MISES AU POINT PRELIMINAIRES

L'atteinte de l'objectif de thèse passe par le recours à plusieurs outils analytiques de microbiologie moléculaire ayant déjà été éprouvés sur des environnements différents (air extérieur, aérosols de compostage, biogaz…). La collecte d'échantillons en aérosols dans des espaces clos, est soumise à de nombreuses contraintes techniques, qui ont été évoquées dans le premier chapitre de ce manuscrit.

Les étapes de collecte, d'extraction et d'amplification ont fait l'objet de mises au point et d'optimisations par le biais d'études environnementales préliminaires qui sont présentées dans cette partie.

I.1 ELABORATION DES STRATEGIES D'ECHANTILLONNAGE

Cevolet avait pour but de développer une méthodologie d'échantillonnage permettant d'étudier la diversité microbiologique de l'air de différents espaces clos et notamment sa variabilité dans l'espace. Les principaux verrous concernaient l'accessibilité des zones à investiguer, l'acceptabilité par les occupants des métrologies employées et la nécessité de disposer d'une matrice suffisante d'ADN pour permettre les analyses moléculaires.

Dans ce contexte, il a été décidé de procéder à deux types de prélèvement :

- une collecte ponctuelle à haut débit permettant d'étudier la variabilité de la diversité microbienne aéroportée dans le temps,
- une collecte intégrée sur près d'un mois permettant d'accéder à une quantité d'ADN suffisante pour l'étude de la diversité spatiale dans différents espaces clos sans géner les activités qui s'y déroulent.

I.2 ARTICLE 1 : PROCEDURE EXPERIMENTALE OPTIMISEE POUR L'ANALYSE MOLECULAIRE DE L'AIR DE TROIS ENVIRONNEMENTS INTERIEURS.

Une étude a été menée afin de développer une procédure expérimentale optimisée pour l'analyse moléculaire de l'air, appliquée aux environnements intérieurs. Le système choisi devait répondre à plusieurs caractéristiques :

- Le temps de prélèvement devait être suffisamment court pour permettre un suivi dans le temps et pour gêner au minimum les occupants (facilité de mise en œuvre),
- La réalisation des analyses moléculaires dans des conditions optimales (aspect qualitatifs et quantitatifs).

Notre approche s'est divisée en deux parties. Il fut tout d'abord nécessaire de valider les protocoles de collecte pouvant être appliqués dans de tels environnements et pour la technique analytique utilisée. Pour y parvenir, des mesures ont été effectuées dans un environnement où les conditions de ventilation et de circulation des personnes lors de l'analyse, étaient contrôlées et maintenues

constantes. La faisabilité de la méthodologie a ensuite été éprouvée en environnement réel dans un bureau paysager et deux musées.

Les résultats de cette étude sont présentés sous la forme d'un article accepté dans *Clean-Soil, Air, Water*.

RELIABLE PROCEDURE FOR MOLECULAR ANALYSIS OF AIRBORNE MICROFLORA IN THREE INDOOR ENVIRONMENTS: AN OFFICE AND TWO DIFFERENT MUSEUM CONTEXTS.

Molecular analysis of airborne microflora in indoor environments

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Bioaerosols, CE-SSCP, indoor air quality, museum, Q-PCR

Abstract

Biological aerosols from air constitute a significant source of exposure to microorganisms in public places. Airborne microorganisms are involved in the development of certain respiratory symptoms, allergies or infections among users and occupants. Various sampling instruments have commonly been used in aerobiology to collect bacteria and fungi suspended in the air. The objective of this study was to develop a reliable procedure for sampling in indoor public environments presenting different levels of occupancy, airborne bacteria and fungi to be subjected to molecular analysis (Bacteria and Fungi Q-PCR, CE-SSCP fingerprinting). Four different sampling devices were tested *in situ* in an office building (open-plan type) and the sampling strategy chosen was tested in two museum contexts. In accordance with the drawbacks involved to our study (quantitative and qualitative aspects, cost and overcrowding), cyclone device appeared to be most suitable. The results underline the effectiveness of this high-volume aerosol sampling device for both qualitative and quantitative molecular analysis. Four *in situ* sampling collections were carried out in one day in the Louvre Museum to study quantitative and qualitative variations of airborne bacterial and fungal diversity. The quantitative results revealed a similar order of magnitude for the numbers of both bacteria and fungi. In the Louvre Museum, the samples yielded between $3.7x10^4$ and $4.1x10^4$ GE (Genome Equivalent) bacteria/m^3 air and between $5.0x10^4$ and $5.9x10^4$ GE fungi/m^3 air and in the Decorative Arts Museum between, $2.1x10^4$ and $2.5x10^4$ GE bacteria/m^3 air and between $1.4x10^4$ and $1.7x10^4$ GE fungi/m^3 air. The results also indicate that the dominant bacterial community displayed a stable structure over a short period of time whereas dominant eukaryotic airborne community appeared more variable.

1. Introduction

In recent years, the increase of epidemic alerts has led to a reconsideration of the risks of microbial aerosols associated with indoor air quality and of human exposure to various pollutants, including microorganisms [1]. Sites with high occupancy rates (transport systems, public buildings, etc.) appear to be particularly sensitive to these risks. Given its proven impact on both health and buildings, the microbiological quality of air in indoor environments has thus become of increasing interest. Airborne microorganisms can cause respiratory symptoms among occupants and studies have demonstrated their role in the degradation (partial or total) of the substrate they colonize (wood, textiles, paper, pigments, varnish, etc.) [2-4]. Despite the importance of this subject, the microbial content of air has been the object of much less studies than other environments such as soil or water.

Different devices are used for collecting microorganisms from the air and are based on different techniques for recovering the microorganisms (filtration, solid impaction or impingment) [5]. The choice of a particular sampling device will be linked essentially to the type of analysis to be carried out and the environment which is involved. However, factors such as the device's cut-off diameter (d_{50}), the non-multiplication of microorganisms in fluid collections and re-aerosolization are often considered when studying microorganisms from the air. While maintaining the viability of airborne microbes in the collection media is essential for analysis of the cultivable fraction, this is not always so when using molecular tools. Using molecular techniques, the factors which need to be taken into account are mainly the d50 and the larger quantities of air that must be collected within a short period. A short sampling period is also required in order to take into account possible rapid variations

in airborne microbial diversity [6]. The collection of microorganisms using sedimentation methods, which is widely used in combination with culture methods, is not be used with molecular tools. In fact, for the molecular analysis of microbial diversity in aerosol [1, 5, 7, 8], the preferred method of collection is solid impaction or impingment or above all filtration. Because the exposure of humans can only be measured in their presence, drawbacks involved to in situ sampling in public places (noise and overcrowding) must be taken into account so as not to interfere with the occupant's activities.

Only about 1% of environmental microorganisms are currently cultivable [9] and airborne microbes in particular are difficult to cultivate because of the stresses linked to collection methods [1, 7-10]. Moreover, the artificial reproduction of a natural growth medium is all but impossible because of our inability to recreate environmental interaction between microorganisms, temperature, moisture or nutrients for all microorganisms at the same time [5]. Because of these different factors, the quantitative and qualitative measurements of bioaerosols are often underestimated [11] and it is now accepted that non-viable or as-yet-unculturable bacteria can induce allergic reactions, be a source of endotoxins and cause diseases [11]. Molecular tools, although rarely used in such studies, make possible a more exhaustive description of airborne microbiology [12].

In 1995, Alvarez et al. assessed the sensitivity of PCR (Polymerase Chain Reaction) and its susceptibility to environmental interference using Escherichia coli as the target organism in air samples containing environmental bioaerosols [13]. Real-time quantitative Polymerase Chain Reaction (Q-PCR) is now widely applied in medical research and is also used to measure total airborne bacteria in industrial working environments [11, 14, 15] as well as in other enclosed environments such as commercial airline cabins [16, 17].

Some molecular fingerprinting techniques have already been applied to analyzing the community structure of bioaerosols in outdoor air [6, 18], in biogas [19] and at composting sites [20], but not in enclosed spaces (houses, offices, museums, hospitals, schools, etc.). Initial descriptions of airborne microbes have revealed their broad diversity and the presence of all the domains: Bacteria, Eukarya and Archaea [1, 7, 8, 21].

In this context, the objective of the present study was to develop a reliable procedure for molecular analysis of bacterial and fungal diversities, in indoor environments occurring in public areas.

Our approach was divided into two parts. First of all, it was necessary to choose protocols for sampling microbial aerosols that would be suitable for public environments and for the analytical tools to be used. The second part consisted of in situ measurements carried out in three different environments, an office and two different museum contexts, in order to evaluate the preliminary results of qualitative and quantitative variations of airborne bacterial and fungal diversity in such enclosed spaces.

2. Materials and Methods

2.1. In situ *sampling sites*

Three sampling sites appropriate to the field of study were investigated based on gradient of occupancy: an office (type open-plan) and two different museum environments (the Louvre Museum and the Decorative Arts Museum).

The sampling protocols were thus applied in:

- An open-plan office (9.0 x 6.8 x 2.8 m, for a total volume of 170 m^3) (10 people during sampling), located at the Scientific and Technical Centre for Building Research in Marne la Vallée, east of Paris (2.4 km south of the nearest highway).
- The Mona Lisa Room of the Louvre Museum, chosen for its huge size (35 x 15 x 12 m, for a total volume of 6000 m^3) and for its high level of occupancy (1000 people during sampling).
- A storage room in the Decorative Arts Museum in Paris, only accessible to museum staff (4.0 x 8.0 x 2.5 m, for a total volume of 80 m^3) (2 people during sampling).

2.2. Sample collection

Three of the most common sampling techniques were used during this study: impaction (Cyclone device and Atmospheric Dust Captor (CAP), impingement (BioSampler SKC Impinger) and filtration (experimental filter device using synthetic polyester filter). The operating conditions and characteristics of the biocollectors are summarized in Table 13. The devices were tested under standard sampling conditions. All samples were collected *in situ* in the open-plan office. The biocollectors were started up simultaneously. They were deployed as follow:

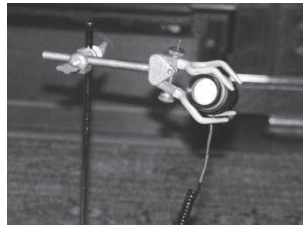
a. An experimental bioaerosol collector (a cyclone device) previously used for the *in situ* sampling of airborne *Legionella* spp. [22] and developed by our laboratory (CSTB). The aerosols penetrate the sampling device tangentially to the wall of the cyclone device where they impact. The wall is washed constantly with a molecular biology-grade water flow circulating in a closed loop in order to concentrate the aerosol particles collected. The cut-off diameter (d_{50}) of the experimental cyclone device is 0.5 µm. Sampling was performed for 30 min under controlled airflow conditions of 1000 L/min, sample 30 m^3 of air in 100 mL of molecular biology-grade water.

b. An SKC Impinger BioSampler (BioSampler), based on the principle of impingement (using sterile molecular biology-grade water), with airflow of 12.5 L/min and a cut-off diameter of 0.3 µm. Sampling is performed for 30 min. A total volume of 0.375 m^3 air is collected.

c. An Atmospheric Dust Captor, Arelco (CAP), coupled to a sampling head (d_{50}< 100 µm) with polyurethane foam, with a collection rate of 10 L/min. In this case, a 5 h of collection period was necessary to sample 3 m^3 air.

d. An experimental filter device developed by the CSTB, with a flow-rate of 80 L/min and used for 5 h to process a volume of 24 m^3 air. Collection was done with a synthetic polyester filter [23].

Table 13 : Synopsis of operating conditions for aerosol sampling.

Collection principle		Solid impaction	Impingment		Filtration
		Arelco CAP	SKC Biosampler	Cyclone-like device	Filtration device
Devices					
Sampling flow rates		10 L/min	12.5 L/min	1000 L/min	80 L/min
Collection time		5 h	15 min	30 min	5 h
Collection substrate		Polyurethane foam	Molecular biology grade water	Molecular biology grade water	Synthetic polyester filter
Analytical methods	**Validation of collection devices (open-plan office)**	Bacterial and fungal Q-PCR Bacterial CE-SSCP	Bacterial and fungal Q-PCR Bacterial CE-SSCP	Bacterial and fungal Q-PCR Bacterial CE-SSCP	Bacterial and fungal Q-PCR
	Applications to microbial communities in two museums			Bacterial and fungal Q-PCR Bacterial and eukaryotic CE-SSCP	

2.3. Sample preparation

Before concentration on a filter, all polyester filters and polyurethane foams were rinsed five times with sterile water up to a volume of 100mL.

The samples were concentrated by filtering the solution through a 47 mm, 0.2 µm polyestersulfone sterile filter (Supor 200, Pall Corporation, USA). The filter was then cut using a sterile scalpel and each half-filter was placed in a 1.5 mL sterile tube and frozen at -80°C.

2.4. Extraction and purification of total genomic DNA

DNA extraction was performed directly on the filter using the protocol described by Moletta [19] but slightly modified. The filter was ground to a powder using a sterile plastic stick while being kept on dry ice. The main steps of the DNA extraction and purification are detailed below.

Volumes of 380 µL 4 M guanidine thiocyanate in 0.1 M Tris–HCl (pH 7.5) and 120 µL 10% N-lauroylsarcosine were added to the ground filter which was frozen at -20°C. After, a volume of 500 µL 5% N-lauroylsarcosine in 0.1 M phosphate buffer (pH 8) was added to the frozen filter and the tube was immediately incubated at 95°C for 10 min. After the thermal treatment, the samples were placed in a vibroshaker with 0.1 mm zirconium beads. Polyvinylpolypyrrolidone was then added to remove any inhibiting substances. DNA was precipitated using isopropanol. Total DNA was then purified using a QiaAmp DNA microkit (Qiagen, Hilden, Germany) and collected in 50 µL DNA-free water. After extraction, the DNA was stored at -80°C. A field control was carried out using molecular biology-grade water for each of the sampling devices and these checked by passing through all extraction and amplification stages.

2.5. Real-time PCR (Polymerase Chain Reaction) amplification and quantification

All PCR assays were performed on a RotorGene 3000 (Corbett Research, Australia). The calibration curve was generated using RotorGene software, version 6.0.

During this study, two different real-time PCR systems were used to assess the concentrations of bacteria and fungi in indoor air. They were all based on the 16S ribosomal RNA gene sequences and 18S ribosomal RNA gene sequences. All primers and the TaqMan® probe were synthesized by Sigma Proligo (Paris, France). The cycle threshold (CT) was calculated as the cycle number at which the reaction became exponential. The cycle threshold of each sample was then compared to a standard curve and the result was expressed as a numerical value of the number of target genomes in the sample. The standard curves were generated by amplifying serial 5-fold dilutions of the total genomic DNA of *Escherichia coli* and *Aspergillus fumigatus*. Concentrations of 4.13×10^7 Genome Equivalent (GE) bacteria/µL and 2.08×10^6 GE fungi/µL of the total genomic DNA solution were determined using the Genequant Pro (Amersham Biosciences). Each standard was tested in triplicate. The current specificity of all real-time amplification systems already published was checked in silico using Probematch software from the ARB database and NCBI Blast [24] against all 16S rRNA gene sequences and 18S rRNA gene sequences available in RDP and/or Genbank.

2.5.1. Evaluation of total bacterial load

A 441 bp (base pair) fragment of the bacterial 16S rRNA gene sequence was amplified with bacterial-directed primers and a probe set (see details in Table 14) [25]. PCR was performed using the Quantitect Probe PCR kit (Qiagen) with 12.5 µL of Master Mix, 0.25 µL of forward primer, 0.25 µl of reverse primer and 0.25 µL of probe, with water added to a final volume of 20 µl. 5 µL of the sample were added to PCR mix. The amplification was carried out under the following conditions: 95°C for 15 min, then 50 cycles at 94°C for 15 s, 60°C for 60 s and 72°C for 20 s. No-template controls with molecular biology-grade water rather than DNA were included in each run.

Serial dilutions of *Escherichia coli* (DSMZ 5695, ATCC 12435) DNA were used as the standard to determine bacterial concentrations with real-time PCR. All samples were included in triplicate in each run. The reaction efficiency of the standard curve for bacterial load evaluations was 0.83 and the correlation coefficient R^2>0.99.

2.5.2. Evaluation of total fungal load

A 379 bp fragment of the fungal 18S rDNA was amplified with fungi-directed primers (see details in Table 14). PCR was performed using the Quantitect Sybr Green PCR kit (Qiagen) with 12.5 µL of Master Mix, 2.25 µL of forward primer and 2.25 µL of reverse primer and water being added to a final volume of 20 µL. 5 µL of the sample were added to the PCR mix. Amplification was carried out under the following conditions: 95°C for 15 min, then 45 cycles at 94°C for 15 s, 52°C for 30 s and 72°C for 45 s. No-template controls with molecular biology-grade water rather than DNA were included in each run.

The Q-PCR system used in this study can amplify the 18S rRNA of the four major fungal phyla: *Ascomycetes*, *Basidiomycetes*, *Zygomycetes* and *Chytridomycetes* [26].

Serial dilutions of the DNA of *Aspergillus fumigatus* (Institut d'Hygiène et d'Epidémiologie de Bruxelles – Mycology Section) were used as the standard to determine fungal concentrations with real-time PCR. All samples were included in triplicate in each run. The reaction efficiency of the standard curve for fungal load evaluations was 0.78 and the correlation coefficient R^2>0.99.

2.5.3. Limits of quantification

The limit of quantification was defined using the lowest concentration of the standard within the linear range at which 95% of PCR results were positive. The limit of quantification of the number of GE bacteria or GE fungi per cubic meter of collected air was then calculated for each sample using these values, taking into account of the equivalent volume of air used for DNA extraction and the dilution factor applied prior to Q-PCR. In the case of bacteria, the limit of detection was $8.8x10^2$ GE/m^3 of air. For the fungal system, the limit of detection was $2x10^2$ GE/m^3 of air and was given by the higher dilution of the standard in the amplified range. A lower limit of detection values was found for fungi than for bacteria, mainly because of the presence in Q-PCR reagents of contaminating 16S rRNA gene sequences arising from the manufacturing process [27]. The dispersion of values was measured for each system based on an analysis of the Q-PCR standards conducted in triplicate, and were found to be those typical in the quantitative experiment; i.e. between 8% and 30%, depending on the collection system. The dispersion observed for Q-PCR triplicate analyses was 3%.

2.6. CE-SSCP (Capillary Electrophoresis Single Strand Conformation Polymorphism) analysis and structure of the microbial community

A nested PCR assay was used to amplify 16S ribosomal fragments of the microbial aerosols with two sets of primers for the bacteria, according the protocol developed by Moletta [19].

2.6.1. First PCR amplification

The first step involved whole bacterial ribosomal DNA and the second PCR produced fragments for CE-SSCP analysis (of about 250 bp in size). The first set of bacterial primers consisted of w18 and w02 primers [28] which amplified whole the 16S rRNA gene sequence. Each tube contained 5 µL of extracted DNA and a PCR mix containing 2.5 units of polymerase Amplitaq Gold LD (Applied Biosystems, Foster City, California), 5 µL Amplitaq Gold LD buffer 10X, 2.5 mM $MgCL_2$, 200 µM dNTPs at 2.5 mM and 200 ng of each primer, adjusted to a total volume of 50 µL molecular biology-grade water. The thermal cycling steps used to amplify bacterial ribosomal RNA genes were as follows: incubation at 95 °C for 10 min and then 20 cycles of incubation at 95°C for 30 s, 50°C for 30 s and 72°C for 1 min.

2.6.2. Second PCR amplification

Five microliters of total DNA or 5 µL of the first PCR were used for each PCR-SSCP amplification. B22 and B23* [29] were used to amplify the V3 16S rDNA bacterial region. The eukaryotic primers used for 18S V7 ribosomal DNA were E04 and E12* (see details in Table 14) [30]. The PCR-SSCP amplification mix contained 1.25 units of *Pfu Turbo* (Stratagene, La Jolla, California), 5 µL of 10X buffer, 200 µM dNTPs, 130 ng of each primer, with water added to a final volume of 50 µL. The thermal profile used for the amplification of ribosomal RNA genes was as follows : incubation at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 30 s, 61°C for 30 s and 72°C for 30 s, and a final elongation of 10 min at 72°C. The same thermal amplification profile was used for Eukaryota, except that the annealing temperature was 51°C.

2.6.3. CE-SSCP analysis

Global microbial communities were studied using CE-SSCP analysis, performed with ABI310 (Applied Biosystems) on all samples with a 1 µL of extracted DNA, in accordance with previously described CE-SSCP amplification methods [31].

2.7. Diversity index

Simpson's diversity index (also known as the species diversity index) is one of the many methods used to measure diversity. In ecology, it is often employed to quantify the biodiversity of a habitat. It takes into account both the number of species present and their relative abundance. Simpson's index measures the probability that two randomly-selected subjects will belong to the same species. This index ranges from 0 to 1, with 1 indicating higher diversity [32]. This index was calculated for all the samples analyzed by CE-SSCP fingerprinting, using StatFingerprints [33-35].

Table 14 : Details of the different primers and probes used for molecular analysis.

Names	Types	Target	Sequences	Positions	References
Real-time Q-PCR					
B01	Primer	Bacteria	5'-TCCTACGGGAGGCAGCAGT-3'	F331-349	[25]
B02	Primer	Bacteria	5'-GGACTACCAGGGTATCTAATCCTGTT-3'	R772-797R	[25]
B14	Probe	Bacteria	5'-CGTATTACCGCGGCTGCTGGCAC-3'	506-528	[25]
E07	Primer	Fungi	5'-TTAGCATGGAATAATRRAATAGGA-3'	F817	[26]
E08	Primer	Fungi	5'-TCTGGACCTGGTGAGTTTCC-3'	R1196	[26]
PCR-SSCP					
B22	Primer	Bacteria	5'-ACGGTCCAGACTCCTACGGG-3'	F329-348	[29]
B23	Probe	Bacteria	5'-TTACCGCGCTGCTGGCAC-3'	R515-533	[29]
E04	Primer	Eukaryotes	5'-CTTAATTTGACTCAACACGG-3'	F955	[28]
E12	Probe	Eukaryotes	5'-GGGCATCACAGACCTGTT-3'	R1195	[30]

Table 15 : Results of the validation of collection devices and of amplification methods for CE-SSCP analysis.

Devices	Volume of air collected (m^3)	Q-PCR		CE-SSCP	
		Bacteria	Fungi	Single PCR	Nested PCR
Cyclone	**30**	+	+	+	+
SKC	**0.3**	-	-	-	-
CAP	**3**	-	+	-	+
Filtration	**24**	+	+	-	+

3. Results and Discussion

3.1. Quantitative and qualitative analyses of microbial diversity in indoor air

Four collection systems (SKC, Cyclone device, Filtration device and CAP) were used under real-life conditions to verify their suitability for the study of occupant's exposure to airborne bacterial and fungal microflora in association with the analytical techniques chosen. The devices were placed in an open-plan office and all samplings were started up simultaneously. The sampling times were adjusted to the constraints of each system in order to respect the standard sampling conditions.

3.1.1. Suitability of the collection devices: quantitative analysis

In order to quantify the total microbial load in air, Q-PCR was employed. Of the four collection devices tested, two yielded positive results by Q-PCR for both bacterial and fungal populations: the cyclone device and the experimental filter device (Table 15).

The use of an impinger appeared to be inadequate for the molecular analysis of samples collected from the indoor air using our assays. Indeed, results of all quantification assays were below the limit of detection (Table 15). The broad range of particle size, and the possible filter saturation due to different principles of collecting and longer sampling times (30 min with the cyclone device and 5 h with the filtration system) could explain these variations. The CAP protocol produced positive results for regarding the quantification of airborne fungi but not bacteria (below the limits of detection of the assays employed).

In the light of these quantitative results, both collectors (cyclone device and experimental filter device) appear to be suitable for the simultaneous measurement of bacteria and fungi in air under our test conditions. The SKC and CAP devices had the lowest flow-rates and volume of samplings. These observations might explain the negative results obtained with these two collectors. The sampling time may not have been sufficient to collect a large amount of DNA required to carry out all molecular analysis in optimal conditions. A longer sampling time was considered but this would not have conformed to the standard use of the devices. The cyclone permitted a shorter sampling time (30 min in contrast to 5 h with the filter) for an equivalent volume of sampled air. It was therefore selected for *in situ* measurements of microbial load.

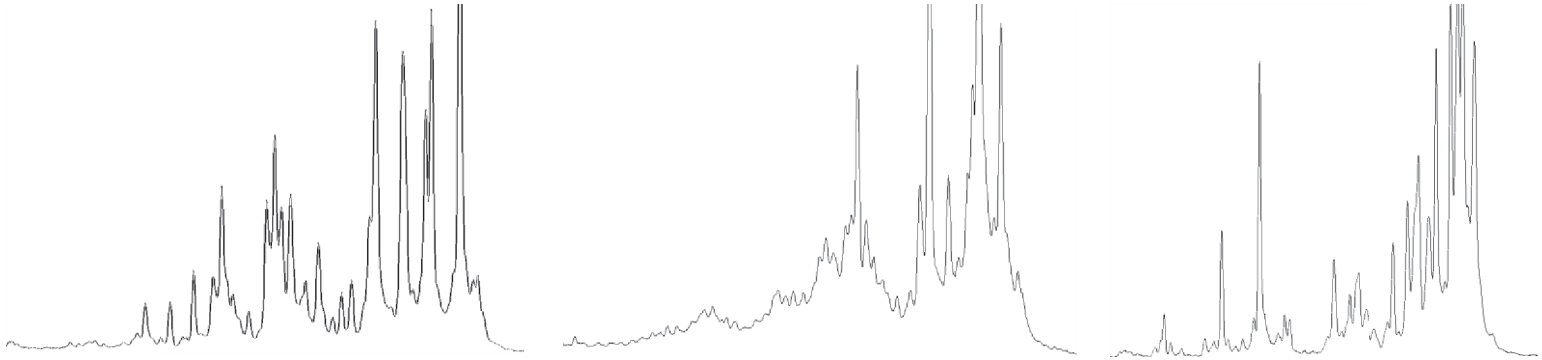
3.1.2. Suitability of the collection devices: qualitative analysis

CE-SSCP is clearly a powerful tool to measuring and comparing microbial diversity in complex ecosystems. In fact, it has been applied elsewhere to the diversity of microbial aerosols from biogas [19] and composting sites [20]. In order to assess microbial diversity in air, a single PCR-SSCP amplification was applied to the samples obtained using the different collectors.

Only samples obtained using the cyclone device permitted obtaining a profile after a single step amplification of 30 cycles. In the light of these results (Table 15), for samples from the three other collectors, a second amplification step was carried out, in accordance with the protocol described by Moletta [19], as applied to biogas samples. Using this method, the sensitivity and, thus, the detection threshold was improved. However, the risk of nonspecific amplifications also increased. To prevent this, no-template nested PCR-SSCP samples were analysed and were subsequently verified by CE-SSCP to be different of the air samples. Positive single PCR-SSCP and nested PCR-SSCP results were

obtained with cyclone device sampling. CAP and filtration samplings produced positive results only using nested PCR (Table 15).

CE-SSCP analysis of the positive results revealed complex patterns, and differences in the structure of the bacterial community were observed as a function of each sampling device (Figure 23). Figure 24 shows the comparison of results obtained with direct PCR-SSCP and nested PCR-SSCP, illustrating how an identical sample could differ if it were treated using a different number of amplification cycles (30 or 50 amplification cycles, respectively). Thus nested PCR-SSCP profiles display supplementary peaks when compared to direct PCR-SSCP profiles. Each sampling device has its own technical characteristics (including collection efficiency and the range of particle size) which could partially explain these differences in CE-SSCP patterns. Indeed, different studies on the performance of bioaerosol sampling devices have shown that particle diameter and type of collection techniques (solid impaction, liquid impaction or filtration) can affect collection efficiency [36, 37]. Other parameters, such as the duration of collection, may also create differences in sampling collection [38, 39]. Under our assay conditions, the cyclone device proved to be the most quantitatively reliable and was thus used during the subsequent phases of our study.



a. CAP **b. Cyclone device** **c. Filtration device**

Figure 23 : Bacterial CE-SSCP profiles obtained with a nested PCR.

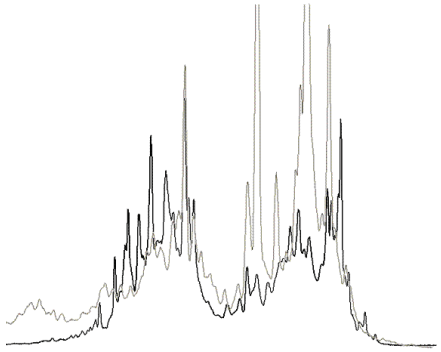


Figure 24 : Comparison of two bacterial CE-SSCP profiles of bioaerosols collected using the cyclone device: Single PCR (black) and nested PCR (grey).

3.2. Application to airborne microbial communities in two museums

The microbial diversity prevailing in the Louvre and Decorative Arts Museums was determined using a cyclone device and analyzed with Q-PCR and CE-SSCP fingerprinting. The aim was to develop this protocol for in situ sampling in such special environments, bearing in mind aspects of public acceptability, sample collection and transport, and to evaluate foremost elements of variability in the structure of airborne microbial diversity found in these enclosed spaces. The collection device was placed in the middle of the room and was accepted by the public despite the fact that it generated some noise which was, however, partially masked by the ambient noise. Q-PCR assays were performed on the four samples collected. All quantitative PCR results are presented with the concentration obtained in GE per cubic meter of air as a function of the collection devices. The quantitative results revealed a similar order of magnitude for the counts of both bacteria and fungi. Indeed, the samples yielded between $3.7x10^4$ and $4.1x10^4$ GE bacteria/m^3 air and between $5.0x10^4$ and $5.9x10^4$ GE fungi/m^3 air in the Louvre Museum and between $2.1x10^4$ and $2.5x10^4$ GE bacteria/m^3 air and between $1.4x10^4$ and $1.7x10^4$ GE fungi/m^3 in the Decorative Arts Museum. Unfortunately, no quantification data obtained using Q-PCR is available in the scientific literature regarding the air in this type of indoor environment, thus preventing a direct comparison of the results.

However, the bacterial and fungal concentrations found in the museums were compared with recent data on the Q-PCR microbial loads determined on air samples from other indoor environments such as poultry houses, commercial airliner cabins and domestic aircraft cabins. In these locations, high concentrations of bacteria were revealed by Q-PCR: values of between $7.7x10^7$ and $1.3x10^9$ cells/m^3 air were monitored for total bacteria in a poultry house using the same bacterial Q-PCR TaqMan system [15]. With regard to flight cabins, the measured values ranged from 10^6 to 10^7 16S rRNA gene copies/m^3 [16, 17]. The results in the literature were expressed as 16S rRNA gene copies/m^3, considering a value of 4.1 16S rRNA gene copies per genome or cells [40]. In outdoor air, several measurements have been made, particularly in waste treatment environments such as sewage plants [11] and anaerobic digestion waste treatment sites [14]. In these two outdoor environments, microflora concentrations in the air ranged from $2.8x10^5$ to $3.2x10^6$ cells/m^3 and 10^5 to 10^6 GE bacteria/m^3 of air, respectively. The airborne microbial concentrations found in the museums were lower than those recorded in other indoor environments such as flight cabins or poultry houses. Higher microbial loads were also observed in outdoor air. Thus, our results justify the use of a high-volume biocollector in order to optimize the initial quantity of DNA matrix.

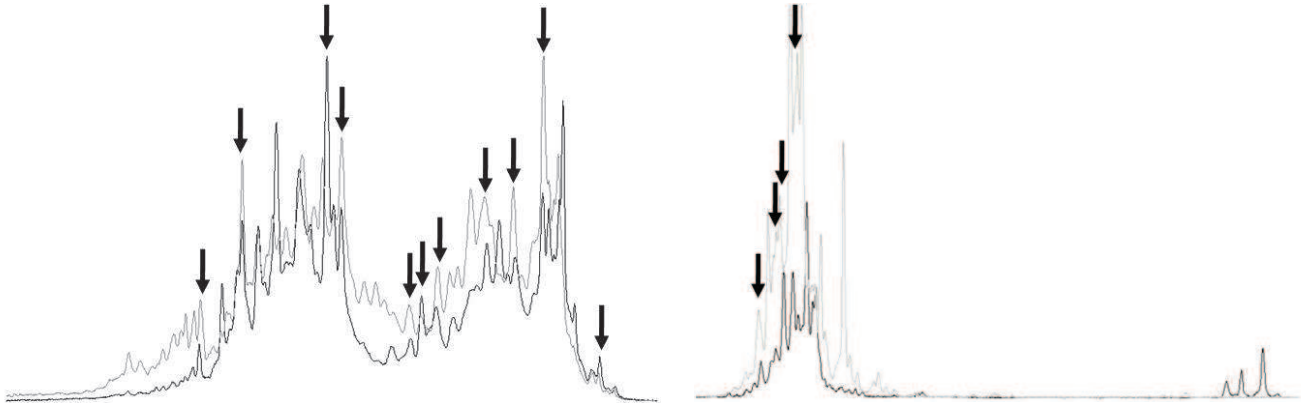
These results can be compared to other measurements obtained using standard microbiological counting methods applied to samples from other European museums. Concentrations of between 10^2 and 10^3 CFU/m^3 for bacteria and 10 and 10^2 CFU/m^3 for fungi have been measured in various museums [41-43]. These values for the cultivable bacterial and fungal fraction of air represent a difference some 1 to 3 logarithms lower than the values obtained using molecular tools. Culture methods have been shown to be time-consuming and to produce an incomplete picture of microbial diversity because certain microbes are preferentially cultivated, such as the particularly resistant species as *Staphylococcus* spp. [5]. In other indoor environments such as dwellings, schools, homes or hospitals, values of between 10 and 10^4 CFU/m^3 have been obtained [44-47]. Using cultivation methods, the indoor airborne concentrations found in museums were similar to those frequently observed in other indoor environments.

In order to assess temporal variations in microbial concentrations, three samples were collected in the Mona Lisa Room of the Louvre Museum every 45 min. The dispersion of fungal Q-PCR values was evaluated at 130%, while that of bacterial Q-PCR values was 27%. We hypothesize that this dispersion may have been linked to the diameter of fungal particles (larger than bacteria) so that fungal aerosols were more subject to variations in the aerodynamics of the room (occupancy, ventilation, etc.). The dominant diversity of bacteria and eukaryotes was studied using CE-SSCP on air from the two museums; the four samples collected produced positive results with PCR-SSCP. The CE-SSCP profiles provided a fingerprint of bacterial and eukaryotic dominant microflora. The profiles revealed a high level of bacterial diversity in indoor air (more than 15 peaks). They also indicated that the dominant bacterial community displayed a stable structure over a short period of time (45 min between each sampling). This means that the molecular analysis was reproducible and, also, that the dominant bacterial community did not vary qualitatively during this period. The CE-SSCP analysis highlighted structural differences in airborne bacterial diversity related to the indoor environments investigated, although it also revealed several common peaks for bacterial profiles (Figure 25). In contrast, dominant eukaryotic community structures exhibited more pronounced differences, with a limited number of common peaks.

One interesting point was highlighted by a comparison of the CE-SSCP patterns obtained in the museums with those from the open-plan office. The bacterial profiles were similar in the museums (Figure 25) but they differed from those found in the office area (Figure 26). The museum profiles were more complex than those from the office (an average of 25 peaks in museums and 15 peaks in the office). Dominant eukaryotic communities displayed different profiles for all the sites studied.

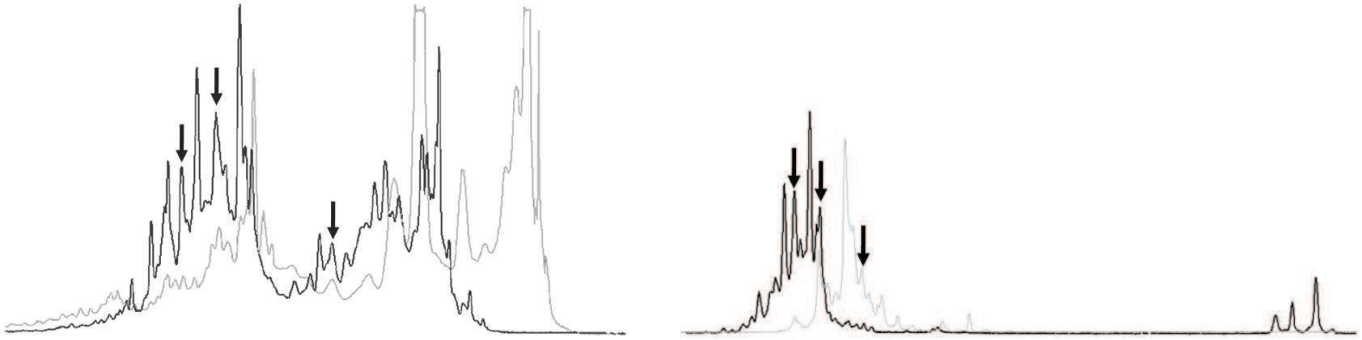
Calculation of the Simpson index revealed a significant diversity within the three sites studied, in terms of both bacterial and eukaryotic diversity. Indeed, values higher than 0.99 were obtained in all cases. By way of comparison, similar values were observed in aerosol samples obtained during different steps of composting (between 0.97 and 0.99) [48], but weaker results (although reflecting a wide diversity) were recorded for samples from a bacterial biofilm in a hot water network : their associated CE-SSCP profiles displayed values ranging from 0.86 to 0.92 [48]. Each indoor environment has specific sources (humans, animals, building and decorative materials…) and, in addition, outdoor air may enter indoors via the ventilation system or doors and windows. All these possible sources could impact bacterial and fungal content of indoor air [49].

The entire protocol (sampling and analysis stages) was found to be satisfactory for assessing the exposure of occupants to airborne microorganisms. The inevitable noise generated by the cyclone device was to a large degree covered up by the ambient noise generated by the visiting public. The collection step required less time than usual; indeed, an initial analysis was done within only two days, thus reducing the overall costs of the study.



a. Bacterial diversity b. Eukaryotic diversity

Figure 25 : Bacterial and eukaryotic CE-SSCP profiles of bioaerosols collected in two museums: the Louvre Museum (black) and the Decorative Arts Museum (grey). Black arrows highlight major common peaks.



a. Bacterial diversity b. Eukaryotic diversity

Figure 26 : Bacterial and eukaryotic CE-SSCP profiles of bioaerosols collected in the open-plan office (grey) and in the Louvre Museum (black). Black arrows highlight major common peaks.

4. Concluding remarks

The principal aim of this research was to develop a reliable procedure for sampling in indoor public environments presenting different levels of occupancy, airborne bacteria and fungi to be subjected to quantitative and qualitative molecular analysis (bacteria and fungi Q-PCR, CE-SSCP fingerprinting). This study has resulted in the development of a methodology that integrates the sampling of airborne bacterial and fungal diversity in indoor air with its subsequent molecular analysis. Based on criteria defined in our study relating to the analysis of the airborne microbes (quantitative and qualitative aspects, cost, and ease of implementation), cyclonic device appeared to be the most efficient. Sampling device with higher flow-rates will be necessary to carrying out molecular analysis under optimum conditions and for studying the dynamics of airborne microbial diversity at the different scale of time (minimum 45 minutes).

For the first time, the variations of micro-organisms present in the air of museums have been studied quantitatively and qualitatively using molecular techniques. Initial results in the two enclosed spaces reveal the relevance of our integrated analytical process (sampling with cyclone device and quantitative and qualitative molecular analysis) for the study of spatial and temporal variations in eukaryotic and bacterial microflora. Our initial results reveal differences between the eukaryotic and bacterial microflora in the two museums, in both quantity and quality. CE-SSCP was able to highlight more pronounced disparities in the dominant bacteria community between the museums and the office area. Based on the DNA extracted by this procedure, molecular analysis can be done for a range of purpose: quantifying total bacteria, fingerprinting for comparisons and measures of microbial diversity as presented in this study. Molecular analysis constitute a powerful tool for describing the airborne microbial diversity prevailing in such indoor areas and for assessing the exposure of their occupants, particular to pathogen related species. Longer sampling campaigns are now being considered in order to obtain data relative to the temporal and spatial diversity of indoor environments. Molecular analysis may provide a new tool in the search for sources of indoor air contamination and in determining the role of air in the transmission of pathogens. These tools could be used to manage air quality indoors and to define specific indicators for microbial quality of air in enclosed spaces.

Acknowledgements

The authors wish to thank all those who contributed to this study and particularly the team from the "Laboratoire de Recherche des Monuments Historiques", Mrs Joëlle Leroux from the Louvre Museum and Mr Maximilien Durant from the Musée des Arts Décoratifs.

They also gratefully acknowledge Valérie Bru-Adan and Anaïs Bonnafous who made a very helpful contribution to the CE-SSCP analysis.

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I.3 ECHANTILLONNAGE INTEGRE

I.3.A Caractéristiques générales

Le recours à un prélèvement intégré se justifie par une meilleure facilité de mise en œuvre (le système est placé sur la gaine de la Centrale de Traitement d'Air (CTA)), une durée de prélèvement plus longue et une totale discrétion. En outre, ce type de système a déjà été mis en œuvre dans d'autres espaces clos (Tringe *et al*., 2008) pour des études ayant recours à des approches indépendantes de la culture. Le principe de collecte retenu a donc été la filtration. En effet, l'étude précédente (§ 1.2) a mis en évidence l'efficacité de ce type de prélèvement.

Le média utilisé est un filtre plan en cellulose (CAMFIL FARR) de type HEPA 13 décontaminé par autoclavage avant d'être mis en œuvre.

Le dispositif est constitué principalement :

- d'un ventilateur en ligne VC200 de marque ALDES fonctionnant à un débit nominal de 1000 m^3/h,
- d'un porte filtre, usiné spécifiquement, qui permet de positionner de façon étanche le filtre plan de 200 mm de diamètre

Le débit de prélèvement effectif du système est de 6,3 m^3/h correspondant à une vitesse frontale de 5,3 cm/s avec une perte de charge de 363 Pa. Des mesures, en continu, de la ΔP et du débit sont réalisées avec un manomètre CP200 (KIMO) et un anémomètre CTV200 (KIMO). La durée de prélèvement est fixée à 4 semaines correspondant à un volume d'air de 4 233 m^3.

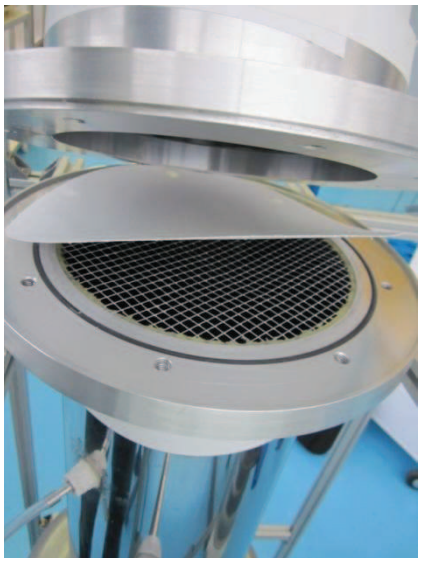

Figure 27 : Photographie du porte filtre avec média filtrant.

Une vérification de l'efficacité de rétention des particules de l'ambiance par leur mesure en amont et en aval du média filtrant de ce dispositif, a été effectuée. Comme attendu, près de 95% des

particules de taille comprise entre 0,3 et 0,4 µm, sont retenues par les filtres neufs ainsi que par les filtres préalablement décontaminés (autoclavés à 120°C pendant 20 minutes).

Nous avons fait le choix de ponctionner l'air directement au niveau de la CTA et plus particulièrement sur la reprise d'air des sites investigués, le réseau assurant, dès lors, le captage et le transport des aérosols vers le dispositif de collecte (Figure 27).

I.3.B Vérification des débits de collecte et de la pression du système de collecte intégré

Le débit de collecte ainsi que la pression est mesurée en continu à l'aide d'une sonde KIMO pendant 4 semaines. Les résultats montrent un maintien du débit de collecte durant les 4 semaines et l'absence d'une diminution de pression durant le prélèvement. Donc, il n'est pas mis en évidence de dégradation du filtre ou de perte de charge durant le temps de collecte.

I.3.C Comparaison prélèvement intégré – prélèvement ponctuel

Différentes vérifications analytiques ont été nécessaires afin de s'assurer de la pertinence d'un système de prélèvement intégré branché sur une centrale de traitement d'air. En effet, l'air investigué lors d'un prélèvement ponctuel dans l'ambiance d'une pièce, est-il équivalent à celui retrouvé dans la gaine aéraulique ?

I.3.C.a *Comptages particulaires et granulométrie*

Deux Compteurs Optiques de Particules Grimm (COP) calibrés ont été placés dans l'enceinte du bureau paysager et au niveau de gaine aéraulique du bureau en présence et en absence d'occupants (respectivement, les 16 et 17 avril 2010) (Figure 28).

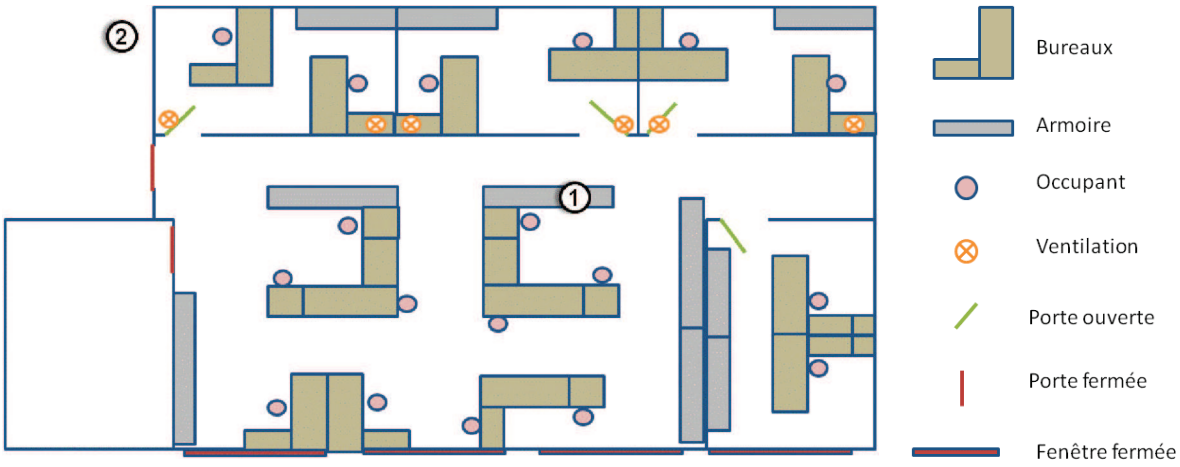

Figure 28 : Schématisation du bureau paysager investigué et positionnement du Compteur Optique de Particules (COP) dans le volume (position 1) et dans la gaine (position 2).

Les vérifications effectuées ont mis en évidence des concentrations en aérosols dans la gaine de reprise comparables à celles de l'ambiance des bureaux (Figure 29).

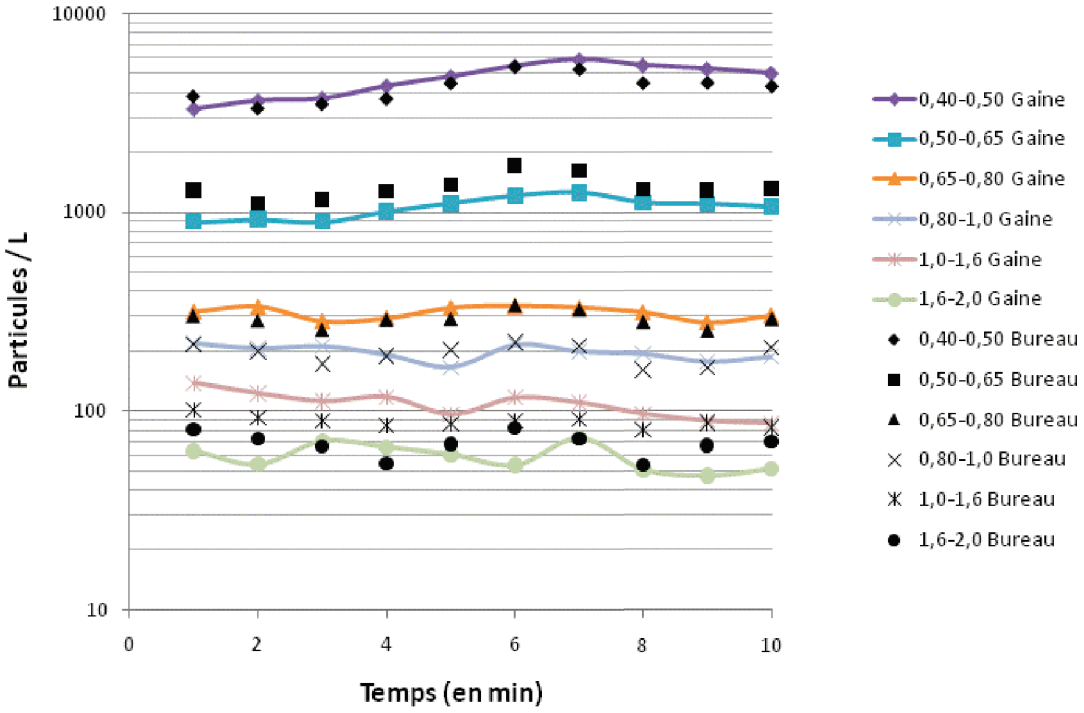

Figure 29 : Suivi de la concentration particulaire par domaine de taille dans le bureau et dans la gaine de reprise d'air.

Entre 93,5 et 94,0% des particules de la gaine et entre 86,4 et 92,4% des particules du volume ont été mesurées dans les deux premiers canaux du COP (diamètre optique des particules entre 0,3 et 0,5 µm). Entre 4,6 et 4,7% des particules de la gaine et entre 5,6 et 9,0% des particules du volume ont été mesurées dans les canaux 3 et 4 du COP (diamètre optique des particules entre 0,5 et 0,8 µm). Moins de 1% de particules observées par le COP étaient entre 2 et 5 µm que ce soit dans la gaine ou dans le volume. Les concentrations totales en particules aéroportées au sein de la gaine variaient entre $7{,}02.10^{9}$ et $1{,}35.10^{10}$ particules/m^3 alors qu'au sein du volume elles étaient comprises entre $2{,}31.10^{9}$ et $6{,}02.10^{9}$ particules/m^3 sur une mesure de 4,5 heures.

La Figure 29 décrit la dispersion granulométrique des particules retrouvées dans la gaine et dans le volume du bureau.

La comparaison deux à deux des différents échantillons montre qu'il n'y a pas de différences significatives entre les 4 profils. En effet, l'ensemble des *p-value* des tests de Wilcoxon est supérieur à 0,05 (p>0,74).

Le COP a montré des profils similaires pour chaque jour d'échantillonnage dans la gaine et dans le volume que ce soit en présence ou en absence d'occupants. On observe notamment la présence d'un mode à un diamètre optique de 2,5 µm correspondant au « mode grossier », dans la gaine et dans le volume (Figure 30).

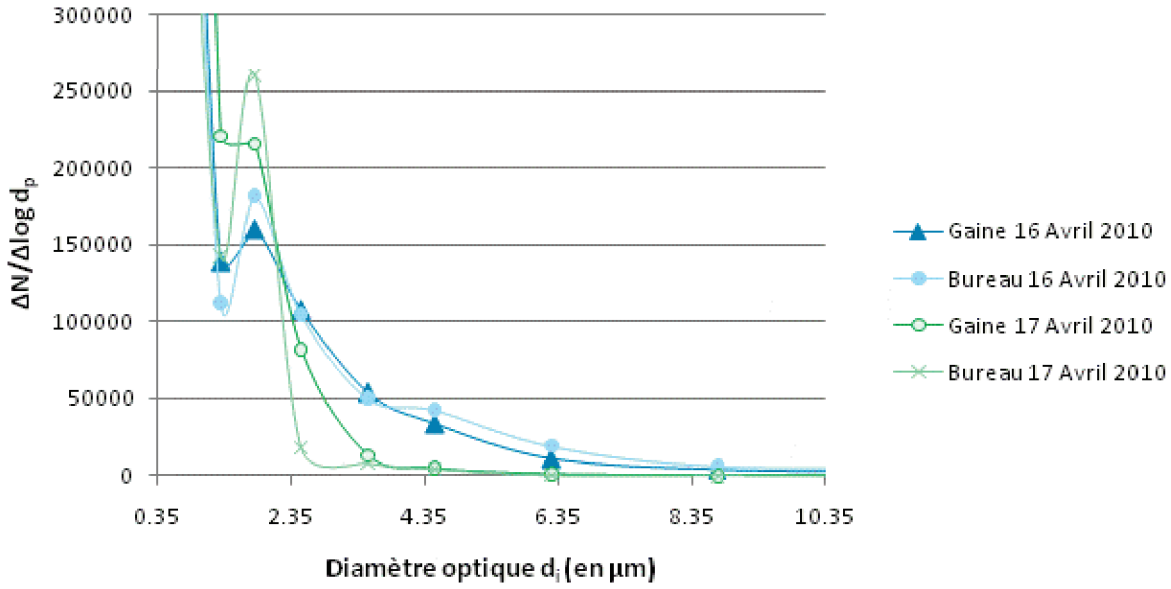

Figure 30 : Profils granulométriques obtenus à partir des mesures faites dans la gaine et dans l'enceinte du bureau les 16 et 17 avril 2010.

I.3.C.b *Diversité bactérienne*

La même démarche a été appliquée sur des prélèvements biologiques afin de s'assurer de la pertinence d'utiliser un système intégré pour l'étude de la diversité microbienne des espaces clos. Pour cela, deux prélèvements ont été effectués : l'un réalisé dans le volume de la pièce au moyen d'un prélèvement cyclonique et le second, au niveau de la gaine aéraulique transportant l'air de la pièce, par le biais du système intégré développé (Figure 31).

A. B.

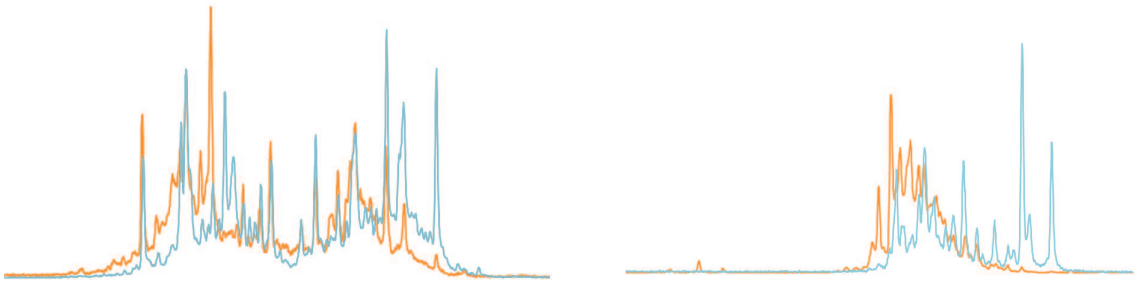

Figure 31 : Profils CE-SSCP obtenus à partir d'un prélèvement cyclonique dans l'enceinte de la pièce (en bleu) et d'un prélèvement intégré dans la gaine aéraulique (en orange). A. Diversité bactérienne globale observée par CE-SSCP. B. Diversité eucaryote globale observée par CE-SSCP.

Les deux profils CE-SSCP Bactéries obtenus sont proches. La similarité mesurée à l'aide du logiciel StatFingerprints indique 97% de similarité entre les deux structures de communauté bactérienne. Pour les eucaryotes, 96% de similarité entre les structures des communautés eucaryotes sont

obtenus pour les deux types de prélèvements. Des valeurs de 98% de similarité ont été observées à partir de deux réplicats d'extraction d'un même prélèvement.

Les résultats obtenus permettent d'envisager une analyse de la microbiologie de l'air pertinente et réaliste par le biais d'un système intégré. Celui-ci a donc été employé pour différents sites d'études : un hôpital, un bureau et un musée.

I.3.C.c *Evaluation de la stabilité du filtre lors d'un prélèvement intégré de quatre semaines*

Dans le cadre de l'étude globale qui vise à déterminer la diversité microbienne de l'air de différents espaces clos sur une période donnée, un système de filtration intégré a été choisi et conçu par le CSTB. Ce type de système permet, entre autre, de concentrer suffisamment de matériel biologique pour des analyses de séquençage très haut débit. Le but de ces expérimentations sera à terme de réaliser un important inventaire moléculaire.

Les filtres HEPA mis en place lors des prélèvements sont utilisés pour la détermination des microorganismes présents dans l'air et ne doivent donc pas interférer sur la dynamique des environnements microbiens prélevés. C'est pourquoi, l'évolution des filtres a été analysée sur le long terme. Pour cela, une étude de la diversité microbienne a été effectuée toutes les semaines par CE-SSCP afin de déterminer la dynamique biologique à la surface du filtre lors d'un prélèvement intégré de quatre semaines.

Toutes les semaines (durant quatre semaines), 2/8^{e} du filtre ont été récupérés et ont subis les étapes du protocole d'extraction. Deux huitièmes d'un filtre témoin ont également été analysés chaque semaine, afin de s'assurer de l'absence de contamination à chaque étape du traitement de l'échantillon (Extraction, PCR-SSCP et CE-SSCP) (Figure 32).

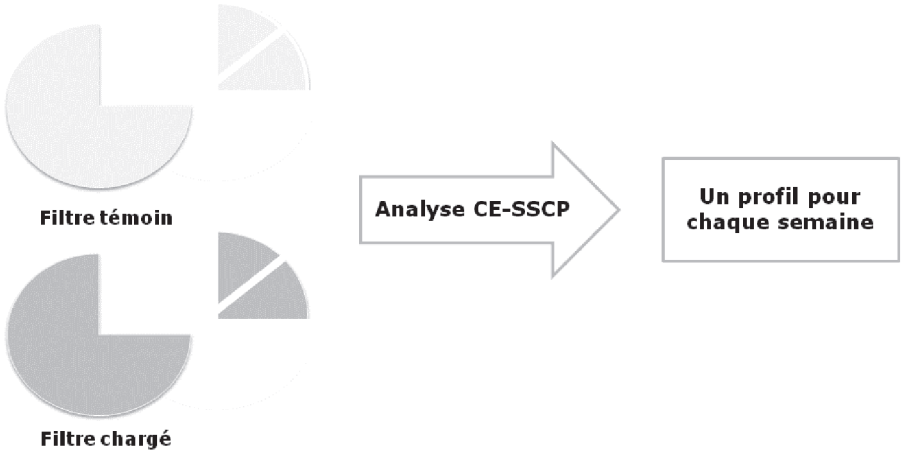

Figure 32 : Représentation schématique de la méthodologie adoptée pour l'étude de la stabilité du filtre sur quatre semaines.

Les diversités microbiennes (Bactéries) de chacun des morceaux de filtre ont été comparées par empreinte moléculaire (analyse CE-SSCP) basée sur la variabilité de l'ADNr 16S (Figure 33).

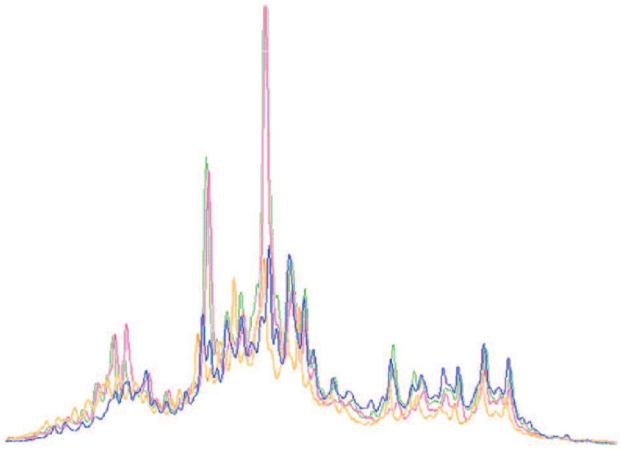

Figure 33 : Suivi des communautés bactériennes à raison d'un profil par semaine.

Toutes les communautés bactériennes ont produit des profils complexes (en moyenne plus de 15 pics).

Les profils obtenus, où chaque pic représente une séquence d'ADNr 16S, sont très proches, montrant des structures de communautés bactériennes similaires et donc l'absence d'une croissance bactérienne contaminante durant les prélèvements. Le filtre ne semble donc pas interférer sur la dynamique des communautés bactériennes présentes à sa surface.

La stabilité des profils obtenus met également en évidence la reproductibilité du protocole expérimental d'analyse à chacune des étapes (extraction, PCR et SSCP).

Au cours des tests en situation du biocollecteur intégré, des échantillons ont été analysés par CE-SSCP toutes les quatre semaines sur une durée d'une année. Les profils obtenus, où chaque pic représente une séquence d'ADNr 16S, sont également très proches (Figure 34).

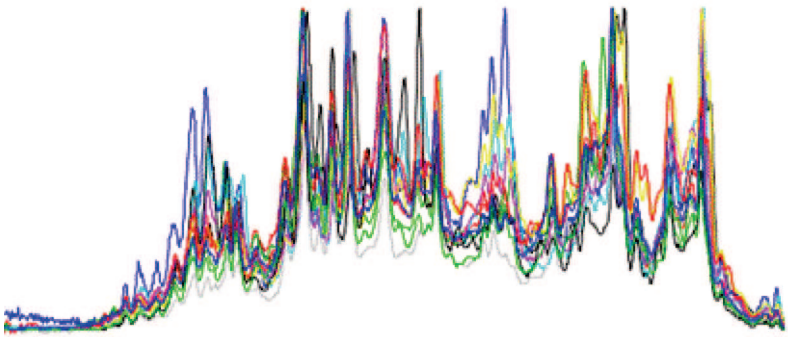

Figure 34 : Suivi des communautés bactériennes sur une durée d'un an.