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Effects of lipid extraction and different collagen extraction methods on archaeological fish bones and its implications for fish bone diagenesis

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ABSTRACT

Gelatin extracted from archaeological fish bones typically exhibits relatively high C/N ratios, presumed to be caused by contamination with lipids or humic substances. The effects of lipid extraction and different collagen extraction methods applied has been studied on modern fish bones but has never been studied systematically on archaeological specimens because of taphonomic and experimental reasons. In this study, the effects of lipid extraction and order of NaOH treatment in collagen extraction method on carbon and nitrogen stable isotope analysis of archaeological fish bone (n = 30) excavated from the Hamanaka 2 site, Hokkaido, Japan (approximately 8th BC–10th AD) is investigated. Gelatin extracted from the same fish bone subsamples with or without lipid extraction procedure indicates neither significant nor systematic differences in carbon and nitrogen stable isotope ratios, elemental concentrations, C/N ratios, and yield. However, reproducibility of stable isotope ratios and elemental concentrations decrease in gelatin extracted from poorly-preserved cod bones ($< 3.5\%$ yield). Gelatin extracted from archaeological fish bones may contain humic contaminants, and its effect becomes greater in gelatin with lower extraction yield. Although there is no significant change in the stable isotope and C/N ratios, change in atomic concentration of carbon and nitrogen suggests that the purity of extracted gelatin increases when NaOH treatment is applied after decalcification. Because this is only a study from one archaeological site, further case studies that evaluate lipids and diagenesis in fish bones are required.

1. Introduction

Although carbon and nitrogen stable isotope analysis of collagen extracted from ancient fish bones is an important research topic in (bio) archaeology (e.g., Barrett et al., 2008; Guiry et al., 2016a; Szpak et al., 2013), there is less technical consideration on the collagen extraction procedures compared with those for ancient mammalian bones (e.g., Guiry et al., 2016b; Nicholson, 1996a, b). Preservation and yield of collagen in fish bones are generally poor compared with that in mammalian bones (Nicholson, 1996a, b; Szpak, 2011). Degradation of collagen and contamination by non-collagenous molecules are the probable causes of the poor status of fish bone collagen. The compact packaging of collagen, protected by the mineral phase of bone, is important for the preservation of collagen (Collins et al., 2002). However, fish bones contain less mineral phase and consist of more loosely-mineralized collagen than mammalian bones (Lee and Glimcher, 1991), which would lead a greater degradation (i.e., biotic attack) of fish bone collagen in the burial environment (Szpak, 2011). Humic substances, the major component of soil contaminant (van Klinken and Hedges, 1995), would easily penetrate loosely-mineralized fish bones and interact with collagen (Szpak, 2011). Furthermore, fish bones generally contain higher amounts of lipids than mammalian bone (e.g., Toppe et al., 2007), and the residual lipid might contaminate extracted gelatin (Szpak, 2011). Therefore, it is important to evaluate the validity and utility of various procedures of collagen extraction in ancient fish bones.

Lipid extraction procedures are important to measure the stable isotope ratios of proteinous tissues (e.g., Logan et al., 2008; Post et al.,

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2007; Sweeting et al., 2006). This is because lipids contain large amounts of carbon but little nitrogen and typically present lower stable carbon isotope ratios (δ^{13}C value) than most proteins (Ambrose, 1990; DeNiro and Epstein, 1977). Therefore, lipid contamination in extracted gelatin results in elevated C/N ratios and lower δ^{13}C values. Szpak (2011) indicated that gelatin extracted from archaeological fish bones typically exhibits relatively higher atomic C/N ratios than modern ones. While the calculated C/N ratios of bone collagen based on amino acid compositions for ray-finned fishes, Actinopterygii (3.15 ± 0.04, $n = 12$), are statistically significantly lower than those for Mammalia (3.23 ± 0.04, $n = 23$), measured actual C/N ratios for gelatin extracted from archaeological bones of Actinopterygii (3.38 ± 0.52, $n = 1127$) are statistically significantly greater than those of Mammalia (3.28 ± 0.33, $n = 1836$) (Szpak, 2011). The difference still remains even if the only gelatin extracted with ultrafiltration is considered (Szpak, 2011). Contamination by lipid is hypothesized to be one of the possible causes of this higher ratio, as well as contamination with humic substances (Szpak, 2011).

However, there has been little study to evaluate the effect of lipid extraction and different extraction methods on archaeological fish bones (but see Guiry et al., 2016b for modern fish bones). This is because: i) the scattered and relatively small size of archaeological fish bones prevent making the replicate measurements required for comparison of different extraction methods; ii) evaluating the effect of lipid extraction *per se* is difficult because the treatment by alkali solution (e.g., NaOH) of bones is essential when extracting collagen from archaeological samples but works like lipid extraction except for phospholipids (Ambrose, 1990); and iii) there is no significant effect of contaminated lipid on the isotope ratios in fish bones with initial lipid concentration of less than 5% (Guiry et al., 2016b), and these species should be omitted for the evaluation of lipid extraction on archaeological fish bones. Different collagen extraction methods produce little systematic difference in archaeological bones with higher collagen yield, but bones with lower collagen yield tend to show inconsistent isotope ratios and elemental concentrations when applying different extraction methods (Dobberstein et al., 2009; Pestle, 2010; Sealy et al., 2014; Tuross, 2012). Fish bones with lower collagen yield would be of special interest for evaluating the isotopic and elemental consistency between different extraction methods.

In this study, the effect of lipid extraction on carbon and nitrogen stable isotope analysis of archaeological fish bone gelatin is investigated. Also, we compared different collagen extraction methods for poorly-preserved fish bones. The following topics are investigated for the extracted gelatin from archaeological fish bones.

- Contamination by lipids: If lipids contaminate gelatin extracted from archaeological fish bones, it is expected that gelatin extracted without lipid extraction will present: i) increased yield because of the presence of lipid in the extracted gelatin; ii) increased atomic concentration in carbon (%C) and C/N ratio because of the high number of carbon atoms in lipid and the absence of nitrogen; and iii) decreased δ^{13}C values because of the typically lower δ^{13}C values of lipid compared to collagen (Ambrose, 1990; DeNiro and Epstein, 1977), as indicated in the study of modern fish bones (Guiry et al., 2016b).
- Unexpected effect of lipid extraction: Although there is no systematic difference in δ^{15}N values of gelatin extracted from modern fish bones with and without lipid extraction (Guiry et al., 2016b), it is possible that δ^{15}N values of tissues alter unexpectedly after the application of lipid extraction possibly because of incidental leaching of tissue proteins (Logan et al., 2008; Sotiropoulos and Tonn, 2004). Collagen in archaeological bones is degraded and contaminated to a certain amount, and needs to be evaluated separately from modern specimens.
- Order of NaOH treatment: Most collagen extraction methods for stable isotope analysis adopt NaOH treatment after decalcification (e.g., Pestle and Colvard, 2012; Sealy et al., 2014; Szpak et al., 2017), but the NaOH treatment is applied before decalcification in some studies (e.g., Yoneda et al., 2004; Tsutaya et al., 2017). Although humic substances are also removed when applying the NaOH treatment before decalcification in well-preserved bones (Yoneda et al., 2004), the efficiency of the NaOH treatment before decalcification in poorly-preserved bones is unclear. Therefore, it is important to evaluate the isotopic consistency between extracted gelatin with the NaOH treatment before or after decalcification especially in samples with lower gelatin yield.

2. Materials and methods

2.1. Fish bone samples

Archaeological fish bones from the Nakatani location of the Hamanaka 2 site, Rebun Island, Hokkaido, Japan were used in this study. Hamanaka 2 site is a large multi-component shell-midden (Sakaguchi, 2007), and has been excavated several times in different locations since 1967 (Kato, 2015). Archaeological excavation of the Nakatani location was led by the Baikal-Hokkaido Archaeology Project (BHAP) and Core-to-Core Project since 2010, and yielded abundant archaeological remains (Kato, 2015; Müller et al., 2016; Weber et al., 2013). Fish bones used in this study were obtained from the Okhotsk (Layer III, 430–960 cal AD: Leipe et al., 2017) and Epi-Jomon (Layers VII and VIII, 2700–1500 cal BP: Weber et al., 2013) occupations. The Okhotsk layer is characterized as a well-stratified shell midden and contains human burials (Okamoto et al., 2016), skeletons of domesticated animals (dog and pig), ceramic and lithic (Lynch et al., 2018) materials, several plant remains (Leipe et al., 2017), and abundant marine mammal, fish, and shellfish remains. The Epi-Jomon layer is found within a densely-packed sand dune formation and contains dog skeletons, ceramic and lithic (Lynch et al., 2018) materials, remains of marine animals, and concentric hearth features. The cool temperature of Rebun Island (mean annual temperature in the period 1978–2002 was 6.6 °C)[1] and the presence of shell both promote good preservation of organic materials at the Hamanaka 2 site (Kato, 2015).

Fish bones were recovered by column sampling and subsequent water floatation with 9.52 and 4 mm mesh. After air drying, fish bones were stored in collection shelves and identified to genus, and, where possible, species by using reference fish collections in the Keio University, Japan. While numerous species were present, we focus on the most frequent fish taxa: vertebrae of cod (*Gadus* spp.), atka mackerel or hokke (*Pleurogrammus* spp.), and rockfish (*Sebastes* spp.), and maxilla/mandible of fugu (Tetraodontidae) were used in this study (Table S1). Although some cod bones could be identified as Pacific cod (*G. macrocephalus*), they are here subsumed into a generic *Gadus* category (Table S1). The total number of fish bone samples is 30 and that of analyzed subsamples is 68. One of the most frequent fish taxa, herring (*Clupea pallasii*), were not used in this study because their small bone size prevents replicate analysis.

2.2. Collagen extraction

In this study, effects of lipid extraction and order of NaOH treatment were evaluated in all samples and in the subset of cod bone samples, respectively. The first batch of fish bones from several taxa was processed with NaOH treatment before decalcification (method A). Taxa with low gelatin yield (< 3.5%: Ambrose, 1990) was used further for the evaluation of the order of NaOH treatment (see Table 1). This

[1] Original data were obtained via the web site of Japan Meteorological Agency. Measurements were made at the nearby Funadomari town in Rebun Island. http://www.data.jma.go.jp/obd/stats/etrn/view/annually_a.php?prec_no=11&block_no=1207&year=&month=&day=&view=p1 (accessed on 2018-04-13).

Table 1
Summary of the stable isotope ratios of gelatin extracted from archaeological fish bones from Hamanaka 2 site.

Fish	Method	Lipid extraction	$\delta^{13}C$		$\delta^{15}N$		%C		%N		C/N		Yield	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cod	A	NLE	−13.91	0.42	16.89	0.86	44.57	0.28	15.81	0.26	3.29	0.06	3.12	0.97
		LE	−13.87	0.44	16.79	0.77	44.52	0.41	15.82	0.31	3.28	0.05	3.19	0.86
	B	NLE	−14.03	0.45	17.26	0.57	37.32	2.32	13.08	0.82	3.33	0.06	1.43	0.49
		LE	−14.05	0.43	17.19	0.70	37.41	2.73	13.26	1.00	3.29	0.06	1.22	0.66
Fugu	A	NLE	−11.35	0.70	13.60	0.84	45.14	0.23	16.33	0.30	3.23	0.05	6.11	1.50
		LE	−11.24	0.87	13.56	0.78	44.97	0.55	16.42	0.34	3.19	0.03	6.94	1.14
Hokke	A	NLE	−12.92	1.23	15.40	1.78	44.87	0.46	16.18	0.52	3.24	0.08	5.31	3.01
		LE	−12.89	1.26	15.34	1.71	44.82	0.58	16.21	0.54	3.23	0.08	5.42	2.76
Rockfish	A	NLE	−12.40	0.35	13.92	0.22	45.31	0.49	16.92	0.27	3.12	0.02	9.78	2.18
		LE	−12.52	0.16	13.94	0.22	45.35	0.63	16.88	0.33	3.13	0.02	9.12	1.71

second batch comprises cod bones, processed with NaOH treatment after decalcification (method B). These two different collagen extraction procedures are described below in detail.

Method A: This extraction method was based on Tsutaya et al. (2017) and Yoneda et al. (2004), and applied to most of the fish bones (Table S1). Fish bones were washed with milli-Q water under ultrasonication, soaked in 0.2 M NaOH for 18 h at 4 °C, and then rinsed with milli-Q water. The fish bones were freeze-dried and halved with a dental drill and diamond disks. After weighing, lipid extraction following Guiry et al. (2016b) was applied to one of the halved subsamples. In lipid-extracted (LE) subsamples, bones were soaked in chloroform and methanol (2:1 in v/v) for 15 min under ultrasonication, and the solvent was refreshed until the solution remained clear. Then, samples were soaked in acetone for 15 min under ultrasonication. The acetone was discarded, and samples were air-dried for overnight under continuous airflow to evaporate any residual solution. In non-lipid-extracted (NLE) subsamples, this lipid-extraction procedure was not applied.
Collagen was extracted from both LE and NLE subsamples. Samples were demineralized with 0.25 N HCl at 4 °C for two or three days until the bone no longer visibly reacted with the HCl solution. The HCl was replaced once. The demineralized samples were gelatinized in weak HCl solution (pH 4.5) at 80 °C for over 48 h, then filtered with a glass fiber filter (Wattmann GF/F), and freeze-dried. Gelatin yield was calculated as the weight of freeze-dried gelatin divided by bone weight of bone after NaOH treatment.

Method B: This extraction method was basically the same as method A except for the order and number of NaOH treatments. This method was applied to the other batch of cod bones, but some cod bones provided sufficient remaining mass after sampling for method A and were included in the batch for method B (Table S1). In this method, bones were washed with milli-Q water, soaked in 0.2 M NaOH for 18 h at 4 °C, dried, halved, weighed, lipid-extracted (only for LE subsamples), and demineralized with 0.5 N HCl. The demineralized samples were soaked again in 0.1 M NaOH at room temperature for 15 h. The NaOH solution was replaced once. After the second treatment with NaOH, samples were washed with milli-Q water, gelatinized, filtered, and freeze-dried. Gelatin yield was calculated with weight of freeze-dried gelatin divided by bone weight after halving but before HCl treatment. The first NaOH treatment was applied to make gelatin yield comparable with that from method A. Because NaOH treatment decrease the weight of sample by removing exogenous organic matters from bone tissue, gelatin yield obtained in method B is not comparable without the first NaOH treatment.

2.3. Determination of lipid concentration in bones

Initial lipid concentration in fish bones was determined by using modern samples. Lipid concentrations of cod and rockfish were taken from previous studies (Guiry et al., 2016b; Toppe et al., 2007). If the values were reported for several bone elements, the mean was used for the representative value of the species. Average of lipid concentrations from several rockfish species was used for genus *Sebastes* (Guiry et al., 2016b). Lipid concentrations of Atlantic cod (*Gadus morhua*) was used for genus *Gadus* (Guiry et al., 2016b; Toppe et al., 2007).

Lipid concentration for *Pleurogrammus* was determined experimentally in this study by following the method presented in Guiry et al. (2016b). Semi-dried Okhotsk atka mackerels (*P. azonus*) caught in Hokkaido ($n = 3$) were purchased at a super market in Okinawa, and defleshed using mild steaming and running tap water. After freeze-drying and weighing, vertebrae were soaked in chloroform and methanol (2:1 in v/v) for 15 min under ultrasonication, and the solvent was refreshed until the solution remained clear. The lipid-extracted bones were dried under continuous air flow for 2 h and then weighed. Lipid concentration was calculated by comparing the weight of a bone before and after the lipid extraction procedure.

Lipid concentration of fugu was not determined because the family Tetraodontidae contains many possible species and it was difficult to obtain fresh samples.

2.4. Stable isotope analysis

The freeze-dried gelatin samples were measured in triplicate, when yields permitted, using an elemental analyzer-isotope ratio mass spectrometry (EA-IRMS: Thermo Flash 2000 elemental analyzer, Finnigan ConFlo VI interface, and Thermo Delta V isotope ratio mass spectrometer; all from Thermo Scientific, Waltham, MA, USA) at the University Museum, the University of Tokyo, Japan. The carbon and nitrogen elemental concentrations (%C and %N, respectively) and isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ values, respectively) were calibrated against laboratory standards (L-alanine [$\delta^{13}C = -19.6 \pm 0.2‰$, $\delta^{15}N = 8.7 \pm 0.2‰$] and L-histidin [$\delta^{13}C = -11.4 \pm 0.2‰$, $\delta^{15}N = -7.6 \pm 0.2‰$]) provided by SI Science Co., Ltd. (Saitama, Japan) whose values are determined by the NBS19 and IAEA Sucrose ANU (calibrated against PDB) and IAEA N1 and IAEA N2 (calibrated against AIR) international standards, respectively. Precision was determined to be less than ± 0.1‰ standard deviation (SD) for both $\delta^{13}C$ and $\delta^{15}N$ on the basis of repeated measurements of calibration standards. Accuracy or systematic error was not determined.

2.5. Statistical tests

All statistical analyses were performed by using the software R,

version 3.4.2 (R Core Team, 2017). The level of significance was set as $\alpha = 0.05$.

3. Results and discussion

3.1. Preservation of bones

Raw data are provided in Tables S2 and S3. Two subsamples of rockfish (A19LE and A19NLE) did not provide sufficient amounts of gelatin for stable isotope analysis, and only atomic carbon and nitrogen concentrations were measured (Tables S2 and S3). These samples provide C/N ratio and elemental concentrations outside the acceptable range for well-preserved collagen (2.9–3.6 for C/N ratio: DeNiro, 1985; 34.8 ± 8.8% for %C and 11–16% for %N: van Klinken, 1999), and are excluded from further analysis. The C/N ratios and atomic concentrations of the other samples were within their respective acceptable ranges. The differences in carbon and nitrogen elemental concentrations for the LE subsample compared to the NLE subsample ($\Delta\%C_{LE-NLE}$ and $\Delta\%N_{LE-NLE}$, respectively) of two subsamples from the same fish bone (B14LE and B14NLE) indicated outlier values (discussed below); these samples are included in the figures but excluded from further analysis.

Because cod gave significantly lower yields compared with other fish taxa for both NLE (Mann–Whitney U-test, $U = 2, p < 0.001$) and LE ($U = 0$, $p < 0.001$) subsamples, mean yield lower than the acceptable limit (< 3.5%) proposed by Ambrose (1990) in method A (Table 1), cod samples were treated separately from the other fish taxa. Although it is possible that cod bone collagen is especially vulnerable to degradation in archaeological deposits, some cooking procedures (e.g., boiling) are known to adversely affect archaeological fish bone preservation (Nicholson, 1996b). It is important to understand the nature of preservation status in cod to investigate whether the cause of its lower yield is natural or artificial.

Compiled data of archaeological cod bone gelatin from previous isotopic studies suggest that their lower yield at the Hamanaka 2 site is likely due to intrinsic factors and not to processing by humans. Previous isotopic studies that report gelatin extraction yields for cod (Gadidae) and other Actinopterygii taxa ($n \geq 2$, respectively) are compiled and summarized in Table 2. Although these data were obtained from several archaeological sites with different cultural backgrounds and chronological periods, Mann–Whitney U-tests indicate significantly lower gelatin yield for cod bones in five out of six datasets (Table 2). This result indicates that archaeological cod bones typically show lower gelatin yield compared with other fishes. Although investigation of the cause of this difference is beyond the scope of this study, the acellular nature of bone growth in cod may be a factor (Nicholson, 1996a). However, it should be noted that cod bones are well preserved in some archaeological sites (e.g., average gelatin yield of 9.0%: Guiry et al., 2012).

3.2. Lipid concentration in bones

Average initial lipid concentrations in modern fish bones were reported as 17.1% for rockfish (Guiry et al., 2016b) and between 1.8% (Guiry et al., 2016b) and 2.3% (Toppe et al., 2007) for cod. Average initial lipid concentrations in bones was experimentally determined as 26.8% for modern hokke in this study. Because the initial lipid concentration is less than 5% in cod, residual lipid would not affect the result of stable isotope analysis irrespective of the application of lipid extraction (see Guiry et al., 2016b). This is not the cause of rockfish and hokke, with substantially higher lipid concentrations.

3.3. Effect of lipid extraction

A summary of the results of the stable isotope analysis is presented in Table 1. Mean $\delta^{13}C$ and $\delta^{15}N$ values of the four fish taxa ranged from −14.1‰ to −11.3‰ and from 13.6‰ to 17.3‰, respectively (Table 1). The differences in $\delta^{13}C$ and $\delta^{15}N$ values ($\Delta^{13}C_{LE-NLE}$ and $\Delta^{15}N_{LE-NLE}$ values), %C, %N, C/N ratio ($\Delta C/N_{LE-NLE}$), and yield ($\Delta Yield_{LE-NLE}$) for the LE subsample compared to the NLE subsample are indicated in Table 3.

Paired t-tests indicated that there is no significant difference in $\delta^{13}C$ and $\delta^{15}N$ values, %C, %N, C/N ratio, or yield between NLE and LE subsamples (Table 4). No systematic shift associated with lipid contamination can be detected in $\Delta^{13}C_{LE-NLE}$ and $\Delta^{15}N_{LE-NLE}$ values (Fig. 1), $\Delta\%C_{LE-NLE}$ and $\Delta\%N_{LE-NLE}$ (Fig. 2), and $Yield_{LE-NLE}$ (Fig. 3). Nor was any significant difference found when only considering fish taxa (i.e., rockfish and hokke) with > 5% of initial lipid concentration in modern bones (Table 4). These results indicate that there is no detectable effect from residual lipid nor unexpected effect with lipid extraction in elemental concentrations and stable isotope ratios of collagen extracted from Hamanaka 2 archaeological fish bones. However, it should be noted that these results do not necessary indicate complete absence of lipids in the archaeological fish bones. NaOH treatment also works like lipid extraction except for phospholipids (Ambrose, 1990). Although they might not affect the stable isotope ratio of bulk bone collagen, small quantities of endogenous lipids have been shown to survive in archaeological bone dating back millennia (e.g., Colonese et al., 2015).

However, F tests indicated greater variance in $\Delta^{13}C_{LE-NLE}$ and $\Delta^{15}N_{LE-NLE}$ values and $\Delta\%N_{LE-NLE}$ of gelatin extracted from poorly-preserved cod bones compared to well-preserved fish bones from other taxa (Table 5). Greater variances in $\Delta^{13}C_{LE-NLE}$, $\Delta^{15}N_{LE-NLE}$, $\Delta\%C_{LE-NLE}$, and $\Delta\%N_{LE-NLE}$ appear in gelatin with lower extraction yield (Figs. 1 and 2). These results indicate that the reproducibility of stable isotope ratios and elemental concentrations of gelatin extracted from the same archaeological bones decreases in samples with lower gelatin yield, especially for bones with approximately < 3.5% yield (Figs. 1 and 2).

Table 2
Differences of gelatin yield (%) in cod (Gadidae) and non-cod fish bone samples reported in previous studies.

Site	Period	Location	Cod			Non-cod			U-test		Reference
			Mean	SD	n	Mean	SD	n	U	p	
Köpingsvik	3400–2400 cal BC	Sweden	1.3	0.7	8	3.1	1.2	5	3	0.016	Eriksson et al. (2008)
Korsnäs	3200–2800 cal BC	Sweden	0.4	0.1	3	2.0	1.0	7	0	0.017	Fornander et al. (2008)
Tissø	6–11th CE	Denmark	1.8	2.5	2	5.5	1.1	9	0	0.044	Jørkov et al. (2010)
Fishergate	8–16th CE	UK	1.2	0.7	6	2.0	1.0	23	38.5	0.106	Müldner and Richards (2007)
Haithabu and Schleswig	9–13th CE	Germany	0.2	0.3	5	2.8	1.1	14	0	0.001	Grupe et al. (2009)
Beverley	14–15th CE	UK	5.2	1.9	12	7.4	2.1	15	36	0.009	Müldner and Richards (2005)

Samples named “whiting” were included in cod in the Fishergate (Müldner and Richards, 2007).

Table 3
Summary of the differences between the stable isotope ratios of gelatin extracted with lipid extraction compared to those without lipid extraction.

Fish	Method	$\Delta^{13}C$		$\Delta^{15}N$		Δ%C		Δ%N		Δ C/N		Δ Yield	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cod	A	0.04	0.38	−0.10	0.27	−0.04	0.39	0.01	0.45	−0.01	0.07	0.06	0.90
	B	−0.09	0.24	−0.12	0.42	0.64	0.92	0.32	0.47	−0.02	0.05	−0.20	0.32
Other	A	−0.01	0.21	0.00	0.08	−0.03	0.52	0.05	0.23	−0.01	0.03	0.24	0.99
> 5% lipid	A	−0.07	0.18	0.02	0.06	0.05	0.49	0.02	0.22	0.00	0.02	−0.08	0.83

Table 4
Results of the paired *t* test for stable isotope ratios between LE and NLE subsamples.

Fish	Method	$\delta^{13}C$		$\delta^{15}N$		%C		Δ%N		C/N		Yield	
		t	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
Cod	A	−0.35	0.733	1.22	0.254	0.35	0.732	−0.07	0.947	0.24	0.817	−0.23	0.826
	B	0.37	0.718	0.45	0.667	−0.33	0.753	−1.09	0.308	1.75	0.118	2.10	0.069
Other	A	0.13	0.902	0.02	0.985	0.23	0.821	−0.76	0.461	1.60	0.133	−0.92	0.374
> 5% lipid	A	1.18	0.273	−1.21	0.259	−0.28	0.783	−0.23	0.824	0.03	0.977	0.30	0.770

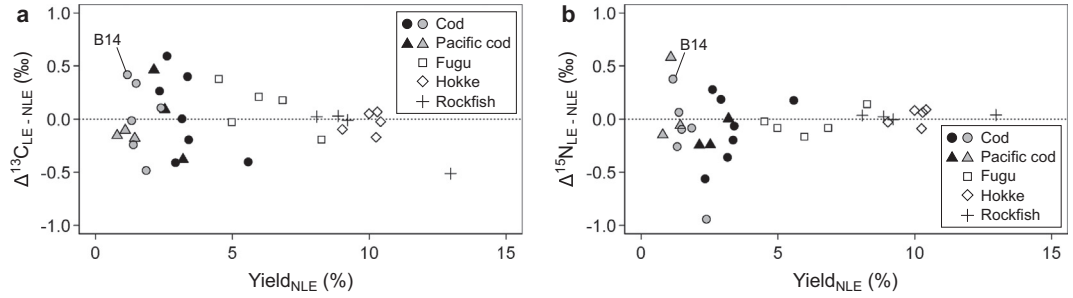

Fig. 1. Difference in $\delta^{13}C$ (a) and $\delta^{15}N$ (b) values between the NLE and LE subsamples against gelatin yield of NLE method. Cod data indicated with solid and gray points were obtained with the methods A and B, respectively. "B14" is an outlier that is not included in statistical analyses.

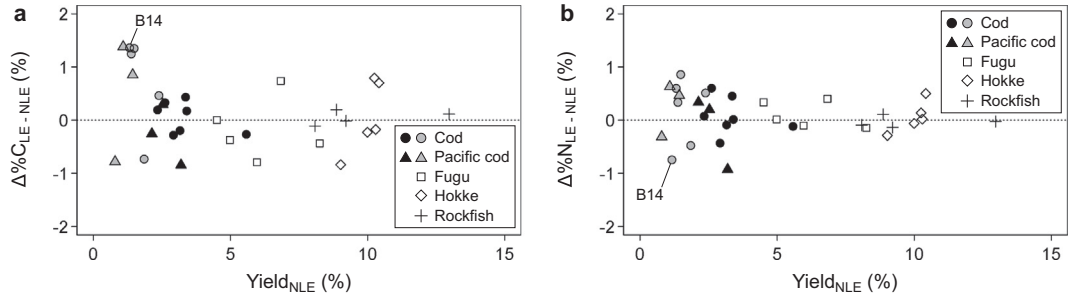

Fig. 2. Difference in %C (a) and %N (b) between the NLE and LE subsamples against gelatin yield of NLE method. Cod data indicated with solid and gray points were obtained with the methods A and B, respectively. "B14" is an outlier that is not included in statistical analyses.

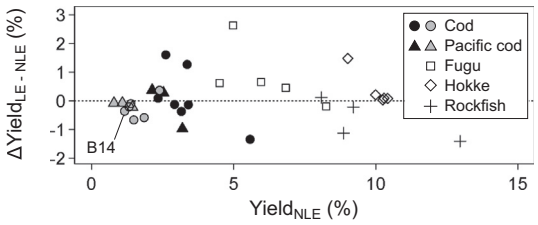

Fig. 3. Difference in gelatin yield between the NLE and LE subsamples against gelatin yield of NLE method. Cod data indicated with solid and gray points were obtained with the methods A and B, respectively. "B14" is an outlier that is not included in statistical analyses.

3.4. Effect of the order of NaOH treatment

The *t*-tests indicate that the %C, %N, and yield of gelatin extracted from cod bones with method B (NaOH treatment after decalcification) are significantly smaller than those with method A (NaOH treatment before decalcification), but no significant difference was found in $\delta^{13}C$, $\delta^{15}N$, or C/N ratio (Table 6).

To investigate the purity of extracted gelatin, the relationship between $\Delta\%C_{LE-NLE}$ and $\Delta\%N_{LE-NLE}$ is plotted in Fig. 4. The difference in concentration of pure collagenous compounds in two subsamples result in the $\Delta\%N_{LE-NLE}$ against $\Delta\%C_{LE-NLE}$ plot on a line with slope of 0.35–0.28 (= 1/2.9–1/3.6) and intersection of 0.0 (Tsutaya et al., 2017), because the atomic C/N ratio of extracted collagen is 2.9–3.6 (DeNiro, 1985). The difference in elemental concentrations in samples B14LE and B14NLE seem to be an outlier (Fig. 4): both $\Delta\%C_{LE-NLE}$ and

Table 5
Results of the *F* test of stable isotopic differences for the LE subsample compared to the NLE subsample.

Comparison	$\Delta^{13}C$		$\Delta^{15}N$		$\Delta\%C$		$\Delta\%N$		Δ C/N		Δ Yield	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Cod-A vs Cod-B	2.42	0.256	0.41	0.212	0.18	0.022	0.89	0.855	1.76	0.469	7.81	0.013
Cod-A vs Other-A	3.31	0.050	10.22	< 0.001	0.58	0.424	3.85	0.028	6.76	0.002	0.83	0.793

Table 6
Summary of the differences between the stable isotope ratios of gelatin extracted with lipid extraction compared to those without lipid extraction.

Comparison	$\delta^{13}C$		$\delta^{15}N$		%C		%N		C/N		Yield	
	t	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
Cod-A-NLE vs Cod-B-NLE	0.55	0.589	−1.07	0.301	8.77	< 0.001	9.03	< 0.001	−1.39	0.184	4.80	< 0.001
Cod-A-LE vs Cod-B-LE	0.84	0.412	−1.16	0.265	7.30	< 0.001	7.00	< 0.001	−0.36	0.724	5.50	< 0.001

Welch's *t* test was applied to the comparison of %C and %N, because these values indicated significantly different variations between the comparative datasets.

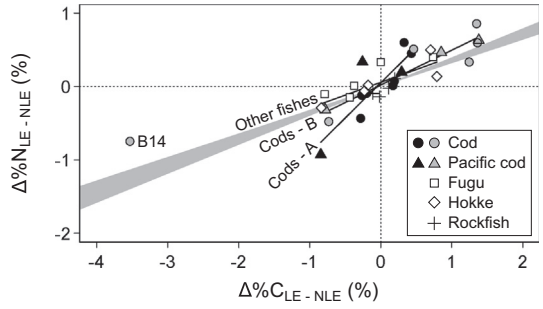

Fig. 4. Relationship between the differences in the values of %C and %N obtained from NLE and LE subsamples. The regression lines of $\Delta\%N_{LE-NLE}$ against $\Delta\%C_{LE-NLE}$ are indicated for extracted gelatin from cod bones with methods A and B and from the other fish bones with method A. The shaded areas indicate the ideal range of C/N ratio of 2.9–3.6 (DeNiro, 1985). Cod data indicated with solid and gray points were obtained with the methods A and B, respectively. "B14" is an outlier that is not included in statistical analyses.

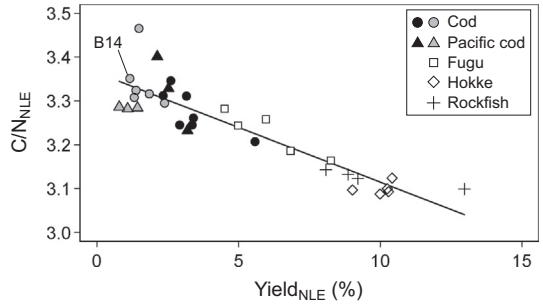

Fig. 5. Relationship between gelatin yield and C/N ratio of NLE subsamples. Cod data indicated with solid and gray points are obtained with the methods A and B, respectively. "B14" is an outlier that is not included in statistical analyses.

$\Delta\%N_{LE-NLE}$ of B14 are outside the 2SD range of the other bones treated with method B. Although the regression line of $\Delta\%C_{LE-NLE}$ and $\Delta\%N_{LE-NLE}$ of gelatin extracted from the other fishes with method A ($\Delta\%N_{LE-NLE} = 0.348 \times \Delta\%C_{LE-NLE} + 0.057$, $R^2 = 0.624$) is almost identical to the ideal range of C/N ratio = 2.9–3.6, that of gelatin extracted from cod with method A ($\Delta\%N_{LE-NLE} = 0.958 \times \Delta\%C_{LE-NLE} + 0.052$, $R^2 = 0.717$) differs in its slope (Fig. 4). The regression line of $\Delta\%C_{LE-NLE}$ and $\Delta\%N_{LE-NLE}$ of gelatin extracted from the cod bones with method B ($\Delta\%N_{LE-NLE} = 0.475 \times \Delta\%C_{LE-NLE} + 0.020$, $R^2 = 0.835$) was intermediate with these two regression lines (Fig. 4).

This result suggests that C- and/or N-containing non-collagenous compounds were contaminated in gelatin extracted from low-yield (i.e., < 3.5%) fish bones, but can be removed to a certain degree by applying NaOH treatment after decalcification. Contamination and removal of these non-collagenous compounds occur irrespective of lipid extraction, but its effect seems to be stronger in gelatin with low extraction yield. Samples with higher gelatin extraction yield contain a larger amount of collagen (signal), and so the relative proportion of the non-collagenous compounds (noise) would be small. On the other hand, the effect of this 'noise' would be relatively large in samples with lower gelatin extraction yield because of a relatively smaller amount of collagen (signal) in the gelatin. The signal/noise ratio in gelatin samples with lower extraction yield is larger than that with higher yield, and thus gelatin with lower yield would be more vulnerable to the random noise resulting from contaminants. This interpretation is supported by the strong negative correlation in yield and C/N ratio of the extracted gelatin (Fig. 5; $C/N = -0.025 \times Yield + 3.364$, $R^2 = 0.816$, for NLE subsamples). It is known that bones with lower gelatin yield tend to show inconsistent isotope ratios and elemental concentrations when applying different extraction methods (Dobberstein et al., 2009; Pestle, 2010; Sealy et al., 2014; Tuross, 2012). Ultrafiltration is believed to decrease the 'noise,' but it does not necessary provide significant improvements on the quality of gelatin in stable isotope analysis (Szpak et al., 2017). However, there is no visible effect in the stable isotope ratios from this contaminants whether NaOH treatment was applied before or after decalcification.

3.5. Implications for fish bone diagenesis

If we assume that the results from Hamanaka 2 site are broadly applicable to other archaeological settings, the results of this study suggest that the relatively higher C/N ratios of gelatin extracted from archaeological fish bones (Szpak, 2011) are not the result of contamination by residual lipid. This is because there is neither a significant nor systematic difference in $\delta^{13}C$ and $\delta^{15}N$ values, %C, %N, C/N ratio, and yield between NLE and LE subsamples of fish bones from the Hamanaka 2 site (Table 3). Residual lipids in fish bone are not only degraded during the burial period, but also removed by NaOH treatment (Ambrose, 1990). Furthermore, $\delta^{13}C$ values of gelatin extracted from fish bones with initial lipid concentration of < 5% do not indicate any measurable effect from residual lipid (Guiry et al., 2016b).

Rather, C- and/or N-containing non-collagenous/non-lipid compounds, especially humic substances, are the possible source contaminants of gelatin extracted from archaeological fish bones. Although the effect of these compounds is negligible in gelatin with higher extraction yield (i.e., higher signal/noise ratio), it becomes prominent in gelatin with lower yield (i.e., lower signal/noise ratio). The differences

in elemental concentrations and stable isotope ratios for the LE subsample compared to the NLE subsample varied in samples with lower extraction yields (Figs. 1 and 2), which indicates a decreased reproducibility in these values extracted from the same archaeological bones. However, because the C/N ratios and elemental concentrations are still within the acceptable range (DeNiro, 1985; van Klinken, 1999), integrity of stable isotope ratios would be secured even for gelatin with lower extraction yield (Tables S2 and S3). Although the elemental concentrations and yield of gelatin (Table 6) significantly decrease, NaOH treatment after decalcification could increase the purity of collagen (Fig. 4). In order to both exclude these contaminants from gelatin (i.e., lowering noise) and to prevent experimental degradation of collagen (i.e., retaining signal), it seems that a novel method of collagen extraction needs to be developed. Unfortunately, however, ultrafiltration is not very effective in removing humic contaminants (Szpak et al., 2017).

Because this is only a study from one archaeological site, it is important to consider the effect of lipid extraction and difference in collagen extraction methods for archaeological fish bones from other archaeological sites of different ages and in different environmental settings. Although the preservation is good and the material is relatively recent in Hamanaka 2 site (i.e., approximately 8th century BC–10th century AD: Weber et al., 2013), it is possible that fish bones from more recent sites contain lipid that affects results of stable isotope analysis. Further case studies that evaluate lipid in, and diagenesis of, fish bones are needed.

4. Conclusions

- There was no measurable effect of residual lipid in stable isotope analysis of archaeological fish bone gelatin from the Hamanaka 2 site.
- No unexpected effects of lipid extraction in $\delta^{15}N$ values were observed in extracted gelatin from Hamanaka 2 archaeological fish bones.
- The contamination of C- and/or N-containing non-collagenous/non-lipid compounds, most probably humic substances, is the probable cause of relatively high C/N ratios of gelatin extracted from archaeological fish bones. Although there is no significant change in stable isotope ratios, this contaminant can be removed to a certain degree by applying NaOH treatment after decalcification.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jasrep.2018.05.026.

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