NATIONAL INSTITUTE
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DISCUSSIONPAPER

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Foreword

Next to homes, schools are indoor spaces in which not only pupils, but also teachers spend a majority of their everyday time. Moisture and dampness problems in Finnish schools are frequent. This research project (acronym MIKOKO) studies associations between moisture damage and microbial exposures in the school environment, using state-of-the-art analyses tools for the characterization of the school 'indoor microbiome'. These efforts will ultimately pave the way for studies on moisture related microbial exposure and related health effects in teachers. MIKOKO also explores the contribution of the school environment to the everyday microbial exposure of teachers. The proposed activities will not only highlight the public health relevance of moisture problems in schools, but also aim at providing tools for objectively assessing adverse microbial exposure situations in the school environment.

The MIKOKO research project is being implemented in two phases: phase 1 (10/2014-12/2015), which has been earlier reported (ISBN 978-952-302-549-3), and phase 2 (1/2016-6/2017), which is the focus of the current final report. The project was funded by Työsuojelurahasto (phase 1: grant no. 114139 and phase 2: grant no. 115425), Academy of Finland (grant no. 252718) and Terveyden ja hyvinvoinninlaitos (THL).

Abstract

Martin Täubel, Hanna Leppänen, Merja Korkalainen, Anne Hyvärinen. Microbial exposures in moisture damaged schools – an occupational health risk for teachers? National Institute for Health and Welfare (THL). Discussionpaper 36/2017. 26 pages. Helsinki, Finland 2017.
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The quality of indoor air is a globally emerging environmental health issue, as we spend over 90% of our time in various indoor environments. One of the major factors impacting on the quality of indoor air is building moisture and dampness, and the associated growth of microbes. While the adverse health impacts of moisture and dampness are well established, the quest for identifying the causal agents and understanding the pathophysiological mechanisms of ill health in damp buildings is still on-going. Next to homes, schools are indoor spaces in which not only pupils, but also teachers spend a majority of their everyday time, and moisture and dampness problems in Finnish school are frequent. This research is dedicated to study associations between moisture damage and microbial exposures in schools, and the related health effects in teachers. Moreover, this study explores the contribution of the school environment to the everyday microbial exposure of teachers. The proposed activities aim to highlight the public health relevance of moisture problems in schools, and to contribute to improving tools for objectively assessing adverse microbial exposure situations in the school environments.

The project has been implemented with the financial support of Työsuojelurahasto in two phases: phase 1 (10/2014-12/2015), which has been earlier reported (ISBN 978-952-302-549-3), and phase 2 (1/2016-6/2017), which is the focus of the current report. This project has performed in-depth characterizations of fungal and bacterial microbiota in moisture damaged and non-damaged schools in Finland, applying next generation sequencing to samples from two recent studies lead by the National Institute for Health and Welfare: SISU (School Intervention Study) and HITEA (Health Effects of Indoor Pollutants). In addition, this research has implemented another field study that compares microbial exposures of teachers in classrooms versus their home environments (SISUhomes study). In total, more than 700 settled dust and air samples have been analysed, using both quantitative and qualitative (next generation amplicon sequencing) DNA-based approaches for characterisation of microbial exposures. With detailed, statistical analyses of the study data still on-going, preliminary results from this research indicate that the approach using next generation sequencing was successful in identifying compositional differences in microbiota of moisture damaged versus reference classrooms. Quantitative PCR assays have been developed in this project targeting specific bacterial groups that have been identified to be relevant based on prior sequencing investigations. General microbial exposure levels appear to be higher in the home environment compared to classrooms in a study among 90 Finish teachers. Clear compositional differences in microbial exposures were visible between these two environments, which is an aspect requiring follow up.

Overall, this research project clearly highlights the importance of considering not only amount but also compositional, qualitative aspects of the microbial exposures in the school environment, when exploring associations with moisture damage and ultimately with teachers' health. Future research will evaluate health associations of microbial factors identified in this research project.

Keywords: moisture damage, mold damage, moisture damage microbes, schools, teachers, respiratory health, indoor air exposures

Tiivistelmä

Martin Täubel, Hanna Leppänen, Merja Korkalainen, Anne Hyvärinen. Mikrobialtistuminen kosteusvaurioituneissa kouluissa – työterveysriski opettajille? Terveyden ja hyvinvoinnin laitos (THL). Työpaperi 36/2017. 26 sivua. Helsinki 2017.
ISBN 978-952-302-929-3 (painettu); ISBN 978-952-302-930-9 (verkkojulkaisu)

Sisäilman laatu on maailmanlaajuisesti kasvava ympäristöterveydellinen kysymys, sillä me vietämme yli 90 % ajastamme erilaisissa sisäympäristöissä. Yksi suurimmista sisäilman laatuun vaikuttavista tekijöistä ovat rakennusten kosteusvauriot sekä niihin liittyvä mikrobikasvu. Kosteus- ja homevaurioihin liittyvät haitalliset terveysvaikutukset ovat hyvin selviä, mutta epäselvää on, mitkä ovat niitä aiheuttavat tekijät sekä mitkä ovat ne patofysiologiset mekanismit, jotka ovat näiden haitallisten vaikutusten taustalla. Kotien ohella koulut ovat sisäympäristöjä, joissa oppilaiden lisäksi myös opettajat viettävät suurimman osan arkipäivistään. Kosteus- ja homevaurioiden tiedetään olevan yleisiä suomalaisissa koulurakennuksissa. Tämä tutkimus pyrkii selvittämään kouluissa havaittujen kosteusvaurioiden ja mikrobialtistuksen yhteyttä opettajien haitallisiin terveysvaikutuksiin. Lisäksi arvioidaan, mikä osa koulussa tapahtuvalla altistumisella on opettajien päivittäiseen mikrobiologiseen kokonaisaltistukseen. Tutkimus korostaa koulujen kosteusvaurioiden kansanterveydellistä merkitystä sekä kehittää työkaluja, joilla haitallista mikrobialtistusta voidaan määrittää objektiivisesti.

Tutkimus on tehty Työsuojelurahaston rahoittamana. Tutkimus koostuu kahdesta osasta, vaiheesta 1 (10/2014–12/2015), joka on raportoitu aiemmin (ISBN 978-952-302-549-3) sekä vaiheesta 2 (1/2016-6/2017), johon tämä loppuraportti keskittyy. Projektissa onnistuttiin tarkastelemaan syvällisesti sekä sienien että bakteerien mikrobistoa kosteusvaurioituneissa ja vauriottomissa suomalaisissa kouluissa käyttäen uuden sukupolven sekvensointitekniikkaa (NGS). Tutkimuksessa käytettiin näytteitä kahdesta Terveyden ja hyvinvoinnin laitoksen johtamasta projektista: SISU (School Intervention Study) ja HITEA (Health Effects of Indoor Pollutants). Lisäksi tutkimukseen kuului kenttätutkimus, joka vertailee opettajien mikrobialtistumista luokissa ja heidän kotonaan (SISUhomes -tutkimus). Tässä tutkimuksessa mikrobialtistumista määritettiin analysoimalla yhteensä yli 700 laskeutuneen pölyn näytettä ja ilmanäytettä käyttäen sekä kvantitatiivisia että kvalitatiivisia (NGS) DNA-pohjaisia menetelmiä. Tutkimuksen yksityiskohtaiset tilastolliset analyysit ovat meneillään ja jatkuvat vielä. Alustavat tulokset osoittavat, että tutkimuksessa onnistuttiin tunnistamaan eroja kosteusvaurioituneiden luokkahuoneiden mikrobiston koostumuksessa verrattuna vauriottomiin luokkahuoneisiin käyttämällä uuden sukupolven sekvensointitekniikkaa. Tässä projektissa kehitettiin kvantitatiivisia PCR –ajoja ilmaisemaan tiettyjä bakteeriryhmiä, joiden on havaittu olevan merkityksellisiä aiemmissa sekvensointitutkimuksissa. Yleiset mikrobipitoisuudet osoittautuivat olevan suurempia tutkimuksessa mukana olevien 90 suomalaisen opettajan kotiympäristöissä kuin luokkahuoneissa. Näiden kahden sisäympäristön välillä havaittiin myös selviä eroja mikrobiston koostumuksessa, mikä vaatii jatkotutkimuksia.

Kaiken kaikkiaan tämä tutkimus osoittaa selvästi, että tutkittaessa kosteusvaurioiden vaikutusta opettajien terveyteen, ei pelkästään altistumisen määrä vaan myös mikrobiston koostumus eli mikrobialtistumisen kvalitatiivinen tarkastelu kouluympäristöissä on huomioitava. Jatkotutkimuksessa tullaan arvioimaan tässä tutkimuksessa havaittujen mikrobitekijöiden yhteyttä terveysvaikutuksiin.

Avainsanat: kosteusvauriot, homevauriot, kosteusvauriomikrobit, koulut, opettajat, hengitysterveys, sisäilma-altistuminen

Contents

Background

The quality of indoor air is a globally emerging environmental health issue. As we spend over 90% of our time in various indoor environments, it is essential to understand and control any health hazards involved. There are pressures to improve the energy efficiency of buildings, and thus there is a need to solve the complex pursuits to both achieve energy saving aims and maintain good indoor air.

A major factor affecting the quality of indoor air is the building moisture and dampness, and the associated growth of "mould", i.e., fungi, bacteria and other microorganisms (1,2). These are strongly linked with ill health, including increased risk of asthma, respiratory, eye and skin symptoms, repeating infections and several airway and autoimmune diseases (2). The prevalence of dampness and moisture damage varies between 20-50% in different countries, partly depending on the metrics used, and the burden of illness and of economy are substantial (3).

The public health importance of 'building mould' is only partially understood since the causal agents of the various health effects as well as their pathophysiological mechanisms are still insufficiently known. The most evident health-related consequence of microbial growth in moisture-damaged indoor environments are increased concentrations of microbial agents, measured as elevated levels of fungi, bacteria and their structural components (e.g. 4), along with alterations of the microbial profile towards potentially more harmful species (5,6). Attempts to identify causal agents have thus far failed, which may be due to limitations in detection methods for microbes that in the past have mostly been restricted to cultivable and viable microbes. It is evident today, that not one single microbe or one single microbial constituent will be able to explain the diverse, adverse health outcomes observed in resident of damp buildings, but that rather specific characteristics within complex exposures trigger ill health. Recent advances in microbial methods towards DNA based approaches with highest resolution – next generation sequencing (NGS) approaches - are likely to advance the field and may allow for the identification of single microbial groups, microbial consortia or overall microbial diversity that link to moisture damage and adverse health outcomes in exposed residents. First studies applying NGS methods to indoor settings have opened our eyes for the enormous richness and diversity of bacteria and fungi in indoor spaces (e.g.7-13). The studies conducted so far were mostly descriptive in nature and attempted to elucidate factors shaping the indoor microbiome. Health implications of indoor bacterial and fungal assemblages and its diversity have been suggested in recent studies (14,15) and interactions between human and indoor microbiota seem evident (16). Further investigations are warranted with a defined focus on the assessment of moisture problems in buildings and its impact on the indoor microbiome.

Next to homes, schools are indoor spaces in which not only pupils, but also teachers spend a majority of their everyday time. The European, multi-centre HITEA study that was coordinated by the applicant has provided minimum estimates for moisture and mould problems in schools with 24% of school buildings being affected in Finland (17). Dampness and mould in the school environment have been associated with respiratory symptoms and asthma among school children (18-20). Recent analyses from the HITEA study in schools in three European countries – Finland, the Netherlands and Spain – indicated that teachers from moisture damaged schools are at increased risk for upper and lower respiratory tract symptoms (21). Teachers from moisture damaged schools reported more asthma symptoms and the effects were found to be stronger among teachers who had been working for more than five years in the same schools. In addition, work-related wheeze and chest tightness and nasal symptoms were more commonly reported from teachers in moisture damaged schools. A study associated with HITEA recently found that endotoxin exposure – a surrogate for general microbial exposure – was several-fold higher in Dutch schools compared to homes and concluded that exposure at school can contribute considerably to environmental endotoxin exposure of children and teachers (22). These findings imply the public health relevance of damp and mould problems and related microbial exposures in schools as a workplace. Intervention studies involving the removal of the source of moisture problems have shown a positive effect of remediation on the occupants' health (23,24).

Aims and objectives

Thus far, causal links between specific microbial exposure situations in damp schools and adverse health effects in teachers have not been established; neither have microbial exposure levels been detailed for school versus home environments in Finland. This research investigates associations between moisture damage and microbial exposures in schools, ultimately facilitating research on the related health effects in teachers. Moreover, this study explores the contribution of the school environment to the everyday microbial exposure of teachers. The proposed activities aim to highlight the public health relevance of moisture problems in schools, and to contribute to developing tools for objectively assessing adverse microbial exposure situations in the school environment. Such tools should support a better identification of schools with moisture damages and related abnormal and potentially health hazardous microbial exposure situations, and ultimately give way to more targeted remediation actions.

The **hypotheses** underlying this research are:

- ***Exposure to environmental microbes in schools exceeds in quantitative terms and/or is different in qualitative terms to exposure situations in residential homes. Schools affected by moisture damage and dampness with a related increase and/or change in microbial content represent an occupational health hazard for teachers working in such schools.***
- ***Novel methods in sampling and in characterizing microbial communities allow for an identification of components within these microbial communities that differentiate indoor situations characterized by moisture damage from non-damage situations.***
- ***Based on this information, quantitative methods can be developed as diagnostic tools to identify abnormal and health hazardous indoor microbial exposure situations.***
- ***Thorough remediation of moisture and mould damage leads to a reduction in microbial exposures and alleviation of adverse health outcomes in teachers.***

The **objectives** defined for this project were:

1) to provide an in-depth characterisation of fungal and bacterial communities in moisture damaged versus non-moisture damaged schools, using next generation sequencing approaches and standardized samples of settled dust and indoor air;

2) to identify fungal and bacterial profiles/factors that are specific to moisture damage situations by using next generation sequencing data;

3) to apply/develop quantitative, DNA based methods (qPCR) for the fungal and/or bacterial targets identified under point 2, in order to provide tools for an objective assessment of abnormal microbial exposure situations relating to moisture and mould damage;

4) to compare - in a subset of study subjects (teachers) – microbial exposure situations in their working and home environment, in order to evaluate the contribution of school exposures to the total exposure to environmental microbes;

5) to compare microbial exposure situations in schools before and after remediation of the moisture damages and its sources

This research links to another TSR funded project, led by Res. Prof. Harri Alenius (formerly Finnish Institute for Occupational Health:"Kosteusvauriopotilaiden diagnosointiin soveltuvien merkkiaineiden kartoittaminen limakalvoilta systeemibiologian keinoin"). This research utilizes study subjects from the same schools, but focuses on developing diagnostic tools to identify patients that suffer from moisture damage related adverse health outcomes. The herein proposed study will focus on characterizing and identifying microbial exposures relating to moisture damage and dampness, and to provide tools for an objective identification and quantitative assessment of adverse microbial exposure situations in schools.

Materials and methods

Study material

This research expands on materials from two studies that have been carried out in Finnish schools. These studies have in common that they include index and reference school buildings, i.e. schools with and without moisture damage and dampness problems. The damage status in these schools has been established via standardized walk through building inspections. In both studies, extensive collection of exposure samples as well as rich datasets on teachers' health will allow for in-depth analyses of the link between moisture damage, microbial exposures and teachers' health.

The ***SISU study (School intervention study)*** has collected severely moisture damaged schools across Finland, with a part of these schools having been repaired and visited before and after moisture damage renovations. The general field work of this study and basic analyses have been financially facilitated by the Academy of Finland (Microbial toxins indoors – gripping an emerging issue; Grant No. 252718). In total, 11 moisture damaged and 5 non-damaged schools have been recruited to the study. Seven of the moisture damage schools have undergone renovations. Detailed exposure and health assessments have been conducted before (all 16 schools) and after remediation (7 schools).

In total, more than 200 teachers are taking part in the SISU study. Exposure samples have been obtained from over 100 classrooms and teachers' lounges in the study schools; airborne settled dust as well as active air samples have been collected. In total, 261 settled dust and 101 active air samples have been utilized for in-depth exposure studies. Questionnaires and symptom diaries have been applied to both, pupils and teachers. Additional clinical sampling (exhaled NO, blood serum, nasal lavage) has been performed from teachers in the course of school visits by nurses and researchers with medical background, to allow determination of immunological parameters. The herein described research is joined with TSR supported research efforts carried out at Työterveyslaitos (TTL, group of Professor Harri Alenius), focusing on immunotoxicological and system-biological aspects of exposures in moisture damaged schools.

The ***SISUhome study*** is an addition to the SISU study in schools and aims to determine the contributions of the home and the school environment to the total microbial exposure indoors for teachers. SISUhome has recruited more than 100 teachers participating in the SISU school study. Electrostatic dust fall collectors have been distributed to these teachers' homes and to their primary teaching classrooms. Questionnaires on home characteristics and health status of these teachers have been collected from the study participants. In total, samples from 89 teachers with paired data of the home and classroom environment were available for analyses of general microbial levels.

The ***HITEA study (Health Effects of Indoor Pollutants)*** is a multi-center, international study that has been implemented during 2008-2013, financed under the EC Framework Programme 7, and that was coordinated by the applicant of this research, Research Prof. Anne Hyvärinen. The study part on indoor exposures in schools has been conducted in identical set-up in Finland, The Netherlands and Spain, representing three climatic regions in Europe. In total, more than 60 schools with and without moisture damage and over 600 teachers participated in the early phases of the study, revealing associations between moisture damage and dampness in schools and adverse respiratory health in pupils and teachers. For this current research, we utilized exposure samples from 6 schools in Eastern Finland (4 index, 2 reference schools) that were followed up in a longitudinal fashion over more than one school year. Exposure samples were taken at three points in time (winter 2009, spring 2009, winter 2010) from approx. 60 classrooms and teachers' lounges. A total of 193 settled dust samples collected with EDCs (see detailed description of EDC samples below) were utilized for in depth characterization of microbial exposures in moisture damaged versus non-damaged Finnish schools. Health data that have been collected from the teachers in these schools include respiratory health questionnaire data, information on lung function (spirometric measurement) and airway inflammation (exhaled NO), inflammatory cytokines in serum and nasal lavage, and total IgG in serum.

Building investigations and microbial sampling

Information on building structures, history, materials and observations of moisture damage and dampness from HITEA and SISU schools have been received from standardized technical investigations that were performed by trained civil engineers. The inspections included walkthroughs utilizing pre-designed checklists for the collection of observations of moisture damage, dampness and visible mould, including their extent and severity. Non-destructive measurements included hand-held moisture detectors, relative humidity, temperature and CO2 monitoring devices. Standardized questionnaires were utilized to collect detailed information on characteristics (building structure, materials, HVAC system, etc.), condition and maintenance of the school buildings. Moisture damage status of homes in the SISUhome study was assessed via questionnaire.

Inhalation exposure to microbes occurs mainly via airborne route, hence air samples in theory characterize best these exposures. Due to the great variations in air concentrations and poor reproducibility, however, settled dust samples with higher reproducibility are preferably collected (25-27). Exposure samples that have been or are being collected in these two school studies and in SISUhome study and that will be utilized in the herein proposed research are:

- Airborne settled dust, passively collected with EDCs (electrostatic dust fall collector; 28): electrostatic dust wipes have been placed on elevated surfaces like shelves or cupboards for a period of 4-8 weeks; the sample collected represents an integrated sample of exposure over a standardized period of time.
- Settled dust from elevated surfaces, collected with a vacuum cleaner into a dust sampling nylon sock (27): the advantage of this sample material compared to EDC dust is that larger sample amounts can be collected, while the age/history of the dust is unknown.
- Active air samples, collected with Button inhalable aerosol sampler (29): active air samples were collected in SISU schools over a period of 2 school days (12hours), at a flow rate of 4L per minute that allows for collection of the actual inhalable dust fraction in indoor air. In each school, samples were collected in three classrooms and one outdoor location.

Microbial analyses methods

- Next generation sequencing on Illumina MiSeq platform (300bp, paired end) was performed for an in-depth characterization of fungal and bacterial communities in indoor air and airborne settled dust. Analyses of the bacterial and fungal assemblages target 16S V4region and ITS1/ITS2 region, respectively. The seque4cning service was outsourced to the DNA sequencing laboratory at LGC Genomics, Germany. Bioinformatics processing and analyses of sequence information largely relied on QIIME software pipeline (www.qiime.org) with built in modules from an in-house pipelines at THL, Department of Health Security. Reference based OTU picking was done using greengenes database for 16S data and UNITE database for ITS fungal data. Alpha diversity was assessed using phylogenetic diversity indices, Chao1 estimates and observed species within each sample. Beta diversity patterns between the samples were explored using PCoA plots, distance histograms, taxonomy charts, rarefaction plots, etc. LDA effect-size (LEfSe) algorithm (30) was used the characterize differences in individual microbial signals between two or more environments (e.g. moisture damage or not) and to assign effect sizes to these differences. MaAsLin (Multivariate Association with Linear Models; http://huttenhower.sph.harvard.edu/maaslin) is used to find associations between building metadata and microbial community abundance or function.

- Quantitative real-time PCR (qPCR) was performed at THL, Environmental Health Unit. QPCR was used to quantify microbial exposures in moisture damaged and non-damaged schools (HITEA, SISU), before and after renovation (SISU), and in homes of teachers (SISUhome). The qPCR analyses targeted a basic set of fungal and bacterial species/groups that represent basic microbial exposure levels indoors, including general assays targeting total fungal DNA, Gram-positive and Gram-negative bacteria, as well as more specific assays targeting *Cladosporium* spp., and *Penicillium/Aspergillus*

group. In addition, 3 qPCR assays were developed in the course of this research project targeting the most promising microbial taxa that have been identified via sequencing to be relevant with respect to moisture damage and dampness indoors.

Health assessment in teachers

The health status of teachers in SISU was assessed using symptom questionnaire information collected prior and post remediation in the schools. In addition, a marker for lung inflammation (exhaledNO), basic blood parameters and levels of inflammatory cytokines in serum were used to assess teachers' health. In SISU home study, the health status of the teachers was assessed via questionnaire. In HITEA, the collected health data include respiratory health questionnaire, information on lung function (spirometric measurement) and airway inflammation (exhaled NO), inflammatory cytokines in serum and nasal lavage, and total IgG in serum. The health questionnaires both in HITEA and SISU were developed including items on demographic characteristics, relevant exposures (at home) and especially on respiratory health. Questions on respiratory health were based on the validated International Study of Asthma and Allergy in Children (ISAAC) Questionnaire (31). The herein described research will focus mostly on the exposure aspects of moisture damage and related microbial proliferation. However, in follow-up efforts we will combine the rich health data sets that are collected in HITEA and SISU studies to the very detailed exposure data generated in the proposed study. Moreover, this application links to research efforts carried out at Työterveyslaitos (TTL, group of Professor Harri Alenius), focusing on immunotoxicological and system-biological aspects of exposures in moisture damaged schools.

Ethical considerations

Ethical issues in this research were considered; both HITEA and SISU studies have received ethical approval by the local ethical committee (Pohjois-Savon Sairaanhoitopiirin Tutkimuseettinen Toimikunta). All teachers participating provided informed consent prior to health measurements.

Implementation and time schedule

Overall implementation of the research

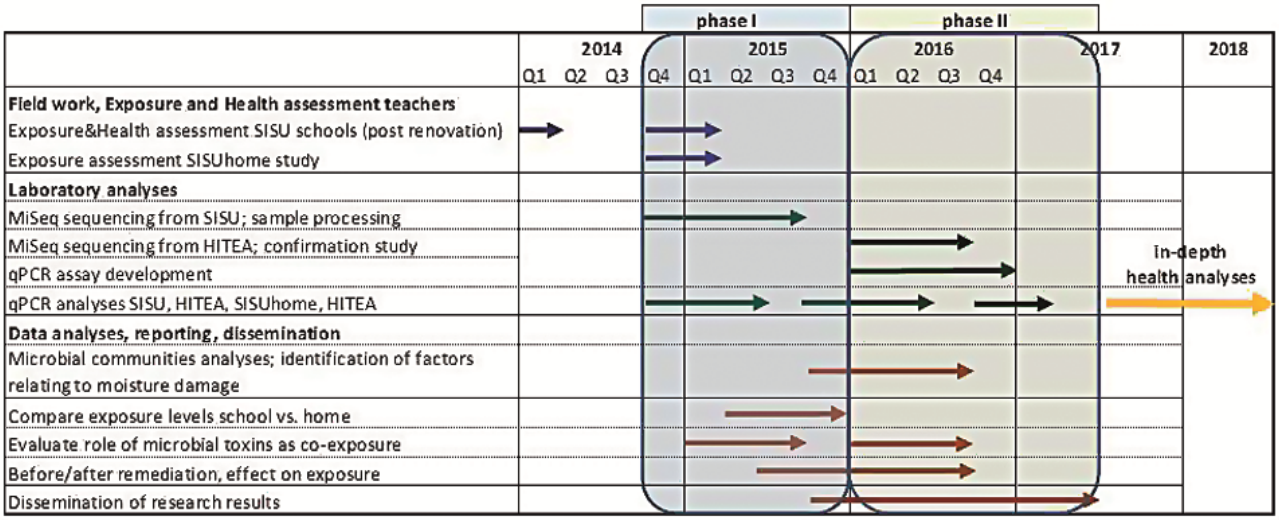
In order to answer **objectives 1 and 2**, we subjected settled dust samples from more than 150 classrooms collected in SISU and HITEA studies to Illumina MiSeq sequencing, to provide an in-depth characterisation of fungal and bacterial communities in moisture damaged versus non-moisture damaged schools. The SISU study provided settled dust and active air samples from classrooms of index (moisture damaged) and reference (non-moisture damaged) schools, as well as from moisture damaged schools before and after renovations. Settled dust samples from the HITEA study were subjected to sequencing in a second, confirmation study. Based on information on bacterial and/or fungal species and groups of species that were found to be linked to conditions of moisture damage and dampness, we developed quantitative PCR assays against some of the promising target groups identified, linking to **Objective 3**. **Objective 4** was answered by the SISU and the SISUhome study, in which we measured microbial exposures of 90 teachers in their workplace (primary teaching classrooms) as well as in their home (living room). Microbe levels of airborne settled dust collected with EDCs were analysed using qPCR assays to enumerate general (total fungal, Gram-negative, Gram-positive) and more specific microbial targets (*Cladosporium* spp., *Penicillium/Aspergillus* spp.). The study design of SISU allowed for answering **objective 5**, which was to compare microbial exposure situations in schools before and after remediation of the damages. Exposure samples (airborne settled dust, active air samples) from the same schools/classrooms were analysed before and after the remediation actions, using quantitative PCR and amplicon sequencing. We used general assays (total fungal, Gram-negative, Gram-positive) to conclude on any changes in total microbial levels, but also target a number of indoor and outdoor relevant fungal and bacterial groups for more specific comparisons. We also included measurements with the novel qPCR assays developed under objective 3.

Time schedule and phases of the project

This research project has been implemented in two core phases, following the funding decisions made by Työsuojelurahasto. Thus, the work has been organized into a phase I (01.10.2014-31.12.2015; period supported by TSR grant No. 114139 and previously reported), and a phase II (01.01.2016-30.06.2017; period supported by TSR grant No. 115425, reported herein). The time schedule, phases and specific tasks referring to this research project are detailed in Table 1 below.

This current project report mainly details phase 2 of the MIKOKO study, but brief information on activities carried out in phase 1 will be complemented to provide a full picture of the project achievements. Following the implementation of phases 1 and 2 and based on the results of the MIKOKO research project we anticipate follow-up studies on this topic that will focus on linking the extensive exposure dataset generated under the current project to rich health datasets that were also collected in SISU and HITEA projects.

Table 1. Time schedule, phases, and specific tasks in the MIKOKO study.



Results and discussion

Major accomplishments during phase 1 of the project have been reported previously (see report under http://urn.fi/URN:ISBN:978-952-302-549-3). In summary, activities under phase 1 refer to:

- **Finalization of field work efforts in SISU schools – exposure and health assessments, before and after renovation**, complementing project fund provided by the Academy of Finland (grant no. 252718).
- **Recruitment and field work in SISUhomes study** – 104 teachers from the SISU schools, were asked to sample their classroom and home environment to allow microbial exposure assessment in these two environments; for 89 study subjects we received valid paired samples. Quantitative microbial analyses were conducted on these sample materials; health questionnaires were additionally collected from the teachers.
- **Sample processing, DNA extraction, and next generation sequencing of settled dust and air samples from the SISU study**. A total of more than 500 settled dust and air samples collected in SISU schools have been processed, DNA has been extracted and all samples have been subjected to quantitative PCR analyses. In addition, more than 370 settled dust and air samples have been subjected to next generation sequencing (NGS) for the characterisation of bacterial and fungal communities in classrooms of the SISU study.
- **Data analyses in SISUhomes and SISU studies had been initiated and partly completed**.

In the following we present and discuss results that build on and continue this previous work from phase 1 and were produced during phase 2 of the project. In-depth statistical analyses of study results are still on-going and peer-review publication in scientific journals is under preparation, prohibiting a detailed presentation of study findings in this open report pre-publication.

Microbial characteristics of moisture damaged and non-damaged classrooms in the SISU study

In this project, the focus was on the analyses that evaluate microbial differences in moisture damaged versus non-damaged classrooms in the SISU study utilizing the newly generated sequencing data. The quantitative microbial analyses of exposure samples from the SISU study are only complementary to the TSR supported research project and therefore are only briefly commented on here. Quantitative PCR analyses of more than 500 settled dust and active air samples collected in 16 SISU schools before and 7 schools after renovation have been performed using both bacterial and fungal quantitative PCR assays. We measured total fungal DNA, *Penicillium* spp./*Aspergillus* spp. group (Pen/Asp group), *Cladosporium herbarum*, as well as Gram-positive and Gram-negative bacteria. Summarizing the results of the analyses based on settled dust samples, we did not identify significantly higher levels of the general microbial markers in index compared to reference schools, and neither did we observe reductions in the general microbial levels post moisture damage remediation in a subsample of study schools. An exception here was the qPCR assay newly developed under this current project, targeting *Phycicoccus* spp., for which we observed a significant reduction in the post compared to pre renovation situation (more details provided in section 5.3). In active air samples, we observed generally higher microbial levels in index versus control schools. These data are currently being further evaluated and analysed.

Next generation, amplicon sequencing was performed on settled dust and active air samples of the SISU study, to characterize the bacterial and fungal microbiota in Finnish classrooms and to evaluate potential associations with moisture damage. These analyses are on-going, but we summarize here some of the preliminary findings. With the aim being to identify microbial signatures of moisture damage, i.e. to identify microbial taxa differentially present and prevalent in moisture damaged and non-damaged classroom samples, we followed an approach that has been recently published by our group (34; we refer to this paper for more detailed description). Using a community structure based approach we first identified principle coordinates from the bacterial community distance metrics with associations to moisture damage, based on the origin of the dust samples from classrooms of a damaged or reference building. In a second step, taxa strongly correlating to the coordinates of interest were identified. We observed that the majority of taxa correlating were phylogenetically allocated to several families within the order of Actinomycetales and to the Acetobacteraceae family within the Rhodospirillales order (Figure 1). These results are partly in line with our previously published findings on the potential relevance of Actinomycetales bacterial taxa as indicators of moisture conditions in residential homes (34). Current analyses are focusing on the evaluation of associations of these and other bacterial taxa with moisture damage in the SISU classrooms, applying different statistical tools.

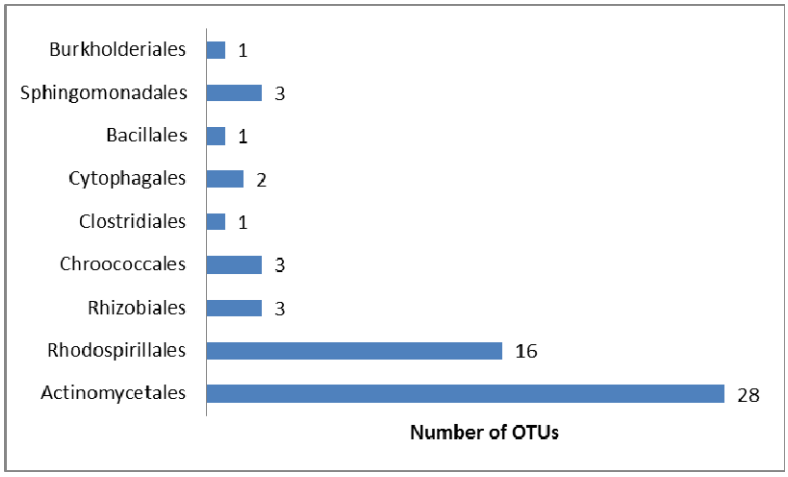


Figure 1. Number of OTUs (by different bacterial orders) that were highly (>0.5 correlation score in Spearman Rank order) correlated with bacterial principle coordinate 2.

Analyses of the fungal microbiota are currently being processed. Initial results are encouraging in that we observe a separation – even if only subtle - of settled dust samples by moisture damage (yes/no/post renovation), based on the dissimilarity in the fungal community composition of these samples (Figure 2). This indicates that identification of fungal moisture damage signatures could be feasible and research on this topic is currently on-going. We anticipate scientific publication of the study findings in SISU based on next generation sequencing until summer 2018.

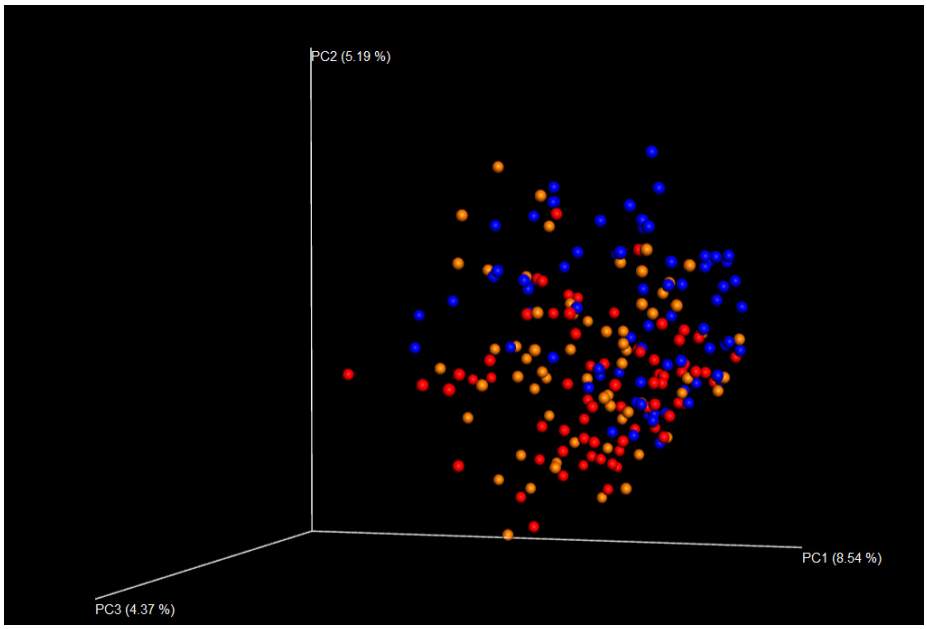


Figure 2. Principle coordinate analysis plot of fungal microbiota based on Bray Curtis distance. Displayed is between sample similarity and dissimilarity based on the fungal community composition in settled dust from reference schools (red), moisture damaged schools (orange) and post-renovation schools (blue).

Microbial exposures in classrooms compared to home environments – the SISUhomes study

From 104 teachers that participated in this study, we received samples from both home and classroom from 89 teachers, in addition to health and home exposure questionnaires. 178 EDC samples were processed to extract dust and subsequently DNA, and quantitative PCR was applied to measure several bacterial and fungal species and groups, in order to compare microbial exposure levels and composition in homes versus schools. We measured total fungal DNA, Penicillium spp./Aspergillus spp. group (Pen/Asp group, including several of the most common indoor fungal species), Cladosporium herbarum (a mold with strong outdoor context).

Fungal and bacterial levels in settled dust were statistically significantly higher in the home environment compared to classrooms (Figure 3). This was true for total fungal content, more outdoor related fungi (*C. herbarum*) as well as for more indoor prominent fungi (Pen/Asp group). For bacteria, the effect was more pronounced for Gram-positive compared to Gram-negative bacteria. We interpret this finding of lower microbial levels observed in classrooms compared to home environments to likely relate to more efficient and fully mechanical ventilation with filtered incoming air in all study schools, but further analyses need to be done to confirm this. Also other factors may contribute to the lower microbial levels generally observed in the school environments, including: less frequent occurrence of so called normal microbial sources or sinks such as vegetables covered with soil, firewood, pets, or rugs on flooring; potentially more frequent cleaning activities; and no shoes and outdoor clothes being worn in classrooms, which are vehicles for tracking in outdoor particles and microbial material.

Interestingly, when comparing the microbial content in classrooms versus homes, there are not only differences in the general levels, but also clear and significant differences in the composition of the exposure. For example, the total fungal content in homes is more dominated by *Penicillium* spp. and *Aspergillus* spp., and the bacterial content of homes is more dominated by Gram-positive bacteria versus Gram-negative bacteria, compared to the situation in classrooms. These findings were statistically significant, and indicate that compositional, qualitative aspects will be relevant in addition to quantitative aspects, when comparing school and home environments with respect to their microbial exposure situations and potential impact on teachers' health. Follow-up studies comparing in detail the bacterial and fungal microbiota in these two environments are warranted.

When comparing the general microbial exposure levels in settled dust in moisture damaged versus non-damaged or renovated schools, we observe mostly higher microbial levels in moisture damaged schools; however, these findings do not reach statistical significance (Figure 4). It is obvious that next to amount also the microbial composition of the exposure may be crucial in both relating to moisture damage and to health effects observed in teachers in moisture damaged schools. We also observe large variation between individual schools with respect to their mean bacterial (up to 10-fold differences) and fungal (up to >30-fold difference) levels, and these data will be analysed also against symptom questionnaire data collected from teachers and pupils in these schools, to investigate effects of microbial exposure on teachers' health, also independent of moisture damage.

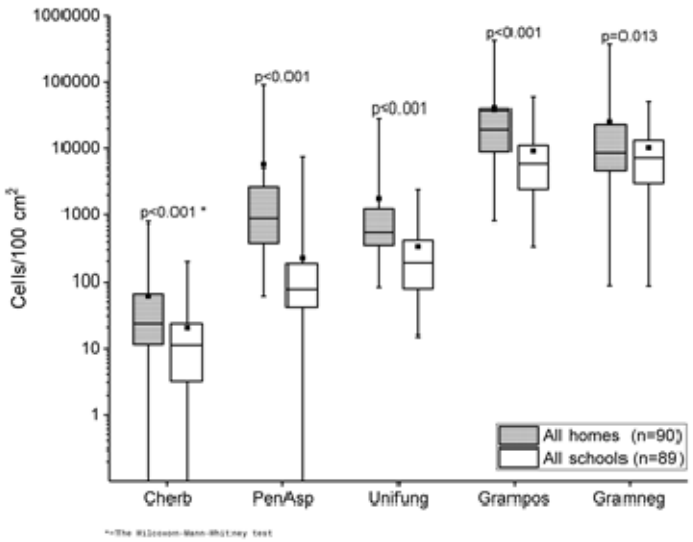


Figure 3. Microbial levels in homes (n=90) vs classrooms (n=89) of teachers in the SISUhomes study (qPCRs for: *Cladosporium herbarum* (Cherb), *Penicillium/Aspergillus* group (PenAsp), total fungi (Unifung) and Gram-positive (Grampos) and Gram-negative (Gramneg) bacteria; whiskers depict min and max, boxplots 25th, 50th and 75th percentiles, squares indicate mean values).

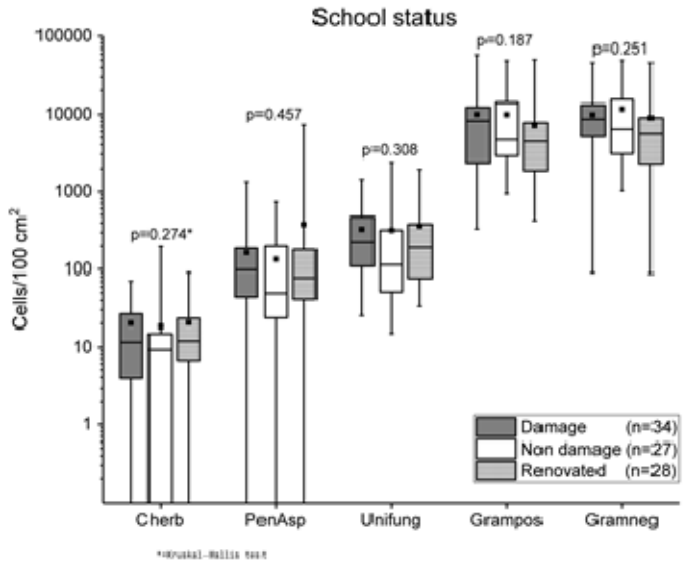


Figure 4. Microbial levels determined in 89 classrooms from moisture damaged, non-damaged and post-renovation schools included in the SISUhomes study (qPCRs for: *Cladosporium herbarum* (Cherb), *Penicillium/Aspergillus* spp. group (PenAsp), total fungi (Unifung) and Gram-positive (Grampos) and Gram-negative (Gramneg) bacteria).

Development of quantitative PCR assays targeting moisture damage indicators

New qPCR assays were developed for selected bacterial groups based on earlier findings that indicated associations of these bacterial groups with moisture damage (34): *Burkholderia* spp., *Pedobacter* spp., *Phycicoccus* spp., *Nakamurellaceae* spp. and *Xanthomonadaceae* spp.. First, the data services of the Ribosomal Database Project (RDP) were used to collect all annotated 16S sequences of bacteria within these phylogenetic groups (86 *Burkholderia*, 53 *Pedobacter*, 5 *Phycicoccus*, 4 *Nakamurellaceae* and 176 *Xanthomonadaceae* sequences). The sequences were aligned and phylogenetic trees were built using EMBL-EBI MAFFT or Clustal Omega Multiple Sequence Alignment tools. The alignment of 5-6 randomly selected sequences were used to design primer pairs for each bacterium, at genus or family level, by the web based primer finder PriFi. This program generated a list of potential primer pairs fulfilling a number of criteria, such as the optimal length of the amplicon, the optimal melting temperature and the length of primers (Figure 5). In some cases, also Primer-BLAST and Primer3Plus tools were used in addition.

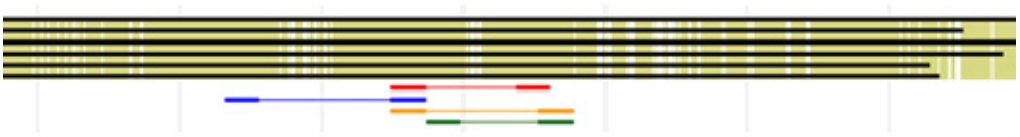


Figure 5. An example of PriFi results showing suggested primers (red, blue, yellow, green) for Burkholderia strains (black lines).

In silico –checking of suggested primer pairs were done using RDP's Probe Match and Basic Local Alignment Search Tool (BLAST). The most promising primer pairs were selected (2-3 primer pairs / assay) for development of *Burkholderia*, *Pedobacter* and *Phycicoccus* qPCR assays.

Gradient PCRs were performed to check the specificity and find optimized PCR conditions for the each primer pair using DNA from bacterial target and non-target strains. Based on these results, the best primer

pair was selected to be used for each assay. The best options for these assays were further tested using temperature gradient and different primer concentrations. Next, standard curves for each target strain were run using real-time PCR (QuantStudio6, Applied Biosystems) and SYBR Green technique (Figure 6).

The performance of these qPCR assays and their potential to differentiate moisture damaged from non-damaged environments was tested on settled dust and air samples of the SISU study. In technical terms, the performance of these three qPCR assays appeared to be good when applied to actual indoor sample materials. The *Phycicoccus* spp. qPCR assay was most promising in that significantly higher levels of *Phycicoccus* bacteria were detected in settled dust of classrooms from moisture damaged schools in the before versus after renovation comparison, i.e. levels were significantly reduced in classrooms following moisture damage renovations. We also observed elevated *Phycicoccus* qPCR levels in air samples in index (moisture damaged) versus reference schools. The suitability of these quantitative measurement approaches to objectively support building investigations for moisture damage and indoor mold and/or to follow-up successfulness of renovation activities will be further evaluated.

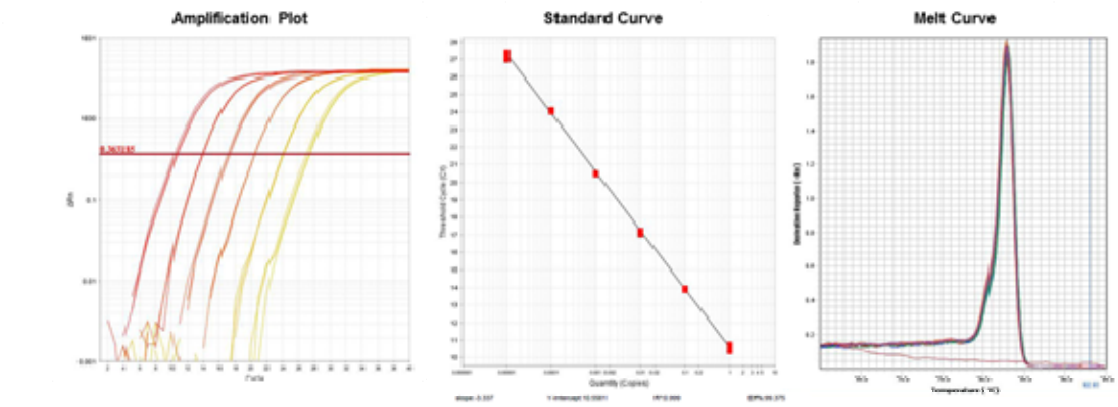


Figure 6. An example of a qPCR experiment showing amplification plot, standard curve and melt curve of one Pedobacter target strain.

Microbial characteristics of classrooms from moisture damaged and non-damaged schools in HITEA

The international HITEA study, originally carried out in school buildings from three European countries, located in three different climatic regions of Europe – Finland, The Netherlands, and Spain – was included in this research project in order to complement, confirm and strengthen findings on microbial signatures associated with moisture damage in the SISU study. Here, we report on the analyses of >180 settled dust (EDC) samples collected in >60 Finnish classrooms in three repeated assessments over one year. The TSR support allowed us to perform new extraction of EDC wipes collected in the HITEA study and perform basic quantitative microbial measurements with qPCR and in-depth qualitative measurements of the bacterial and fungal microbiota in classrooms using next generation sequencing.

In the quantitative measurements we observe clear seasonal trends in the microbial exposure in classrooms, with significantly higher levels of total fungi, *Penicillium*/*Aspergillus* spp. group, and Gram-negative bacteria in the spring assessment as compared to the winter measurements. We did not observe such significant seasonal difference for Gram-positive bacteria; this makes sense, given that the major source of Gram-positive bacteria are the human occupants, i.e. pupils and teachers, in classrooms, and this source is rather constant throughout occupied periods of the school year.

In this analysis we found microbial levels to be significantly higher in classrooms from moisture damaged compared to non-damaged, reference school buildings in Finland. This was true for all microbial

parameters quantitatively assess here, i.e. Gram-positive and Gram-negative bacteria, total fungal DNA and *Penicillium*/*Aspergillus* spp. group. We moreover found dose-response relationships between microbial measurements in the classrooms and a three-level moisture damage score based on location, extent and severity of moisture damage observations made in the study schools. These findings are highly relevant and also somewhat surprising, as earlier microbial measurements have not shown such clear associations of microbial levels with moisture observations in the Finnish school buildings in this same study. We assume that the sample type that was used in this current study – settled dust collected with electrostatic dust collectors – was better compared to earlier approaches in that sample collection occurred passively for a known period of time on a standardized area, while the samples were less prone to resuspension of particles from the sampling surface. Thus, this TSR supported initiative for re-analysis of the sample materials will have a major impact on data analysis and reporting of the HITEA study.

In addition to the quantitative measurements, we also performed amplicon sequencing on the same sample materials in order to characterize the bacterial and fungal microbiota in Finnish classrooms and to evaluate potential associations with moisture damage. These analyses are on-going, but we summarize here some of the preliminary findings. We observed a significant difference in both bacterial and fungal communities in Finnish classrooms from moisture damaged versus non-damaged buildings (using ADONIS statistical analysis). However, the effect size of this finding was very small, i.e. moisture damage as such explained only a small part of the variation in microbiota composition. We then set out to further evaluate differences in individual taxa between moisture damaged and non-damaged classroom samples, i.e. to identify microbial signatures of moisture damage. We followed an approach as recently published in our group and refer to this paper for more detailed description (34). This was a community structure based approach that identifies principle coordinates from the bacterial and fungal community distance metrics with associations to moisture damage (yes/no), based on the origin of the dust samples from classrooms of a damaged or reference building. In a second step, taxa strongly correlating to the coordinates of interest are identified, representing potential moisture damage indicator taxa. Similar to our findings from the SISU study, members of the *Actinomycetales*, *Rhodospirillales* and *Rhodobacteraceae* order are prominent among such taxa. These preliminary findings are encouraging and more detailed analyses of bacterial and also fungal taxa indicative of moisture damage are ongoing; results from this study will be published in the form of a scientific paper (timeline for submission: spring 2018).

Conclusions and outlook

The MIKOKO research project has been successfully implemented in a major effort that included state of the art microbial analyses of sample materials from three different studies, in order to evaluate teachers' microbial exposure situations. The objectives defined prior to the start of the research project have been fulfilled.

The current state of the analyses indicates that the approach using next generation sequencing was successful in identifying compositional differences in microbiota of moisture damaged versus reference classrooms. Additional analyses to pinpoint specific microbial features that are differently present in damaged versus reference classrooms are currently on-going. Quantitative PCR assays have been developed in this project targeting specific bacterial groups that have been identified to be relevant in moisture damage based on prior sequencing investigations. Initial results suggest that some of these quantitative measurement approaches may prove useful in supporting moisture damage investigations, but further evaluation will be needed. General microbial exposure levels appear to be higher in the home environment compared to classrooms in a study among 90 Finnish teachers, but clear compositional differences in exposure were visible between these two environments, which is an aspect of interest to follow up on.

This research project clearly highlights the importance of considering not only amount but also compositional, qualitative aspects of the microbial exposures in the school environment, when exploring associations with moisture damage and ultimately with teachers' health. The approach proposed and followed in this project to analyse in depth the fungal and bacterial communities in moisture damaged versus non-damaged schools using next generation sequencing is highly promising. Initial results are encouraging in that they indicate associations of specific microbial taxa with moisture damage, even in situations where differences in general microbial levels were absent.

Future efforts based on the work accomplished in this project will focus on further statistical analyses and scientific publication of the study findings. Importantly, we plan on linking these results with teachers' health data available from the same studies, so to explore associations between moisture damage, microbial exposures and teachers' health.

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