Journal of Archaeological Science 132 (2021) 105416

Contents lists available at ScienceDirect

Journal of Archaeological Science

journal homepage: www.elsevier.com/locate/jas

Improved quality control criteria for stable carbon and nitrogen isotope measurements of ancient bone collagen

Eric J. Guiry [a,b,*], Paul Szpak [b]

[a] School of Archaeology and Ancient History, University of Leicester, Mayor's Walk, Leicester, LE1 7RH, United Kingdom
[b] Department of Anthropology, Trent University, 1600 West Bank Dr., Peterborough, ON, K9L 0G2, Canada

ARTICLE INFO

Keywords:
Humic acids
Bone
Collagen
Taphonomy
Stable isotopes
Diagenesis
C:N ratio

ABSTRACT

The carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic compositions of bone and tooth collagen provide a powerful tool for studying past biological, environmental, and cultural phenomena. Collagen has a well-understood chemical composition that has enabled the development of invaluable quality control (QC) criteria for isotopic data – something that is extremely rare among biogeochemical research on ancient biomolecules as a whole. The most important of these collagen QC criteria is atomic C:N ratio (ratio of carbon to nitrogen atoms), which provides an indicator of the extent to which the amount of carbon and nitrogen present in a sample matches the known composition of pure collagen, thereby indicating whether contamination or diagenesis may be influencing a sample's isotopic compositions. We present a model describing the relationship between the carbon and nitrogen isotopic and elemental compositions that accounts for the isotopic composition of the endogenous collagen and exogenous contaminants as well as taxon-specific information about the collagen's amino acid composition. In some cases the traditional C:N QC parameters are applicable, while in others they can result in the inclusion of unreliable (altered) isotopic data primarily due to contamination from humic substances. Using new and previously published data on taxa commonly encountered in ancient studies, we further illustrate how using traditional C:N QC parameters may lead to the inclusion of altered isotopic compositions in real archaeological and paleontological scenarios. We argue that the traditional 'one size fits all' approach to the C:N QC criterion should be avoided and we outline new collagen QC criteria specific to certain taxa and environments on the basis of the results of our model. These revised criteria will help to improve the interpretation of isotopic data by more accurately identifying samples with isotopic compositions altered by contamination.

1. Introduction

The carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic compositions of bone and tooth collagen[1] from archaeological and paleontological deposits can provide a powerful tool for studying past biological, environmental, and cultural phenomena. Use of these isotopic compositions for investigating dietary patterns and ecological relationships has grown rapidly over the past thirty years and now forms a cornerstone of many investigations of past ecosystems and human societies. Aside from their utility as a tool for reconstructing past events, the widespread adoption of $\delta^{13}C$ and $\delta^{15}N$ analyses stems, at least in part, from their applicability to collagen, a highly stable (and thus often abundant) molecule capable of preserving biogenic isotopic compositions for millennia (Dobberstein et al., 2009). What is more, and arguably as important, collagen has a well characterized chemical structure, which has enabled the development of various quality control (QC) indicators (Ambrose, 1990; DeNiro, 1985). The availability of collagen QC indicators provides an independent measure of the reliability of data at a level that is rarely available for isotopic analyses of other organic or inorganic materials, particularly ancient ones.

Collagen QC criteria (%C, %N and elemental C:N ratio) are based on a detailed understanding of the amino acid compositions of collagen

* Corresponding author. School of Archaeology and Ancient History, University of Leicester, Mayor's Walk, Leicester, LE1 7RH, United Kingdom.
E-mail addresses: ejg26@leicester.ac.uk (E.J. Guiry), paulszpak@trentu.ca (P. Szpak).

[1] We recognize that what is referred to throughout the literature as 'collagen' may actually represent a mixture of substances and may more aptly be defined as 'gelatin' or 'acid insoluble residue'. The proteinaceous component of whole bone is ~90% collagen by weight; however, because non-collagenous proteins (NCPs) are likely removed during the chemical extraction process, the actual collagen content by weight is well in excess of 90%. We therefore believe that it is still reasonable to call the residue this material collagen.

https://doi.org/10.1016/j.jas.2021.105416
Received 31 October 2020; Received in revised form 17 May 2021; Accepted 18 May 2021
Available online 8 June 2021
0305-4403/

Table 1
Proportion of recent archaeological isotopic research (published 2017/01 to 2020/06; sample include 25319 analyses from 356 studies; see ESM 1 for survey parameters) utilizing common collagen QC criteria. "Total sample n = " varies between indices as some studies provided insufficient detail to assess which QC criteria were used. Among studies that explicitly use C:N as a collagen QC criterion, 16.9% (same percentage for analyses and studies) do not provide enough detail to identify the specific range used.

QC criterion	Total sample n =	Proportion of analyses		Total sample n =	Proportion of studies	
		n =	%		n =	%
C:N	25319	24393	96.3	356	331	93.0
Using DeNiro's range 2.9–3.6[a]	20036	19042	95.0	269	250	92.9
Using an alternative range[a]	20036	994	5.0	269	19	7.1
C:N & Elemental %	24689	16083	65.1	351	201	57.3
C:N & Collagen Yield %	24798	8207	33.1	352	113	32.1
C:N, Elem. %, & Col. Yld. %	24689	6297	25.5	351	86	24.5

[a] Denotes figures calculated from the fraction of studies in which the specific C:N range deemed acceptable was reported.

across vertebrate species (Neuman, 1949). Collagen extracts from ancient samples that are found to have elemental compositions deviating from those expected based on the known amino acid compositions of collagen have necessarily been contaminated and/or degraded, both of which may result in skewing of their isotopic compositions.

DeNiro (1985) was the first to develop a systematic collagen QC criterion based on observations of anomalies in the relationship between the isotopic and elemental compositions of ancient and modern bone collagen extracts. In particular, he proposed that samples with atomic C:N ratios (hereafter C:N) falling between 2.9 and 3.6 are less likely to have isotopic compositions that have been altered by *post mortem* processes. Following a similar approach, additional criteria have been added including concentrations of C (%C) and N (%N), being greater than 13% and 4.8% (Ambrose, 1990), respectively, as well as collagen concentration (as % yield of collagen extract relative to that of the initial sample), being greater than 0.5–2% (Ambrose, 1990; DeNiro and Weiner, 1988; Dobberstein et al., 2009; Van Klinken, 1999). Although further amendments to these criteria, including a narrower C:N range of 3.1–3.5 (Van Klinken, 1999), have also been suggested, the vast majority of recent archaeological research (n = 356, published 2017/01–2020/06; 93% of studies and 95% of analyses, Table 1) have evaluated collagen QC against DeNiro's (1985) C:N criterion (by comparison, elemental % and collagen yield % were used for 65 and 33% of analyses, and only in conjunction with C:N; see Table 1).

While these collagen QC indicators provide a clear envelope outside of which isotopic data are more likely to be unreliable, they do not necessarily certify the reliability of isotopic data from samples with QC criteria falling inside the envelope. This much was clearly outlined by DeNiro (1985), who noted that samples with C:N falling between 2.9 and 3.6 may be still have isotopic compositions altered by *post mortem* processes. In this context, however, DeNiro (1985) and others (e.g., Ambrose, 1990; Van Klinken, 1999) believed that the impacts on the isotopic compositions of these samples would be inconsequentially small, such that identification of the human or animal's dietary behaviour would still be possible.

Following these and other early studies (e.g., Masters, 1987; Schoeninger et al., 1989; van Klinken and Hedges, 1995), relatively little work, in comparison to the volume of isotopic measurements of ancient collagen, has been done on further refining collagen QC criteria for ancient samples. However, a number of papers have improved our understanding of variation in the amino acid composition of collagen. Studies synthesizing data on collagen amino acid compositions from hundreds of animals (Fig. 1; Guiry and Szpak, 2020; Szpak, 2011) have provided new insights on variation in key QC criteria occurring between animals at broad inter-class levels (i.e., fish *vs.* mammals and birds, see Fig. 1a) and across taxa living in different kinds of environments (cold water *vs.* warm water fish, see Fig. 1b). These analyses provide more accurate estimations of the fundamental parameters (i.e., the range of % C, %N, and C:N occurring in vertebrate collagen) upon which the most widely used collagen QC criteria are based and can therefore serve as a framework for their refinement.

In this paper, we investigate the extent to which ancient bone collagen isotopic compositions may become significantly altered and yet still produce C:N values falling within the envelope of acceptable QC criteria. This issue is explored in four thematic sections. First, in the context of more recent information about the chemical compositions of collagen, we outline how the ancient collagen QC criteria (namely C:N) that are currently widely accepted may not be sufficiently sensitive for detecting major contaminants in some cases (Section 2). Second, we describe the extent to which these issues may occur in archaeological samples by simulating how the most prevalent ancient bone collagen contaminant, humic acids, affects isotopic compositions of different kinds of samples and under different conditions (Section 5.1). Third by comparing isotopic compositions and collagen QC indicators among large datasets derived from single species we demonstrate that data from ancient collagen extracts with otherwise acceptable QC indicators can have substantially skewed isotopic compositions (Section 5.2). Finally, we propose some 'best practices' for more nuanced interpretation of existing QC criteria (Section 5.3).

2. Context

2.1. Biogenic variation in C:N

Ancient collagen QC criteria were initially defined based on a relatively small number of early observations of the elemental concentrations in modern and ancient vertebrate collagen (Ambrose, 1990; DeNiro, 1985). Recent larger scale surveys of collagen amino acid compositions and elemental concentrations of modern bone collagen extracted with techniques widely used in archaeology and related disciplines (Guiry and Szpak, 2020; Szpak, 2011) offer more insight into variation in the elemental compositions of collagen among different taxa and environments. These data can, therefore, provide a framework for improving the most widely utilized (Table 1) of ancient collagen QC parameters – C:N. Amino acid compositions from 436 vertebrates, representing 193 species (collated in Guiry and Szpak 2020), indicate that the range of C:N among vertebrate collagen is between 3.00 and 3.33, less than half of the acceptable C:N envelope for collagen defined by DeNiro (1985). This C:N envelope was not based solely on theoretical ranges for collagen C:N, but also on observations of how this parameter varied with isotopic compositions for taxa with known feeding habits (DeNiro, 1985). Fig. 1a shows how variation within this narrower range differs significantly among taxa, with mammals and birds falling between 3.11 and 3.28 (although 3.00 to 3.28 is deemed acceptable for modern samples; Guiry and Szpak, 2020) and fish between 3.00 and 3.33. Moreover, among fish, C:N varies systematically between environments (Gustavson, 1955; Rigby, 1967; Rigby and Spikes, 1960), with cold- and warm-water adapted fish falling at the lower and higher ends of the range, respectively (Fig. 1b). These higher resolution observations for the chemical compositions of collagen can provide valuable reference points for detecting unreliable isotopic compositions in ancient bone collagen extracts, nearly half of which (46%) fall outside the observed range for modern collagen (Fig. 1c and Electronic Supplementary Materials [ESM] 1). These observations could have important implications for defining what we should consider an acceptable elemental composition for ancient collagen. For example, should the same criteria be used for two taxa with C:N of 3.11 and 3.28?

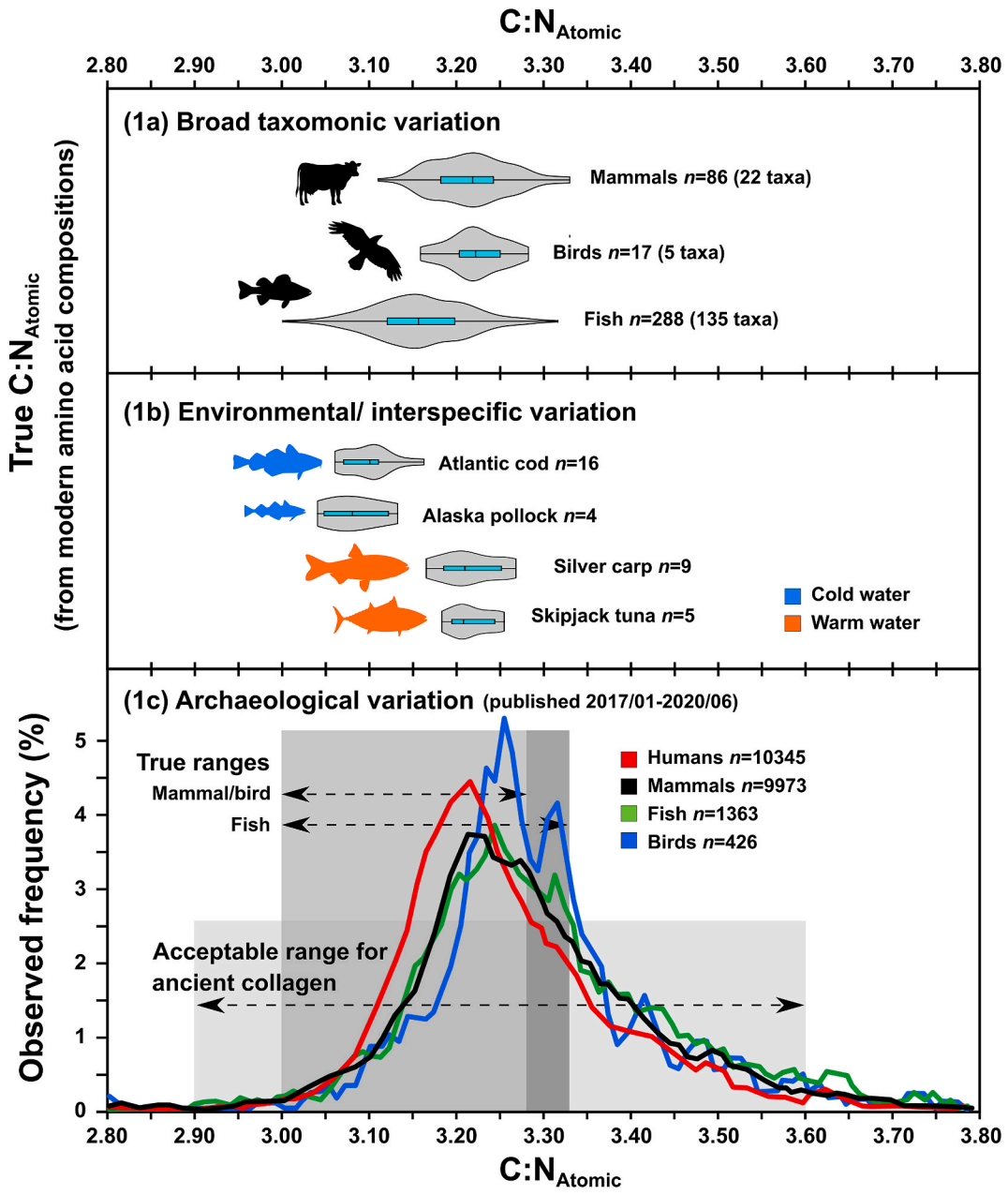

Fig. 1. Violin and box plots (1a and 1b) showing that the range of variation in C:N observed among vertebrate taxa (data from Guiry and Szpak, 2020 and ESM 1) is less than half that allowed in current collagen QC criteria (i.e., C:N 2.9–3.6). Variations in C:N differs broadly among fish (Actinopterygii) *vs.* mammals (Mammalia) and birds (Aves; see 1a) and fish species adapted to colder and warmer waters (see 1b). Percent frequency histogram (1c) showing the distribution observed in archaeological C:N (n = 25319 analyses, binned in 0.01 increments) by taxa collected from 356 recently published studies (see ESM 1 for survey parameters; only data published with elemental percent or C:N reported to >1 decimal place shown here; n = 22197). Roughly half (53.8%) of recently published archaeological C:N fall within the known ranges for modern vertebrates.

Before exploring this possibility, it is first necessary to consider the main processes by which collagen C:N can be altered and their relative importance among ancient specimens. These fall broadly under two categories: contamination (endogenous and exogenous) and degradation.

2.1.1. *Endogenous contaminants*

Although derived from same raw materials (i.e., diet and nutrients) as collagen, endogenous contaminants (i.e, biogenic, but non-collagenous materials) may have differing isotopic compositions due to the biochemical processes associated with their sourcing and synthesises. The main endogenous sources of contamination of bone collagen extracts are likely to come from lipids, humic acids (those derived from the breakdown of the organism's tissues), and non-collagenous proteins (NCPs).[2] Lipids and humic acids are C-rich and N-poor and therefore can result in: 1) higher C:N and, 2) skewed $\delta^{13}C$ (largely lacking N, they are less likely to influence $\delta^{15}N$, although see Section 5). While less is known about potential contributions from endogenous humic acids (van Klinken and Hedges, 1995), to the extent that they are derived from the breakdown products of collagen itself,

[2] We are aware that carbohydrates attached to collagen via glycolization (for review see Hennet 2019) are an additional source of endogenous contamination, but do not consider them here as they are thought to make up only a minute fraction (c. 1%) of the mass of collagen (Grassmann and Schleich 1935).

they may be less likely to have a significant impact on the measured $\delta^{13}C$ of collagen extracts. Lipids, on the other hand, have significantly and systematically lower $\delta^{13}C$ (DeNiro and Epstein, 1977) than bone collagen (by ca. −5 to −12‰; Ambrose, 1990; Guiry and Hunt, 2020; Vogel, 1978) and are widely regarded as a major potential source of contamination in modern bone collagen extracts (Guiry et al., 2016b). This is particularly so for fish, which may have bone lipid contents surpassing 50% by weight (Ambrose, 1990; Guiry et al., 2016b; Liden et al., 1995). The extent to which lipids survive in ancient bone in quantities that would impact bone collagen isotopic compositions, however, is poorly understood (although see Collins et al., 2002; Colonese et al., 2015; Evershed et al., 1995; Scott, 2020) but it is broadly assumed that, aside from special circumstances, such as permafrost deposits or for fish bones, they typically are not abundant in ancient collagen extracts.

Relative to collagen, most NCPs are richer in C and poorer in N (e.g., Fisher et al., 1987; Guiry and Szpak, 2020; Gundberg et al., 1984) and therefore co-extraction of NCPs with bone collagen could result in: 1) higher C:N and, 2) shifted $\delta^{13}C$ and $\delta^{15}N$ (Guiry and Hunt, 2020; Masters, 1987). Among archaeological and palaeontological studies, however, the potential for NCP contamination is not routinely considered (although see Collins et al., 2000; Smith et al., 2005) when interpreting collagen isotopic compositions. This limited focus on NCPs may result, at least in part, from the fact that common protocols for collagen extraction (i.e., removing the inorganic fraction through acidification or chelation; e.g., Ajie et al., 1990; Gundberg et al., 1984; Wadsworth and Buckley, 2018) should remove NCPs that have a lower affinity for binding to collagen (for discussion on increases in NCP concentration in poorly preserved bone see Dobberstein et al., 2009; Masters, 1987).

2.1.2. Exogenous contaminants

Exogenous sources of contamination can be highly varied and result from failure to remove any C- or N-bearing materials that have become incorporated into a bone from the burial environment. Because common collagen extraction protocols are efficient at removing the majority of other burial residues (e.g., Ambrose, 1990), the most common of these will be humic acids (for review see Kendall et al., 2018; van Klinken and Hedges, 1995). Exogenous humic acids are typically derived from the breakdown of organic matter from local vegetation and will therefore have isotopic compositions reflecting dominant photosynthetic processes (i.e., C_3 with lower $\delta^{13}C$ *vs.* C_4 with higher $\delta^{13}C$) used by plants in the local area (e.g., Hiradate et al., 2004). While the presence of exogenous humic acid contamination in bone collagen extracts will result in proportionately higher C:N, their influence on $\delta^{13}C$ is less predictable. In contrast to other common C-rich contamination sources (e.g., lipids, which have isotopic compositions that are only moderatley, but consistently offset from proteins in the same organism; Post et al., 2007), the isotopic compositions of exogenous humic acids and collagen vary independently and can differ by a smaller or larger margin. Consider two collagen extracts from samples derived from the same burial context with different collagen $\delta^{13}C$ values. If these samples are contaminated with the same humic acid source and in the same proportions, they will have $\delta^{13}C$ values that are altered to different degrees. This potential for variation in the extent to which humic acids will influence resulting isotopic compositions makes assessment of the relative importance of this contaminant contextually-dependent, especially for humans, who may have diets rich in marine resources or C_4 plants, but their remains may end up deposited in environments dominated by C_3-plants. These scenarios decouple isotopic variation in the collagen from that of the burial environment.

2.1.3. Degradation

A wide range of chemical (e.g., peptide hydrolysis) and biological (e.g., microbial heterotrophy) processes occurring during the taphonomic history of ancient bones can result in the loss of collagen (for reveiws see Collins et al., 2002; Kendall et al., 2018; Nielsen-Marsh et al., 2007; Smith et al., 2007). Collagen loss is widely observed in ancient bones but, in and of itself, does not necessarily cause changes to a sample's elemental or isotopic composition. In fact, samples that have retained very small quantities of their original collagen can still produce C:N and elemental concentrations matching biogenic parameters (e.g., Dobberstein et al., 2009). Although, rarely observed archaeologically, degradation could in theory result in meaningfully altered collagen QC parameters and isotopic compositions if selective amino acid loss occurs (e.g., Grupe and Turban-Just, 1998). In a similar fashion to NCP contamination (Masters, 1987), selective loss of amino acids could alter elemental or isotopic compositions by changing the relative quantities of certain amino acids, which may be isotopically distinctive from the bulk collagen (Hare et al., 1991).

Collagen QC criteria are not well suited to differentiating between the presence of selective amino acid loss and contamination as the source of unreliable isotopic compositions. However, as most sources of collagen contamination will have a C:N that is higher than that of collagen, and selective amino acid loss could in some cases result in lower C:N (i.e., loss of those with higher relative C content such as phenylalanine and leucine), unusually low C:N may provide some indication. Because low C:N are rarely observed in ancient collagen extracts (2.6% of 25,319 C:N published between 2017–01 and 2020–06 fall below 2.90, see Fig. 1c), broad scale analyses of the kinds of taphonomic conditions that result in selective amino acid loss have not been possible. Studies of ancient and experimentally degraded collagen, however, demonstrate that selective amino acid loss is likely rare, due to the stabilizing relationship between collagen and bone mineral, and occurs mainly when collagen yields are extremely low (Dobberstein et al., 2009; Hare, 1980; Masters, 1987). These observations suggest, therefore, that selective amino acid loss is probably limited to a narrower range of contexts, such as those where samples are geologically old (i.e., having lost much of their collagen), and therefore are likely not a relevant factor for considering assessing QC for many ancient samples.

2.2. Considering more sensitive collagen QC criteria

If the main source of variation in collagen C:N is contamination, rather than degradation, it should be possible to use known variation in vertebrate collagen C:N (i.e., between taxonomic classes and environments) to develop more sensitive collagen QC criteria. The currently accepted C:N of 2.9–3.6 is more than two times wider than the observed range in vertebrates (3.00–3.33) and therefore, assuming that selective amino acid loss is rare, means that isotopic compositions of ancient collagen extracts with higher C:N (ca. 3.30–3.60 for fish and 3.28–3.60 for mammals) could be unreliable. This was demonstrated by a recent study (Guiry and Szpak, 2020) using modern bone, contaminated to varying degrees with endogenous lipids, showing that the isotopic composition of collagen extracts can be substantially altered within the acceptable C:N range. In particular, for every shift of +0.25 in C:N, lipid-contaminated sample $\delta^{13}C$ was shifted by −0.5 ‰, which, for taxa with lower C:N could result in collagen extract $\delta^{13}C$ values that are shifted −1.2 ‰ by the time C:N reaches 3.6. Humic contaminants, which can have a $\delta^{13}C$ that is far lower than lipids, could have an even larger impact (particularly for collagen from C_4 or marine feeding individuals that have been deposited in a C_3 environment).

It is therefore likely that the widely accepted C:N criterion of 2.9–3.6 is not suitable for all ancient samples, but rather should be evaluated relative to context. For example, assuming that a specimen's collagen has not undergone selective amino acid loss, any C:N greater than 3.30 in fish and 3.28 in mammals indicates that collagen extracted from the sample is contaminated with non-collagenous C. As shown in Fig. 1c, nearly half (46%) of published isotopic compositions from ancient collagen fall into this category. Although this does indicate that a large fraction of ancient collagen $\delta^{13}C$ values are likely at least partly derived from contamination, assessment of whether isotopic data are unreliable requires a more nuanced approach. The susceptibility of a collagen

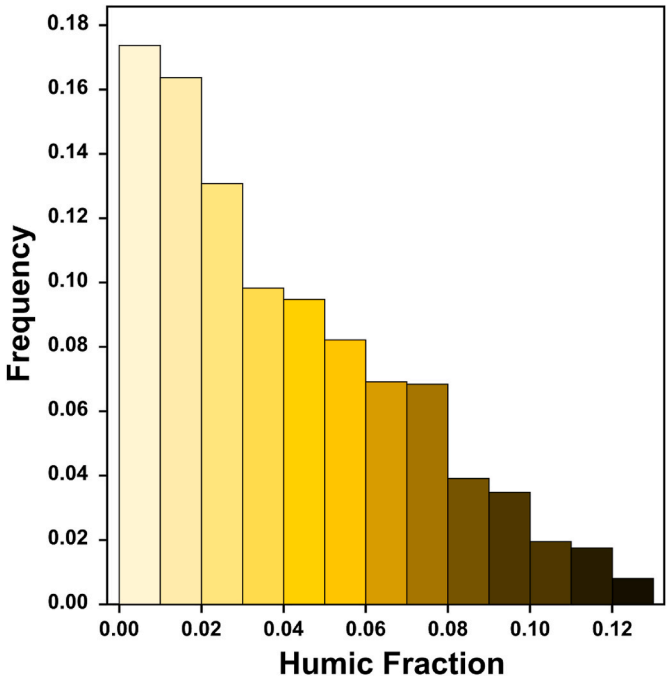

Fig. 2. Histogram showing the frequency of different levels of humic contamination used in the modeling.

extract's $\delta^{13}C$ to be altered will vary based on: 1) species (with cold water fish being more affected than warm water fish, mammals and birds; Fig. 1a and b), and 2) the difference between the $\delta^{13}C$ of the likely contaminant (humics) and the collagen. The extent of any difference also depends on the relative amounts of contamination and collagen. These relative differences are explored using simulated data and experimental comparisons in Sections 5.1 and 5.2, respectively.

3. Methods

3.1. Collagen/humic acid contamination model

We developed a quantitative model describing how variable levels of humic acid contamination influence the isotopic and elemental compositions of bone collagen with the following assumptions:

1. There are two quantitatively significant sources of carbon and nitrogen in the sample: endogenous protein (primarily collagen with the elemental composition of collagen) and exogenous humics. In other words, we must assume that endogenous humics from *in situ* humification and residual lipids are not important sources of carbon and nitrogen.
2. Related to the first assumption, it stands to reason that variation in the bone collagen isotopic composition for a particular sample is driven by the relative proportions of endogenous protein and exogenous humics.
3. The elemental composition (and C:N) of the collagen is known and can be assumed to be constant for a given taxon. This is a reasonable assumption as the amino acid composition of collagen is highly conserved within a species (Buckley, 2018).
4. Humic acids or compounds with similar elemental compositions are the primary exogenous contaminant. These compounds have reasonably predictable elemental and isotopic compositions for a given environment.

The relationship between the elemental and isotopic compositions of a sample exhibiting various levels of humic contamination can be described by the following system of mass balance equations (Schwarcz, 1991):

Table 2
Elemental and isotopic compositions for different taxa used in the simulation.

Species	wt% C	wt% N	C:N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Atlantic cod	41.45	15.60	3.10	−13.0	+17.0
Ringed seal	42.20	15.25	3.23	−13.0	+17.0
Cattle	42.24	15.29	3.22	−20.0	+4.0
Human (Global mode)	42.25	15.27	3.23	−19.5	+9.7
Human (C_4 Diet)	42.25	15.27	3.23	−10.0	+9.0

$$f_{c_{\delta^x}} + f_{h_{\delta^x}} = 1 \quad \text{Equation 1}$$

$$\delta^x s = \left(f_{c_{\delta^x}}\right)(\delta^x c) + \left(f_{h_{\delta^x}}\right)(\delta^x h) \quad \text{Equation 2}$$

$$f_{h_{\delta^x}} = \frac{\left(f_{h_{\delta^x}}\right)([z_h])}{\left(f_{h_{\delta^x}}\right)([z_h]) + \left(f_{c_{\delta^x}}\right)([z_c])} \quad \text{Equation 3}$$

$$f_{c_{\delta^x}} = \frac{\left(f_{c_{\delta^x}}\right)([z_c])}{\left(f_{c_{\delta^x}}\right)([z_c]) + \left(f_{h_{\delta^x}}\right)([z_h])} \quad \text{Equation 4}$$

where $f_{C\delta x}$ is the fraction of the sample represented by the endogenous collagen and $f_{h\delta x}$ is the fraction of the sample represented by the exogenous humic contaminants. $\delta^x s$ represents the isotopic composition of the mixture where x refers to the isotope system (e.g., C or N). $\delta^x c$ and $\delta^x h$ represent the isotopic composition of the collagen (c) and humics (h) for a given element (z). $[z_h]$ and $[z_c]$ refer to the elemental concentration (i.e., wt%) for a given element in the humics (h) and collagen (c). To generate the model, we simulated different levels of humic contamination at 1% intervals and recorded the impact of this contamination on the isotopic compositions of the collagen-humic mixture ($\delta^X s$).

Following assumption 4, for most simulations we assumed that the contaminating humic acids were derived from C_3 plant organic matter, which is true in most environments. From a survey of published literature (Francioso et al., 2005; Romero et al., 2007), we estimated the wt% C and wt% N of humic acids to be 51.67% and 1.20%, respectively. The $\delta^{13}C$ and $\delta^{15}N$ values for humic acids were estimated to be −25.53 ‰ and +1.52 ‰, respectively. Using these elemental and isotopic compositions for humic acids, we ran the simulation on four different types of collagen (Table 2). The elemental compositions of the cod, cattle, and humans were based on average amino acid compositions measured for these species. The elemental compositions for seal represent the mean of all mammalian bone collagen (Guiry and Szpak, 2020). The isotopic composition of the 'global human mode' represents the most common $\delta^{13}C$ and $\delta^{15}N$ values measured on ancient human bone collagen based on our review of the literature. For one example, we simulated humic contamination in a C_4-dominated environment. All other parts of the simulation were the same, but we changed the $\delta^{13}C$ of the humic contaminants to −12.50 ‰. Under these circumstances, we simulated only contamination of a human bone from an individual with a diet rich in C_4 plants ($\delta^{13}C = -10.00$ ‰) as a situation in which animals with very low bone collagen $\delta^{13}C$ values would be deposited in a C_4-dominated environment is not realistic.

3.1.1. Testing the collagen-humic acid contamination model

Using the above model, we attempted to simulate real world conditions for ancient bone collagen contaminated with various proportions of humic acids for the same set of taxa outlined in Table 2. Specifically, we sought to determine which variables (e.g., collagen isotopic composition, collagen elemental composition, amount of isotopic variance within a population) influenced the relationship observed between C:N and $\delta^{13}C$ or $\delta^{15}N$.

For each species, twelve simulations were performed, each of which

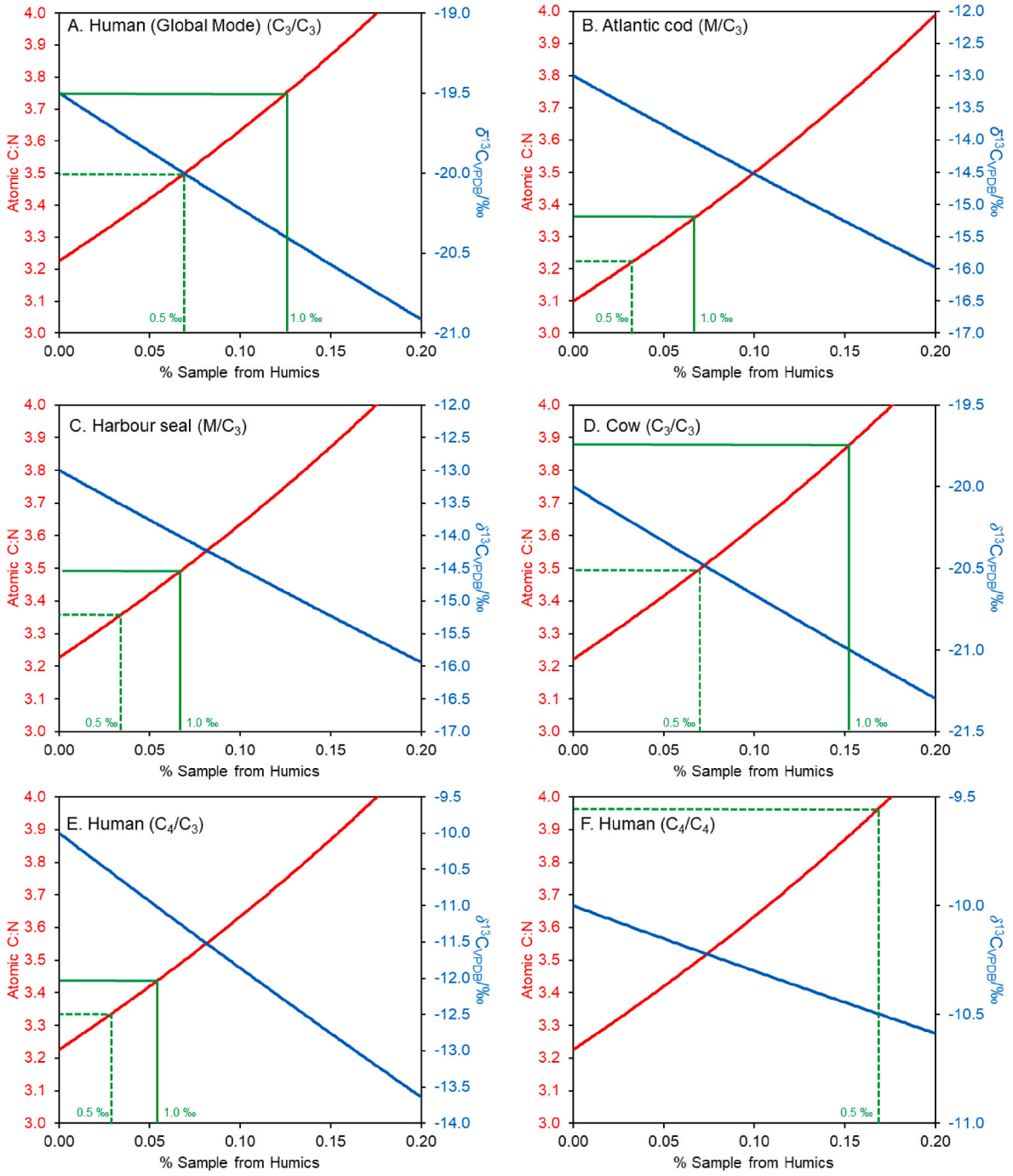

Fig. 3. Modeled C:N and $\delta^{13}C$ for each of the bone collagen specimens, highlighting only C:N between 3.0 and 4.0. The broken lines on each panel represent the fraction of humic contamination and the C:N that would result in a shift of 0.5 ‰ and 1.0 ‰. Panel F does not present broken lines for 1.0 ‰ because this shift would require a C:N of >4.0.

consisted of a randomly generated Gaussian distribution of 10,000 individuals. In each of these simulations, the isotopic and elemental compositions of the humic contaminants was kept constant ($\delta^{13}C$ = −25.53 ‰ or −12.50 ‰, $\delta^{15}N$ = −1.52 ‰; wt% C = 51.67%, wt% N = 1.20%). The elemental compositions of the bone collagen within a single taxon were also kept constant. The twelve simulations differed in terms of the amount of isotopic variation in the uncontaminated bone collagen, with standard deviations for $\delta^{13}C$ and $\delta^{15}N$ being: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0 ‰. Each individual was subjected to a randomized amount of humic contamination, wherein the fraction of humics in the analyzed sample would be between 0 and 0.125. These levels were selected based on the results of the modeling as they likely represent realistic levels observed in ancient bone collagen *and* would still produce, in most cases, C:N in the analyzed samples that could be considered acceptable by traditional QC criteria. Low levels of humic contamination occurred with a higher probability than high levels of humic contamination (Fig. 2). The same distribution of humic contamination was sampled across all taxa and individuals. Using the system of equations outlined previously, we were able to determine the isotopic and elemental compositions for the resulting mixed sample consisting of bone collagen and humic contaminants.

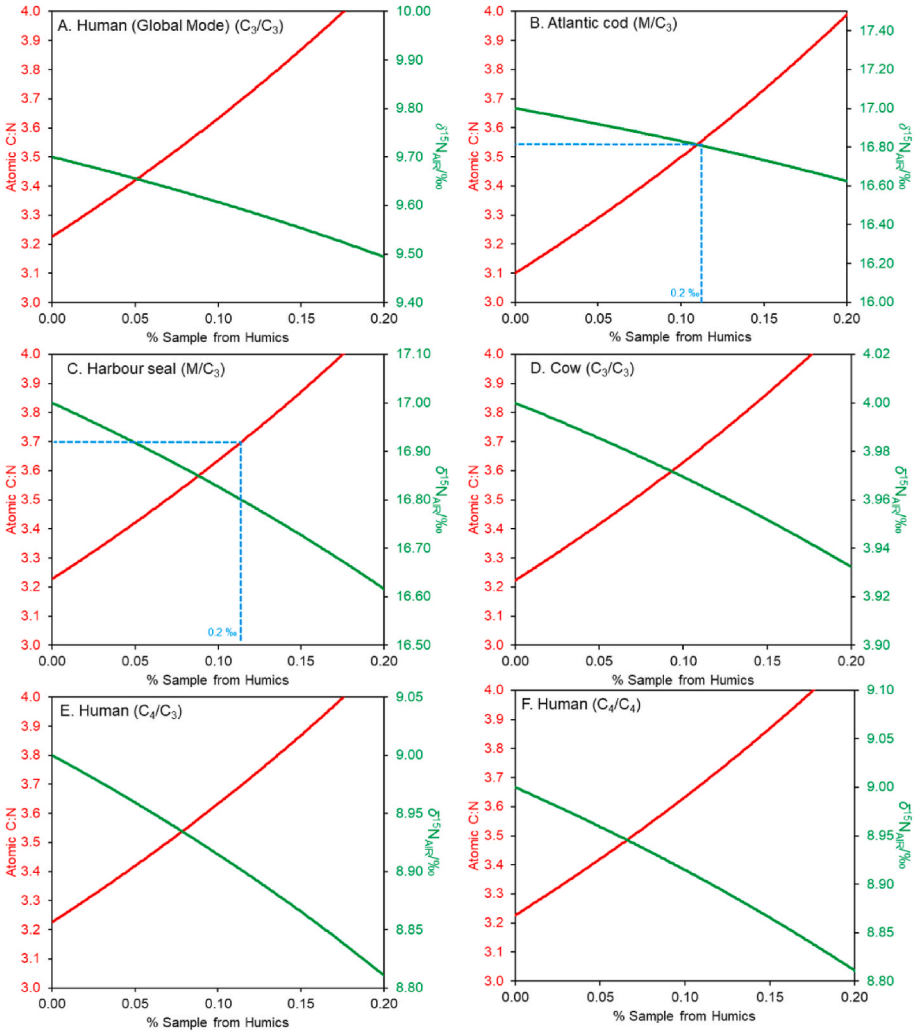

Fig. 4. Modeled C:N and δ^{15}N for each of the bone collagen specimens, highlighting only C:N between 3.0 and 4.0. The broken lines represent the fraction of humic contamination and the C:N that would result in a shift of 0.2 ‰. The fact that there are no broken lines in most panels indicates that C:N < 4.0 does not cause an appreciable shift in δ^{15}N for most sample types.

3.2. Isotopic comparisons

In order to assess the extent to which isotopic compositions of ancient samples with acceptable QC criteria may be skewed, we compared δ^{13}C and δ^{15}N against C:N for collagen extracted from large numbers of samples within a single species from a narrowly defined geographic region. Our rational was that, in making comparisons within a single species, differences driven by *post mortem* alteration (rather than dietary or baseline variation) would be more easily detectable than if comparisons included multiple species with variable ecological niches. Because there are no physiological or environmental processes, other than *post mortem* alteration, that should result in a correlation between collagen isotopic compositions (δ^{13}C, δ^{15}N) and QC indicators (C:N, %C, and %N) the presence of any correlation between these data provides a clear indication that isotopic compositions have been, to some extent, skewed.

We compared bone collagen isotopic compositions and QC indicators from archaeological Atlantic cod (*Gadus mourha*; hereafter "cod", n = 657; 605 from this study, 52 from; Guiry et al., 2012; Hutchinson et al., 2015 from sites in eastern Canada Newfoundland and Nova Scotia). We also made comparisons using previously published archaeological data from terrestrial (cattle, *Bos taurus* for Scottish sites, n = 149; Fraser et al., 2017; Jones and Mulville, 2016; Jones and Mulville, 2018) and marine (ringed seal, *Pusa hispida* from the Canadian Arctic, n = 759; Szpak et al., 2019b) mammals. These comparisons were designed to highlight how measured isotopic compositions can vary in relation to: 1) differences in C:N that occur among taxa and environments (e.g., lower in cold water fish and higher in mammals); with, 2) collagen isotopic compositions (δ^{13}C lower in terrestrial mammals and higher in cod and marine mammals) that differ relative to potential local humic acid contaminant sources.

3.2.1. Sample treatment

Samples from 605 cod were collected from 10 sixteenth-to-nineteenth-century sites as part another project aimed at developing a regional survey of isotopic variation in historic cod populations. Samples included specimens representing a wide range of preservation contexts within (e.g., varying taphonomic preservation) and between (e.g., including underwater and terrestrial excavation) sites (ESM 1, Table S1).

Collagen was extracted from all samples following the same protocols. Samples were cut into small cubes and soaked in 2:1 chloroform-methanol solution in an ultrasonic bath (solution refreshed every 15 min until solution remained clear) to remove residual lipids. Samples were then demineralized in 0.5 M HCl and neutralized in Type I water. Demineralized samples were then treated with 0.1 M NaOH in an ultrasonic bath (solution refreshed every 15 min until solution remained clear). Sample were neutralized in Type 1 water, refluxed in 0.01 HCl at

Table 3
Shifts in isotopic compositions ($\Delta^{13}C$ and $\Delta^{15}N$) and C:N caused by variable amounts of humic contamination. Values in boldface indicate those >0.5 ‰). Asterisks indicator the initial C:N for a given sample type when there was no humic contamination.

	C:N	*f* Humics	$\Delta^{13}C$ (‰)	$\Delta^{15}N$ (‰)
Human (Global Mode)	3.23*	–	–	–
	3.30	0.018	−0.14	−0.02
	3.40	0.044	−0.32	−0.04
	3.50	0.069	**−0.50**	−0.06
	3.60	0.092	**−0.76**	−0.08
Atlantic cod	3.10*	–	–	–
	3.20	0.026	−0.40	−0.04
	3.30	0.052	**−0.80**	−0.08
	3.40	0.076	**−1.17**	−0.13
	3.50	0.100	**−1.52**	−0.17
	3.60	0.122	**−1.85**	−0.21
Harbour seal	3.23*	–	–	–
	3.30	0.018	−0.27	−0.03
	3.40	0.044	**−0.67**	−0.07
	3.50	0.068	**−1.03**	−0.11
	3.60	0.091	**−1.37**	−0.16
Cattle (C_3 Diet, C_3 Environment)	3.22*	–	–	–
	3.30	0.020	−0.13	−0.01
	3.40	0.045	−0.30	−0.01
	3.50	0.070	−0.47	−0.02
	3.60	0.093	**−0.62**	−0.03
Human (C_4 Diet, C_3 Environment)	3.23*	–	–	–
	3.30	0.019	−0.36	−0.01
	3.40	0.044	**−0.83**	−0.04
	3.50	0.069	**−1.29**	−0.06
	3.60	0.092	**−1.71**	−0.08
Human (C_4 Diet, C_4 Environment)	3.23*	–	–	–
	3.30	0.024	−0.06	−0.01
	3.40	0.046	−0.14	−0.04
	3.50	0.069	−0.21	−0.06
	3.60	0.092	−0.28	−0.08

70 °C for 36 h, and then centrifuged to separate out remaining solids. Solubilized collagen was transferred to a fresh tube with a Pasteur pipette, frozen, and lyophilized.

Isotopic and elemental compositions were measured on 500 μg subsamples of collagen on a Vario MICRO elemental analyzer coupled via continuous flow to an Isoprime Isotope ratio mass spectrometer at the Archaeology Chemistry Laboratory at The University of British Columbia (Vancouver, Canada). Stable carbon and nitrogen isotope compositions of samples as well as three check standards were calibrated relative to VPDB and AIR, anchored to USGS40 and USGS41a (Qi et al., 2003, 2016). Accepted and observed long-term averages for all check and calibrations standards are provided in ESM 1, Table S2. Replicate numbers, means, and standard deviations for check standards (Table S3), calibration standards (Table S4), and sample replicates (Table S5) are also provided in ESM 1. Following Szpak et al. (2017b), for $\delta^{13}C$ and $\delta^{15}N$, respectively: systematic errors ($u_{(bias)}$) were ±0.11‰ and ±0.13‰; random errors ($uR_{(w)}$) were ±0.10‰ and ±0.10‰; standard uncertainty was ±0.15‰ and ±0.16‰.

4. Results

4.1. Collagen/humic acid contamination model

The modeled isotopic and elemental compositions for the humic-contaminated bone collagen for the human (global mode) are presented in full in ESM 1, Fig. S2. Figs. 3 and 4 highlight the area of concern for studies of ancient collagen, specifically C:N between 3.0 and 4.0. These data are presented in full for the different taxa in ESM 2. Humic contamination had a profound effect on sample $\delta^{13}C$ (Fig. 3), but a negligible effect on $\delta^{15}N$ (Fig. 4) owing to the high [C] and low [N] in humic substances. The effect of humic contamination was greatest when the $\delta^{13}C$ or $\delta^{15}N$ of the bone collagen and humics differed to the greatest extent (Table 3). In other words, in an environment where the humic acids were derived from C_3 plant matter, the bone collagen of marine organisms with high $\delta^{13}C$ and $\delta^{15}N$ were more susceptible to having altered isotopic compositions than terrestrial organisms with low $\delta^{13}C$ and $\delta^{15}N$ (Figs. 3 and 4). The same was true for the $\delta^{13}C$ values of organisms consuming a diet rich in C_4 resources when the surrounding environment was dominated by C_3 plants, as reflected in the human with the C_4 diet (Fig. 3e).

Of the two factors that might impact the extent to which isotopic compositions of collagen could be altered by humic contamination (initial isotopic compositions and initial C:N ratios), the initial isotopic compositions had a much greater impact than the initial C:N of the collagen (Table 3, ESM 2). Both the ringed seal and cod had the same initial isotopic compositions, but the C:N ratio of cod was 3.10 whereas for ringed seal it was 3.23. When humic contamination made up 3% of the analyzed sample by mass, the $\delta^{13}C$ of cod and ringed seal were both shifted by −0.5 ‰ and the C:N was 3.21 for cod and 3.34 for seal. When humic contamination made up 10% of the analyzed sample, $\delta^{13}C$ for both taxa were shifted by −1.5 ‰ and the C:N was 3.50 for cod and 3.63 for seal. Therefore, taxa with low bone collagen C:N will have slightly more altered $\delta^{13}C$ near the upper limit of the traditional acceptable range. Notably, a C:N of 3.6 equates to shifts in $\delta^{13}C$ of −0.28 ‰ (human with a C_4 diet in a C_4 environment), −0.62 ‰ (cattle with a C_3 diet in a C_3 environment), −0.76 ‰ (human with global mode values), −1.37 ‰ (ringed seal), −1.71 ‰ (human with a C_4 diet in a C_3 environment), and −1.85 ‰ (Atlantic cod). This result calls into question the universality of the 2.9–3.6 range for C:N as a QC metric for ancient collagen.

In the simulated ancient populations, those taxa that had collagen isotopic compositions that differed substantially from those of the humics were most affected by contamination as reflected in the steeper slopes and stronger correlations when C:N and $\delta^{13}C$ were compared (Fig. 5). Specifically, the two marine animals (cod and ringed seal) and the humans with the C_4 diet living in a C_3-dominated environment were most impacted, while those groups that had $\delta^{13}C$ values more similar to the humic contaminants were less impacted (Fig. 5). The $\delta^{15}N$ values did not vary significantly with C:N across the range of taxa and environments examined (Fig. 6).

The strength of the relationship between C:N and $\delta^{13}C$ varied according to the amount of isotopic variance in the bone collagen of each population. At the lowest level of variance (standard deviation of 0.1 ‰ for $\delta^{13}C$), the relationship between C:N and $\delta^{13}C$ was strongest, and the strength of this relationship steadily decreased as the amount of variation increased (Fig. 7, ESM 3–7). This pattern held true for all species.

4.2. Isotopic analyses

Isotopic and elemental compositions from cod are shown in ESM 1, Table S6 and are summarized in Table S7. Isotopic analyses of cod (n = 605; 657 including data for literature; see Fig. 8) produced a range of $\delta^{13}C$ (mean = −14.4 ± 0.1 ‰; range = −16.3 to −12.9 ‰) and $\delta^{15}N$ (mean = +15.4 ± 0.8 ‰; range = +19.1 to +13.1 ‰). Sample C:N for cod ranged between 3.03 and 3.64 (mean = 3.24) with nearly all (604 of 605, or 99.8%) falling within the envelope of acceptability defined by DeNiro (1985). While not the focus of this paper, these data also provide a basis for exploring inter- and intra-site variation in the relationship between C:N, isotopic compositions, and taphonomic context (for detailed discussion see ESM 1).

Fig. 8 highlights variation in the relationship between C:N and collagen isotopic compositions among taxa that are expected to have different relationships between isotopic compositions and C:N on the basis of our modeling results (Fig. 5). For cod (n = 657, including previously published data, n = 52 ;Guiry et al., 2012; Hutchinson et al., 2015) from eastern Canada, $\delta^{13}C$ was strongly and significantly negatively correlated with C:N (n = 657, Pearson's r = −0.558, p < 0.001) while $\delta^{15}N$ was weakly, but also significantly negatively correlated with C:N (n = 657, Pearson's r = −0.110, p = 0.004, see Fig. 8). A strong

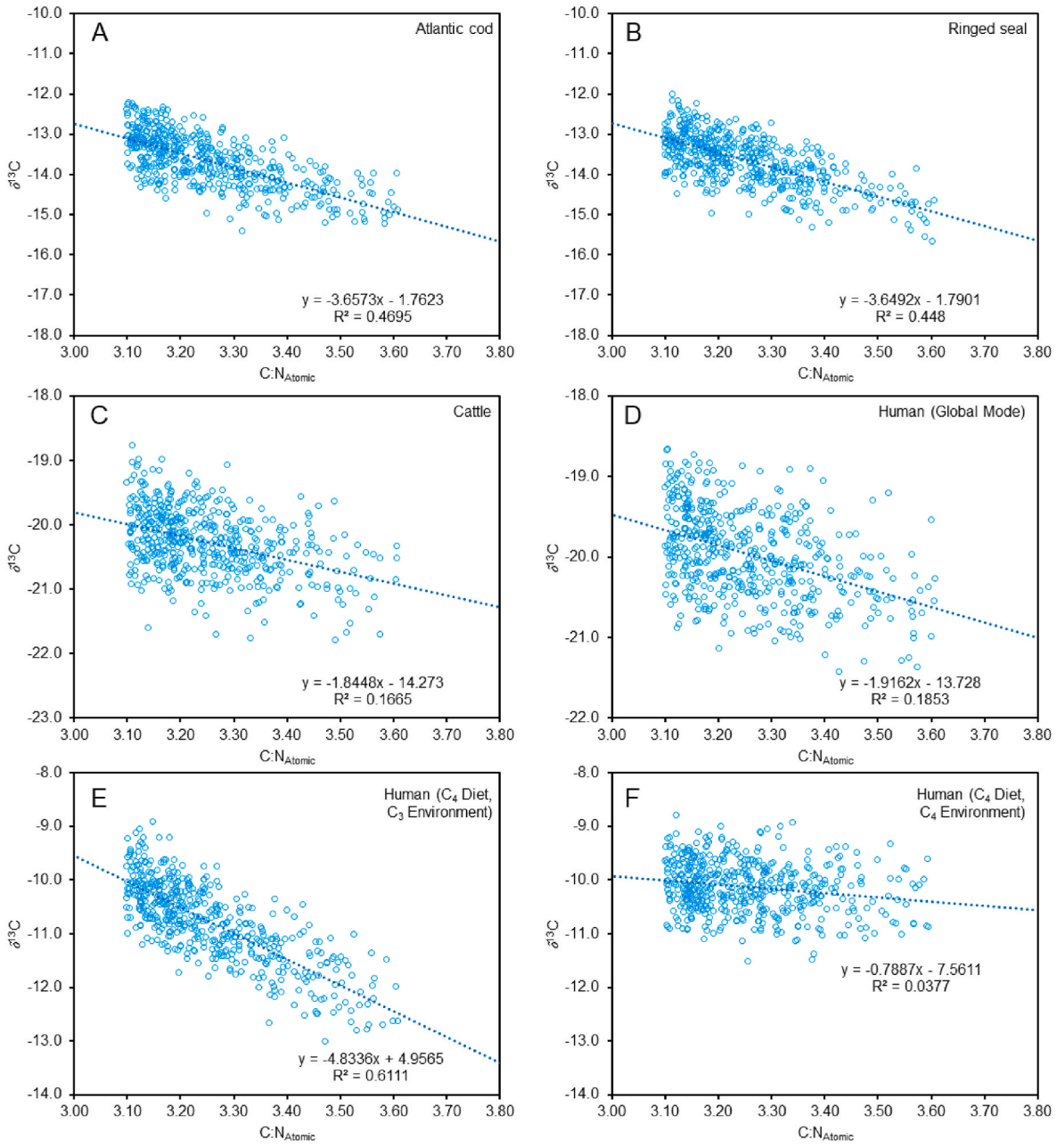

Fig. 5. Relationship between $\delta^{13}C$ and C:N for each of the modeled scenarios for 500 randomly selected samples. For each case, the standard deviation of the $\delta^{13}C$ values was set to 0.5 ‰.

negative correlation between C:N and $\delta^{13}C$ was also observed among published data from ringed seals from the Canadian Arctic ($n = 759$, Pearson's $r = -0.493$, $p < 0.001$; Szpak et al., 2019b) and cattle from Scotland ($n = 149$, Pearson's $r = -0.431$, $p < 0.001$; Fraser et al., 2017; Jones and Mulville, 2016; Jones and Mulville, 2018). In contrast to cod, a significant correlation between C:N and $\delta^{15}N$ was not found among either ringed seals (Pearson's $r = -0.054$, $p = 0.135$) or cattle (Pearson's $r = +0.048$, $p = 0.599$).

5. Discussion and conclusion

5.1. Simulated humic contamination

The results of the modeled data allowed us to make several predictions about the relationship between elemental and isotopic compositions of ancient collagen. These relationships are important because stronger correlations imply a more profound impact of humic contamination on the isotopic compositions of the samples. First, for a dataset of ancient collagen from a single species and context, we would expect a stronger relationship between $\delta^{13}C$ and C:N than for $\delta^{15}N$ and C:N. This expectation is based on the fact that humic acids contain more carbon and much less nitrogen than bone collagen. The presence of humics will therefore have a much stronger impact on C:N than it will on $\delta^{15}N$ until a point at which the proportion of humics in the measured sample is far beyond what would be realistically expected for ancient samples (Fig. 4), considering that C:N > 5 are very rare (Fig. 1). The difference in isotopic compositions between the endogenous collagen and the humic acids should have a strong impact on the strength of the relationship between $\delta^{13}C$ or $\delta^{15}N$ and C:N. Within individual taxa, we should also see the strongest relationship between $\delta^{13}C$ and C:N for marine organisms or organisms consuming a high proportion of C_4 plants (or animals consuming C_4 plants) whose bones were deposited in a terrestrial

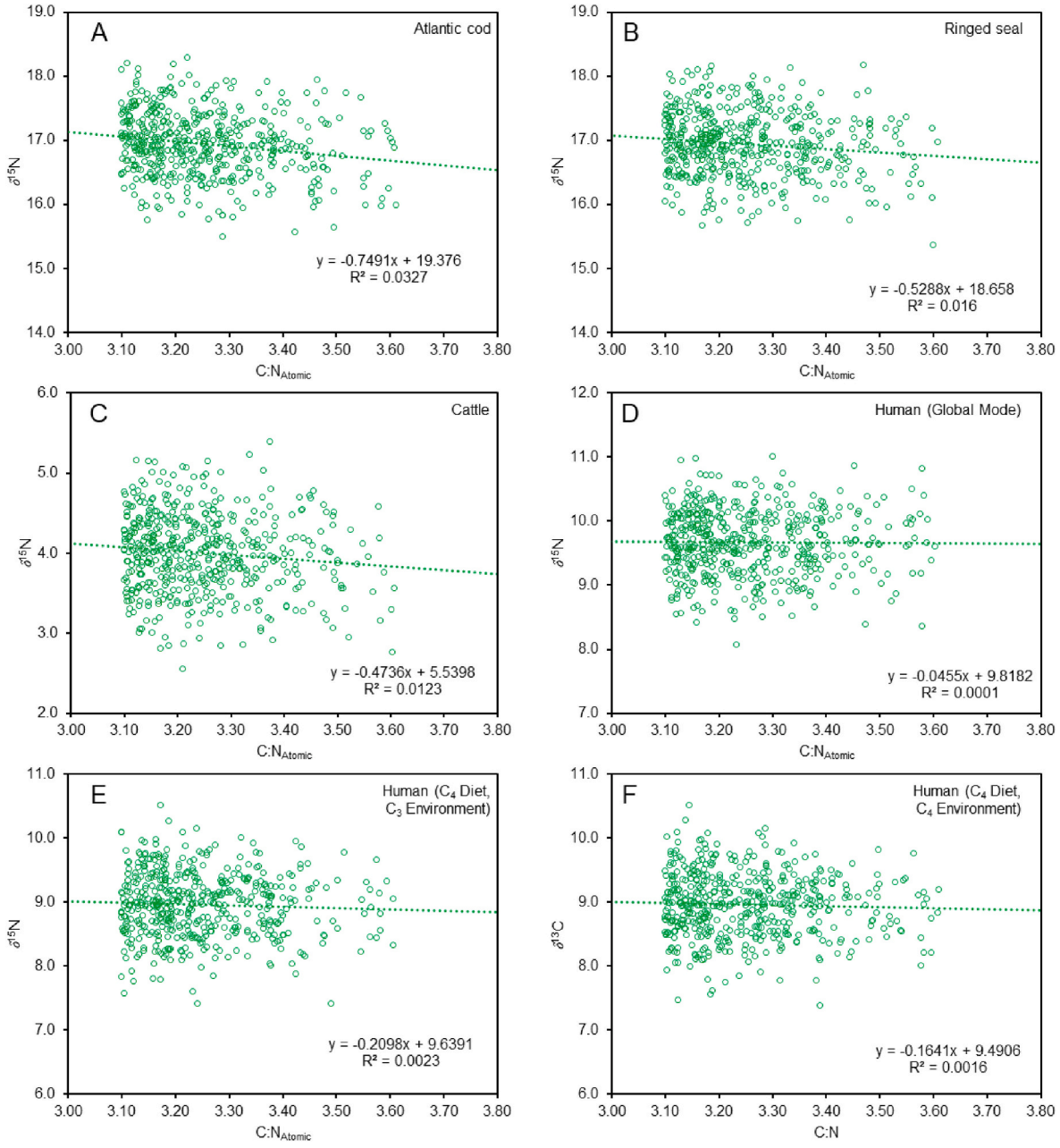

Fig. 6. Relationship between $\delta^{15}N$ and C:N for each of the modeled scenarios for 500 randomly selected samples. For each case, the standard deviation of the $\delta^{15}N$ values was set to 0.5 ‰.

environment dominated by C_3 plants (Fig. 5). Although we do not expect to see strong correlations between $\delta^{15}N$ and C:N, this should occur for marine organisms or terrestrial organisms with high $\delta^{15}N$ whose bones were deposited in terrestrial environments (Fig. 6). Species with collagen amino acid compositions that result in relatively low C:N should be characterized by stronger relationships between $\delta^{13}C$ or $\delta^{15}N$ and C:N than species with higher initial C:N. The effect of the initial C:N of the collagen should not be as strong as the effect of the difference in isotopic compositions between the humics and the collagen.

5.2. Isotopic and collagen QC comparisons

Findings from modeled data were supported by our comparisons of C:N and isotopic compositions measured on archaeological bone collagen from different taxa and provide a useful illustration of how biological and behavioural factors can influence the extent to which isotopic data derived from bone collagen will be altered while still producing C:N that fall within the acceptable envelope. In particular, knowledge of species' behavioural ecology, variation in regional isotopic baselines, and the amino acid compositions of collagen from different taxa have allowed us to anticipate the interspecific patterns observed in the relationship between bone collagen C:N and isotopic compositions (Fig. 8). Consistent with the results of the modelling, cod bone collagen, with a lower true C:N (c. 3.10) and higher $\delta^{13}C$, has the greatest potential for contamination within the C:N envelope of 2.90–3.60 and had the strongest relationship between C:N and $\delta^{13}C$. This shows that NaOH pre-treatment protocols used during collagen extraction were not effective at fully removing humic acid contamination. In this context, it is possible that that cod samples may have benefited from additional washes in NaOH, which may have resulted in further reductions in C:N. In contrast to cod, cattle, with a higher true C:N (c. 3.22) and lower $\delta^{13}C$, show a much smaller but still significant relationship.

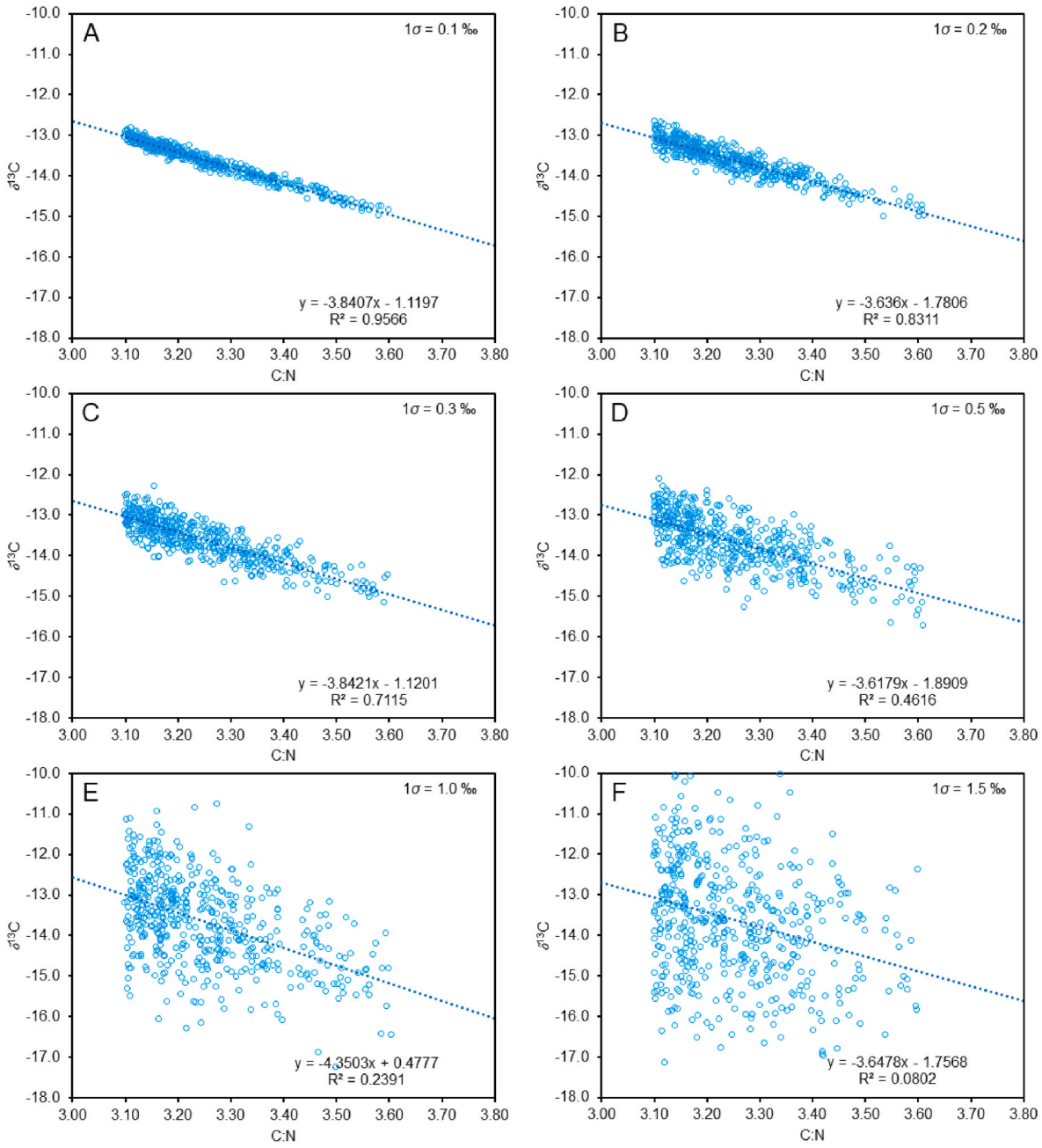

Fig. 7. Relationship between $\delta^{13}C$ and C:N for Atlantic cod at six different levels of initial variation in collagen $\delta^{13}C$ (standard deviations of 0.1 ‰ [A], 0.2 ‰ [B], 0.3 ‰ [C], 0.5 ‰ [D], 1.0 ‰ [E], and 1.5 ‰ [F]) for 500 randomly selected samples.

Ringed seals, with a higher $\delta^{13}C$ and true C:N (c. 3.23), fall between these two endpoints of susceptibility to humic acid contamination-related skewing of bone collagen $\delta^{13}C$.

In contrast to $\delta^{13}C$, significant correlations between C:N and $\delta^{15}N$ were rare among our comparisons (see also ESM 1). The fact that cod showed a significant correlation between C:N and $\delta^{15}N$, while ringed seals and cattle did not, may reflect the additive effects of their lower true C:N and the high $\delta^{15}N$ of the collagen. Because archaeological cod bone collagen with a C:N close to 3.60 will contain proportionally more contamination derived from humic acids than will seal or cattle (Fig. 4), the cod are more likely to have altered $\delta^{15}N$ due to humic contamination (see Section 5.1). This hypothesis is, however, difficult to test with real data.

5.3. Best practices for using collagen QC

The results of the modeling as well as the real ancient bone collagen data demonstrate that the most widely used QC criterion for ancient collagen (DeNiro's C:N range of 2.9–3.6, see Table 1) is not equally effective across a range of species and environments. In this section, we outline a series of best practices aimed at maximizing the number of useable data and rejecting those that may be characterized by altered isotopic compositions.

Taking steps to prevent the presence of humic contaminants in the extracted collagen is the best defense. Based on previous experimental studies, the use of centrifugal filters ('ultrafilters') is not effective at removing humic contaminants and because these compounds have high molecular weights, may actually selectively retain them (Szpak et al., 2017a). Treatment of the collagen with sodium hydroxide appears to be

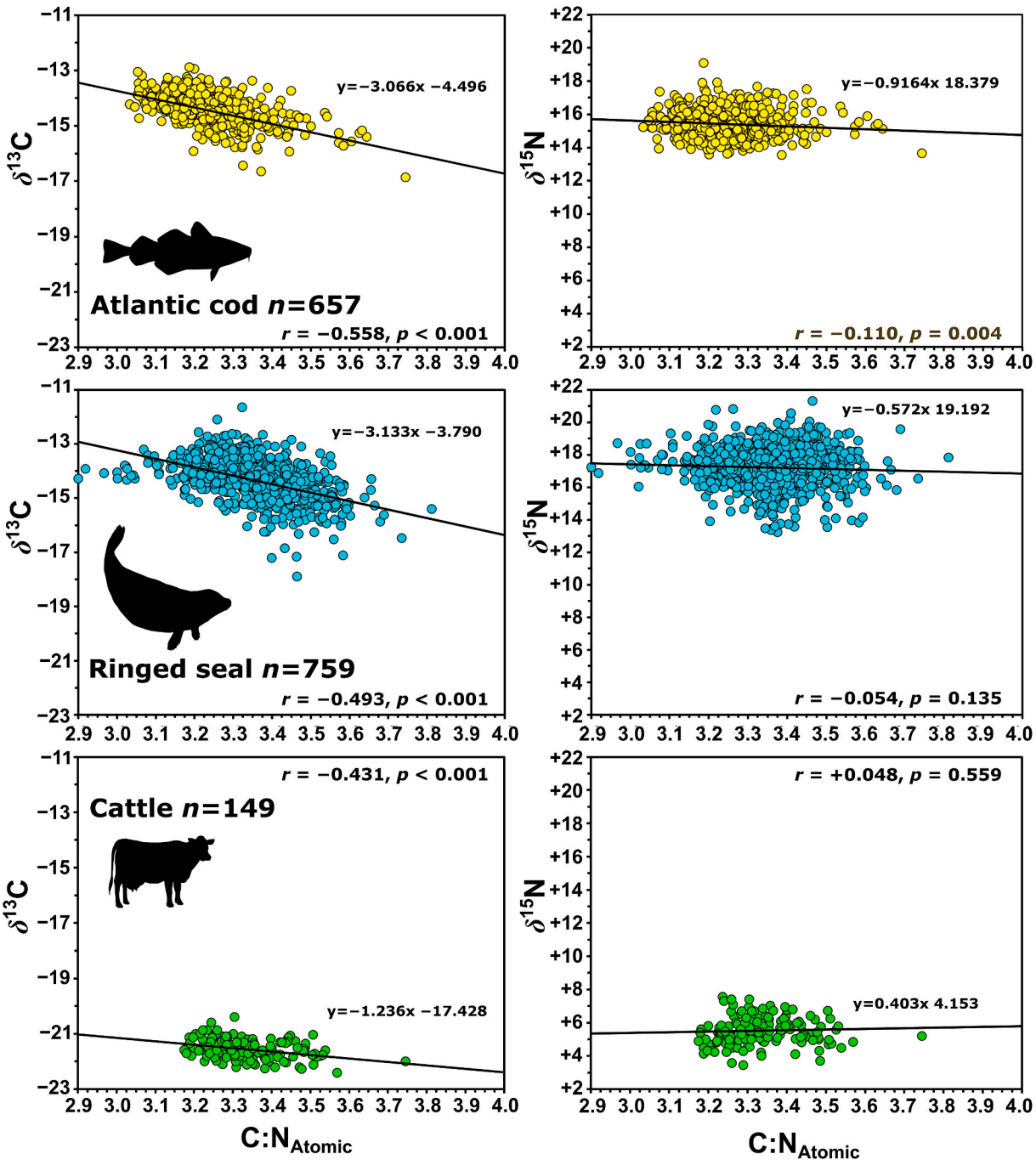

Fig. 8. Bone collagen isotopic compositions compared with C:N from archaeological Atlantic cod from eastern Canada (data from this study, $n = 657$, and published sources, $n = 52$; Guiry et al., 2012; Hutchinson et al., 2015), ringed seals from the Canadian Arctic (Szpak et al., 2019b), and cattle from the Scotland (Fraser et al., 2017; Jones and Mulville, 2016, 2018).

the most effective technique for removing humics (Szpak et al., 2017a), but the efficacy of this technique must be balanced against the fact that strongly alkali solutions are detrimental to the collagen itself (Rudakova and Zaikov, 1987). The use of XAD resins may also be effective at removing humics (Stafford et al., 1988), but such treatments may be cost or time prohibitive, particularly in the context of studies using EA-IRMS and producing hundreds of analyses.

A standard practice for exploratory data analysis should be to plot the C:N against the $\delta^{13}C$ and $\delta^{15}N$ for an individual taxon. The presence of a correlation signals the presence of humic contamination as discussed in previous sections. Such a plot should also be considered as a QC metric for ancient collagen. There are, however, some limitations to this approach. First, it is limited to analyses of a single taxon. Second, this approach works best when the isotopic variation within that taxon is relatively low (Fig. 6). In some archaeological contexts, single wild or livestock species may be characterized by $\delta^{13}C$ values across a 10‰ range (Guiry et al., 2016a, 2017; Pearson et al., 2007; Szpak et al., 2019a) and this high level of variation would serve to limit or completely obscure the existence of any correlations between C:N and $\delta^{13}C$.

The C:N remains a robust indicator of collagen preservation, particularly when paired with elemental compositions and collagen yield (Ambrose, 1990). We suggest, however, that the upper limit of this range be modified in some circumstances, although individual

Table 4
Suggested alternative upper limits for ancient collagen C:N.

Collagen C:N	Example Taxa	Expected Animal Bone Collagen $\delta^{13}C$	Conservative Upper Limit of C:N (0.5 ‰ Tolerance)	Liberal Upper Limit of C:N (1.0 ‰ Tolerance)
>3.15	Mammals, birds, warm-water fish	−20.0‰	3.50	3.90
		−18.0‰	3.45	3.70
		−16.0‰	3.40	3.60
		−14.0‰	3.35	3.50
		−12.0‰	3.35	3.45
		−10.0‰	3.35	3.45
		−8.0‰	3.30	3.40
<3.15	Cold water fish	−20.0‰	3.40	3.70
		−18.0‰	3.30	3.55
		−16.0‰	3.25	3.45
		−14.0‰	3.25	3.40
		−12.0‰	3.20	3.35
		−10.0‰	3.20	3.30
		−8.0‰	3.20	3.30

investigators may use some level of discretion depending on their research questions. For example, in a study that relies on $\delta^{15}N$ values of bulk bone collagen to interpret changes in environmental conditions or foraging behaviour over time, samples characterized by high C:N ratios are unlikely to negatively impact the interpretations since the presence of humic contaminants minimally impacts $\delta^{15}N$ (Table 4, Fig. 8). We would caution, however, that samples with low collagen yields or low carbon and nitrogen concentrations (Ambrose, 1990) should still be excluded. As far as $\delta^{15}N$ is concerned, the existing QC criterion of 3.60 for C:N is sufficient and if anything, may be too conservative as our worst case scenario (cod with a C:N of 3.60, had $\delta^{15}N$ that was only shifted by 0.2 ‰). With respect to $\delta^{13}C$, however, our data indicate that a narrower range should be used in some circumstances. How much narrower this range should be will depend on the kinds of questions being asked by the individual researchers and the degree of resolution required in a particular study. Accordingly, we propose two sets of criteria, one more conservative than the other (Table 4). The more conservative metric uses a cut-off point where the $\delta^{13}C$ values are expected to be shifted by 0.5 ‰ or less. The less conservative metric uses a cut-off point where the $\delta^{13}C$ values are expected to be shifted by 1.0 ‰ or less. By way of comparison, under commonly-encountered scenarios (i.e., excluding the C_4 human from a C_4 environment), DeNiro's upper limit of 3.60 for C:N results in shifts of between 0.62 ‰ and 1.85 ‰ (Table 4). Since it is has been fairly common for studies presenting isotopic measurements of modern bone collagen to cite the 2.9–3.6 range for C:N as 'representative of the range observed in modern collagen', we must emphasize that the criteria outlined here should only be applied to ancient samples where exogenous humic contamination is suspected. For modern samples, the primary concern is lipid contamination and an alternative set of criteria should be followed (Guiry and Szpak, 2020).

C:N ratios are reported variably to one or two decimal places in the literature. Reporting C:N to two, rather than one, decimal places can have a small but meaningful impact on whether samples meet QC criteria (Table 5). While the uncertainty on elemental compositions as measured by EA-IRMS is typically high, the uncertainty on the resulting C:N ratios is much lower. For example, in surveying our previously published data wherein samples had been analyzed in duplicate, the mean differences between duplicate pairs were: 1.11% for wt% C, 0.40% for wt% N, and 0.09 for C:N (n = 3,646 analyses). While these metrics will certainly vary among instruments, we suggest that C:N ratios be reported to two decimal places and carbon and nitrogen elemental compositions be reported to one decimal place.

One potential solution to the problem of data characterized by a high degree of humic contamination is to generate a correction for $\delta^{13}C$ based on the relationship between C:N and $\delta^{13}C$. Post et al. (2007) presented a similar method for lipid-contaminated samples. This approach should be used with great caution and only under certain circumstances. First, the sample size must be large. Second, there should be a high representation of samples with both normal and high C:N. If both of these criteria are not met, the probability of introducing systematic bias in the accuracy of the measurements significantly increases. In those instances where a large number of data have been collected, but a fairly small number have high C:N, a more prudent approach would be to simply reject those few samples.

We applied these new QC criteria to the published data that we surveyed to produce Fig. 1. The conservative criterion (expected shift in $\delta^{13}C < 0.5$ ‰) excluded large numbers of samples that would otherwise have been accepted across the board (Table 5). The liberal criterion (expected shift in $\delta^{13}C < 1.0$ ‰) excluded a similar, or even lower, number of analyses than the DeNiro criterion when the $\delta^{13}C$ of the

Table 5
Impact of different collagen QC criteria on the number of accepted isotopic compositions shown as percentages of samples that would need to be rejected. For conservative (0.5 ‰ tolerance) and liberal (1.0 ‰ tolerance) guidelines see Table 4 (>3.15 category). For DeNiro (1985) a range of 2.9–3.6 was applied uniformly to all groups calculated at 1 decimal place (i.e, cut-off set to 3.6) 2 decimal places (i.e., cut-toff set at 3.60). Color coding shows difference relative to DeNiro (1985) calculated to 1 decimal place (the mostly widely used approach) as follows: red = 20–30% more samples rejected; orange = 10–20% more samples rejected; yellow = 0.1–10% more samples rejected; green = fewer samples rejected. Data are from survey presented in Fig. 1 using only mammal/bird/reptile samples with C:N > 2.90 and for which C:N could be re-calculated using %C and %N.

$\delta^{13}C$ Range	***n*=**	**% of Analyses Failing Collagen QC Criteria**			
		Conservative	**Liberal**	**DeNiro 1985**	
				2 decimal	1 decimal
< −20 ‰	6059	12.9	2.7	5.4	4.2
−20 to −18 ‰	6047	7.4	1.1	2.8	1.5
−18 to −16 ‰	1752	20.7	5.9	5.9	4.1
−16 to −14 ‰	1503	41.3	9.4	3.0	2.3
−14 to −12 ‰	1047	21.6	7.8	2.0	1.7
−12 to −10 ‰	575	17.7	7.1	2.8	2.4
> −10 ‰	837	35.5	19.2	3.0	1.6
All Analyses	17820	15.9	4.2	4.0	2.8

samples were low. When the sample $\delta^{13}C$ were high, however, both the liberal and conservative criteria excluded far more samples than the DeNiro criterion. The application of these new criteria will decrease the number of samples included in some analyses but will improve the quality of the data.

Declaration of competing interest

We have no conflicts of interest, financial or otherwise, to declare.

Acknowledgements

For samples we thanks the Lori Temple at the Rooms Museum, Charles Dagneau at Parks Canada, Jonathan Kyte at Seahorse Cultural Resource Management Services, Barry Gaulton at Memorial University, and Stéphane Noël. Thanks also to Jonathan Duelks and Rani Bevilacqua for laboratory assistance. Funding: Social Science and Humanities Research Council of Canada, the University of British Columbia's Department of Anthropology, and the Canada Research Chairs Program. Both authors contributed equally to all aspects of the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jas.2021.105416.

References

Ajie, H.O., Kaplan, I.R., Slota, P.J., Taylor, R.E., 1990. AMS radiocarbon dating of bone osteocalcin. Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. Atoms 52, 433–437.
Ambrose, S.H., 1990. Preparation and characterization of bone and tooth collagen for isotopic analysis. J. Archaeol. Sci. 17, 431–451.
Buckley, M., 2018. Zooarchaeology by mass spectrometry (ZooMS) collagen fingerprinting for the species identification of archaeological bone fragments. In: Giovas, C.M., LeFebvre, M.J. (Eds.), Zooarchaeology in Practice. Springer, Cham, pp. 227–247.
Collins, M.J., Gernaey, A.M., Nielsen-Marsh, C.M., Vermeer, C., Westbroek, P., 2000. Slow rates of degradation of osteocalcin: green light for fossil bone protein? Geol. 28, 1139–1142.
Collins, M.J., Nielsen–Marsh, C.M., Hiller, J., Smith, C., Roberts, J., Prigodich, R., Wess, T.J., Csapo, J., Millard, A.R., Turner–Walker, G., 2002. The survival of organic matter in bone: a review. Archaeometry 44, 383–394.
Colonese, A.C., Farrell, T., Lucquin, A., Firth, D., Charlton, S., Robson, H.K., Alexander, M., Craig, O.E., 2015. Archaeological bone lipids as palaeodietary markers. Rapid Commun. Mass Spectrom. 29, 611–618.
DeNiro, M.J., 1985. Postmortem preservation and alteration of in vivo bone collagen isotope ratios in relation to palaeodietary reconstruction. Nat 317, 806–809.
DeNiro, M.J., Epstein, S., 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Sci 197, 261.
DeNiro, M.J., Weiner, S., 1988. Chemical, enzymatic and spectroscopic characterization of "collagen" and other organic fractions from prehistoric bones. Geochem. Cosmochim. Acta 52, 2197–2206.
Dobberstein, R.C., Collins, M.J., Craig, O.E., Taylor, G., Penkman, K.E., Ritz-Timme, S., 2009. Archaeological collagen: why worry about collagen diagenesis? Archaeological and Anthropological Sciences 1, 31–42.
Evershed, R.P., Turner-Walker, G., Hedges, R.E., Tuross, N., Leyden, A., 1995. Preliminary results for the analysis of lipids in ancient bone. J. Archaeol. Sci. 22, 277–290.
Fisher, L.W., Hawkins, G.R., Tuross, N., Termine, J.D., 1987. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J. Biol. Chem. 262, 9702–9708.
Francioso, O., Montecchio, D., Gioacchini, P., Ciavatta, C., 2005. Thermal analysis (TG–DTA) and isotopic characterization (^{13}C–^{15}N) of humic acids from different origins. Appl. Geochem. 20, 537–544.
Fraser, S., Elsner, J., Derek Hamilton, W., Sayle, K.L., Schlumbaum, A., Bartosiewicz, L., 2017. Matrilines in Neolithic cattle from Orkney, Scotland reveals complex husbandry patterns of ancestry. J Archaeol Sci Rep 14, 46–54.
Grassmann, W., Schleich, H., 1935. Uber den Kohlenhydratgehalt des Kollagens II. Biochem. Z. 277, 320–328.
Grupe, G., Turban-Just, S., 1998. Amino acid composition of degraded matrix collagen from archaeological human bone. Anthropol. Anzeiger 213–226.
Guiry, E., Szpak, P., 2020. Quality Control for Modern Bone Collagen Stable Carbon and Nitrogen Isotope Measurements. Methods in Ecology and Evolution.
Guiry, E.J., Hunt, B.P., 2020. Integrating fish scale and bone isotopic compositions for 'deep time' retrospective studies. Mar. Environ. Res. 160, 104982.
Guiry, E.J., Needs-Howarth, S., Friedland, K.D., Hawkins, A.L., Szpak, P., Macdonald, R., Courtemanche, M., Holm, E., Richards, M.P., 2016a. Lake Ontario salmon (*Salmo salar*) were not migratory: a long-standing historical debate solved through stable isotope analysis. Sci. Rep. 6.
Guiry, E.J., Noël, S., Tourigny, E., Grimes, V., 2012. A stable isotope method for identifying transatlantic origin of pig (Sus scrofa) remains at French and English fishing stations in Newfoundland. J. Archaeol. Sci. 39, 2012–2022.
Guiry, E.J., Szpak, P., Richards, M.P., 2016b. Effects of lipid extraction and ultrafiltration on stable carbon and nitrogen isotopic compositions of fish bone collagen. Rapid Commun. Mass Spectrom. 30, 1591–1600.
Guiry, E.J., Szpak, P., Richards, M.P., 2017. Isotopic analyses reveal geographical and socioeconomic patterns in historical animal trade between predominantly wheat- and maize-growing agricultural regions in eastern North America. Am. Antiq. 82, 341–352.
Gundberg, C., Hauschka, P., Lian, J., Gallop, P., 1984. Osteocalcin: Isolation, Characterization, and Detection, Methods Enzymol. Elsevier, pp. 516–544.
Gustavson, K., 1955. The function of hydroxyproline in collagens. Nat 175, 70–74.
Hare, P., 1980. Organic geochemistry of bone and its relation to the survival of bone in the natural environment. In: AK, B., AP, H. (Eds.), Fossils Making: Vertebr. Taphonomy Paleoecol. Chicago University Press, Chicago, pp. 208–219.
Hare, P.E., Fogel, M.L., Stafford, T.W., Mitchell, A.D., Hoering, T.C., 1991. The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. J. Archaeol. Sci. 18, 277–292.
Hennet, T., 2019. Collagen glycosylation. Curr. Opin. Struct. Biol. 56, 131–138.
Hiradate, S., Nakadai, T., Shindo, H., Yoneyama, T., 2004. Carbon source of humic substances in some Japanese volcanic ash soils determined by carbon stable isotopic ratio, δ13C. Geoderma 119, 133–141.
Hutchinson, W.F., Culling, M., Orton, D.C., Hänfling, B., Handley, L.L., Hamilton-Dyer, S., O'Connell, T.C., Richards, M.P., Barrett, J.H., 2015. The globalization of naval provisioning: ancient DNA and stable isotope analyses of stored cod from the wreck of the Mary Rose, AD 1545. R Soc Open Sci 2, 150199.
Jones, J.R., Mulville, J., 2016. Isotopic and zooarchaeological approaches towards understanding aquatic resource use in human economies and animal management in the prehistoric Scottish North Atlantic Islands. J Archaeol Sci Rep 6, 665–677.
Jones, J.R., Mulville, J.A., 2018. Norse animal husbandry in liminal environments: stable isotope evidence from the scottish north atlantic islands. Environ. Archaeol. 23, 338–351.
Kendall, C., Eriksen, A.M.H., Kontopoulos, I., Collins, M.J., Turner-Walker, G., 2018. Diagenesis of archaeological bone and tooth. Palaeogeogr. Palaeoclimatol. Palaeoecol. 491, 21–37.
Liden, K., Takahashi, C., Nelson, D.E., 1995. The effects of lipids in stable carbon isotope analysis and the effects of NaOH treatment on the composition of extracted bone collagen. J. Archaeol. Sci. 22, 321–326.
Masters, P.M., 1987. Preferential preservation of noncollagenous protein during bone diagenesis: implications for chronometric and stable isotopic measurements. Geochem. Cosmochim. Acta 51, 3209–3214.
Neuman, R., 1949. The amino acid composition of gelatine, collagens and elastins from different sources. Arch. Biochem. 24, 289–298.
Nielsen-Marsh, C.M., Smith, C.I., Jans, M.M., Nord, A., Kars, H., Collins, M.J., 2007. Bone diagenesis in the European Holocene II: taphonomic and environmental considerations. J. Archaeol. Sci. 34, 1523–1531.
Pearson, J.A., Buitenhuis, H., Hedges, R.E.M., Martin, L., Russell, N., Twiss, K.C., 2007. New light on early caprine herding strategies from isotope analysis: a case study from Neolithic Anatolia. J. Archaeol. Sci. 34, 2170–2179.
Post, D.M., Layman, C.A., Arrington, D.A., Takimoto, G., Quattrochi, J., Montana, C.G., 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152, 179–189.
Qi, H., Coplen, T.B., Geilmann, H., Brand, W.A., Böhlke, J., 2003. Two new organic reference materials for $\delta^{13}C$ and $\delta^{15}N$ measurements and a new value for the $\delta^{13}C$ of NBS 22 oil. Rapid Commun. Mass Spectrom. 17, 2483–2487.
Qi, H., Coplen, T.B., Mroczkowski, S.J., Brand, W.A., Brandes, L., Geilmann, H., Schimmelmann, A., 2016. A new organic reference material, l-glutamic acid, USGS41a, for $\delta^{13}C$ and $\delta^{15}N$ measurements – a replacement for USGS41. Rapid Commun. Mass Spectrom. 30, 859–866.
Rigby, B., 1967. Correlation between serine and thermal stability of collagen. Nat 214, 87–88.
Rigby, B., Spikes, J., 1960. Hydroxyproline and the shrinkage temperature of collagen. Nat 187, 150–151.
Romero, E., Plaza, C., Senesi, N., Nogales, R., Polo, A., 2007. Humic acid-like fractions in raw and vermicomposted winery and distillery wastes. Geoderma 139, 397–406.
Rudakova, T., Zaikov, G., 1987. Degradation of collagen and its possible applications in medicine. Polym. Degrad. Stabil. 18, 271–291.
Schoeninger, M.J., Moore, K.M., Murray, M.L., Kingston, J.D., 1989. Detection of bone preservation in archaeological and fossil samples. Appl. Geochem. 4, 281–292.
Schwarcz, H.P., 1991. Some theoretical aspects of isotope paleodiet studies. J. Archaeol. Sci. 18, 261–275.
Scott, M., 2020. Chew the Fat: an Examination of the Preservation of Fatty Acids in Archaeological Bone, Anthropology. Trent University, p. 181.
Smith, C.I., Craig, O.E., Prigodich, R.V., Nielsen-Marsh, C.M., Jans, M.M.E., Vermeer, C., Collins, M.J., 2005. Diagenesis and survival of osteocalcin in archaeological bone. J. Archaeol. Sci. 32, 105–113.
Smith, C.I., Nielsen-Marsh, C.M., Jans, M.M.E., Collins, M.J., 2007. Bone diagenesis in the European Holocene I: patterns and mechanisms. J. Archaeol. Sci. 34, 1485–1493.
Stafford, T.W.J., Brendel, K., Duhamel, R.C., 1988. Radiocarbon, ^{13}C and ^{15}N analysis of fossil bone: removal of humates with XAD-2 resin. Geochim Cosmchim Acta 52, 2257–2267.

Szpak, P., 2011. Fish bone chemistry and ultrastructure: implications for taphonomy and stable isotope analysis. J. Archaeol. Sci. 38, 3358–3372.
Szpak, P., Krippner, K., Richards, M.P., 2017a. Effects of sodium hydroxide treatment and ultrafiltration on the removal of humic contaminants from archaeological bone. Int. J. Osteoarchaeol. 27, 1070–1077.
Szpak, P., Metcalfe, J.Z., Macdonald, R.A., 2017b. Best practices for calibrating and reporting stable isotope measurements in archaeology. J Archaeol Sci Rep 13, 609–616.
Szpak, P., Millaire, J.-F., Chapdelaine, C., White, C.D., Longstaffe, F.J., 2019a. An integrated isotopic study of early intermediate period camelid husbandry in the santa valley, perú. Environ. Archaeol. 25, 279–295.
Szpak, P., Savelle, J.M., Conolly, J., Richards, M.P., 2019b. Variation in late holocene marine environments in the Canadian Arctic Archipelago: evidence from ringed seal bone collagen stable isotope compositions. Quat. Sci. Rev. 211, 136–155.
Van Klinken, G.J., 1999. Bone collagen quality indicators for palaeodietary and radiocarbon measurements. J. Archaeol. Sci. 26, 687–695.
van Klinken, G.J., Hedges, R.E.M., 1995. Experiments on collagen-humic interactions: speed of humic uptake, and effects of diverse chemical treatments. J. Archaeol. Sci. 22, 263–270.
Vogel, J.C., 1978. Isotopic assessment of the dietary habits of ungulates. South Afr. J. Sci. 74, 298.
Wadsworth, C., Buckley, M., 2018. Characterization of proteomes extracted through collagen-based stable isotope and radiocarbon dating methods. J. Proteome Res. 17, 429–439.