The bacterial composition of ventilation filter dust in Norwegian pre-school nurseries

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Original Article

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Abstract
The microbial content of dust collected from intake and exhaust air filters in six Norwegian nurseries was determined using 16S rRNA pyrosequencing and plate count analyses. The concentration of endotoxins was also estimated. About 96% of the sequences were classified as *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Cyanobacteria*. At the genus level, about 30% of sequences from the exhaust filter were classified as bacteria of probable human origin, such as *Streptococcus* and *Corynebacterium* species. These were close to absent in intake dust samples (<1%). This suggests that occupancy shapes the indoor microbiota, creating an environment relatively rich in genera of potential health significance. There were significantly greater counts of culturable bacteria in exhaust samples, indicating that passage of air through the nursery causes deterioration in the general air quality. Although there was significantly more endotoxin in exhaust dust, the endotoxin levels per colony forming unit were similar in both samples. The present study is the first of its kind in Norwegian nurseries, and is important as it reveals which groups of microorganisms nursery occupants are exposed to. In addition to possible direct health issues, the nature of our early exposure to microbes may be significant in the development of immunological conditions.

Keywords
Air quality, pyrosequencing, colony counts, endotoxins, ventilation systems, nursery, indoor ecology

Introduction

People spend much of the day indoors where they are exposed to the microorganisms that are present in buildings. In Norway, about 90% of children aged between 1-5 years attend nurseries, and 92% of this group spend up to 41 hours per week there.[1] Nursery attendance has been associated with an increased risk of upper respiratory illnesses, including bacterial ones such as otitis media.[2, 3]

The culturable fraction of indoor microorganisms has been relatively well-documented.[4-6] However, the majority of publications have focused on molds and their spores, and there is comparatively little information on bacteria in the indoor climate. The bacteria most often found in indoor environments such as schools and nurseries are gram-positive genera such as *Micrococcus* and *Staphylococcus*.[5] *Actinomyces* are also commonly grown from samples taken in schools and nurseries.[7] However, often only 1% of environmental microorganisms are easily cultured on standard media[8], a discrepancy which has been referred to as the "great plate count anomaly".[9] The advent of culture-independent methods has revealed far more complex microbial communities than those revealed by culture-based methods. Culture-independent methods based on amplification and sequencing of 16S rRNA genes can, when combined with high-throughput DNA sequencing, enable the identification of thousands of different bacteria within a single environmental sample. These methods are for the first time enabling a comprehensive, culture-independent mapping of the bacteria that surround us in our everyday life.[10, 11]

Understanding the origins of indoor microorganisms and how they are disseminated in buildings is important. Such knowledge could provide a framework for the implementation of dedicated measures to reduce exposures to detrimental microorganisms and their toxins. Indoors, bacteria are emitted and resuspended from humans, pets, plants, carpets, and other building surfaces.[12, 13] It has been shown that human occupants leave a distinct human microbial fingerprint indoors, and different room types and surfaces will have different fingerprints depending on their specific use.[14-16]

Heating, ventilation and air conditioning (HVAC) systems are necessary for meeting energy and air quality demands in modern buildings. These systems transport large quantities of air into and out of buildings and have extensive inner surface areas in the form of ventilation ducts and filters. Airborne constituents such as dust, plant particles, and microorganisms are also transported and spread by HVAC systems. One study that compared rooms ventilated via a HVAC system to naturally ventilated rooms, found that building design had a large impact on the indoor bacterial composition and the abundance of certain taxa.[17] An integral component of HVAC systems are air filters. Usually both air entering and leaving buildings is filtered. Filter capture depends on particle size, filtration efficiency, and running parameters. In Europe, filters are classified based on the lowest filtration efficiency in accordance with the European EN779:2012 standard.[18] In the United States the ASHRAE Standard 52.2 is most widely used.[19] HVAC units in public buildings in Norway are normally equipped with F7 filters. F7 class HVAC filters capture on average 80-90% of all 0.4 μm sized particles[18], and should, therefore, also retain most bacteria and other microorganisms entering and leaving buildings. As a consequence, the analysis of filter dust has been proposed as a viable approach for long term sampling of airborne microbial contaminants.[20]

In the present study microbial populations in filter dust were studied in order to:

a) indicate the types and numbers of bacteria that enter and leave Norwegian nurseries and thereby identify the taxa to which children and staff are routinely exposed.
b) study microbial capture by HVAC filters, and thereby provide an indication of how filters affect air quality.

The Norwegian climate shows great annual variation and is unlike that investigated in most previous studies.[21, 22] Few studies have been dedicated to the microbiome of nurseries[23], and the present study is the first to our knowledge that documents the types and number of microbes entering and leaving Norwegian nurseries via HVAC systems.

Materials and methods

Sampling sites and sample preparation

Samples were collected from HVAC units in six nursery buildings situated in the Oslo and Akershus area in Norway (Table 1). Sampling was conducted in the period fall 2013 to fall 2015. All the nurseries were situated in urban areas and exposed to traffic in the form of nearby roads or railways. For all nurseries, the air intake for the HVAC units was placed either on the roof or high up on an external wall. Each HVAC unit was programmed to run from approximately two hours prior to opening hours, and to shut down one hour after closing time.

Table 1. Sampling sites. Samples were collected at six nurseries. Two of the nurseries were serviced by more than one heating, ventilation and air conditioning (HVAC) unit.

Nursery (N)		**HVAC Unit**			**Filter dust sample ID**	
Site	# of HVAC units	Unit ID	Heat exchanger	Filter Class[a]	Intake	Exhaust
N1	3	N1.1	Rotary heat exchanger	F7	N1 (pooled)[b]	N1.1
		N1.2	Rotary heat exchanger	F7		N1.2
		N1.3	Rotary heat exchanger	F7		N1.3
N2	2	N2.1	Rotary heat exchanger	F7	N2 (pooled)[c]	N2.1
		N2.2	Rotary heat exchanger	F7		N2.2
N3	1	N3	Rotary heat exchanger	F7	N3	N3
N4	1	N4	Plate heat exchanger	F7	N4	N4
N5	1	N5	Rotary heat exchanger	F7	N6	N5
N6	1	N6	Rotary heat exchanger	F7	N6	N6

[a] According to EN779:2012 Standard[18]

[b] Intake dust samples from HVAC unit N1.1, N1.2 and N1.3 were pooled together to make one intake dust sample for nursery N1.

[c] Intake dust samples from HVAC unit N2.1 and N2.2 were pooled together to make one intake dust sample for nursery N2.

Collection of dust samples from filters

Figure 1 shows the general construction of the HVAC units examined and position of the filters sampled (Figure 1(a) and (c)). At each of the sampling sites dust was collected from the inside surface of HVAC filters by vacuuming using a Flite2 area sampling pump fitted with a sterile plastic 0.4 μm polycarbonate micro-vacuum cassette (SKC Inc, PA, USA). In nurseries that had more than one HVAC unit, one exhaust dust sample was collected for each HVAC unit. This was because the exhaust air originated from different rooms with different use, occupancy and size. Intake dust samples from each HVAC unit were pooled together into one sample before testing (Table 1). Samples were prepared for subsequent analyses as summarized in Table 2. All dust samples were analyzed by 16S rRNA gene sequencing. Dust samples collected from four nurseries were further analyzed for colony forming units (CFU) counts and endotoxin (EU) concentrations.

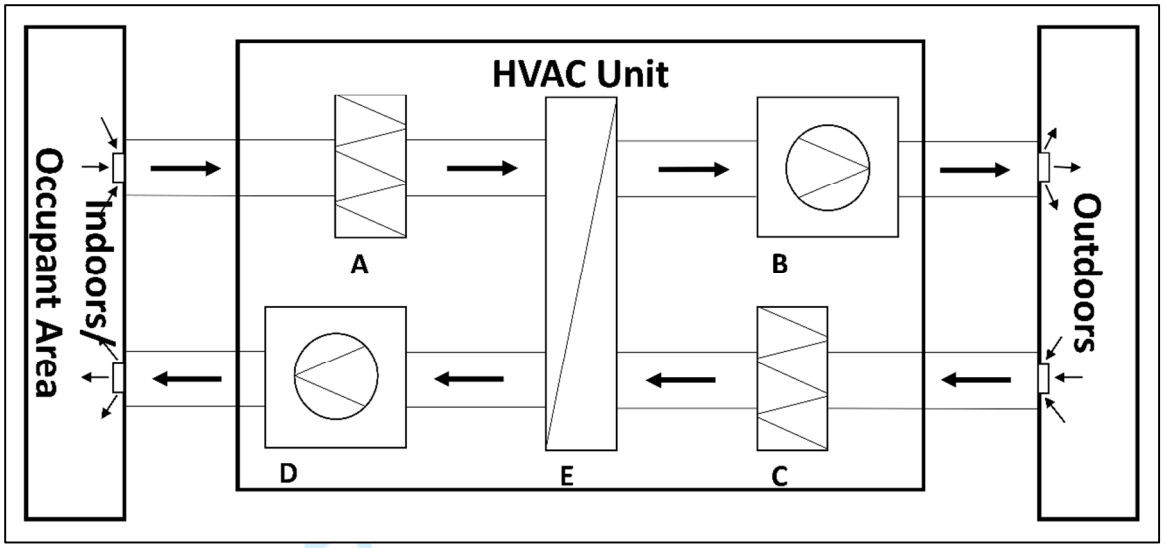

Figure 1. Simplified schematic diagram of the nursery heating, ventilation and air conditioning (HVAC) units and the major components. a: exhaust filter (sampling site), b: exhaust air fan, c: intake filter (sampling site), d: intake air fan, e: rotary or plate heat exchanger depending on the specific HVAC unit.

Isolation of bacteria from dust samples

Samples were transported to the laboratory and bacteria were extracted from dust samples in the same working day using the following procedure. Twenty mg dust was aseptically transferred to a sterile 1.5 ml Eppendorf tube containing a spatula of acid-cleaned, sterile glass beads (Ø =0.5 mm). The dust was suspended in 2 ml molecular biology grade water amended with 0.002% ultrapure Tween 20 (Rockland Immunochemicals, PA, USA). The contents were vortexed vigorously in 3 × 1min. rounds with intermittent ice bath cooling using a Vortex Genie (Scientific Industries Inc., LA, USA). After vortexing, the tubes were allowed to stand in a vertical position for 15 min. after which the upper 1 ml was removed for plate count analyses.

Table 2. Sample analysis. Tests performed and number of samples analyzed.

Nursery (N)	**Filter dust samples analyzed per nursery**					
	16S rRNA analysis		Culture analysis		Endotoxin	
Site	Intake	Exhaust	Intake	Exhaust	Intake	Exhaust
N1	1	3	1	3	1	3
N2	1	2	1	2	1	2
N3	1	1	1	1	1	1
N4	ND[a]	1	1	1	1	1
N5	1	1	-	-	-	-
N6	1	1	-	-	-	-

[a] No data, not possible to collect sufficient quantities of dust for analysis.

Culture analysis

Sample portions of 100 μl, diluted where necessary, were spread onto some or all of the following agar types. Incubation conditions and the suppliers' details are given in parentheses. Tryptone soya agar (TSA) (37 °C; 36h; Oxoid, Basingstoke, UK); R_2A agar (27°C; 14d; Oxoid); Sabouraud dextrose agar containing 0.1 mg/ml chloramphenicol

(SAB) (27°C; 4d; Oxoid); Dichloran Rose-Bengal Chloramphenicol agar containing 0.1 mg/ml chloramphenicol (DRBC) (27°C; 4d; Oxoid). TSA and R_2A were supplemented with 10 mg/l amphotericin B (Sigma-Aldrich, St. Louis, Missouri, USA) to prevent fungal growth. The final counts were recorded as CFU per mg dust.

Endotoxin analysis

Endotoxins present in dust samples were measured as EU/mg dust using an end-product chromogenic test based on Limulus Amebocyte Lysate (LAL) (QCL-1000 assay kit; Lonza, Basel, Switzerland). All glass tubes, pipette tips and other materials were certified endotoxin-free. Approximately 100 mg dust was added to 2 ml endotoxin-free water amended with endotoxin-free tween 20 at 0.05% in 10 x 75 mm tubes (Lonza; catalogue N201). Dust samples were thoroughly suspended by vortexing at maximum speed using a Vortex Genie for 30s. Samples were visually inspected for the absence of clumps in the suspended material. Capped tubes were subsequently shaken in the horizontal position at 300 rpm. for 90 minutes to release endotoxin, and then centrifuged at 1000 × g for 15 min. to separate out the dust. From each tube, 1 ml of the upper portion of supernatant was transferred to a clean tube using a heat-treated boro-silicate Pasteur pipette. The samples were immediately placed vertically at 4°C and allowed to stand for an additional 30 min. to allow further precipitation of larger particles. Testing was performed on 50 μl portions taken from the surface layer of the extracted endotoxin samples. The control *Escherichia coli* endotoxin (supplied) was suspended in endotoxin-free water at the prescribed concentration. Dilutions in the range of 0.1 – 1.0 EU/ml were made as described in the protocol for the production of standard curves. Thereafter 50 μl of samples, dilutions of the standards (in the range 10^{-1} – 10^{-5}), and water as negative control were tested for the presence of endotoxin in pre-warmed microtiter plates held without shaking at 37 ± 1°C (Thermoshaker, Quantifoil GmbH, Jena, Germany). Measurements were performed exactly as described in the manufacturer's protocol. In brief, after final incubation in the presence of the chromogenic substrate, the reaction was stopped by the addition of 25% acetic acid. The absorbance in each well was measured at 405 nm using a Victor 1420 Multilabel Counter (Perkin Elmer, Turku, Finland) and the concentration of endotoxin in samples was measured by reference to a standard curve generated from the supplied endotoxin control. For the samples, the average value of endotoxin concentrations falling within the linear range of the standard curve was calculated. Experiments were repeated once and the average toxin concentration of the two runs was calculated. The coefficient of correlation (r) for the standards was greater than the value of 0.98 stipulated for acceptance of the standard curve. Finally, results were converted to EU/mg dust.

Isolation of DNA from dust samples

DNA was extracted from approximately 100 mg dust using the PowerWater® DNA isolation kit (MO BIO, CA, USA). Prior to chemical extraction, dust samples were pretreated using a μ-MiniBeadbeater (BIOSPEC, OK, USA) as follows: Dust was weighed into a bead-beater tube (BIOSPEC), and then thoroughly mixed into 1 ml of prewarmed (55 °C) PW1 from the kit using a pipette tip. Tubes were placed at 55°C for 10 min. to aid lysis. The remainder of the tube was then filled by the addition of beadmix from the kit. The dust/bead mix was homogenized using the beadbeater set at maximum

effect for 5 min. The liquid fraction of the tube (about 1ml) was transferred to an Eppendorf tube and centrifuged at 13000 × g for 1 min. Taking care to avoid the precipitate the aqueous phase was transferred to a new Eppendorf tube. Thereafter 200 μl of PW2 from the kit was added, and the kit protocol was followed to completion. Final elution of the DNA was with 75 μl of molecular biology grade water prewarmed to 55°C. Eluted DNA was quantified and the 260/280 ratio was measured using a ND-1000 spectrophotometer (NanoDrop, DE, USA). DNA was stored at -20 ºC until required for downstream applications.

16S rRNA gene sequencing

PCR amplification was performed using the universal bacterial 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 338R (5-TGCTGCCTCCCGTAGGAGT-3) primers, which amplify the V1–V2 regions of the 16S rRNA gene.[16] Primers were designed for use with the GS Junior system (454 Life Sciences, Branford, CT, USA). Adaptors and multiplex identifiers (MIDs) were included in primer sequences which were purchased from Eurofins Genomics laboratories (Ebersberg, Germany). PCR reactions were carried out in a total volume of 50 μl with the following components: 10 μl 5X Flexi buffer (Promega, Fitchburg, WI, USA), 2 μl dNTPs (10 mM, Promega), 0.2 μl GoTaq polymerase (5U/μl, Promega), 3 μl $MgCl_2$ (25 mM, Promega), 31 μl certified nucleic-acid free water (Invitrogen, Waltham, MA, USA), 1 μl (25 μM) forward primer (Eurofins), 1μl (25 μM) reverse primer (Eurofins), 1.5μl template DNA. PCR-conditions were as follows: 5 min. initial denaturation at 94°C; 35 cycles of 60 seconds at 94°C, 45 seconds at 55°C and 90 seconds at 72°C; and 10 min. at 72°C for final extension. PCR amplicons were size controlled and quantified by gel electrophoresis using Low Mass DNA Ladder standards (Invitrogen). PCR amplicon concentrations were calculated based on the standards using a Bio Rad Gel Doc XR+ system with Bio Rad Image Lab software (Hercules, CA, USA). Triplicate reactions for each sample were pooled at equimolar concentrations prior to sequencing. DNA was sequenced unidirectionally at Eurofins Genomics laboratories with Roche/454 GS Junior system.

Analysis of 16S rRNA gene sequences

Sequences were processed and analyzed using the default parameters in QIIME (Quantitative Insights into Microbial Ecology) version 1.8.0.[24] Briefly, raw sequences were first assigned to their dust sample of origin using their respective MIDs. Low quality sequences were removed. Quality filtering settings were as follows: sequences were required to be minimum 200 basepairs in length, have no less than a quality score of 25, contain no more than six ambiguous bases and have no primer mismatches. After barcode assignment and quality control, the remaining high-quality sequences were denoised using QIIME denoiser using default settings, and assigned into operational taxonomic units (OTUs) at a 97% sequence similarity cutoff using UCLUST.[25] Representative sequences were then aligned using PyNast[26] against the reference sequences from Greengenes[27] (2013 release), and assigned taxonomically with UCLUST. To eliminate potential bias due to sampling depth, all samples were rarified to 19 000 sequences per sample. FastTree[28] was used to generate phylogenetic trees. For community analysis, Fast UniFrac[29] was utilized to produce principal coordinate analyses (PCoA) to compare the phylogenetic distances between samples. The unprocessed DNA

sequences obtained in this study have been deposited in the NCBI SRA archive under accession number SRP059818.

Statistical analysis

The paired t-test was used to compare colony counts, endotoxin concentrations, and normalized endotoxin values (EU/CFU_{R2A}) in intake and exhaust dust samples.

Results

Bacterial diversity

Fourteen dust samples from six nurseries, five from intake filters and nine from exhaust filters (Table 2) were analyzed for their bacterial composition by pyrosequencing. After quality control, 12 636 OTUs defined at a 97% sequence similarity cut-off were detected in a total of 800 14716S rRNA sequences. After adjusting the sampling depth to 19 000 sequences per sample, 10 504 distinct OTUs from 266 000 sequences were obtained. Rarefaction analysis showed that additional sampling depth would be unlikely to greatly increase the number of unique sequences detected (results not shown). Exhaust samples were generally the most diverse with an average of 2415 OTUs detected per sample. Intake samples had on average 1835 OTUs per sample (Table 3).

Table 3. Bacterial diversity. Alpha diversity in analyzed sequencing samples.

Nursery (N)		**Alpha diversity metrics at 19 000 sequences**		
Sample	**Site**	Number of OTUs	CHAO1	Faith's Phylogenetic Diversity
Exhaust	**N1**	2703	3697	110
		2787	3685	115
		2824	3737	114
	N2	2231	3162	96
		2577	3552	104
	N3	2244	3183	106
	N4	2346	3111	111
	N5	2273	3194	110
	N6	1753	2352	88
Exhaust average		**2415**	**3297**	**106**
Intake	**N1**	2748	3872	109
	N2	2382	3403	96
	N3	1523	2305	81
	N5	2281	3129	91
	N6	240	456	21
Intake average		**1835**	**2633**	**79**

Weighted Unifrac PCoA. The bacterial composition of the exhaust and intake samples were compared quantitatively using principal coordinate analysis (PCoA) on a weighted UniFrac basis (Figure 2). The first two principal components (PC1 and PC2) combined explained approximately 85 % of the variation in the weighted Unifrac distances between samples. The exhaust and intake samples form distinct clusters, with the exhaust samples grouping more closely. The PCoA reveals that the bacterial communities recovered from the exhaust filter dust were significantly distinct from those of the of the intake dust ($p<0.01$). This indicates that the origin of the HVAC unit is less important than the nature of the sample (i.e. intake or exhaust) as a source of variation.

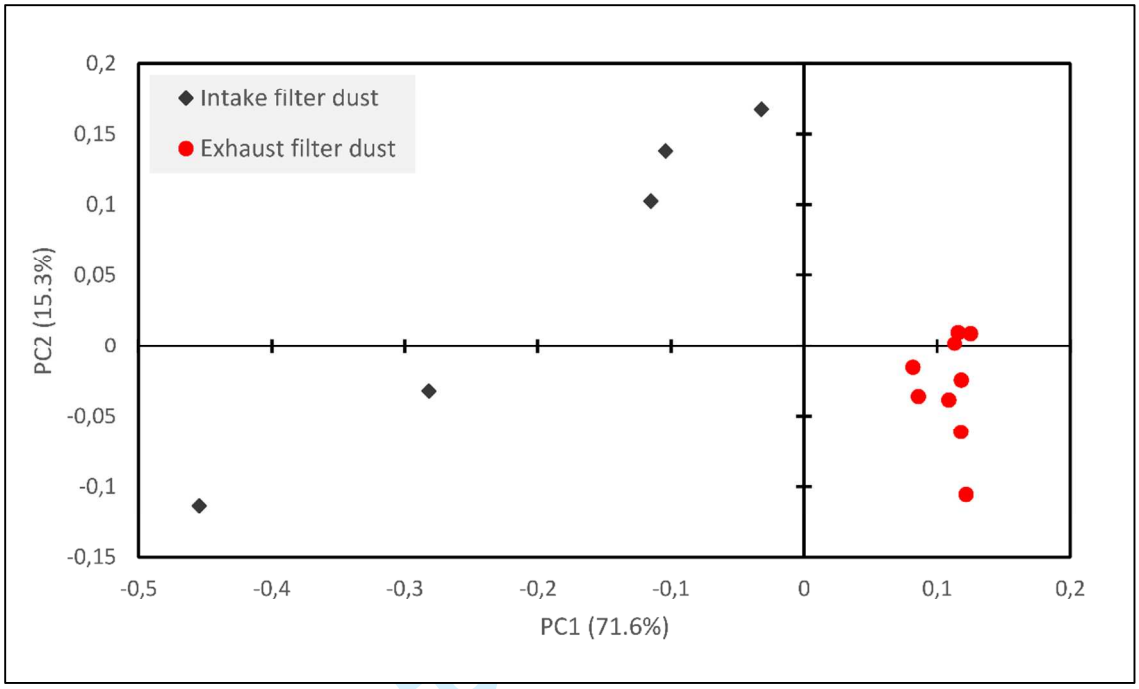

Figure 2. Comparison of bacterial populations in ventilation filter dust. Weighted UniFrac-based bacterial diversity principal coordinate analysis of exhaust filter dust (red circles) and intake filter dust (gray diamonds) from this study. Each point represents a dust sample.

Taxonomic composition of filter dust. Bacterial communities on HVAC filters were analyzed for taxonomic affiliation by pooling the 16S rRNA gene sequences from each nursery into exhaust and intake sample populations. Dust samples were largely dominated by *Actinobacteria, Proteobacteria, Firmicutes, Bacteroidetes* and *Cyanobacteria* (Figure 3(a)). These five phyla together made up approximately 96 % of the relative sequence abundance in the samples. *Actinobacteria* was the most dominant phylum in exhaust samples, whereas *Cyanobacteria* was the most dominant phylum in intake samples. *Proteobacteria* was the second most dominant phylum in both sample sets.

Major bacterial genera present in dust samples. The most commonly detected OTU in exhaust samples showed greatest similarity to *Micrococcus* sp., whereas the most common OTU in intake samples was *Streptophyta.* The 20 most prevalent genera in exhaust and intake samples and their relative sequence abundances are shown in Figure 3(b) and (c). These figures show clear differences between the bacterial communities of exhaust and intake filter dust. Several human-health related genera that are known to include potential pathogens were identified in exhaust dust samples and comprised 29% of all sequences in these, whereas they were close to absent in intake dust samples (<1%). The genera indicated were *Micrococcus*, *Streptococcus*, *Corynebacterium, Propionibacterium,* and *Staphylococcus*.[14, 17, 30-32] Sequences classified as *Micrococcus* and *Streptococcus* were dominant in exhaust samples making up 16% and 8% of the sequences respectively. Some genera which include primary pathogenic species, such as *Legionella* sp., *Burkholderia* sp., *Neisseria* sp., and *Mycobacterium* sp. were also indicated, but with low relative sequence abundance (<1%). Intake dust samples were

strongly dominated by *Streptophyta* sequences, which made up 38% of all the sequences. *Streptophyta* DNA accounted for less than 5% of the sequences in exhaust samples.

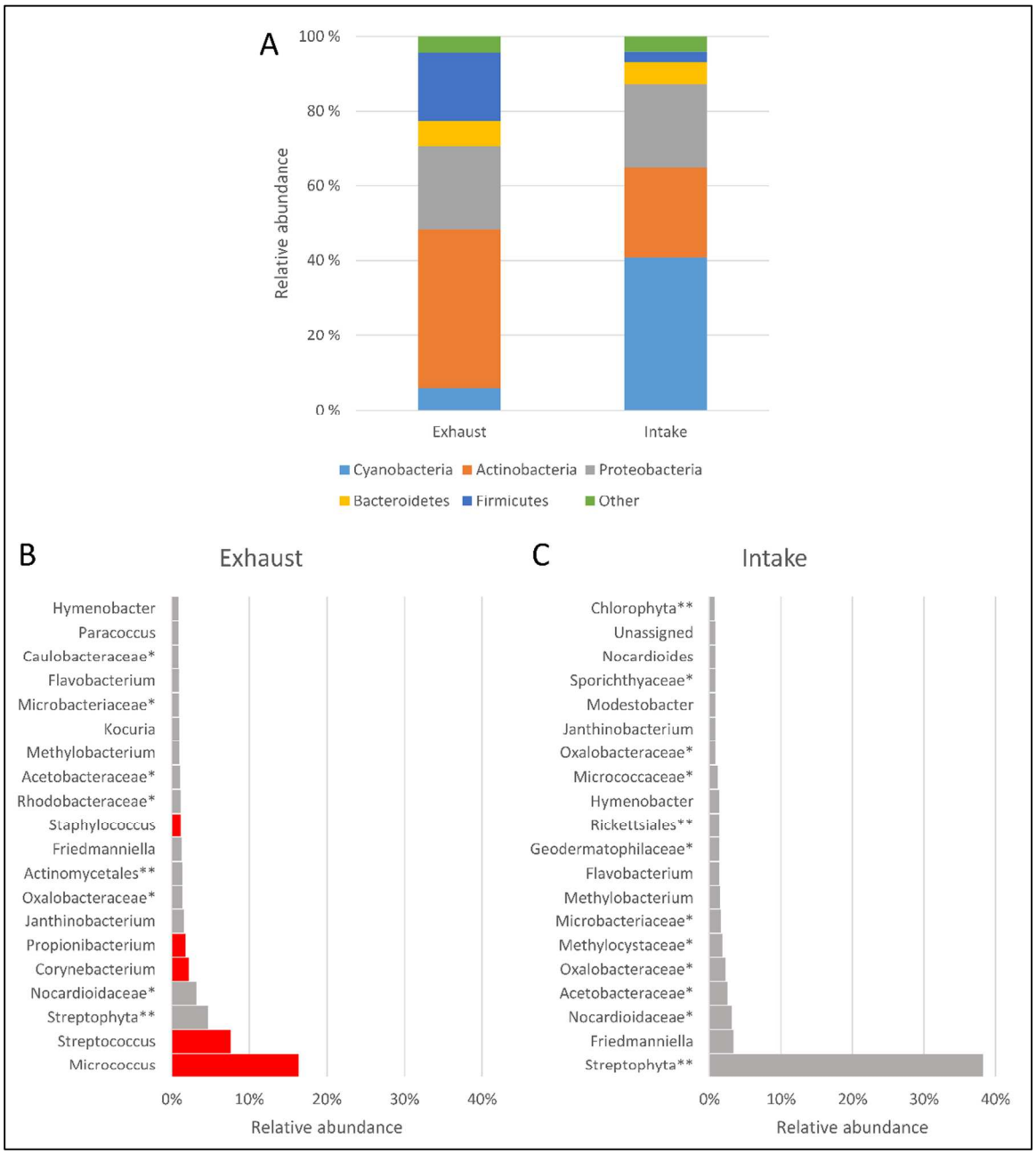

Figure 3. Taxonomic composition of bacteria in ventilation filter dust samples. Relative abundance of the five most abundant bacterial taxa identified at the phylum level in exhaust filter dust and intake filter dust (**a**). The relative abundance of the 20 most abundant bacterial groups identified at the genus level in exhaust filter dust (**b**) and intake filter dust (**c**). All taxa are classified to the genus level, or the highest taxonomic level to which they could be confidently assigned (*family, **order). Genera that are highlighted in red are considered human-health related.

Culture analysis

CFU measurements were carried out on the dust samples to estimate the readily culturable fraction of bacteria and fungi in these. Regardless of the agar type, the CFU/mg dust for exhaust samples was greater than that of intake samples (Table 4).

However, the difference was only significant for bacterial counts (TSA and R_2A) ($p < 0.05$). For both the fungal agars (SAB and DRBC) the CFU/mg values were not found to be significantly different ($p > 0.05$). The counts on TSA at 37°C with a short incubation period were similar to those obtained after prolonged incubation on R_2A at 27°C. The results would be in keeping with the presence of large numbers of human-associated bacterial genera in exhaust dust.

Endotoxin

Table 4 shows the concentration of endotoxins in intake and exhaust dust samples. There was a significantly greater endotoxin concentration in exhaust samples compared to intake samples ($p < 0.05$). However, when endotoxin concentrations were normalized against CFU counts on R_2A, the difference between exhaust and intake was no longer found to be significant ($p >> 0.05$). This suggests that exhaust samples are not enriched for endotoxin producing genera.

Table 4. Colony forming units (CFU)/mg and endotoxin concentrations in ventilation filter dust.

Nursery (N)			**Agar type (CFU/mg dust)**				**Endotoxin**
Site	Dust sample	Sample ID	TSA	R_2A	SAB	DRBC	EU/mg dust
N1	Intake	N1	80	210	23	34	0.18
	Exhaust	N1.1	15682	15036	357	1995	7.78
		N1.2	20240	41400	322	1380	4.84
		N1.3	1728	19968	360	840	7.13
N2	Intake	N2	33	12	216	314	0.80
	Exhaust	N2.1	22560	20832	2280	4440	6.75
		N2.2	16899	8736	4264	3718	6.05
N4	Intake	N4	6	5	7	6	0.53
	Exhaust	N4	860	2348	163	267	4.08
N5	Intake	N5	138	31	43	32	ND[a]
	Exhaust	N5	11250	9000	340	490	8.65

[a] Not detected

Discussion

The major aim of the present study was to provide a first comprehensive culture-independent mapping of bacteria that enter and leave Norwegian nurseries through HVAC systems. The results bring us closer to understanding how air quality is changed with respect to its content of microbes on passage through the indoor environment of Norwegian nurseries. The study provides information on the exposure of staff and children to microbes of potential health significance.

Bacterial composition of filter dust

Filter dust was analyzed for its bacterial content by both culture (see below) and culture independent methods. Filter dust is readily available, and has the advantage of representing airborne microorganisms entering and leaving the whole building volume. Several studies have found that indoor environments have increased concentrations of human-related microorganisms.[14, 30, 31, 33] Dust taken from HVAC filters in Norwegian nurseries provides support for this. Sequence analysis of intake and exhaust filter dust samples showed that these were composed mainly of five phyla that together made up more than 96% of the relative sequence abundance in each sample. These five phyla have also been identified as the dominant ones in other indoor microbiome studies.[30, 33] Of these five phyla, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* are also the proposed main constituents of the human microbiota[34, 35], thus providing support for an origin of exhaust filter bacteria in human activity. The results are in keeping with occupancy being the major factor in the shaping the indoor microbiota in Norwegian nurseries. Due to energy efficiency demands, staff are often instructed to open windows as little as possible, thus restricting natural ventilation. This practice is probably a contributing factor to the high proportion of human-health related bacteria in exhaust dust.[36]

Genus level classification supports the conclusions drawn from phylum level identification. A high proportion of the sequences from exhaust dust samples represented genera that contain potential pathogens. Daycare attendance has previously been associated with an increased risk of upper respiratory illness. Bacterial genera that are known to inhabit the human microflora, especially the skin, such as *Micrococcus, Streptococcus, Corynebacterium, Propionibacterium,* and *Staphylococcus*[34, 37] comprised about 30% of all sequences in exhaust samples and yet were almost absent in intake samples. This is of potential relevance for the health of nursery occupants. Some etiological agents of respiratory disease are bacterial. These include agents of tonsillitis and otitis media (*e.g.* group A S*treptococci*), and other upper respiratory tract infections (*e.g. Haemophilus influenzae* and *Streptococcus pneumoniae*). *Streptococcus* is a common constituent of the human microbiome, and several *Streptococcus* species are associated with diseases in humans, including respiratory tract infections.[34, 37-39] Several bacterial genera commonly associated with otitis media, including *Moraxella,* and *Haemophilus* as well as *Streptococcus*[40], were detected in the sequence analysis of exhaust dust samples. The genus *Corynebacterium* contains several medically important species that are pathogens or opportunistic pathogens.

Micrococcus, which was the most predominant genus in exhaust samples, has also been found to be most prevalent in other comparable studies.[30, 41]

Streptophyta sequences dominated in the intake dust samples and were much less prevalent in exhaust samples (Figure 3(b) and (c)). This is a chloroplast 16S rRNA encoding gene found in green plants, algae, and certain bacteria. Our findings are supported by previous studies which found less chloroplast DNA in mechanically ventilated rooms than those with natural ventilation.[17] Mechanical ventilation systems might then be beneficial to those suffering from pollen allergies. It has been found that HVAC systems equipped with MERV13 filters, the ASHRAE equivalent to F7 filters, effectively reduced indoor levels of common asthma and allergy triggers such as cat allergens and airborne $PM_{2.5}$.[42] Eleven of the 13 most prevalent taxa in intake dust were also found to be among the 20 most prevalent taxa in exhaust dust. These included genera commonly isolated from outdoor environments, such as soil and water. These were *Oxalobacteraceae, Janthinobacterium, Flavobacterium* and *Friedmanniella.*

Culture analyses

Viable bacteria and fungi from the dust samples were cultured on several agar media under different conditions to reveal the presence of a wide range of microbial groups. Most culture-based studies on microbes indoors have focused on fungi, owing to their association with dampness. However, as table 4 shows, the CFU/mg dust counts for bacteria were far greater than those for fungi. The presence of bacteria in the indoor environment has probably not received enough attention. In addition to their importance as agents of respiratory disease (see above), some genera such as *Streptomyces* sp. are strongly associated with moisture damage. *Streptomyces* have, furthermore, been found to stimulate the production of pro-inflammatory mediators even more strongly than many problem fungi[43], and this is of potential relevance in the development of immunological disorders.

Most earlier comparable studies have typically used only nutrient-rich media such as MEA, DG18, Rose Bengal, Czapek-Dox, or TSA[4, 5, 7, 41, 44], which limit the detection of many environmental species. TSA is a nutrient-rich general purpose agar used in the isolation and cultivation of a wide-variety of fastidious and nonfastidious microorganisms. It is used in standard quality control tests of both water and wastewater.[45] R_2A is primarily used for the isolation of bacteria in potable water.[46] It contains a wide-range of carbon sources and is more nutrient-poor than TSA. Many environmental species are readily overgrown by fast growing microbes on nutrient-rich, agars making their enumeration difficult.[46] It was hypothesized that the combined use of TSA and R_2A at the given incubation temperatures in the present study would lead to the detection of a wider range of bacterial strains, providing a truer picture of microbial numbers and diversity in dust. Generally, similar numbers of bacteria where obtained on the nutrient rich agar (TSA) at 37°C/48 hrs. and the relatively nutrient poor R_2A at 27°C/14 d. (Table 4). This supports the notion that the majority of bacteria exiting the nurseries were of human rather than of environmental origin, and is in keeping with the conclusions drawn from the sequencing data, i.e. that room occupants primarily shape the indoor microbiome. The fungal count on DRBC were greater than on SAB, and the former should be favored in future studies.

The exhaust air dust was far more heavily contaminated with microorganisms than the intake air dust. It was shown that exhaust dust samples could have up to 1000-times higher concentrations of viable bacteria than intake dust samples at the same

nursery (Table 4). This tendency was seen regardless of agar type and culture conditions, suggesting that the difference applies to a wide range of bacterial and fungal taxa. Comparable studies on the readily culturable fraction of domestic dust samples, typically find the culturable fraction ranges from 100 to 20 000 CFU/mg dust.[41, 47] The present findings are in a similar range, suggesting that filter dust is comparable in this respect to dust collected from indoor surfaces. A study by Hospodsky et al.[14] also shows that rooms, when occupied, had 66 times more airborne bacterial DNA than when they were vacant..

Endotoxin in dust samples

There were significantly higher concentrations of endotoxins in the exhaust dust than in the intake dust (Table 4). Possible explanations are that airborne particles from the indoor environment carry higher levels of endotoxins, or endotoxin producing bacteria, than those from outdoor areas. The main source of gram-negative bacteria from indoor environments is settled dust.[41] Comparisons of the proportions of gram-negative bacteria in the intake and exhaust dust are complicated due to the large proportion of sequences typing as cyanobacteria. However, the two main phyla containing gram-negative species (*Bacteroidetes* and *Proteobacteria*) make up approximately equal proportions of both the intake and exhaust sequences (Figure 3(a)). Furthermore, the readily culturable bacterial load in exhaust dust samples was much higher than in intake samples (Table 4). This is the most likely explanation of the difference in endotoxin levels. When the endotoxin concentrations were normalized with respect to colony counts, it was seen that there was no significant difference between the content of intake and exhaust dust samples. This result is important as it suggests that most gram negative species are not generally enriched in the indoor nursery environment. To our knowledge this has not previously been investigated. Filters did not show signs of moisture during inspection, and are probably dried by the continual airflows. Notwithstanding, indoor air by virtue of high CFU counts may be a more important source of exposure to endotoxins than outdoor air. Endotoxin concentrations in floor dust samples have been found to range from <1 to 300 EU/mg dust, but are typically around 20 EU/mg dust.[41, 48] In the present study it was found that endotoxin concentrations in HVAC filter dust, maximally about 8 EU/mg (Table 4), were lower than what can be expected to be found in domestic floor dust samples, which are typically between 16-18 EU/mg dust.[41, 48] This suggests that HVAC filters, when properly maintained (*e.g.* avoiding dampness) do not accumulate endotoxins. It is important to measure endotoxin concentrations because it is suspected that endotoxin in the indoor environment could have effects on the development and exacerbation of asthma and allergies and other symptoms.[49, 50] One study found that endotoxin levels in schools were up to four times higher than in homes, and that high exposure to endotoxins at school is more likely to correlate with asthma than high endotoxin levels at home.[51, 52] Some studies suggest that nurseries could have endotoxin levels even greater than schools.[51, 53] Exposure to endotoxin at an early age has also been found in some studies to have a protective effect with regards to the development of allergic disease.[3, 54, 55] One study found that higher endotoxin levels in domestic dust could partly explain the lower prevalence of atopic disease among Estonian two-year olds when compared to Swedish two-year old.[48]

Conclusion

This study used pyrosequencing to identify bacteria present in HVAC filter dust and is, to our knowledge, the first of its kind in Norwegian nurseries. As most previous work on the indoor climate has focused on fungi, indoor exposure to bacteria is not well characterized. The results provide a comprehensive overview for future reference of the bacteria which children and staff are exposed to, and could be combined with epidemiological data on disease and ill health in nursery children.
Pyrosequencing showed that the composition of bacterial taxa retained on HVAC filters was similar to those found in indoor dust in other studies. This suggests that filter dust sampling could be an appropriate, simple method for collecting samples that are representative of long term exposures in the indoor environment. Many recent studies have relied only on culture-independent analysis methods and not included culture analysis. Although not detecting all taxa, culturing should indicate the relative numbers of microorganisms present under different conditions and settings. A striking difference in bacterial numbers leaving and entering nurseries was found and this probably also influences exposure to endotoxins. Colonies that have grown on the media in this study have been isolated in pure culture and will be identified in future work. As HVAC systems are increasingly important for the shaping of our indoor environment, the present study is important as it shows which bacterial taxa are retained and collected on HVAC filters. This could have practical implications for future ventilation practices.

Authors' contribution

All authors contributed equally in the preparation of this manuscript.

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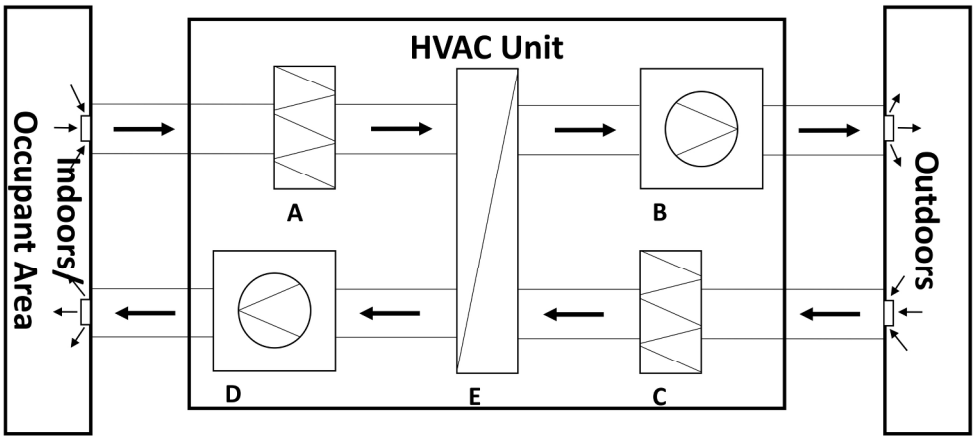

Figure 1. Simplified schematic diagram of the nursery heating, ventilation and air conditioning (HVAC) units and its major components. A: exhaust filter (sampling site), B: exhaust air fan, C: intake filter (sampling site), D: intake air fan, E: rotary or plate heat exchanger depending on the specific HVAC unit.

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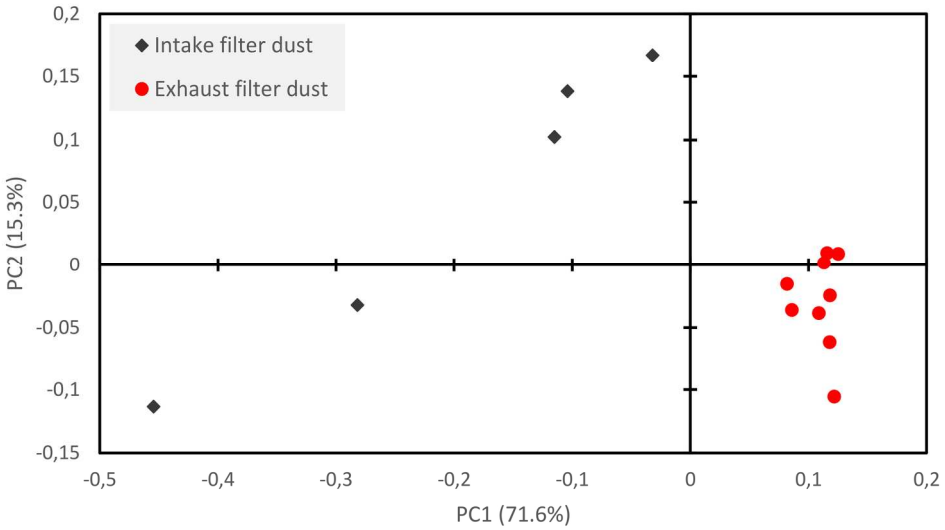

Figure 2. Comparison of bacterial populations in ventilation filter dust. Weighted UniFrac-based bacterial diversity principal coordinate analysis of exhaust filter dust (red circles) and intake filter dust (gray diamonds) from this study. Each point represents a dust sample.

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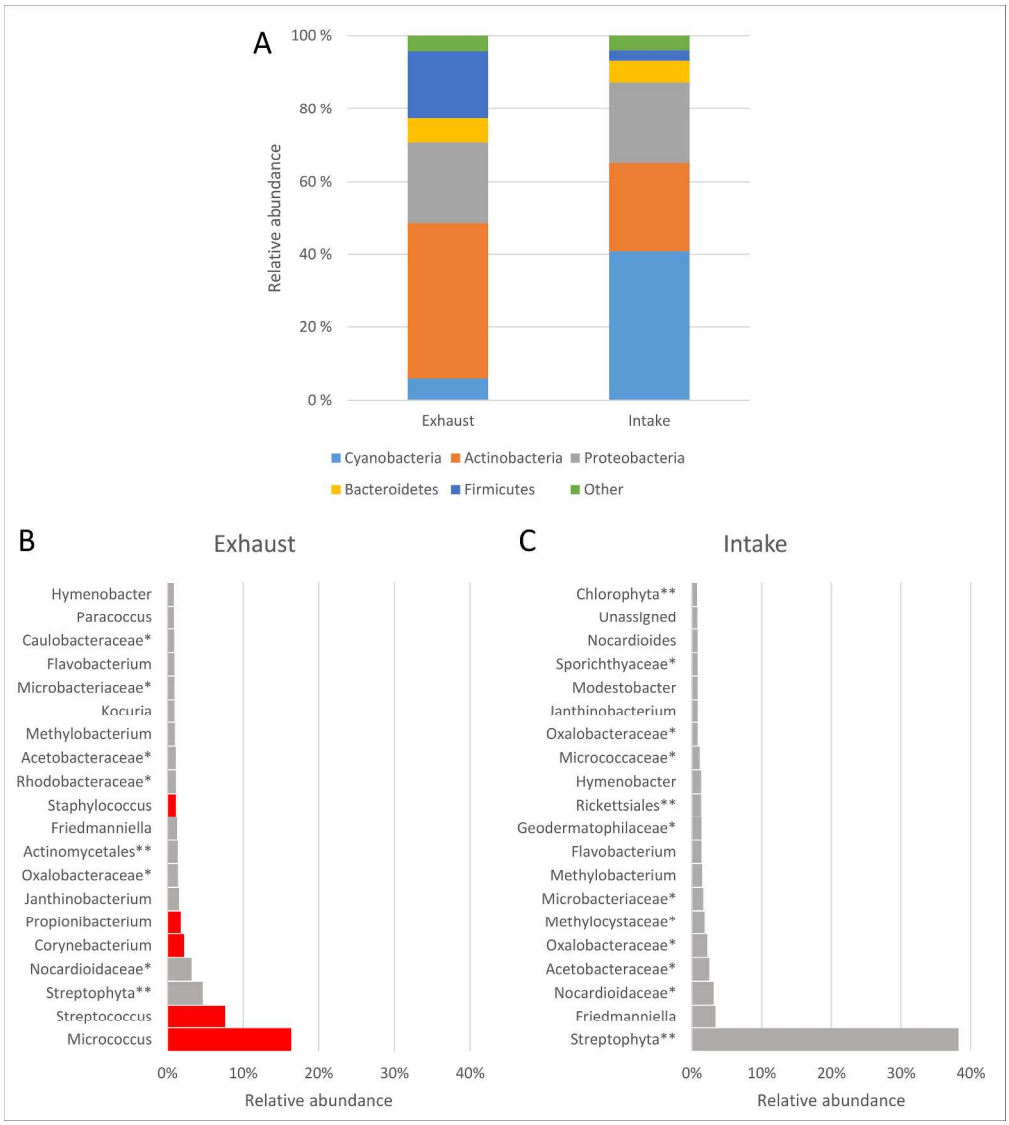

Figure 3. Taxonomic composition of bacteria in ventilation filter dust samples (all nurseries). Relative abundance of the five most abundant bacterial taxa identified at the phylum level in exhaust filter dust and intake filter dust (A). The relative abundance of the 20 most abundant bacterial groups identified at the genus level in exhaust filter dust (B) and intake filter dust (C). All taxa are classified to the genus level, or the highest taxonomic level to which they could be confidently assigned (*family, **order). Genera that are highlighted in red are considered human-health related.

208x232mm (600 x 600 DPI)