Reducing microbial and human contamination in DNA extractions from ancient bones and teeth

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Although great progress has been made in improving methods for generating DNA sequences from ancient biological samples, many, if not most, samples are still not amenable for analyses due to overwhelming contamination with microbial or modern human DNA. Here we explore different DNA decontamination procedures for ancient bones and teeth for use prior to DNA library preparation and high-throughput sequencing. Two procedures showed promising results: (*i*) the release of surface-bound DNA by phosphate buffer and (*ii*) the removal of DNA contamination by sodium hypochlorite treatment. Exposure to phosphate removes on average 64% of the microbial DNA from bone powder but only 37% of the endogenous DNA (from the organism under study), increasing the percentage of informative sequences by a factor of two on average. An average 4.6-fold increase, in one case reaching 24-fold, is achieved by sodium hypochlorite treatment, albeit at the expense of destroying 63% of the endogenous DNA preserved in the bone. While both pretreatment methods described here greatly reduce the cost of genome sequencing from ancient material due to efficient depletion of microbial DNA, we find that the removal of human DNA contamination remains a challenging problem.

Bones and teeth excavated from archeological sites frequently contain fragments of endogenous ancient DNA, enabling direct inferences about the genetic composition of organisms that died tens to hundreds of thousand years ago. However, DNA preservation is highly variable and depends not only on the age of the material but also on environmental parameters at the excavation site (1,2). DNA preservation is thought to rely on the interaction of nucleic acids with hydroxyapatite and collagen, the main components of the inorganic and organic fractions of bones and teeth, respectively (1,3). DNA adsorption to hydroxyapatite is mediated through electrostatic interactions of positively charged calcium ions and the negatively charged phosphate groups of the DNA backbone. Hydroxyapatite-bound DNA shows a decreased rate of depurination compared with free DNA (4). In addition, hydroxyapatite binds and inactivates nucleases (5). The interaction of DNA with collagen and its role in long-term preservation of DNA in ancient tissue is less well understood, but it has been hypothesized that DNA-collagen complexes, which form spontaneously in aqueous solutions in vitro, might play a protective role (3,6).

Ancient DNA analysis is complicated by the fact that DNA interaction with the bone/tooth matrix is not restricted to endogenous DNA (i.e., DNA present in the organism before or at death) but also occurs with extraneous DNA, such as that of microorganisms entering the bone/tooth matrix during decomposition. With few exceptions (7–9), microbial DNA usually constitutes >95% of the molecules in ancient DNA extracts, preventing cost-efficient sequencing using high-throughput technologies. This problem can be partially alleviated by hybridization-based enrichment of parts of the genome (10–12) or of the complete genome (13). However, this strategy requires substantial effort and introduces biases in the representation of sequences along the genome (14,15). The situation is further complicated when analyzing ancient human remains, which are frequently contaminated with modern human DNA. In this case, endogenous ancient sequences must be separated from modern DNA contamination by making

METHOD SUMMARY

We systematically compared 3 DNA decontamination procedures for ancient samples using a set of 15 animal and Neanderthal bone and tooth samples: (*i*) phosphate treatment of bone powder, (*ii*) hypochlorite (bleach) treatment of bone powder, and (*iii*) repeated extraction of bone powder with an EDTA- and proteinase K-containing lysis buffer. Significant depletion of microbial and human DNA from samples is observed when a phosphate or hypochlorite treatment is used prior to regular extraction, but not when re-extraction is performed.

use of uracils (U) in ancient DNA, which are induced by cytosine (C) deamination and accumulate over time, preferentially in single-stranded overhangs at the ends of the DNA molecules (16). DNA polymerases read uracils as thymines (T), creating C to T differences to the reference genome and allowing ancient DNA molecules to be identified *in silico*. DNA molecules carrying uracils can also be physically separated during library preparation (17). However, both approaches are wasteful as they discard the majority of ancient DNA fragments not carrying deaminated cytosine residues.

A more straightforward solution to the problem of microbial and human contamination of bones and teeth is the selective removal of contaminant DNA by chemical or enzymatic treatment prior to DNA extraction. Numerous such procedures have been described, including treating bones or bone powder with proteinases (18), EDTA, sodium hypochlorite (bleach), or acids (see Reference 19 for an overview). However, previous studies primarily aimed at removing human contamination prior to amplification of short DNA segments by PCR. The suitability of these methods for decontaminating samples prior to library preparation and whole genome sequencing remains largely unexplored, the only exception being reports of an increase in the proportion of endogenous DNA relative to microbial and human contamination when performing two rounds of DNA extraction on the same bone powder (20–22). Here, we present an evaluation of three pretreatment methods for the removal of DNA contamination from ancient bones and teeth: (*i*) the release of surface-bound DNA by phosphate treatment, (*ii*) treatment with bleach, and (*iii*) repeated extraction of DNA from bone pellets using an EDTA/proteinase K lysis buffer. We compare these methods using powder from 14 ancient bones and 1 tooth preserved under different environmental conditions and show that they can substantially increase the ratio of endogenous to contaminant DNA.

Materials and methods

Binding of DNA to hydroxyapatite and bone powder

Suspensions in water were prepared from 200 mg Bio-Gel HTP hydroxyapatite (Bio-Rad, Hercules, CA) or 200 mg bone powder (prepared using a dentistry drill from an ancient North Sea whale sample) by adding 1 mL water and rotating at room temperature (RT) overnight. To each tube, 50 µg of GeneRuler Ultra Low Range DNA Ladder (Thermo Scientific, Waltham, MA) was added, and the suspensions were incubated with rotation at RT for 2 h, as modified from a previously described protocol (5). Hydroxyapatite or bone powder was then pelleted in a benchtop centrifuge, washed 3 times with 1 mL of water, resuspended in 1 mL of water, and split into 50 µl aliquots, each containing approximately 10 mg of suspended hydroxyapatite/bone powder. One aliquot was incubated with rotation 3 times for 30 min with 500 µl TT buffer (10 mM Tris-HCl, pH 8.0, 0.1% Tween-20), and a second was incubated with 500 µl 0.5 M sodium phosphate buffer, pH 7.0 (Alfa Aesar, Ward Hill, MA). Buffers were replaced after each incubation step. Pellets were washed with 1 mL water, and DNA was extracted by overnight incubation in 900 µl ET buffer (0.45 M EDTA, pH 8.0, 0.05% Tween-20) at RT. All relevant incubation buffers and final extracts were concentrated and desalted twice with 450 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) using Microcon Ultracel YM-3 concentrators (Millipore, Darmstadt, Germany) following the manufacturer's recommendations. Electrophoresis was carried out on 4% agarose gels.

Sampling, pretreatment of bone powder, and DNA extraction

Bone powder was removed from 14 ancient bones and 1 tooth in an ancient DNA laboratory using a dentistry drill at the lowest speed. The powder was then evenly distributed to 2.0 mL tubes. Ceramic beads (Precellys 2.8 mm zirconium oxide beads; Peqlab, Erlangen, Germany) that had not been pretreated in any way were added to each tube to facilitate resuspension of the bone pellets after centrifugation steps. Final amounts were approximately 30 mg (Neanderthal samples) and 50 mg (animal samples) of powder per tube. Bleach treatment was performed by adding 1 mL of a 0.1%, 0.5%, 2% or 6% sodium hypochlorite solution (Roth, Karlsruhe, Germany) and incubating with rotation for 15 min at RT. Bone pellets were then washed 3 times with 1 mL water for 3 min to remove residual bleach. Sodium phosphate treatment was performed by adding 1 mL 0.5 M sodium phosphate buffer supplemented with 0.1% Tween-20 to facilitate penetration of the buffer into the matrix and incubating for 15 min with rotation at RT. This step was repeated twice for a total of three phosphate washes. Bone pellets were then washed with 1 mL of TT buffer. One milliliter of lysis buffer (0.45 M EDTA, 0.25 mg/mL proteinase K, 0.05% Tween-20) was added to all pellets, and the suspensions were incubated with rotation at 37°C overnight. At least one extraction blank (no powder) was included in each set of DNA extractions. DNA was extracted using a guanidine hydrochloride-based binding buffer and silica columns, as described previously (23) with 2 modifications: (*i*) Tween-20 was added to the binding buffer at a concentration of 0.05%, and (*ii*) silica columns from the High Pure Viral Nucleic Acid Large Volume Kit (Roche, Basel, Switzerland) were used instead of MinElute columns (Qiagen, Venlo, The Netherlands). For DNA extractions from 30 mg bone powder, lysis and binding buffer volumes were reduced by half. Re-extraction of bone pellets was performed by adding 1 mL lysis buffer to the bone pellet remaining after the first extraction, incubating overnight at 37°C and following the procedure described above. DNA was extracted from the phosphate wash buffers (individually or pooled by sample) by adding them directly to the binding buffer. We verified experimen-

tally that this procedure is effective for recovering DNA from phosphate buffer (Supplementary Figure S1).

Library preparation, quantification, amplification, and enrichment

DNA libraries were prepared from 20% of the extract volumes using a single-stranded library preparation method described elsewhere (24), with the following modifications: (*i*) The single-stranded adaptor oligonucleotide (CL78) was cleaned from human contamination and synthesis artifacts by *E. coli* exonuclease I treatment. For this purpose, 7.5 µM of oligonucleotide was incubated for 20 min at 37°C in 1× CircLigase II buffer with 46 U exonuclease I (New England BioLabs, Ipswich, MA) in a reaction volume of 23 µl. The exonuclease was then heat-inactivated for 1 min at 95°C. (*ii*) Primer extension and blunt-end repair were united into a single reaction. To achieve this, phosphorothioate linkages (denoted with *) were introduced into the extension primer (5´-GTGACTGGAGTTCAGACGT GTGCTCTTCC*GA*TC*T-3´), and primer extension was carried out in Klenow reaction buffer supplemented with 2 µM primer, 200 µM dNTPs, and 20 U Klenow fragment of *E. coli* DNA polymerase I (Thermo Scientific), incubating for 5 min at 25°C and then 25 min at 35°C. One microliter of each library was used to measure the number of library molecules by digital PCR (QX200 system; Bio-Rad) using primers IS7 and IS8 (25) and EvaGreen chemistry following the manufacturer's protocols. The remaining libraries were amplified and tagged with pairs of sample-specific barcodes (26) using AccuPrime Pfx DNA polymerase (Life Technologies, Carlsbad, CA) as described elsewhere (27), but using higher primer concentrations (1 µM). Neanderthal libraries were enriched for mitochondrial DNA (mtDNA) using human mtDNA probes and bead-based hybridization capture (28).

Sequencing and sequence analysis

Libraries from each experiment were pooled and sequenced on Illumina's MiSeq or HiSeq2500 platforms using a 76+7+76+7 cycles recipe (26). Base calling was performed using Illumina's Bustard software (MiSeq runs) or freeIbis (HiSeq runs) (29). Sequences that did not perfectly match the expected index combination were discarded. Forward and reverse reads were overlap-merged to reconstruct full-length sequences (30), which were then aligned to suitable reference genomes [polar bear, horse, cow, dolphin, human nuclear (hg19), and mitochondrial (NC_012920)] using BWA (31) with parameters tailored for ancient DNA (32). Sequences shorter than 35 bp were discarded, and PCR duplicates were removed by calling a consensus from sequences with the same alignment start and end coordinates (bam-rmdup; https://github.com/udo-stenzel/biohazard). Alignments retained after duplicate removal were used to determine the frequency of substitutions for each position in the alignments as well as the base composition of the reference genome around alignment start and end points (16). Bam-rmdup was also used to extrapolate the total number of Neanderthal/human molecules in the libraries enriched for mtDNA.

Results and discussion

Our first experiment focused on the interaction of DNA and hydroxyapatite. It is known that free phosphate ions compete with the phosphate groups in the DNA backbone for binding to hydroxyapatite (33,34), a principle often exploited in liquid chromatography for the separation of nucleic acids (33). Phosphate has also been occasionally included in buffers for ancient DNA extraction (35,36). To determine whether a 0.5 M sodium phosphate buffer is suitable for releasing DNA from hydroxyapatite, we incubated a water suspension of hydroxyapatite with a double-stranded DNA size marker. As determined by agarose gel electrophoresis, no DNA is detectable in the supernatant after a 120 min incubation, indicating that all DNA was efficiently adsorbed (Figure 1A). The hydroxyapatite was then split into 2 aliquots, 1 of which was incubated 3 times for 30 min in TT buffer and the other in 0.5 M sodium phosphate buffer, and then dissolved with EDTA. As expected, no DNA was released into the TT buffer, and DNA release occurred only through the action of EDTA. In contrast, complete DNA release was achieved in the first phosphate incubation, with no additional marker DNA being recovered through a second exposure to phosphate or EDTA. Results are identical when replacing pure hydroxyapatite with powder from an ancient whale bone, demonstrating that ancient bone exhibits a high binding affinity for DNA in aqueous solutions (Figure 1B) and that this binding can be reversed with 0.5 M sodium phosphate.

Bleach treatment of bones or bone powder (19,37) is the most common method for removing human contamination in forensic research and has also been used in ancient DNA research (38). The reported efficiency of bleach as a decontaminating agent varies between studies, from complete human contamination removal using a ≥ 3% bleach solution (19), to incomplete contamination removal regardless of the bleach concentration used (39). However, it has

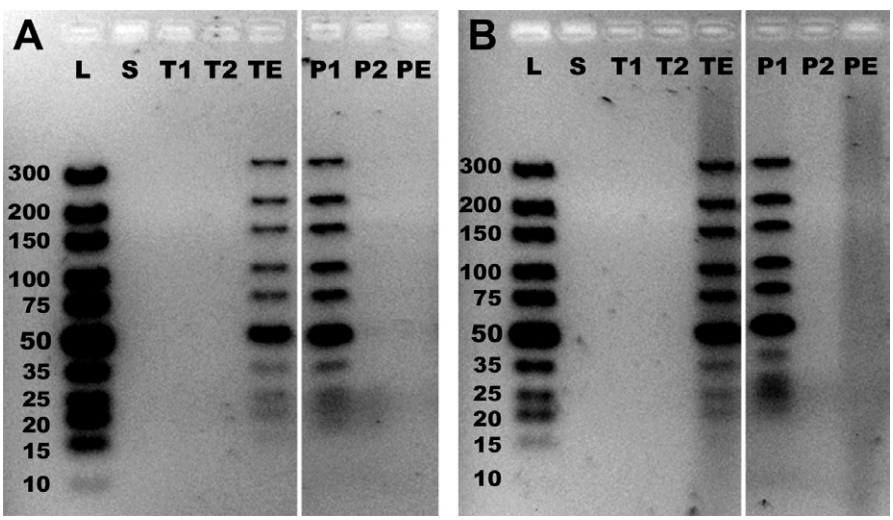

Figure 1. Adsorption and release of DNA from hydroxyapatite and ancient bone powder. DNA release was detected by agarose gel electrophoresis (panel A: hydroxyapatite; panel B: ancient whale bone). L: DNA ladder (size marker and substrate for binding experiment); S: Supernatant after incubating the DNA in either a hydroxyapatite or bone powder suspension; T1 and T2: TT buffer incubation; P1 and P2: phosphate buffer incubation; TE and PE: EDTA buffer incubation. Smears in the TE and PE lanes in panel B possibly originate from DNA preserved in the bone. Differences in band intensities are due to difficulties in evenly splitting the hydroxyapatite/bone powder suspensions.

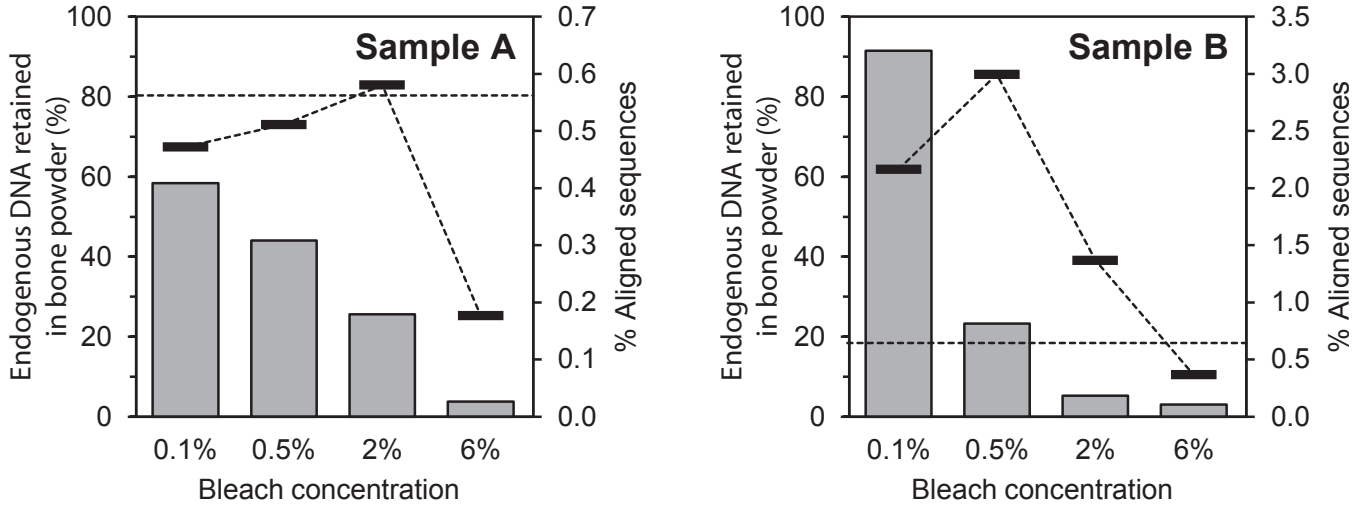

Figure 2. Effect of different bleach concentrations on DNA present in bone powder from two ancient animal samples. Plotted are the percentage of endogenous DNA retained in the bone powder after bleach treatment relative to the untreated control (gray bars) and the percentage of sequences aligning to the reference genome in the treated powder (horizontal bars) and the untreated control (dashed horizontal line).

been reported that approximately three quarters of the total endogenous DNA is lost by bleach treatment of powdered bone (39). To determine how bleach treatment of bone powder alters the characteristics of DNA libraries generated from ancient bones, we sampled 250 mg of powder from 2 cave bear bones (Samples A and B), which we then split into five 50-mg aliquots. Four aliquots were treated with different concentrations of bleach (0.1%, 0.5%, 2%, and 6%), and 1 aliquot remained untreated as a control. The bone powder was then incubated with an EDTA/proteinase K lysis buffer, and the DNA was purified using a silica-based technique (23). We then characterized the DNA in two ways: first by generating DNA libraries and measuring the number of library molecules by digital droplet PCR, and second by shallow shotgun sequencing of the libraries on Illumina's MiSeq or HiSeq platforms. Combining both data allows for accurate quantification of the number of endogenous DNA fragments, which are identified through their similarity to an appropriate reference genome. In Sample A, bleaching had a solely destructive effect, causing endogenous DNA loss, most severely at higher bleach concentrations, without substantially altering the percentage of sequences that could be aligned to the reference genome (Figure 2 and Supplementary Table 1S). For Sample B, bleaching also destroyed a considerable fraction of the endogenous DNA, but we observed a substantial increase in the percentage of aligned sequences. This increase is highest at a bleach concentration of 0.5% (from 0.6% to 3.0% aligned sequences). DNA sequences generated from bleached and untreated bone powders exhibit very similar fragment size distributions and frequencies of deamination-induced substitutions (Supplementary Figures S2 and S3), indicating that bleach does not cause damage in addition to DNA loss at a level that would affect downstream analyses. In contrast to a previous study (40), we also see no evidence for depurination-induced strand breaks when analyzing the base composition around DNA break points (Supplementary Figures S4 and S5).

We next evaluated the effects of bleach treatment, phosphate treatment, and re-extraction on a set of eight Late Pleistocene bones and one tooth from three cave sites in Eurasia, a permafrost site in the Yukon (Canada), and an underwater site in The Netherlands. In this set, we included replicates from Sample A of the previous experiment to assess the reproducibility of our experiments. We obtained three aliquots of bone powder from each specimen and used the first for regular DNA extraction. The second was treated with 0.5% bleach, and the third was treated 3 times with 0.5 M sodium phosphate buffer prior to DNA extraction. In addition, we re-extracted DNA from the first bone powder aliquot as suggested in previous publications (20,21) and extracted DNA from all phosphate wash buffers. We observed an increase in the percentage of aligned sequences after bleach treatment for all specimens except for both replicates of Sample A (Figure 3A, Samples A2 and A3, and Supplementary Table S1), which is consistent with the results obtained from this specimen in the previous experiment. Averaged across all specimens, the increase in the percentage of aligned sequences is 3.4-fold, with the biggest increases (up to 8.2-fold) seen in the North Sea specimens (Samples H and I). The only tooth (Sample C) shows a moderate 3.2-fold increase. However, bleach treatment comes at the expense of an average 57% endogenous DNA loss (range 11%–84%) (Figure 3B). Phosphate treatment led to a slightly smaller but consistent increase in the percentage of aligned sequences (2.1-fold on average, range 1.1- to 3.1-fold) and a smaller release of endogenous DNA from the sample powder (35% on average, range 4%–83%). In agreement with the initial experiment, most contaminant DNA is released during the first phosphate incubation (Supplementary Figure S6). In regards to size distribution and nucleotide misincorporation patterns, sequences obtained after bleach and phosphate pretreatment are similar to the untreated control (Supplementary Table S1; Supplementary Figures S7 and S8). The increases in percentage of aligned sequences following the two treatments correlate poorly (Supplementary Figure S9), suggesting that both methods should be tried whenever possible.

In contrast to bleach and phosphate treatment, very little DNA is recovered when re-extracting bone pellets remaining after a first round of extraction. On average, 91% of the DNA is released in a single round of DNA extraction, and most samples show a decrease in the percentage of aligned sequences after this treatment. However,

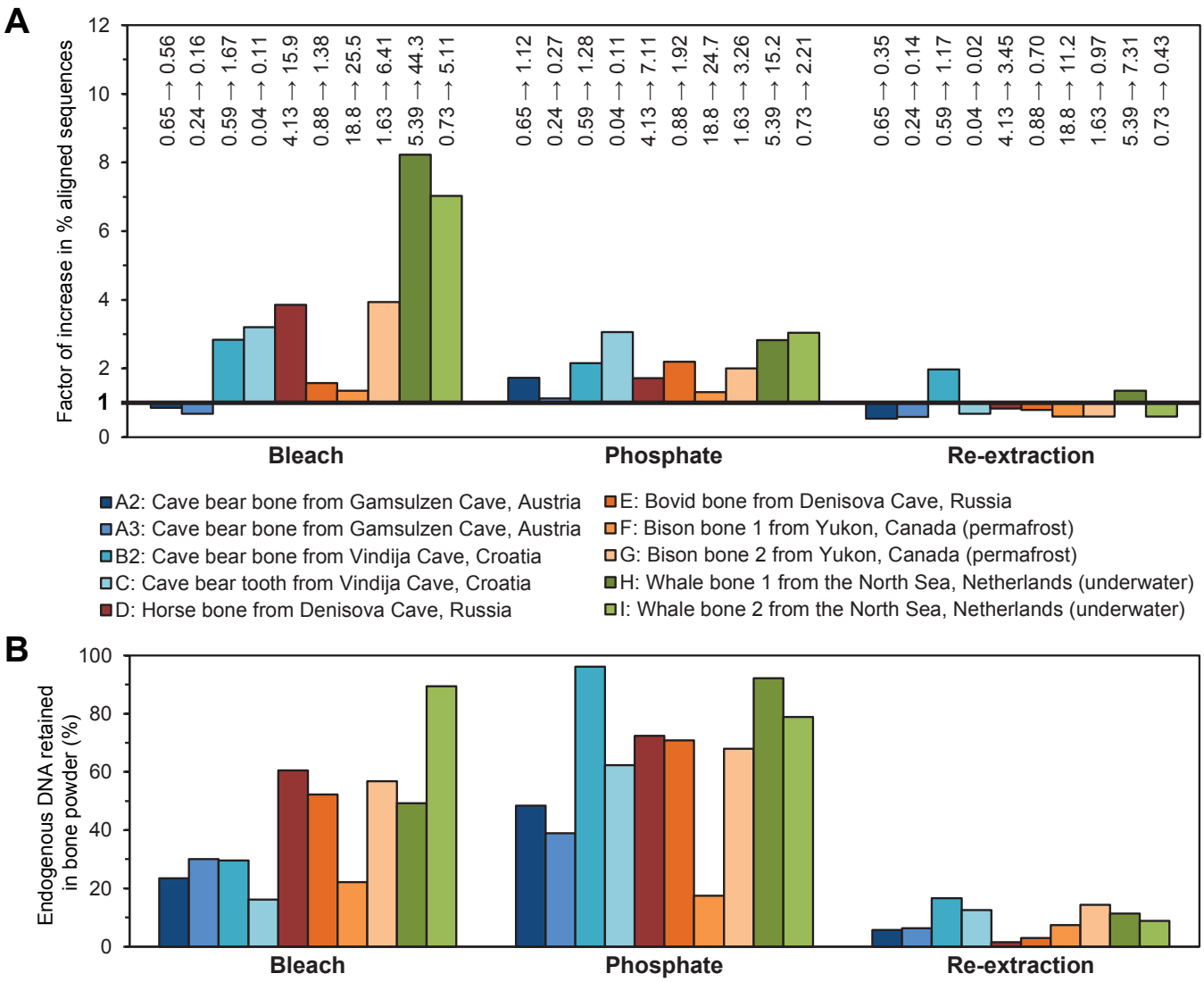

Figure 3. Comparison of bleach treatment, phosphate treatment and, re-extraction using 10 ancient animal samples. Plotted are (A) the increase in percentage of aligned sequences relative to the untreated control (absolute percentages denoted above the bars) and (B) the percentage of endogenous DNA retained in the bone/tooth powder. For bleach treatment, the latter value was inferred by comparison to the untreated control; for phosphate treatment, it could be directly inferred using the number of endogenous DNA molecules recovered from the phosphate and lysis buffers.

we note that a recent study suggests more favorable results, similar to those obtained by the phosphate treatment described here, may be obtained when shortening the incubation time of the first extraction (22).

A potential depletion of human contamination is difficult to assess using shotgun sequences from animal bones due to the similarity of mammalian nuclear genomes. To specifically address this problem, we applied bleach and phosphate treatment to a set of Neanderthal bones previously found to be either moderately or severely contaminated with human DNA and enriched the libraries for mtDNA, in addition to direct sequencing. In line with the previous experiment, we observed an increase in the percentage of aligned sequences for 4 of 6 specimens (range 2.8- to 24-fold) following bleach treatment (Figure 4A). In 2 bones (Samples L and M) from the same site, bleach treatment caused a severe loss of endogenous DNA (96% and 92%, respectively) (Figure 4B) and an enrichment of non-endogenous DNA, while phosphate treatment resulted in a moderate increase in the percentage of aligned sequences (1.5- and 2.1-fold) and a smaller release of endogenous DNA (58% and 39%). Using the sequences enriched for mtDNA, we then determined the percentage of human contamination at positions where all published Neanderthal mitochondrial genomes differ from those of 311 present-day humans (17). In 4 of 6 samples (Samples K, L, N, and O), we detected a significant reduction of human contamination after bleach treatment (Figure 4C), although this reduction was relatively small (less than 2-fold) in 2 samples (K and L). It is important to note that the percentage of human contamination in the library does not necessarily reflect the level of contamination present in the bone powder before DNA extraction and library preparation, as some human DNA is introduced during laboratory work (Supplementary Table S1). This is particularly true for bleach-treated bone powder, where a substantial loss of endogenous DNA molecules counterbalances the effect of human contami-

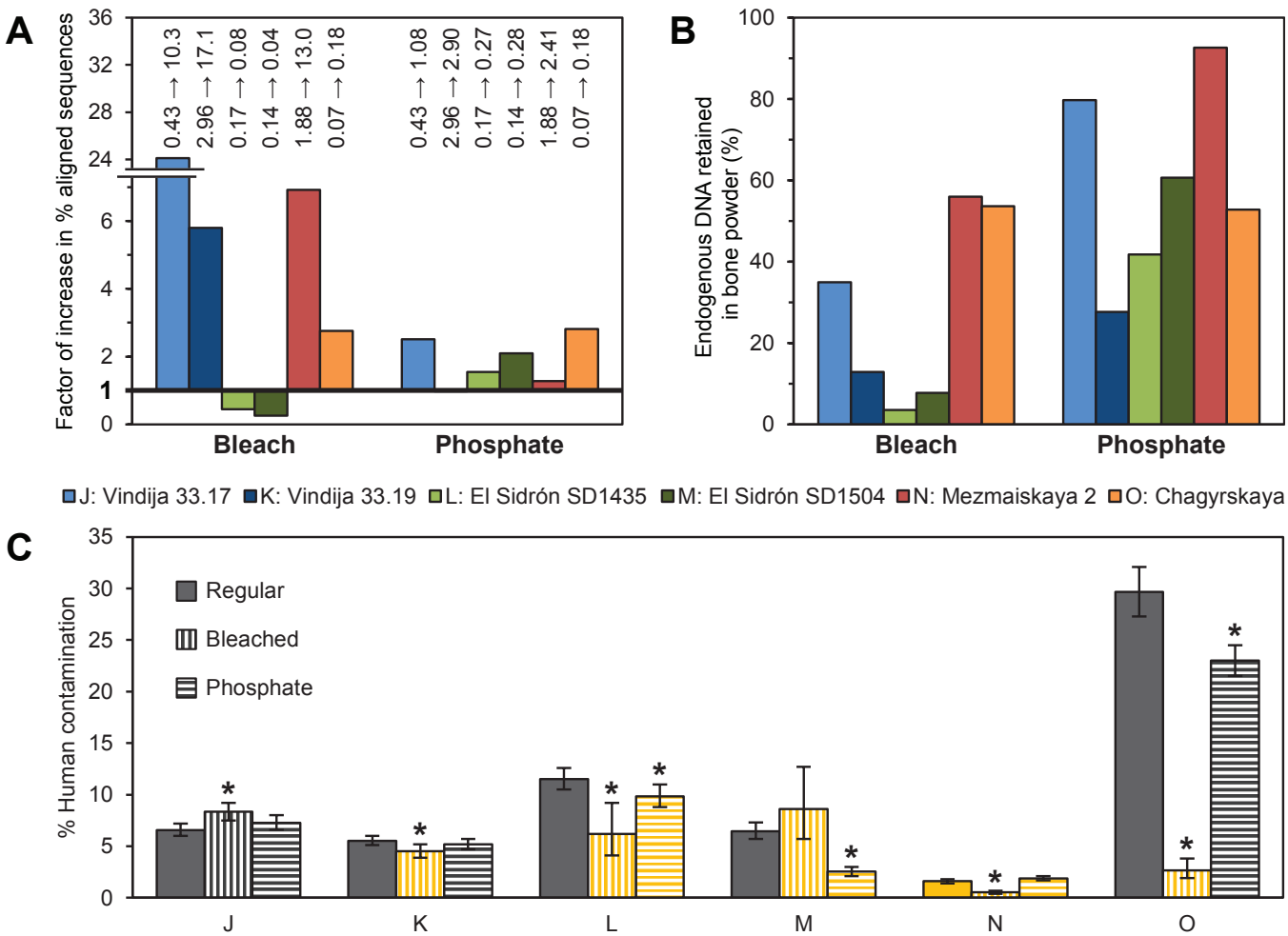

Figure 4. Removal of microbial and human contamination from six Neanderthal bones. Plotted are (A) the increase in percentage of aligned sequences relative to the untreated control (absolute percentages denoted above the bars), (B) the percentage of endogenous DNA retained in the bone powder, and (C) the percentage of human contamination in the libraries (with 95% confidence intervals) inferred from mtDNA sequences. A chi-square test was used to determine significant differences (denoted with *) compared to the untreated controls ($\alpha = 0.05$) . Note that the number of human contaminant molecules in some sample libraries (highlighted in yellow) is less than 4× higher than in the extraction and library blank (Supplementary Table S1).

nation depletion, in one case leading to an increased percentage of human contamination in the library (Sample M). In one sample (Sample J), bleach treatment clearly failed to reduce human contamination in the bone powder, even though it led to a substantial depletion of microbial DNA, indicating that bleach treatment provides no guarantee for human contamination removal, which is in agreement with some previous studies (37,39).

Phosphate treatment proved less effective for the removal of human contamination, with only a single sample (Sample M) showing a substantial decrease in contamination (2.6-fold). The relative ineffectiveness of phosphate treatment in removing human relative to microbial contamination may indicate that human contamination—or at least a fraction thereof—is protected inside particles such as skin flakes or cell debris, whereas microbial DNA, which seems to be largely of ancient origin in most specimens (17), is adsorbed directly to hydroxyapatite.

In conclusion, two of the three methods tested here caused a substantial depletion of microbial DNA contamination. The first, incubation in phosphate buffer, can be safely implemented as a routine step prior to ancient DNA extraction for reducing the load of microbial contamination, as all DNA released into the buffer remains accessible for DNA extraction, library preparation, and sequencing. Furthermore, phosphate treatment requires no optimization of incubation time, as nearly all of the DNA is released in a single incubation step. The second method, exposure of ancient bone powder to bleach, shows even greater potential for depleting microbial contamination and is also more effective at removing human contamination. However, bleach treatment is difficult to control, as it can lead to severe losses of endogenous DNA and might thus be unsuitable for small and precious specimens that cannot be repeatedly sampled. While our results are of immediate relevance for attempts to generate genome-wide data from ancient bones or teeth, further studies are needed, especially to address the problem of human contamination removal from ancient bones as well as the reagents used in DNA extraction and library preparation.

Author contributions

P.K. and M.M. designed the experiments and analyzed the data. P.K., S.N., and A.A.P. performed the experiments. T.G., M.T.G., and M.H. improved the single-stranded library preparation. P.K. and M.M. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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