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RESEARCH ARTICLE

Linear polyacrylamide is highly efficient in precipitating and purifying environmental and ancient DNA

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Abstract

1. Nucleic acid extraction from complex environmental and ancient tissue material is prone to co-extract inhibitory substances that make further molecular analysis difficult or impossible. This co-extraction occurs in both solid-phase and liquid-phase/organic nucleic acid extraction protocols. Currently, the widely used method to overcome inhibition includes the addition of bovine serum albumin (BSA) to the downstream enzymatic reactions or the dilution of the nucleic acid extracts. BSA, however, seems to reduce the inhibitory effect of certain compounds only, and excessive dilution may change the original DNA composition.
2. In this study, we introduce an innovative new method using linear polyacrylamide (LPA) to efficiently precipitate and purify nucleic acids extracted from complex environmental and ancient tissue samples in one working step. The LPA method replaces the precipitation step in classic liquid-phase/organic extraction protocols or can be easily applied as an additional post-extraction step on impure DNA extracts. As a proof of concept, we experimented with this method on different ancient human mummy samples (bones, soft tissues and gut contents) from different time periods (5000 BC–1800 AD), as well as on complex environmental samples (e.g. soil, activated sludge and animal faeces) known to contain inhibitory compounds.
3. We demonstrated that LPA precipitates nucleic acids, even in an aqueous ethanol solution without the addition of chaotropic salts, resulting in the recovery of highly pure DNA from all tested samples that displayed inhibition with previously published extraction protocols. Compared to the current, most widely used silica-based extraction method for ancient and sedimentary DNA, our LPA method resulted in comparable DNA qualities and overall DNA compositions (human endogenous content and microbial diversity).
4. In conclusion, our LPA method with its high purifying capacity provides an important alternative to the commonly used DNA extraction protocols in the environmental and ancient DNA (aDNA) fields.

KEYWORDS
ancient DNA, endogenous DNA content, environmental DNA, linear polyacrylamide, nucleic acid extraction, nucleic acid purification, PCR inhibitors

1 | INTRODUCTION

Nucleic acid extraction is the crucial step for DNA and RNA-based molecular downstream applications. Protocols generally aim to extract nucleic acids from different starting material in high quantity and quality. For complex environmental samples and ancient tissue material, however, the retrieval of DNA that can be successfully subjected to molecular analysis is often challenging due to the presence of inhibitory substances (Schrader et al., 2012; Wilson, 1997) or due to highly fragmented DNA present only in low quantities (Orlando et al., 2021). Currently, most protocols in both the environmental and aDNA field are based either on solid-phase or on liquid-phase/organic nucleic acid extraction. During solid-phase extraction, nucleic acids selectively absorb to a matrix (e.g. magnetic beads, silica) due to specific hydrophobic, polar and/or ionic interactions (Ali et al., 2017; Bowien & Dürre, 2003). For example, silica matrices display a high binding affinity for nucleic acids under increased concentrations of chaotropic salts such as guanidine hydrochloride or guanidine isothiocyanate (Boom et al., 1990; McCormick, 1989; Melzak et al., 1996; Vogelstein & Gillespie, 1979). The efficient and selective binding of nucleic acids hallmarks the silica-based extraction procedure, and due to its reproducibility and easy performance, it became part of most commercial environmental nucleic acid extraction kits (Taberlet et al., 2018). In addition, customised silica-based DNA extraction protocols are currently widely used in the aDNA field (Dabney et al., 2013; Gamba et al., 2014; Glocke & Meyer, 2017; Rohland et al., 2018). One drawback of this method is that small DNA fragments tend to bind tightly to the silica matrix and are therefore difficult to elute, unless the right DNA binding buffer is chosen (Gerstein, 2004; Green & Sambrook, 2018). This, however, increases the risk that inhibitory substances become co-extracted on the silica matrix (Baar et al., 2011; Dong et al., 2006; Rohland et al., 2018). The principle of the liquid-phase/organic nucleic acid extraction lies in the separation of proteins and other cellular components from the nucleic acid molecules by adding the organic solvent phenol (Kirby, 1956), usually mixed with chloroform and isoamyl alcohol (Sambrook & Russell, 2006). This results in two phases: an upper aqueous phase that contains the nucleic acids, and a lower organic phase where most proteins and cellular debris remain. The nucleic acids in the aqueous phase become further purified and concentrated by either using molecular weight cut-off columns (Leonard et al., 2000; Norén et al., 2013) or classically via precipitation with 100% ethanol, isopropanol, spermine or polyethylene glycol in the presence of high chaotropic salt concentrations (Dowhan, 2012; Green & Sambrook, 2016, 2017; Hoopes & McClure, 1981; Paithankar & Prasad, 1991). The precipitation step is essential to yield a high recovery of nucleic acids (Ali et al., 2017). Moreover, in case of precipitation with ethanol and cations, the yields can be substantially improved by adding neutral carriers such as yeast tRNA, glycogen or linear polyacrylamide (LPA) (Green & Sambrook, 2016). With the addition of LPA as a carrier during salt-based ethanol precipitation, a recovery of even picograms of nucleic acids can be achieved (Gaillard & Strauss, 1990; Strauss & Varshavsky, 1984). This increased DNA yield could facilitate the molecular analysis of ancient skeletal and mummified specimens where highly fragmented ancient DNA is normally present only in minute amounts. The main drawback of the alcohol/salt-based nucleic acid precipitations, however, is the co-precipitation of polymerase chain reaction (PCR) inhibitors such as blood or collagen degradation products in ancient human remains (King et al., 2009; Scholz et al., 1998). This is also a well-known phenomenon in environmental samples where polyphenolic compounds such as humic and fulvic acids co-precipitate with the DNA and then strongly inhibit polymerase activity or other enzymes used in molecular downstream processes (de Bruijn, 2011; Matheson et al., 2010).

The aim of our work was to overcome this major limitation of the classic nucleic acid precipitation protocols by introducing a new precipitation/purification method that is solely based on the presence of LPA and aqueous ethanol solution. In this study, we tested the LPA method on three environmental samples known to be challenging to obtain PCR inhibitor-free extracts: fen soil, animal faeces and activated sludge. In addition, we applied the new method on four ancient bones (e.g. petrous bone, rib, vertebra) and five soft tissue samples (e.g. muscle, lung, stomach content), known to contain minute amounts of short DNA fragments and PCR inhibitors. We were able to show that the LPA method is highly applicable to a variety of ancient samples, as demonstrated through the hereby presented sample selection of different skeletal and mummified specimens from various origin and times (Neolithic to Modern Period). We assessed the PCR amplification before and after the LPA purification of environmental and aDNA extracts. Furthermore, by applying a next generation sequencing (NGS) approach using metagenomic shotgun sequencing on selected ancient human and environmental samples, we compared the DNA yield, fragment length recovery and DNA composition of extracts obtained by the current most widely used silica-based extraction method for ancient and sedimentary DNA versus our LPA method.

2 | MATERIALS AND METHODS

2.1 | Sample description

In this study, we submitted three environmental and nine ancient human tissue samples to different nucleic acid extraction protocols, including the new LPA precipitation/purification method (as described below). The nucleic acid extracts were tested for inhibitors

using a PCR-based approach, and in selected samples the DNA composition was assessed using an NGS-based approach (Figure S1). For the environmental samples, we included activated sludge, bovine faeces and fen soil material that are known to contain PCR inhibitors such as humic and fulvic acids. For the ancient human tissue samples, we selected four bone tissue and five soft tissue samples from different origin and times (Neolithic to Modern Period). All the ancient samples contained DNA that was highly fragmented and of low concentration, as well as additional substances that inhibited further molecular downstream processes. For further details to the samples and the applied nucleic acid extraction protocols, please refer to Tables S1 and S2.

2.2 | DNA precipitation with LPA in an aqueous 'salt-free' DNA solution

Previous studies have shown that LPA is an efficient neutral nucleic acid carrier during salt-based ethanol precipitation (Gaillard & Strauss, 1990; Strauss & Varshavsky, 1984). We tested first the capacity of LPA to precipitate DNA (5 µg of an ultra-low-range DNA ladder) in an aqueous salt-free ethanol solution. The LPA results were compared to the DNA ladder precipitates obtained by classical methods using isopropanol (Nair et al., 2014) or 0.3 M sodium acetate and 100% ethanol (Green & Sambrook, 2016). The precipitated DNA ladders were visualised on a 4% agarose gel.

In the following text, we describe the LPA preparation and LPA precipitation step in an aqueous salt-free ethanol solution (Figure 1). A 0.5% linear polyacrylamide (LPA) solution was prepared as described by Gaillard and Strauss (1990) with minor modifications. LPA synthesis is performed in a sterile 50 ml disposable polypropylene tube: 250 mg acrylamide (without bis-acrylamide) is dissolved in 4.5 ml TE buffer (pH = 8) and the polymerisation reaction is started by adding 50 µl of 10% (w/v) ammonium persulfate solution and 5 µl TEMED and left for 3 hr at 37°C. In the subsequent precipitation with 20 ml ice-cold (−20°C) 100% ethanol, a white polymer clump is formed. The ethanol is thoroughly removed with a sterile serological pipette by squeezing the white polymer several times with the pipette tip. Next, the white polymer is subjected to a short washing step with 10 ml ice-cold (−20°C) 70% ethanol, which must be thoroughly removed with a sterile serological pipette immediately after the addition. The residual ethanol is further evaporated from the polymer pellet by keeping the polypropylene tube with the lid open for 5 min at RT under a sterile PCR workstation. Finally, the LPA pellet is re-dissolved in 50 ml sterile PCR-grade water overnight at RT. The obtained 0.5% LPA solution is aliquoted and stored at 4°C.

To precipitate and purify nucleic acids in solutions with a volume up to 200 µl, add 40 µl of 0.5% LPA and 6 volumes ice-cold (−20°C) 70% ethanol. The content is mixed gently by inverting the tube and then centrifuged for 10 min at 16,000 × *g* at RT. The supernatant is carefully removed until only a distinct translucent pellet remains at the bottom of the tube. The residual ethanol is further evaporated by keeping the tube with the lid open for 1 hr at RT under a sterile PCR workstation. Finally, the pellet is re-dissolved in 40–60 µl sterile PCR-grade water or TE buffer and stored at −20°C (Figure 1).

2.3 | Application of the novel LPA precipitation protocol on different environmental and ancient human samples

2.3.1 | Assessment of the LPA precipitation/purification capacity in comparison to classic nucleic acid extraction protocols

Next, we tested the capacity of LPA to precipitate and purify nucleic acid extracts from environmental and ancient samples that displayed PCR inhibition with previously applied extraction methods

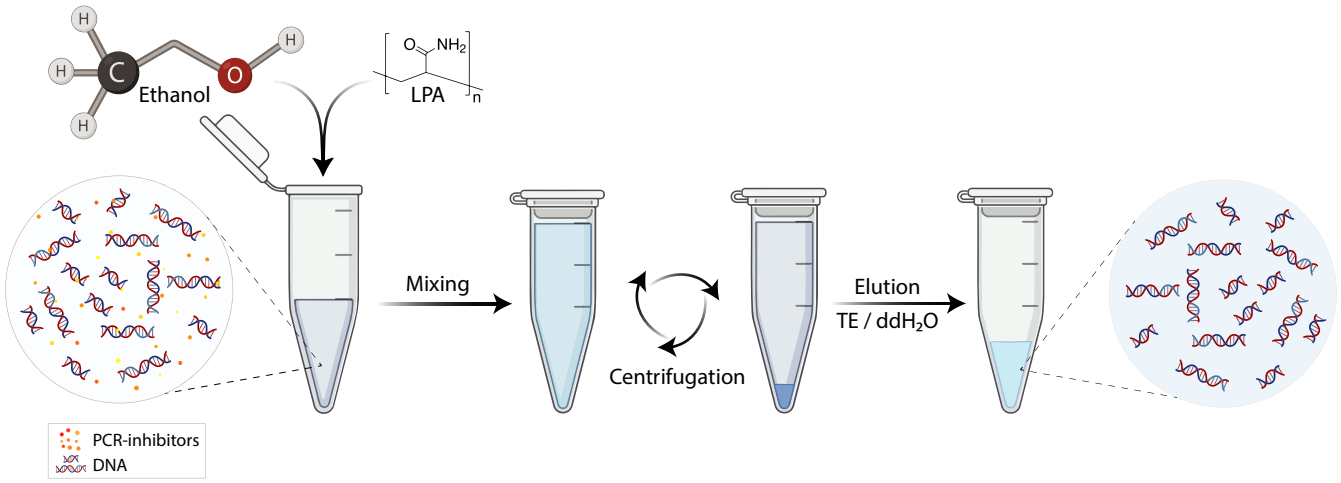

FIGURE 1 Schematic illustration of the linear polyacrylamide (LPA)-based nucleic acids precipitation and purification in aqueous salt-free ethanol solution. After mixing the content (LPA, 70% ethanol, nucleic acid solution) and centrifugation, a distinct translucent pellet remains at the bottom of the tube. The residual ethanol is removed, and after a 1-hr drying step the nucleic acid containing pellet is re-dissolved in sterile PCR-grade water or TE buffer. Illustrations were made using BioRender

(Table S1, Figure S1). The samples were subjected to different nucleic acid extraction protocols including various nucleic acid precipitation and purification methods (Table S2). These classic nucleic acid extracts were compared to the extracts obtained by the precipitation/purification using LPA. Therefore, nucleic acids from 100 mg sample material were extracted using both liquid-phase/organic extraction and solid-phase extraction methods commonly used in the environmental and aDNA research fields. The nucleic acid extractions of the environmental samples were performed in a modern DNA laboratory. The nucleic acid extraction and molecular analysis of the ancient samples were conducted at the aDNA laboratory of the Eurac Research Institute for Mummy Studies in Bolzano, Italy. Sample preparation and DNA extraction were performed in a dedicated pre-PCR area following the strict procedures required for studies of aDNA: use of protective clothing, UV-light exposure of the equipment and bleach sterilisation of surfaces, use of PCR workstations and filtered pipette tips. For more information on the different nucleic acid extraction protocols applied to the various samples, please refer to Table S2.

Both a phenol–chloroform–isoamyl alcohol extraction (P/C/I), as described by Griffiths and colleagues (Griffiths et al., 2000), and a chloroform–isoamyl alcohol extraction (C/I) following the protocol of Tang and colleagues (Tang et al., 2008), provided the basis for the liquid-phase/organic nucleic acid extraction protocols. Environmental and soft tissue samples were lysed using CTAB-based lysis buffers known to denature cell wall lipopolysaccharides and proteins. Bone samples were subjected to EDTA lysis to demineralise the bone matrix. In the Griffiths protocol, the samples were lysed in 1 ml CTAB extraction buffer (10% [wt/vol] CTAB, 0.7 M NaCl, 240 mM potassium phosphate buffer, pH 8) overnight at 55°C. In the Tang protocol, samples were either lysed in (a) 1 ml CTAB buffer (100 mM Tris–HCl [pH 8.0], 2.0 M NaCl, 20 mM EDTA, 2% [wt/vol] CTAB, 10 μl Proteinase K [10 mg/ml]), at 55°C for 24 hr, with subsequent addition of 1% (vol/vol) PVP and 2% (vol/vol) β-mercaptoethanol and overnight incubation at 50°C (for details, please refer to the Supplementary Methods) or (b) in 1 ml EDTA-based lysis buffer (0.5 M EDTA, pH 8, 0.25 mg/ml proteinase K and 0.05% [vol/vol] Tween 20). Soft tissue and soil samples were subjected at the beginning of the lysis to an additional homogenisation/mechanical disruption step using Qiagen PowerBead Tubes in a bead beating instrument (Retsch®, settings: 25 Hz, 30 s). The protocols were used in combination with different nucleic acid precipitation and purification methods. Nucleic acids in the P/C/I protocol were precipitated and purified with one of the following methods: (a) precipitation with 2 volumes of 30% (wt/vol) polyethylene glycol 8000–1.6 M NaCl (P/C/I/PEG) and washing with ice-cold (−20°C) 70% (vol/vol) ethanol, modified from Griffiths (2000); (b) overnight precipitation with 1 volume of ice-cold isopropanol (P/C/I/Iso) at −20°C and washing with ice-cold (−20°C) 70% (vol/vol) ethanol, as described by Nair et al. (2014); (c) precipitation with 0.1 volume 3 M sodium acetate and 2 volumes ice-cold (−20°C) ethanol (P/C/I/EtOHAc) at room temperature (RT) for 3 hr and washing with ice-cold (−20°C) 70% (vol/vol) ethanol, as described by Green and Sambrook (2016); (d) precipitation with 0.1 volume 3 M sodium acetate and 2 volumes ice-cold (−20°C) ethanol at RT for 3 hr, washing with ice-cold (−20°C) 70% (vol/vol) ethanol, as described by Green and Sambrook (2016), which is followed by a second precipitation and purification of the nucleic acid extracts using spermidine (P/C/I/EtOHAc-Sp) as described by Hoopes and McClure (1981). Nucleic acids in the chloroform–isoamyl alcohol extraction protocol (C/I) based on Tang et al. (2008) were precipitated with 0.1 volume 3 M sodium acetate and 2 volumes ice-cold (−20°C) ethanol (CTAB-C/I/EtOHAc) at RT for 3 hr and subsequently washed with ice-cold (−20°C) 70% (vol/vol) ethanol, as described by Green and Sambrook (2016).

Aside from the above-described organic extraction protocols, we also subjected two ancient human specimens (bone and soft tissue) and one environmental sample (bovine faeces) in the precipitation/purification test to a solid-phase nucleic acid extraction using a silica-based DNA extraction method (EDTA-Silica-a) as described by Rohland et al. (2010) and modified by Gamba et al. (2014). In brief, this included an initial lysis step in 3.5 ml lysis buffer (0.5 M EDTA, pH 8, 0.25 mg/ml proteinase K) followed by a concentration of the lysate in an Amicon© 10 kDa column, and a final DNA binding to a silica column (Qiagen MinElute purification kit).

The LPA precipitation/purification step was either applied to nucleic acid extracts that showed PCR inhibition or it was implemented in organic nucleic acid extraction protocols, thereby replacing the last precipitation and purification step (please refer to the Supplement file for a modified chloroform–isoamyl alcohol extraction protocol that includes the LPA precipitation/purification step).

The quantity and quality of the nucleic acid extracts were compared before and after nucleic acid precipitation/purification using LPA. Therefore, we measured the DNA concentration using a fluorescence-based nucleic acid quantification method (Quantus™ Fluorometer). In addition, we tested the nucleic acid extracts for the presence of inhibitory substances using different PCR assays targeting human, animal, bacterial or fungal genomic fragments. All PCR assays were conducted in 50 μl volume using the AmpliTaq Gold 360 Master Mix, 0.2 mg/ml bovine serum albumin (BSA), 1 mM primer and 5 μl DNA template. For details to the PCR targets and the PCR conditions, please refer to Table S3.

2.3.2 | Effect of the novel LPA method on the overall DNA composition

To further systematically test the effect of the novel LPA precipitation/purification step on the DNA quantity, quality and overall composition (human DNA content and microbial diversity), we compared the NGS shotgun sequencing results of four ancient human specimens (two bone tissue and two soft tissue samples) and one environmental sample (fen soil) (Table S1, Figure S2), after replicated nucleic acid extraction using both the novel LPA method and the current most widely used silica-based extraction method (Silica-b) for ancient and sedimentary DNA (Rohland et al., 2018). Importantly, for this comparison, we selected samples that showed

no PCR inhibition in both extraction methods. In our replicated study design, we performed each extraction protocol three times on one sample to obtain representative results. First, we digested for both protocols (LPA, Silica) the bone powder and soil samples (50 mg for each replicate) in an EDTA-based lysis buffer (0.5 M EDTA, pH 8, 0.25 mg/ml proteinase K and 0.05% [vol/vol] Tween 20; 1 ml for each replicate) overnight at 37°C. The ancient human soft tissue samples (50 mg for each replicate, cut in small pieces) were once lysed for the LPA protocol in a CTAB-based lysis buffer (for details, please refer to the CTAB-C/I/LPA extraction protocol in the Supplementary methods) and for the EDTA-Silica-b protocol the soft tissue was digested in the same EDTA-based lysis buffer as described above (Figure S1). Both soft tissue and soil samples were subjected at the beginning of the lysis to an additional homogenisation/mechanical disruption step using Qiagen PowerBead Tubes in a bead beating instrument (Retsch®, Settings: 25 Hz, 30 s). After the lysis step, the lysate was first separated from the undigested sample material by centrifugation (2 min at 16,000 g). Next, 1 ml of lysate (for each replicate) was subjected once to organic purification and the DNA was precipitated/purified using LPA (EDTA-C/I/LPA protocol, see Supplementary Methods) and the lysate was transferred one time to a 10.4 ml binding buffer D (5 M guanidine hydrochloride, 40% [vol/vol] 2-propanol, 0.12 M sodium acetate and 0.05% [vol/vol] Tween 20) and bound to a silica column as described by Rohland et al. (2018) (EDTA-Silica-b). For this, the lysate/binding buffer mixture was poured into the 20 ml tube extender (QIAGEN Inc.) of the silica spin column assembly and centrifuged for 4 min at 500 g. After the washing and drying step, the DNA became eluted from the silica column with the same elution buffer volume as previously used for the LPA protocol. The concentration of all replicated DNA extracts was measured using a fluorescence-based nucleic acid quantification method (Quantus™ Fluorometer). For details on the DNA concentrations of the extracts and libraries, please refer to Table S4.

For sequencing, double-stranded DNA libraries for all samples were prepared according to the published protocol of Meyer and Kircher (2010). Thereby, 10 µl nucleic acid extract of the sample replicates (three replicates per sample and extraction method) was used as DNA input volume, and unique P5 and P7 index combination was added during the indexing PCR (14 cycles) to each library. The DNA extracts of the environmental soil sample were subjected, before the library preparation, to a DNA fragmentation step using the Covaris® Ultrasonicator device (Settings: Peak power 50; duty factor 20; 200 cycles; and 175 s). This DNA fragmentation step was implemented in the library preparation to also recover most high-molecular-weight DNA of the modern sample. A library preparation control (PCR-grade water) was included for each of the five samples. DNA libraries were quality checked by determining the length distribution and quantity of DNA fragments using the Agilent Bioanalyzer system. Libraries were sequenced on the Illumina HiSeqX platform using the 151-base pair paired-end sequencing kit. In addition to the sample replicates, DNA extraction blank controls of the LPA and Silica-b method were converted into Illumina libraries and subjected to shotgun sequencing. For details on the metagenomic datasets, please refer to Table S5. Sequencing data are available from the European Nucleotide Archive under accession no. PRJEB45999.

Paired Illumina reads were processed (adapter removal and read merging) and quality checked using AdapterRemoval (Schubert et al., 2016) and fastp (Chen et al., 2018), respectively. First, we assessed the read length distribution profile on the pre-processed reads by combining the data of the sample replicates, removing the duplicated sequences using the SeqKit tool v0.8.1 (https://bioinf.shenwei.me/seqkit/) (Shen et al., 2016), and subsampling the data to 13,766,042 randomly selected reads using the Seqtk tool (https://github.com/lh3/seqtk). The subsampling was performed in order not to bias the comparative analysis by the variable sequencing depth. Next, we determined the library complexity by plotting the fraction of unique reads (kmers) produced by a sequencing run, as a function of the number of sequenced reads, using a random read subsample (n = 3,000,000) and the CalcUniqueness option in the BBTOOLS package (sourceforge.net/projects/bbmap/; Bushnell et al., 2017). In the following step, we removed from the initial pre-processed data all reads <35 bp using the SeqKit tool and then aligned reads ≥35 bp to the human full genome (build Hg19, Rosenbloom et al., 2015) and the human mtDNA reference genome rCRS (Andrews et al., 1999) using Bowtie2 (v1.2.1.1) and the 'end-to-end' parameter (Langmead & Salzberg, 2012). To deduplicate the mapped reads, we used the DeDup tool v0.12.8 (https://github.com/apeltzer/DeDup). The minimum mapping and base quality were both 30. The resulting bam files were used to check for characteristic aDNA nucleotide misincorporation frequency patterns using DamageProfiler v1.1 (Neukamm et al., 2021). The sex of the individual was assigned using a maximum likelihood method, based on the karyotype frequency of the mapped human X and Y chromosomal reads (Skoglund et al., 2013). In samples with sufficiently high mitochondrial read numbers, the rate of human contamination was estimated using Schmutzi v1.5.1 (Renaud et al., 2015). Variants in the mitochondrial genomes were called using SAMtools mpileup and bcftoools (Li et al., 2009) with stringent filtering options (quality > 30). The haplogroups were identified by submitting the variant calling files to the HaploGrep 2.0 website (Weissensteiner et al., 2016). To make sure that the comparison between the two methods (LPA and Silica) was not biased by the variable sequencing depth between the samples and sample replicates, we performed once more the same human analysis as outlined above on samples that displayed the presence of endogenous human DNA with previously deduplicated and subsampled datasets (Table S6). For this, we removed read duplicates from the datasets and subsampled all datasets to 3,607,686 randomly selected reads using the SeqKit and the Seqtk tools, respectively.

To compare the samples based on the overall microbial composition, we used DIAMOND (v2.0.7) blastx search (Buchfink et al., 2021) to check the deduplicated subsampled reads against the NCBI nr database (Release 237, April 2020). Then, we used MEGAN6 software (Huson et al., 2016) to assign the reads to the

lowest common ancestor (LCA) and calculate the Shannon and Inverse Simpson (InvSimpson) diversity indices (Bağcı et al., 2021). Statistical tests were performed using the R package 'STATS' (https://cran.r-project.org/) and visualised with the 'GGPLOT2' (https://ggplot2.tidyverse.org/).

3 | RESULTS

3.1 | DNA precipitation and purification using LPA in an aqueous ethanol solution

Our initial precipitation test with a DNA ladder revealed that even in the absence of cations (salts) using an aqueous ethanol solution only, LPA still efficiently precipitates DNA (Figure 2a). In contrast to classical precipitation methods using isopropanol or 0.3 M sodium acetate and 100% ethanol, LPA in aqueous 70% 'salt-free' ethanol solution showed a higher efficiency in precipitating DNA oligonucleotides as small as 35 bp.

When we further applied this novel precipitation method to environmental DNA extracts, we could additionally show that this minor change in the LPA precipitation protocol, using an aqueous ethanol solution without the addition of chaotropic salts, not only helps to efficiently recover DNA, but seems to have also major purifying effects on the DNA extracts. Fen soil DNA that was obtained by a classical P/C/I/PEG extraction method initially contained high concentration of brownish humic acid substances that inhibited further PCR-based downstream molecular analysis (Figure 2b, Figure S2). After DNA precipitation of the fen soil DNA extract using LPA in aqueous 70% 'salt-free' ethanol, we could not only recover the high molecular weight DNA but in parallel also efficiently remove most brownish humic acid substances (Figure 2b). Importantly, our novel LPA precipitation method successfully purified replicated fen soil DNA extracts from PCR inhibitory substances and allowed further downstream molecular analysis (Figure S2). We observed the same DNA purifying effect of LPA with replicated DNA extracts of bovine faeces samples that initially showed PCR inhibition after silica-based DNA extraction EDTA-Silica-a (Figure S3), where the inhibitory substances were successfully removed with an additional LPA precipitation/purification step.

Next, we tested whether the novel LPA method could be also implemented in classic nucleic acid extraction protocols as a final precipitation/purification step. For this, we submitted activated sludge samples from a wastewater treatment plant in Germany to both a classical P/C/I/PEG extraction protocol (Griffiths et al., 2000) and a modified version of this method, where we replaced the polyethylene glycol 8000–1.6 M NaCl precipitation and 70% ethanol washing steps with the 'one-step' LPA precipitation/purification in aqueous 70% 'salt-free' ethanol solution. The newly implemented LPA precipitation step resulted in highly pure high-molecular weight DNA that showed less PCR inhibition than the DNA extracts of the classical PEG-based methods (Figure S4).

Based on these first promising results that we obtained with the 'one-step' LPA precipitation/purification method in difficult environmental samples, we further applied our novel method on ancient human remains and compared the result with DNA extracts from other already existing nucleic acid precipitation and purification protocols. Thereby, we subjected the lung tissue of an 18th- to 19th-century human church mummy from Piraino, Italy, to a P/C/I extraction protocol combined with different nucleic acid precipitation and purification steps. All previously published methods using the precipitation agents isopropanol, 0.3 M sodium acetate with 100% ethanol and 0.3 M sodium acetate with 100% ethanol, followed by purification using spermidine and 30% polyethylene glycol 8000 with 1.6 M NaCl, co-precipitated in this sample substantial amounts of brownish coloured decomposition products that inhibited the subsequent PCR assay (Figure 2c).

Only the 'one-step' LPA precipitation/purification in aqueous 70% 'salt-free' ethanol solution resulted in a clear nucleic acid solution that showed no PCR inhibition. The enhanced capacity of LPA to precipitate and purify nucleic acids in aqueous salt-free ethanol solution could be additionally demonstrated on other mummified human remains including visceral tissue from an embalming jar (1292), a bone sample of a human salt mummy (1152), a rib bone sample of an Egyptian human mummy (854) and the Iceman's stomach content (1054). All those samples initially showed PCR inhibition after classical organic (P/C/I/PEG, CTAB--C/I/EtOHAc) or solid-phase (EDTA-Silica-a) nucleic acid extraction (Figure S5, Tables S1 and S2). Using the LPA precipitation/purification step, we could successfully submit the previously inhibited DNA extracts from all human mummified tissues in this study to molecular downstream processes.

To provide a first understanding for the purifying effect of LPA on nucleic acid extracts containing PCR inhibitors (e.g. humic acids), we tested different nucleic acid precipitation/purifications methods on a DNA solution containing the acidic azo dye Ponceau S (Figure S6). Ponceau S acts in concentrations higher than 0.1% w/v as a PCR inhibitor. Interestingly, similar to what we observed with the 18th- to 19th-century Italian lung tissue, all previously published precipitation/purification methods co-precipitate substantial amounts of the reddish coloured artificial PCR inhibitor, whereas the novel LPA precipitation/purification step purifies the DNA solution from the low molecular weight acidic azo dye Ponceau S.

3.2 | DNA composition after LPA precipitation/purification

To systematically assess the influence of the novel LPA method on the DNA quantity, quality and overall composition (human DNA content and microbial diversity), we compared the shotgun sequencing results of four ancient human specimens (two bone tissue and two soft tissue samples) and one environmental fen soil sample (Table S1, Figures S1 and S7) after triplicated nucleic acid extraction using (a)

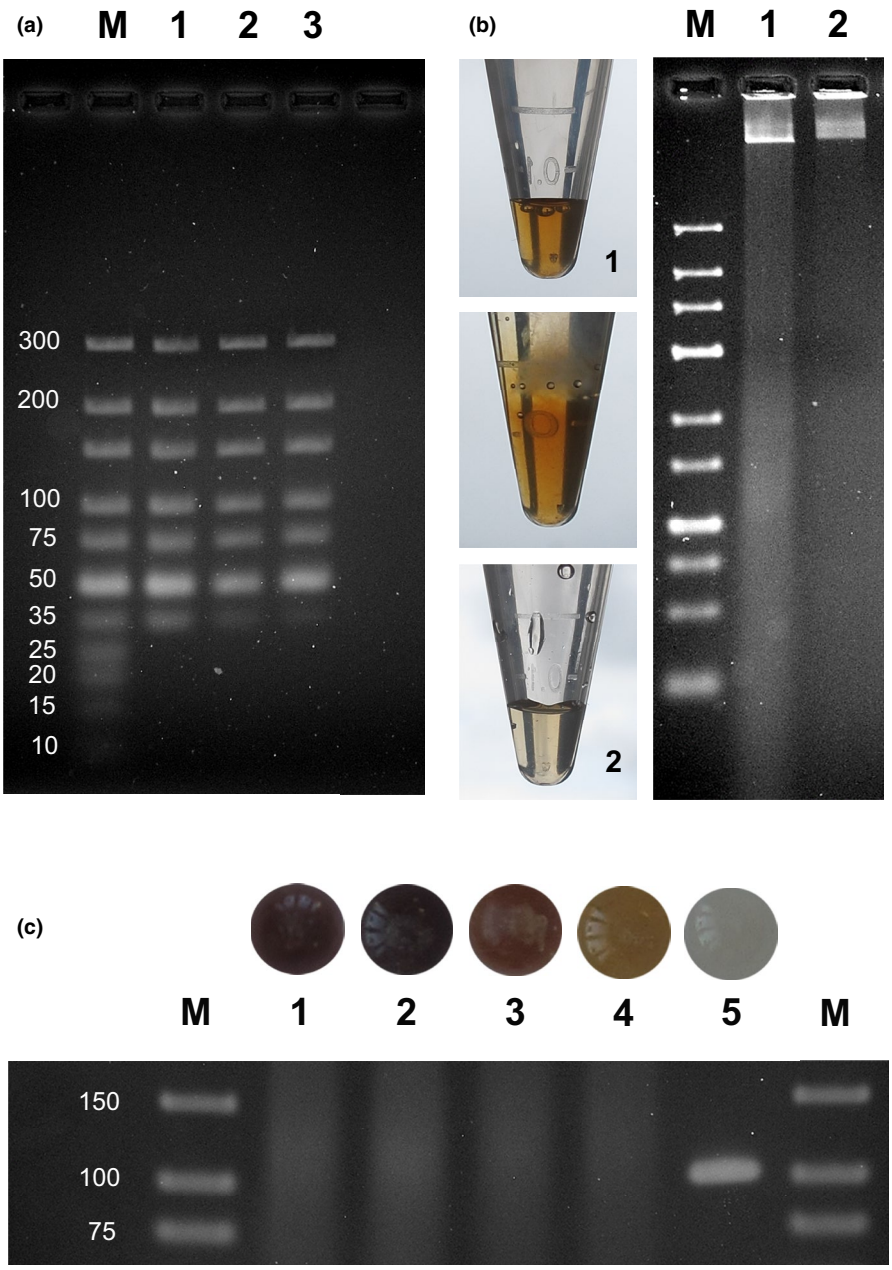

FIGURE 2 Capacity of linear polyacrylamide to precipitate and purify nucleic acids in aqueous salt-free ethanol solution. (a) Precipitation of 0.5 μg DNA ladder (M) using linear polyacrylamide (LPA) and 70% ethanol (1), 0.3 M sodium acetate and 100% ethanol (2), or isopropanol (3). (b) Fen soil nucleic acid extracts obtained with the P/C/I/PEG extraction method before (1) and after (2) precipitation/purification with LPA. The DNA Ladder (M) consists of DNA fragments ranging from 25 to 700 bp (please refer to Figure S3b for more details on the marker). The reaction tube in the middle shows the white LPA cloud after addition of ice-cold (−20°C) 70% ethanol and before the centrifugation step (c) Comparison of nucleic acid extracts obtained from an 18th- to 19th-century lung tissue using the P/C/I extraction method in combination with different precipitation and purification steps: isopropanol (1), 0.3 M sodium acetate and 100% ethanol (2), precipitated with 0.3 M sodium acetate and 100% ethanol and purified with spermidine (3), 30% polyethylene glycol 8000–1.6 M NaCl (4), LPA and 70% ethanol (5). The dots display the colour of the nucleic acid extracts after the different precipitation and purification steps. All nucleic acid extracts were tested for molecular inhibition using a PCR assay targeting a short fragment of the hypervariable region 1 in the human mitochondrial genome

our modified chloroform–isoamyl alcohol extraction protocol with the implemented LPA precipitation/purification step (CTAB-C/I/LPA, EDTA- C/I/LPA) and (b) the current most widely used silica-based extraction method (EDTA-Silica-b) for ancient and sedimentary DNA (Rohland et al., 2018).

Generally, both methods revealed highly reproducible results in our replicated approach (Figures 3–5). The DNA yield in the LPA method was approximately 4–10 times higher in nearly all samples in comparison to the silica extracts, except for one tissue sample (2653), which revealed comparable DNA concentrations in both extraction methods (Figure 3a, Table S4). After transforming the DNA extracts into Illumina libraries using the same input volume (10 μl) and indexing PCR conditions (14 cycles), there were no more major differences observable in the DNA libraries concentrations of both methods (Table S4). Shotgun sequencing of the Illumina libraries resulted in 4,228,392–30,003,133 pre-processed reads in almost all sample replicates (Table S5). Two replicates showed no (2653-S3) or only minute amount of data (2634-SL1a) after sequencing and were therefore excluded from the subsequent analysis. Both methods displayed a highly similar read length distribution profile in all samples, indicating a comparable good performance in precipitating/binding highly fragmented DNA present in the specimens (Figure 3b). The LPA extracts of the Neolithic petrous bone sample (2053) contained slightly more highly fragmented DNA (<40 bp) in comparison to the silica extracts. The opposite effect can be seen in the fen soil sample (3039) where the silica extract is shifted towards fragments below 40 bp. In this environmental sample, however, we submitted the high molecular weight DNA to a fragmentation step using ultrasonication, which could explain the observed differences. Next, we assessed the library complexity of the replicated

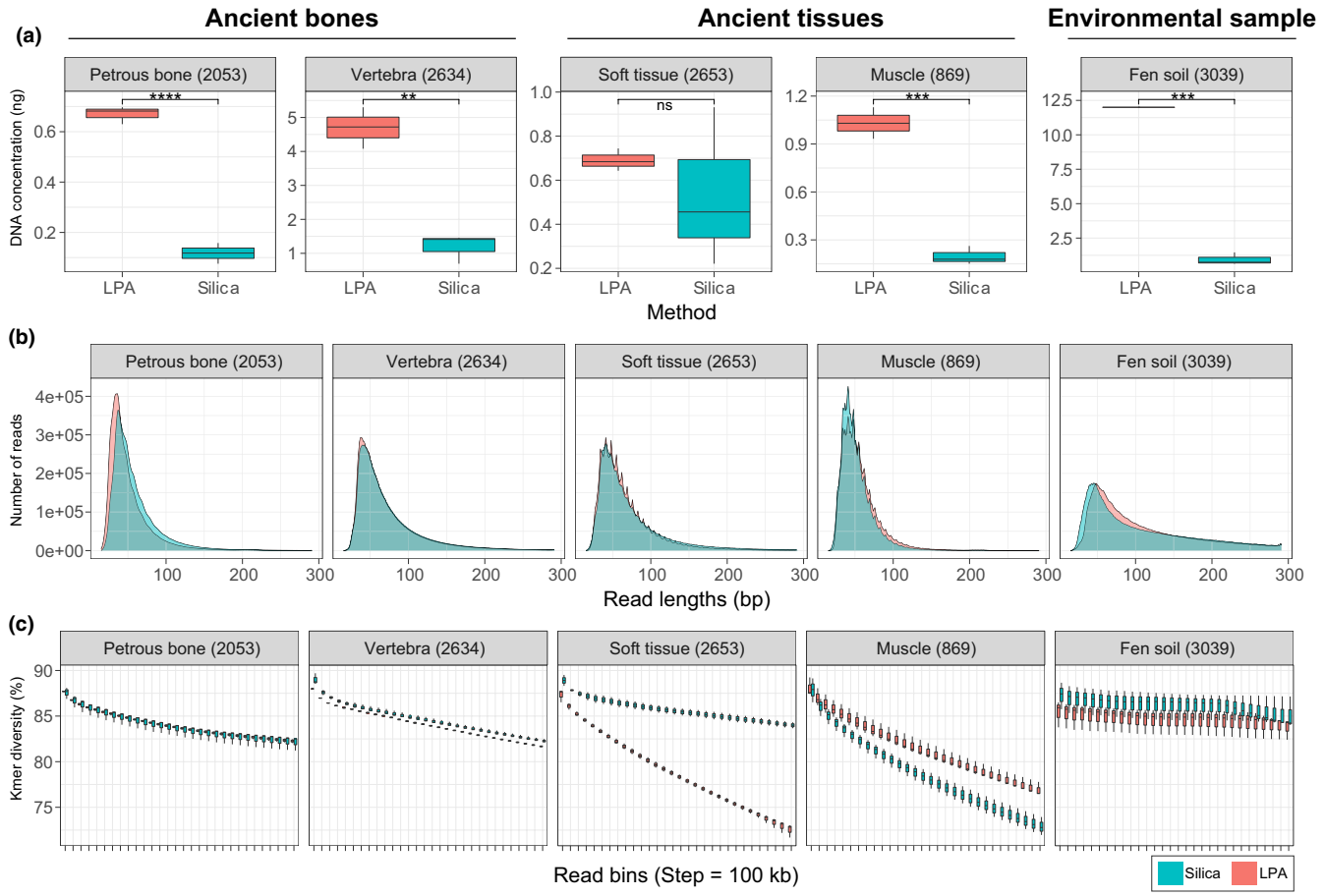

FIGURE 3 Basic characteristics of the nucleic acid extracts and the shotgun sequencing data of the five samples after replicated extraction using both methods (linear polyacrylamide, Silica). (a) Box plots showing the DNA concentration of the replicated DNA extracts (ng/µl). *p*-values are indicated on the top of each pair based on homoscedastic Student's *t* test ($^{ns}p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$). (b) Density plot showing the read fragment length distribution in base pairs (bp). (c) Library complexity displayed as fraction of unique reads (kmers = 25 bp) produced by a sequencing run, as a function of the number of reads sequenced (subsampled to 3,000,000 reads), where the *x*-axis represents the cumulative number of reads with a moving step of 100 kb. The boxplots represent three replicates ($n = 3$). 2053: temporal bone, petrous part, Hungarian skeleton, 5th millennium BC; 2634: vertebra bone, Bolivian mummy, 11th–14th century AD; 2653: soft tissue, Bolivian mummy; 869: muscle tissue, Egyptian mummy, 2nd century BC; 3039: fen soil, Germany. For further details on the samples, please refer to Table S1

shotgun datasets by plotting the fraction of unique reads (kmers), produced by a sequencing run, as a function of the number of reads sequenced (Figure 3c). These 'inverse' rarefaction curves show highly similar library complexities in both the LPA and silica extracts of the bone tissues and the fen soil sample. Interestingly, the tissue samples had one sample with a higher library complexity in the silica extracts (sample 2653) and one in the LPA extracts (sample 869). In contrast to the bone and environmental samples, these two soft tissue samples were treated with different lysis buffers (EDTA- and CTAB-based) in the two extraction methods (Table S1, Figure S7), which could explain the observed differences. After this first assessment of the general characteristics of the shotgun datasets, we next analysed in more detail the endogenous human DNA in the metagenomes. Alignment of the metagenomic reads to the human genome (hg19) revealed that three of the four human specimens contained sufficient human endogenous DNA for further comparative sequence analysis (Table S5). The muscle tissue (869) of the Egyptian mummy contained only minute amounts of human DNA and was therefore excluded from the subsequent analysis. The retrieved human sequence reads of the bone (2053, 2634) and soft (2653) tissues were highly fragmented and displayed an increased C to T misincorporation pattern at the 5′end, indicative for aDNA (Orlando et al., 2021). Low contamination rates of the mitochondrial DNA obtained with the software Schmutzi further supported the authenticity of the ancient human DNA (Table S5). The only exception was the silica extracts of the soft tissue sample 2653, where the human reads showed very low DNA damage and a higher mitochondrial contamination rate (Figure S8), possibly due to a contamination with modern human DNA. Since both extraction (LPA and silica) blanks were free of human DNA, this contamination most likely occurred during the sampling and handling of this specific sample for the silica extraction. The authentic ancient human DNA enabled us to further molecularly sex the individuals and to assign specific mitochondrial haplogroups

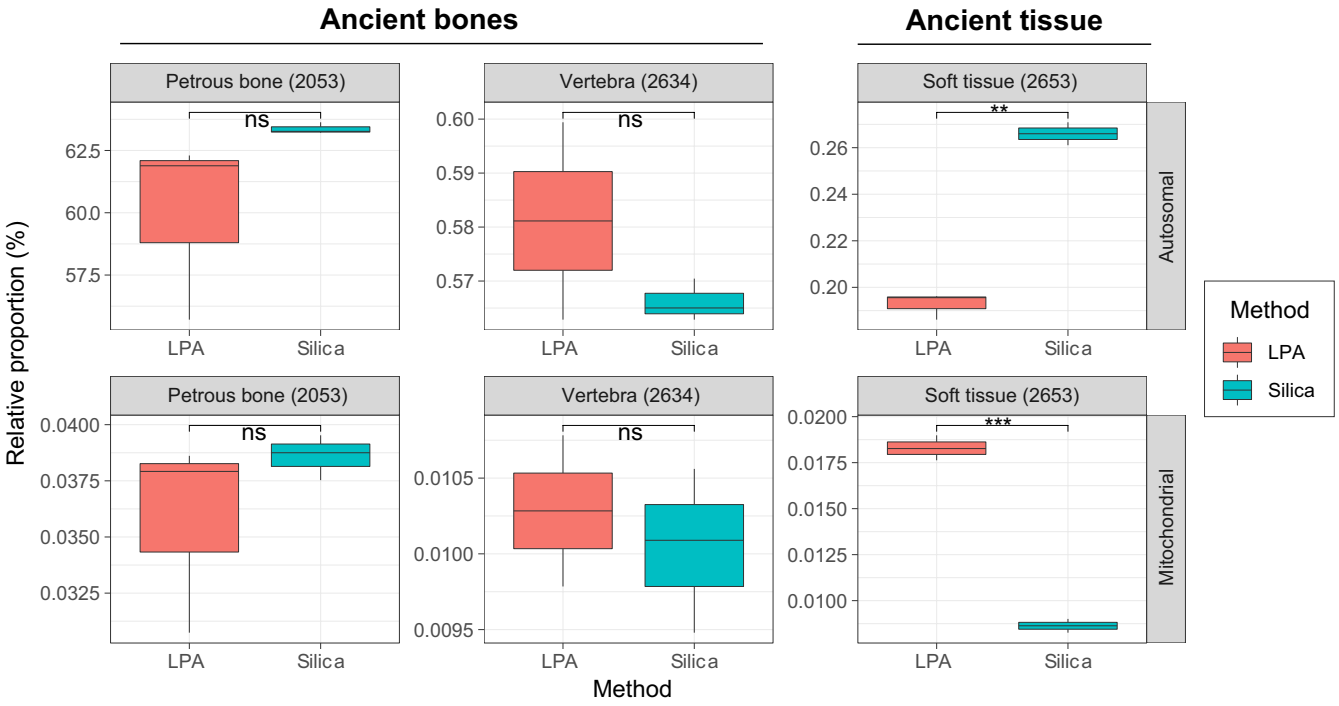

FIGURE 4 Boxplots displaying the relative proportion (%) of human autosomal and mitochondrial sequence reads in three samples containing ancient human endogenous DNA. *p*-values are indicated on the top of each pair based on homoscedastic Student's *t* test. For details of the *p*-values and the samples, please refer to the caption of Figure 3

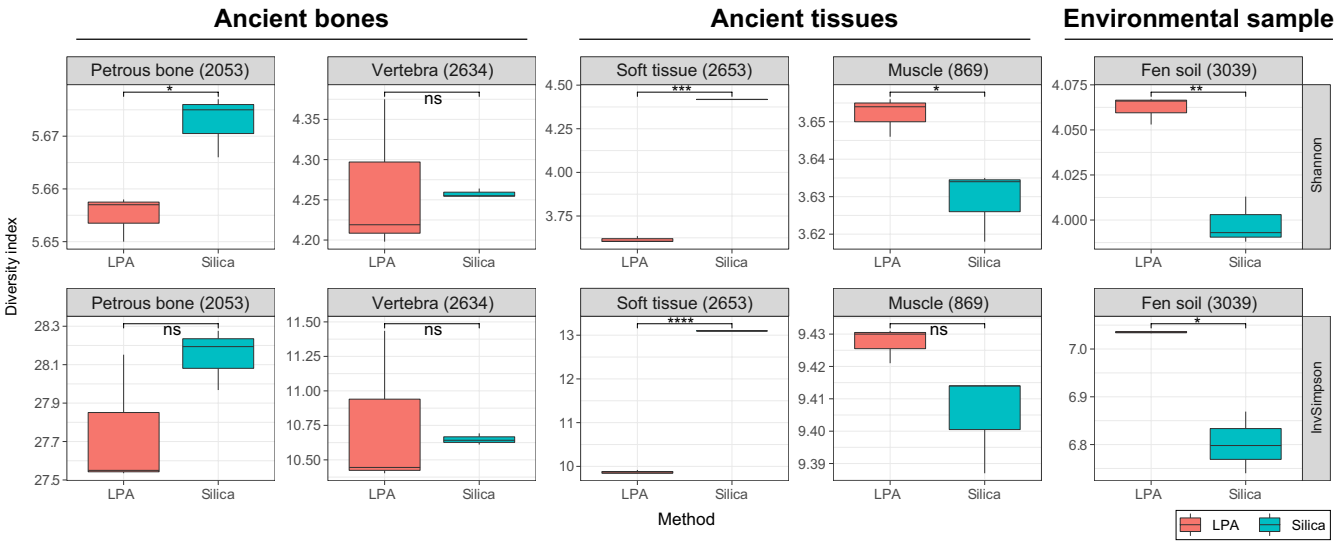

FIGURE 5 Boxplots displaying the alpha diversity of the microbial communities in the samples after replicated linear polyacrylamide and silica extraction. Both Shannon and InvSimpson diversity indices are provided. *p*-values are indicated on the top of each pair based on homoscedastic Student's *t* test. For details of the *p*-values and the samples, please refer to the caption of Figure 3

to each specimen. Thereby, we obtained an additional proof for the authentic nature of this material, since we could assign both Bolivian samples (2634, 2653) to mitochondrial haplogroups (A2, B2) that belong to the four main pan-American mtDNA lineages (Achilli et al., 2008; Bodner et al., 2012; Perego et al., 2010; Tamm et al., 2007). Furthermore, both haplogroups have been detected in previous studies in ancient human remains from Meso- and South America (Fehren-Schmitz et al., 2015; Posth et al., 2018).

The three samples with authenticated human endogenous DNA were further subjected to a systematic comparison of the human autosomal and mitochondrial DNA content (Figure 4, Table S6). Therefore, the filtered and normalised shotgun reads (≥35 bp, de-duplicated, subsampled) were once more aligned against the human autosomal genome (hg19 without the mitochondrial genome) and against the mitochondrial genome (rCRS). In both cases, the bone tissue samples (2053, 2634) revealed the LPA and the silica DNA

extraction were comparable to relative proportions of the human autosomal and mitochondrial DNA. In contrast to the bone tissue, the soft tissue of the Bolivian mummy (2653) displayed significant differences in the endogenous human DNA content in the two extraction methods. Whereas the silica extracts contained more autosomal DNA, the LPA extracts were higher in mitochondrial DNA content. However, it is important here to underline that the soft tissue samples were subjected to different lysis buffer treatments in the two extraction methods, and that the silica extracts of this sample show indications for a modern human DNA contamination (see above).

Finally, we wanted to evaluate whether the two extraction methods influence the DNA-based assessment of microbial community structures. Therefore, we assessed the microbial alpha diversity (Shannon and InvSimpson) in the subsampled metagenomic datasets (Figure 5). For the bone samples, the alpha diversity was similar among the different extraction methods, except for sample 2053, which showed a higher Shannon alpha diversity in the silica extracts. Tissue sample 2653 displayed a higher alpha diversity for both indices in the silica extracts, whereas tissue 869 had a higher Shannon alpha diversity in the LPA extracts. In the environmental soil sample 3039, both alpha diversity indices were higher in the LPA extracts.

4 | DISCUSSION

Nucleic acid extraction in complex environmental and ancient tissue material is often linked to an increased co-extraction of inhibitory substances. This co-extraction occurs in both solid-phase and liquid-phase/organic nucleic acid extraction protocols (Baar et al., 2011; de Bruijn, 2011; Dong et al., 2006; King et al., 2009; Matheson et al., 2010; Rohland et al., 2018; Scholz et al., 1998). Widely used measures to overcome the inhibitory effect of these substances include the addition of BSA or the increase in concentration of the polymerase enzyme in the PCR (Hedman & Rådström, 2013; King et al., 2009; Kreader, 1996; Sidstedt et al., 2020; Sutlović et al., 2005). The BSA effect, however, appears to be sample specific and only helps to reduce the inhibitory effect of certain substances (Opel et al., 2010; Rohland & Hofreiter, 2007; Schrader et al., 2012). A more general method to overcome inhibition is the dilution of the extracted nucleic acid, which results in a reduction of PCR inhibitors (King et al., 2009; Schneider et al., 2009). Excessive nucleic acid dilution, however, may lead to a change in the overall DNA composition and complexity (Castle et al., 2018; Wang et al., 2017).

In this study, we introduced a novel method with linear polyacrylamide, which efficiently precipitates and purifies nucleic acids from complex environmental and ancient tissue samples in one working step. Previous protocols used LPA as a neutral carrier in combination with classic alcohol/salt-based precipitation methods to recover minute amounts of DNA (Gaillard & Strauss, 1990; Green & Sambrook, 2016). We demonstrated that LPA precipitates nucleic acids even in aqueous ethanol solution without the addition of chaotropic salts (cations). Our small, yet important change in the precipitation protocol resulted in the recovery of highly pure DNA that showed no inhibition in contrast to DNA extracts obtained by previously published extraction and precipitation protocols. This DNA-purifying effect could be observed in all environmental samples (fen soil, activated sludge and bovine faeces) and ancient human bone and soft tissues. The challenging mummified specimens of different origin and from different times (Neolithic to Modern Period) included historic lung tissues, visceral tissue from a Medici embalming jar, a rib bone of an Egyptian mummy that was soaked with bitumen material, a bone sample of a human mummy from the Chehrābād salt mine and the Iceman's stomach content. In general, human mummified soft tissue is considered to be a particularly challenging material known for the highly fragmented, low concentrated DNA and the presence of decomposition products that inhibit further molecular downstream processes (Aufderheide, 2003; Neukamm et al., 2020; Pääbo, 1989; Scholz et al., 1998; Schuenemann et al., 2017). Our results indicate that the LPA method not only successfully removes these decomposition products, but also other inhibitors such as salts in high concentrations (salt mummy) or yet unknown compounds (Iceman stomach) that prevented up to now all further molecular analysis. Currently, we regularly use the novel LPA precipitation/purification step in our laboratory implemented in a chloroform–isoamyl alcohol extraction protocol modified from Tang et al. (2008) (Supplementary Methods). We effectively applied this new protocol on different mummified human and animal remains (e.g. soft tissue, palaeofaeces, intestinal contents, leather artefacts), which allowed us to retrieve important new information on ancient specimens that is best exemplified by the molecular reconstruction of the Iceman's *Helicobacter pylori* (Maixner et al., 2016) and his last meal (Maixner et al., 2018) from the stomach content.

Importantly, our method resulted in comparable DNA quality and overall composition (human DNA content and microbial diversity) when we systematically confronted metagenomic datasets of four ancient human specimens (two bone tissue and two soft tissue samples) and one environmental fen soil sample after triplicated nucleic acid extraction using (a) the LPA method and (b) the current most widely used silica-based extraction method for ancient and sedimentary DNA (Rohland et al., 2018). Even though the LPA extraction resulted in higher DNA yields in comparison to the silica method, these higher concentrations seem to have no effect on the library preparation and the overall DNA composition. We assume that this could be due to depletion of all reagents during the library preparation protocol step, for example, adapter ligation, which might have led to an equalisation/normalisation. Both methods revealed highly similar DNA fragment length distribution profiles and library complexities. The normalised replicated data allowed further in-depth analysis of the endogenous human DNA content in three samples. Following the ancient human DNA authentication of Orlando et al. (2021), damage pattern analysis, contamination checks and mitochondrial haplogroup assignment supported the authenticity of the ancient human DNA in these samples. Both LPA and the silica DNA extraction revealed comparable relative proportions of the human autosomal and mitochondrial DNA. Hence, the LPA method

could display an interesting alternative to silica-based protocols in the aDNA field, which had been optimised for the recovery of short DNA fragments but occasionally co-extract inhibitory substances (Glocke & Meyer, 2017; Rohland et al., 2018). Nonetheless, it remains to be determined whether this efficient recovery of endogenous DNA using LPA is generally evident for all ancient specimens. Further comparative analysis of the metagenomic data of the ancient bone tissue samples revealed high similarity in the overall microbial taxonomic composition between the two extraction protocols (LPA vs. Silica). Variations in the microbial diversity of the soft tissue samples may be explained by the application of different lysis buffers. Lastly, we also tested the influence of the LPA precipitation/purification step on the recovery of high-molecular-weight DNA in a modern environmental sample by subjecting the fen soil DNA before library preparation to a fragmentation step using ultrasonication (Head et al., 2014). Thereby, the LPA method efficiently recovered a more diverse microbial community composition in comparison to the silica extracts.

Overall, samples treated with different lysis buffers (EDTA- and CTAB-based) in the two extraction methods show slight differences in the DNA composition (human DNA content and microbial diversity). To better understand the effect of the lysis step on the recovery of high-quality DNA, we suggest future comparative metagenomic studies on ancient and environmental samples using LPA in combination with different lysis buffers and digestion times. Previous studies already discovered a considerable impact of the lysis step on the recovery of ancient endogenous DNA (Boessenkool et al., 2017; Damgaard et al., 2015) and the overall microbial composition in modern environmental samples (Fidler et al., 2020). In addition, we recommend comparative nucleic acid extraction studies with mock microbial communities helping to further assess the capacity of the novel LPA method (Highlander, 2013; Sui et al., 2020).

To understand the observed purifying effect of LPA when precipitating nucleic acids in the aqueous salt-free ethanol solution, we have to consider first the principle behind nucleic acid precipitation using alcohol. In brief, nucleic acids can be 'salted-out' and concentrated from aqueous solutions by adding ethanol and cations (Green & Sambrook, 2016; Li et al., 2020). In aqueous solution, highly polar nucleic acids are surrounded by a hydration shell of water molecules. By adding alcohol, the hydration shell becomes disrupted and the free phosphate residues form ionic bonds with cations, which results in the precipitation of nucleic acids. This 'salting-out' effect only occurs if the cations are present in sufficient quantity to neutralise the charge on the phosphate residues. In general, alcohol precipitation of nucleic acids requires the presence of at least 0.1 M monovalent cations in the initial aqueous solution (Dowhan, 2012). Since common PCR inhibitors, like humic acids, have physical and chemical properties similar to those of nucleic acids, they become co-precipitated in this widely used precipitation protocol (Schrader et al., 2012). By adding the LPA and aqueous ethanol only to the nucleic acid solution, we overcame such limitation of the classical precipitation approach (Figure 1). Without the addition of salt, LPA still precipitates in the aqueous ethanol solution while most of the inhibitors stay in the solution (Figure 2b). The nucleic acids, however, appear to be specifically pulled down by the precipitated LPA.

The most likely explanation for this effect is that the nucleic acids become at least partially precipitated in the aqueous ethanol solution and then get carried along with the long-chained LPA molecules during centrifugation. Simultaneously, we think that the purifying effect of this novel method is due to both a reduced 'salting-out' effect of inhibitors in aqueous ethanol solution and a size exclusion by LPA during centrifugation. The latter size exclusion effect would also explain the absence of DNA oligonucleotides smaller than 35 bp when we precipitated a DNA ladder with LPA (Figure 2a). The original publication of Gaillard and Strauss already reported this size exclusion effect of LPA on DNA oligonucleotides below 20 bp (Gaillard & Strauss, 1990). If we now consider a cut-off value of 35 bp oligonucleotides, this will correspond to the size exclusion of all molecules with a molecular weight (Mw) below 21 kDa. This would explain the purifying effect of LPA we observed on DNA solutions containing the acidic azo dye Ponceau S with a Mw of 0.76 kDa (Figure S6). Furthermore, it could be the main reason for the removal of humic and fulvic acids from nucleic acid solutions, since the Mw of these inhibitory compounds ranges between 4 and 30 kDa with a mean Mw below 19.2 kDa (Perminova et al., 2003). Hence, the purifying effect of LPA when precipitating nucleic acids in aqueous salt-free ethanol solution is most likely due to the size exclusion of inhibitory substances with a molecular weight below 21 kDa.

In summary, and compared with the current established ancient and environmental DNA purification methods, our innovative method offers the following advantages: (a) exclusion of post-extraction inhibitory substances by combining the nucleic acid precipitation and washing step, thereby overcoming of the co-precipitation of inhibitors in difficult environmental and ancient human samples; (b) economic feasibility, that is, no need for additional disposable materials, for example, silica columns, collections funnels or Amicon filters; and finally (c) ease of handling and suitability for low-tech laboratories. Additionally, the LPA precipitation/purification can be easily applied as an additional post-extraction step to nucleic acid extracts that display PCR inhibition; alternatively, classic precipitation/purification steps in liquid-phase/organic nucleic acid extraction protocols can be replaced by it. Therefore, our LPA method with its high purifying capacity provides an important alternative to the commonly used silica-based nucleic acid extraction protocols in the environmental and aDNA field.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHORS' CONTRIBUTIONS

F.M., C.M. and A.Z. conceived the ideas and designed the methodology; F.M., C.M., H.Y.J., M.S.S., S.L., D.P.-M., I.S., E.M., G.P., I.P., G.C. and G.V. collected the data; F.M., C.M., H.Y.J., M.S.S., S.L., D.P.-M., I.S., E.M., G.P., I.P. and G.C. analysed the data; F.M., H.Y.J., M.S.S. and A.Z. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Sequencing data are available at the European Nucleotide Archive (ENA) under ENA: PRJEB45999.

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