

A new sub-sampling method for analysis of air samples collected with the Andersen single-stage sampler

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Abstract A protocol for bioaerosol collection was developed that provides not only accurate predictions of fungal concentration, but also improves species recovery. Random transfer of a subset of 50 of the 400 impaction points from Andersen single-stage bioaerosol sampling plates results in subcultures that are accurate predictors of fungal concentration (CFU/m³), when

compared to duplicate untouched Andersen plates. A linear regression model was developed to estimate CFU/m³ from the colonies counted on the Random-50 plates. The random transfer to five plates (“Random-50” plates), allows large numbers of fungi to be recovered and identified, including slow-growing fungi that otherwise would be masked by fast-growing fungi.

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Abbreviations

ACCURATE	Accurate Assessment of Bioaerosols in Indoor Environments
ANOVA	Analysis of Variance
AUDIT	Assessment of Urban Dwellings for Indoor Toxics
CFU/m ³	Colony Forming Units per cubic meter
MEA	Malt Extract Agar
NYIEQ	New York Indoor Environmental Quality Center
USEPA	United States Environmental Protection Agency

1 Introduction

It was the goal of a bioaerosol study of urban homes in Syracuse, NY, to perform a comprehensive

evaluation of the microbial population including not only total fungal concentration in air samples but also, the identification of as many species of airborne fungi as possible. The homes recruited for this study were selected by the *Assessment of Urban Dwellings for Indoor Toxics* (AUDIT) program at Upstate Medical University, Syracuse, NY (Abraham et al., 2004). This bioaerosol survey (*Accurate Assessment of Bioaerosols in Indoor Environments* (ACCURATE)) was one component of the AUDIT study that collected measurements of environmental variables within homes of infants with a predisposition of asthma. In order to achieve the overall objective, it was important to develop protocols to isolate and identify possible under-represented fungi from air samples.

From 1980 to 1993, the prevalence of asthma in the U.S. increased by 75% with the largest increase occurring in children under 5 years of age (CDC, 1994). Exposure to fungi such as *Penicillium* spp. (Licorish, Novey, & Kozak, 1985), *Aspergillus* spp. (Garrett, Abrahamson, & Hopper, 1998), and *Alternaria* spp. (Negrini, Berra, & Campi, 2000) is one identified risk factor for asthma development along with excessive allergen and tobacco smoke (Utell & Looney, 1995); gas stove exposure and nitrogen dioxide (Ciuk, Volkmer, & Edwards, 2001). Although links have been established between fungi and asthma (Stark, Burge, & Ryan, 2003), there was a perceived need to improve protocols in order to isolate possible slow-growing fungi. Recovery of bioaerosols with the Andersen sampler is influenced by spore size limitation (Martinez, Todd, & Fishbach, 1991), the type of growth media (Burge & Otten, 1999; Duchaine, Mériaux, & Comtois, 2002) and possibly by the varying growth rates of fungi. Slow growth of particular species of fungi may cause difficulty in separating isolates for identification, especially when they are competing with ubiquitous fast-growers such as *Aspergillus* and *Penicillium*. Andersen samples provide a discrete “snapshot” of fungal concentration in a measured volume of air. The term “total fungal concentration” or “total CFU/m³” expresses the count of the number of all colonies that grow on the plate obtained in that discrete, measured amount of air (typically 28 l/min for 3 minutes). This study describes a method that was developed

to improve conditions for growth of individual isolates by transferring Andersen impaction points to sub-culture plates. The main objective of this report was to determine if fungal concentrations estimated with these sub-cultured plates (Random-50 plates) are representative of the fungal concentration determined with the standard protocol of analyzing the typical Andersen plate that serves as the basis for determining fungal concentrations. The usefulness of the Random-50 plates as a means to estimate fungal concentration in Andersen samples is presented here. A second objective was to compare the fungal concentrations that were obtained on two different times on the same day and to compare those samples collected on two consecutive days of sampling in the same location to determine to what degree, if any, they are reproducible.

2 Methods

From January 2002 to December 2002, the AUDIT program at Upstate Medical University, Syracuse, NY, collected indoor and outdoor air samples from 130 homes, as part of a larger study comprising home-sampling visits over a period of 16 months. Samples were transported to State University of New York College of Environmental Science and Forestry (SUNY-ESF), Syracuse, NY, for analysis for fungal concentrations and identification of isolates. Analysis of the Random-50 method described in Sect. 2.3 was applied to the sampling visits in 2002.

2.1 Media and plate preparation

Malt extract agar medium (MEA) (3% BD Bacto Malt Extract, 1% BD Bacto Agar) was aseptically prepared for on-site sampling and culturing. In order to observe impaction dimples to facilitate their transfer to subculture plates, impaction dimple retention tests were performed. Better retention of dimples was obtained with 1% agar than with other concentrations (1.50, 1.10, and 0.75%). Dimple retention appeared to be affected by the plate’s age, relative humidity, and temperature. Twenty ml of MEA was aseptically poured in each Petri plate using an automatic dispenser (Wheaton Unispense II). The plates of media were

then allowed to gel on a confirmed level surface (bubble level placed on surface). Petri plates without any condensation on the lid and inside edge were sealed with parafilm to maintain sterility and freshness, and stored at room temperature. For 3-min sampling, up to 3-day-old plates were used while up to 1-week-old plates were used for 6-min sampling. During periods of high humidity fresh plates were used when possible.

2.2 Bioaerosol collections

To collect and measure viable microorganisms, forced air (28 L/min) was impacted for 3 or 6 minutes onto MEA plates using an Andersen N6 Air Sampler. Samples were taken indoors and immediately outside the home at the beginning (Day 1) and end (Day 2) of a 24-hour period. For each sampling period two replicate plates were collected sequentially (Plates 1 and 2 in Fig. 1). Plate 1 was the typical Andersen plate normally used to count colonies to determine total fungal concentration. Plate 2 is a replicate of Plate 1 and serves here as the plate to be sub-cultured with the Random 50 method. For each home sampling visit, there were three indoor sampling periods (Day 1 Time 1, Day 1 Time 2, and Day 2). During each of these sampling periods, replicate plates (Plate 1 and Plate 2) were collected. Although not presented here, one outdoor sample was taken on each day.

2.3 Random-50 sub-sampling isolation procedure

Within 3 hours after collection, a sub-sample of 50 randomly selected “plugs” was aseptically removed from Plate 2, the “sub-cultured” plate and placed into five Random-50 plates; 10 per plate (Fig. 1). This Random-50 (R-50) isolation procedure used templates for locating 50 random impaction points, or dimples among the 400 impaction dimples on each plate representing one-eighth of the total sample. Locations for the random points were assigned based on numbers generated using a random number generator; numbering was done concentrically from the inside circle to the outermost circle. One template was used for each field sample plate. Each template had 400 “dots”, 350 black “dots” and 50

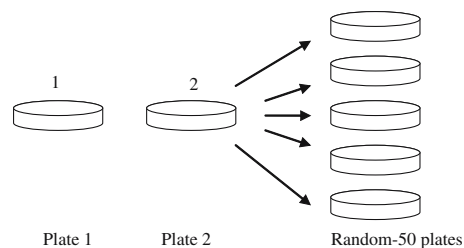


Fig. 1 Three types of sample plates were used in this study. Plate 1 is the typical Andersen plate that was left untouched and was fully counted; Plate 2, the sub-cultured plate that is a replicate of Plate 1; and the Random-50 plates are five plates containing sub-cultures of Plate 2. Fifty impaction points were randomly removed from Plate 2 and inoculated onto the Random-50 plates (10 per plate)

“random” red dots corresponding to the pattern of dimples produced on the sample plate by the Andersen N6 Air Sampler. The impaction dimples were aligned to the pattern on the template placed under the Petri plate and visible through the agar (Fig. 2a). The 50 impaction dimples that were found over the red marks on the template were cut using one sterile cylindrical cutter per dimple (Fig. 2a). These cutters, manufactured at SUNY-ESF, were designed to allow cutting to be restricted to one dimple. Cutters were used one time then re-sterilized in the autoclave. Each cut dimple or “plug” was removed from the plate using a sterile full spear dissecting needle, 10 mm long by 3.5 mm wide (Carolina Biological Supply Company). Each plug was transferred to a fresh plate of MEA (Fig. 2b). Ten plugs were placed approximately equidistant from other plugs on each plate resulting in five R-50 plates for each original. All plates were sealed around the edges with two 2 layers of parafilm and incubated at room temperature. Using a dissecting microscope, colony-forming units (CFUs) were counted on Plate 1, Plate 2 and the five R-50 plates at 2–3 and 7–10 days after field sampling. R-50 plates with no growth were kept for a maximum of 1 month at room temperature and periodically checked for fungal growth.

2.4 Preparation of isolates for identification

As cultures appeared on each Random-50 plate, growing from the 10 inoculum plugs, colonies were transferred to slides for microscopic observation and identification to taxon. From all

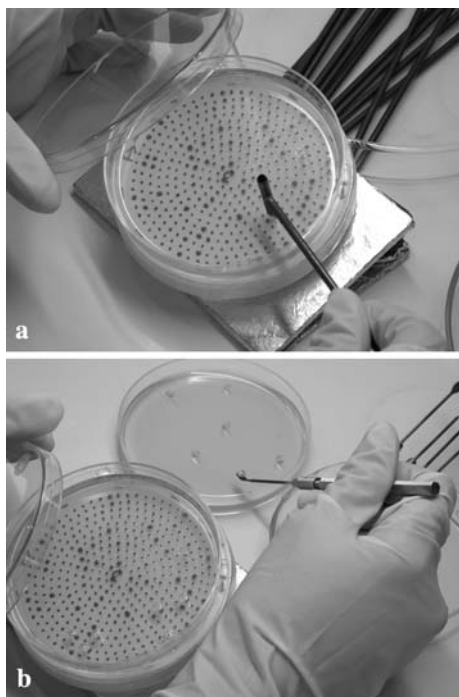


Fig. 2 The transfer of randomly-selected impactation points from the Andersen N6 collection plate to one of five sub-culture plates. **(a)** A plug of agar centered around one impactation point is cut with a cylindrical plug extractor. Fifty of the 400 impactation points are selected visually by aligning with the red dots (slightly larger) on the template beneath the plate. **(b)** Transfer of 10 impactation plates to a “Random-50” plate. A total of 50 impactation points are transferred to 5 plates from each sample plate

homes, a total of 12,456 slides were prepared (Anagnost et al., 2004). These slides were stored as vouchers for microscopic study of fungal morphology. In addition to voucher slides, for about 2300 isolates, transfers were also made to culture plates or tubes for later study. The Random-50 plates were stored up to one month and isolates were prepared as they appeared.

2.5 Statistical analysis

The concentration of viable fungi in each discrete air sample was calculated as the number of colony-forming units per the amount of air sampled:

$$\text{CFU/L} = \frac{\text{the number of colonies/}}{((\text{sampling time (min)})(\text{flow rate (l/min)}))}.$$

For each Plate 1 plate, the fully-counted, untouched, typical Andersen plate, this value was

converted to CFU/m³ by multiplying by 1000 to convert from l to m³ of air:

$$\text{CFU/m}^3 = \left[\frac{\text{number of colonies observed in the Plate 1}}{(\text{air flow rate (l/min)})(\text{minutes})} \right] * (1000 \text{ l/m}^3)$$

To compare the fungal concentrations obtained with the R-50 plates to that of the concentration obtained with the Plate 1 plates, the sum of the number of colonies in the five R-50 plates was multiplied by a factor of 8 (50 impactation points represent 1/8 the total 400 impactation points).

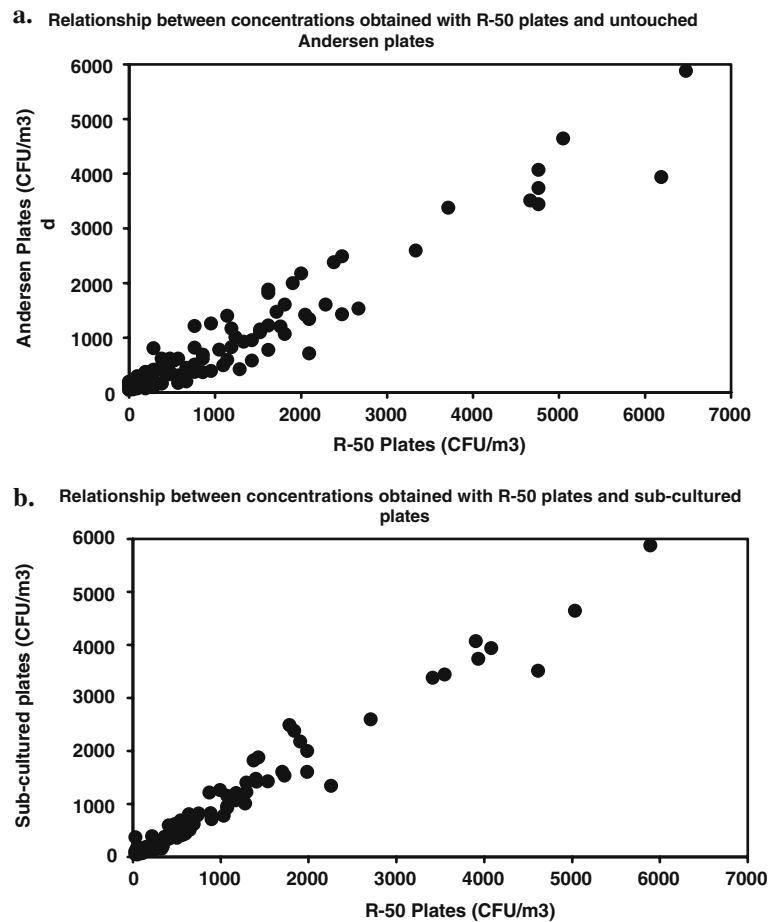
For Plate 2, the “sub-cultured plate”, colonies were counted that grew from 350 impactation points that remained after sub-culturing. The CFU/m³ was multiplied by a factor of 400/350 or 1.14 for comparison with the untouched plate (Plate 1).

2.5.1 Estimation of total fungal concentrations from R-50 plates and development of the linear model

The colony counts obtained from one sampling period (Day 1 Time 1) for each of 98 houses were analyzed to determine if the 50 impactation points were representative of the total 400 impactation points in the untouched collection plate in determining fungal concentration (CFU/m³). The hypothesis is that the concentration obtained with the 50 sub-sampled impactation points (sum of 5 Random-50 plates) represents one-eighth of the concentration obtained with the fully-counted plate (Plate 1 plates) and is the same as the concentration obtained with Plate 1 (400 impactation points) when multiplied by a correction factor of 8. A second hypothesis is that the counts obtained with the sub-cultured plates (Plate 2 plates), those from which the random 50 impactation points were removed, represent 350/400 of the counts obtained from the typical, untouched Andersen plates (Plate 1 plates).

Since the relationships between the three sampling types, the untouched Andersen plate (Plate 1), the subcultured plate (Plate 2), and the R-50 plates (five Plate 3 plates) were linear in the CFU/m³ variables (Fig. 3), a linear model was used to regress R-50 CFU/m³ to those of un-

Fig. 3 The relationship between fungal concentrations obtained by counting colonies in the sub-cultured plates to that of the untouched Andersen plate that was fully counted. **(a)** The relationship between fungal concentrations obtained with “Plate 1” plates and R-50 Plates for Day 1 Time 1. Regression analysis of the 98 sampling points gives an R^2 of 0.92. **(b)** The relationship between fungal concentrations obtained with “Plate 2” plates and R-50 plates for Day 1 Time 1. Regression analysis of the 98 sampling points gives an R^2 of 0.94



touched plates and sub-cultured plates. Again, only the CFU/m³ in the sampling period Day 1 Time 1 was used to fit the two models.

2.5.2 Comparison between sampling types within each sampling period

The relationships between the sample types within each indoor sampling period (Day 1 Time 1, Day 1 Time 2, and Day 2) were evaluated by determining Pearson correlation coefficients.

2.5.3 Comparison between sampling periods within one sampling type

In order to test our second objective, to determine reproducibility of samples when collected at different times of day or on different days, the

three sampling periods (Day 1 Time 1, Day 1 Time 2 and Day 2) were compared using Pearson correlation coefficients. To determine if similar degrees of reproducibility could be obtained with counts of R-50 plates or sub-cultured plates as the Andersen Plate 1 plates, Pearson correlation coefficients between each sampling period for each sample type (Andersen, sub-cultured and R-50) were determined.

3 Results and Discussion

3.1 Estimation of total fungal concentrations from R-50 plates

A regression analysis comparing the Random 50 plates to the untouched Andersen plates from each sample collected Day 1 Time 1 (98 collections) indicated a linear relationship ($R^2 = .92$)

(Fig. 3a). A similar comparison between the R-50 plates and the sub-cultured plates also indicated a linear relationship and a high degree of correlation ($R^2 = .94$) (Fig. 3b). Indoor fungal concentration (CFU/m³) of the untouched and sub-culture plates can be estimated with high confidence from the R-50 concentrations ($R^2 = .92$ and $.94$, respectively) using the following linear regression models, respectively:

1. Estimated untouched Andersen CFU/m³ = $22.33 + .78$ (indoor R-50 CFU/m³)
2. Estimated sub-cultured CFU/m³ = $-6.29 + .82$ (indoor R-50 CFU/m³)

The ability to estimate total CFU/m³ from the R-50 plates indicates their value as a quantitative representation of the total fungal concentration obtained in each air sample.

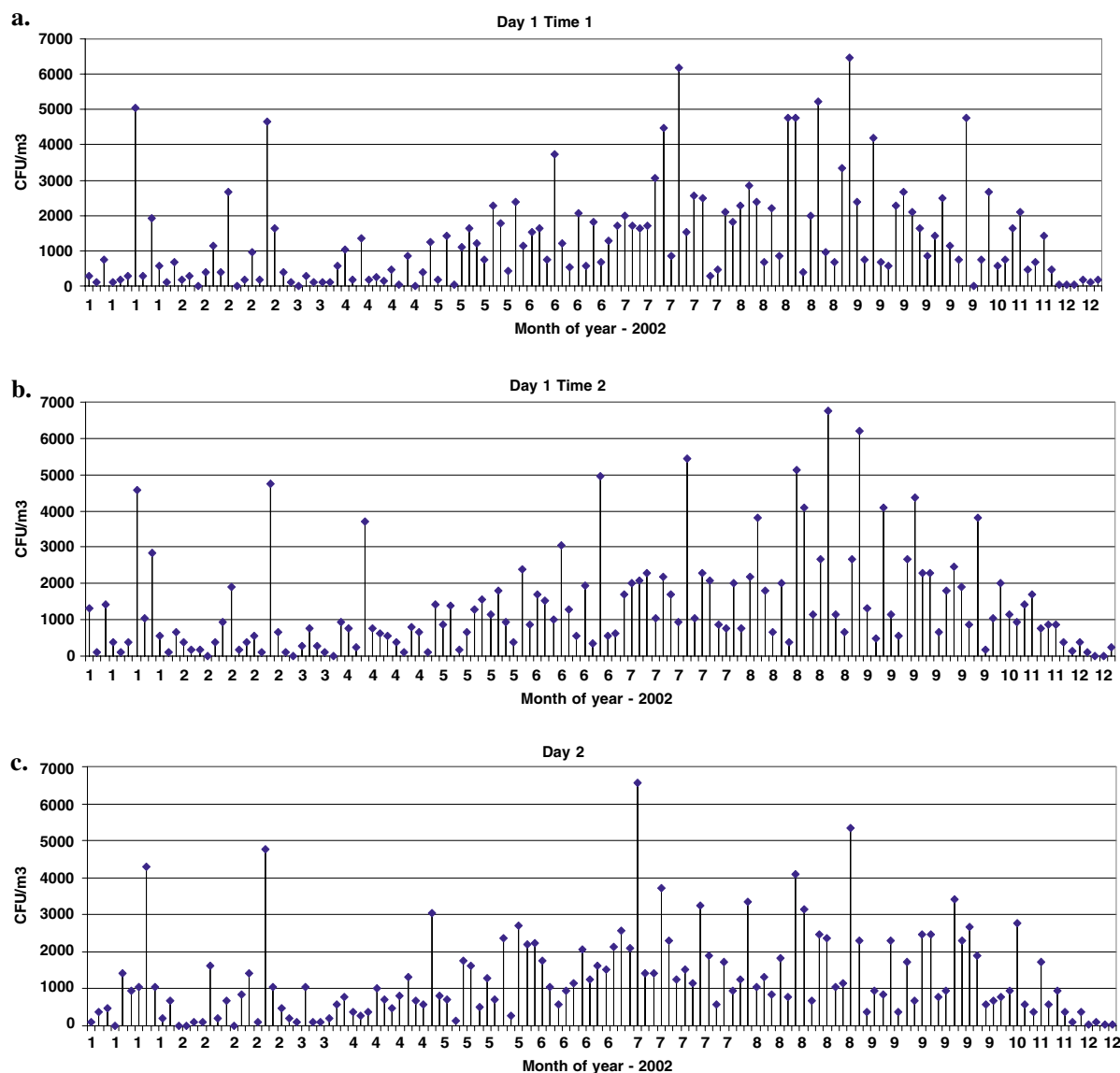


Fig. 4 Total fungal concentration of R-50 plates collected with the Andersen sampler from 130 homes presented in order of sample collection from January through December 2002. (a) Samples collected during the Day 1 Time 1

sampling period, (b) Samples collecting during the Day 1 Time 2 sampling period, and (c) Samples collected during the Day 2 sampling period

Fungal concentrations in these homes determined with the R-50 method ranged from 0 to 6500 CFU/m³ (Fig. 4). Much of the variation appeared to be related to season of collection, although many other factors could be considered to influence variation within each home.

3.2 Comparison between sampling types within sampling periods

To evaluate this non-standard sampling protocol for bioaerosols, comparisons were made between sampling types within each sampling period. Pearson correlation coefficients between the CFU/m³ obtained with the R-50 plates against the Plate 1 plates (untouched Andersen plates) and Plate 2 plates (sub-sampled plates) were slightly higher for the sampling period Day 1 Time 1 than the other two sampling periods (Table 1). Pearson correlation coefficients were also higher between R-50 and sub-sampled plates (Plate 2 plates) than those between R-50 and typical Andersen replicate plate (Plate 1 plates) (Table 1). All Pearson correlation coefficients were statistically significant (p value < 0.001).

3.3 Comparison between sampling periods within one sampling type

Pearson correlation coefficients were also computed between the sampling periods for a given sampling type. It was evident that the relationships between the two sampling times in Day 1 were stronger than those between the first day and second day sampling periods (Table 2). These results indicate that samples taken on the same day are somewhat reproducible, while those taken on consecutive days are not.

Table 1 Pearson correlation coefficients, R values, comparing the concentrations obtained with each of the three sampling types for each sampling period (Day 1 Time 1, Day 1 Time 2 and Day 2)

Sampling period	Correlation with R-50 Plates	
	Plate 1	Plate 2
Indoor Day 1 Time 1	.96	.97
Indoor Day 1 Time 2	.89	.94
Indoor Day 2	.89	.94

Table 2 Pearson correlation coefficients, R values, comparing the concentrations (CFU/m³) between samples collected Day 1 Time 1 and Day 1 Time 2, and between samples collected Day 1 Time 1 and Day 2, for each of the three sampling types

Sample type	Day 1 Time 1 versus Day 1 Time 2	Day 1 Time 1 versus Day 2
Andersen plate (Plate 1 plates)	.87	.60
Sub-cultured plates (Plate 2 plates)	.93	.59
R-50 plates (Plate 3 plates)	.91	.59

3.4 Facilitation of isolate identification with the Random -50 transfers

One of the goals of the ACCURATE project was to isolate as many fungi as possible from study homes in order to better understand relationships between bioaerosols and infant health, manifested as wheeze. By transferring 50 impaction points from each original Andersen plate to five plates, the fungi were readily observable for isolation. From these plates more than 12,000 isolates were obtained as voucher slides that were studied for identification, eventually resulting in 170 taxa (Anagnost et al., 2004). Among these taxa were slow-growing fungi that were rarely or never-before reported in air surveys (Fernando et al., 2005). The Random-50 sub-culturing method facilitated the ability to isolate a large number of fungi. It also allowed the recovery of slow-growing fungi, such as *Acrodontium intermissum*, *Acrodontium myxomyceticola*, and *Myxotrichum deflexum*, probably by lessening competition from other isolates (Fernando et al., 2005). Because of the high degree of correlation between total fungal concentrations obtained with the R-50 and untouched replicate plates, it is possible that the concentrations of individual species identified from the R-50 plates can be calculated and used for further analysis of bioaerosol populations in these homes.

4 Conclusions

This R-50 sub-sampling method of analyzing samples obtained with the Andersen N6 sampler

enables the estimation of CFU/m³ with high confidence from the R-50 plates. An advantage to this method is the more inclusive microbe isolation that allows a more comprehensive evaluation of cultures for identification of individual isolates. Results reported here indicate that this method has the possibility of providing comprehensive evaluation of air samples for the identification of fungi, with high level of confidence that accurate fungal concentrations can also be estimated.

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References

- Abraham, J. L., Crawford, J. A., Hall, G., Hargrave, T., Hunt, A., Lane, S. D., Liu, C., Naishadham, D., Rosenbaum, P., & Anagnost, S. E. (2004). *The Syracuse AUDIT Study: Risk factors for wheeze in the first year of life: Study design, characteristics, range of exposures, and preliminary analysis of outcomes*. New York, USA: Center of Excellence in Environmental and Energy Systems Syracuse University Syracuse.
- Anagnost, S. E., Catranis, C. M., Wang, C. J. K., Zhang, L., Fernando, A. A., Morey, S. R., DeStefano, P., Garback, C., LaMoy, M., Hall, G., Crawford, J. A., Naishadham, D., Hunt, A., & Abraham, J. L. (2004). *Survey of fungi found in Syracuse AUDIT study homes*. New York, USA: Center of Excellence in Environmental and Energy Systems Syracuse University Syracuse.
- Burge, H. A., & Otten, J. A. (1999). Fungi. In: J. Macher (Ed.), *Bioaerosols: Assessment and Control* (pp. 19-8 to 19-9). Cincinnati, OH: American Conference of Governmental Industrial Hygienists.
- CDC (Centers for Disease Control and Prevention) (1994). Asthma mortality and hospitalizations among children and young adults, 1980–1993. *Morbidity and Mortality Weekly Report*, 45, 350–353.
- Ciuk, J., Volkmer, R. E., & Edwards, J. W. (2001). Domestic nitrogen oxide exposure, urinary nitrate, and asthma prevalence in preschool children. *Archives of Environmental Health*, 56(5), 433–438.
- Duchaine, C., Mériaux, A., & Comtois, P. (2002). Usefulness of using three different culture media for mold recovery in exposure assessment studies. *Aerobiologia*, 18, 245–251.
- Fernando, A. A., Anagnost, S. E., Zhou, S., Morey, S. R., Catranis, C. M., & Wang, C. J. K. (2005). Noteworthy microfungi from air samples. *Mycotaxon*, 92, 322–338.
- Garrett, M. H., Abrahamson, M. J., & Hopper, B. M. (1998). Indoor environmental risk factors for respiratory health in children. *Indoor Air*, 8, 236–243.
- Licorish, K., Novey, H. S. & Kozak, P. (1985). Role of *Alternaria* and *Penicillium* spores on the pathogenesis of asthma. *Journal of Allergy and Clinical Immunology*, 76(6), 819–825.
- Martinez, K. F., Todd, W. F., & Fishbach, T. J. (1991). *Evaluation of alternative samplers for bioaerosols, phase I: physical sampling efficiency, Report No. CT-160-04A*. Cincinnati, OH: National Institute for Occupational Safety and Health.
- Negrini, A. C., Berram, D., & Campi, P. (2000). Clinical study on *Alternaria* spores sensitization. *Allergologia et immunopathologia*, 28(2), 71–73.
- Stark, P. C., Burge, H. A., & Ryan L. M. (2003). Fungal levels in the home and lower respiratory tract illnesses in the first year of life. *American Journal of Respiratory and Critical Care Medicine*, 168, 232–237.
- Uttell, M. J., & Looney, R. J. (1995). Environmentally induced asthma. *Toxicology Letters*, 82, 47–53.