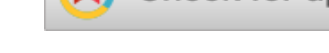

Received: 27 January 2021 | Revised: 20 April 2021 | Accepted: 19 May 2021

DOI: 10.1111/ina.12865

ORIGINAL ARTICLE

WILEY

Microbial exposures in moisture-damaged schools and associations with respiratory symptoms in students: A multi-country environmental exposure study

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Funding information
European Commission, Grant/Award Number: 211488; Academy of Finland, Grant/Award Number: 252718, 339666 and 296587; The Finnish Work Environment Fund, Grant/Award Number: 115424 and 114139; Yrjö Jahnssonin Säätiö; Juho Vainion Säätiö; California Department of Public HealthCalifornia Department of Public Health

Abstract

Moisture-damaged buildings are associated with respiratory symptoms and underlying diseases among building occupants, but the causative agent(s) remain a mystery. We first identified specific fungal and bacterial taxa in classrooms with moisture damage in Finnish and Dutch primary schools. We then investigated associations of the identified moisture damage indicators with respiratory symptoms in more than 2700 students. Finally, we explored whether exposure to specific taxa within the indoor microbiota may explain the association between moisture damage and respiratory health. Schools were assessed for moisture damage through detailed inspections, and the microbial composition of settled dust in electrostatic dustfall collectors was determined using marker-gene analysis. In Finland, there were several positive associations between particular microbial indicators (diversity, richness, individual taxa) and a respiratory symptom score, while in the Netherlands, the associations tended to be mostly inverse and statistically non-significant. In Finland, abundance of the *Sphingomonas* bacterial genus and endotoxin levels partially explained the associations between moisture damage and symptom score. A few microbial taxa explained part of the associations with health, but overall, the observed associations between damage-associated individual taxa and respiratory health were limited.

KEYWORDS
bacteria, built environment, classrooms, fungi, indoors, microbiome, mold, mycobiome

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1 | INTRODUCTION

Associations of building dampness, visible mold, and moisture damage with adverse respiratory health effects have been reviewed and conclusively documented.[1-3] The causes of indoor dampness and moisture damage are varied, including failures in structures such as leakage on roofs or water pipes, water accumulation and condensation due to poor ventilation and inadequate insulation, or flooding. Wetting of materials may lead to microbial growth and indoor biological exposures, including fungal and bacterial spores, cell fragments, and secondary metabolites.[3]

School environments are important when considering the impact of indoor air pollution on population health. Pupils and teachers spend extended periods of time inside school buildings, most days of the week, most weeks of the year, and over multiple years. Moreover, pupils are considered to be a vulnerable population and are more susceptible to the effects of air pollution.[4] While many studies have examined microbial exposures in school buildings and respiratory health, fewer have done so in the context of water damage status of those buildings.[5] The prevalence of moisture damage and indoor dampness in school buildings is not well known but may be as high as those estimated for the overall building stock,[6] which is thought to be between 10% and 50%.[3,6,7]

While microbial factors are thought to contribute significantly to adverse health effects observed in occupants of moisture-damaged buildings, the quest to find actual causative agents and to clarify underlying mechanisms is ongoing.[2] It is common, but not universal, to find that general markers of microbial biomass are higher in water-damaged schools and other buildings compared with non-damaged ones.[8-14] Several studies focused on microbial exposures have found both negative and positive associations between exposures to microbial markers in schools and respiratory health.[15-17] Recently, there have been efforts to integrate these two approaches by investigating whether pre-selected microbial markers are linked to building damage and also examining the health associations with those markers.[18,19] For instance, Holst et al.[18] found higher levels of airborne microbial markers (such as endotoxin and cultivable fungi) in damaged classrooms and that classroom conditions associated with moisture damage were associated with lung function and wheezing. However, the health associations could not be attributed to levels of the general microbial markers. In another study, Simoni et al.[19] found that viable molds and total fungal DNA were higher in damaged classrooms, but total fungal DNA was not related to respiratory symptoms. On the other hand, the DNA concentrations of various fungal genera were significantly associated with respiratory health in this study, but these were not reported to be associated with school dampness. In both these studies, the often observed increase in general microbial biomass markers in moisture-damaged buildings does not link directly to adverse health outcomes, but together the results suggest that the more resolved the environmental exposure, the more relevant it may prove for health outcomes.

Here, we take that exact next step by employing indicator analysis on high-throughput sequencing data to identify the fungal and bacterial taxa associated with moisture damage in school buildings in an untargeted approach, and then look for associations with the abundance of those identified fungal and bacterial taxa with respiratory health. We used an index-reference study design, comparing school with moisture damage (index) to those without (reference), in different geographic/climatic regions of Europe. The objectives of this work, conducted in the framework of Health Effects of Indoor Pollutants (HITEA) study,[20] were (i) to study associations of classroom microbiota with moisture damage in Finnish and Dutch primary schools, (ii) to investigate associations of the identified moisture damage indicators with respiratory symptoms reported by the pupils; and (iii) to explore whether exposure to specific taxa within the indoor microbiota may explain the association between moisture damage and respiratory health previously reported in the HITEA school study.[11,21]

Practical Implications

- This study identified specific microbial species related to inspection-based building moisture damage and then investigated links between those microbial exposures and health effects.
- Bacterial markers were more strongly associated than fungal markers with respiratory symptoms in occupants.
- We suggest that refinement of the taxa indicative of moisture damage and increased sample size of damaged buildings will strengthen this approach.

2 | MATERIALS AND METHODS

2.1 | Study design and building assessment

The recruiting, building investigations, and selection of the participating schools for the HITEA study have been described previously.[20,21] The study protocols of the HITEA school study were approved by the local ethical committees of the participating study centers. In brief, more than 700 schools in three European countries—the Netherlands, Spain, and Finland—were assessed through a screening questionnaire focusing on current and past moisture damage, dampness, and mold observations in the school buildings. While the original HITEA study compared three countries, the environmental samples from Spain meant to be included in this current analysis could not be located at the time of processing. Thus, only samples from Finland and the Netherlands are used in the present analysis. The contacted schools were located in convenient geographical proximity to the participating study centers. In the Netherlands, schools were located within tens of kilometers of Utrecht, in Finland, schools were more spread out, located within 250 km of Kuopio. Considering only schools with completed questionnaire information, with at least 200 pupils, and with no major

repairs planned in the following two years, schools were selected to include about half schools with and half without self-reported moisture problems.

Centrally trained personnel carried out walk-through school building investigations, collecting extensive background information on the school building characteristics and specifically on observations of dampness, moisture, and mold using a standardized protocol.[22] This multi-phase assessment of schools in the three European countries, the protocols used, and information collected during the walk-through building inspections, as well as the selection of eligible school buildings, have been reported by Haverinen-Shaughnessy et al.[20] Ultimately, 16 schools and 28 individual school buildings were included for the detailed, longitudinal exposure assessment described in the current analysis. Reference school buildings were those that had no signs of dampness problems, moisture or water damage, or mold growth in the building nor a history with such problems. Index schools had these problems, and they were widespread, affected several classrooms, or were observed within the past 12 months.[20] Within each country, the selected schools represented the largest available exposure contrast available with respect to extent and severity of moisture and dampness observations, specifically in classrooms. Ten schools in the Netherlands (five index and five reference) and six schools in Finland (four index and two reference) were included in this study (Table 1). Details with respect to selection criteria and the final selection of schools are described in Borràs-Santos et al.[21]

TABLE 1 Sampling period, numbers of schools, school buildings, and sampled classrooms as well as outdoor and indoor environmental assessments during three exposure assessment periods

	Exposure Assessment 1 (2009)	Exposure Assessment 2 (2009)	Exposure Assessment 3 (2010)
the Netherlands			
Measuring period[a]	Feb–April	May–June	March–April
Number of schools ($n_{index}/n_{reference}$)	10 (5/5)	10 (5/5)	10 (5/5)
Number of school buildings ($n_{index}/n_{reference}$)	17 (7/10)	17 (7/10)	17 (7/10)
Settled dust samples in classrooms ($n_{index}/n_{reference}$)	86 (36/50)	85 (37/48)	99 (40/59)
Mean outdoor temperature (°C)[b]	6.2	15.9	9.2
Mean precipitation (mm)[b]	1.1	2	0.5
Mean (range) indoor temperature (°C)[c,d]	21.1 (18.1–22.8)	n.a.	21.0 (19.7–23.3)
Mean (range) indoor relative humidity (%)[c,d]	44 (35–52)	n.a.	36 (28–48)
Mean (range) indoor CO_2 (ppm)[c,d]	921 (686–1250)	n.a.	981 (739–1120)
Mean (range) air exchange rate (h^{-1})[c,e]	2.4 (1.4–4.6)	n.a.	2.7 (1.1–5.4)
Finland			
Measuring period[a]	Jan–March	March–May	Feb–March
Number of schools ($n_{index}/n_{reference}$)	6 (4/2)	6 (4/2)	6 (4/2)
Number of school buildings ($n_{index}/n_{reference}$)	11 (9/2)	11 (9/2)	11 (9/2)
Settled dust samples in classrooms ($n_{index}/n_{reference}$)	62 (44/18)	62 (44/18)	69 (49/20)
Mean (range) outdoor temperature (°C)[b]	−5.9 (−5.8 to −6.0)	4.2 (3.2–5.6)	−8.6 (−8.1 to −8.8)
Mean precipitation (mm)[b]	0.6 (0.5–0.7)	1.3 (1.2–1.4)	1.4 (1.3–1.6)
Mean (range) indoor temperature (°C)[c,d]	22.0 (20.5–23.4)	n.a.	21.6 (20.0–22.8)
Mean (range) indoor relative humidity (%)[c,d]	15 (11–17)	n.a.	13 (8–25)
Mean (range) indoor CO_2 (ppm)[c,d]	646 (526–900)	n.a.	595 (537–676)
Mean (range) air exchange rate (h^{-1})[c,e]	4.5 (1.9–13)	n.a.	5.2 (2.7–8.2)

n.a., not assessed.

[a]Each individual school was sampled for 8 weeks; interval provided indicates in which period all schools of one country were assessed in the respective exposure assessment.

[b]Average of the 8 weeks of sampling period for each school (exposure assessments in Dutch schools were started and ended on the same day, thus no variation between schools).

[c]Performed in one classroom per school during exposure assessments 1 and 3; presented are means of all schools as well as the range of the means of individual schools.

[d]Daytime (Monday–Friday, 08.00–16.00), averaged measurements per classroom.

[e]Air exchange rates [h−1], median of build-up events during one school week.

2.2 | Environmental sampling

Each school was sampled three times over the period of approximately 15 months: during late winter/early spring 2009 (exposure assessment 1 occurred January–March in Finland, February–April in the Netherlands), late spring/early summer 2009 (exposure assessment 2 occurred in March–May in Finland and May–June in the Netherlands), and during late winter/early spring 2010 (exposure assessment 3 occurred in February–March in Finland and March–April in the Netherlands). Table 1 provides details on the sampling periods in each country, including basic outdoor and indoor environmental conditions, the latter derived from one representative classroom per school (modified from ref. [11]). Within each country and exposure assessment, the sample collection periods were made parallel between the study schools as much as feasible (ie, start and stop dates of assessments in different schools within each country were typically within 2 weeks). The sampling campaigns were also made parallel between the two countries; however, school-free periods were avoided. Sampling locations in the schools were primarily full-time occupied classrooms (attended by children aged 4–12 years), but also other indoor locations where pupils and teachers spend a considerable amount of their time were considered (eg, part-time classrooms, such as music or arts classes; hallways; teachers' lounges; libraries; and similar). In each school, approximately 15 locations (target: 10 classrooms, 5 other locations) were sampled, with emphasis on representing the whole school building and study population, including both pupils and teachers. The sampling strategy also matched the proportions of rooms with and without damage observations as determined in the building inspections. The same locations were sampled throughout the repeated assessments, whenever possible.

The current analysis was restricted to classrooms and was based on settled dust samples, collected using electrostatic dustfall collectors (EDCs[23]; holding two electrostatic wipes per sampler). The EDCs were placed by a trained field worker on elevated surfaces at a height of 1.5–2.5 m, typically on top of cupboards, shelves, etc., and avoiding locations with major airflow disturbances such as close to doors, frequently opened windows, or ventilation ducts. Following an 8-week collection period, EDCs were transferred to the local study center and stored in dry and dark conditions at room temperature for a maximum of 2 weeks before being transferred to a −20°C freezer. Measurements of endotoxin and glucan in dust collected with one of the two wipes of the EDCs were reported previously by Jacobs et al.[11] The DNA in the dust from the second wipe was extracted and used for the sequence-based analyses reported here.

2.3 | Assessment of the fungal and bacterial microbiome

The wipes were stored frozen until analysis and shipped on dry ice to the Finnish Institute for Health and Welfare, in Kuopio, Finland, to perform dust and DNA extraction. Dust extraction from the two electrostatic clothes was performed following Shorter et al.,[24] with minor modifications. Each wipe was transferred into a sterile bag (Rollbag 1300, Interscience) and extracted two consecutive times with 25 ml sterile water with 0.05% Tween20. For the extraction, we used a stomacher (Bagmixer 400V, Interscience), operated at maximum speed for 10 min. The two extracts per wipe were combined, concentrated to approximately 1.5 ml via centrifugation (6000 *g*, 15 min, 4°C), aliquoted, and stored at −20°C until DNA extraction.

DNA extraction and purification were performed from measured amounts of approximately 1.5 ml of the EDC dust extract. The extracts were pelleted using an Eppendorf microcentrifuge at maximum speed for 15 min. After removal of supernatant, pellets were resuspended in 400 µl lysis buffer of the Chemagic DNA Plant Kit (PerkinElmer chemagen Technology GmbH). Salmon testis DNA (Sigma-Aldrich Co)[25] was added to the samples as an internal standard. Cells were disrupted in an initial bead-milling step (MiniBeadbeater-16, BioSpec Products, Inc.) at maximum speed for 1 min. DNA was then purified following the Chemagic DNA Plant Kit protocol, using KingFisher™ ml DNA extraction robot (Thermo Fisher Scientific Inc.). Reagent controls as well as bacterial and fungal mock communities were extracted along the actual samples. DNA was stored at −20°C until processing.

Quantitative PCR (qPCR) was performed from sample DNA utilizing previously established qPCR assays: Gram-positive and Gram-negative bacteria[26]; group of *Penicillium* spp., *Aspergillus* spp., and *Paecilomyces variotii*[27]; total fungal DNA[28]; and the internal standard salmon testis DNA.[25] qPCRs were performed as detailed previously.[29] Positive (bacterial and fungal mock communities) and negative reagent controls, as well as no template controls, were included in the qPCR runs. Numbers of microbial cell equivalents (CE) in the samples were calculated using relative quantification, as described in Haugland et al.,[27] utilizing the salmon DNA internal standard to assess and correct for the presence of inhibitors and the performance of the DNA extraction. qPCR results from a total of 27 samples were excluded from statistical analysis due to elevated internal standard CT (threshold cycle). For nine samples with non-detects in the *Penicillium/Aspergillus* assay, we imputed ½ theoretical detection limit values based on standard curves. qPCR results were normalized for sampling area and are presented as cell equivalents per m^2 sampling surface area.

Sample DNA was shipped frozen to sequencing service partner LGC Genomics (Germany), where fungal ITS and bacterial 16S PCR, library preparation, and sequencing on Illumina Miseq v3 were carried out, as described in detail in Jayaprakash et al.[30] Raw fungal and bacterial sequences were deposited in the National Center for Biotechnology Information's Sequence Read Archive under BioProject accession PRJNA635510.

Processing into "amplicon sequence variants" (ASVs) was implemented in the DADA2 package[31] in the R environment[32] along with additional software described within. Analyses for ASVs account for sequencing error, and thus, ASVs have greater taxonomic resolution than operational taxonomic units (OTUs).[33] For the bacterial sequences, forward and reverse reads were filtered (no ambiguous sequences,max error rate 2 for the forward reads, 5 for the reverse

reads, and quality truncation 2 and truncated to lengths of 200 bps). Reads were dereplicated, ASVs inferred, merged, and then bimeras removed. Taxonomy was assigned using the Silva v128 database.[34] For fungi, forward reads were filtered (max error rate 2, quality truncation 2) and truncated to a length of 200 bps, while reverse reads were simply truncated to a length of 200 bps. Paired forward and reverse reads were identified using fastq-pair[35] and then paired using PEAR.[36] Returning to DADA2, sequences with N's were removed, dereplicated, and then sequence variants inferred. Bimeric sequences were removed, and taxonomy was assigned against the UNITE database,[37] version 7.2 2017-12-01.

Quality filtering included processing both positive and negative controls. For bacteria, a reference (a mock community of known taxon input) was nearly completely recovered. Seven taxa were input and seven ASVs inferred, although with incomplete matching: *Pseudomonas aeruginosa* was not recovered, and there were two ASVs classified as *Escherichia/Shigella*. The prevalence method in the decontam package,[38] using a threshold of 0.5 that will identify as contaminants all sequences that are more prevalent in negative controls than in positive samples, identified 19 taxa as contaminants (range of relative abundances: 0.00057%–0.54%), with the dominant ones classified as *Ralstonia*, *Bradyrhizobiaceae*, and a particular ASV of *Sphingomonas*. For fungi, 43 taxa were in the reference mock community, and 43 ASVs were inferred, although with incomplete matching as with bacteria. All but four taxa (*Chaetomium globosum*, *Eurotium chevalieri*, *Mucor racemosus*, and *Rhizopus stolonifera*) had ASVs represented by taxa of the genus but not necessarily to the precise species. The prevalence method in the decontam package, using a threshold of 0.5, identified 34 mostly low-abundant taxa (range of relative abundances: 0.00055%–0.57%) as contaminants, and these taxa were removed.

Microbiome community tables were processed as phyloseq objects[39] in R. Heat trees were generated using the "metacoder" package.[40] The samples were rarefied to a common number of sequences (n = 1000 for fungi, n = 2000 for bacteria; Figure S1) for composition and diversity analyses, unless otherwise indicated. Differences in community composition across environmental factors were determined using permutational multivariate analysis of variance (PERMANOVA) with the Bray-Curtis index. Taxonomic richness (number of observed taxa) and Shannon diversity (an index that takes into account the number of different taxa and their proportions) were calculated in phyloseq. To identify taxa associated with water damage, we relied on two statistical tools that use different approaches for detecting differential abundance, ANCOM[41] and *selbal*.[42] Within ANCOM, we looked for taxa (ASVs and genera) associated with building damage using the unrarefied dataset and adjusting for the sampling period. We used the "moderate" approach to account for multiple comparisons, which relies on adjusting p-values within a sample using the Benjamini-Hochberg (BH) procedure.[43] With *selbal*, we looked for ASVs associated with building damage after removing low-abundance taxa from the unrarefied dataset: those taxa in less than two samples for Finland and less than seven samples in the Netherlands. Separate tests of taxonomic associations with water damage were conducted for both countries. In a confirmation step, associations of taxa identified in ANCOM and *selbal* with moisture damage status of the school building were tested in country-specific logistic regressions using tertiles of exposure variables, or "0," "≤median," and ">median" in the case of large number of zeros in the data LOGISTIC procedure, adjusted for study phase, implemented in SAS 9.3 (SAS Institute).

2.4 | Respiratory health assessment

Information on the respiratory health status of pupils of the study schools was collected through a parent-administered questionnaire, as described in detail in Borràs-Santos et al.[21] and Jacobs et al.[11] The assessment used questions from the validated International Study of Asthma and Allergies in Childhood questionnaire[44] (see Supplementary Material). Questionnaires were administered during late fall/early winter 2008 (majority of questionnaires answered in mid-November to end November in Finland and late November to end December in the Netherlands), that is, approximately two months prior to exposure assessment 1. In line with Jacobs et al.,[11] we drew from the following three symptoms reported retrospectively for the past 12 months: wheeze, nocturnal dry cough, and rhinitis. Associations between microbial exposure measurements and respiratory symptoms were examined using a respiratory symptom score.[45] These symptoms were each scored 0 (no) or 1 (yes) and added together, resulting in a score from 0 to 3. The score was then used as a binary variable (0 = 0; 1 = 1–3), that is, analyzing "yes" to one or more of the three symptoms versus "no" to all.

Country-specific analyses of DNA-based microbial markers and moisture indicators with the symptoms score were adjusted for potential confounders in line with our earlier analysis described in Borràs-Santos et al.,[21] including confounders based on previous evidence (gender, age, and moisture damage in the home) and others based on association with respiratory health and exposure (educational level). The DNA-based microbial markers and moisture indicators included microbial concentrations in settled dust (cell equivalents/m^2 collection surface) from qPCR measurements, as well as specific taxa derived from sequence analysis (ASVs and genera) and diversity estimates (richness and Shannon diversity index). In addition, for the analysis of associations with health in the Finnish data, the specific moisture indicators were also analyzed separately using absolute abundance,[46] determined by multiplying the relative abundance of each taxon by the total biomass as measured with qPCR.

The analysis was conducted using building-level exposure estimates, that is, the mean of individual classroom measurements in a given school building. Exposure measurements derived from exposure assessment 1 were used, as this exposure assessment was closest in time to the respiratory health assessment. Exposure measurements were put in tertiles (or, in case of large number of zeros, in the respective variable in categories "0," "≤median," ">median"). Logistic regression with generalized estimating equations with an exchangeable correlation structure to account for correlation between measures in the school buildings within subjects (i.e., inclusion of school building as group variable) was used to determine

TABLE 2 Study population in respiratory health analyses comparing prevalence of respiratory symptoms during previous year to school building-level exposure during exposure assessment 1

	All	Damaged buildings	Non-damaged buildings
the Netherlands			
Number of participants in questionnaire survey	1402	510	892
Boys, *n* (%)	660 (47%)	242 (47)	418 (47)
Age, years (SD)	9.1 (1.8)	9.4 (1.8)*	8.9 (1.8)*
Years of parental education, years (SD)	16.2 (3.4)	15.6 (3.7)*	16.5 (3.2)*
Moisture damage at home *n*(%)	362 (26)	167 (33)*	195 (22)*
Respiratory symptoms, *n* (%)			
Wheeze in last 12 months	132 (9)	52 (10)	80 (9)
Nasal symptoms, no cold in last 12 months	318 (23)	121 (24)	197 (22)
Nocturnal dry cough, no cold in last 12 months	260 (19)	98 (20)	162 (18)
Symptom Score[a]	480 (35)	175 (35)	305 (35)
Finland			
Number of participants in questionnaire survey	1332	757	575
Boys, *n* (%)	617 (46)	352 (46)	265 (46)
Age, years (SD)	9.9 (1.8)	10.0 (1.8)*	9.7 (1.8)*
Years of parental education, years (SD)	15.8 (2.9)	15.5 (3.0)*	16.1 (2.7)*
Moisture damage at home *n*(%)	102 (8)	72 (10)*	30 (5)*
Respiratory symptoms, *n* (%)			
Wheeze in last 12 months	171 (13)	111 (15)*	60 (11)*
Nasal symptoms, no cold in last 12 months	424 (33)	268 (37)*	156 (29)*
Nocturnal dry cough, no cold in last 12 months	162 (13)	101 (14)	61 (11)
Symptom Score[a]	524 (42)	321 (45)*	203 (38)*

SD: standard deviation

[a]Respiratory symptom score[45] calculated as binary variable with 1 being "yes" in the last 12 months to any single one or multiple of the three symptoms: wheeze, nasal symptoms, and nocturnal dry cough.

*$p<0.05$ for difference between pupils from damaged and non-damaged schools ($\chi 2$ test or Mann-Whitney *U* test).

associations between microbial markers and respiratory symptom score using the GENMOD procedure in SAS. This analysis hypothesizes that the microbial exposures may be an intermediate step in the pathway between exposure to moisture damage and respiratory symptom score and tests how much of this association may be attributable to the microbial exposure. A similar approach has been used, for example, to explore the beneficial role of microbial exposures in farming environments.[47]

3 | RESULTS

3.1 | Study overview

A total of 463 classroom dust samples were collected in the three assessments using EDCs (Table 1). In the Netherlands, there was an average number of 250 samples (out of approximately 270) successfully processed for subsequent qPCR and fungal and bacterial amplicon sequencing data analyses, with approximately 80 of those occurring during exposure assessment 1. In Finland, there was an average number of 160 samples (out of approximately 190) processed for each of the microbial measurements, and an average of 50 classroom dust samples occurring during exposure assessment 1. Sample losses mostly refer to inhibition detected in DNA extracts subjected to qPCR analysis, or low sequence read counts (below sequence count threshold; see Methods section) produced during amplicon sequencing. Low sequence read counts were specifically an issue for fungal sequencing of Finnish classrooms and particularly for the winter seasons (Figure S1).

An overview of the study population, including some basic descriptive statistics as well as respiratory symptom prevalence, is presented in Table 2. More detailed descriptive analysis of the demographics of the study population and respiratory health outcomes with comparisons between countries and between damaged

TABLE 3 Summary information about the sequencing depth, taxonomic classification, and taxonomic richness of fungi and bacteria in classroom dust samples, rarefied to a common sequencing depth, in Finland and the Netherlands

	Fungi		Bacteria	
	Finland	the Netherlands	Finland	the Netherlands
Total number of sequences	857 492	2 194,970	1 814 994	1 515 108
Median number of sequences per sample	1430	5721	8394	5747
Total number of ASVs in all samples	2210	5596	3061	2717
Mean ASV richness in each sample	28	114	50	73
Total number of genus-level taxa in all samples	407	666	507	481
Mean genus-level taxa richness in each sample	15	61	33	43

and non-damaged schools has been presented previously by Borrás-Santos et al. (2014) and Jacobs et al.[11] Moisture damage in the home environment was more frequently reported by Dutch than by Finnish study participants. Wheeze and nasal symptoms were more prevalent among Finnish schoolchildren, and a symptom score of one or higher was also more frequently assessed for Finnish than for Dutch schoolchildren. The previous paper by Jacobs et al.[11] including almost exactly the same study population reported comparable current asthma prevalence between the Netherlands (6%) and Finland (8%).

3.2 | Classroom microbiota

Summary information on sequence depth, depth of taxonomic classification, and mean ASV and genus-level taxon richness in Finnish and Dutch classrooms is provided in Table 3. Median relative abundance of the dominant bacterial and fungal genera in Finland and the Netherlands is presented in Figure S2. There was large phylogenetic diversity observed in the fungal and bacterial microbiota of school classrooms (Figure 1A,B). Within fungi, there was a greater richness of taxa within the Ascomycota (70.7% of rarefied taxa) than the Basidiomycota (27.4% of rarefied taxa) (Figure 1A). Many of the most abundant genera, including *Aspergillus*, *Epicoccum*, and *Cladosporium* within the Dothideomycetes and Eurotiomycetes classes, were common to both Finland and the Netherlands (Figure 1A). However, there were regional differences, and many of the abundant taxa that differed between countries were yeasts. For instance, *Mrakia* and *Apiotrichum* are among the most abundant genera in the Netherlands, while *Cyberlindnera* and *Phaeococcomyces* are among the most abundant genera in Finland. Within bacteria, the Bacteroidetes and Proteobacteria were both common and abundant (Figure 1B). Many of the detected genera are human-associated and thus commonly detected in buildings, with a few key exceptions: *Dietzia* in Finland, as well as *Truepera*, *Nocardioides*, and *Rubellimicrobium* in the Netherlands. The microbiota of Finnish schools showed a greater influence of human occupants than the Dutch schools (Figure S3). The abundance of 12 human-associated bacterial families[48] comprised 11.9% of the entire community in Finland but only 3.6% in the Netherlands. The environmental factors of country, school, and season had the strongest measured impact on this diverse community composition, although most of the variation remained unexplained in both fungi and bacteria (Table S1).

Complementing the qualitative sequence analysis with quantitative measurement of bacterial and fungal groups using qPCR, we observed significantly different levels of both bacterial and fungal groups between countries, with levels being consistently lower in Finnish classrooms compared with those in the Netherlands (Figures 2 and 3; Table S2). This was also true for the taxonomic richness and diversity estimates in classroom dust. Seasonal effects varied within countries. In Finland, microbial communities had higher richness, diversity, and biomass in the spring than in the winter. In Dutch classrooms, however, the seasonal effect was less consistent, with higher total fungal DNA levels in samples collected during spring/early summer, but lower Gram-positive, Gram-negative and *Penicillium/Aspergillus* group levels, compared to samples collected during late winter/early spring. Three taxa (*Epicoccum nigrum*, *Mycosphaerella tassiana*, and *Aspergillus piperis*) dominated the spring samples in the Netherlands, making up over 80% of the sequences, a pattern which is known to bias estimates of richness.[49]

Because the microbial composition in the school dust samples was strongly influenced by geographic location and seasons (Figures 2 and 3, Table S2), tests of how building damage influences richness, diversity, and biomass were explored within country and season. In Finland, both fungal and bacterial markers were consistently higher in damaged buildings than in undamaged buildings for both seasons (Table S2). In the Netherlands, relationships between microbial markers and building damage status were variable.

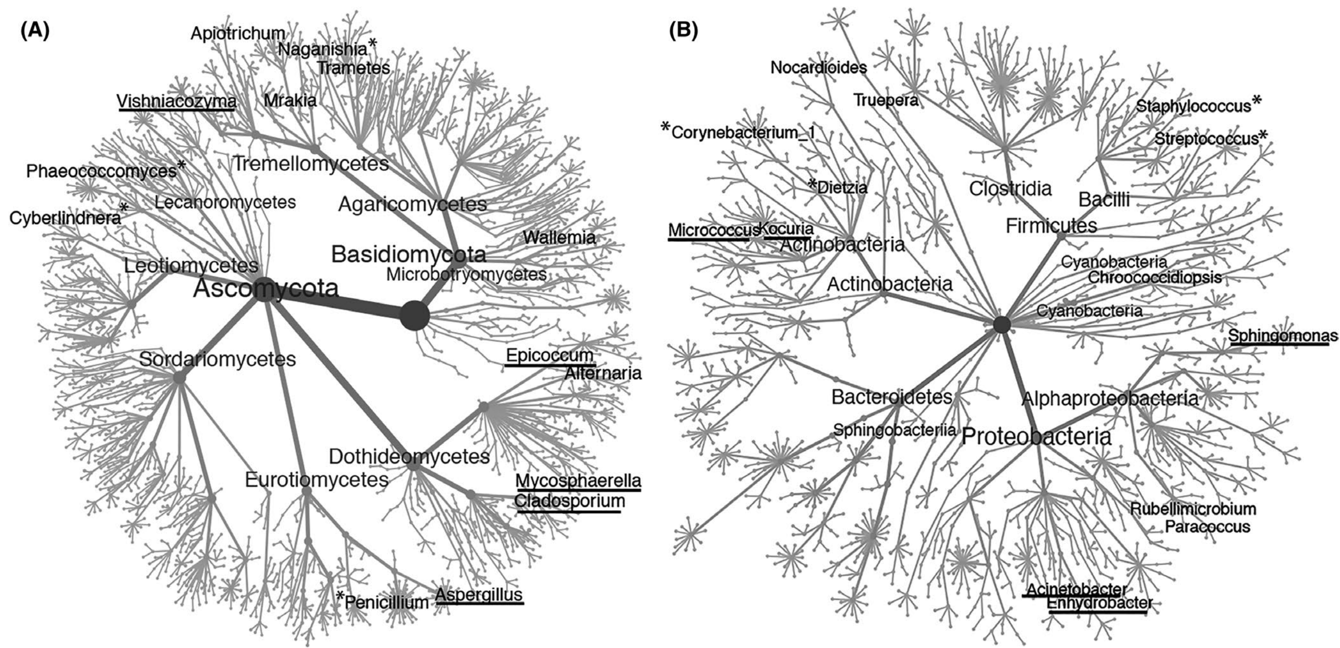

FIGURE 1 Phylogenetic diversity of fungi (A) and bacteria (B) in indoor dust samples of schools in Finland and the Netherlands. The size and color of nodes represent the number of ASVs in each group, where larger and darker nodes have more ASVs than smaller and lighter nodes. For fungi (A), label nodes are phyla, classes with greater than 30 ASVs, and the 10 most abundant genera in either Finland or the Netherlands. For bacteria (B), label nodes are the 5 largest phyla and classes as well as the 10 most abundant genera in each Finland and the Netherlands. The most common genera in both Finland and the Netherlands are underlined, while those most common in Finland are starred (*) and those in the Netherlands are unadorned

While fungal richness, diversity, and *Penicillium/Aspergillus* DNA were higher in damaged Dutch schools in both seasons, total fungal DNA was not increased. With respect to bacteria, Gram-positive and Gram-negative DNA loads were both higher in damaged Dutch schools in both seasons, while bacterial richness and diversity showed no consistent patterns (Figure 3, Table S2).

Table 4 lists the individual taxa, at the ASV level derived from the sequencing analysis, that were associated with building damage based on ANCOM—these are referred to here as indicator taxa. Note that the sequence types indicative of moisture damage differ between the two countries, even if pointing toward the same genera in some cases (eg, *Sphingomonas*, *Vishniacozyma*). A full list of taxa identified by both ANCOM and *selbal*, including descriptive statistics of ASVs and genera associated with building damage, is detailed in Table S3. Logistic regression analysis of the indicator taxa (in tertiles) confirmed significant (p trend-test <0.05) association of indicator taxa with moisture damage status of the school building in all cases (data not shown).

3.3 | Microbial associations with respiratory symptoms

To review our approach, first we detailed the links between particular microbial markers and health effects in students using a symptom score. As the exposure measurement, we used microbial levels (qPCR markers), microbial taxon richness, and diversity as well as moisture damage indicator taxa as determined during exposure assessment 1 that being the exposure assessment closest to the respiratory health assessment. Next, we modeled effects of moisture and mold damage with respiratory symptoms and adjusted those models for individual microbial markers. In this novel approach, we show that part of the association between moisture damage and respiratory health could be explained by some of the specific microbial exposures.

In Finnish schools, we found associations between fungal and bacterial microbial markers determined from classroom dust and respiratory symptom score (Table S4). Higher quantities of total fungal load and *Penicillium/Aspergillus* detected through qPCR were both significantly associated with increased symptom score, while the bacterial loads showed no significant associations. Total fungal and *Penicillium/Aspergillus* loads were highly correlated in this school building-level analysis, and ORs (95% confidence intervals [CIs]) for the middle and highest categories were 1.43 (1.27–1.61) and 1.26 (1.09–1.46), respectively. However, when examining the bacterial composition, as detected through marker-gene analysis, it was bacterial markers that were more strongly linked with symptom score. Bacterial richness and diversity (for middle category: 1.43 [1.36–1.50]; for highest category: 1.26 [1.09–1.46]), as well as a bacterium *Sphingomonas* sequence variant (for middle category: 1.14 [1.07–1.20]; for highest category: 1.41 [1.34–1.49]) and the *Sphingomonas* genus (for middle category: 1.04 [0.95–1.14]; for highest category: 1.41 [1.32–1.50]), were positively associated with symptom score. Unexpectedly, fungal taxa and diversity were generally inversely, but non-significantly, associated with respiratory symptoms. Specifically, the adjusted odds ratio for symptom score

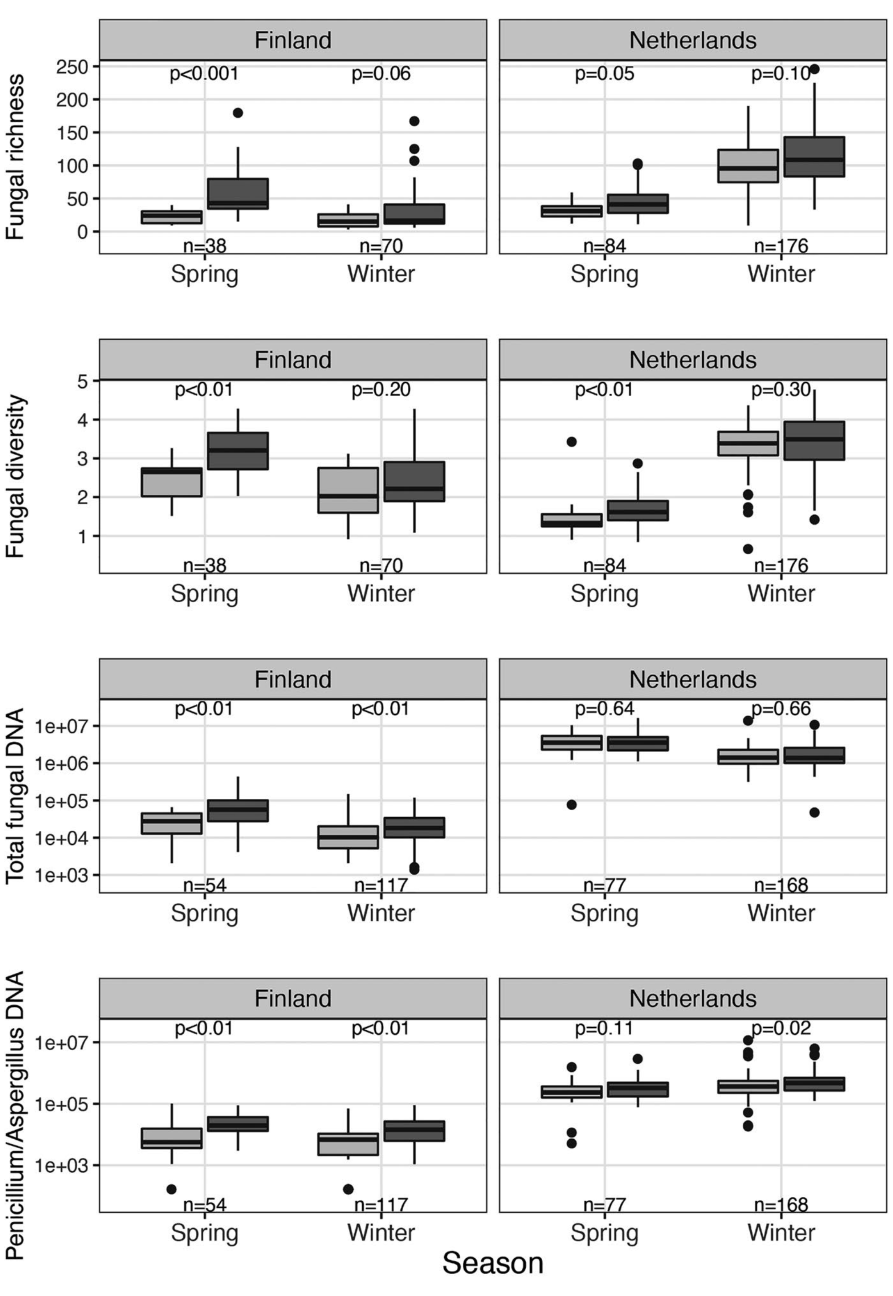

FIGURE 2 Fungal richness, diversity, total fungal DNA, and *Penicillium/Aspergillus* DNA in Finland (left column) and the Netherlands (right column) across season and building damage in samples rarefied to a common sequencing depth. Note that the axes for the qPCR data (bottom two rows) are log-scale. Shown are the *p*-values of the Wilcoxon test of differences between the Undamaged and Damaged groups within each geographic location and sampling season

decreased as the abundance of an *Aspergillus* ASV and the *Aspergillus* genus increased (Table S4).

For the fungal and bacterial indicator taxa in Finland, we performed additional analysis based on an estimate of their quantity (in contrast to the values based on relative abundance in a sample used above) by considering their absolute abundances, an approach that combines quantitative biomass PCR with relative abundance of taxa. Using absolute abundance did not increase the strength of the respiratory health associations (Table S5).

In Dutch schools, we did not observe any significant associations (*p* trend-test <0.05) of bacterial and fungal qPCR markers, richness/diversity, indicator taxa, and respiratory symptom score (Table S6). In total, we tested associations of the relative abundance of 27 fungal ASVs and 18 fungal genera, as well as 14 bacterial ASVs and 7 genera in Dutch classroom dust, all of those found to be moisture damage indicators (Table S6). Associations, if any, tended to be inverse, although none of these were statistically significant.

As shown previously for individual respiratory symptoms,[11] there exists an association between moisture damage and respiratory health in Finnish, but not in Dutch pupils in the HITEA study (Table S7). Based on the results of the analyses presented here, we used the association between respiratory symptom score and moisture damage in the school—with basic adjustment including gender, moisture damage at home, school, parental education—and additionally adjusted those models in Finland for the individual microbial exposures that we observed to be associated with respiratory

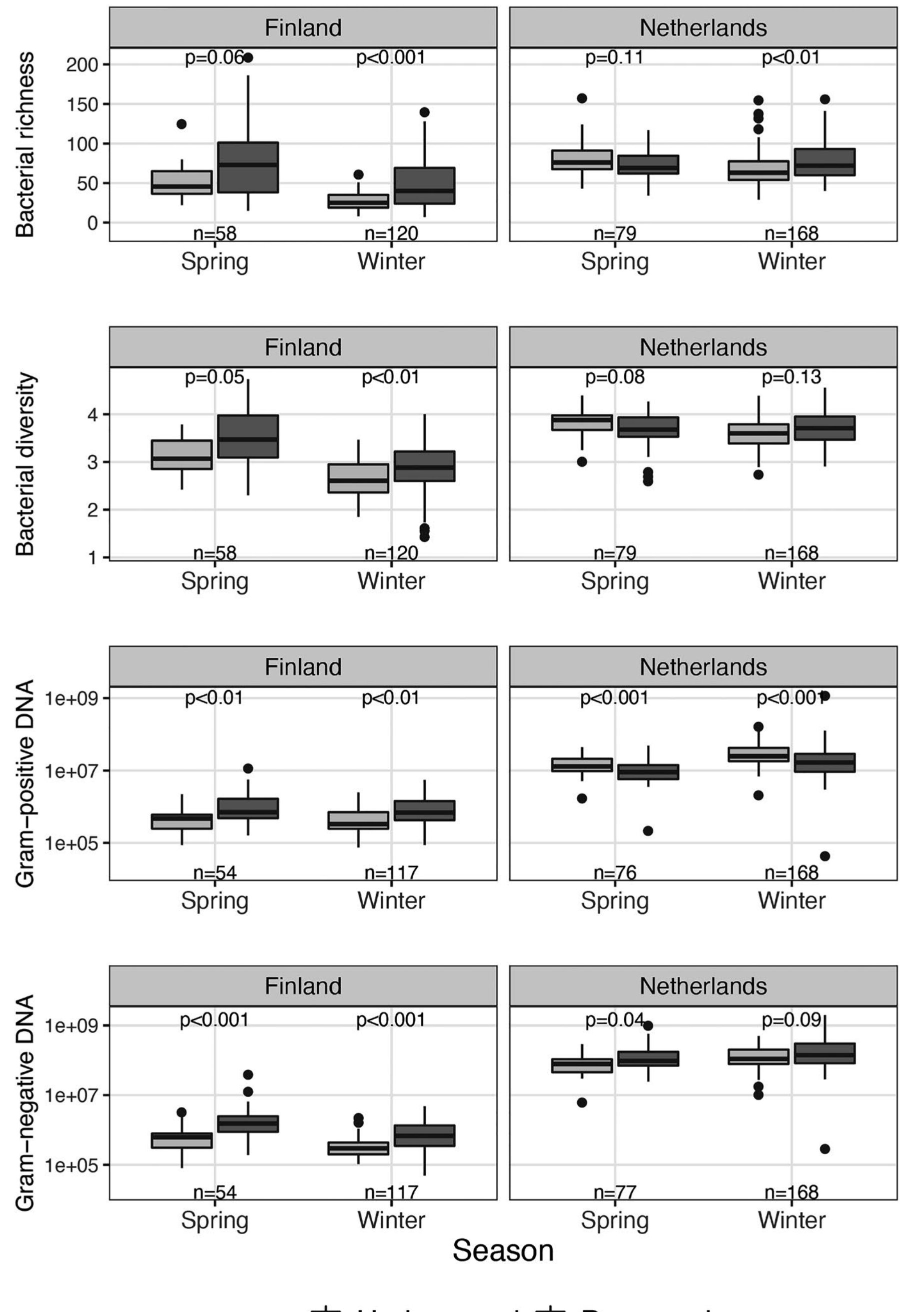

FIGURE 3 Bacterial richness, diversity, Gram-positive DNA, and Gram-negative DNA in Finland (left column) and the Netherlands (right column) across season and building damage in samples rarefied to a common sequencing depth. Note that the axes for the qPCR data (bottom two rows) are log-scale. Shown are the *p*-values of the Wilcoxon test of differences between the Undamaged and Damaged groups within each geographic location and sampling season

symptoms (Table 5). The relative abundance of *Sphingomonas* genus in classroom dust and levels of endotoxin explained approximately 30% of the respiratory health risk between moisture damage in the school and increased symptom score.

4 | DISCUSSION

4.1 | Overview of study findings

The starting point for this work was a large school study carried out in an identical design in two European countries, and specifically designed to evaluate moisture damage-associated exposures and health effects, strongly supported by detailed building investigations. In taking this approach, we examined whether the negative health effects so often associated with damp and moldy buildings could be explained not by predefined microbial targets but instead by the particular species that showed increased prevalence and abundance in moisture-damaged schools. We found that in this dataset, part of the association between moisture damage and respiratory health could be explained by some of the specific microbial exposures, in particular bacterial rather than fungal exposures, but overall, the contribution of individual taxa to the observed health effects was limited. In this regard, the results of this study align with many other studies that find inconsistent, weak, or no associations between microbial markers linked to moisture damage and health

TABLE 4 Taxa indicative of moisture damage in schools in Finland and the Netherlands. Separate taxa given the same taxonomic identification are differentiated by their ASV number

	Fungi	Bacteria
Finland	*Aspergillus proliferans*, *Vishniacozyma victoriae*	*Sphingomonas* spp. (ASV9), *Sphingomonas* spp. (ASV42)
the Netherlands	*Guehomyces pullulans*, *Cladophialophora* spp., *Tetracladium marchalianum*, *Endophoma elongata*, *Devriesia pseudoamericana*, *Cystofilobasidium capitatum*, *Vishniacozyma carnescens*, *Gibberella baccata* (ASV36), *Cladosporium delicatulum*, *Vishniacozyma dimennae*, *Vishniacozyma foliicola*, *Vermiconia calcicola*, *Gibberella baccata* (ASV90), *Mrakiella aquatica*, *Helotiales* spp., *Buckleyzyma aurantiaca*, *Knufia* spp., *Fusicolla aquaeductuum*, *Vishniacozyma victoriae*, *Ascomycota* spp., *Didymellaceae* spp., *Capnodiales* spp., *Nectria ramulariae*, *Mycoarthris corallina*, *Chaetothyriales* spp., *Epicoccum nigrum*, *Acremonium alternatum*	*Sphingomonas* spp. (ASV37), *Rubellimicrobium* spp. (ASV27), *Sphingomonas* spp. (ASV167), *Hymenobacter* spp., *Rubellimicrobium* spp. (ASV94), *Pleurocapsa* spp., *Marmoricola* spp., *Amaricoccus tamworthensis*, *Parafilimonas* spp., *Sphingomonas* spp. (ASV14), *Chamaesiphon* spp., *Cyanobacteria* (ASV313), *Cyanobacteria* (ASV309)

TABLE 5 Associations between moisture damage and symptom score in Finnish schools, using the basic adjustment (school, gender, moisture damage in the home, and education) and additionally adjusting the model for the individual microbial markers

	Symptom Score odds ratio (CI)	Change in additional risk relative to basic adjustment
Basic adjustment	1.33 (1.22, 1.45)	–
+ Fungal richness	1.35 (1.28, 1.43)	6%
+ Bacterial richness	1.37 (1.23, 1.53)	12%
+ *Sphingomonas* ASV_9	1.31 (1.22, 1.40)	−6%
+ *Sphingomonas* genus	1.22 (1.13, 1.32)	−33%
+ *Penicillium/Aspergillus* group	1.28 (1.10, 1.50)	−15%
+ Gram-negative bacteria	1.40 (1.35, 1.46)	21%
+ Endotoxin	1.22 (1.13, 1.32)	−33%
+ Glucan	1.34 (1.2, 1.49)	3%

CI, confidence interval.

outcomes.[2,5] However, there are some indications that with further refinement of the methods, discussed below, there would be greater power to detect the specific microbial agents that induce health effects.

4.2 | The study approach: novelty and development

The general identification of the microbial agents consistently and quantitatively associated with building damage is a persistent challenge.[50] Here, we used statistical tools to flag those taxa that show increased prevalence and/or abundance in certain types of environmental samples compared to others, without prior assumptions about the relevant microbial factors.[41,42] A few taxa were identified by both approaches, but the majority were only identified by one, suggesting that the choice of indicator taxon analysis can have a strong influence on which taxa are tested against health outcomes. While the differences in microbial markers between damaged and non-damaged schools were greater and more consistent for Finland than for the Netherlands, indicator analysis identified few individual taxa associated with damage in the Finnish school buildings. Thus, there were very few taxa to test against health associations in Finland, the very location where schoolchildren seem to be at higher respiratory health risk from exposure to moisture damage in the school-environment, compared to other European countries.[21]

There are several explanations, not mutually exclusive, as to why indicator taxon analysis identified few damage indicators in Finland. One, the overall microbial biomass in classrooms is much lower in Finland than the Netherlands—typically two orders of magnitude. With the lower overall biomass and lower taxonomic richness in the Finnish classrooms (Table 3, Figures 2 and 3), it is less likely to find statistical associations between taxa and building damage. The difference in biomass and taxon richness between Dutch and Finnish classrooms is more pronounced for fungi, but also true for bacteria. Two, many studies indicate that there are strong stochastic effects in determining which fungi grow in response to moisture in buildings.[51] Indeed, a recent study showed that dust collected from different homes within the same state and exposed to elevated relative humidity in the laboratory each had unique microbial consortia that proliferated, and so looking across homes or buildings would find limited commonalities.[52] Additionally, different types of building damage may drive different microbial ecological patterns that, again, limit common species for statistical tools to identify. There were differential types of building damage observed between the Finnish and Dutch schools. Specifically, water damage and mold odor were more common in the Finnish school buildings, which were also older in general, while observations of dampness problems,

including condensation of windows, were more prevalent in Dutch schools.[20] As such, no two moisture damages in buildings are identical in building failure (type of damage, duration, water source, and amount), microbial inoculum, and maintenance response. All these factors contribute to the challenge of identifying uniform patterns of microbial response to moisture damage in buildings, and this study affirms the need for improvements in the analytical approaches to identify the taxa associated with water damage that can account for these building-level variations.

The quantity of the relevant microbial markers is likely an important component of health outcomes. In this study, we found that more often it was quantitative microbial markers (e.g., *Penicillium/Aspergillus* group as measured by qPCR, endotoxin) that explained health associations. Amplicon sequencing data in its nature are not quantitative but describes the relative abundance of individual bacterial and fungal taxa detected in a sample; thus, it is differences in relative abundances that can be statistically compared across samples. There are ways—though imperfect—to circumvent this apparent limitation in estimating actual exposure. Following such approach, we examined the absolute abundance[46] of individual taxa with respiratory health,however, associations were no stronger than when considering their relative abundance-based metrics. While it could be that the microbes we identified are good moisture damage indicators but are in fact not relevant for health, it is more likely that we have not yet identified the relevant building-associated microbes to analyze in a quantitative way.

Despite the ambitious nature of the study design, we did have to account for limited statistical power in two specific instances. One, limitations in statistical power guided the analysis toward using a symptom score rather than evaluating more specifically associations of multiple individual respiratory symptoms and a multitude of microbial exposure variables, in order to avoid challenges associated with multiple testing. The exploratory analysis of identifying indicator taxa associated with moisture damage was controlled for multiple testing; however, results of that analysis using statistical tools tailored to deal with microbiome type datasets were confirmed with logistic regression analysis. Two, there were only a limited number of buildings to explore building damage, microbial indicators, and health effects. Consistent with the assessment that Finnish schoolchildren showed a stronger health response to school microbial exposures than Dutch schoolchildren,[21] these results show that individual microbial exposures explain some of the differences in Finland but not in the Netherlands. However, this study is based on data from six Finnish buildings, assessing the link between the exposures and health. We might expect relationships to be stronger between classroom-level exposure and health effects. Unfortunately, the classroom-level analyses would have included a highly reduced dataset in our analysis, since only pupils with both questionnaire response and dust samples plus valid sequencing result from their "home" classroom would have been included, thus reducing statistical power to identify relationships. However, there is evidence that averages of group exposure can be more meaningful than individual-based exposure estimates when the exposures vary across time and when capturing the true exposure is complicated by imprecision in microbial sampling and variation in analytical approaches.[53] Increased representation and building assessment at a finer spatial scale—in this case, in the number of classroom-level damage assessments—may reveal further patterns between environmental microbes and respiratory health. Moreover, this study focused on microbial exposures in school environments, which represent one potential environmental exposure affecting respiratory health.

Taken together, our results indicate that with improved methods to identify the taxa associated with water damage in the locations where there are observed health effects of water damage, and with a robust quantitative measure of those identified taxa, we may be able to identify stronger direct links between specific microbial taxa and symptoms.

4.3 | Health effects of fungi and bacteria

Somewhat surprisingly, in our study bacterial taxa showed stronger relationships with health in the context of moisture damage than fungal ones. Fungi, because they are the visible growth on wet building materials and because they are able to grow at lower water activity levels,[51,54] are generally thought to be more strongly associated with water damage and, consequently, the drivers of water-damaged health associations. However, we observed only weak links between environmental levels of individual fungal species and health outcomes. Along similar lines, a previous study within the HITEA framework showed that muramic acid, a marker of Gram-positive bacteria, and not fungal cell components, was the most important predictor of the immunotoxicological potential of the classroom settled dust.[55] From what we observed here, fungal loads and moisture indicators were less relevant in the respiratory health context than bacterial ones. In fact, the abundance of two fungal moisture indicator taxa in Finland was inversely associated with health symptoms. Similar observations were made in the Dutch samples, where fungal taxa were very abundant and consistent indicators of moisture damage, but almost exclusively associated inversely with respiratory health of exposed pupils.

This counterintuitive relationship, where moisture damage is associated with negative health effects, but the microbial taxa indicative of moisture damage are associated with protective health effects, has been observed previously.[56] In that study, increases in moisture observations were associated with increased fungal diversity, while increased fungal diversity was protective against childhood asthma development.[56] On the other hand, Lai et al.,[57] working in inner-city classrooms, report that an increase in microbial diversity in classroom dust was associated with increased asthma symptoms, similar to the results we found here in Finland. We hypothesize that the timing of the environmental exposures is important and could drive different relationships for asthma development and for symptoms once asthmatic. A review of microbial exposures in school buildings showed that reported associations between these microbial measurements in schools and students' respiratory

health have been protective, detrimental, and absent.[5] However, exposures from moisture-damaged buildings are generally considered to be negative, and thus, the tendency toward a protective relationship observed with moisture-associated fungi in this study remains unexplained.

Many of taxa indicative of moisture damage in this study (Table 4) have not been previously shown to grow in indoor environments, and whether these taxa are actually growing on damp building materials was not determined here. Connections between individual bacteria in the built environmental and occupancy health are limited and are mostly positive[54]: Individual bacterial species encountered indoors from dogs[58] and farms[59] have been shown to have protective effects in mouse and people, respectively. *Sphingomonas* bacteria has previously not been specifically reported in moisture-damaged environments, while in Finland, it explained some of the association with symptom score and wheeze. Yet to be published work by Täubel et al. does show, however, that *Sphingomonas* is a bacterial taxon that is frequently detected on wet building materials and occurs significantly more frequently on damaged materials compared with non-damage ones. Others have reported links with *Sphingomonas* in the microbiome of the respiratory tract with health.[60,61] An ultimate goal is to elucidate the mechanisms by which environmental exposures induce health effects.

5 | CONCLUSIONS

The study took an agnostic approach to identify taxa that were associated with moisture damage and then to examine health effects of those taxa. Rather than relying on *a priori* hypotheses on which taxa or microbial markers should be measured, we utilized the strength of high-throughput sequencing to characterize the diverse microbial milieu and statistical tools to identify the individual taxa of interest. Although limited associations were found, the results indicate that there is promise in this approach to the ongoing endeavor to identify the causative agent(s) behind the health effects of dampness and mold. The associations to microbial exposures should be studied in a high number of buildings assessed for damage, close in both space and time to the occupants for which respiratory health is assessed. Specifically, we suggest looking for strong quantitative responses of microbial taxa or groups, considering both fungi and bacteria.

ACKNOWLEDGMENTS

The authors wish to extend special thanks to the teachers, pupils, principals, and cleaning personnel of the participating study schools for their support and cooperation. We acknowledge the HITEA team of field workers in Finland, the Netherlands, and Spain for sample and data collection, and the laboratory personnel at THL for sample processing and analysis. We are grateful to Asko Vepsäläinen for statistical support and data management, and we thank Pekka Tiittanen for developing data management practices for the HITEA study. We thank Zachary Foster for help in generating the heat tree figure. This work was supported by the European Commission as part of HITEA (Health Effects of Indoor Pollutants: Integrating microbial, toxicological, and epidemiological approaches), Grant agreement no. 211488 under the Seventh Framework Programme, Topic ENV.2007.1.2.1.1. "Indoor air pollution in Europe: An emerging environmental health issue." Additional funding was granted to the project by the Finnish Work Environment Fund (grant numbers 115424, 114139), Juho Vainion Säätiö, Yrjö Jahnssonin Säätiö, the Academy of Finland (grant numbers 252718, 339666, 296587), and the California Department of Public Health

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

Rachel I. Adams contributed to methodology, formal analysis, curated the data, wrote—original draft, and visualized the study. Hanna Leppänen contributed to methodology, investigated the study, and wrote—review & editing. Anne M. Karvonen contributed to methodology, formal analysis, and wrote—review & editing. José Jacobs investigated the study, contributed to resources, curated the data, and wrote—review & editing. Alicia Borràs-Santos investigated the study, contributed to resources, curated the data, and wrote—review & editing. Maria Valkonen investigated the study and wrote—review & editing. Esmeralda Krop investigated the study, contributed to resources, curated the data, and wrote—review & editing. Ulla Haverinen-Shaughnessy investigated the study and wrote—review & editing. Kati Huttunen investigated the study and wrote—review & editing. Jan-Paul Zock conceptualized, investigated the study, contributed to resources, wrote—review & editing, supervised, and involved in funding acquisition. Anne Hyvärinen conceptualized, investigated the study, contributed to resources, wrote—review & editing, involved in project administration, supervised, and involved in funding acquisition. Dick Heederik conceptualized, investigated the study, contributed to resources, wrote—review & editing, supervised, and involved in funding acquisition. Juha Pekkanen conceptualized, investigated the study, contributed to resources, supervised, wrote—review & editing, curated the data, and involved in funding acquisition. Martin Täubel conceptualized, contributed to methodology, investigated the study, contributed to resources, curated the data, wrote—original draft, wrote—review & editing, supervised, and involved in project administration and funding acquisition.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/ina.12865.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Adams RI, Leppänen H, Karvonen AM, et al. Microbial exposures in moisture-damaged schools and associations with respiratory symptoms in students: A multi-country environmental exposure study. *Indoor Air*. 2021;31:1952–1966. https://doi.org/10.1111/ina.12865