or those ground to small size fractions (such as powdered samples), or samples treated with NaOH for extended periods of time (20 h). There is no evidence, however, based on our data to support the claim that EDTA treatment reduces collagen loss relative to NaOH treatment. Although NaOH may be more likely to reduce collagen yields through damage to the collagen molecule, reductions in yield through increased sample handling may present an equal problem for EDTA-treated samples. To ensure that EDTA does not contaminate the samples, 15 rinses post-treatment are often implemented (Tuross et al., 1988), which has the potential to reduce collagen yield if any solid bone is removed by human error during the rinsing process.

## 5 | CONCLUSION

Treatment of ancient bone collagen samples with EDTA for the purpose of humic removal does not improve the probability of generating reliable isotopic and elemental data that pass accepted quality control criteria and are representative of the endogenous collagen. Our results demonstrate that under the experimental conditions examined, EDTA cannot effectively remove humic contaminants, and therefore is not a superior humic removal agent than NaOH. In future isotopic research on ancient bone collagen, NaOH should be used rather than EDTA if there is concern that the sample may be contaminated by humic acids.

### AUTHOR CONTRIBUTIONS

**Tess Wilson:** Conceptualization (equal); data curation (lead); formal analysis (lead); funding acquisition (equal); investigation (lead); methodology (lead); project administration (supporting); writing – original draft (lead); writing – review and editing (supporting). **Paul Szpak:** Conceptualization (equal); data curation (supporting); formal analysis (supporting); funding acquisition (equal); investigation (supporting); methodology (supporting); project administration (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (lead).

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### CONFLICT OF INTEREST

The authors do not have a conflict of interest to declare.

### DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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