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Examination of total cell count in building materials by acridine orange direct count (AODC)

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ABSTRACT

The Acridine orange direct count (AODC) enables the determination of total cell count (TCC) in building materials. The AODC technique is considered as an analytic standard in many different areas but is not common in the indoor environment. In this paper AODC was tested with various building materials to investigate TCC. Colony forming units (CFU) only detect viable cells in contrast to the TCC assay which estimates total microbial contamination. The results show that the total cell count is a reliable and sensitive method to investigate the indoor environment.

1. Introduction

The investigation of indoor material and air samples for moulds is widespread, with the aim to estimate both remediation costs and health risks. The sampling strategy, sampling itself, preparation and cultivation of the sample, and the identification of colony forming units (CFUs), are described in the Deutsches Institut für Normung, or German institute of Standardization (DIN), the International Organization for Standardization (ISO) Number 16000–17, and the following guidelines. There is thus a broad consensus about the evaluation of CFU of moulds and bacteria from indoor environments and verified by a corresponding round robin test from the Baden-Württemberg Department of Health.

In recent years, the microscopic evaluation of samples has been established alongside CFU analysis. In air samples, adhesive microscope slides are coated via impaction and analysed microscopically (DIN ISO 16000-18, 2012). Surfaces of materials are sampled using clear strips of adhesive film and evaluated via light microscopy. This methodology has not yet been validated, but the Verein Deutscher Ingenieure, or Association of German Engineers (VDI), is currently developing a guideline on sampling and evaluation. In spite of the lack of validation, there are methods for microscopic analyses of air and of material surfaces.

CFUs from material samples are analysed by the suspension method, which takes the complete sample into account rather than just the surface. Spores, hyphae, and bacteria are transferred into a sterile suspension when preparing the sample (DIN ISO 16000-17, 2010).

Adhesive film samples represent only the surface of a material. Hence, adhesive film samples do not correlate with the number of CFUs identified using the suspension method for the same material. To obtain comparable results, the same suspension must be used for the CFUs and microscopic investigation. The microscopic determination of spores, hyphae, and bacteria in the suspension is the analysis of TCC (Trautmann and Meider, 2018). The literature contains many studies of suspensions using fluoresce molecules such as 3,6-bis[dimethylamino] acridinium chloride, or acridine orange (AO), and 4′,6-diamidino-2-phenylindole (DAPI). Kepner Jr and Pratt (1994) investigated the staining properties of two dyes on bacteria from environmental samples and determined that epifluorescence direct counting is the best available method for the counting of total bacteria in environmental samples. Kepner Jr and Pratt (1994) also described that more cells in environmental samples were stained with AO than with DAPI. It is also determined that the epifluorescence direct counting method for the determination of bacteria numbers using AO was recognized as the standard method by the American Public Health Association (American Public Health Association, 1989).

Hobbie et al. (1977) described the staining of the suspension on Nuclepore filters to count bacteria. With AO, the RNA and DNA strands of cells contained in the suspension are stained and then counted via fluorescence microscopy. This publication was the basis for many additional investigations and the method came to be known as acridine orange direct count (AODC). This staining and counting method has been used in different areas and has quickly come to be considered the standard method for determining microorganisms in suspensions. It has also been used as a comparative measurement (Cragg and Parkes, 2014). In 1988, Negrón-Alvíra et al. (1988) investigated water samples for Legionella using the AODC method. In other publications, the AODC method was used to investigate clay samples (Stroes-Gascoyne et al., 2007), foodstuffs (Strayer et al., 2007), water samples (Cragg and

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Parkes, 2014), and rock samples (Vestergård et al., 2011) for bacteria. The counting of the total cell count of moulds using AODC was published by Yamamoto and Uchida in 1982 and by Houtman et al. (2016). Other areas in which epifluorescence microscopy is used involves the investigation of algae and other microorganisms on building facades (Messal, 2009) and medical research of tumour cells (Liu et al., 2014, 2017).

To determine the TCC of moulds and bacteria in air samples (filtration method), AODC was used and published as the CAMNEA method (Palmgren et al., 1986). This method was taken up and described as VDI Guideline 4253 Part 4. In this method, however, DAPI is used as the dye (VDI 4253 Blatt 4, 2013).

The analysis of a suspension with the CFU method and the TCC method shows not only the viable microorganisms of a sample that can be cultivated, but also the moulds and bacteria that do not grow under laboratory conditions. Kepner Jr and Pratt (1994) have shown that there are also viable bacteria that did not produce as CFUs. Sherachan et al. (2018) also state that viability and the ability to be cultivated are not synonymous, and that the ability to be cultivated is reduced considerably by stress (e.g. lack of moisture). Investigations of indoor samples have revealed that the TCC can be > 100 times higher than the CFU (Trautmann and Meider, 2018).

Indoor environment samples are being investigated for microbial loading to estimate the level of health risk to inhabitants. In most studies on the topic of interior space contamination, the CFU is used as the reference method. However, it is difficult to establish a link between health effects and microorganisms through qualitative detection of viable microorganisms (WHO, 2009; Järvi et al., 2018). Newer investigations have shown that a health-endangering effect may also arise from microorganisms that cannot be cultivated (Croston et al., 2018; Korkalainen et al., 2017). It is thus becoming ever clearer that the health risk determination is independent of the viability of spores. Different cellular components and metabolic products can initiate inflammatory processes in the body (Croston et al., 2018; Korkalainen et al., 2017). The total amount of microorganisms is therefore more important than the composition of microflora when assessing the level of health risk.

The objective of this study is to investigate whether the AODC method is suitable for determining the total cell count for moulds and bacteria on construction materials. In addition to microorganism composition, therefore, the total number of microorganisms is of great importance in the assessment of health risk.

2. Materials and methods

2.1. Sample preparation

The sample was measured and weighed, and all other specifics, such as appearance and humidity, were recorded on the laboratory report. The suspension was prepared analogously to ISO DIN 1600–17 and 16,000–21. The sample was reduced to small pieces and 1–10 g of the sample was transferred into a baffled flask, covered with a specific amount of buffered saline solution (5–100 ml), and shaken at 200 rpm for 15 min. The sterile saline solution was buffered so that the pH affected the microorganisms as little as possible. In addition, the solution Tween 80 was added to lower the surface tension. For the TCC counting, one part of the suspension was fixed with 37% formaldehyde (formalin) in order to avoid a change in the number of microorganisms in the samples through lysis or cell growth.

2.2. Filter preparation

100 µl of the suspension was filtered with 2 ml of demineralized and sterile filtered water and four drops of sterile filtered AO on a black polycarbonate filter with a pore size of 0.4 µm and a diameter of 25 mm. A cellulose filter that was moistened with sterile filtered and demineralized water was used as a support filter on the filter holder. The dyed suspension was sucked by a pump from the funnel through the polycarbonate filter.

After the dyeing process, the filter was transferred to an object plate, wetted with a drop of immersion oil, and covered with a coverslip. Before counting, one more drop of immersion oil needed to be applied to the coverslip.

For some mineral samples (e.g. plaster) the dye can lead to strong background lighting. Antifading reagent (e.g. Citifluor AF 2) or hydrochloric acid may be used in this case.

2.3. Microscope set-up

The total cell count of the filter surface was counted using a magnification of x1250 with an Olympus BH2 set-up for Epifluorescence and a filter set for acridine orange (excitation filter BP450–490, a DB2 dichroic beam splitter, and an LP barrier filter). The objectives were a plan 100× oil and x12.5 eyepiece, one containing an eyepiece graticule indexed 10 × 10 grid.

2.4. Counting procedure

Only qualified personnel can perform this counting. It is very important to distinguish spores and hyphae from other particles and fibers. To ensure the qualification, internal and external proficiency testing are necessary for quality assurance.

After staining, spores, hyphae, and bacteria were distinguished by morphological features and counted separately. The counting was based on fluorescently labelled cells per grid area. During counting, the height-adjustment was often insufficient to capture all cells per grid. The individual grids were also examined with transmitted light to identify mould spores that were difficult to stain with AO due to their dark colour.

Initially, the entire filter was viewed with x10 magnification to ensure a homogeneous distribution of the microorganism on the filter.

Subsequently, 100 fields on the filter were selected uniformly, randomly, and systematically, and all cells were counted separately for spores, hyphae, and bacteria. The counting was noted in the laboratory report as well as any anomaly.

Depending on the sample, cell aggregates or cell density may be too high, so counting is not reliable. In these cases, a 1:10 or 1:100 dilution of the suspension must be created, which is also dyed and counted. The dilution level must be considered when calculating the results. Sporadic cell aggregates must be counted as accurately as possible and mentioned in the lab report classified as follows:

Aggregate class 1: approx. 25–50 cells
Aggregate class 2: approx. 50–100 cells
Aggregate class 3: > 100 cells

2.5. Calculation

The evaluated microorganisms were initially recorded and evaluated per counted grid square and subsequently converted per grams or cm^2 as follows:

$$TCC = (n/g)K_{MF}(V/P)/M$$

TCC:Total Cell Count
n:total number of detected cells on the evaluated area
g:number of evaluated counting squares
K_{MF}:microscope factor (grid surface mm^2/effective filter area mm^2)
V:volume of phosphate buffer solution
M:amount of Sample (gram or cm^2)

The result is the average value formed from the repeated

Table 1
Categories of concentration ranges (based on Trautmann and Meider, 2018).

Categories	Mould	Bacteria
1) slightly loaded	$> 3{,}0 \times 10^5$–$1{,}0 \times 10^6$	$> 3{,}0 \times 10^6$–$2{,}0 \times 10^7$
2) loaded	$> 1{,}0 \times 10^6$–$1{,}0 \times 10^7$	$> 2{,}0 \times 10^7$–$2{,}0 \times 10^8$
3) heavily loaded	$> 1{,}0 \times 10^7$	$> 2{,}0 \times 10^8$

determination as TCC specified per gram or per cm^2. The limit of detection of the TCC depends on the weighed amount of material, the amount of buffer solution used, and the volume of the fixed sample. Each sample should be calculated so as to come up with the corresponding limit of determination.

3. Results

3.1. Method validation

In this study, the AODC examined real material samples from indoor areas. The selected samples were grouped into three categories based on the load of microorganism. The concentration ranges were divided into the following categories (Table 1):

In each category 10 samples were examined separately for moulds and bacteria, and a triplicate determination was made. An accuracy test showed a higher standard deviation with a lower mould and bacteria concentration. In the lightly loaded area, the standard deviation for mould varied between 14 and 17%, and for bacteria between 6 and 9%. In the loaded range, the standard deviation of the moulds reduced to 5–10% and increased slightly in bacteria to 7–13%. In the heavily loaded concentration range, the samples showed a standard deviation of 4–12% in the moulds and 4–18% in the bacteria.

Accuracy was examined by analysing 10 samples from category 2 for six consecutive days. The results show a low standard deviation of the mean in moulds between 9 and 11%, and in the bacteria between 7 and 9%.

To examine the precision, 10 samples from category 2 were examined six times in triplicate and the results were compared. The method was very robust across all concentration ranges with a low standard deviation of 10% in moulds and 14% in bacteria.

3.2. Comparison of TCC to CFU

In addition to the standard method review, a wide variety of indoor material samples were randomly selected and examined using the AODC and CFU methods. Fig. 1 shows the comparison of TCC and CFU in mould and Fig. 2 shows the same comparison in bacteria. Figs. 1 and 2 demonstrate that the TCC is always higher than the CFU, which confirms the stability of this method.

3.3. Retrievability of microorganisms

In a further experimental set-up, a suspension was prepared and dyed with the AODC method. A suspension with a concentration of 3.6 * 10 ˆ 7 of mould spores was made with real samples and analysed with AODC. This suspension “X” was the basis of this experiment. Three different materials [expanded polystyrene (EPS), wallpaper, and plasterboard] were inoculated with suspension “X” and stored for three weeks at 26 °C with 80% relative humidity. After an incubation period of three weeks, each of the mouldy materials was examined by the AODC method 5 times in triplicate. As a control, each material without inoculation was also examined five times. As shown in Fig. 3, the AODC method was able to reproduce the applied microorganisms. The goal of this experiment was to show that the AODC Method can regain the microorganisms of the primary suspension.

4. Conclusion

The analytic of TCC by AODC is a stable detection method. The microscopic analysis of the suspension enables the comparison of TCC and CFU in a sample. This allows for a far-reaching evaluation of material samples from an indoor environment. The TCC analysis provides more detailed information about the indoor damage and the course of the damage. Together with the CFU analysis, a detailed assessment of the samples can be carried out and could lead to sustainable remediation.

Especially when the microorganisms are stressed (e.g. due to

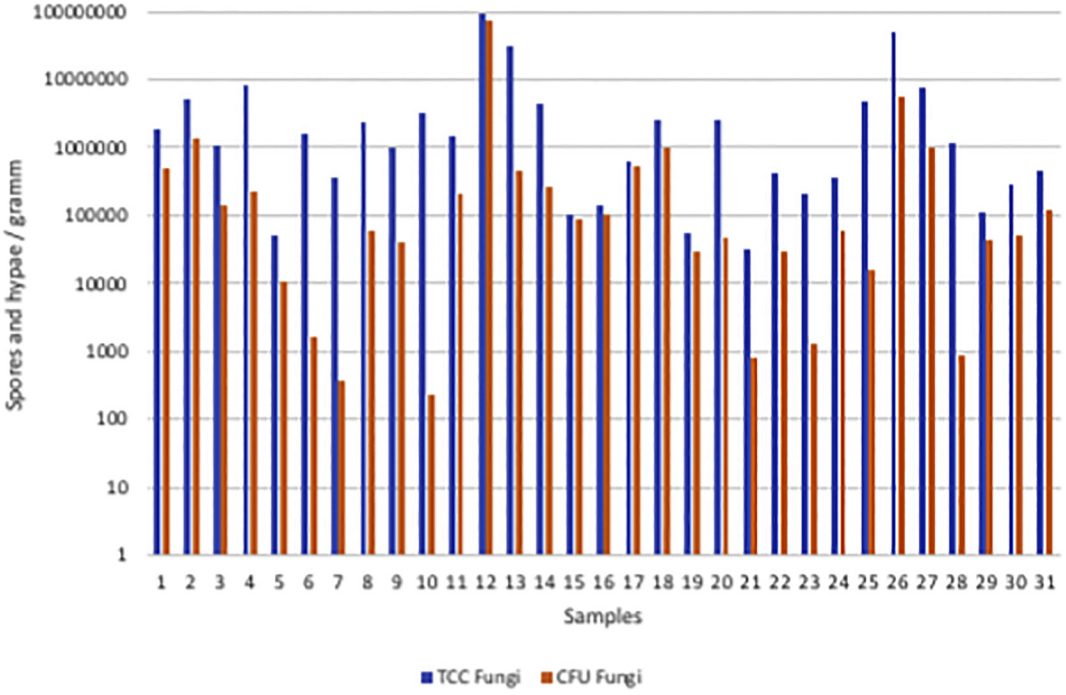

Fig. 1. Comparison TCC/CFU fungal spores in different samples.

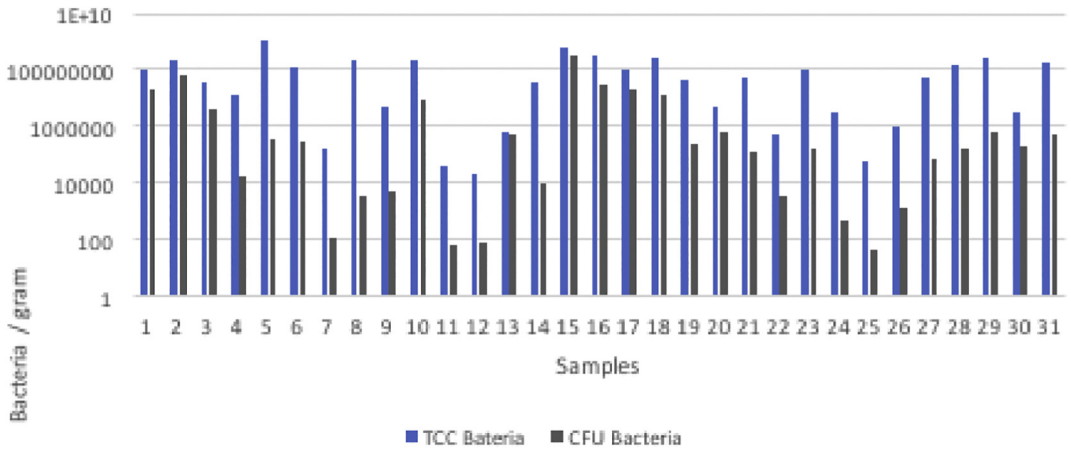

Fig. 2. Comparison TCC/CFU Bacteria in different samples.

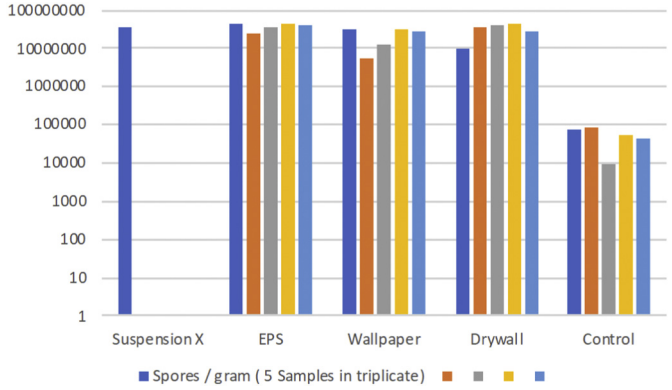

Fig. 3. Detection of spores on drywall, EPS, and wallpaper.

dryness or biocides), microbiological damage can be overlooked by a single CFU analysis. A hygienic evaluation of indoor environments is possible through the use of the AODC method. This more accurate assessment is also important in terms of the health effects of indoor moulds and bacteria. The latest guideline of the German Federal Environment Agency (UBA, 2017) states that viable and non-viable microorganisms have an irritating and toxic effect. Therefore, the determination of the TCC also makes sense from a medical point of view as preventive health protection.

Declaration of Competing Interest

None.

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