Recognition Errors in the Quantification of Micro-Organisms by Fluorescence Microscopy

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Objectives. An interlaboratory comparison of fluorescence microscopic counting of micro-organisms was carried out to assess counting errors in the measurement of micro-organisms in bioaerosols generated during handling of municipal waste.

Methods. Series of 27 replicate samples were collected in the work environment with a modified field exposure chamber. The preparation methods of three Scandinavian laboratories were compared. Four microscopists from these laboratories performed the counts which were also compared. Duplicate counting of identical microscopic fields allowed the assessment of recognition errors.

Results. The field exposure chamber collected replicate samples with a relative standard deviation of 5% when particles ≤15 μm aerodynamic diameter were collected. Storage time of 40–200 days had no significant influence on the total micro-organism count. Differences between preparation methods were from 2 to 35% for bacteria, and from 15 to 35% for fungal spores when samples were analysed in Oslo; the results for fungal spore counts were significantly different \( P < 0.01 \). These differences were not confirmed when samples were analysed in Umeå, Copenhagen and Oslo using those laboratories methods. These results can be explained by less efficient redispersion of aggregates when the Umeå and Copenhagen methods were recreated in Oslo yielding a greater number of innumerable aggregates. Differences between microscopists were minor for fungal spores (2–12%) but substantial for bacteria (4–53%). A major source of error was the recognition of bacteria which had a relative standard deviation (rsd) of 37% although a lower size limit of 0.75 μm was adopted for counting of bacteria. Fungal spores were recognised with much better precision (rsd 9%).

Conclusions: Recognition errors of bacteria may be substantial and more specific fluorochromes are needed for fluorescence microscopic counting of micro-organisms.

Keywords: fluorescence microscopy; counting errors; bacteria; fungal spores; field exposure chamber

INTRODUCTION

Inhalation of non-infectious micro-organisms is known to cause respiratory diseases such as asthma, chronic obstructive pulmonary disease, hypersensitivity pneumonitis and organic dust toxic syndrome in various working populations (Lacey and Dutkiewicz, 1994). Health effects typical for exposure to non-infectious microbial agents are usually not dependent on viability of the micro-organisms but are probably caused by microbial components and products such as allergens, other antigens and toxins. Exposure to the total microbial burden should therefore be measured, including viable and non-viable bacteria or fungi, rather than only the culturable fraction (Eduard and Heederik, 1998). Among the non-culture-based methods for measurement of micro-organisms fluorescence microscopy has become popular as both bacteria and fungal spores can be counted (Palmgren et al., 1986). The fluorescent stain facilitates the recognition of micro-organisms among other particles and micro-organisms present in complex aggregates. As the fluorochromes that have been used for this pur-
pose also may stain non-microbial particles, shape is an important criterion for the recognition of microorganisms. However, the shape of small bacteria is difficult to observe as their size can be close to the optical resolution of light microscopes (approximately 0.2 µm).

In a Nordic study of microbial exposure in waste sorting plants fluorescence microscopy was selected to measure exposure to fungal spores and bacteria. A preliminary comparison of three Scandinavian laboratories using duplicate samples collected by side-by-side sampling had shown large differences between the laboratories, especially in one plant. This was ascribed to the presence of large numbers of small fluorescent particles which were counted as bacteria in one laboratory but not in the other laboratories. The present study was conducted with replicate samples from the latter plant in order to study differences between microscopists and between methods used at the laboratories.

**METHODS**

**Experimental design**

In the first part of the study a field exposure chamber was developed for collection of replicate filter samples. The homogeneity of the replicate samples was assessed in a series of samples collected with a maximum particle size of 15 µm at a manual sorting plant for waste paper situated in Oslo. These samples were prepared by the Oslo method and counted by two microscopists in Oslo. The effect of extended storage time was also assessed.

In the second part of the study a series of samples was analysed in Oslo by the Copenhagen, Umeå and Oslo methods by four microscopists, and also in Copenhagen and Umeå using those laboratories’ methods. These samples were collected with a maximum particle size of 5 µm at a waste sorting plant situated in southern Sweden. A waste water treatment plant emitting unpleasant odour and bacteria was situated nearest to this plant.

**Field exposure chamber**

A previously described field exposure chamber (Skogstad et al., 1996) was further developed for simultaneous collection of 27 replicate filter samples of particles with sizes up to 15 µm aerodynamic diameter. The chamber was designed with an annular chamber in order to achieve a close to symmetrical flow field at the filter cassette inlets (Fig. 1). An average ascending air velocity of three times the sedimentation velocity of 15 µm particles was achieved at a total flow rate 62 l. min⁻¹, and a flow rate per filter cassette of 2.3 l. min⁻¹. The maximum size of the collected particles was restricted to aerodynamic diameters of 5–15 µm by using nozzles with different diameters and adjusting the total flow rate. The nozzle diameters were calculated with a Model 201 Impactor Calculator (V.A. Marple, Sierra Instruments, Inc., Carmel Valley, CA, USA). Samples were collected on polycarbonate filters with pore-size 0.4 µm (Poretics, Osmonics, Livermore, CA, USA) in open-face 25 mm diameter filter holders made of graphite-filled polypropylene (Millipore, Bedford, MA, USA). The filter cassettes were threaded and mounted in the stainless steel chamber by brass sockets which assured conductive connection between the filter cassettes and the chamber wall to minimise static charging.

**Sample storage**

Samples were stored in a refrigerator at 4°C at the laboratories. The samples for evaluation of the homogeneity of replicate samples were stored for 40–200 days. Samples used in the method, microscopist and laboratory comparisons in Oslo were stored for 2 days
before analysis, and samples analysed in Copenhagen and Umeå for 6 days.

**Sample preparation — Umeå and Copenhagen method**

Samples were prepared with acridine orange as fluorochrome by the method described by Palmgren *et al.* (1986). To each filter cassette 2 ml of an aqueous 0.05% Tween 80 solution was added. The collected micro-organisms were resuspended using a shaking table for 10–15 min. To 1 ml of the suspension 1.5 ml (Umeå method) and 1 ml (Copenhagen method) of a commercial 0.01% w/v acridine-orange solution (Bio Merieux, Marcy l’Etoile, France) was added together with 8 drops of a 0.01% (w/v) acetic acid buffer with pH 4. The staining time was 30 s (Copenhagen method) and 3 min (Umeå method). The suspension was filtered through a black-stained polycarbonate membrane filter with pore-size 0.2 µm (Poretics, Osmonics, Livermore, California, USA). The filter was then air dried and mounted on a microscope slide with immersion oil. All reagent solutions were filtered through 0.22 µm sterile Millipore filters (Millipore S.A., Molsheim, France) before use.

**Sample preparation — Oslo method**

Samples were prepared with acridine orange as fluorochrome by a modified method described by Heldal *et al.* (1996). To each filter cassette 2 ml of an aqueous 0.1% Tween 80 solution was added. The collected micro-organisms were resuspended by sonication in an ultrasonic bath for 3 min. To 1 ml of the suspension 2 ml of a 0.025% (w/v) acridine-orange solution was added (Merck, Darmstadt, Germany) and 2 ml of a 0.1 M citrate buffer with pH 6.6. The staining time was 2 min and filters were rinsed with 2 ml of a 0.1 M citrate buffer with pH 3 and quickly with 2 ml of 2-propanol in order to remove excess fluorochrome from not specifically stained particles. The suspension was filtered and the filter was mounted for microscopy as described above.

**Counting of fungal spores and bacteria**

Samples were counted with a Nikon Labophot epifluorescence microscope fitted with a BP 450-490 exciter filter, a FT 510 dichromatic beam splitter, a LP 520 barrier filter, and a HBO 100 W high-pressure mercury source (Nikon Corporation, Tokyo, Japan) at a magnification of 1250×. A total of 50 fields evenly spaced on the filter area were enumerated. The detection limit of the methods as described here was 6×10³ micro-organisms/filter based on counting four single micro-organisms and zero blanks. The proportion of the suspension that is analysed can be varied, however, usually resulting in a 2–3× higher detection limit.

In the homogeneity test all samples were counted consecutively by two microscopists counting all micro-organisms recognisable by shape. Micro-organisms crossing the margins were counted if their centre was within the viewing field. Micro-organisms in aggregates were counted if possible.

In the method and microscopist comparison study, two microscopists counted identical fields using a teaching head (Nikon TH-L) mounted on the epifluorescence microscope. One sample was analysed by all microscopists for training purposes and to establish counting criteria. It was decided that only micro-organisms larger than 0.75 µm should be enumerated as morphological recognition of smaller micro-organisms was considered uncertain. Fungal spores and bacteria were discriminated by shape and size (fungi always greater than 1.5 µm) and counted separately. Bacteria and fungal spores in aggregates were counted if possible while complex, uncountable, aggregates were recorded by the number of such aggregates.

**Method, microscopist and laboratory comparisons**

One microscopist from each laboratory prepared four randomly selected samples in the Oslo laboratory using their own methods, chemicals and equipment as far as possible, a total of 12 samples. One microscopist from Umeå, one microscopist from Copenhagen and two microscopists from Oslo each counted six samples, two samples prepared by each method. All six possible combinations of two microscopists were used twice in duplicate counting of the 12 samples. As the methods used at the laboratories in Umeå and Copenhagen could not be recreated completely in Oslo, the microscopists from Umeå and Copenhagen also analysed six samples from the same series of replicate samples in their own laboratory.

**Statistical analysis**

The homogeneity of replicate samples was estimated by the relative standard deviation (≡coefficient of variation or CV) obtained from the variance component between samples. The effect of storage time on the total micro-organism count was tested with linear regression. Differences between methods and microscopists were analysed by two-way ANOVA. The recognition error was estimated by the relative standard deviation of duplicate counts on samples obtained by counting of identical fields. This error was computed by the equation:

\[
\text{rsd} = \sqrt{\frac{\text{d}_i^2}{\frac{1}{2}\sum d^2}}
\]

where \(d_i\) is the difference between the \(i\)th pair of duplicate counts, \(x_i\) is their average, and \(n\) the number of duplicate counts. The comparisons of results from microscopists analysing samples at their own labora-
Table 1. Homogeneity of 26 replicate samples collected with the field exposure chamber estimated by two-way ANOVA. Each sample was counted once by two microscopists

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Mean square</th>
<th>Estimated variance</th>
<th>F-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
<td>25</td>
<td>6.49×10^{11}</td>
<td>2\sigma_{\text{samples}}^2 + \sigma_e^2</td>
<td>1.06</td>
<td>0.4</td>
</tr>
<tr>
<td>Microscopist</td>
<td>1</td>
<td>3.73×10^{12}</td>
<td>26\sigma_{\text{microscopist}}^2 + \sigma_e^2</td>
<td>6.07</td>
<td>0.02</td>
</tr>
<tr>
<td>Error</td>
<td>25</td>
<td>6.15×10^{11}</td>
<td>\sigma_e^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The homogeneity is given by \( \text{rsd} = \text{sd samples}/\text{arithmetic mean} = 1.30×10^5/2.45×10^6 \approx 5.3\% \). The source of variation DF = \( \sqrt{(6.49×10^{11} - 6.15×10^{11})/2} \) = 1.30×10^5.

The results were done with the non-parametric Kruskal–Wallis and Mann–Whitney tests as large differences occurred between standard deviations of results obtained at different laboratories.

RESULTS

Homogeneity of the field exposure chamber

Two microscopists at the laboratory in Oslo counted micro-organisms in 26 replicate samples as described above (Table 1). One sample was lost due to a large change in flow rate during sample collection. The samples were difficult to count due to small bacteria and the presence of numerous not specifically stained particles. The arithmetic mean concentration of all micro-organisms was 2.4×10^6 m^{-3}. The homogeneity estimated by the relative standard deviation of replicate samples was 5.3%. A significant difference between microscopists of 20% was observed (\( P/\chi^2 0.05 \)). Linear regression analysis revealed no significant influence of storage time on the results.

Method and microscopist comparisons

The method and microscopist comparisons of samples prepared and analysed in Oslo are shown in Tables 2–4. The arithmetic mean concentrations of micro-organisms were 1.7×10^5 fungal spores m^{-3}, 1.6×10^5 bacteria m^{-3}, and 0.55×10^5 microbial aggregates m^{-3}. The counting of fungal spores was more precise than the counting of bacteria with relative standard deviations of 21 and 51%, respectively (Tables 3 and 4). Fungal spore counts showed only small differences between microscopists which were not significant. However, two-way ANOVA of the fungal spore counts showed that the preparation method had a significant influence (\( P<0.01 \)); the preparation method as used in Oslo yielded 24% higher counts than the Umeå method and 43% higher counts than the Copenhagen method. Bacterial counts differed by a factor of more than 2 between microscopists (\( P<0.05 \)). The method used in Copenhagen yielded 33–35% lower bacterial counts than the other methods, but these differences were not significant.

Microscopist differences were further elucidated by the variability of duplicate counts that were performed on the same microscopic fields. Relative standard deviations of 9 and 37% were found for fungi and bacteria, respectively.

Laboratory comparisons

The results from samples analysed by microscopists from Oslo, Umeå and Copenhagen at their...
Recruitment of micro-organism quantification

Recognition errors in micro-organism quantification

Table 3. Two-way analysis of variance of relative fungal spore concentrations in 12 replicate samples prepared by three different methods for fluorescence microscopy and enumerated by four different microscopists

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Mean square</th>
<th>F-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation method</td>
<td>2</td>
<td>427.2</td>
<td>6.31</td>
<td>0.008</td>
</tr>
<tr>
<td>Microscopist</td>
<td>3</td>
<td>28.4</td>
<td>0.42</td>
<td>0.7</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>67.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The relative experimental error $sd_e = (\sqrt{\text{mean square error}})/\text{grand mean} = 21%$

*Degrees of freedom

Table 4. Two-way analysis of variance of relative bacterial concentrations in 12 replicate samples prepared by three different methods for fluorescence microscopy and enumerated by four different microscopists

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>DF</th>
<th>Mean square</th>
<th>F-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation method</td>
<td>2</td>
<td>605.7</td>
<td>1.66</td>
<td>0.2</td>
</tr>
<tr>
<td>Microscopist</td>
<td>3</td>
<td>1309.0</td>
<td>3.59</td>
<td>0.03</td>
</tr>
<tr>
<td>Error</td>
<td>18</td>
<td>364.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The relative experimental error $sd_e = (\sqrt{\text{mean square error}})/\text{grand mean} = 51%$. The relative standard deviation of the variability between microscopists was estimated from mean square microscopist = $3sd_{\text{microscopist}}^2 + sd_e^2 \rightarrow sd_{\text{microscopist}} = 47%$ as in Table 1.

*Degrees of freedom

Own laboratories results are shown in Table 5. The relative concentration of fungal spores was significantly higher in samples analysed in Umeå compared to Copenhagen and Oslo, Kruskal–Wallis test and Mann–Whitney tests, $P<0.05$. The relative concentration of bacteria was higher in Oslo compared to the other laboratories but the differences between laboratories was not significant ($P = 0.4$). The relative concentration of complex aggregates was lower in Copenhagen compared to the other laboratories and the differences were of borderline significance ($P = 0.07$).

**DISCUSSION**

The relative standard deviation of replicate samples collected with a modified field exposure chamber using an impactor with a cut-off diameter of 15 µm was approximately 5%. The chamber was designed in order to collect particles with relative large aerodynamic size as we wanted to study the influence of complex aggregates on the counting accuracy. Such aggregates occur naturally in the aerosol as fungal spores can be liberated as chains and clumps, and bacteria attach firmly to plant fragments and skin scales. Aggregates can be difficult to disperse (Heldal *et al*., 1996), and increase counting errors (Eduard and Aalen, 1988). However, preliminary results indicated that the enumeration of small micro-organisms represented an even greater challenge for microscopists. It was therefore chosen to reduce counting errors from the presence of large aggregates in the comparative study by removing particles larger than approximately 5 µm from the aspirated aerosol.

The results of the comparative study showed significant differences of up to 30% between preparation

Table 5. Analysis of micro-organisms by fluorescence microscopy in inter-laboratory comparison. Relative concentrations of micro-organisms in samples prepared and analysed in Oslo, Umeå and Copenhagen

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Fungal spores</th>
<th>Bacteria</th>
<th>Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM $^b$</td>
<td>SD $^c$</td>
<td>AM</td>
</tr>
<tr>
<td>Umeå</td>
<td>64</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>Copenhagen</td>
<td>64</td>
<td>7</td>
<td>36</td>
</tr>
<tr>
<td>Oslo</td>
<td>52</td>
<td>9</td>
<td>52</td>
</tr>
</tbody>
</table>

$^a$Counts adjusted for differences between air volume and preparation method

$^b$Arithmetic mean

$^c$Standard deviation
methods when fungal spores were counted with the highest counts obtained by the Oslo method. Similar differences were observed for bacterial counts, but these differences were not significant, probably due to the larger experimental error. In the Oslo method a higher detergent concentration in the dispersion fluid is used, ultrasonic treatment instead of shaking, and the filter is rinsed after staining to remove acridine orange from not specifically stained particles. This has been shown to increase the number of countable micro-organisms compared to the standard method (Heldal et al., 1996). However, the Umeå and Copenhagen methods could not be exactly recreated at the Oslo laboratory. The samples were probably less intensively agitated during the resuspension procedures as the number of complex aggregates with innumerable micro-organisms found in samples analysed in Oslo was larger than in samples analysed in Umeå and Copenhagen by the Umeå and Copenhagen methods. The larger number of aggregates may in turn explain the observed differences between preparation methods as they may have obscured micro-organisms. When replicate samples were analysed by the microscopists from Umeå and Copenhagen at their own laboratory fungal spore counts were similar or higher than those obtained by the microscopists from Oslo. Counts of bacteria were lower but not significantly. It is not clear from these data which method should be preferred. Samples prepared by the Oslo method were easier to count, however.

The comparison of microscopists is valid as the microscopists counted the same number of samples prepared by each method. The fungal spore counts by different microscopists were quite similar in contrast to bacteria of which two microscopists counted twice as many as the other two. The differences between bacterial counts were mainly due to microscopists recognising bacteria differently among other fluorescing particles. The recognition error could be studied separately by counting identical fields. If random fields had been counted by each microscopist large errors would have been introduced from counting randomly distributed aggregates of micro-organisms (Eduard and Aalen, 1988). The recognition error was estimated to 37% for bacteria compared to 9% for fungal spores. This error explains the greater part of the total between microscopist variability of bacterial counts which had a relative standard deviation of 47%. Thus, in spite of the constraint to count bacteria with size 0.75 µm or larger, recognition of bacteria was still difficult. It should be noted, however, that the site for collection of replicate samples was chosen as a ‘worst case’ as preliminary results had shown even larger differences between laboratories.

Some information was obtained on the effect of extended storage at 4°C on the total micro-organism count as samples that were analysed to test the homogeneity of the field exposure chamber were stored for 40–200 days before analysis. No significant linear trend was observed indicating that the samples did not change during this period.

In conclusion, recognition of small bacteria may represent a major source of error in the enumeration of bacteria by fluorescence microscopy when their size approaches the optical resolution of the fluorescence microscope and their shape cannot be observed satisfactorily. Fungal spores are more easily recognised and can be counted more precisely. However, not all spore types are stained by commonly used fluorochromes (Burge, 1995). Thus, better fluorochromes are needed for the analysis of bacteria and fungal spores by fluorescence microscopy. Other challenges are training of microscopists, standardisation of analytical methods, and dispersion of aggregates of micro-organisms.

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