Does DNA extraction affect the physical and chemical composition of historical cod (*Gadus morhua*) otoliths?

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Archived otoliths constitute an important source of historical DNA for use in temporal genetic studies, but such otoliths are also valuable for other research applications, e.g. growth or microchemistry studies, where information about the past is of relevance. Consequently, there are potentially conflicting interests regarding how the limited and irreplaceable otolith collections should be used. To resolve this, it is important to find out whether DNA extraction damages otoliths such that they can no longer be used for other research purposes or whether individual otoliths can be used in multiple applications. We examined the effects of three different DNA extraction methods on the elemental composition, the morphology, and the clarity of annual growth increments for successful age estimation of Atlantic cod (*Gadus morhua*) otoliths that had been archived for 0–31 years. The three extraction methods yielded DNA of comparable quality, and none of the methods caused major damage to the otoliths. Of the element concentrations measured, only Mg and Rb showed considerable changes resulting from DNA extraction. The physical properties of the otolith (morphology and clarity of annual growth increments) were not affected. Hence, cod otoliths can be used for several research purposes after DNA extraction.

Keywords: archived otoliths, Atlantic cod, DNA extraction, *Gadus morhua*, historical analysis, microchemistry, otolith research.

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Introduction

Since the early 1900s, many research institutions have collected otoliths and scales from commercially exploited fish to monitor age composition and other aspects of population biology. Some of these collections have been retained. Today, this archived material is an important source of historical DNA that can be used to gain valuable insights into the temporal genetic variation in the populations. Examples of such applications include comparisons between past and present levels of genetic diversity to estimate effective population sizes (Hauser *et al.*, 2002; Hutchinson *et al.*, 2003; Poulsen et al., 2006), evaluation of possible interdecadal changes in allele frequencies at candidate loci suspected to be under selection (Nielsen *et al.*, 2007), and inferences about the long-term stability of migration patterns and population structure (Ruzzante *et al.*, 2001). Many further opportunities remain to be explored in this field (Nielsen and Hansen, 2008).

Archived otoliths used to recover DNA for temporal genetic studies are also valuable for retrospective or historical analyses in other fields of fish biology and fisheries science, such as different tracer applications, e.g. microchemistry (Thresher, 1999; Campana *et al.*, 2000) and otolith shape analysis (Campana and Casselman, 1993; Stransky *et al.*, 2008), as well as reconstruction of historical age distributions and growth rates (Swain *et al.*, 2007; Limburg *et al.*, 2008). As historical otolith collections are limited and irreplaceable, it is important that we learn as much as possible from them and, where possible, use them for multiple research purposes. This is particularly important for efforts to link phenotypic information to genetic data for the same individuals (Naish and Hard, 2008).

Otoliths are acellular, and DNA is recovered from dried blood and tissue still adhering to their surface. Hence, DNA extraction should ideally be conducted before any other analysis, because the samples should be reasonably untouched for successful and reliable extraction avoiding cross-sample contamination. However, some concern has been expressed whether DNA extraction may damage otoliths, thereby preventing subsequent research opportunities. DNA extraction from otoliths typically involves incubating them in a lysis solution to disrupt the cellular structure of the dried tissue on their surface, and thereby release the DNA. The solution often contains EDTA, which could decalcify the otoliths and cause alterations of trace element composition (Campana and Neilson, 1985; Miller *et al.*, 2006), a protease (typically proteinase K), which could digest the proteinaceous matrix of the otolith (Shiao *et al.*, 1999), and salts and detergent that could add chemical contaminants (Rooker *et al.*, 2001; Swan *et al.*, 2006). The question is whether the concentrations of these substances and the exposure times involved in DNA extraction result in significant changes to the otolith structure or composition.

Two recent studies have assessed the effect of DNA extraction on age estimation, with contrasting results. Heath *et al.* (2007) found that DNA extraction did not affect ageing success in coral trout (*Plectropomus leopardus*) otoliths, whereas Cuveliers *et al.* (2009) found that common sole (*Solea solea*) otoliths could not be used for age determination following unmodified DNA extraction methods. Here, we tested three different DNA extraction methods on otoliths of Atlantic cod (*Gadus morhua*) and examined the impact not only on the features important for age estimation, but also on morphology (weight, size, and shape) and the elemental composition of the otoliths. The purpose was to identify the extraction protocol that yielded the highest quality DNA for subsequent polymerase chain reaction (PCR) analysis, while minimizing potential alteration to the otoliths. We also investigated whether otolith size affected the vulnerability to DNA extraction, and how DNA recovered from recently collected otoliths performed in PCR analysis compared with DNA extracted from samples that had been stored for decades.

Methods

Design

The effects of the three DNA extraction methods were tested on pairs of sagittal cod otoliths. For assessment of physical effects, such as changes in size, shape, and clarity of annual growth increments, 36 otolith pairs were obtained from the archives at the National Institute of Aquatic Resources (DTU Aqua) in Denmark (18 pairs from fish caught in 1976 and 18 pairs from fish caught in 2007, all from the North Sea). Changes in chemical composition attributable to DNA extraction were measured on a separate set of 18 otolith pairs, nine from the DTU Aqua archives (fish caught in 1976) and nine from juvenile cod (0-group) reared in captivity (fish sacrificed in 2007).

After sampling, the otoliths from the juveniles were stored at room temperature in plastic well trays (nunc trays) for several months before being transferred to plastic vials. Otolith pairs obtained from DTU Aqua had been stored individually in paper envelopes at room temperature since removal from the fish (and small quantities of dried mucus or blood were visible on most of them). One otolith from each of these pairs was intact, but the other had previously been used for age reading and manually broken in half to expose the annual growth increments in accordance with accepted age-estimation protocols for cod. The otoliths from the juveniles were all $<$0.6 cm long (11–23 mg), whereas half the otolith pairs from adults were 1–1.5 cm long (140–260 mg, from small adults of $\sim$40 cm) and the other half of the pairs were $>$1.5 cm long (320–1020 mg, from large adults of $>$60 cm).

The otolith pairs were divided into nine groups with an even distribution of otolith sizes and year of capture, and the groups were allocated randomly to a combination of one of three DNA extraction protocols and one of three lysis times. The three DNA extraction methods used were (i) a commercial kit (EZNA Tissue DNA kit, Omega Bio-Tek) following the manufacturer's instructions, (ii) the method published by Hutchinson *et al.* (1999), and (iii) a modified version of the Hutchinson *et al.* (1999) protocol used by Cuveliers *et al.* (2009), with a lower concentration of EDTA (1 nM) and SDS (0.5%) in the lysis solution, but otherwise following the Hutchinson *et al.* (1999) method. For all methods, lysis was allowed to proceed for 3, 5, or 8 h with intermittent vortexing. After lysis, otoliths were removed from the digestion solution, rinsed thoroughly in deionised water, and then stored individually in clean paper bags until further analysis. Negative controls (reactions with no otoliths) were used for all DNA extractions, and otoliths used for the microchemical assessment were only touched with plastic forceps.

Quality of the DNA

The quality of the extracted DNA samples ($n = 63$) was evaluated by testing PCR amplification success for two microsatellite loci and for fragments of the aromatase and the Pan-I locus. For Pan-I, primers designed specifically for degraded DNA and spanning a 142-bp segment were used, and amplification conditions followed Nielsen *et al.* (2007) using 40 PCR cycles with an annealing temperature of 55°C. The microsatellites Gmo34 (87–119 bp; Miller *et al.*, 2000) and Tch5 (184–268 bp; O'Reilly *et al.*, 2000) were amplified over 39 cycles with an annealing temperature of 50°C. For the 512-bp aromatase fragment, PCRs were completed over 40 cycles with an annealing temperature of 60°C [primers: 5′-TATTTTCCGTCTGCGAGTGG (forward) and 5′-AGTTACT GGCTGTCCCGATG (reverse), designed based on sequence data available at GenBank Accession number DQ402370]. For these markers, amplification was conducted in 12.5 μl total reaction volume containing 0.2 μM (Gmo34) or 0.4 μM (Tch5 and aromatase) of each primer, 0.8 mM dNTP, 1× Taq buffer (HT Biotechnology Super Taq PCR Buffer), 0.65 units of Taq polymerase (Enzyme Technologies Ltd Super Taq), 0.5 mM (Tch5 and aromatase only) $MgCl_2$, and 0.5 μl DNA extract.

The microsatellite PCR products were analysed on a Basestation51 automated sequencer (MJ Research/BioRad), according to the manufacturer's recommendations, and genotyped using the Carthographer Sequencing and Genotyping Analysis software. The Pan-I was analysed as described by Nielsen *et al.* (2007), and amplification of the aromatase PCR products was analysed on 3% agarose gels. Both negative and positive (individuals of known genotype) controls were used in each PCR batch and analysis run. For each marker, the amplification success was measured as the percentage of samples that could be genotyped within three PCR attempts. Some 80% of the samples were reanalysed in additional independent PCRs to verify the consistency of the results (because of the risk of large allele dropout in microsatellites with large allele sizes, 100% of the Tch5 genotypes were validated, and only for samples where the same genotype was observed in at least two independent PCRs was the amplification recorded as successful). The effects of protocol, lysis, storage time of sample (recent or from 1976), and otolith weight on the amplification success were analysed with a logistic regression in which the response variable for each sample was 1 if it successfully amplified for a given marker in at least one of up to three attempts, and 0 if it did not. Two-way interactions between protocol and age of sample and protocol and size were included in the initial model. The significance of the different factors and interactions was assessed with likelihood ratio tests, and the final model was fitted with only the significant terms.

Effects on the physical structure

Possible effects on the physical structure of the otoliths were assessed by comparing photographs of whole otoliths and polished cross sections taken before and after DNA extraction. The photographs were taken with the software Analysis from SIS Gmbh and a stereomicroscope equipped with an Olympus DP50 digital camera attached to a monitor. Identical camera settings with a frame of

2776 × 2074 pixels were used for all images taken with either ×3, ×5, or ×8 magnification (depending on otolith size), and a standardized intensity and angle of reflected light. The intact otolith of each pair was weighed to the nearest milligramme, and its external shape (two-dimensional silhouette) photographed with the concave side down (sulcus up). The DNA extracted from these intact otoliths was used for subsequent PCR analysis.

The pattern of annual growth increments in cod otoliths is most clearly revealed in a cross section through the core, perpendicular to the dorso-ventral axis. As the pattern may look slightly different depending on the exact location of the cross section, the impact on clarity of annual growth increments was compared using images of the same half otolith before and after the DNA extraction procedure. The half otoliths were mounted in plasticine with the cross section facing up and, before capturing the image, a thin layer of water was brushed onto the surface to increase the visibility of the growth increments. The half otoliths were then subjected to the same DNA extraction procedure as their intact counterparts. Following DNA extraction, another set of images was taken of both the intact and the half otoliths. Six additional otoliths were photographed on both days with no treatment in between, as procedural controls. The intact otoliths were also weighed again after extraction, following 1 week of air-drying.

The images taken before and after DNA extraction were analysed using ImageJ software (Rasband, 1997–2009). From the whole otoliths, the silhouette area and the perimeter of the otolith silhouette were measured on both images of each otolith. To test for an overall effect of DNA extraction, the measurements before and after were compared using a Wilcoxon paired-samples test. Kruskal–Wallis tests were applied to assess whether the difference between images taken before and after extraction for each otolith was affected by the protocol or lysis time.

The readability of the age of an otolith depends on the clarity of annual growth increments, i.e. the contrast between translucent and opaque zones in the cross section. To assess whether this contrast was affected by DNA extraction, matching transect lines between the core and a clearly identifiable mark on the edge were drawn on the two greyscale images of each half otolith (taken before and after extraction). ImageJ was then used to measure the greyscale value (light intensity) of each pixel (on a 0–255 scale) along this transect on the two photographs. It was not possible to compare these transect profiles directly, because inevitable slight differences in the positioning of the otoliths between photographs meant that comparisons pixel by pixel would be biased. Instead, summary statistics were used. The measured profiles were smoothed using a simple moving average filtering over five points, and local maxima and minima in these smoothed curves, corresponding to each opaque zone followed by a translucent zone (one annual growth increment), were recorded. The "contrast" for each annual growth increment was defined as the difference between the greyscale value at each local maximum and the greyscale value at the following local minimum. The otoliths had between 2 and 6 annual growth increments. The "average contrast" for each otolith image was calculated as the mean of the contrasts measured for all its annual growth increments.

The data were $\log_{10}$-transformed to achieve normality and homogeneity of variance (as assessed through visual inspection of data coupled with Shapiro–Wilk and Levene's tests) and analysed first with a paired *t*-test on the average contrast before and after DNA extraction for each otolith (excluding the controls) to test for an overall effect of DNA extraction. An ANOVA was then used to test for differences between control pairs and treated otoliths, and whether any of the protocol types or lysis times affected the otoliths more than others. Further, to assess whether annual growth increments near the otolith edge were more prone to fading than those near the core, the difference in contrast before and after extraction was compared between the outermost (near the edge) and the innermost (near the core) annual growth increment of each otolith, using paired *t*-tests.

Effects on the microchemistry

To identify possible alterations to the chemical composition, elemental concentrations were compared within otolith pairs where the intact otolith (otolith A) was used for DNA extraction and the broken otolith (two halves) was left untouched (otolith B). All otoliths (including three additional pairs where both otoliths had been left untouched to control for the natural variation in trace elements between the left and the right otolith) were then relabelled for blind analysis of elemental concentrations using solution-based inductively coupled plasma mass spectrometry (ICPMS).

In preparation for the chemical analysis, the otoliths were weighed, then dipped in 2% HNO_3 (Romil Ultrapure) for 15 s to remove any surface contamination, rinsed in 18 MΩ purified water (Mili-Q), and air-dried. Sample digestion and dilution protocols followed Swan *et al.* (2006) to obtain final dilution factors of ~200 000 for major elements (Na, Ca, and Sr) and 2500 for trace elements. The quantities of acid used and final volumes of the digests were proportional to the sample weights, so all resulting solutions were of a similar concentration. Two sets of fish otolith reference material were processed in parallel to the cod otolith samples for quality control, FEBS-1 (Sturgeon *et al.*, 2005) and NIES-022 (Yoshinaga *et al.*, 2000).

A Thermo Finnigan Element 2 ICP-MS, operated in scanning mode with an acquisition time of 60 s and a carrier gas of argon, was used to determine elemental concentrations in the otoliths. The ICP-MS analysis was carried out over two sessions, one for each dilution range. It was calibrated at the beginning of each session using an external set of standards, and drift-monitored with a non-matrix matched multi-element solution at concentrations of 1 and 20 $\mu g\,l^{-1}$. Instrumental limits of detection (LOD) were calculated from 3 × the standard deviation of mean concentrations in the procedural acid blank. Only one measurement (of Pb) was below the LOD, and this was removed from further analysis.

Concentrations of 12 isotopes were measured, ^{7}Li, ^{23}Na, ^{24}Mg, ^{43}Ca, ^{55}Mn, ^{63}Cu, ^{85}Rb, ^{88}Sr, ^{137}Ba, ^{138}Ba ^{206}Pb, and ^{208}Pb, and the resulting data were expressed as fully quantitative element concentrations ($\mu g\,g^{-1}$ otolith) based on isotope measurements and calculated using natural abundance ratios.

Elemental concentrations for the otolith samples were $\log_{10}$-transformed to achieve normality and homogeneity of variance. To assess whether DNA extraction in general caused a directional change in the microchemical composition, a paired *t*-test was conducted for every element, comparing the A and B otoliths of each pair (excluding the control pairs). This was followed up with an ANOVA to investigate whether treatment groups showed different effects. For that analysis, the response variable was $\log_{10}$(concentration in otolith A/concentration in otolith B), and the explanatory variables were the protocol, the lysis time, and the average weight of each otolith pair. No interactions

between factors were considered because of the small sample size. Significant effects were further investigated with a Games–Howell *post hoc* test. Separate tests were carried out for each element, and the control otolith pairs (both A and B untreated) were included in this analysis.

Results

Quality of the DNA products

The amplification success rates for the different DNA fragments are listed in Table 1. For Gmo34 and Pan-I, all but one sample successfully amplified. For aromatase, all the samples extracted from recently collected otoliths from adult fish amplified to produce PCR products adequate for sequencing, but none of the samples extracted from historical or juvenile otoliths did, confirming that the extracted DNA originates from the historical samples rather than from contemporary contamination with fresh DNA.

Tch5 was the only locus with variable amplification success across treatment groups (resulting from more than a single sample failing to amplify, as was the case for Gmo34 and Pan-I). The samples extracted with the Omega protocol gave the highest amplification success rate at this locus, but the difference between the protocols was not significant ($p = 0.183$), nor was there an overall effect of lysis time ($p = 0.965$). All protocols appeared to work equally well with different types of otolith, because there were no interaction effects between protocol and either storage time ($p = 0.701$) or otolith weight ($p = 0.452$).

Across protocols, amplification success at this locus was, however, affected by otolith size and storage time. Overall, DNA samples from large otoliths amplified significantly better than small ones ($p = 0.001$). The samples extracted from juvenile otoliths showed the lowest success rate, and when the data were analysed without these samples, there was no significant effect of size ($p = 0.064$), although otoliths from larger adults tended to give greater success. Samples that had been collected recently amplified better than samples that had been archived for decades ($p = 0.024$), and this effect was even more significant ($p < 0.001$) when the juvenile samples (that were collected recently but very small in size) were excluded from the analysis.

Effects on the physical structure

One small lobe (always <2% of the total area) had chipped off five of the whole otoliths during DNA extraction, probably a result of vortexing. Those otoliths were excluded from the analysis of effects on morphometrics and weight. For the remainder of the otoliths, the mean difference between otoliths A and B within each pair was <1% (s.d. <3%) for total area and weight and <3% (s.d. <19%) for perimeter, and these differences were not significant ($p \geq 0.170$; Figure 1). The Kruskal–Wallis tests also suggested that there was no differential effect between the protocols, lysis times, and size of the otoliths ($p \geq 0.221$).

Visual inspection of the pairs of images of each cross-sectioned otolith suggested that no reduction in the contrast of annual growth increments had resulted from DNA extraction (Figure 2). On average, the growth increments in images taken after DNA extraction showed a slightly greater contrast (on average 3%, s.d. 32%, higher contrast) than those in images taken before the treatments. However, this difference was not significant ($p = 0.775$). The ANOVA showed that there was no difference between the control group and any other treatment groups for either protocol or lysis ($p \geq 0.332$). There was also no significant effect of otolith size ($p = 0.167$), and there was no differential impact on contrast between annual growth increments near the core and those close to the edge ($p = 0.297$).

Effects on microchemistry

The measured elemental concentrations in the otoliths are summarized in Table 2 and Figure 3. Not all samples could be processed (because of small sample volumes), but there were at least 23 otolith pairs for each element. For six elements, Li, Na, Ca, Cu, Sr, and Pb, there were no significant concentration differences ($p \geq 0.107$) between otoliths that had been exposed to DNA extraction and their untreated counterparts. Of these, Li, Na, Ca, and Sr varied only slightly and randomly within pairs, but for Cu and Pb, there was a clear trend of a higher concentration in the otoliths exposed to DNA extraction. However, for those two elements, there was generally a very large variation in concentration measurements within otolith pairs, even for the controls (Table 2, Figure 3). Mn and Ba concentrations were significantly higher in the A otoliths (treated; $p = 0.021$ and 0.044, respectively), but the relative differences were very small. The average change in these elements was <2.5% of the concentration in the untreated otolith (s.d. <5%; Table 2), and no more than 2 of the 26 pairs differed by more than the variation measured between the otoliths in the control pairs, where both were untreated. Only two elements were notably influenced by DNA extraction: the Mg concentration was significantly higher in the otoliths exposed to DNA extraction ($p = 0.002$; mean ± s.d. percentage difference relative to untreated otolith was 40.8 ± 81.6%), and the Rb concentration was significantly lower in the otoliths used for DNA extraction ($p = 0.003$; mean ± s.d. percentage difference relative to the untreated otolith was 14.0 ± 32.0%; Table 2).

Table 1. Amplification success rate (%) for each locus.

			Year of capture[a]		Size			Protocol		
Locus	Length (bp)	Overall (63)	1976 (36)	2007 (18)	Juv (9)	Small (27)	Large (27)	Ome (21)	Hut (21)	Cuv (21)
Gmo34	87–119	98	100	100	89	100	100	100	95	100
Tch5	184–268	70	64	100	33	63	89	81	62	67
Pan-I	142	98	100	100	89	100	100	100	95	100
Aromatase[b]	512	29	0	100	0	33	33	29	29	29

Results are presented both for all samples combined (overall) and for the different treatment groups (the number of otolith pairs for each group is given in parenthesis). Abbreviations used for the protocol are: Cuv, the Cuvellier *et al.* (2009) method; Hut, the Hutchinson *et al.* (1999) method; and Ome, the commercial Omega kit.

[a]Excluding juveniles.

[b]Amplification has only been counted as successful for samples where a PCR product adequate for sequencing was produced. For an additional seven samples, there was a low level of amplification.

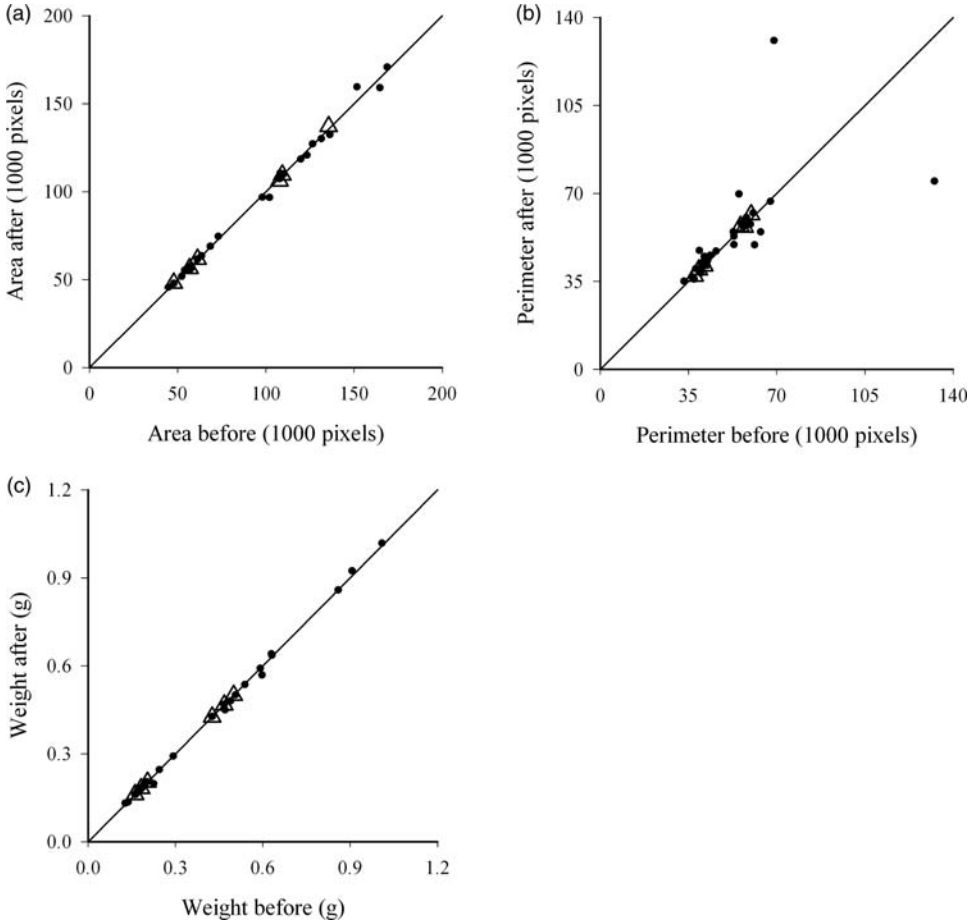

Figure 1. Comparisons of the (a) area, (b) perimeter, and (c) weight of each otolith before and after DNA extraction (dots). The open triangles depict measurements on untreated otoliths as controls. The diagonal lines represents identical measurements before and after DNA extraction, indicating no effect of the treatment.

The ANOVA showed that there were no significant differences in the effects of the three protocols for any of the elements ($p \geq 0.109$). Otolith weight was a significant factor for Na concentrations where the small (especially the juvenile) otolith pairs showed a significantly greater difference in concentration between the treated and untreated otoliths than did the larger pairs ($p = 0.002$). For all other elements, otolith weight was not a significant factor ($p \geq 0.119$). Lysis time significantly influenced the difference in concentration between pairs for Li, Na, Mg, Mn, Cu, and Ba ($p \leq 0.044$). However, *post hoc* tests revealed that this effect was mostly caused by the group exposed to lysis for 5 h having a significantly smaller difference between pairs than the groups exposed for 3 and 8 h, and in most cases none of the groups were significantly different from the control group. Incorporating lysis as a covariate instead of a factor in the model showed that the difference in concentration within pairs did not increase with lysis time for any element ($p \geq 0.242$). Lysis time was only a significant covariate for Mn ($p = 0.044$), but for that element the relationship was inversely proportional, so it was the shortest lysis time that caused the greatest change.

Discussion

This study has demonstrated that DNA can be extracted successfully from otoliths with several different methods and that the otoliths can still be used for other purposes following the DNA extraction. There was no difference in amplification success for the DNA extracted with the three protocols tested, nor did the protocols have different effects on otolith composition, shape, or visual appearance. Therefore, any of the protocols could be used in future studies based on combining different types of information that can be obtained from otoliths. There was no evidence of longer lysis times producing a better quality DNA product, but also no indication that longer lysis times (maximally 8 h) would result in more damage to the otoliths.

DNA yield

The PCR amplification success rate was in general very high for the short fragments. Hence, although extraction from otoliths only provides a small amount of DNA, which is often degraded, it is sufficient for a relatively consistent amplification of many genetic markers. However, the DNA extracted from juvenile otoliths exhibited a much lower success rate for the short fragments (more attempts needed to get a successful amplification; data not shown), and amplification almost always failed for the longest fragment. These otoliths were up to 50 times smaller (by weight) than the adult otoliths used in this study, and perhaps they simply did not contain enough tissue on the surface to provide a reasonable quantity of DNA (although small stains

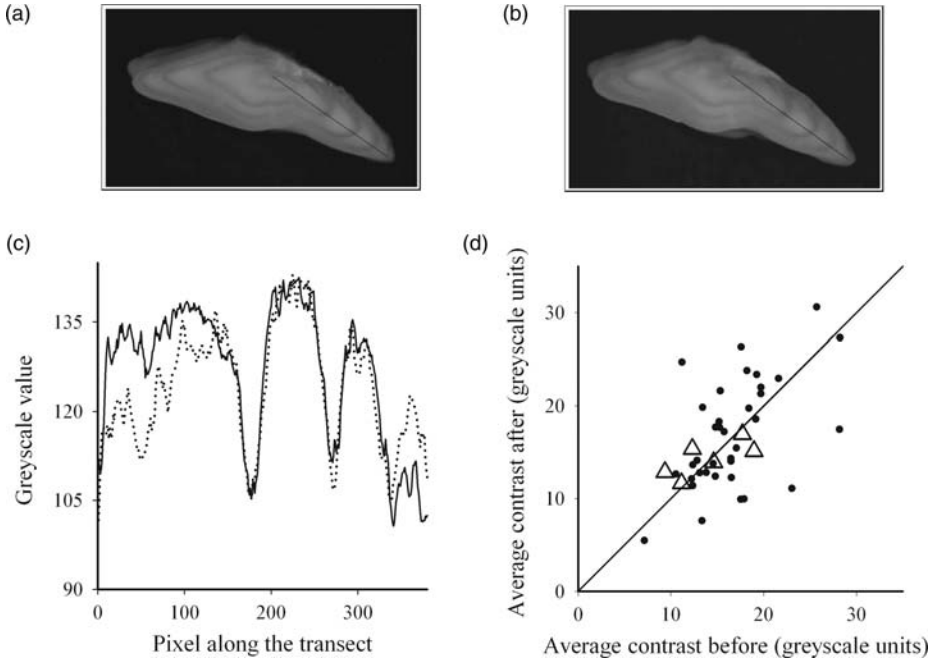

Figure 2. The effect of DNA extraction on contrast of annual growth increments: (a) photograph of the cross section of an otolith taken before DNA extraction. The transect line used to measure greyscale values is depicted by a black line; (b) same otolith cross section after DNA extraction; (c) measured greyscale values along the transect of the otolith cross section photographed in (a) (dotted lines) and (b) (solid line); (d) correlation between the average contrast (over annual growth increments) of individual otoliths (dots) before and after DNA extraction. The open triangles represent control otoliths that were not exposed to DNA extraction. The diagonal line represents identical measurements before and after DNA extraction, indicating no effect of the treatment.

Table 2. Summary statistics on the relative difference in element concentrations between the two otoliths of each pair.

	Control pairs ($n = 3$)		Treated vs. untreated				
Element	**Mean (%)**	**Max (%)**	***n***	**Mean (%)**	**s.d. (%)**	**% A > B**	***p*-value**
^{7}Li	0.5	9.1	26	1.5	4.5	65.4	0.107
^{23}Na	0.5	8.6	20	1.0	7.2	50.0	0.625
^{24}Mg	0.8	7.1	26	40.8	81.6	84.6	0.002
^{43}Ca	0.5	9.2	20	−0.6	3.0	50.0	0.348
^{55}Mn	2.8	5.9	26	2.4	5.0	65.4	0.021
^{63}Cu	52.7	97.4	26	192.8 (64.0[a])	689.4	53.8	0.221
^{85}Rb	10.7	29.2	26	−14.0	32.0	19.2	0.003
^{88}Sr	1.8	8.5	20	−0.2	2.8	45.0	0.719
^{137}Ba	5.6	12.3	26	1.5	3.0	73.1	0.025
^{138}Ba	4.4	9.0	26	1.4	3.2	76.9	0.044
^{206}Pb	38.9	231.8	25	100.4 (54.1[a])	283.7	60.0	0.723
^{208}Pb	6.8	124.0	25	144.2 (60.0[a])	451.4	56.0	0.421

The difference was calculated as the concentration in the treated otolith (A) minus the concentration in the untreated otolith (B), divided by the concentration in the untreated otolith (B) to give the percentage difference from the untreated otolith. A positive value indicates that the concentration was highest in the otolith subjected to DNA extraction (for the control pairs, both otoliths A and B were untreated). The % A > B indicates the proportion of pairs showing increased element concentrations after DNA extraction or contamination by the procedure. The *p*-values are from the paired *t*-test comparing concentrations in the treated and untreated otoliths of each pair.
[a]With exclusion of a single outlier >25 times greater than the average of the rest.

from dried blood or mucus were visible on some of them). Adult otoliths provided a much better source for DNA in this study.

The DNA extracted from the 1976 samples displayed a lower amplification success rate for the medium-sized fragments than did the recent samples, and they could not support adequate amplification of the longest fragment (>500 bp) at all. This result was expected, because DNA becomes fragmented and degraded over time, and the relationship between fragment size and amplification success in historical DNA is consistent with that reported in other studies (Nielsen and Hansen, 2008). Using large otoliths and genetic markers with alleles shorter than a few hundred base pairs is likely to improve the genotyping success (and decrease the risk of errors attributable to large allele dropout) with historical samples.

Effects on physical structure

DNA extraction did not appear to change the physical properties measured in this study. The similarity in weight, silhouette area,

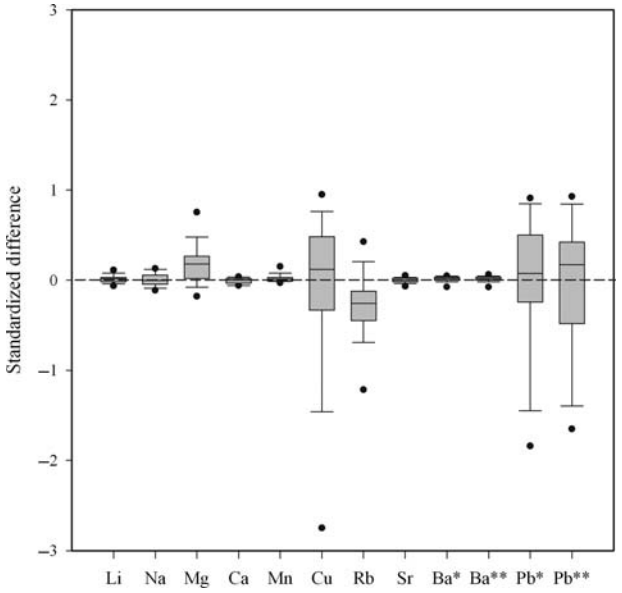

Figure 3. The relative difference in element concentrations between the treated (A) and untreated (B) otoliths of each pair [(otolith A − otolith B)/otolith B]. The horizontal band in each box represents the median, the bottom and top of the boxes represent the 25th and 75th percentiles, and the error bars define the 5th and the 95th percentiles. Outlier data points are marked by dots. Three extreme outliers for the Pb isotopes are not shown (asterisk, the light isotope; double-asterisk, the heavy isotope).

and perimeter suggests that the extraction solution does not dissolve the otolith, at least not within the 8-h lysis time tested here. The only observed alteration of otolith intactness was that the lobes were chipped off five of the whole otoliths. Such physical breakage could most likely be avoided by using gentle rocking or rotation during lysis, rather than vortexing as was done here.

There was no reduction in the clarity of annual growth increments; if anything, the DNA extraction may have caused a slight (though not significant) improvement in clarity. This result is consistent with a recent study that found that DNA extraction did not influence age-determination success in coral trout otoliths (Heath *et al.*, 2007). In contrast, however, the core area of sole otoliths became less visible when they were exposed to DNA extraction with the Hutchinson *et al.* (1999) protocol or a commercial kit (Cuveliers *et al.*, 2009). Perhaps sole otoliths, being thin and flat, are more susceptible to damage by the extraction procedure than the thicker, rounder cod otoliths. The difference could also, in part, be methodological, because the sole study assessed the visibility of the nucleus in the intact otolith. Any slight etching of the outer surface may have had a greater impact on this than on the visibility of annual growth increments in a cross-sectioned otolith, as tested here and in the study with coral trout otoliths. In this respect, the current study may be viewed as conservative, because the reading surface on the cross section was directly exposed to the digestive solution, and DNA is often extracted from whole otoliths so the cross section is typically not directly exposed to the lysing solution. Therefore, our results clearly demonstrate that otoliths, at least from cod, can be used for both morphometric and growth-based studies after DNA extraction.

Effects on microchemistry

Our results also suggest that otoliths can be used for microchemical analyses after DNA extraction, because the concentrations of most elements were similar or varied only slightly between treated and untreated otoliths of matching pairs, except for Mg and Rb. An underlying assumption of these experiments is, of course, that the two otoliths of a fish have similar elemental concentrations. The control pairs showed that although there was some variation, there was a reasonably good correspondence between the concentrations in left and right otoliths from individual fish (mean difference $<3\%$, but up to a maximum of 9% for some pairs) for Li, Na, Mg, Ca, Mn, and Sr. For Rb and Ba, the variation in concentration between the left and the right otoliths was slightly greater, and for Cu and Pb, the variation was very large, up to $>100\%$. These differences may represent natural variation between the two otoliths of an individual (i.e. asymmetric deposition of elements), but it is also likely to result from the data-treatment protocol, because Cu and Pb are often present at very low concentrations. The variation observed here between the left and the right otoliths is generally comparable with that reported in other studies (e.g. Proctor and Thresher, 1998; Campana *et al.*, 2000; Rooker *et al.*, 2001; Swan *et al.*, 2006).

The variation within the control otolith pairs, whether natural or a result of analytical protocol, could define the functional detection limit or a baseline for evaluating the effect of DNA extraction. Although there was a significant trend for higher concentrations in the otolith subjected to DNA extraction for Mn and Ba, these differences were so small that they fell well within the variation observed in the control otolith pairs. The observed differences were $<3\%$ on average, much lower than the differences typically used in the discrimination between components of different cod stocks (Campana *et al.*, 2000; Jonsdottir *et al.*, 2006). Therefore, perhaps otoliths subjected to DNA extraction could be used for microchemical studies with these elements without significantly compromising the accuracy of stock-structure delineation, although the possible bias resulting from slightly greater concentrations of these elements should be kept in mind.

Most notably, there was no evidence of changes in Ca or Sr concentrations as a result of DNA extraction. This finding is reassuring, because these two elements are crucial for otolith microchemistry studies. In several analytical techniques (including LA-ICPMS), Ca is used as an internal calibration standard (Thresher, 1999; Campana *et al.*, 2000; Hedges *et al.*, 2004). Sr concentrations are related to temperature, salinity, and fish growth characteristics (Townsend *et al.*, 1995; Elsdon and Gillanders, 2002; Zimmerman, 2005), so provide one of the strongest signals for stock delineation in marine fish (Swan *et al.*, 2006).

The only elements that showed differences exceeding the variation in the control otolith pairs was Mg, which appeared to increase with DNA extraction, and Rb, which appeared to decrease. It is unclear what mechanism caused these changes. Mg belongs to the group 2 metals that can replace Ca in the $CaCO_3$ matrix of the otolith, so is expected to be fairly stable. However, Mg concentration in otoliths appears to be much more susceptible to alteration than other group 2 elements, and experiments have shown that it might be influenced by a number of frequently used handling and storage methods, such as freezing and storage in ethanol (Milton and Chenery, 1998; Hedges *et al.*, 2004). It has therefore been suggested that some of the Mg is bound in the proteinaceous matrix of otoliths and

hence is more labile (Hedges *et al.*, 2004). This could also be an explanation for the changes observed in Rb. However, regardless of the specific mechanism, the results of this study suggest that it is imprudent to include Mg and Rb in microchemical studies on otoliths previously used for DNA extraction.

Conclusion

Overall, the study shows that although DNA extraction can change the concentration of certain elements, cod otoliths may still be used for a number of microchemical applications after DNA extraction. It is, however, not completely certain that these results are valid for other species, because otoliths vary in composition between species (Proctor and Thresher, 1998; Swan *et al.*, 2006). Given that the physical properties were also not affected, historical genetic studies are not likely to conflict with other research activities on valuable and irreplaceable archived cod otolith collections, even in cases where only one otolith from each individual remains.

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