

Electronic Nose for the Early Detection of Moulds in Libraries and Archives

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Key Words

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Abstract

The development of sensor technology has stimulated interest in the use of characteristic volatile and odorous compounds produced by fungi as early indicators of deterioration in materials. Sensor arrays to measure traces of volatile chemicals could detect early fungal growth in libraries and archives.

In this study we tested an electronic nose to detect fungi actively growing on paper samples. The main aim was to verify whether or not a device, currently used to detect fungal activity in stored grain, might be suitable for detecting mould activity on paper. The findings indicate that it is possible to discriminate "*in vitro*" between paper samples affected by moulds and those unaffected, both at 100% RH (relative humidity), and at 75% RH, simply by measuring their odour fingerprint with an electronic nose. The sensors used in this study discriminated for each paper type three different species of actively growing fungi. Cluster analysis (CA) showed that it was possible to differentiate between specific species. Different paper types influenced the emission of odorous signals by

moulds. When considering data from all the paper types, principal component analysis (PCA) indicated that only samples analysed at 100% RH could be separated.

This study suggests that, before electronic-nose technology can be applied to the early detection of mould growth in libraries, archives, museums or in display cases, more information will be required on the influence of substrata and of other environmental parameters in the production of volatile chemicals by fungi.

Introduction

Fungal bio-deterioration in archive and library collections depends mainly on environmental conditions. The main factors that limit fungal spore germination and growth are unfavourable temperatures and the relative humidity (RH) in the environment, and a very low water activity (a_w) or equilibrium RH of the substrate (paper, parchment, glues, textiles or any other organic materials in the book) [1,2,3].

To prevent fungal growth, books and archives are commonly stored in controlled environments, with temperature and humidity maintained in a defined range, and periodically cleaned of dust [4]. Prevention of extensive fungal contamination is our only weapon against bio-deterioration. Early detection of fungal activity on

materials in libraries and archives is therefore fundamental to avoid serious damage to our cultural heritage.

The relationship between poor conservation and the musty background odour often encountered in libraries and archives is well known to all people that work with books and documents.

The recent development of sensor technology has stimulated interest in the use of characteristic volatile and odorous compounds produced by fungi as early indicators of deterioration in materials [5,6]. Moreover, the development of a system for the detection of the growth of moulds in libraries, archives and museums long before it becomes perceptible to the human olfactory sense would be of great interest for preservation and conservation purposes [7].

Microbial volatile organic compounds (MVOCs) are produced as metabolic by-products of bacteria and fungi and may be detected before any visible signs of microbial growth appear. The most commonly identified volatile microbial metabolites include 3-methyl-1-butanol, 1-hexanol, 1-octen-3-ol, 2-heptanone, and 3-octanone [8,9]. In a few cases, these odorous compounds are fruity or flowery and may be adapted to attract arthropod dispersers (e.g. insects carrying the mould conidia to new growth sites). Usually they are musty or earthy and are probably adapted to deter grazing and feeding invertebrates and vertebrates, or at least to give a distinct "not food" odour to mould colonies and their underlying nutritional substrates. A few such volatiles have been found to be directly irritating to vertebrates [10].

MVOCs can serve as early indicators of potential biocontamination problems. A specific technology (the electronic nose or e-NOSE) for the use of MVOCs to detect spoilage of organic materials has already been developed for grain storage. The e-NOSE technology is actually used for the early detection of quality changes in cereal grain [11].

The electronic nose is a device that replicates the structure and principles of the natural olfactory sense. It is composed of a chamber containing receptors (after the nose of vertebrates), a system that can amplify signals, and a data analysis system, which allows us to transpose the information contained in the odour into a "volatile pattern", which summarises the most important elements and identifies the pattern when compared to others. It has been proposed that sensor arrays capable of measuring small amounts of volatile compounds could be used in the early detection of fungal activity in library and archive environments, although such a development of the technology to the field of cultural heritage conserva-

tion has, to our knowledge, not been commercially considered as yet. It should be noted that the electronic nose does not provide detailed chemical information on the volatile compounds produced by active fungi, but rather gives a qualitative picture ("volatile pattern") of the mixture of compounds present in the sample [12]. The electronic nose classifies an odour as a complex, showing the synergic activity of different molecular species in a single spot that could be identified by the sensation of a smell.

The range of volatile compounds produced by materials, employees and moulds in libraries and archives is obviously very wide and it is probably peculiar according to the specific environment considered [13,14]. Great care should therefore be taken when developing a suitable combination of sensors that could be used for detecting changes in the "odorous fingerprint" of each environment monitored. A first approach would be the *in vitro* detection of a range of volatile compounds produced by fungi that grow on library materials, in addition to the detection of a range of odours that could be used as quality markers for library materials.

The objectives of this study were: (a) to verify whether or not the electronic nose device currently used to detect fungal activity in stored grain might be suitable for detecting mould activity on paper; (b) to check the intensity of the signals and to see if the fungal activity on paper is quantifiable; (c) to see if different species of fungi growing on the same paper type are discriminated by the sensors; (d) to see if the substrate (different types of paper) influenced the emission of odorous signals by moulds; and (e) to see if different metabolic states of the moulds (i.e. growth at different RH) yield different signals.

Materials and Methods

Paper Types

Three types of paper with different compositions, including the origin of the fibres and the chemical treatments undergone during the manufacturing process, were used in this study. Paper characteristics strongly influence the intensity and the consequences of fungal attack [1], and a minimum of three types of paper are required to verify if the sensors can detect differences in fungal growth.

- Paper type A was a Whatman 1CHR (for chromatography) Cat. No. 3001 917 made of pure cellulose.

- Paper type B was produced by Fedrigoni Mills (Italy) and was called “Freelife vellum”. It is a type of paper with a long lifetime (properties according to ISO CD 9706). It was made with 80% recycled fibre, 15% cellulose, chlorine free, 5% cotton fibres; its surface pH was 7.2.
- Paper type C was produced by Fedrigoni Mills (Italy) and was called “Old mill” ivory. This was an un-coated paper with a long lifetime (properties according to ISO CD 9706). It was made with 100% cellulose fibre, and its surface pH was 6.9.

The paper samples were cut into 2×6cm strips. Both the sides of the strips were exposed to UV light for 45 minutes, in order to sterilise the surface from airborne fungal and bacterial cells [15].

Fungal Strains Used for the Test

Three fungal strains were used to inoculate the paper strips: *Aspergillus terreus* Thom (strain No. 3) was obtained from the ATCC culture collection, *Aspergillus hollandicus* (Anam.: of *Eurotium amstelodami*, (Mangin) Thom and Church) and *Eurotium chevalieri* L. Mangin (strains No. 1 and No. 2 respectively) were from the culture collection of the Istituto Centrale di Patologia del Libro, and had been isolated from deteriorated paper materials [16,17]. All these three species are frequently associated with bio-deterioration of library material [18].

Mycelial Cultures and Inoculum of Fungi on Paper

Mycelial cultures were inoculated on MEA (Malt Extract Agar, Raper and Thom, 1949, prepared according to Pitt and Hocking, 1985). The agar medium was poured into 10cm Petri dishes and the strains were inoculated in the centre. The Petri dishes were then kept in the dark, at 25°C, before their use for the inoculation on paper strips. The spore suspensions were obtained by gently scraping the surface of the 7-day-old cultures with a swab, and washing it with 30ml of sterile, distilled water containing 0.02% Tween 80 (Merck-Schuchardt–Germany). The spore suspensions were filtered through a sterile cotton cloth to remove impurities. The spore density was defined for each strain by counting the elements in a Thoma Chamber by optical microscopy. A defined volume of each spore suspension was diluted with nutritive broth (Sabouraud Broth by DIFCO, Becton Dickinson, USA) in order to inoculate the paper strips. All the inoculations were performed

under a laminar flow hood to assure sterility in the procedures.

We used 100µl of broth for each paper strip, distributing 8 dots of this onto the same. Control samples were strips of each type of paper inoculated with 100µl of broth without fungal spores. Four replicates (a–d) for each treatment were prepared. Each inoculated paper strip was placed in a 50ml polystyrene vial. During the incubation the vials were closed with ventilated caps. The experiments were run under two values of RH: 100% RH and 75% RH. The conditions were realised using double-bottom glass containers; the 100% RH was obtained with distilled water; the 75% RH with a saturated solution of sodium chloride. The vials containing the samples were placed in the glass containers. The glass containers were kept in a thermostatic cell at T=27°C for 7 days. A sensor to register the internal RH (Hygrolog-D Rotronic AY – Swiss) was placed in each container throughout the incubation time.

Electronic Nose Device Used in this Study

The analysis of the odorous pattern of the samples was performed using an e-NOSE 4000 (Neotronics). The operating principle of the e-NOSE 4000 is to measure the resistance change of a group of conducting polymer sensors [12] when exposed to the vapour being analysed. This is achieved by monitoring a constant current through the sensors and monitoring the voltage across its terminals required to produce that current. Each of the sensors in the e-NOSE 4000 head is an electrochemically grown, conducting polymer-based material most commonly based on a polypyrrole. When volatile molecules are adsorbed, the electrical resistance of the material constituent of the sensors changes. Thus the change in the monitored voltage is directly proportional to the change in each sensor resistance. During the sampling time, continuous measurements are taken of the percentage change in the baseline resistance. Then, once per second, the preceding measurements are averaged and the stored results updated to provide a plot of percentage change (positive or negative) against time in 1-second increments. It is the resultant data from these measurements that allow for the analysis of the vapour being tested. The analogue voltage measurement is digitised and fed to a microprocessor. The microprocessor measures each sensor’s response, if necessary applying a calibration factor. Each of the 12 sensor channels therefore essentially comprises a constant current circuit and an analogue-to-digital converter (ADC).

Sampling with the Electronic Nose, and Data Analyses

The 50ml vials containing the samples were used directly in the head space sampler of the e-NOSE 4000. The vials were placed in a glass sampling vessel on the sampling stage and allowed to equilibrate at 25°C for 15 minutes. The headspace was then sampled for a period of 7 minutes. Between each sample nitrogen gas (analytical quality) was used to purge the system. The data were collected and analysed by the e-NOSE software package system. RH and temperature sensors are incorporated in the instrument head. Before the sampling, the RH of the head space of each vial was measured with the electronic nose system at 25±1°C. The RH data for vials containing paper samples with moulds grown after the incubation at 100% and 75% RH are shown in Table 1 and Table 2, respectively. A calibration facility in the software employs a set of correction factors, one for each sensor. This facility ensures that test results are repeatable and that comparison with previously stored data remains valid. Normalised e-NOSE data were analysed using the xlStat programme (Microsoft Excel add-in program) [19]. Principal component analysis (PCA) and cluster analysis (CA) techniques were applied to differentiate samples and variables [20,21]. Hierarchical ascending clustering dendrograms were built on Mahalanobis' squared distances between groups obtained in discriminant analysis at the 95% confidence limit [22,23]. The Mahalanobis' squared distance [22] scales the difference in each co-ordinate by the inverse of the variance in that dimension,

and could be used to measure the dissimilarity between two samples according to the formula: $D^2 = (X_i - X_j)' \text{inv}(\text{COV})(X_i - X_j)$.

Results

Figure 1 shows the data for the first two principle components, which account for 82% of the data. This shows that it is possible to discriminate between the three groups which correspond to the three paper types (Types A, B and C) at 100% RH. The PCA at 75% RH showed less discrimination between paper samples (data not shown), and the two main principle components accounted for only 62% of the variability. A possible explanation for the results obtained in Figure 1 is that the substrate (different paper types) influenced the emission of odorous signals by moulds. This was in contrast to what has been observed by other authors [24] who found that the volatile metabolites produced by the same fungal species on different substrata (different cereal types) were identical.

Figure 2 shows the PCA plot run between the data obtained from the different samples of paper "A" at 100% RH. In the plot the discrimination between the control sample and the samples with the actively growing fungi is clear. The CA shown in Figure 3 also allows a clear discrimination between control and samples with moulds, and between the three fungal species growing on paper type "A". The *Eurotium* species was separated

Table 1. Relative humidity, measured with the electronic nose system at 25±1°C, of paper samples incubated for 7 days at 100% RH.

	Paper type A		Paper type B		Paper type C	
	Mean	STDV	Mean	STDV	Mean	STDV
Control	30.55	±9.85	14.25	±5.55	25.10	±5.91
<i>Aspergillus hollandicus</i>	20.23	±2.97	12.80	±2.26	18.23	±2.65
<i>Eurotium chevalieri</i>	17.48	±5.07	12.60	±4.04	20.35	±4.08
<i>Aspergillus terreus</i>	14.70	±4.65	10.73	±2.98	14.83	±4.86

Table 2. Relative humidity, measured with the electronic nose system at 25±1°C, of paper samples incubated for 7 days at 75% RH.

	Paper type A		Paper type B		Paper type C	
	Mean	STDV	Mean	STDV	Mean	STDV
Control	13.15	±1.61	14.75	±3.01	17.68	±6.08
<i>Aspergillus hollandicus</i>	14.4	±3.37	12.95	±0.78	16.98	±3.12
<i>Eurotium chevalieri</i>	13.73	±2.04	10.98	±9.92	15.43	±3.38
<i>Aspergillus terreus</i>	14.75	±0.83	11.25	±2.43	14.08	±2.56

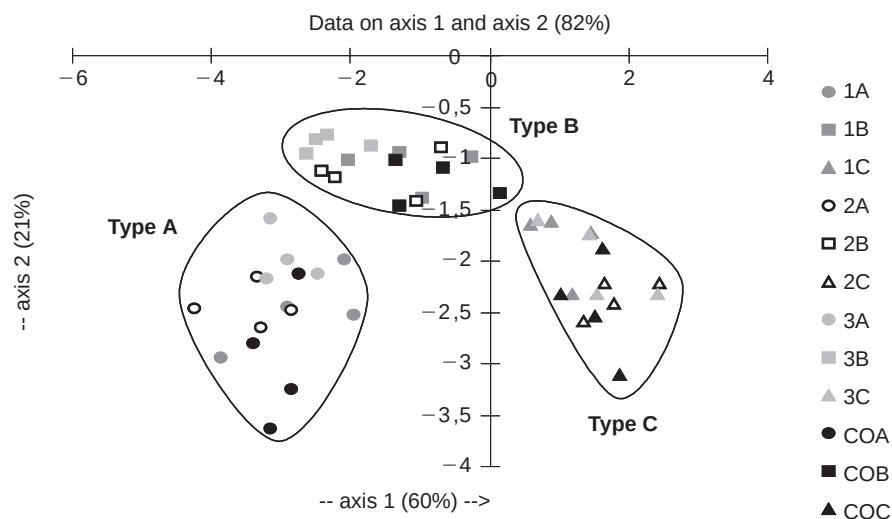


Fig. 1. Principal component analysis (PC1, PC2) of the e-NOSE data relevant to the paper types (types A, B and C), the fungal species (Species 1, 2 and 3) and the control (CO), at 100% Relative Humidity.

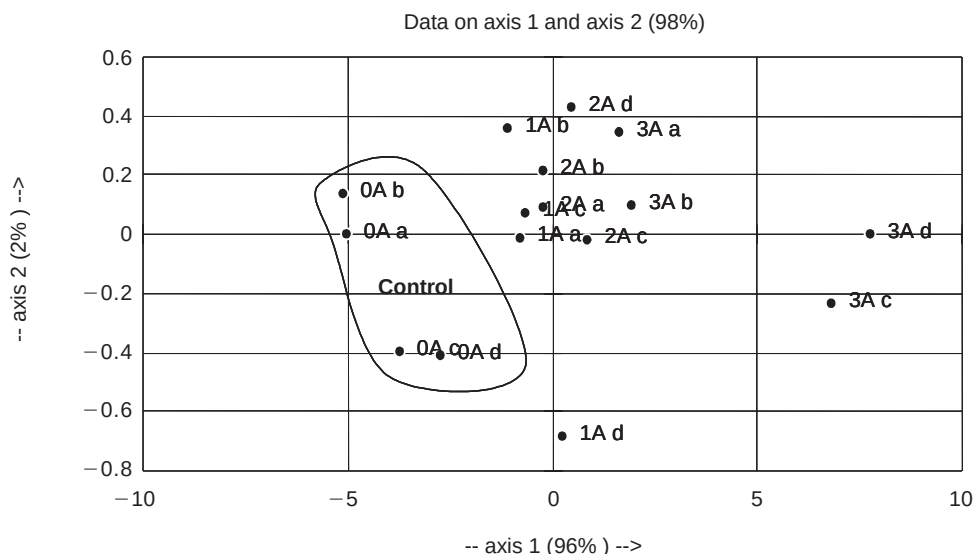


Fig. 2. Principal component analysis between the different samples of paper “A” (Whatman 1CHR) at 100% Relative Humidity. Key for treatments: a, b, c, d (replicates); 0, Control; 1, *Aspergillus hollandicus*; 2, *Eurotium chevalieri*; 3, *Aspergillus terreus*.

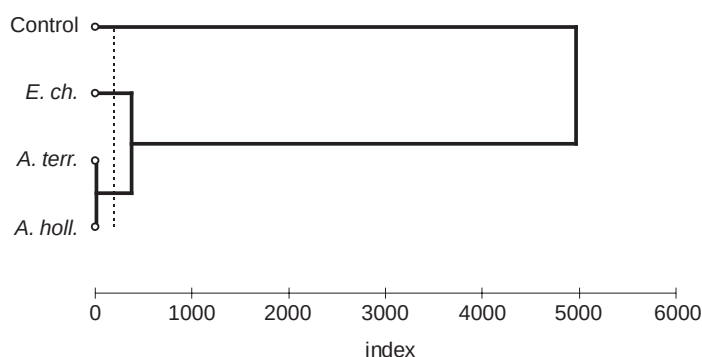


Fig. 3. Cluster analysis ($P=0.05$) of the data showing discrimination between the different samples of paper “A” (Whatman 1CHR), at 100% Relative Humidity. The index indicates the relative distance between treatments.

from the *Aspergillus* species, whilst the two *Aspergillus* species overlapped. The results are encouraging since they demonstrate that paper samples carrying actively growing fungi and control samples can be differentiated.

Figures 4 and 5 show PCA and CA from data obtained for paper “A” at 75% RH. Again, samples with fungi were grouped together while the control was separated out. *A. terreus* was separated from the other two species. This output suggests that, although the odorous fingerprint obtained for fungi growing on paper are significantly different at different values of RH, odour cluster groups always differentiate between mould-damaged paper types and control samples independent of environmental RH.

Figures 6 and 7 show PCA data obtained for paper “B” and “C” respectively, at 100% RH. The plots show the first two principle components, which accounts for

98% of the data for paper type B and 99% for paper type C. It is possible to discriminate between the control sample and the samples with the actively growing fungi in both the plots. The PCA of data obtained for paper samples B and C, at 75% RH, gave a statistically significant discrimination between the control samples and the samples with the fungal inoculum (data not shown).

Discussion

Previous studies have shown that there are marked differences in the production of volatile metabolites between closely related species and even between strains of the same fungus [25]. Further studies demonstrated that, besides being influenced by species and substrate composition, the production of volatile metabolites could

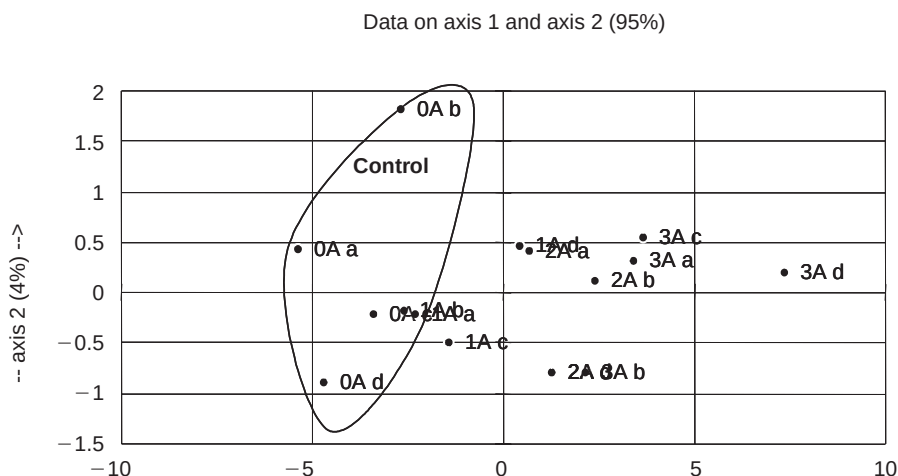


Fig. 4. Principal component analysis between the different samples of paper “A” (Whatman 1CHR) at 75% Relative Humidity. Key for treatments: a, b, c, d (replicates); 0, Control; 1, *Aspergillus hollandicus*; 2, *Eurotium chevalieri*; 3, *Aspergillus terreus*.

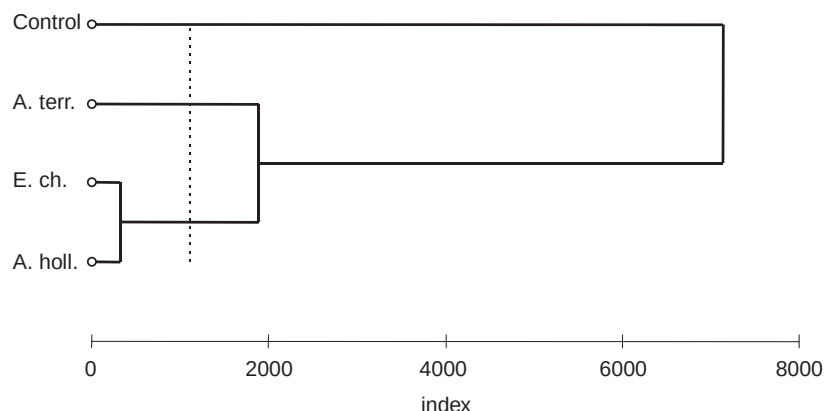


Fig. 5. Cluster analysis ($P=0.05$) of the data showing discrimination between the different samples of paper “A” (Whatman 1CHR) at 75% Relative Humidity. The index indicates the relative distance between treatments.

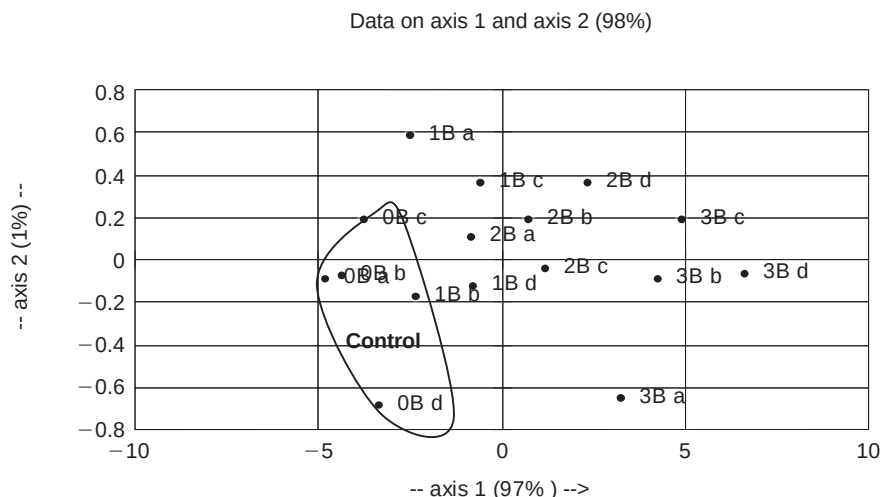


Fig. 6. Principal component analysis between the different samples of paper “B” (Freelife vellum) at 100% Relative Humidity. Key for treatments: a, b, c, d (replicates); 0, Control; 1, *Aspergillus hollandicus*; 2, *Eurotium chevalieri*; 3, *Aspergillus terreus*.

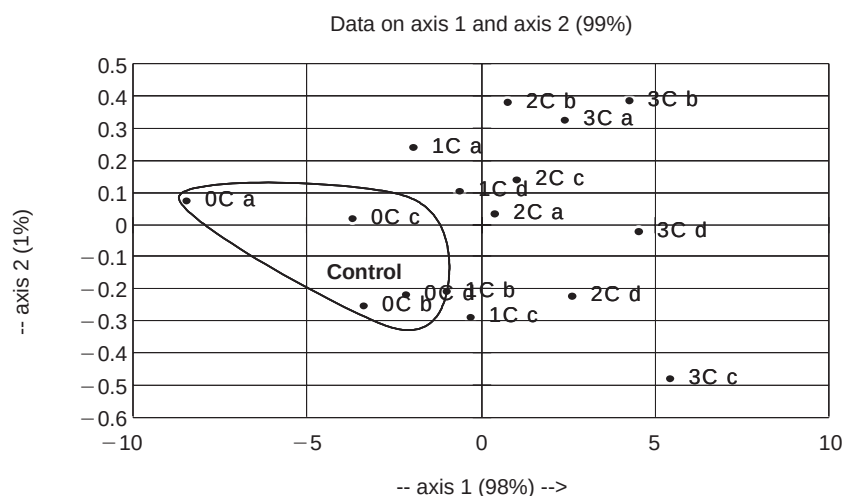


Fig. 7. Principal component analysis between the different samples of paper “C” (Old Mill) at 100% Relative Humidity. Key for treatments: a, b, c, d (replicates); 0, Control; 1, *Aspergillus hollandicus*; 2, *Eurotium chevalieri*; 3, *Aspergillus terreus*.

be influenced by the duration of fungal growth [26]. Furthermore, the amounts of volatile metabolites produced can be influenced by a particular stage of the organism’s life cycle [26].

It is very clear that the variables defining the quality and quantity of volatile metabolites produced by filamentous fungi are numerous and difficult to control. However, there are some compounds that have been reported as common to most of the fungi and for a wide range of metabolic and environmental conditions. These are detectable by the sensors on which the e-NOSE technology is based, and are mainly represented by alcohols and terpenes. The ability of e-NOSEs to discriminate between different volatile patterns is, in fact, very

good [25]. Some authors succeeded in distinguishing between different genera of fungi, although the technology will require further optimisation to detect subtle variation in volatile production patterns in species, within a single genus [25,26]. In the field of the early detection of fungal growth, the discriminating ability of the e-NOSE should be mainly addressed to distinguishing uncontaminated from contaminated material. This means the overall threshold of the instrument is more important than its resolving power among different volatile compounds.

In conclusion, the findings indicate that it is possible to discriminate “*in vitro*” between paper samples affected by moulds and those unaffected, at both 100% RH and at

75% RH, simply by measuring their odorous fingerprint with an electronic nose. Although control samples were always grouped separately from samples with moulds, greater overlapping occurred between samples incubated at 75% RH. Results were more reliable at 100% RH because of a higher growth rate together with a better development of fungal mycelium on paper. Paper samples incubated at 75% RH showed much less evidence of mycelial growth and maturation, compared to the samples incubated at 100% RH. It is quite clear that, in all the PCA plots obtained for each paper type (Figures 2, 4, 6 and 7), the main component can be attributed to the degree of maturity of the mycelia developed by each fungal species on the paper samples. This is demonstrated by the fact that, in all plots, the control samples (with no fungal growth) are found on the left side (negative values) of the first component axis, whilst the samples inoculated with *A. terreus* are found on the right side (positive values). In fact *A. terreus* showed, among the three fungal strains, the highest growth and spore production, both at 75% and 100% RH. Statistical analysis was carried out on normalised data showing that the concentration of volatile compounds produced by the samples could not be responsible for the trend seen in the PCA plots.

Moreover, the sensors used in this study discriminated for each paper type the three different species of actively growing fungi, although the grouping differed according to paper type. In addition, fungal species yielded different odorous patterns at different RH values, thus confirming what has been demonstrated by other authors [25,26] on the influence of fungal metabolic state on production of volatile compounds. When considering data obtained from all the paper types, the PCA indicated that only samples analysed at 100% RH could be separated. This finding indicates that the variability of odorous signals should account both for the equilibrium RH of materials and differences in paper grades. This suggests that the e-NOSE may need further optimisation to detect variations in volatile production patterns in different species growing on several paper types.

New sensor arrays are continuously being developed and this will lead to better differentiation, regardless of RH [27]. Furthermore, if the aim is to discriminate between the presence or absence of mould, then it is possible to do this regardless of RH. However, RH may influence sensitivity in relation to discrimination at a species level.

A further advance in the detection of moulds in libraries or archives would be a study of the pattern of

volatile compounds originating from communities of paper bio-decomposers at different stages of their development. The profiles of microbial volatile organic compounds (MVOCs) of single microbial species cultured on different building materials have been examined in several studies [28,29], but little is known about production of MVOCs by mixed microbial cultures. Microbial colonisation of materials is a dynamic process in which population composition changes in response to the equilibrium RH of the materials.

This study suggests that, before e-NOSE technology can be applied to the early detection of mould growth in libraries, archives, museums or display cases, more information will be required on the influence of substrata and of environmental parameters on volatile production by fungi. Indeed, before MVOCs analysis could be used as a reliable indicator of microbial contamination in buildings, it will be necessary to identify the volatile organic compounds (VOCs) expected from growth of microbial species and communities frequently found in moisture-damaged libraries and archives, and to verify that these VOCs derive from no other major sources in the buildings.

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