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SHORT COMMUNICATION

MEASUREMENT OF THE FUNGAL DETERIORATING POTENTIAL IN THE DUST OF INDOOR ENVIRONMENTS

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Several methods that can help in defining either the amount of biological contaminants present in the air or the species diversity of the microbial communities inhabiting the dust are available. Unfortunately, none of these methods provides information on the metabolic potential of the microrganisms, and therefore they are often useless in terms of forecasting the potential dangerousness. An enzymatic method developed for the evaluation of the risk to stored library materials in association with the metabolic diversity of the fungal community inhabiting dust deposits is applied and discussed here. The procedure consists in the direct inoculation of dust samples into commercial micro-titre plates and involves colour formation by reduction of a tetrazolium dye to assess utilization of 95 separate sole carbon sources during a 4–10 days incubation period. The protocol utilized in this study was specifically developed to analyse and compare the metabolic diversity of fungal communities present in the dust of different conservative situations in an Italian public library. The samples of settled dust, each corresponding to a different conservative situation, were successfully discriminated based on the metabolic potential of the fungal communities inhabiting the shelves where samples were collected.

1 Introduction

Information on the relationship between exposure to airborne fungal spores and their potential effects is often needed to understand and manage health problems to humans and damage problems to materials, especially in the environments destined to the conservation of valuable objects. In most cases, this information is not available because of the difficulties in identifying and quantifying fungal spores in air samples. Analytical techniques based on culturability are widely accepted and employed to evaluate microbial contamination in an indoor environment.[1] Conventional techniques use either microscopy

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or culture methods. Both approaches are time consuming, require experienced personnel, and may be unreliable. Culturing can be equally difficult if a suitable selective medium is not available or the spores are not viable in vitro. To overcome these drawbacks, molecular techniques are now extensively used for the detection and identification of microorganisms in environmental samples and methods for purifying DNA from conventional airborne spore samplers have been widely discussed and described.[2] Detection and quantification of microbiological markers and/or agents of disease, such as ergosterol, endotoxins, β-glucans and extracellular polysaccharides (EPS), were also used as methods to assess microbial contamination and to more accurately evaluate microbial exposures in the indoor environment.[3,4] All these methods can help in defining either the amount of biological contaminants present in the air, or the quality of the microbial communities inhabiting the dust, in terms of species diversity. Besides, they do not provide information on the functional diversity (metabolic potential) of the species, and therefore are often useless in terms of forecasting the potential dangerousness of dust to the different materials stored in each environment. Microbial communities affecting the different habitats offered by stored organic materials (libraries, archives, deposits, etc.) are varied and characterised by metabolic peculiarities. Fungal and bacterial spores and propagules found in indoor dust can be considered as active inhabitants of dust, or as elements passively introduced by other sources.[5] Moreover in indoor environments, dustborne and airborne biological agents may represent different ecological niches, and according to Rao[1] surface dust sampling can represent a possible historical marker for cumulative exposures.

A method was developed by Pinzari and Montanari[6] to be applied in the evaluation of the risk to stored library materials in association with the metabolic diversity of the fungal community inhabiting dust deposits on storage space surfaces. Here a specific data treatment first proposed by Buyer[7] and collaborators for soil samples was applied to distinguish samples based on a different ability in degrading clusters of compounds. This approach provides an evaluation of the biological dangerousness of dust to classes of materials. The procedure consists in the direct inoculation of dust samples into commercial microtiter plates and involves colour formation by reduction of a tetrazolium dye to assess utilization of 95 separate sole carbon sources during a 4–10 days incubation period. The single time-point results are indicative of heterotrophic fungal population density and total potential heterotrophic activity, while the multiple time-point data detect differences between communities in metabolic potential on various classes of substrates. The method is far less time-consuming and technically much simpler than either identification of isolates or phylogenetic analysis.[7]

The protocol utilised in this study is based on the sampling of settled dust, and was specifically developed to analyse and compare the metabolic diversity of fungal communities present in the surfaces of three different conservative situations in an Italian public library.

2 Materials and Methods

The test kits used in this study were originally designed and produced to identify single isolates of microbial species[8]. The microtiter plate by Biolog contains 96 wells with different carbon substrates. In the FF microplates substrate selection was optimized for differentiation of filamentous fungi. The substrate set includes sugars (monosaccharides, disaccharides, oligosaccharides and polysaccharides) amino acids and organic acids as well as alcohols, glycerides, esters, amines, amides, nucleotides, etc. A full list of the substrates assemblage in the different types of Biolog plates produced is reported by Preston-Mafham et al.[9]

The plates rely on a redox dye (tetrazolium violet for bacteria and iodonitrophenyltetrazolium for filamentous fungi) to detect respiration (NADH formation) of sole carbon sources; 95 wells contain the substrate and the dye, while the control well (the first, at top left on the plate) contains the dye alone. The content of the wells is supplied in a dry state; the medium is reconstituted when the wet sample is added.

The direct inoculation of the environmental sample provides information on the metabolic abilities of a “microbial community” or, at least, of a combination of strains.[10,11]

Samples of dust were collected in the library depository of the National Library of Rome. The depository examined is located in an eight-floor modern building that was built between 1965 and 1975. Although the library and its depository is located in the centre of Rome, a wide space with green yards divide the building from traffic. On each floor of the depository, the books are organised into metal stacks. The environmental monitoring during the 24 months of the study, indicated a generally good maintenance of standard climatic conditions (the average of relative humidity values ranged between 45 and 60%, and the temperature

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from 19 to 22 °C). Due to the uncertainty associated with bioanalytical research of this kind, particular care was taken in the choice of sampling areas.

Dust was sampled using sterile cotton swabs with sterile frames made of plastic in order to delimit the sampled area in accordance with a standard procedure. The frames measured 5x5 cm^2, and for each swabbing, a new frame was used. Three different shelves holding books were sampled: the first had undergone a dusting procedure three months earlier (Shelf 1); the second 9 months earlier (Shelf 2); the third was coated with a 2-year old dust deposit (Shelf 3). The sampling of dust was performed controlling most of the possible sources of variability (type of surface sampled, distance of the shelf from the floor and the air conditioning units, dimension of the sampled areas, homogenisation of the samples, etc.). The main differences between the compared samples of dust were linked to the time passed after the last dusting procedures operated by conservators.

In order to obtain representative average samples, the dust was taken from large areas of each shelf and then homogenised. For the inoculation of Biolog FF plate, each shelf was sampled 9 times to obtain 3 groups of 3 swabs. Each group of three swabs was put into a sterile glass tube containing 20 ml of a solution made with 0.03% Tween 40 (Sigma P1504), 0.25 % Gellan Gum (Sigma P8169), 50 mg/ml gentamycin, and 100 mg/ml streptomycin in order to limit the analysis to fungal community. The solution prepared for the inoculation is the same used in the manufacturer protocol for identification purposes. While the gellan gum is not a potential substrate for fungi, the Tween 40 additive has proven to not influence the selectivity of colour formation in the plates since its final concentration in the single well is very low.[12]The mixture was gently vortexed at 22 °C and then left to settle for 3 h.

In our experimental trials, we used the Biolog (Hayward, CA, U.S.A.) FF plate which contains substrates and dyes particularly suitable for the evaluation of fungal growth.[12] Each well on the plate was inoculated with 100 µl of suspension. Individual plates were placed in plastic bags to prevent drying in the external wells (the bags were not tightly closed in order to allow O_2 and CO_2 to circulate), and incubated at 26 °C. Optical densities of the wells were measured reading the absorbance at both 490 nm and 750 nm using a microtiter plate reader (Vmax, Molecular Devices, CA, USA). Measurements were effected immediately following inoculation (time zero), and subsequently at 24, 48, 72, 96, 168 and 240 h intervals, in order to obtain a well colour development curve for each well. Data from the different samples were compared at each incubation time but the best discrimination corresponded to the point when all the plates reached a plateau in the curve, corresponding to 168 h of incubation in all the samples. Raw data were transferred to an Excel (Microsoft) sheet according to sample (3 samples), replicate (12 replicates each), and reading time (10 reading points, one every 24 h). Two separate sets of data were obtained according to the double wavelength used in scanning the plates.

The average well *colour* development (AWCD) was calculated for each plate at each reading time simply by summing the optical densities obtained at 490 nm for all the wells of the plate and dividing the obtained amount for 96. Similarly, the average well *turbidity* development (AWTD) was calculated using the optical densities obtained for each plate at 750 nm. Moreover, the 96 substrates (95 + water) were divided into six guilds:[7] polymers, carbohydrates, carboxylic acids, amines and amides, amino acids, miscellaneous and the average absorbance for all wells within each category calculated.

Analysis of Variance (ANOVA)[13,14] was employed to investigate the differences in dust fungal assemblages. In the ANOVA each comparison is considered significant (the difference is significant) if the probability exceeds the confidence interval on the basis of Tukey's HSD test (HSD, Honestly Significant Difference).[15] The test is a multiple comparison procedure generally used in conjunction with an ANOVA (analysis of variances) to find which means are significantly different from one another. The statistical package XLStat (Addinsoft 2007-Pro, Paris, France)[16] was used.

3 Results and Discussion

The ANOVA was performed on the AWCDs and the AWTDs of the three samples for each reading time in order to evaluate the statistical significance of the difference between the averages. The best discrimination between samples was obtained at 168 h of incubation (Figures 1 and 2). Optical densities at 490 nm (AWCDs) are proportional to fungal cells' respiration, dye reduction, and substrate use[8], while optical densities at 750 nm (AWTDs) are proportional to fungal biomass and the initial cell density of the inoculum[17]. Results of the ANOVA are presented in Table 1. According to substrate use of the fungi isolated in the dust, the Shelf 2 does not differ significantly from Shelf 1 and Shelf 3, whereas Shelf 1 and Shelf 3 show significant differences when compared. The differences between samples based on fungal growth (AWTD)

Contrast	Difference	Standardized difference	Tukey's critical value	Probability of the difference being insignificant	Significant
Shelf 1 vs Shelf 3	0.230	2.631	2.414	0.030	Yes
Shelf 1 vs Shelf 2	0.152	1.738	2.414	0.201	No
Shelf 2 vs Shelf 3	0.078	0.892	2.414	0.648	No

Table 1: In the table, the results of the one-way analysis of variance performed in the multiple comparison of the three samples based on the AWCD values are reported (a=0.05; test used: Tukey's, HSD). Both significant and insignificant differences between samples are listed. The critical value is the value that (for a given number of replicates) the standardised difference must achieve to reach statistical significance (at the 0.05, considering the number of replicates used, its level is 2.414).

Guilds of substrates	450 nm (dye reduction), use of substrates		
	Shelf 1	Shelf 2	Shelf 3
Carbohydrates	0.70 a	0.57 ab	0.48 b
Polymers	0.54 a	0.40 b	0.44 ab
Amines and Amides	0.35 a	0.35 a	0.29 a
Amino Acids	0.57 a	0.44 ab	0.43 b
Miscellaneous	0.33 a	0.39 a	0.33 a
Carboxylic acids	0.48 a	0.37 b	0.35 b

Table 2: Summary of all pairwise comparisons of the three samples based on the average values obtained for the six guilds of substrates (a=0.05; test used: Tukey's, HSD) at the optical density at 490 nm. The values are shown as bars in the histogram of Figure 3. Analysis of the differences between the categories with a confidence interval of 95%. Samples signed with different letters (a or b) are significantly different. Where both the letters are present, the values are not significantly different from the two other samples.

were not significant according to Tukey's test (data not shown). The fungal community settled or developed in three-month time on Shelf 1 showed a higher respiration to biomass ratio with respect to the communities inhabiting the older dust (9 and 24 months respectively for Shelf 2 and 3). Comparisons between samples according to average values of optical densities calculated on the substrate guilds have also been tested with ANOVA and are summarised in Figure 3 and in Table 2. The growth of the mycelium in the wells (AWTDs data, measured at 750nm) was not significantly different between the samples (data not shown). A different usage of polymers discriminated Shelf 1 from Shelf 3 in terms of use of the substrates. Amines and miscellaneous guilds did not discriminate the fungal assemblages in the dust samples, while a slightly different use of amino acids was observed between Shelf 1 and the other shelves. Nitrogen (in the form of nitrogen-containing carbon sources, like amines and amino acids) probably represents a limiting factor in dust. Nitrogen-containing substances are utilised almost in the same way, both in a newly deposited dust and in an old one, indicating that its usage by fungal metabolism is not strictly correlated with the usage of the other classes of compounds. The metabolic activity of the fungal community on the different carbon sources is directly proportional to the ability of the fungal spores to germinate and degrade a given range of substrates.[7,12] The single time-point results are indicative of heterotrophic fungal population density and total potential heterotrophic activity on various classes of sub-

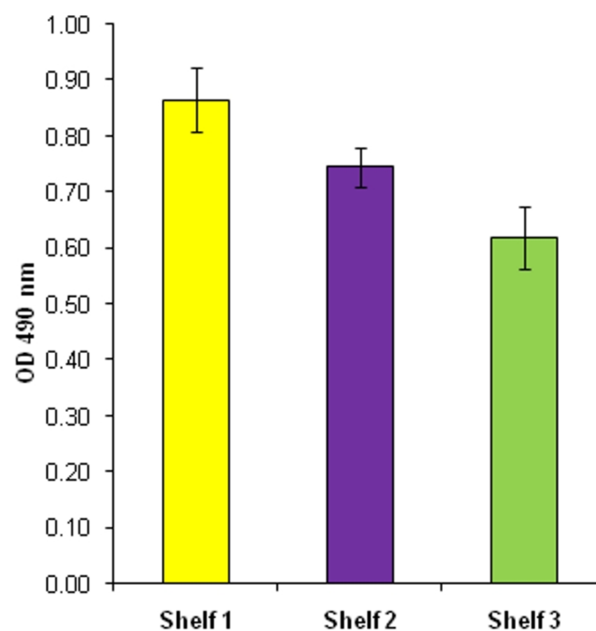

Figure 1: Average well colour development (AWCD) values after 168 h incubation. Average values of 12 replicates are shown. The bars indicate the standard deviation values.

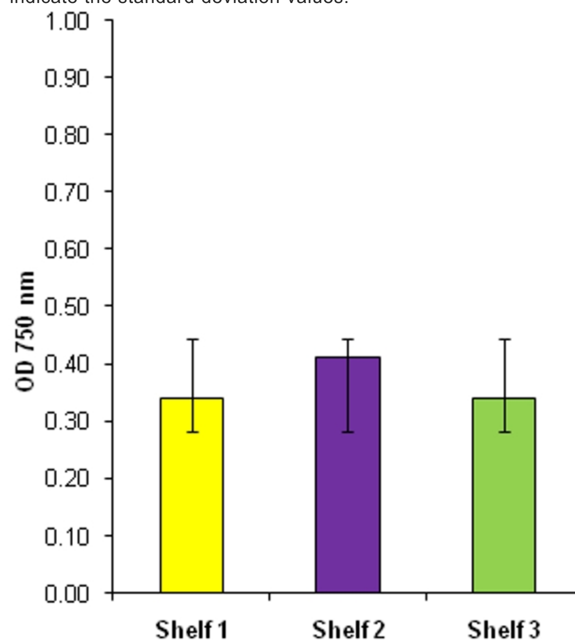

Figure 2: Average well turbidity development (AWTD) values after 168 h incubation. Average values of 12 replicates are shown. The bars indicate the standard deviation values.

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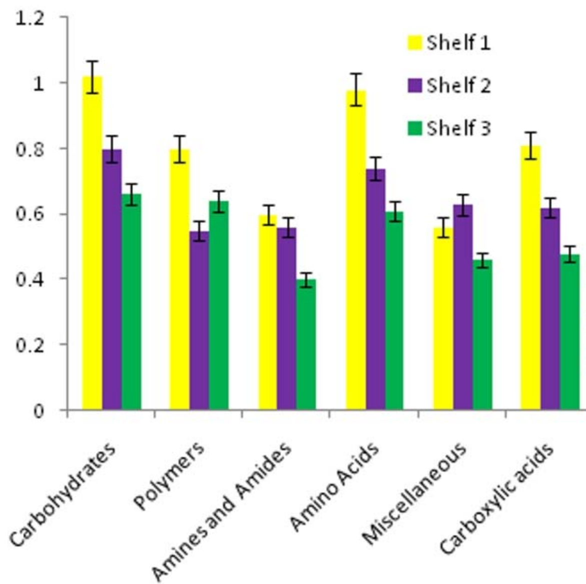

Figure 3: Average well colour development (AWCD) values after 168 h incubation for each guild of substrates. The bars indicate the standard deviation values. The statistical significance of the differences between the bars is reported in Table 2.

strates. The method is far less time-consuming and technically much simpler than either identification of isolates or phylogenetic analysis. A fast and intense colour development in the plates corresponds to a higher catabolic versatility of the fungal community in the dust sample. Moreover, the lack of colour development in a specific well or in a group of related substrates indicates that in the fungal community represented in the dust sample, none of the viable strains can attack and degrade certain compounds. This sort of results can be translated to dangerousness for the affected objects since they directly indicate the presence in the dust of potential decomposers, skilled with specific enzymatic abilities (i.e. the specific ability of degrading polymers in the new dust). The comparison of the effects of different conservation treatments on airborne fungi assemblages in a given environment can be expressed as differences in the pattern of colour development in the wells of the microplate.

4 Conclusions

The three samples of settled dust studied here, each corresponding to a different conservative situation, were successfully discriminated based on the metabolic potential of the fungal communities inhabiting the dust settled on the shelves where samples were collected. Moreover, according to the results presented, a recently deposited dust, like that collected from shelf 1, can have a higher catabolic potential when compared with an “old dust”, as resumed by the significantly higher value of the AWCD in the histogram in Figure 1.

The lower catabolic activity of an "old dust" respect to a "new dust" can be due to the presence of inhibiting or biostatic substances in the old dust that makes the spores less viable. A further possible interpretation of the significant difference evaluated between old and new dust is that the former contains substances that, once inoculated in the wells together with spores, can alter some enzymatic abilities of the fungi. In the study-case here exemplified, the shelves sampled were in the same room, at the same distance from windows and air conditioning units. Moreover all the dust samples were taken from the same type of surface (metal stacks), and although it was not possible to weight the sampled dust, the same standard areas were considered. The differences observed in deposited dust can reasonably be attributed to the differences in dusting procedures applied to the three set of shelves. A further source of information that can help in understanding the results is the relationship between the overall fungal respiration (substrate usage = colour development = optical density measured at 490 nm) and the fungal growth (biomass development in the wells = turbidity of the wells= optical density measured at 750 nm). The fungal growth was not significantly different between the three samples of dust, while the fungal community present in the dust sample of shelf 1 showed a significantly higher average respiration rate. This means that the fungal community in the “new” dust from shelf 1 was characterised by a higher metabolic quotient (respiration to biomass ratio) when compared to that present in the “old” dust, which developed the same amount of biomass in the wells of the microplate, but consumed a lower amount of substrates.

Changes in fungal community physiological profiling in the dust deposited on surfaces can be studied, and correlated with specific problems in conservation environments. Fungal cells and propagules in dust deposits can be analysed by examining their functional aspect and better evaluated for their potential harmfulness to materials.

The different classes of organic compounds present in the plates showed different capacities in distinguishing samples, further studies have recently been addressed to the definition of a set of substrates more suitable for fungi that cause damage on specific materials. The grouping of substrates into guilds represents a step in this direction. This approach was initially used in this study just to reduce data, but then it appeared informative and capable of distinguishing samples based on a different ability in degrading clusters of compounds, and therefore on a specific dangerousness to classes of materials.

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