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Anionic nanocellulose as competing agent in microbial DNA extraction from mine process samples

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ABSTRACT

Microorganisms in flotation and minerals processing may significantly affect the grade and yield of metal concentrates. However, studying the phenomena requires working techniques to detach microorganisms and their DNA from mineral particles to which they strongly adhere. We developed a new method utilizing the competitive properties of anionic nanocellulose to block sorption of DNA to and detach microbial cells from mineral particles from ore processing. In general, up to one ng DNA mL^{-1} sample was obtained with the custom anionic nanocellulose method (CM) compared to DNA amounts below the Qubit assay's detection limit for extractions with a commercial kit (KIT). Similarly, 0.5–4 orders of magnitude more bacterial 16S and fungal 5.8S rRNA gene copies were detected by qPCR from CM treated samples compared to KIT extractions. A clear difference in the detected microbial community structure between CM and KIT extracted samples was also observed. Commercial kits optimized for mineral soils are easy to use and time efficient but may miss a considerable part of the microbial communities. A competing agent such as anionic nanocellulose may decrease the interaction between microorganisms or their DNA and minerals and provide a comprehensive view into the microbial communities in mineral processing environments.

1. Introduction

Microorganisms in mineral processing may have great consequences for e.g. the grade and yield of metals in concentrates from flotation (De Mesquita et al., 2003; Mhonde et al., 2020; Evdokimova et al., 2012). However, studying microbial communities in these types of mineral and metal-rich materials is challenging, because microorganisms and nucleic acids may attach strongly to minerals and with different strength depending on mineral type and environmental conditions, e.g. (Miettinen et al., 2021; Direito et al., 2012; Cao et al., 2011; Tan and Chen, 2012).

Microorganisms may adhere to mineral particles in several ways, such as through hydrophobic interactions or electrostatic attraction (i.e. surface charge) with the solid surface (Yee et al., 2000) and with substances such as extracellular polymeric substances (EPS) (Hong et al., 2013). Yee et al. (2000) showed with pure cultured *Bacillus subtilis* that adsorption of the bacterial cells to corundum increased with decreasing pH and was lowest at alkaline pH, whereas adsorption to quartz to which the bacterium generally showed lesser affinity, was not greatly affected by pH. Hong et al. (2013) showed also with *B. subtilis* that absence of EPS did not affect the adhesion to clay particles but enhanced the adhesion to goethite by increasing chemical interactions. The adhesion of typical bioleaching bacterial species, i.e. *Acidithiobacillus ferrooxidans*, *At. thiooxidans* and *Leptospirillum ferrooxidans* to chalcopyrite decreased significantly when the EPS was removed (Zhu et al., 2012). These bacteria also showed different adhesion levels to pyrite, chalcopyrite and quartzite depending on the growth conditions, with the lowest affinity to attach to quartzite and other typical gangue minerals (Africa et al., 2013).

Detachment of microbial cells from mineral soil and minerals processing tailings is a continued challenge. Methods including pH adjustments of the samples, use of detergents, sonication, density gradient centrifugation, dilution, redox adjustments, ionic strength adjustments, competitive compounds, such as BSA, skim milk, nucleotides etc. have been developed and tested, but in general with poor results for mining environment samples, e.g. (Direito et al., 2012; Yee et al., 2000; Ehlers et al., 2008; Le et al., 2020). In addition, protocols using metal chelating agents, such as EDTA have been designed (e.g. Nkuna et al., 2022; Krsek and Wellington, 1999; Ettenauer et al., 2012), but EDTA may have a negative effect on downstream nucleic acid based assays. Release or

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separation of nucleic acids from mineral samples has also been tested by different means, such as using ethanol in the lysis buffer for the purpose of precipitating the nucleic acids leading to conformational changes to the secondary structure of the nucleic acid and to decrease the effect of water (Direito et al., 2012). In addition, phenol:chloroform:isoamyl alcohol extractions have been used in many protocols to separate the DNA from the minerals by forcing the DNA into the aqueous phase (Le et al., 2020; Zeng et al., 2008). However, the success of the chosen protocol depends on the physicochemical characteristics of the sample, such as pH, acidity, salinity, and metal content, as well as on the type of minerals in the samples (Direito et al., 2012; Mojarro et al., 2017; Morris Jr, 2014; Sagova-Mareckova et al., 2008).

In this study, a novel DNA extraction method aimed for mine process samples containing high concentrations of different metal containing minerals was developed. The method is based on using a competing substance that binds to the positive charges on the mineral surfaces and thus preventing DNA from lysed cells to adsorb to the minerals. Water, mineral and tailings samples from the mineral processing plants from two different mines were tested. We compared the DNA extraction efficiency of the custom method (CM) to that of a universally used DNA extraction kit designed for soil samples (KIT), estimated the extracted DNA amounts with fluorometer as well as the abundance of microorganisms in the samples by qPCR and characterized the microbial communities by amplicon sequencing.

2. Materials and methods

2.1. Samples

The samples used in this study originated from two different mines. Mine 1 is situated in the south of Europe in Portugal (Miettinen et al., 2021) and Mine 2 in the subarctic region of Finland (Bomberg et al., 2020). Process water was collected on site into sterile plastic bottles (Nalgene, Rochester, NY, USA) and was filtered on site onto 0.2 μm pore-size Sterivex™ filtration units using sterile 60 mL disposable syringes. The Zn Rougher Feed, Zn RZ Tailings, Cu Rougher Feed and Cu RC Final Tailings samples were collected in clean plastic buckets and filtered on Sterivex™ units using a peristaltic pump (Miettinen et al., 2021) on site. The Cu Tailings, Sulfur Tailings, Zn Rougher Feed, Zn RZ Tailings, Cu Rougher Feed and Cu RC Final Tailings slurry samples were collected on site directly into 250 mL or 1000 mL sterile plastic Nalgene bottles and frozen directly after sample collection. All samples were transported frozen to the laboratory. The physicochemical properties of the samples are presented in Table 1.

2.2. DNA extraction with kit

Previously (Le et al., 2020) we tested four different commercial DNA extraction kits, i.e. the Macherey-Nagel NucleoSpin Soil DNA extraction kit with lysis buffers SL1 and SL2, and NucleoBond RNA soil + DNA kit, MoBio Laboratories Inc. PowerMax Soil DNA kit, Zymo Research Corp. ZR SoilMicrobiome DNA Midiprep, designed for soil samples on samples Zn Rougher Feed (Mine 1) and Cu Tailings 2 (Mine 2). The test resulted in very poor DNA amounts with all kits and variations of the extraction buffers. For the present work the NucleoSpin Soil DNA extraction kit (KIT) (Macherey-Nagel GmbH and Co, KG, Düren, Germany) was chosen to represent the commercial kits as it is widely used, has good modification properties provided in the kit (two different lysis buffers, SL1 and SL2 and the possibility to use or not to use enhancer solution SX), has good scalability properties (includes adequate amounts of buffers to allow for larger extraction volumes) and is the most affordable of the kits tested in [4]. Altogether, 10 different samples were tested with three replicate DNA extractions (Table 2). The samples collected from 250 to 600 ml of water (Table 2) on 0.22 μm pore-size Sterivex™ polyethersulfone (PES) filters (Merck, Burlington, MA, USA) were aseptically opened in a laminar flow hood and the membrane cut out with sterile scalpels and tweezers and inserted in to 5 mL Eppendorf tubes before DNA extraction. The slurry samples were thawed at +4 °C, thoroughly mixed and 5 mL pipetted into a 5 mL Eppendorf tube, centrifuged at 3184 xg for 5 min in an Eppendorf 5810R benchtop centrifuge

Table 1
The physico-chemical composition of the samples used in this study. nd = not determined, bdl = below detection limit. The data is compiled from Miettinen et al., 2021; Miettinen et al., 2023; and Bomberg et al., 2023.

	unit	Zn Rougher Feed	Zn RZ Tailings	Cu Rougher Feed	Cu RC Final Tailings	Process water	Cu Tailings 1	Cu Tailings 2	Sulfur Tailings 1	Sulfur Tailings 2	Sulfur Tailings 3	Method
pH		6.8	6.7	8.8	9.4	7.5	9.9	9.7	9.7	9.3	7.1	
ORP	mV/SHE	259	276	141	133	104	169	202	202	225	123	
SP Cond.	mS/cm	4.6	6.3	4.4	5.2	2.2	2.7	2.6	2.7	2.6	2.2	
T	°C	33.3	33	30.6	32.2	17	12.3	23	12.4	23	19	
TDS	g/L	6.3	77.3	5.8	5.9	nd	2.2	2.1	2.0	2.0	nd	Evaporation, 105 °C
Sulfate	mg/L	2744	2892	2498	2872	480	530	600	620	660	410	
Thiosulfate	mg/L	22	13	414	472	12	73	58	70	44	38	
Stot	mg/L	1675	1739	1233	1571	180	210	310	280	300	190	ICP-MS
Cl	mg/L	1225	1169	1137	1412	360	640	570	580	520	360	
Na	mg/L	970	100	929	973	210	350	260	270	240	200	ICP-MS
K	mg/L	102	7.2	100	101	49	66	54	57	52	57	ICP-MS
Ca	mg/L	644	6.5	743	914	130	180	150	160	140	110	ICP-MS
Mg	mg/L	109	0.8	31	8.3	63	61	54	74	61	61	ICP-MS
P	μg/L	<500	560	<500	890	64	150	160	110	130	59	ICP-MS
Cu	μg/L	<100	<100	<100	<100	<100	<3.0	<3.0	<3.0	<3.0	<100	ICP-MS
Fe	μg/L	130	1300	2300	360	25	<50	<50	270	<50	bdl	ICP-MS
Zn	μg/L	50	930	8000	450	nd	<5.0	<5.0	<5.0	6.8	nd	ICP-MS

Table 2
Sample sizes and DNA amounts of the different sample types extracted in this study. The biomass collection method is indicated by asterixes * and ** in the Sample size column. Each DNA extraction was done in three replicates.

Sample	Sample size	DNA extracted ng/mL	Total DNA ng	DNA conc. ng/mL sample	Sample size	DNA extracted ng/mL	Total DNA ng	DNA conc. ng/mL sample
Zn Rougher Feed	500 mL water*	<0.5	0	0	32.5 mL slurry**	135–184	13.5–18.4	0.42–0.57
Zn RZ Tailings	500 mL water*	<0.5	0	0	32.5 mL slurry**	<0.5–126	0–12.6	0–0.39
Cu Rougher Feed	600 mL water*	<0.5	0	0	32.5 mL slurry**	128–154	12.8–15.4	0.40–0.47
Cu RC Final Tailings	600 mL water*	<0.5	0	0	32.5 mL slurry**	<0.5–137	0–13.7	0–0.42
Process water	250 mL water*	724–1280	72.4–128	0.29–0.51	17 mL water**	248–268	24.8–26.8	1.46–1.58
Cu Tailings 1	5 mL slurry**	<0.5	0	0	32.5 mL slurry**	194–232	19.4–23.2	0.60–0.71
Cu Tailings 2	5 mL slurry**	<0.5	0	0	27 mL slurry**	212–228	21.2–22.8	0.79–0.84
Sulfur Tailings 1	5 mL slurry**	<0.5	0	0	32.5 mL slurry**	184–233	18.4–23.3	0.57–0.72
Sulfur Tailings 2	5 mL slurry**	<0.5	0	0	32.5 mL slurry**	154–230	15.4–23.0	0.47–0.71
Sulfur Tailings 3	250 mL water*	<0.5	0	0	17 mL water**	149–177	14.9–17.7	0.88–1.04
Negative DNA extraction controls		<0.5	0	0		<0.5–86.4	0–68.8	

* collected on 0.2 μm pore-size Sterivex filters.
** Centrifugation.

(Eppendorf, Hamburg, Germany), supernatant discarded, and the pelleted solids were used for DNA extraction. The DNA extractions were performed according to the manufacturer's protocol with the exception that the lysis was done in 5 mL Eppendorf tubes with double amounts of lysis buffer SL1 and enhancer solution and the beads from one bead tube. The DNA extractions proceeded as previously described (Miettinen et al., 2021; Bomberg et al., 2020). The samples were vortexed horizontally at full speed using a Genie v2 vortexed for 5 min, followed by a centrifugation at 3184 x*g* for 5 min in an Eppendorf 5810R benchtop centrifuge, whereafter the supernatant was collected and used for the proceeding DNA extraction according to the manufacturer's instructions. The DNA was eluted in 100 μL elution buffer SE. The extracted amount of DNA was measured using a Qubit 2.0 Fluorometer (Fisher Scientific, Loughborough, UK) with the High Sensitivity kit for double stranded DNA.

2.3. DNA extraction with the custom method

A custom DNA extraction protocol (CM) was developed to be used in parallel with the KIT. The method is based on using anionic nanocellulose as a competitive substance to compete with microorganisms and nucleic acids for the positive charges on mineral particles and possibly also cause the microorganisms/nucleic acids to detach from the surfaces. Frozen samples were thawed at +4 °C, mixed well and triplicate 17–32.5 mL aliquots (Table 2, Fig. 1) were collected into sterile 50 mL screw capped plastic test tubes (Corning, New York, USA). Samples were centrifuged 15,000 x*g* for 20 min in a Multifuge X3 FR centrifuge (Thermo Scientific, Waltham, Massachusetts, United States) and the supernatant was removed. The pellet was dissolved in lysis buffer (0.5 M EDTA (Sigma-Aldrich) in 10 mM PBS with 140 mM NaCl, 2.7 mM KCl, 0.05% Tween20 (PBS-Tween tablets, Medicago AB, Uppsala, Sweden) with final concentration of Tween20 (Sigma-Aldrich, Saint Louis, MO, USA) 0.4% and pH 7.8 at a ratio of 1:1 volume pellet:buffer, sterile acid washed bead beating beads (150–212 μm, Sigma-Aldrich, Saint Louis, MO, USA) was added at a 0.25:1 volume ratio beads:pellet, and autoclaved 121 °C, 20 min, sterile anionic nanocellulose was added at 10% of the final volume (1.1% dry solids content). The anionic nanocellulose was prepared according as described (Skogberg et al., 2017). Shortly, the raw material was bleached and never dried softwood kraft pulp, which was chemically pre-treated before fibrillation (Fig. 1). The anionic pulp was produced using 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) mediated oxidation according to (Saito et al., 2006). Some 150 g of oxidized pulp was soaked at 1.5% solids and dispersed using a high-shear Ystral X50/10 Dispermix (Ystral GmbH, Ballrechten-Dottingen, Germany) mixer for 10 min at 2000 rpm. The pulp suspension was then fibrillated twice with the microfluidizer type MF7115–30 (Microfluidics International Corporation, Westwood, MA) at 1800 bar pressure. The equipment was equipped with 400 μm and 100 μm processing chambers. After the first pass the gel was further diluted to 1% consistency before the second cycle. The final product had a final dry material content of 1.1% and a charge value of 1.3 mmol g^{-1} dry pulp.

The samples with nanocellulose were homogenized using the FastPrep (MP Biomedicals, Solon, OH, USA) bead beater, using three cycles consisting of 45 s at 1600 rpm. Thereafter, 300 μl of lysozyme (Sigma-Aldrich, Saint Louis, MO, USA, 10 mg mL^{-1}) and 100 μl of proteinase K (Qiagen, Hilden Germany, 20 mg L^{-1}) were added to the extraction reactions, which were then incubated for one hour at 37 °C followed by one hour at 50 °C. Sterile filtered 10% sodium dodecyl sulfate (SDS, Sigma-Aldrich, Saint Louis, MO, USA) was added to the extractions to a final concentration of 0.03% followed by an additional incubation for 2 h at 65 °C. After the incubation, the samples were centrifuged at 10000 x*g* for 5 min, and the supernatant was collected and stored for further processing. The remaining pellet was frozen overnight, thawed and submitted to re-extraction using the same volume of lysis buffer as the remaining pellet, nanocellulose was added equal to 10% of the pellet-lysis buffer suspension volume and SDS to a final concentration of 0.03%. The reactions were thoroughly mixed by vortexing until homogenous and incubated for 2 h at 65 °C. After the incubation the reactions were centrifuged at 10000 x*g* for 5 min, and the supernatant was collected and combined with the earlier supernatant. Phenol:chlroform: isoamyl alcohol (25:24:1, Sigma-Aldrich, Saint Louis, MO, USA) was added to the supernatant at 1:1 ratio and the reactions were gently mixed in a fume hood. The samples were centrifuged for 15 min, 15,000 x g whereafter the aqueous phase was collected and extracted with chloroform:isoamyl alcohol (24:1, Sigma-Aldrich, Saint Louis, MO, USA) at a ratio of 1:1. After gentle mixing, the tubes were centrifugated for 15 min, 15,000 x g, and the aqueous phase was collected. DNA was precipitated by adding a 0.7 volume of isopropanol (Merck KGaA,

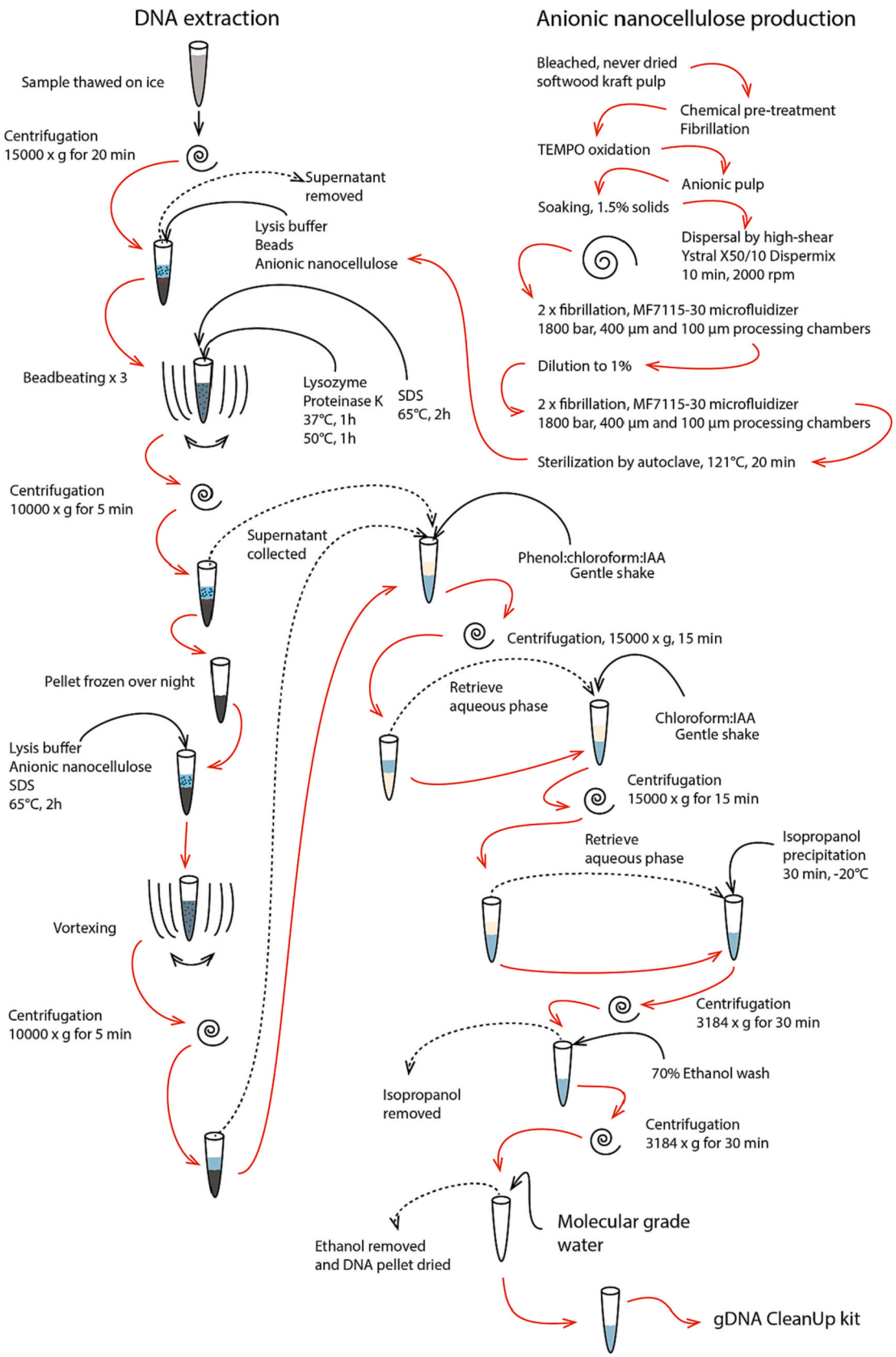

Fig. 1. The schematic procedure of the custom method (CM) DNA extraction and anionic nanocellulose production. Red arrows indicate flow direction, black arrows input to and dashed arrows extraction from sample tubes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Darmstadt, Germany) to the samples, followed by thorough mixing and incubation for 30 min at −20 °C. The precipitated DNA was then pelleted by centrifugation for 30 min at 3184 xg in an Eppendorf 5810R benchtop centrifuge and the supernatant was removed. The pellet was washed with 70% ethanol, centrifuged as above, the ethanol was removed and the pellet was air dried in a laminar flow hood for a minimum of 10 min. The DNA was dissolved in 100 μl of molecular grade water, further purified with the gDNA CleanUp kit (Macherey-Nagel GmbH and Co, KG, Düren, Germany) and finally eluted in 2 × 50 μl elution buffer EB. The extracted amount of DNA was measured using a Qubit 2.0 Fluorometer with the HS kit for double stranded DNA using a total of 5 μl DNA extract for each measurement.

2.4. Quantitative PCR

The amounts of bacteria and fungi in the samples were estimated using qPCR. Bacterial 16S rRNA genes were targeted using the primers Bact_341F and Bact_805R (Herlemann et al., 2011) using the SensiFAST™ Real-Time PCR Kit (Bioline, London, UK). The fungi were detected using a TaqMan probe-based method with primers 5.8F1 and 5.8R1 and the FAM-labeled probe 5.8P1 specific for the fungal 5.8S rRNA gene (Haugland and Vesper, 2002). The probe-based qPCR assay was performed using the SensiFAST Probe No-ROX kit (Bioline, London, UK).

All qPCR amplification reactions were done in triplicates in 10 μL reaction volumes in 1× master mix using 1 μL template or standard DNA per reaction. The bacterial reaction mixtures contained 0.25 μM of each primer, whereas the fungal assay was run with 0.5 μM of each primer and 0.2 μM probe. All qPCR runs contained negative control reactions, i.e. reactions prepared with PCR grade water instead of DNA. The qPCR assays have been thoroughly described previously (Miettinen et al., 2021; Bomberg et al., 2018). All qPCR was performed using a LightCycler LC480 (Roche, Basel, Switzerland).

2.5. Amplicon library sequencing

The bacterial and fungal communities in the samples were characterized with amplicon sequencing of the bacterial 16S rRNA gene and fungal ITS1 region. The bacterial were targeted with the Bact_341F/Bact_805R (Herlemann et al., 2011) primers, and fungi with primers ITS1 and ITS2 Gardes and Bruns, 1993; White et al., 1990). The primers were equipped with adapters for the Iontorrent PGM platform containing 9-nucleotides long barcodes (most samples) and prepared and sequenced on the Iontorrent PGM platform at Bioser (Oulu, Finland) as previously described (Miettinen et al., 2021; Bomberg et al., 2018).

In addition to the previous samples sequenced on the Iontorrent PGM platform, the new Process water and Sulfur Tailings 3 samples were prepared for in-house sequencing using the iSeq 100 platform (Illumina, San Diego, CA, USA). The same primers were used as described above using the same reaction conditions as for the Iontorrent amplicons and the procedure is described in detail in Appendix A. All sequence data has been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under Project accession number PRJEB61213.

2.6. Sequence analysis

The sequence data was analyzed using the mothur software version 1.43.0 (Schloss et al., 2009) and all commands in this section refer to mothur unless otherwise stated. The Iontorrent sequence data was first demultiplexed using the fastq.info command together with an oligos file. The sequences were quality filtered using the trim.seqs command allowing for a maximum of 2 nucleotide differences in the primer sequences and no differences in the barcode sequences, no ambiguous nucleotides, maximum length of homopolymer stretches of 8 nucleotides and minimum sequence length of 200 nt, qwindowaverage = 20 and qwindowsize = 40. The iSeq 100 paired end sequence data was quality trimmed using the make.contigs command with default settings. All sequence data was merged for downstream analysis. The whole sequence data was dereplicated using unique.seqs. The bacterial sequences were aligned to the silva 138 reference database (Quast et al., 2012; Yilmaz et al., 2014; Glöckner et al., 2017) that had first been optimized to cover only the effective area enclosed by the primers according to the recommendations by Werner et al. (2012). The aligned sequence data was screened (screen.seqs) to exclude sequence reads falling outside of the defined alignment area, whereafter the alignmnent was filtered (filter.seqs) to remove uninformative columns (i.e. without sequence data) throughout the alignment. The sequence data was again dereplicated (unique.seqs) before pre-clustering (pre.cluster). The bacterial sequence data was chimera checked using chimera.vsearch against itself and the chimeric sequences were removed. The remaining sequence reads were classified with classify.seqs against the optimized silva 138 database, after which sequence reads not belonging to the domain Bacteria were removed. A distance matrix was calculated in the bacterial sequences (dist.seqs) using a cutoff value of 0.03, whereafter the sequence reads were clustered into OTUs sharing a minimum of 97% sequence similarity using the cluster command.

The fungal ITS sequence data was not aligned, but proceeded directly to chimera screening (chimera.vsearch) after which sequence reads deemed chimeric were removed. The sequence reads were then classified with the classify.seqs command using the Unite version 8.2 ITS database covering all eukaryotes as reference (dated 2020-02-04, UNITEv8_sh_dynamic_s_all) (Kõljalg et al., 2020; Nilsson et al., 2019). Sequences in the fungal ITS1 data that were not identified as Fungi were removed whereafter the unaligned ITS1 sequence reads were clustered into OTUs with 97% sequence homology.

The bacterial and fungal OTUs were classified using the curated sequence read taxonomy and the data was transformed into a biom table, counts and taxonomy tables for further processing.

Alpha- and betadiversity calculations were performed with Phyloseq (McMurdie and Holmes, 2013) in R (R Computing, 2013), excluding contaminant OTUs based on sequences detected in the negative reagent controls and singleton OTUs. Principal coordinate analysis (PCoA) was calculated on relative abundances of OTUs in the different samples with Bray Curtis dissimilarity model using phyloseq in R. Ballonplots showing the relative abundances of the 40 genera with highest relative abundances in the samples were visualized using ggpubr (Kassambara and Kassambara, 2020) and ggplot2 (Villanueva and Chen, 2019) in R according to (https://github.com/alex-bagnoud/OTU-table-to-bubble-plot) (SupplementB.R). Statistical analysis (Mann-Whitney pairwise test) and boxplots were performed using PAST4 (Hammer et al., 2001).

3. Results

3.1. DNA extraction

The DNA amounts obtained from the samples using the commercial kit (KIT) were generally below the detection limit of the Qubit High Sensitivity assay in all samples except the Process water samples, where the amount of DNA obtained was up to 0.5 ng/mL original sample (Table 2). Nevertheless, the amount obtained from Process water using the custom method (CM) was threefold, up to 1.5 ng/mL original sample water. The other sample types consisting of slurry yielded DNA between 0 and 1.04 ng/mL original sample with most samples within a range of 0.3–0.8 ng DNA mL^{-1} original sample. However, the CM also showed some background DNA signal in the Qubit HS assay for some negative control extraction sets, and in one individual case the measured DNA amount was quite high compared to the samples.

3.2. Bacterial and fungal amounts

In all samples the bacterial 16S rRNA and fungal 5.8S rRNA gene

copy numbers detected from the CM samples were higher, even up to four orders of magnitude, compared to the KIT extracted counterparts (Figs. 2 and 3) apart from the Cu RC Final Tailings sample where neither method detected fungal genes. The number of bacterial 16S rRNA gene copies mL^{-1} sample was 0.02–2.7 × 10^3 in most KIT extracted samples, with the exception of the Process water with almost 10^6 16S rRNA gene copies mL^{-1} sample, and Cu Tailings 2 and Sulfur Tailings 2, where the number of bacterial 16S rRNA gene copies was below the detection limit of the assay. In contrast, the lowest number of bacterial 16S rRNA gene copies mL^{-1} detected in the CM samples was 6.0 × 10^2 from Cu Rougher Feed and the highest 2.5 × 10^5 and 1.3 × 10^6 copies mL^{-1} in Sulfur Tailings 3 and Process water, respectively.

The fungal 5.8S rRNA gene copy numbers detected with the KIT extracted DNA was between 0 and 1.5 × 10^1 copies mL^{-1}, whereas the numbers detected from the CM samples were 0.1–6.4 × 10^3 copies mL^{-1} with the highest number detected in Cu Tailings 1.

Generally, the number of bacterial 16S rRNA gene copies detected directly in the CM negative extraction control qPCR reactions was clearly lower than in the sample qPCR reactions. However, two negative controls had bacterial 16S rRNA gene copy numbers similar to some of the samples (Fig. 4). A negative control extraction was included in every DNA extraction event, and it was detected that negative controls were not related to a certain extraction event but they can be regarded as individuals. This is based on the finding that qPCR results of samples extracted in separate events were not affected, but were exactly at the same level, even though one of the negative controls in a specific extraction event was clearly contaminated. This highlights the importance of triplicate extractions for each sample in order to notice possible contamination due to the extraction process. In addition, in the melting curve analysis following the bacterial qPCR assay, the amplicon peaks in the negative controls had clearly lower melting temperature than the amplicons in the samples. In the case of fungal contamination, the detected number of 5.8S rRNA gene copies in the negative controls was clearly lower than that of the bacterial 16S rRNA gene copies and was mostly negligible (Fig. 4).

3.3. Sequence analysis

The number of bacterial sequence reads obtained from the KIT samples varied between 0 and 2555, with the highest number obtained from the Process water samples (Table 3, Fig. S1). With the CM samples, the number varied between 1 and 5467 reads, with the highest number obtained from Zn RZ Tailings. The average number of bacterial sequence reads from the KIT extracted samples was 564 (STD 781) and CM samples 1673 (1316). The average number of OTUs, Chao1 estimated number of OTUs and Shannon's diversity index was statistically significantly higher in the CM samples ($p < 0.0005$) compared to KIT extracted samples according to the Mann Whitney pairwise test (Table 3, Figs. S2-S4).

The Principal coordinates analysis (PCoA) showed that the bacterial communities differed between the DNA extraction protocols within sample type in all samples except Process water and Sulfur Tailings 3 (Fig. 5). In most sample types, the identified communities formed distinct groups based on the extraction method. Generally, when the DNA extraction was successful in a sample, the resulting diversity was wide. This shows as multitude of small spheres in the relative abundance images such as Fig. 6 but also in the Shannon's diversity index (Table 3). Another aspect of the same phenomenon was the low diversity in the Cu and Sulfur Tailings 2 KIT samples (Fig. 6), which was logic since these samples had very low bacterial 16S rRNA gene counts (Fig. 2). There was notably higher diversity in most CM samples compared to the KIT samples, especially in the Copper and Sulfur Tailings 1 and 2 (Fig. 6). The detected bacterial communities differed within sample types depending on which DNA extraction method was used, whereas the communities were very similar between replicate samples extracted with the same method (Fig. 6). When the relative abundance of a certain genus was high with either extraction method, the other method detected the same genus at least most of the time, but the relative abundances could be quite different. For example, there was a clearly higher relative abundance of unclassified Actinobacteria in samples Zn Rougher Feed, Zn RZ Tailings, Cu Rougher Feed, Cu RC Final Tailings and Cu Tailings 1 detected with the KIT method, compared to the CM. In addition, *Ralstonia* appeared more frequently in the Cu Tailings 2, S Tailings 1 and 2 when the KIT was used, compared with the CM. In contrast, e.g. Unclassified *Microbacterium*, *Sulfuricurvum*, *Thiobacillus, Thiovirga* and many other taxa were more frequently detected in the CM samples compared to the KIT samples. In general, a higher number of bacterial taxa were detected in the CM extracted samples compared to the KIT, with the exception of the Process water sample, where no great difference was seen between the DNA extraction methods verifying both extraction methods successful in this type of clean water sample with low amounts of solids.

The number of fungal ITS1 sequence reads obtained from the KIT samples was 0–25,768, with an average number of sequence reads 2099 (STD 5010) per sample, whereas the CM samples yielded 0–18,961 sequence reads, with an average of 2820 (STD 4136) reads per sample (Table 3, Fig. S5). The great standard deviation from the average with both extraction methods also reflects the great variation in sequence numbers obtained between samples. As with the bacterial communities, the average number of OTUs and Chao1 estimated number of OTUs

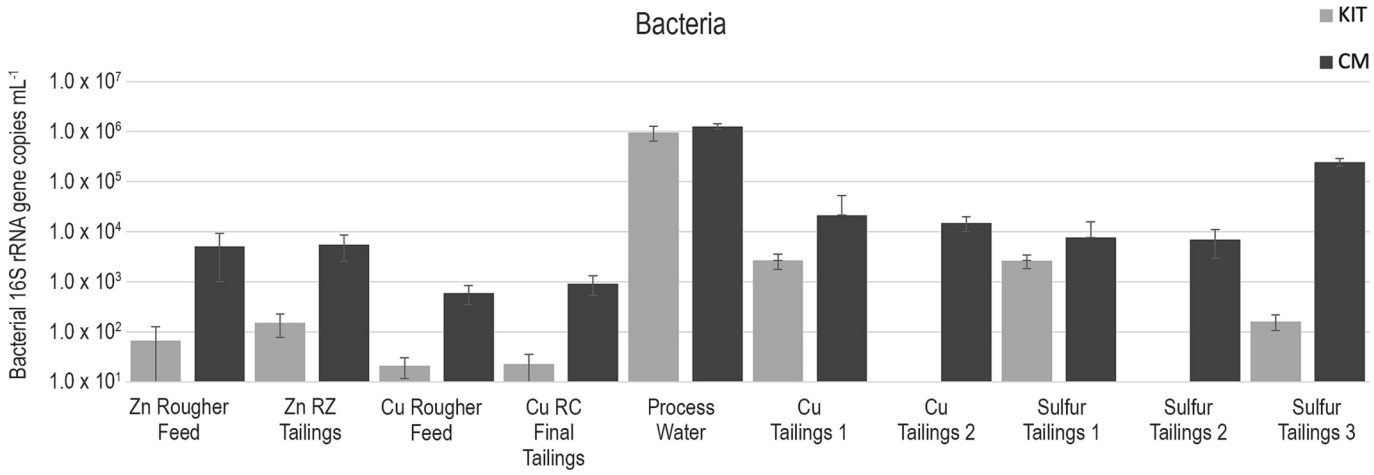

Fig. 2. Number of bacterial 16S rRNA gene copied mL^{-1} sample estimated by qPCR. Each column represents the average of three replicate samples run in three replicate reactions each, i.e. $n = 9$. The error bars represent standard deviation.

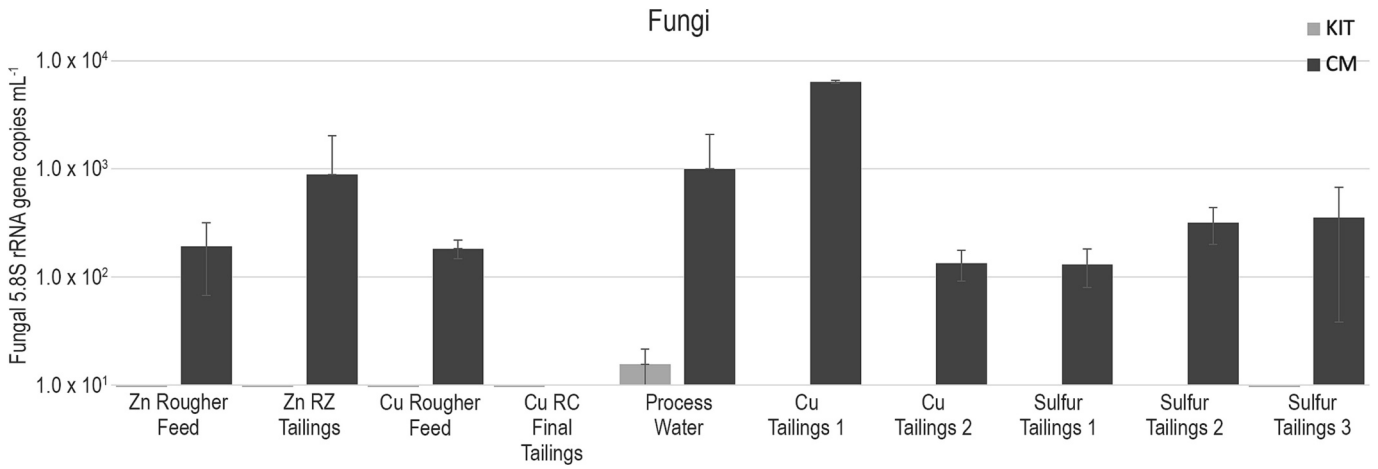

Fig. 3. Number of fungal 5.8S rRNA gene copied mL^{-1} sample estimated by qPCR. Each column represents the average of three replicate samples run in three replicate reactions each, i.e. $n = 9$. The error bars represent standard deviation.

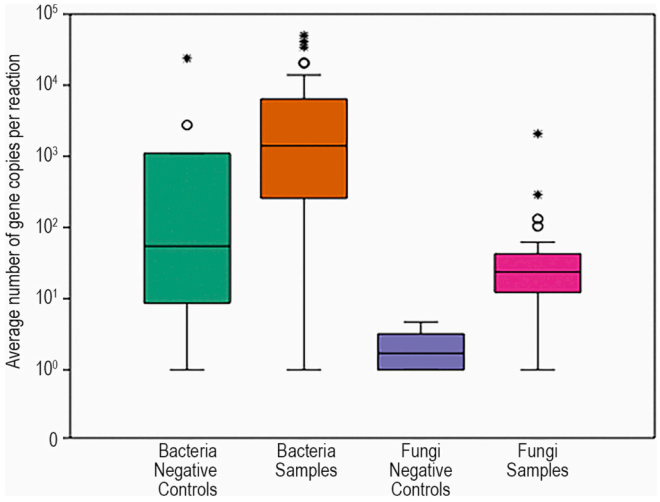

Fig. 4. Box plots indicating the minimum, maximum and median number of bacterial 16S and fungal 5.8S rRNA gene copies detected directly in the qPCR reactions and calculated from the average of three replicate qPCR reactions per sample. Open circles and stars indicate outlier samples falling >1.5 x and 3 x of the box length outside the minimum or maximum whiskers, respectively. A total of 10 negative control extractions and 30 individual sample extractions are included in the box plots.

Table 3
Average number of bacterial and fungal sequence reads, OTUs, Chao1 estimated number of sequence reads and Shannon's diversity index of the samples extracted with the commercial kit (KIT) and the custom DNA extraction method (CM). SDT stands for standard deviation.

		Sequence number	OTU number	Chao1	Shannon
Bacteria	KIT	592	47	82	2.22
	STD	760	48	100	0.87
	CM	1635	165	236	3.34
	STD	1308	95	114	0.93
Fungi	KIT	2099	37	48	1. 24
	STD	5011	40	50	0.90
	CM	2820	46	69	1.29
	STD	4136	54	92	0.77

identified was higher in the CM samples compared to the KIT extracted ones, but the difference was not as pronounced as for the bacteria (Table 3, Figs. S6 and S7). The average Shannon diversity index was only slightly higher in the CM vs. KIT samples (Table 3, Fig. S8). However, there was no statistically significant difference between the alphadiversity measurements from the KIT vs. CM samples.

In agreement with the bacterial data, the fungal communities also differed between DNA extraction method in almost all samples, with the exception of Sulfur Tailings 3, and mixed partly only in Process water and Sulfur Tailings 1 (Fig. 7). In all other sample types the communities identified from the KIT extracted samples were clearly different from the ones detected in the CM samples.

The fungal communities appeared very patchy and inconsistent with only *Malassezia* and unclassified Malasseziales occurring consistently in all KIT extracted Zn Rougher Feed, Zn RZ Tailings, Cu Rougher Feed and Cu RC Final Tailings samples, but only at very low relative abundance in the CM counterparts, if found at all (Fig. 8). Unclassified fungi were more commonly detected in some of the CM samples, but not exclusively so.

Bacterial 16S rRNA gene sequences were detected from all 10 CM negative control samples and fungal ITS1 sequence reads from four out of the 10 negative controls. The number of bacterial sequence reads varied between 28 and 5714, and fungal sequence reads between 870 and 19,405. In the KIT extraction controls, bacterial sequences were obtained from all 8 controls and fungal sequences from three out of 8 controls. The number of bacterial sequence reads obtained from the KIT controls varied between 3 and 4408 and the fungal sequence reads between 35 and 17,098. The taxonomy of the most prominent OTUs of the CM and KIT extraction controls differed from the OTUs identified from the samples and could readily be filtered from the data. Of the contaminants in the CM represented by on average at least 10 sequence reads in the controls, in total 12 bacterial OTUs, most belonged to *Pseudomonas* (Fig. 9A). In addition, *Janthinobacterium*, unclassified Sphingomonadaceae, *Burkholderia-Caballeronia-Paraburkholderia* cluster, *Delftia*, unclassified Oxalobacteriaceae and unclassified Actinobacteriaceae OTUs were among the 12 prominent OTUs in the CM controls. The most common fungal contaminant belonged to *Myceliophthora*, followed by *Penicillium*, *Phaeotheca*, unclassified Ascomycota, *Mycosphaerella*, *Trametes*, *Cadophora*, unclassified Sclerotiniaceae, *Cryptococcus*, *Resinicium* and unclassified Fungi (Fig. 9B).

In the KIT controls, the most prominent bacterial contaminants were unclassified Sphingomonadaceae, *Pseudomonas*, unclassified Ruminococcaceae, unclassified Bacillaceae, *Staphylococcus*, *Diaphorobacter*, *Limnohabitans*, *Methylobacterium-Methylorubrum* cluster, *Enhydrobacter*, *Burkholderia-Caballeronia-Paraburkholderia* cluster and *Polaromonas* (Fig. 9C), whereas the fungal contaminants belonged to unclassified Sclerotiniaceae and *Fusarium* (Fig. 9D). It should be noted that the genera *Pseudomonas* and *Polaromonas* were also detected from the

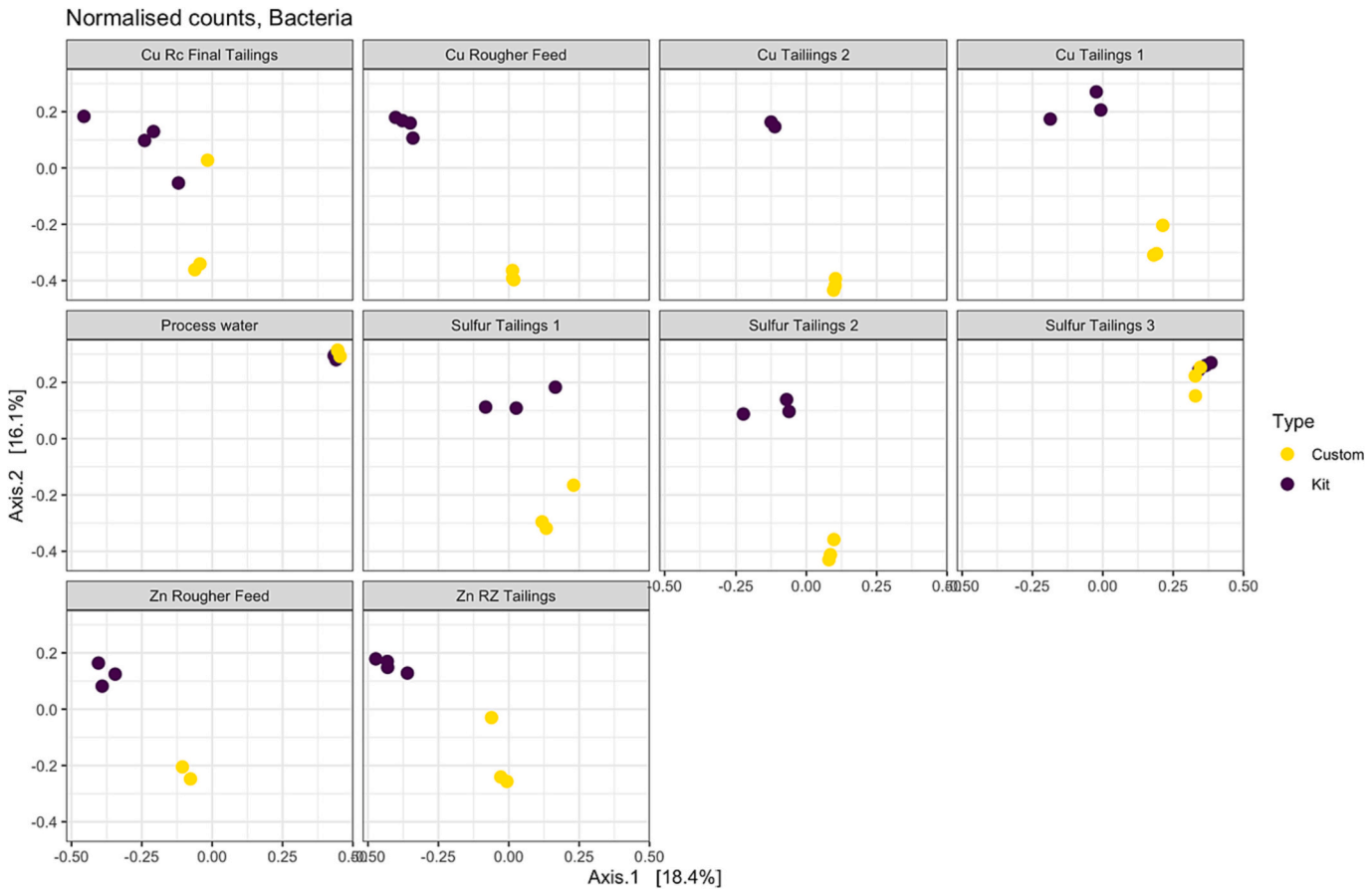

Fig. 5. Principal coordinates analysis of the bacterial communities identified in the different sample types. Yellow dots represent samples extracted with the custom DNA extraction method, black dots samples extracted with the kit. The X and Y axes describe percent variance between the different samples. The analysis was calculated using relative OTU abundance and Bray Curtis dissimilarity model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

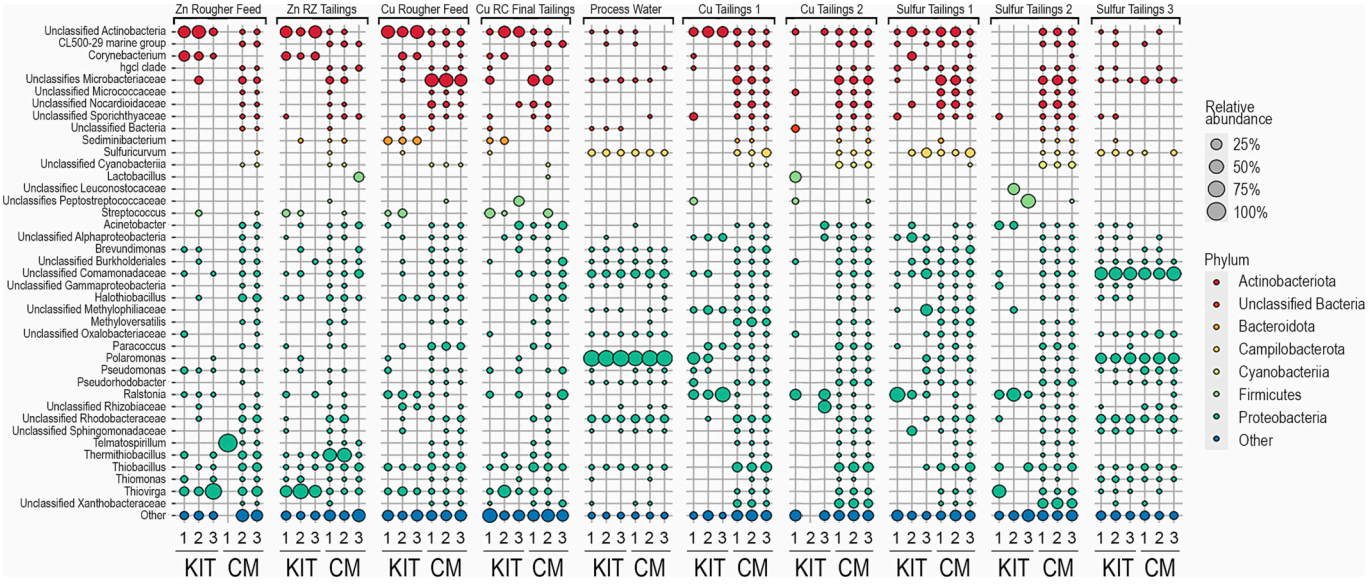

Fig. 6. The relative abundance of the 40 most prominent bacterial genera detected from the different sample types. The size of the spheres indicates relative abundance according to the legend to the right, and the colours indicate taxonomical affiliation on phylum level. KIT – DNA sample extracted with commercial kit, CM – extraction with custom method. Samples for which no sequence data was obtained are shown as empty columns in the graph.

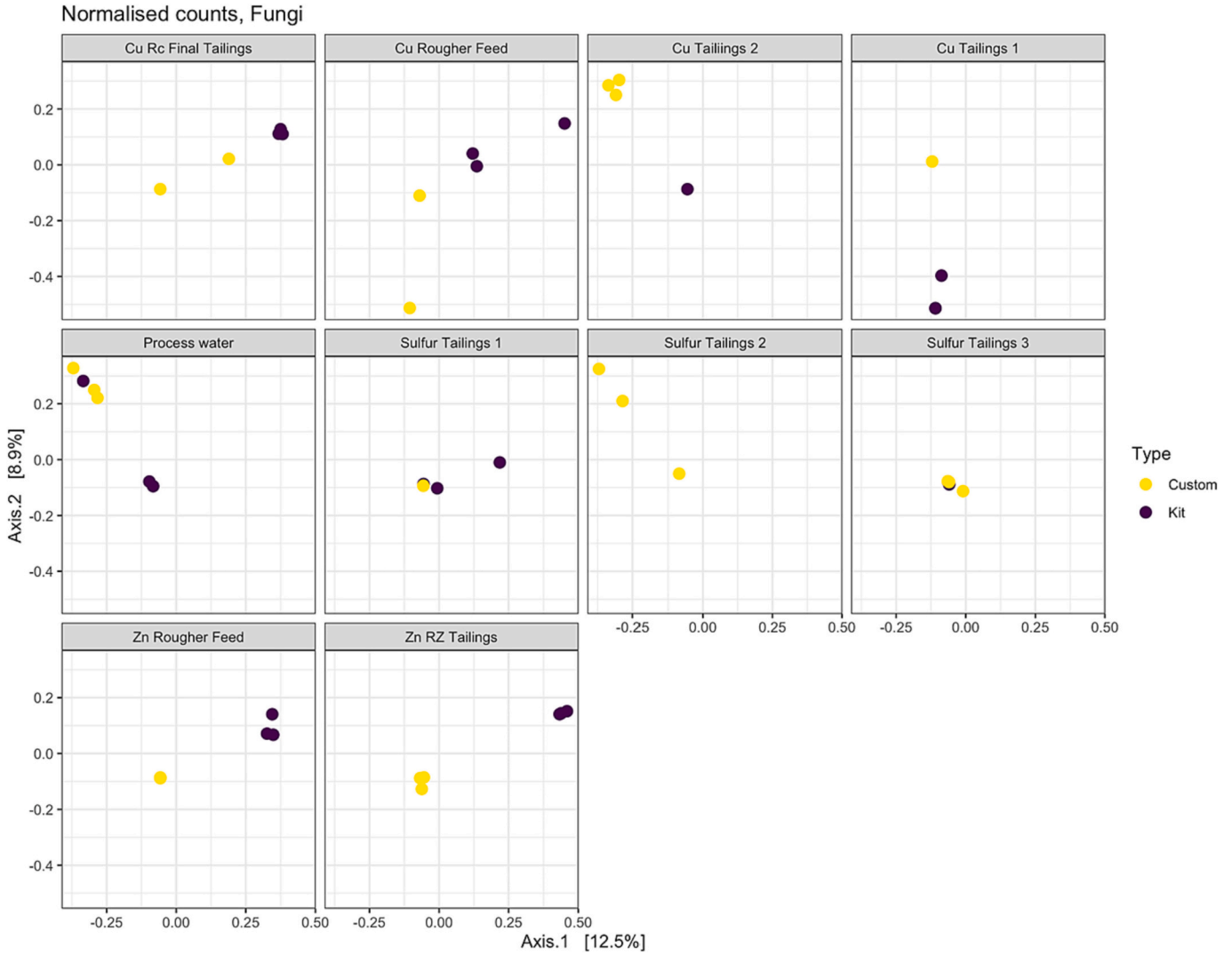

Fig. 7. Principal coordinates analysis of the fungal communities identified in the different sample types. Yellow dots represent samples extracted with the custom DNA extraction method, black dots samples extracted with the kit. The X and Y axes describe percent variance between the different samples. The analysis was calculated using relative OTU abundance and Bray Curtis dissimilarity model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples but were represented by different OTUs than in the negative controls and the clearly sample related OTUs were thus deemed to be part of the microbial communities in the samples, whereas the control related OTUs were removed. Likewise, *Myceliophtora*, *Trametes* and *Cadophora* were detected in the samples as well as in the negative DNA extraction controls but were represented by different OTUs in the samples and controls.

All contaminant OTUs were not present in all negative control extractions (Fig. 9), and in general, OTUs were not shared between the CM and KIT negative controls. The only exceptions were bacterial OTUs Sphingomonadaceae_unclassified_OTU3 and *Burkholderia-Caballeronia-Paraburkholderia*_OTU8, which were found in both CM and KIT controls. Of these Sphingomonadaceae_unclassified_OTU3 was also detected from 5 out of 7 PCR non-template controls as the most prominent PCR reagent contaminant, whereas the *Burkholderia-Caballeronia-Paraburkholderia*_OTU8 was not detected in any of the PCR non-template controls and may be a laboratory contaminant.

4. Discussion

Mine and mineral processing environments often contain quite large amounts of microorganisms when samples are examined by microscopy (Miettinen et al., 2021; Le et al., 2020; Kinnunen et al., 2020), or by plate counts (Levay et al., 2001), but are challenging to directly extract microbial cells or nucleic acids from, because both cells and nucleic acids adsorb strongly to the minerals. Here, we developed a protocol using anionic nanocellulose as a competitive compound to adsorb to the positive charges of the minerals, preventing released DNA to adsorb to and potentially also detach adsorbed microbial cells from mineral particles for more efficient DNA extraction from these samples. This is of importance when studying the effect of microorganisms on e.g., flotation, metals recovery efficiency and grade of metal concentrates. The knowledge of specific microorganism types and quantities present in each process step can assist in the process control, as various microbial species have been shown to e.g. depress different minerals in different ways, function as biocollectors for specific minerals, change surface chemical properties of minerals (reviewed by Kinnunen et al., 2020). In addition, microorganisms have profound effects on water fouling in the formation of biofilms and slimes, when water is being recycled in mines and mineral processing (Pihlajakuja et al., 2017; Ayache et al., 2013; Bereschenko et al., 2011). Studying the microbiology of these types of environments is essential, and thus successful recovery of indigenous

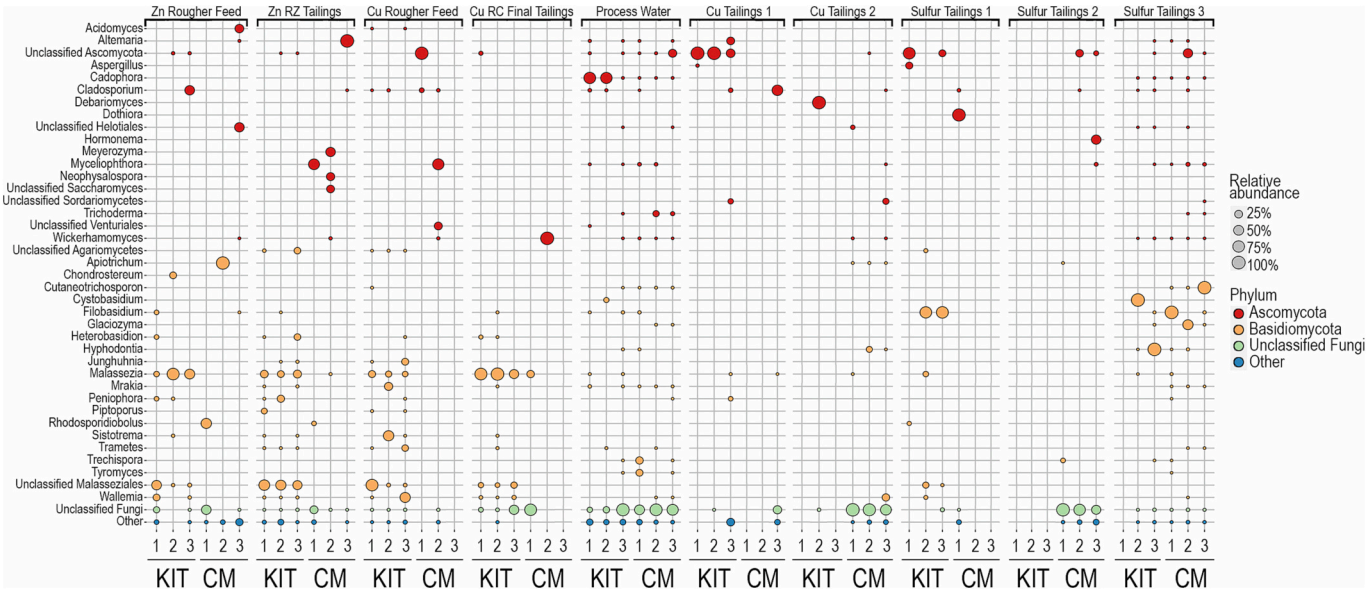

Fig. 8. The relative abundance of the 40 most prominent fungal genera detected from the different sample types. The size of the spheres indicates relative abundance according to the legend to the right, and the colours indicate taxonomical affiliation on phylum level. KIT – DNA sample extracted with commercial kit, CM – extraction with custom method. Samples for which no sequence data was obtained are shown as empty columns in the graph.

microbial cells and/or their DNA is important.

The choice of DNA extraction method may greatly impact the detected microbial community composition, as was also shown in this study (Figs. 5 – 8). Similar results have been reported with different commercial DNA extraction kits on e.g. soil samples (Zielińska et al., 2017; Iturbe-Espinoza et al., 2021), sediment samples (Ramírez et al., 2018) and water and bioreactor samples (Walden et al., 2017). In addition, Carrigg et al. (2007) also showed that the bacterial community profiles in soil and sediment samples detected with several custom DNA extraction methods, one including a phenol:chloroform extraction phase, and a commercial kit differed greatly between the extraction procedures. This result is in agreement with our study where the microbial communities detected from the CM and KIT extracted DNA were clearly distinctive from each other.

There was a clear difference in the amount of DNA extracted with the CM versus the KIT (Table 2). Measurable amounts of DNA were obtained from all sample types with the CM protocol, but only from the Process water with the KIT (Table 2). This is likely due to the Process water being relatively clean and devoid of mineral particles to which microorganisms can attach. However, all other samples contained high proportions of mineral particles. Previous studies have shown that different minerals attract microorganisms in different ways and that factors such as pH affects the adsorption (Tan and Chen, 2012; Dong et al., 2013; Jia et al., 2008). We show that the developed CM protocol was functional for samples originating from two different mineral processing plants across Europe emphasizing its suitability for different mineralogy and chemistry.

A statistically significantly higher bacterial community diversity was revealed with the CM compared with KIT protocols, showing the importance of the DNA extraction method for obtaining as wide as possible DNA representation of the microbial diversity of a specific sample. Nevertheless, assuming that a bacterial cell contains 0.35–32 fg DNA, depending on the size and replication status of the chromosome (Table 4), the CM extracted DNA can be estimated to originate from approximately 10^4–10^6 cells mL^{-1}. However, previous data has shown that these sample types may contain up to the order of 10^8 cells mL^{-1} (Le et al., 2020). Thus, only a minor part of the microbial community in these samples was attained with the new, more efficient CM DNA extraction protocol. However, the amounts of DNA extracted was significantly higher than that extracted with the KIT, with the exception of the Process water samples.

Using different strategies of serial detachment of microbial cells with e.g. two washes with MilliQ water (Le et al., 2020), two washes with MilliQ water containing detergent (Le et al., 2020), or 3–8 washes with 0 K Basal Salts Medium (BSM) (pH 1.6) + detergent (Makaula et al., 2020) and other similar studies on acidic bioleaching applications (Govender et al., 2013; Chiume et al., 2012) have shown that each wash step extracts a specific part of the mineral colonizing microbial community. The most loosely attached microbial fraction is likely harvested in the first washes and more and more strongly attached cells harvested in the later extraction solutions (Makaula et al., 2020). Makaula et al. (2020) showed that 8 washes with BSM extracted most of the cells from the minerals from acidic bioleaching applications, whereas the data from 6 washes introduced an error of 10% for the number of cells detached and only 3 washes introduced errors of >30% with great variation between replicate samples. The authors also showed that the number of loosely attached cells, i.e., harvested in the first wash, represented at most 10% of the total number of attached cells. Nevertheless, not even 8 washes could remove all attached microbial cells from the minerals, which was shown by SEM imaging and activity analysis of the mineral samples. However, it should be kept in mind that the conditions differ markedly in bioleaching and chemical flotation processes. The time frame is different from weeks in bioleaching to hours in flotation process, the mineral particles are freshly ground and attract both chemical and microbial reactions in flotation and the pH conditions enable diverse microbial survival strategies and hence much more versatile microbial communities compared to bioleaching. All these parameters affect the physicochemical characteristics of the sample and thus the DNA extraction.

Following Makaula et al. (2020), the CM protocol repeated the DNA or cell extraction step in the beginning of the CM protocol. However, likely due to differences of the bioleaching and flotation processes, DNA extraction proved to be more difficult from the flotation samples than from the bioleaching as still only a fraction of the microbial communities present was collected from the flotation process samples. This is of particular interest, because the microorganisms that are undetachable from the minerals may be the ones having the greatest effects on flotation efficiency and ultimately affect the metal grade and recovery. It has been shown that bacteria adhere to minerals in flotation experiments within minutes of exposure (Zheng et al., 2001; Arias et al., 2023) and

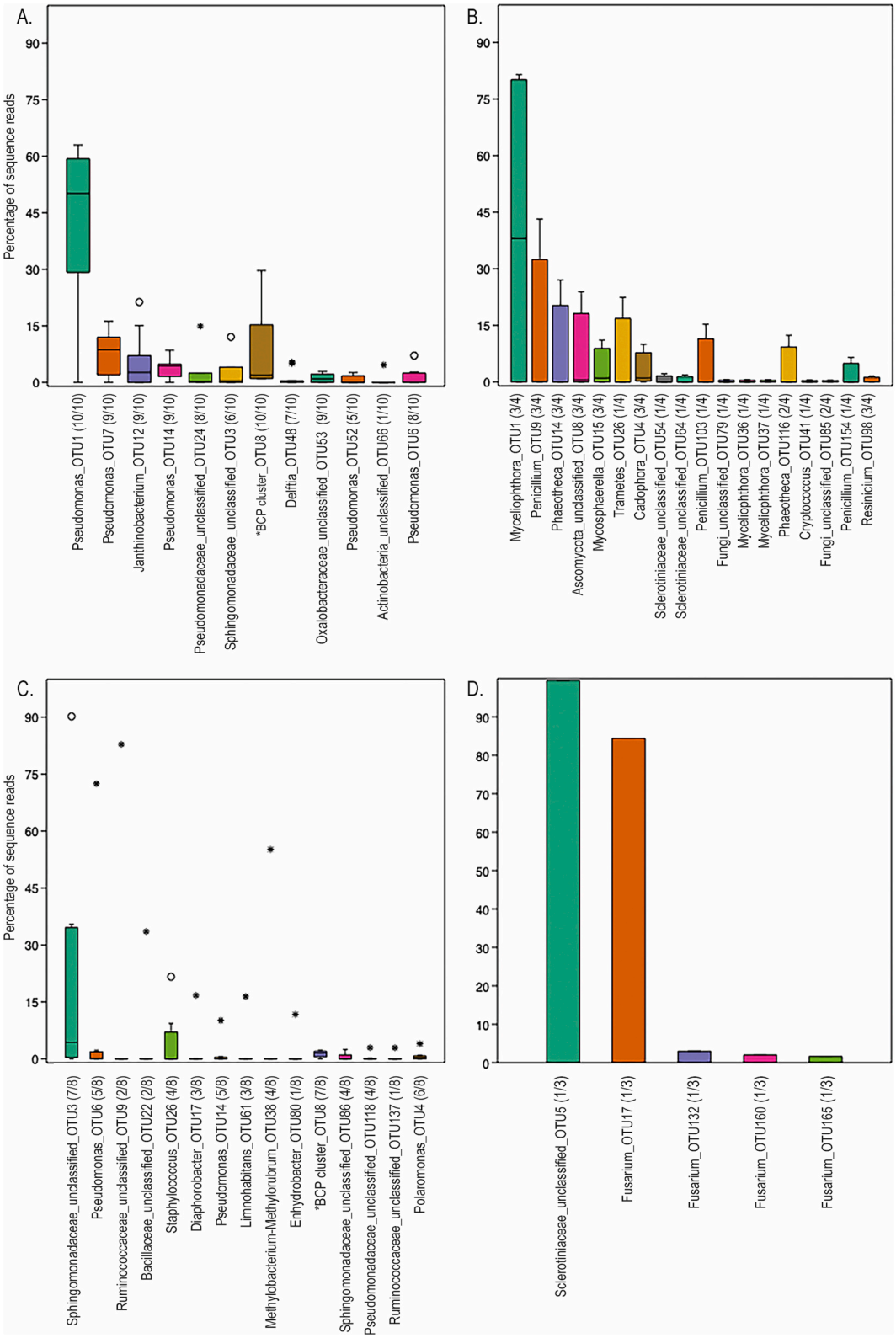

Fig. 9. Box plots over the relative abundance of the most prominent A, C) bacterial and B, D) fungal OTUs identified from the CM DNA extraction control samples (A, B) and KIT extraction controls (C, D). The whiskers indicate the minimum and maximum values and the horizontal line in the box the median of the relative abundance in the CM negative controls. Open circles and stars indicate outliers falling >1.5 x and 3 x of the box length outside the minimum or maximum whiskers, respectively. Bacterial and fungal sequences were obtained from 10/10 and 4/10 CM negative controls, and 8/8 and 3/8 KIT negative controls, respectively, which were included in the box plots. OTUs representing on average at least 10 sequence reads in the negative controls are included in the graph. Values in parentheses indicate the number of negative controls each OTU has been detected from. *BCP = *Burkholderia-Caballeronia-Paraburkholderia* cluster.

Table 4
Examples of the genome size in mega base pairs (Mbp) and estimated amount of DNA in femtograms (fg) in bacterial cells with one chromosome and in cells with double chromosomes at the end of replication and cell division. The DNA mass was calculated according to (Genome size bp Average mole mass of a nucleotide bp 660 g/mol)/Avogadro's constant N_A 6.02214076 × 10^{23} mol) = mass of DNA in cell.

Bacterium	Genome size Mbp	fg DNA/cell, single chromosome	fg DNA/cell at the end of cell division	Reference to genome size
Carsonella ruddii	0.16	0.17	0.35	Nakabachi et al., 2006
Mycoplasma genitalium	0.58	0.64	1.3	Su and Baseman, 1990
Candidate phyla SR1, WWE3, TM7, and OD1	0.7–1.2	0.77–1.32	1.5–2.6	Kantor et al., 2013
Pelagibacter ubique	1.3	1.42	2.9	Giovannoni et al., 2005
Escherichia coli	4.5–5.5	4.9–6.0	9.9–12.1	Rode et al., 1999
Myxococcus xanthus	9.4	10.2	20.5	López-Rojo et al., 2023
Sorangium cellulosum	14.8	16.2	32.4	Han et al., 2013

that pH strongly affects the adsorption time, the strength of the attachment and which mineral the bacteria most readily adsorb to (Zheng et al., 2001). Zheng et al. (2001) showed that *Bacillus subtilis* and *Mycobacterium phlei* affected the oleate flotation differently at different pH by having dissimilar adsorption affinity to dolomite and apatite as well as dissimilar dolomite and apatite depression efficiency depending on pH. In addition, Natarajan and Deo (2001) showed that different bacterial compounds, such as proteins and polysaccharides, showed different affinity for different minerals and e.g. bacterial proteins had a flotation promoting effect on quartz and kaolinite, while depressing hematite and corundum.

Only few studies exist where the naturally existing microbial communities in mineral flotation processes have been studied, despite the impact the microorganisms may have on the flotation process. Thus far DNA based studies have been reported from Finland, Portugal, and Chile (Miettinen et al., 2021; Bomberg et al., 2020; Arias et al., 2023; Natarajan and Deo, 2001; Liu et al., 2013). The DNA extraction from the Finnish and Portuguese samples relied on commercial DNA extraction kits, whereas the Chilean samples were extracted using a phenol-chloroform extraction method. However, in all these studies the DNA extraction was performed on the liquid part of the samples. Immediately as the amount of mineral particles increase the DNA extraction efficiency declines. In a previous study, we tested the NucleoBond® RNA soil + DNA kit (Macherey-Nagel GmbH and Co, KG, Düren, Germany) for samples Zn Rougher Feed and Cu Tailings 2, a protocol containing a phenol:chloroform:isoamyl alcohol step (Le et al., 2020), as well as the classic phenol extraction protocol, where the above mentioned samples were mixed 9:1 with 0.5 M EDTA (pH 8) and 1:1 volume of phenol: chloroform:isoamyl alcohol combined with mechanical lysis by rigorous bead beating (unpublished). With the NucleoBond® kit we obtained 0.25 ng DNA mL^{-1} sample from Cu Tailings 2, but the DNA amounts remained below the detection limit of the Qubit HS assay for the Zn Rougher Feed, as well as for both samples using the EDTA -phenol: chloroform:isoamyl alcohol protocol, leaving the results clearly below the performance of the CM protocol. Thus, an agent competing with the DNA for adsorption sites on the mineral surfaces is beneficial and needed.

Improved DNA extraction protocols are of broader interest than only in mineral processing applications and have been optimized for different sample types, such as iron-rich clays (Hurt Jr et al., 2014), bentonite clay (Miettinen et al., 2022), rock and sediment samples (Lever et al., 2015). When considering taking new DNA extraction protocols into use, one should also consider the source of the material. In mineral processing applications the mineral surfaces are freshly exposed and processed immediately, thus decreasing the risk of relic DNA being present in the samples. However, in different sample types, such as different types of soil, it has been found that on average 40% of the extractable prokaryotic and fungal DNA is extracellular and may inflate the detected species richness of the sample (Carini et al., 2016).

Additionally, the use of the CM method demands special focus on carrying out the protocol avoiding contamination during each step. The nanocellulose itself may be a source of contamination (Fig. 9), and it was clear from the uneven detection of different contaminant OTUs in the CM controls that the occurrence of contaminants was not stable. In comparison, commercial DNA extraction kits have also been shown to contain contaminating DNA and specific common lineages have been identified (Salter et al., 2014; Sheik et al., 2018; Eisenhofer et al., 2019), some of which were also detected in the KIT controls in this study. This highlights the importance of including negative reagent control extractions in every sample batch as well as including non-DNA control reactions in all downstream applications to identify and correctly filter contaminants from the data. Our work is a proof-of-concept for using anionic nanocellulose in DNA extraction procedures designed for mineral samples as a competing agent that prevents DNA from adsorbing to minerals. In this first experiment the nanocellulose was custom made and autoclaved before use. The DNA extraction procedure was shown to outcompete the performance of commercial DNA extraction kits in general, and in the future attention needs to be put on the strictly aseptic manufacturing of the nanocellulose targeted for DNA extraction procedures.

5. Conclusions

Naturally occurring microorganisms and their DNA adhere strongly to mineral surfaces and may have significant effects on mineral processing applications, such as flotation, affecting both the grade and yield of the metal concentrate. We have developed a technique using anionic nanocellulose as an agent competing with the microorganisms for binding sites on the mineral surfaces thus preventing microbial DNA from attaching. With this method more DNA was extracted and a more wholistic picture of the microbial communities inhabiting mineral processing samples was obtained. The DNA amounts extracted with the commercial KIT method was generally below the detection limit of the DNA measurement assay, whereas up to 1.5 ng DNA per mL original sample slurry was obtained with the CM method. In consequence, up to three orders of magnitude higher bacterial 16S rRNA gene copy numbers and a higher microbial diversity were detected using the CM method compared to the KIT. However, all DNA present in the samples was not successfully extracted indicating that method optimization is still needed.

Declaration of generative AI and aiassisted technologies in the writing process

No generative AI and AI-assisted technologies were used in the writing process of this paper.

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Ethics statement

This work does not contain studies with human participants of live

animals, or hazardous or sensitive material.

CRediT authorship contribution statement

Malin Bomberg: Conceptualization, Investigation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hanna Miettinen:** Conceptualization, Investigation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors have no conflicts of interest associated with this research work.

Data availability

Sequence data has been deposited in ENA and study number provided in the manuscript. All other data is included in the methods and results. The authors confirm that all supporting data, code and protocols are provided within the article and supplementary data file. All sequence data has been deposited in the European Nucleotide Archive (https://www.ebi.ac.uk/ena/) under Project accession number PRJEB61213.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2023.106850.

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