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Optimization of bacterial DNA and endotoxin extraction from settled airborne dust

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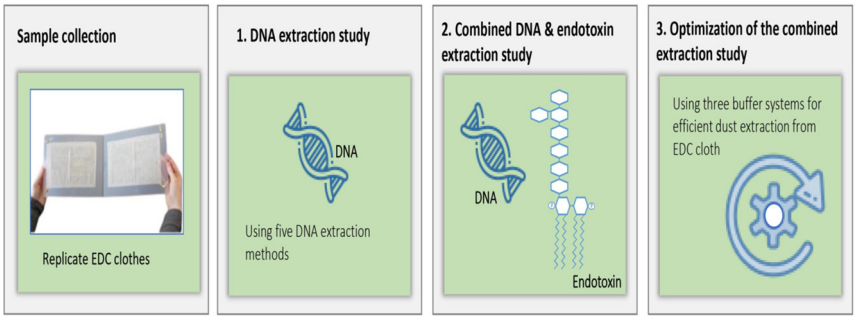
HIGHLIGHTS

- The DNeasy Power soil Kit had the best DNA extraction yield and time invested.
- Rinsing EDC cloth with pyrogen-free water promoted removal of dust particles.
- A protocol for DNA and endotoxin extraction using a single EDC was developed.

GRAPHICAL ABSTRACT



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ABSTRACT

Collecting and obtaining sufficient amount of airborne particles for multiple microbial component assessments can be challenging. A passive dust sampling device, the electrostatic dust fall collector (EDC) has been established for assessing airborne exposures including endotoxin and glucans. Recently, with advances in next-generation sequencing techniques, EDCs were used to collect microbial cells for DNA sequencing analysis to promote the study of airborne bacterial and fungal communities. However, low DNA yields have been problematic when employing passive sampling with EDC. To address this challenge, we attempted to increase the efficiency of extraction. We compared DNA extraction efficiency of bacterial components from EDCs captured on filters through filtration using five extraction techniques. By measuring the abundance, diversity and structure of bacterial communities using qPCR and amplicon sequencing targeting 16S rRNA genes, we found that two techniques outperformed the rest. Furthermore, we developed protocols to simultaneously extract both DNA and endotoxin from a single EDC cloth. Our technique promotes a high quality to price ratio and may be employed in large epidemiological studies addressing airborne bacterial exposure where a large number of samples is needed.

1. Introduction

Active air sampling is thought to provide an accurate representation of airborne bioaerosol populations due to the high volume of air that is sampled. Active sampling, on the other hand, underestimates microbial diversity by saturating the filter and desiccating the microorganisms, and requires skilled handling of materials and equipment, making it difficult

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to collect a large number of samples at different locations (Normand et al., 2009; Ghosh et al., 2015; Eduarda and Heederik, 1998). Unlike active air sampling, sampling of settled dust allows for simple, non-invasive, cumulative sampling of bioaerosol over a period of several days. While short-term temporal dynamics of bioaerosols cannot be assessed due to low temporal resolution, the extended period is beneficial for assessing a representative sampling of microbial exposure (Normand et al., 2009). Due to its simplicity, settled dust sampling is among the most popular approaches for measuring microbiological airborne exposure in the indoor environment (Viegas et al., 2018; Viegas et al., 2019a; Viegas et al., 2019b; Viegas et al., 2020a; Viegas et al., 2020b).

Among the different techniques, EDCs (Electrostatic Dust fall collectors) have been employed as a standard for passive sampling of settled airborne dust. The EDC is a sampling device consisting of a polypropylene folder holding 2 or 4 electrostatic cloths (Kilburg-Basnyat et al., 2016; Noss et al., 2008). They are easy to use and are ideal for large population studies because they can be mailed to, deployed by, and returned by study participants without the need for elaborate equipment and field staff (Kilburg-Basnyat et al., 2016; Kilburg-Basnyat et al., 2015). Since the introduction of EDCs, the dust collected on EDC cloths has been used to study traditional markers of biological exposure, like allergens, endotoxins and glucan exposure (Jacobs et al., 2014a; Samadi et al., 2010; Jacobs et al., 2014b; Sander et al., 2018; Krop et al., 2014; Schlünssen et al., 2015), as well as culturing of bacteria and fungi (Hoppe et al., 2012; Spilak et al., 2015). More recent molecular markers have been used to identify microbial communities (Vestergaard et al., 2018; Adams et al., 2021) and to identify antimicrobial resistance genes in occupational environments (Luiken et al., 2020; Van Cleef et al., 2014; van Cleef et al., 2015; Van Cleef et al., 2016). Recently EDCs have also been used to collect settling dust to study environmental contamination of SARS-CoV-2 infection in mink farms, meat processing plants, nursing homes and secondary schools (de Rooij, 2021a; de Rooij, 2021b; Jonker, 2022; Linde, 2022).

After EDCs were established to assess indoor air quality, the feasibility of EDCs for endotoxin measurements were validated by several studies of urban and farm homes. Factors that can affect EDC sampling efficiency have been assessed and include heating, mailing, electrostatic charge, storage, and deployment time (Kilburg-Basnyat et al., 2016; Kilburg-Basnyat et al., 2015). In addition, the effect of the extraction procedure and the effect of extraction media on the concentration of endotoxin from EDC has been studied. Noss et al. (2010) and Spaan et al. (2008) recommend using 0.05 % Tween 20 (surfactant) in pyrogen-free water (PFW) as the extraction medium rather than PFW alone for the extraction of endotoxin from settled dust. However, Hoppe Parr et al. (2017) showed that higher endotoxin concentrations were obtained by extraction of organic dust with Tris-HCL EDTA buffer compared to Tween 20 buffer. Shin et al. (2018) revealed the mechanism of soil particle removal from fibrous materials cloth by diffusiophoresis, the directed motion of soil particles by chemical gradients of surfactant. The authors emphasized the importance of surfactant gradients that establish via rinsing with fresh water for soil particle removal. They concluded that rinsing with fresh water is the key to the effective cleaning.

The use of culture-independent methods and in particular the use of 16S rRNA gene and ITS sequencing techniques for the characterization of bacterial and fungal communities has received increasing attention in airborne exposure studies due to the ability to identify a large range of bacterial and fungal taxa that elude culture-based studies (Vestergaard et al., 2018; Adams et al., 2021; Dannemiller et al., 2014; Lai et al., 2018). Thus, culture-independent methods together with EDCs became an important tool in epidemiological studies that aim to establish a link between airborne microbial exposure and human health. However, many of the DNA extraction techniques employed so far have resulted in low DNA yields, Therefore, several amplification steps are required for sufficient starting material for DNA sequencing with the risk of introducing sequence artifacts and contamination from the environment or the reagents used (Salter et al., 2014; Castelino et al., 2017). Therefore, increasing the amount of dust extracted from EDC cloth and finding an optimal method for DNA extraction from settled dust are needed.

In the current study, we first aimed to compare the efficiency and comparability of five DNA extraction methods using replicate EDC samples collected in parallel and standardized bacterial community. Secondly, we aimed to combine bacterial DNA and endotoxin extraction. Thirdly, we tested three buffer systems to increase the amount of dust extracted from EDC cloth to achieve an optimal amount of DNA and endotoxin extraction.

2. Material and methods

2.1. Study design

The settled airborne dust was collected using EDCs from the living room and the bedrooms of ten participants in Aarhus, Denmark. For each participant eight EDC replicates per sampling location (16 EDC cloths per participant) were collected; four EDCs in the bedroom and four EDCs in the living room. Each EDC cloth had an exposure area of 0.0209 m^2 and were placed about 150 cm above the ground for a period of 14 days. We successfully collected 155 out of 160 EDC cloths that were used for the analysis.

2.2. Dust extraction

Dust extraction was performed in a clean lab for nucleic acid work to avoid contamination. All the tools used were either sterilized before use or were single use sterile tools. Dust extraction from EDC cloths was performed as described previously by Adams et al. (2015). Briefly EDC cloth were placed in sterile stomacher bag and mixed with 20 ml extraction buffer consisting of 0.05 % Tween 20 (Sigma Aldrich, Missouri, United States) in pyrogen free water (PFW, Milli-Q® A10 Ultrapure Water). The samples were then processed in stomacher (VWR type Star Blender LB400) for 10 min at maximum speed. The extracted fluid was collected in a 50 ml Falcon tube and kept on ice. This procedure was repeated once more until a total volume of 40 ml was extracted from the filter cloth. The dust suspended in Tween 20 buffer was collected into a 25 mm 0.22 μm pore size polyethersulfone membrane filter (Merck, New Jersey, United States). The glass-vacuum filtration device was rinsed with hydrochloric acid and ethanol and autoclaved between runs. Clean autoclaved glass funnel was used for each filter cloth extract. The membrane filter containing the concentrated dust samples was aseptically transferred into pre-filled bead tubes/Falcon tubes and stored at −20 °C until DNA extraction. An overview figure represents steps of extraction of the settled airborne dust from the EDC cloth presented in Fig. 1. For the DNA extraction study (Section 2.3), stomacher Star Blender LB400 (VWR, Radnor, Pennsylvania, USA) was used and for the remaining part of the study, low noise Smasher™ (bioMérieux, Marcy-lÉtoile, France) was used with adjustments: the samples were processed for 3 min at the fast mode (620 stroke/min). Further modifications were added to the dust extraction process to improve its efficiency (Sections 2.4 and 2.5).

2.3. DNA extraction

Earlier methods used centrifugation to concentrate dust extracted from the EDC cloths prior to DNA extraction (Vestergaard et al., 2018; Adams et al., 2015). In this study we instead collected the dust extracted from EDC cloths onto 0.22 μm polyethersulfone filters to improve the efficiency of particle retention, as we in a pilot study revealed a higher number of 16S rRNA gene copies using a filtration-based approach compared to a centrifugation-based approach (Supplementary Fig. 1). Five DNA extraction methods were then used on these filters to compare their performance. We compared three commercially available DNA extraction kits, DNeasy PowerSoil Pro Kit (named PowerSoil kit), DNeasy PowerWater Kit and MagAttract PowerWater DNA/RNA Kit (MO BIO Laboratories, a Qiagen Company, Hilden, Germany) with two previously described non-commercial DNA extraction methods, the first was used before to extract DNA from Sterivex filter columns (named protocol A) (Lever et al., 2015) while the second was used to extract DNA from sediment samples (named protocol B) (Xiao, 2017).

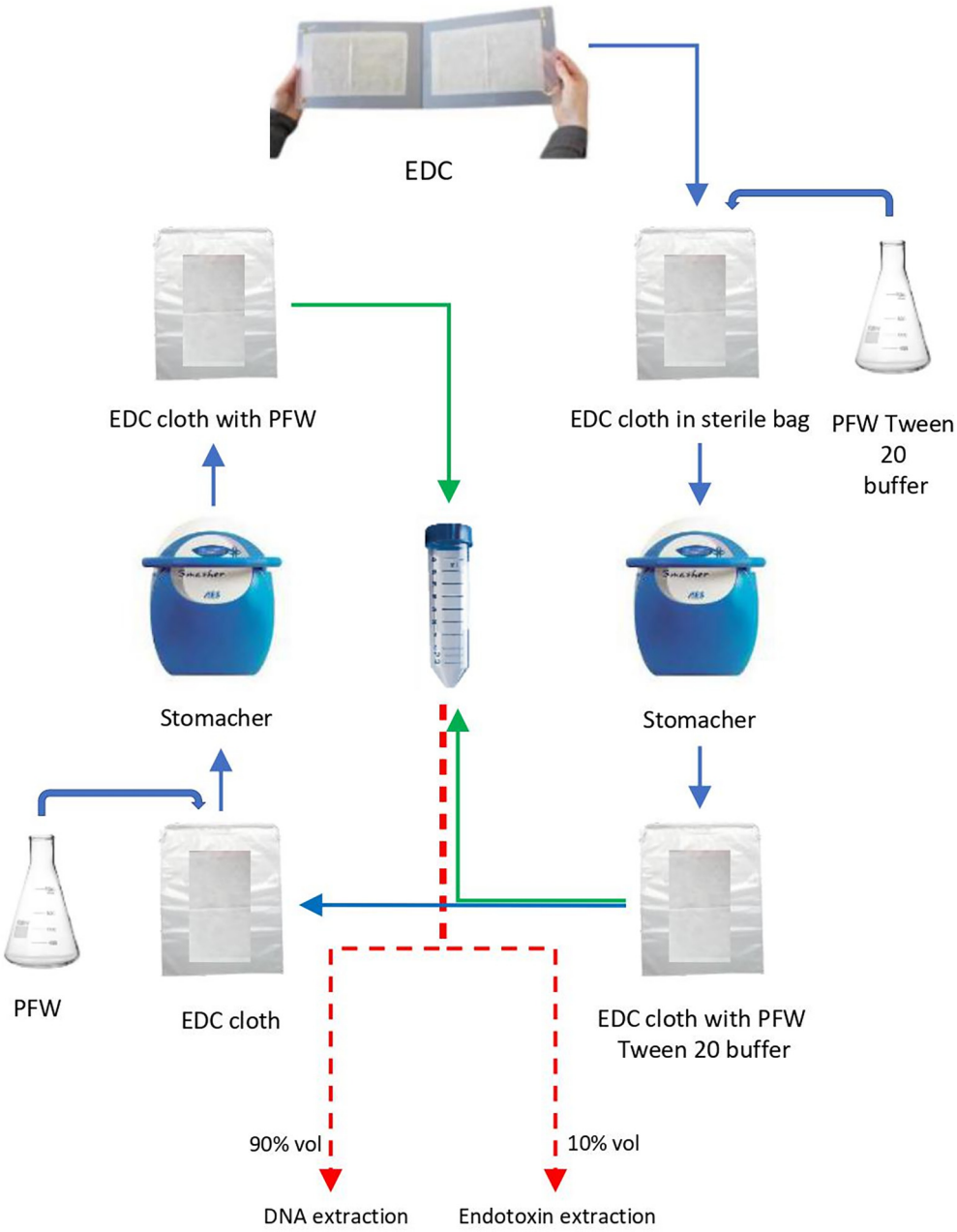

Fig. 1. Overview figure represent steps of extraction of the settled airborne dust from single EDC cloth. Bule arrows represent the direction of dust extraction from EDC cloth, green arrows represent collection of extracted fluid, and red arrows represent dividing of extracted fluid for downstream analysis.

In protocol A, membrane filters were placed into 15 ml Falcon tubes and entirely soaked with 0.1 ml of 10 mM dNTP and 1 ml of cell lysis solution consisting of: 30 mM Tris-HCl, 30 mM EDTA, 1 % Triton X-100, and 800 mM guanidium hydrochloride. The tubes were subjected to vortexing for 10 min at maximum speed. Following the lysis step, the tubes were frozen completely at −80C (at least 40 min). Then they were incubated on a thermoshaker (600 rpm) for 1 h at 50 °C. DNA was purified twice with 1 × sample volume of chloroform:isoamyl alcohol (24:1, vol:vol; Sigma Aldrich, Missouri, United States) with centrifugation steps at 10,000 ×*g* for 10 min in between the washes. Following DNA purification, 20 μg/ml of Linear polyacrylamide, 1.5 × volume isopropanol, and 0.1 volume 5 M NaCl were used to precipitate DNA overnight at −20 °C, then centrifuged at 4 °C for 30 min at 14,000 ×*g*. The pellets were washed with 70 % ethanol and dried for 5–7 min with a SpeedVac pre-heated to 50 °C. After that, the pellets were resuspended in 80 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and purified with the Clean All RNA/DNA Clean-Up and Concentration Kit (Norgen Biotek, Ontario, Canada) to remove PCR inhibitors.

In the protocol B, membrane filters were placed at 2 ml screw cap micro tubes containing 0.25 ml zirconia beads (0.1 mm diameter, BioSpec, Oklahoma, United States) and lysis buffer mixture containing 200 ul TNS buffer (50 mM Tris, 150 mM NaCl, 10 % Sucrose, pH 8.0) and 650 ul sodium phosphate buffer solution (112.9 mM Na2HPO4, 7.1 mM NaH2PO4), the tubes were subjected to bead beating at 50 oscillations s^{-1} for 1 min using a TissueLyser LT 2500 (Qiagen Company, Hilden, Germany), followed by incubation in a thermomixer with 600 rpm at 50 °C. After lysis step, the mixture was centrifuged for 10 min at 19,000 ×*g* at 4 °C. The nucleic acids from the supernatant were purified with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol:vol; Sigma Aldrich, Missouri, United States),

followed by purification with an equal volume of chloroform: isoamyl alcohol (24:1, vol:vol; Sigma Aldrich, Missouri, United States). One ml polyethylene glycol 8000 (Sigma Aldrich, Missouri, United States) was used to precipitate DNA at 4 °C overnight, then centrifuged at 19,000 ×*g* for 30 min. The precipitates were rinsed with an ice cold 70 % ethanol solution, dried in the air, and dissolved in 80 ul TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) before being kept at −20 °C.

The three commercial kits were used according to manufacturers' instructions with the following refinement: the bead-beating step was carried out in a TissueLyser bead-beating machine for 10 min at 50 s^{-1}.

Two DNA extraction studies were performed. In the first DNA extraction study, the dust collected from EDC cloth simultaneously was used for the five DNA extraction methods and DNA yields were determined by quantitative PCR (qPCR). In the second DNA extraction study, the two most efficient methods were compared using qPCR and MiSeq sequencing of the V3-V4 region of bacterial 16S rRNA genes.

2.4. Combined DNA and endotoxin extraction

After achieving the first aim of the study, improving the amount of DNA extraction from settled dust. In this study we tested co-extraction of DNA and endotoxin from a single EDC cloth and compared the endotoxin yield to the established method described by Noss et al. (2008).

In the Combined DNA and endotoxin extraction, 10 % of 40 ml liquid containing the dust extracted from the EDC cloth were transferred to a 15 ml Falcon tube for endotoxin analysis. The cell debris was removed by centrifugation at 1000 ×*g* for 15 min and the supernatants were stored in glass vial as aliquots at −20 °C until analysis.

Following the classical method described by Noss et al. (2008), the EDC cloth was placed in glass Erlenmeyer flasks containing 20 ml 0.05 % Tween 20 buffer and shaken on a horizontal shaker (160 reciprocations/min) for 60 min at room temperature. 10 % of the extraction volume (2 ml) was harvested, centrifuged at 1000 ×*g* for 15 min, and supernatants were stored as aliquots in glass vials at −20 °C until analysis. The supernatants from the two extraction methods were analysed with the quantitative kinetic chromogenic LAL assay (Lonza, Basel, Switzerland).

2.5. Optimization of the combined extraction method

After establishing the combined extraction method, we aimed at optimizing the method by changing extraction buffers. Dust was extracted from replicate EDC clothes collected in the living room. Replicate EDCs were extracted by (i) double washing of the EDC with 100 mM Tris-10 mM EDTA buffer, (ii) double washing the EDC with 0.05 % Tween 20 buffer, (iii) first washing EDC with 0.05 % Tween 20 buffer followed by rinsing the EDC with 30 ml PFW. To compare between different buffers used, the DNA was extracted by PowerSoil kit and DNA yield was determined by qPCR. The endotoxin concentrations were measured by the kinetic chromogenic LAL assay.

2.6. Mock community

The ZymoBIOMICS™ Standardized microbial community (D6300) (mock community) (Zymo Research,Irvine, California,USA) with total cell concentration of ~1.4×10^{10} cells/ml was used to assess the DNA extraction efficiency a of different buffer system used. The bacterial cell numbers were estimated using qPCR targeting16S rRNA genes. The 16S rRNA operon copy number of 4.2 (Větrovský and Baldrian, 2013) was used to convert 16S rRNA gene copies obtained from by the qPCR into bacterial cell numbers. Across protocols that were tested, the DNA was eluted in 80 ul of TE buffer. The efficiency of the DNA extraction methods as a function of bacterial cell number was calculated according to the formula below. The expected bacterial cell number loaded on the unexposed EDC cloth were compared to actually retrieved bacterial cell number calculated by the formula.

$$\textit{Bacterial cell number} = \frac{\text{16S rRNA gene copies per ul} \times 80}{4.2}$$

First, the mock community was used to assess efficiency of DNA extraction as a function of different cell loads by double washing EDC cloths with 0.05 % Tween 20 buffer. The efficiency was determined using qPCR targeting 16S rRNA genes. The unloaded EDC cloths were spiked with known bacterial cell loads spanning between 7.71×10^4 and 7.71×10^8 cells per EDC cloth. Three EDC clothes for each cell load were extracted. Secondly, the mock community was used to test the effect of additional rinsing with PFW on DNA yield, therefore 12 blank EDC cloths were spiked with 7.71×10^6 bacterial cells and extracted using the following two buffer systems: i) double washing only with 0.05 % Tween 20 buffer or ii) single washing with 0.05 % Tween 20 buffer followed by 1–3 rinses with 30 ml PFW. Thirdly the mock community was used to test the efficiency of PowerSoil kit to extract DNA by adding 7.71×10^6 bacteria directly into pre-filled bead tubes.

Additionally, the mock community was used to test the ability of the combined extraction method (single washing EDC cloth with 0.05 % Tween 20 buffer followed by rinsing once with PFW) to extract DNA across bacterial taxa using 16S RNA sequencing.

2.7. Quantification of bacterial abundance

The bacterial abundance was measured by 16S qPCR as previously described by Vestergaard et al. (2018). The reactions were performed using a MX3005p qPCR machine (Agilent, Santa Clara, California, United States) and carried out in a 20 ul reaction volume containing 10 ul SYBR Green 1Master-2×, 2 ul bovine serum albumin (BSA; 10 mg/ml), 1 ul forward primer Bac908F (50-AAC TCA AAK GAA TTG ACG GG-30), 1 ul reverse primer Bac1075R (50-CAC GAG CTG ACG ACA RCC-30) (10 pmol/ml) (Ohkuma and Kudo, 1998), 4 ul dH2O, and 2 ul template DNA. Controls were obtained by substituting DNA template with ddH2O (double-distilled water). Standard curves were obtained using serial dilutions of a plasmid containing a full-length 16S rRNA gene related to Sphingomonadales. The thermal cycling conditions were one cycle of initial denaturation for 5 min at 95 °C, followed by 45 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 20 s, and 80 °C for 7 s.

2.8. 16S rRNA amplicon sequencing

Using Bac341F (5′-CCT ACG GGN GGC WGC AG-3′) and Bac805R primers (5′-GAC TAC GGT ATC TAA TCC-3′), the 16S rRNA gene V3 and V4 region was amplified. (Krop et al., 2014). The Illumina protocol (16S Metagenomic Sequencing Library Preparation) was followed with changes described in Vestergaard et al. (Kilburg-Basnyat et al., 2015). The library preparation included three PCR reactions. The first PCR amplified the 16S rRNA gene's V3 and V4 regions using bacteria-specific primers. The Illumina overhang adaptors were added in the second PCR, and the Nextera XT Index primers were used for the third PCR. AMPure XP magnetic beads were employed to clean the PCR products after each PCR step. The Quant-iTTM dsDNA assay kit and a FLUOstar Omega fluorometric microplate reader (BMG LABTECH, Ortenberg, Germany) were used to measure the concentration of the PCR products. Following that, the samples were diluted to around 3 ng/ml DNA and pooled together prior to being sequenced with MiSeq sequencing (Illumina, San Diego, California, United States).

2.9. Bioinformatic and statistical analysis

The sequencing data was processed in R (version 4.1.2) first by primer trimming using the cutadapt wrapper (Martin, 2011). The trimmed sequences were further processed using DADA2 (Divisive Amplicon

Denoising Algorithm 2) pipeline version 1.18.0 (Callahan et al., 2016) which used to infer true bacterial sequences from reads following the online tutorial. We added one modification into the DADA2 analysis pipeline using the shortread package version 1.48.0 (Morgan et al., 2009) to randomly subsample all sequences to 50,000 reads following quality filtering in order to make richness comparisons accurate. The ASVs were taxonomically classified up to the species level. The reference database used in the current study was the SILVA database version 138 (Quast et al., 2012). The decontam package version 1.10.0 (Davis et al., 2018) was used to eliminate ASVs from contaminating reads. For contaminate detection, the decontam package employed the "prevalence" method, where the identification of contaminates was based on the presence or absence taxa in the true positive samples (exposed EDC cloths) compared to the prevalence of these taxa in negative controls (unexposed EDC clothes and template-free PCR controls).

Heatmaps depicting the relative abundance of distinct bacterial taxa were created using the Ampvis2 package version 2.6.8. (Andersen, 2018) Phyloseq version 1.27.6 (McMurdie and Holmes, 2013) was used to generate two diversity measures for alpha diversity observed and the Shannon index. The Aitchison dissimilarity matrix was constructed using the "dist" function in the coda.base package version 0.3.1 and used to compare the microbial communities between different samples. PCoA ordination was carried out using the ape package version 5.5 (Paradis et al., 2004). According to Shapiro-Wilks test, the data were not normally distributed, so the Wilcoxon Rank Sum test which is included in the "wilcox.test" function in R version 4.1.2, was used to investigate the differences in bacterial abundance measured by qPCR and endotoxin yield measured by the LAL assay.

2.10. LAL assay

The supernatants from the two extraction methods (combined and classical method) were analysed without prior dilution. To study the effect of Tween 20 on LAL assay 10 supernatants were measured in three dilutions as 25×, 50× and undiluted. The 25× and 50× dilution showed higher endotoxin yield than undiluted supernatants. However, no significant differences were seen (Supplementary Fig. 2). The supernatants from the other experiments were diluted 50-times in PFW and analysed with the quantitative kinetic chromogenic LAL assay (Kinetic-QCL 50-650 U kit, Lonza, Walkersville, Maryland, USA). *Escherichia coli* O55:B5 reference standard endotoxin was used. Twelve concentrations spanning between 25 EU/ml and 0.012EU/ml were used for the standard curve. The detection limit for Vmax obtained by the kinetic LAL Assay was defined as the average of the assay blanks plus two times the standard deviation of these blanks. Results were given in EU m-2.

3. Results

3.1. DNA extraction studies

In the first DNA extraction study we compared amount of DNA yield by qPCR between the five DNA extraction methods. We found that protocol A and PowerSoil kit resulted in the highest DNA yields among the five DNA extraction methods (Fig. 2). The second DNA extraction study was conducted to compare the two best performing extraction techniques using qPCR and 16S rRNA bacterial gene sequencing. Protocol A resulted in more consistent DNA yields than PowerSoil kit when the first and the second DNA extraction study were compared (Fig. 3). After the decontamination procedures, samples retained on average (mean) 97 % of their reads, with a minimum of 85 % and a maximum of 100 %. The PowerSoil kit produced less contamination by fraction reads compared to protocol A, on average samples extracted by the PowerSoil kit retained 98 % of their reads compared to 95 % for protocol A. One sample extracted by the PowerSoil kit was removed from subsequent analysis due to a high (>50 %) contamination rate. (Supplementary Fig. 3).

In terms of bacterial composition, all samples from a specific location clustered together, while there was no clustering based on the method used. This indicates that variation between samples is greater than the variation caused by the two methods (Supplementary Fig. 4). The community composition at the phylum level was reproducible across the two methods for each home and contained a mixture of *Actinobacteria*, *Bacteroidetes*,

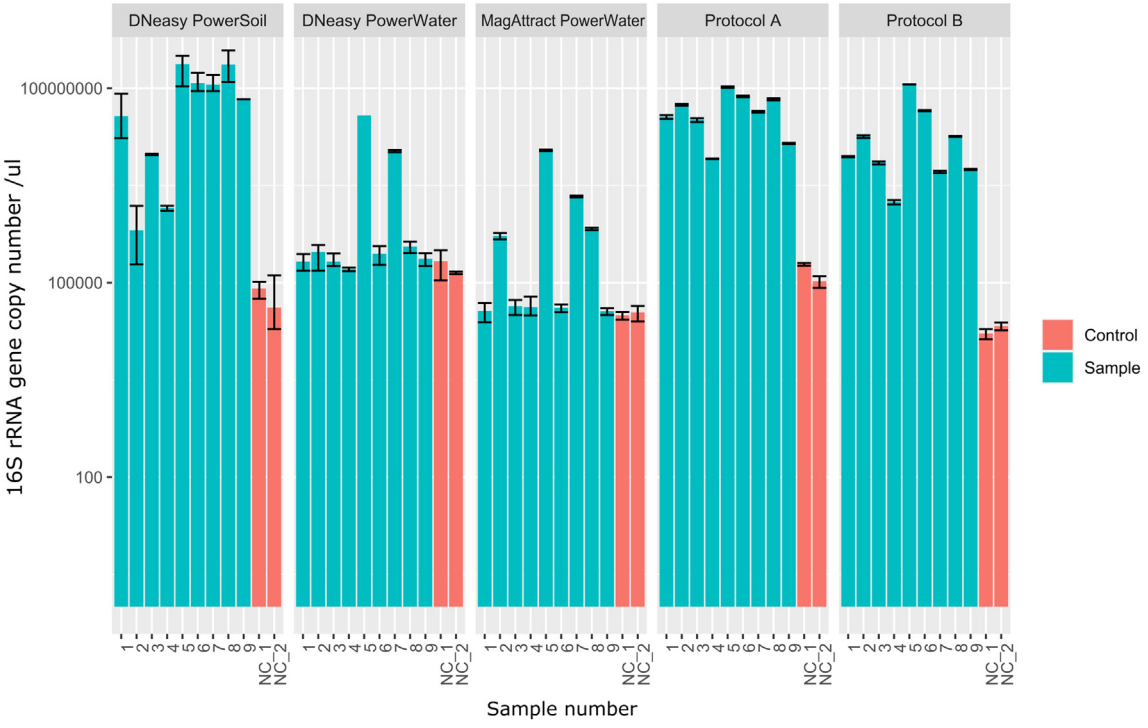

Fig. 2. Quantitative PCR measurements of the 16S rRNA of five EDC batches (each contain 9 EDC clothes) extracted by five DNA extraction methods. NC_1 and NC_2: Negative controls (unexposed EDC cloth).

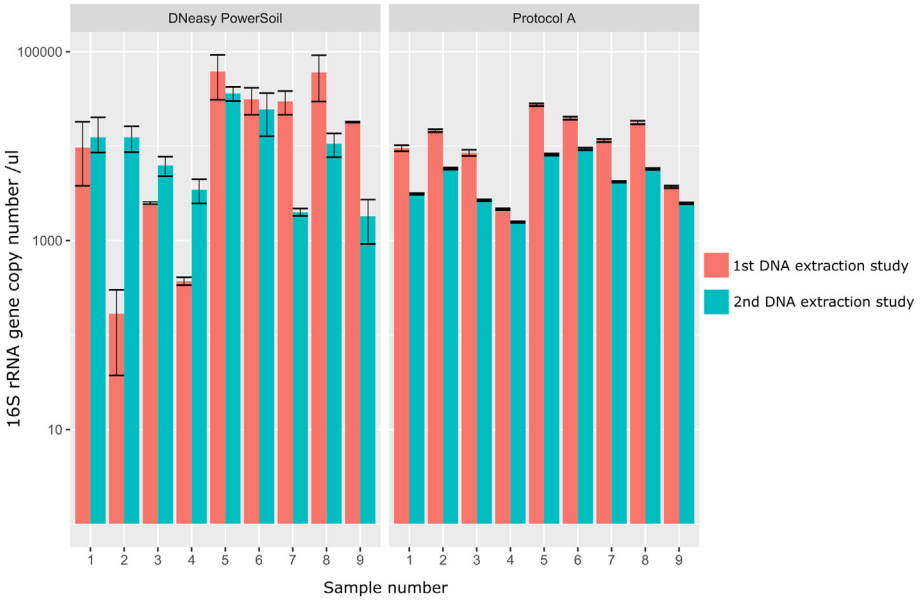

Fig. 3. Quantitative PCR measurements of the 16S rRNA genes using two replicate EDC batches extracted by PowerSoil kit and protocol A.

Cyanobacteria, *Firmicutes*, and *Proteobacteria*. The PowerSoil kit yielded a higher fraction of *Actinobacteria* and *Firmicutes* extraction, while protocol A generally yielded a higher fraction of *Proteobacteria* (Supplementary Fig. 5).

Protocol A consistently resulted in a higher Shannon diversity index and raw richness and in terms of reproducibility protocol A also performed better (Fig. 4). Contamination did not explain the higher bacterial diversity, or the higher bacterial richness obtained by protocol A as compared to the PowerSoil kit (Supplementary Figs. 6 and 7).

Comparing DNA yield obtained from the centrifugation-based protocol where the dust pellet resuspends in 1.5 ml 0.05 % Tween 20 before DNA extraction using the PowerSoil kit in Vestergaard et al. (2018) with the filtration-based protocol using the same DNA extraction kit showed that collecting the dust pellet on a membrane filter before DNA extraction significantly increased DNA yield (P value = 0.001) (Supplementary Fig. 1). Both samples from the two studies were from suburban homes.

3.2. Combined DNA and endotoxin extraction study

The combined method generally yields higher endotoxin concentrations, although the difference was not statistically significant (P = 0.51)

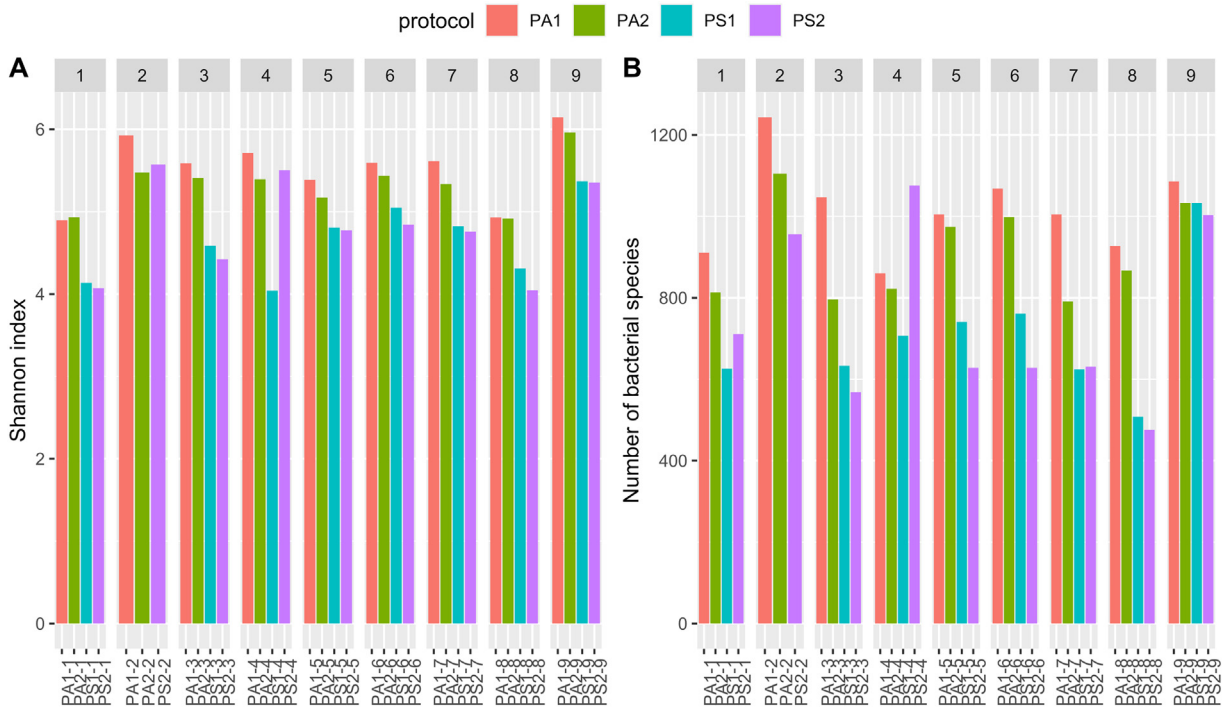

Fig. 4. Diversity measures calculated for two replicate EDC batches extracted by protocol A (PA) and PowerSoil kit (PS). PA1 & PA2: batch one and two extracted by protocol A, PS1 & PS2: batch one and two extracted by PowerSoil kit. (A) Bar plots of Shannon index considering both the richness and evenness (B) Bar plots of richness in term of number of bacterial species (OTUs).

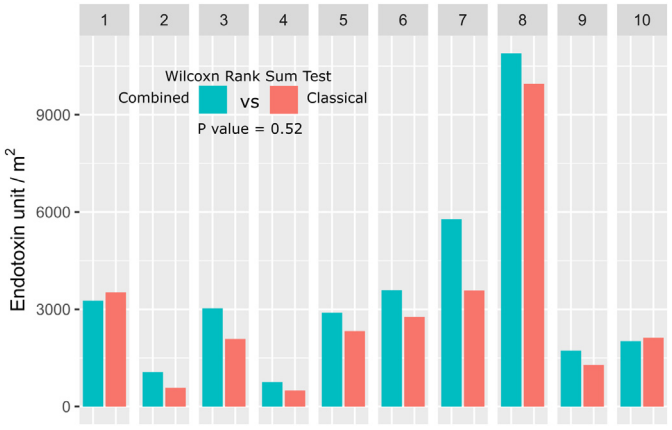

Fig. 5. Bar-plot of endotoxin concentration extracted by classic endotoxin extraction and combined extraction method.

compared to method commonly used for endotoxin extraction by Noss et al. (2008). However, the patterns were preserved (Fig. 5).

3.3. Optimization of the combined extraction study

We obtained a higher DNA and endotoxin yield when the EDC cloths were extracted using 0.05 % Tween 20 buffer in comparison to 100 mM Tris-HCl 10 mM EDTA buffer (Fig. 6). The 50-x dilution for Tris-HCl EDTA supernatants were just below detection limit of the LAL assay (0.012 EU/ml) compared to the 50-x dilution for Tween 20 supernatants that was above the detection limit of the LAL assay. We also showed that exchanging 0.05 % Tween 20 buffer with PFW during the second wash increased both DNA and endotoxin yield.

3.4. Mock community

Using a standardized mock community, we found that the efficiency of DNA extraction by double washing the EDC with 0.05 % Tween 20 buffer ranged from 2 to 4.4 %, with higher bacterial cell numbers giving higher DNA yields (data not shown). In a second mock community study, rinsing with PFW after single wash with 0.05 % Tween 20 buffer resulted in higher DNA yields (7.3 %) than washing twice with 0.05 % Tween 20 (5.8 %). A second rinsing step with PFW resulted in same range DNA yield (7.4 %), and a third rising step led to a decreased DNA yield (5.4 %) (Fig. 7). We also tested the efficiency of the PowerSoil kit to extract DNA directly from a standardized bacterial community and found that the PowerSoil kit was able to extract 57 % of the mock community's bacterial DNA.

Using the final proposed combined extraction method which included single washing of the EDC cloth with Tween 20 buffer, rising with PFW and DNA extraction using the PowerSoil kit, we could extract DNA from all bacterial taxa from the mock bacterial community (Fig. 8).

4. Discussion

The aim of this study was to optimize dust and DNA extraction from airborne dust collected using EDCs and to establish a method to jointly extract DNA and endotoxin from a single EDC cloth. We optimized DNA yields by rinsing an EDC cloth with PFW after the wash with 0.05 % Tween 20 buffer. We also show that deploying a single EDC cloth is sufficient to accurately determine DNA and endotoxin exposure. This possibility promotes cost and time-efficient analysis in large epidemiological studies.

Previously, DNA has been extracted from settled dust collected by EDC cloths as described by Adams et al. (2015): these were centrifugation-based protocols where the dust from the EDC was collected as pellets and subsequently resuspended in 0.05 % Tween extraction buffer for DNA extraction. This method, however, results in low DNA yields, especially in studies of suburban homes that characterized by low dust exposure compared to farmers homes and stables as showed by Amin et al. (n.d.). In this study,

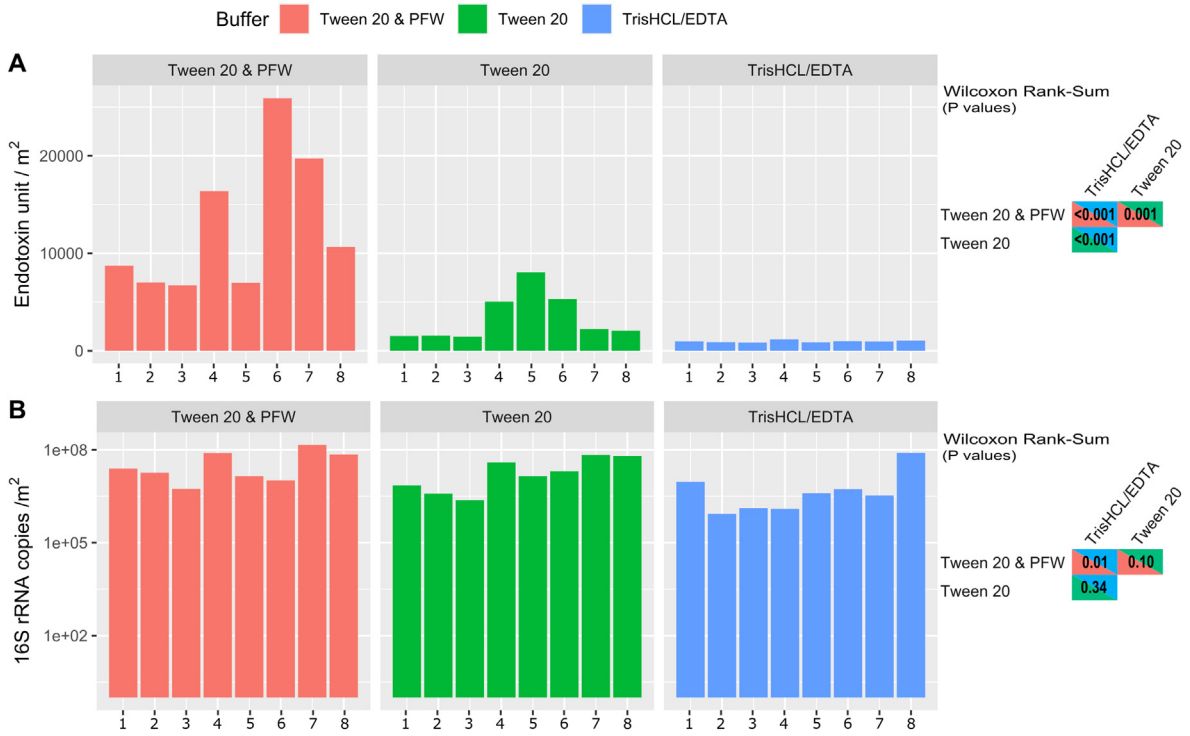

Fig. 6. A) Quantitative endotoxin measurement. B) quantitative PCR measurements of the 16S rRNA genes. Extracted by 1) Single washing with Tween 20 buffer followed by rinsing with PFW (Tween 20 & PFW), 2) Double washing 0.05 % Tween 20 buffer (Tween 20), 3) Double washing with 100 mM Tris 10 mM EDTA buffer (TrisHCL/EDTA). The significance of the differences depicted in this figure is demonstrated by the Wilcoxon Rank Sum test results.

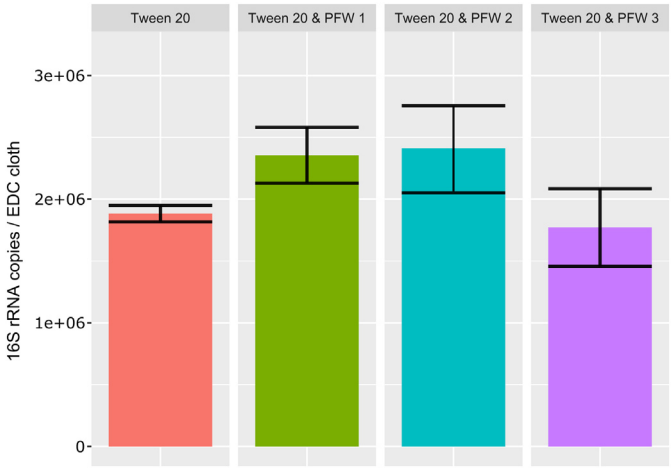

Fig. 7. 16S rRNA genes copy numbers obtained from EDC cloths spiked with 7.71 × 10^6 bacterial cells (32.3 × 10^6 16S rRNA gene copies) and extracted with four ways: Double washing with 0.05 % Tween 20 buffer, single washing with Tween 20 buffer followed by 1–3 rinsing with PFW. Each bar represent average of three replicates.

35 out 100 samples were removed after the filtration quality step duo to low number of reads. Switching to a filtration-based DNA extraction protocol, i.e., by collecting extracted dust pellets on membrane filters, showed a significant increase in DNA yield measured by quantitative PCR using similar DNA extraction methods.

In the DNA extraction study, the PowerSoil kit and DNA extraction method (protocol A) described by Lever et al. (2015) showed the highest DNA yields. Therefore, we performed another round of the DNA extraction using these two methods on replicate EDCs to study reproducibility of the DNA extraction in terms of DNA yield, bacterial richness, and bacterial composition. Protocol A resulted in more consistent DNA yields compared to the PowerSoil kit. However, the DNA yield obtained with the PowerSoil kit was sufficient and above the detection limit. Protocol A showed higher

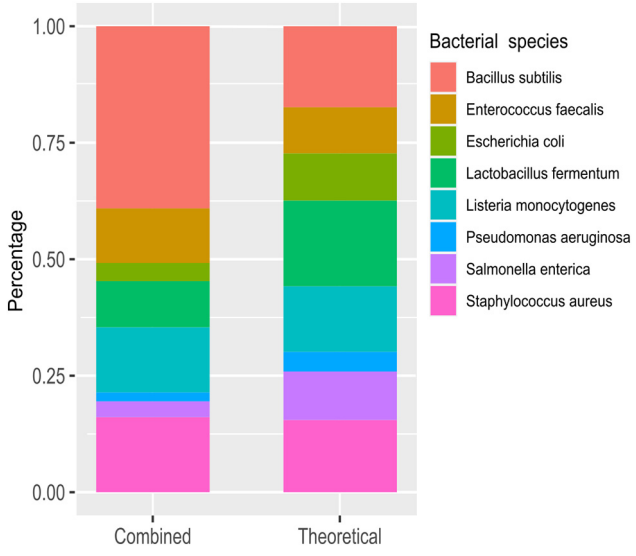

Fig. 8. Comparing the community composition of combined extraction method with the theoretical composition of the ZymoBIOMICS™ Microbial Community Standard. The combined stacked bar plot shows average community-level composition of species retrieved from 6 EDC clothes spiked with 10^6 bacterial cells from the standardized mock community and extracted by PowerSoil kit. For comparison, we show the theoretical composition of the 16S rRNA gene abundance for the standardized mock community.

diversity and richness for all EDC cloths, which indicates the ability of the method to extract more bacterial taxa from EDC cloths compared to the PowerSoil kit. Community composition obtained through both methods was comparable. However, the PowerSoil kit seemed to be better in detecting gram-positive bacteria such as *Firmicutes* and *Actinobacteria* than protocol A, suggesting that PowerSoil is able to break down the harder cell wall of gram-positive bacteria which is likely due to the bead-beating procedure. Depending on the downstream analysis and the scale of the study, we would recommend prioritizing one of the two protocols. Protocol A is better suited for deep metagenomic sequencing and for smaller studies where absolute quantification and reproducibility is of major importance. On the other hand, the PowerSoil kit's straightforward and fast procedure makes it amenable to large-scale epidemiological studies that may involve a large number of samples.

Comparing the combined extraction of DNA and endotoxin with the classical method for extraction of endotoxin (Noss et al., 2008) using replicate EDCs showed largely similar results. This is likely due to same extraction buffer used in the two methods (0.05 % Tween 20). Therefore, we conclude that a single EDC cloth can be used for extraction of both DNA and endotoxin allowing more flexibility in study design for epidemiological studies and reduced time required for sample analysis.

Optimization of the combined extraction method testing different buffers showed that Tween 20 resulted in a higher DNA and endotoxin yield compared to Tris HCL EDTA buffer. This could be due to the ability of surfactant (Tween 20) to separate dust particles from fibrous material such as the EDC cloths. Hoppe Parr et al. (2017) reported approximately two-fold increase in endotoxin units measured by LAL assay when using Tris HCL EDTA buffer compared to Tween 20 buffer (Hoppe Parr et al., 2017). However, they used house and barn dust collected by a high-volume small surface sampler and brushed off horizontal surfaces, respectively, while our study used airborne dust passively settled on the EDC cloths and therefore a low volume of material (Hoppe Parr et al., 2017). This indicates that Tween 20 is better for extracting dust from electrostatic EDC cloths compared to Tris HCl EDTA buffer.

Using a standardized mock community, we discovered that exposing EDC clothes to more bacterial cells result in higher relative DNA yields. However, in general, washing the EDC cloth with 0.05 % Tween 20 buffer twice produced a low DNA yield. Therefore, we further optimized the extraction for higher DNA yields. The dust extraction consists of two rounds washing the EDC cloth with 0.05 % Tween 20 buffer to extract the dust particles out of the EDC cloth. Recently, Shin et al. (2018) revealed the mechanism behind the removal of soil particles from fibrous materials using surfactant gradients produced via rinsing with fresh water that drives diffusiophoresis of soil particles out of fibrous materials (Shin et al., 2018). Using replicate EDC cloths, we showed that rinsing EDC cloth with PFW instead of second wash with Tween 20 buffer increases both DNA and endotoxin yields. This suggests that the washing step with PFW increases diffusiophoresis of dust particles out of the EDC cloth as explained by Shin et al. (2018). Subsequent washing steps did not further increase the yield and excessive washing with PFW actually decreased the DNA yield. Therefore, we chose to rinse the EDC cloth once with PFW after one single Tween 20 buffer wash to save time and decrease risk of contamination.

EDC cloths analysed using our developed protocol were successfully able to detect all bacterial taxa in the standardized mock community indicating the ability of the developed protocol to identify different bacterial taxa on the EDC cloths. However, the PowerSoil method revealed more gram-positive bacteria such as *Bacillus subtilis*. Previous studies have demonstrated that the microbial composition is primarily influenced by the efficacy of cell lysis rather than DNA recovery (Salonen et al., 2010; Scupham et al., 2007). A higher representation of *Bacillus subtilis* could be due to a prolonged bead beating (cell lysis) step, which could result in gram-negative genomic DNA shredding and thereby lower their representativeness. This is also in line with our observation that PowerSoil extraction of indoor dust resulted in a higher proportion of gram-positive Phyla *Firmicutes* and *Actinobacteria*. Thus, further optimization of the PowerSoil

DNA extraction method by decreasing the duration of the bead beating step might lead to a better species abundance representation.

Despite the ability of the developed protocol to extract different bacterial cell types without affecting the endotoxin quantification, one current limitation of the current study is that it does not test for the viability of bacteria collected on the membrane filter. Therefore, if the method is to be extended to studies of pathogen exposure, which rely on understanding the viability of pathogenic microorganisms, techniques such as cultivation or differential staining coupled with flow cytometry or fluorescent microscopy should be included. The main focus of the study was to address our ability to analyse airborne bacterial loads, diversity and community composition simultaneously with endotoxin analysis. Hence, we have not focused on other parts of the microbiota, such as fungi and glucans.

The developed protocol for simultaneous extraction of DNA and endotoxin from a single EDC cloth was planned as part of a large epidemiological study where hundreds of EDCs were collected. The detailed final protocol and materials used for combined DNA and endotoxin extraction from a single EDC cloth can be found in the supplementary material (see also Fig. 1).

5. Conclusion

After a series of pilot studies using different DNA extraction methods and different buffer systems, we finalized a joint protocol for DNA and endotoxin extraction using a single EDC cloth. Combined extraction of DNA and endotoxin would notably reduce the time and cost required for analysis of samples as well as allow other EDC cloths to be used in studying other analytes. Overall, in large epidemiological investigations addressing airborne exposure, that require a large number of samples, our technique supports a high quality to price ratio.

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Credit authorship contribution statement

VS, TS, IM, RB, and TŠ-T designed and carried out sample collection. IM, and TŠ-T planned the DNA extraction study. All authors planned optimization of DNA and endotoxin from a single EDC. HA extracted dust from EDC filters and carried out qPCR, LAL assays and the sequencing. HA, IW, and IM analysed the data from sequencing, qPCR and LAL assay. HA, RB, IM, and TŠ-T wrote the manuscript. All authors revised the manuscript.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Randi J. Bertelsen reports financial support was provided by European Research Council.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159455.

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