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# One-year follow-up of microbial diversity in bioaerosols emitted in a waste sorting plant in France

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## ABSTRACT:

Bioaerosols emitted in waste sorting plants (WSP) can induce some adverse health effects on the workers such as rhinitis, asthma and hypersensitivity pneumonitis. The composition of these bioaerosols is scarcely known and most of the time assessed using culture-dependent methods. Due to the well-known limitations of cultural methods, these biodiversity measurements underestimate the actual microbial taxon richness. The aim of the study was to assess the airborne microbial biodiversity by using a sequencing method in a French waste sorting plant (WSP) for one year and to investigate the main factors of variability of this biodiversity. Static sampling was performed in five areas in the plant and compared to an indoor reference (IR), using closed-

face cassettes ( $10 \text{ L} \cdot \text{min}^{-1}$ ) with polycarbonate membranes, every month for one year. Environmental data was measured (temperature, relative humidity). After DNA extraction, microbial biodiversity was assessed by means of sequencing. Bacterial genera *Staphylococcus*, *Streptococcus*, *Prevotella*, *Lactococcus*, *Lactobacillus*, *Pseudomonas* and fungal genera *Wallemia*, *Cladosporium*, *Debaryomyces*, *Penicillium*, *Alternaria* were the most predominant airborne microorganisms. Microbial biodiversity was different in the plant compared to the IR and seemed to be influenced by the season.

Keywords: bioaerosol; biodiversity; 16S rDNA; Illumina sequencing; ITS rDNA; next-generation sequencing; occupational exposure; household waste sorting; determinants

#### Abbreviation list

CFC closed-face cassette

(r)DNA (ribosomal)desoxyribonucleic acid

HTS high-throughput sequencing

WSP waste sorting plant

IR Indoor Reference

ITS internal transcribed spacer

OEL Occupational Exposure Limit values

OTU Operational taxonomic unit

QS Sorensen-Dice coefficient

50 RH relative humidity

## 51 **1 Introduction**

52 The management of waste-limiting landfill disposal is an important issue in our  
53 society. Thus, industrial structures and treatment channels are developing all  
54 over the world in order to collect, sort, recycle waste or produce energy from  
55 residual materials. Of these channels, the sorting of household, commercial  
56 and industrial solid waste is one of the challenges all countries face. For 2020,  
57 the European Union plans to increase the amount of domestic waste recycled,  
58 to represent 50% of overall waste mass (Council of the European Union,  
59 2008). In France, the number of waste sorting plants increased from 265 to  
60 410 installations between 2000 and 2014 (ADEME, 2015). They treat several  
61 million tons of non-hazardous household or industrial waste every year and  
62 employ an increasing number of workers involved in sorting waste manually,  
63 cleaning and maintaining facilities, driving handling or loading machines, etc.

64 Published studies revealed that workers in waste sorting plants (WSP) were  
65 exposed to complex bioaerosols including dust, bacteria and fungi, as well as  
66 microbial compounds and metabolites (Madsen et al., 2016; Park et al., 2013;  
67 Schlosser et al., 2015; Tolvanen, 2001; Van Tongeren et al., 1997). The  
68 corresponding exposure has been associated to health disorders among  
69 workers, and respiratory, gastrointestinal or dermatologic symptoms have  
70 been reported for many situations that involve the handling of household  
71 waste (Poole and Basu, 2017; Walser et al., 2015). However, data provided  
72 by epidemiological studies is not sufficient to be able to establish a link  
73 between exposure and health issues, and there are no Occupational

Exposure Limit values (OELs) available for airborne biological agents. As the health issues caused by bioaerosols are linked to the type of species and compounds to which workers are exposed, one of the reasons explaining the current lack of OELs may be linked to the complex composition of bioaerosols in the workplace atmosphere, such as WSP. Most of the existing published studies provide biodiversity data in WSP by culture and identification of microorganisms on nutrient media. They revealed that predominant airborne microorganisms in WSP belonged to *Aspergillus* and *Penicillium* for fungi and to *Bacillus* and *Staphylococcus* for bacteria (Breum et al., 1999b; Lehtinen et al., 2013; Madsen et al., 2019; Rahkonen, 1992; Viegas et al., 2014). A recent study has provided new data based on high-throughput sequencing (HTS) measurements, confirming previous culture-dependant findings, as well as highlighting new microbial taxa such as *Wallemia*, for fungi (Degois et al., 2017). Indeed, HTS has provided a new approach for environmental microbial studies over the last decade. The technique was already being used to characterize microbial communities in swine houses (Kumari et al., 2016), composting facilities (Mbareche et al., 2017b) or wastewater treatment plants (Lin et al., 2014). It provides a solution to some of the limitations of the culture method but it also introduces other types of biases such as DNA extraction efficiency or errors of sequencing (Duquenne, 2018; Mbareche et al., 2017a). Nevertheless, the use of HTS in WSP has enabled a more complex bioaerosol composition to be revealed, with more microbial taxa detected, as opposed to culture (Degois et al., 2017).

The evolution of microbial biodiversity over time in bioaerosols has also been investigated in different environments. Thus, geographical location,

temperature, relative humidity and, more generally, the seasons were found to be potential determinants of biodiversity of airborne microbial communities for wastewater treatment plants (Ding et al., 2016), swine houses (Kumari et al., 2016) and outdoor air (Bowers et al., 2012). As the impact of each of these factors on biodiversity varies from study to study, no conclusions can be drawn regarding the mode of action for environmental factors. To date, such an investigation has not yet been carried out for bioaerosols emitted in French WSPs. Yet, a better understanding of factors affecting airborne communities would improve knowledge about microbial exposure for workers and would provide useful data for establishing further sampling strategies as well as for managing biological risk. Biodiversity measurements were taken in the working environment of a waste sorting plant (WSP) over the period of one year.

The aims of the present study were: 1) to characterize the bacterial and fungal biodiversity in bioaerosols in six sampling areas of a WSP using HTS, 2) to assess temporal variations of the microbial biodiversity in emitted bioaerosols and 3) to investigate the environmental parameters which may affect the microbial biodiversity in the bioaerosols emitted in a waste sorting plant.

## **2 Material and methods**

### **2.1 Description of the waste sorting plant**

The waste sorting plant (WSP) is in eastern France and employs about 50 workers for the sorting activities. The plant treats non-hazardous household

and industrial waste collected from 44 towns (representing approximately 235,000 inhabitants). An overview of the general organization of the plant and the sorting activities is provided in Figure 1. Household waste comprised various materials (cardboard, different types of plastic packaging, cartons and metals) unloaded directly onto the ground by trucks in a waste unloading area (Figure 1 ❶). Waste was then selected at random to be fed into the sorting chain to then be manually sorted by workers in sorting cabins (Figure 1 ❷ and ❸) before being stored in pits. Industrial waste such as furniture or wood items was fed into a wood chipper (Figure 1 ❹) and sent to an incineration unit adjacent to the WSP along a conveyor belt to supply the hot water system of the neighbouring town. As mentioned above, incoming waste was treated at random but the mode of treatment was mostly based on a “last in, first out” approach. However, it was not possible to specify the storage time and the nature of the waste treated on the days of sampling.

The company daily recorded the amount of waste delivered to the unloading area, shredded or squeezed by the press. The median mass was 71,640 kg (59,200-89,920 kg) in the unloading area, 16,320 kg (5,040-24,500 kg) for the shredder, and 23,296 kg (18,800-39,151 kg) for the press. Even if it mainly included food packaging, most of the treated waste was of varying materials (between 59% and 86% of daily mass of waste) and the remaining fraction consisted of large cardboard items and papers/magazines processed through a separate sorting chain, which was not examined in this study.

## 2.2 Sampling methods and strategy

Stationary bioaerosol samples were collected at five sampling points positioned near significant working areas inside the WSP, known as “waste exposure areas” in the following sections of the paper: point 1 in the unloading area; point 2 in the first sorting cabin; point 3 in the third sorting cabin; point 4 near the shredder and point 5 near the press (Figure 1). In addition, a sample was taken from an IR (indoor reference) (point 6) in a meeting room in another administrative part of the building, hermetically separated from waste sorting activities. Samples were collected every month from January to December 2016 for all the sampling points in order to assess the variation of microbial biodiversity (12 samples collected per sampling location).

To perform bioaerosol sampling, three-piece 37 mm closed-face cassettes (CFC) with a polycarbonate membrane filter (Nuclepore®, 0.8 µm pore size) and a cellulose support pad (Millipore®, thick cellulose absorbent pad) were attached to constant flow pumps (Bravo, Tecora, France) calibrated at a flow rate of 10 L/min using a flow meter (Mass Flowmeter 4040, TSI Inc., USA). Flow rates were measured before and after sampling. CFCs were placed at the approximate height of the human respiratory tract and sampling was performed for five hours during the work shift. In parallel to bioaerosol sampling, temperature and relative humidity (RH) of the air were measured using a real-time measuring device (Labguard® 3D Mobiguard, Biomérieux, France) positioned near the sampling device. All samples were transported to the laboratory in a cold box, stored at 4°C and analyzed within 24 hours of being collected.



### **2.3 Pre-treatment of samples and DNA extraction**

To elute the particles from the filter, a volume of 5 mL of a sterile solution (0.01 % Tween 80 in ultra-pure water) was introduced into each CFC through the inlet. Then, the cassette inlets and outlets were closed with caps and shaken (Heildolph®, Multi-Reax shaker) at 2000 rpm for 20 min and at room temperature. DNA extraction was performed using a commercial kit (FastDNA® SPIN kit for soil, MP Biomedicals, Illkirch, France), according to the manufacturer's instructions, with 500 µL of eluted particle solution. The DNA samples were stored at -20°C until sequencing.

### **2.4 DNA sequencing and sequence data analysis**

The DNA samples were purified, amplified and sequenced by the INRA Transfert Environnement (Narbonne, France) sequencing platform. The bacterial biodiversity was examined by sequencing the 16S rDNA (v3-v5 variable region) using primers F-5'-GTGYCAGCMGCCGCGGTA-3' and R-5'-CCCGYCAATTCMTTTRAGT-3'. The primers used to amplify the specific fungal barcode ITS2 were w404 5'-CTTCCCTACACGACGCTCTTCCGATCTTGTGARTCATCGAATCTTTG-3' and w40 5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTCCCTCCGCTTATTGATATGC-3'. For the DNA amplification by PCR, the mixture contained 5 µL of DNA template, 4 µL of dNTPs (25 mM), 1 µL of each primer (100 ng/µL), and 0.5 µL of Taq polymerase in a total volume of 50 µL. The PCR cycle was 2 min at 95°C (denaturation step), 1 min at 56°C or 65°C for ITS2 or 16S rDNA amplification respectively (annealing step) and 1 min at 72°C (elongation step). The two genetic targets were sequenced using paired-end sequencing

with Miseq technology (Illumina, San Diego, USA). Sequence analysis was performed according to Mothur's SOP (Kozich et al., 2013). Simply put, the reads were trimmed; barcodes, primers and homopolymers longer than 8 pb were removed using Mothur (v.1.37.4) software (Schloss et al., 2009). Chimera were detected and removed using UNCHIME (Edgar et al., 2011). After alignment, the reads with 100% identity were clustered into a single sequence. The bacterial reads were assigned by the Wang method (Wang et al., 2007) that was executed in Mothur with SILVA database. Operational taxonomic units (OTUs) were defined using the traditional method. The ITS2 reads were processed in the same way as bacterial sequences. Fungal OTUs were assigned at the genus level using the massBLASTer algorithm in the UNITE database (<https://unite.ut.ee/analysis.php>) with an e-value of over  $10^{-60}$  and more than 95% identity.

## **2.5 Statistical analysis**

### **2.5.1 Sample labelling**

The names of the samples follow a letter code (B or F for bacterial or fungal samples respectively) with a first digit to indicate the month of sampling and a second digit that represents the sampling location. For example, the bacterial analysis for the sample collected in February in the sorting cabin is named B23 in figures, which represents Bayesian's results.

### **2.5.2 Diversity analysis**

Alpha diversity, which estimates the diversity within a sample, was calculated according to the Shannon index and Specific Richness (SR) to measure variations in the first sorting cabin, the sorting cabin, and the shredder. To

assess the statistical differences, Mann-Whitney and Kruskal-Wallis tests were performed using Statgraphics Centurion XVI software (Statistical Graphics Corp.,USA).

The Sorensen-Dice coefficient (QS) (Tuomisto, 2010) was calculated to compare the beta diversity between measurements and the similarity in composition between the bioaerosols emitted inside the plant and in the IR. The more the microbial compositions between two samples have in common, the closer the index value is to 1 (identical composition). This index was calculated using the Mothur software for bacterial and fungal biodiversity, over a period of four months: January (winter), April (spring), August (summer) and October (fall). These months were chosen because of the success of sequencing for the two genetic targets (ITS and 16S rDNA) at all the sampling points.

### **2.5.3 Correlation calculation**

To establish a relation between the QS and the temperature or the RH, the value of the Spearman' rs statistic was calculated using Statgraphics Centurion XVI software (Statistical Graphics Corp.,USA).

### **2.5.4 Bayesian networks analysis**

Bayesian networks were used to identify the similarities between measurements (Pearl and Russell, 2002). The software used for this technique is BayesiaLab ([www.bayesia.com](http://www.bayesia.com)). For each measurement, the biodiversity data is summarized into the presence (value 1) or absence (value 0) of the genus. Each measurement is then a vector whereby each coordinate

symbolizes the presence or absence of a specific genus (Table S1). The data table used displays measurements as columns and genera as rows.

Based on this data, the idea is to automatically build a network that highlights the statistical correlations between the measurements, based on the frequency of shared genus between samples. Each measurement becomes a node and whenever a statistical correlation is strong enough ( $p$  value  $< 0.0001$ ), then a link is established between the nodes (Figure S1).

In a second step of the statistical analysis, a data clustering method is applied to form groups of nodes with strong enough similarity. In Figure S1, the nodes bearing the same color belong to the same group. This analysis can be viewed through a dendrogram (Figure S2), the highest level of which shows the best compromise between the overall number of groups formed and the average similarity within the groups. It is expected that the standard statistical settings for forming the groups will identify patterns of biodiversity between measurements, particularly when taking into account the season in which measurement was carried out.

### **3 Results**

#### **3.1 Sampling conditions**

During the sampling campaign (twelve days in the year 2016), 72 bioaerosol samples were collected (representing 5 sampling points and an IR for each day) for microbial biodiversity analysis. The number of workers in the WSP was representative of the previous years but the plant had recorded an increase in the amount of waste sorted. No production dysfunctions were observed during sampling periods except a halt in production in November

with no activity for the two sorting cabins. The temperature was monitored at the different sampling sites over the period of the campaign. It varied from 0°C to 27°C (Figure S2 A and B). Temperatures measured in waste management areas were at the maximum in summer (about 27°C in July) and the minimum in winter (about 0°C in the hall and 12°C inside the cabins in January and February). Measured values were similar between areas located in the hall (unloading waste area, shredder and press/storage) and varied between cabins. The temperature monitored at the IR was more stable (about 20°C) and significantly higher than in waste management areas. The recorded values of relative humidity (RH) varied from 28% to 60% and were similar between the two cabins and the IR; they were higher in areas located in the hall especially during fall and winter (Figure S3 A and B).

### **3.2 General sequencing data**

A total of 63 samples (87% success rate) and 68 samples (94% success rate) were successfully sequenced for bacterial and fungal biodiversity, respectively. Samples with DNA concentrations lower than 3 ng.µL<sup>-1</sup> were not sequenced (winter samplings or IR). The number of reads (a read is a sequence analyzed by the sequencing machine) after sequencing varied extensively depending on the location and on the month of sampling. The median of the reads was approximately 25,000 except for 16S rDNA reads for the IR, which contained less reads than the other sampling locations (Table 1). Two samples with a very low number of bacterial reads were not considered.

### **3.3 Bacterial biodiversity and monthly variation**

#### **3.3.1 *Dominant bacterial phyla and their variations***

Thirty bacterial phyla were observed but 96% of the reads represented the five most prominent phyla in all the samples (Figure 2A, B, and C): Actinobacteria, Bacteroidetes, Cynaobacteria, Firmicutes and Proteobacteria. Among them, Firmicutes and Proteobacteria were the two most dominant specimens in samples collected in waste treatment areas (median=41.5% and 24.6% respectively) and in the IR (median= 20.0% and 48.6% respectively), followed by Actinobacteria and Bacteroidetes. The variation of the main bacterial phyla over the course of the study is illustrated in Figure 2A, B, and C for three sampling locations: unloading waste area, in the first-sorting cabin and in the IR (for the other sampling points, see Figure S4A, B, and C in the supplementary materials). A high proportion of Cyanobacteria was recorded in February in the unloading waste area with 65.7% of the bacterial reads attributed to this phylum. This result was not observed at any other sampling site or at any other time during the sampling campaign.

#### **3.3.2 *Dominant bacterial genera and their variations***

At the genus level, 605 bacterial genera were observed in our samples. The more abundant bacteria are known as core microbiome in biodiversity studies. In the current project, the core microbiome was composed of 45 genera with 6 Actinobacteria, 7 Bacteroidetes, 15 Firmicutes, 1 Nitrospinae, and 16 Proteobacteria genera. These main genera represented between 9.3% (waste unloading area in February) and 83.8% (IR in September) of the bacterial reads.

As for phyla, the description of the results is focused on three sampling points: the unloading waste area, the first-sorting cabin and the IR (Figure 2D, E, and F). Results from the other sampling plants are presented in Figure S4 of the supplementary materials. One of the most dominant bacterial genera found at each sampling point, including the IR, was *Staphylococcus* (Figure 2D, E and F) with a median of approximately 9% of relative abundance in all sampling points, except near the press in March. *Prevotella* and *Pseudomonas* were present throughout the whole year in substantial proportion in the two waste treatment locations (about 3%) and in lower proportion in the IR (less than 0.6%). *Streptococcus* was also found every month but in lower proportions with a maximal value of 11.3%, observed in the IR in January. Other bacterial genera were frequently found during the year in the bioaerosols collected in the unloading area and in the first sorting cabin: *Lactobacillus* (3%), *Bacillus* (1%), *Leuconostoc* (2%) which was absent in the IR, as well as *Aerococcus*, *Lactococcus*, *Acinetobacter*, and *Corynebacterium*.

### **3.4 Fungal biodiversity and its variation**

#### **3.4.1 Dominant fungal phyla and their variations over the year**

Among the 6 phyla detected in the bioaerosols, Ascomycota, Basidiomycota, and Mucoromycota were the dominant ones (Figure 3A, B, and C and Figure S5 A, B and C in the supplementary materials). Ascomycota reads represented more than 50% of the fungal reads (56.3% in the waste treatment areas and 53.2 % in the IR). The main fungal phyla were present at each period and at each sampling point with varied proportions depending on the location and the time of the sampling.

### 3.4.2 Dominant fungal genera and their variations over the year

A total of 592 fungal genera were identified when all the collected bioaerosol samples were considered. The fungal core microbiome was composed of 45 genera. This core microbiome varied from 58.7% (in October near the press) to 91.2 % (in January in the first-sorting cabin) with a median value of 77.2%.

The relative abundance of the fungal microbiome found in the unloading waste area, the first-sorting cabin and the IR and its variation over the year is described in Figure 3. The fungal biodiversity found in the sorting cabin, near the shredder and near the press is illustrated in Figure S5 of the supplementary materials. Among the core microbiome, 8 genera were found in huge proportions throughout the whole year: *Cladosporium*, *Alternaria*, *Debaryomyces*, *Penicillium*, *Candida*, *Wallemia*, *Cryptococcus*, and *Mucor* (Figure 3 and Figure S5 of the supplementary materials). *Wallemia* was the predominant genus in the two occupational sampling points (Figure 3 D, E, and F). It represented 17.3% (up to 66.4% in February) and 21.4% (up to 54.8% in November) of the ITS2 reads in the unloading waste area and in the first sorting cabin respectively. In the IR, *Wallemia* was also recorded in substantial proportion (6.5%). *Cladosporium* and *Debaryomyces* were also dominant genera found at the three sampling locations throughout the year. *Cladosporium* was the predominant genus in the IR. In the unloading waste area and in the first-sorting cabin, about 5% of fungal reads were attributed to *Penicillium* and *Candida* (5.2% and 6.1% in the unloading waste area; 5.2% and 4.5% in the first-sorting cabin). They only represented about 1% in the IR.



### **3.5 Variation of alpha biodiversity**

The sampling location had no statistical impact on Shannon indexes for bacteria and fungi. The Shannon index values were higher for the bacterial biodiversity than for the fungal biodiversity. The number of microbial genera did not depend on the period of sampling, neither for bacteria nor fungi.

### **3.6 Season and humidity as the main factors of microbial diversity**

The similarity of microbial (fungal and bacterial) composition between the IR and the sampling points inside the WSP was measured by calculating the Sorensen-Dice coefficient (QS). For bacterial biodiversity, this coefficient varied from 0.07 in winter to 0.6 in spring in the sorting cabin (Figure 4A).

There was more similarity between bacterial communities in waste treatment areas and IR in fall-summer than in winter-spring. A positive correlation was observed between the QS values and temperature values (Spearman  $r_s$  p-value=0.51, n=20). The higher the temperature was, the closer the bacterial composition of bioaerosols was to that of IR.. The sampling location had an impact on bacterial biodiversity regarding Sorensen-Dice coefficient (Kruskal-Wallis test: p=0.003). For fungal biodiversity, the values varied from 0.12 to 0.64 (Figure 4B). The coefficient was minimal (about 0.15) in summer and maximal in spring with values superior to 0.5. The fungal composition in bioaerosols collected at the IR and at the sampling points in the WSP showed closer similarities in spring and in fall. The fungal biodiversity was negatively correlated with the relative humidity of the air (Spearman  $r_s$  p-value=-0.67, n=20), the higher the RH, the more the composition of fungal bioaerosols differed, as compared to the IR.

### 3.7 Results from Bayesian networks statistics

The Bayesian networks were used to group bioaerosol samples according to the similarities in their microbial composition at the genus level. This operation is called “clustering” and is only based on statistical data. The groups were then analyzed to determine whether the composition of bioaerosol can be attributed to an environmental parameter (e.g. a given pattern of bioaerosol is more often present during a specific season). A first clustering provided 20 clusters with statistical similar composition for fungal biodiversity and 17 for bacterial biodiversity (Figure 5 and Figure S6 in the supplementary materials). The clusters represent the sets of bacterial or fungal genus that are frequently identified together in a sample. After clustering, the composition of each cluster was analyzed to determine the common patterns between clusters: the day or location of sampling, the intensity of activity (amount of treated waste), the number of workers, the monitored climatic conditions at each sampling point, the atmospheric conditions during and three days before the collection. The clusters were named according to the parameters (bacterial diversity Figure S6 in the supplementary materials; fungal biodiversity Figure 5A). For fungi, 18 factors were attributed to a parameter that was most of time associated with the day of sampling. After the first clustering, a second Bayesian analysis was performed to establish relationships between the renamed clusters (Figure 5B). This analysis shows that the composition of microbial aerosols is related with the seasons of the year. For bacteria, no relation with identified parameters was found to link the samples. In a same group, they were bioaerosols sampled at different locations and at different time (Figure S6 in supplementary materials).

## 4 Discussion

### 4.1 Overview of the sampling campaign

During the sampling campaign, the climatic conditions were those of the semi-continental climate usually found in the North East of France. The year 2016 in particular was characterized by very marked seasons, with very low temperatures in winter and rather high temperatures in summer. The production as well as working activities were considered representative for the investigated WSP.

### 4.2 Dominant airborne microorganisms in the WSP

The biodiversity data revealed that some microbial taxa was present in substantial proportions throughout the whole year and at all the sampling locations. For bacteria, the Firmicutes was the dominant bacterial phylum followed by Proteobacteria and Bacteroidetes, with an exception for the unloading waste area in February (Figure 2). About 605 bacterial genera were found but only few of them (45) account for most of the 16S rDNA reads in collected bioaerosol samples. Among these genera, *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Lactococcus* but also *Prevotella*, *Pseudomonas*, *Acinetobacter* and *Bacillus*, were present and dominant in all samples. For fungi, the dominant phyla were Ascomycota and Basidiomycota. Among the 592 fungal genera, only five genera (*Wallemia*, *Cladosporium*, *Debaryomyces*, *Candida*, and *Penicillium*) constituted more than half of the ITS2 reads.

The present study corroborates our previous findings in another WSP located in France, using HTS technologies (Degois et al., 2017). In this previous

study, the same genera (*Staphylococcus*, *Streptococcus*, *Penicillium*) were also found to be in abundance. *Staphylococcus* was isolated from bioaerosol samples collected in truck cabs for waste collection (Madsen et al., 2016) and during cardboard sorting (Madsen et al., 2019). *Staphylococcus* and *Bacillus* were found by culture in bioaerosols emitted during paper sorting (Breum et al., 1999). In the present study, the genus *Prevotella* was also found in high relative abundance throughout the whole year. To our knowledge, this genus was not reported in studies measuring the bacterial composition in bioaerosol emitted in waste sorting plant. The fungal genera *Penicillium* and *Aspergillus* were also isolated by culture from samples collected in truck cab samples (Madsen et al., 2016) and in a waste treatment plant (Breum et al., 1999; Lehtinen et al., 2013; Tolvanen, 2001; Viegas et al., 2014). In addition to *Penicillium*, *Cladosporium* was the predominant fungal genus in a sorting paper plant (Breum et al., 1999). *Candida* and *Alternaria* were also found in high proportion, using HTS, in a domestic compost heap and in the bioaerosols emitted from it (Mbareche et al., 2017a; Wéry, 2014).

The results from our study indicate that *Wallemia* was one of the predominant genera in the bioaerosols emitted in the investigated WSP. This genus belongs to the order Wallemiales (Wallemiomycotina, Basidiomycota) and includes several species (*W. ichthyophaga*, *W. muriae*, *W. sebi*, *W. mellicola*, *W. tropicalis*, *W. Canadensis*, *W. hederæ* and *W. peruviana*). These species are salty environments (Zalar et al., 2005) and are known to be spoilage organisms of food items with low water activity (aw) (Zajc and Gunde-Cimerman, 2018). But some are also found in the airborne state and in settled dust in occupational and indoor environments, as well as dwellings

(Fröhlich-Nowoisky et al., 2009; Li et al., 2019; Zeng et al., 2004). They usually differ in their xerotolerance and tolerance to solutes (NaCl, MgCl<sub>2</sub>), growth rate and temperature, enzymes and metabolites production profiles as well as in their size of conidia (Jančič et al., 2015; Jančič et al., 2016). The predominance of *Wallemia* in bioaerosols from the WSP can be explained by the presence of food packaging among waste (trays, yoghurt pots and sauce jars, beverage bottles, etc.) on which food residue remains, creating favorable conditions for their growth and survival. This residue may come from food items with the water activity reduced initially or that has been lowered by dehydration during the period prior to sorting. Similar hypotheses can be put forward to explain the predominance of other fungal genera such as *Cladosporium* and *Penicillium*, as several species belonging to these genera were found to be able to grow in the presence of low water activity (Gunde-Cimerman et al., 2003). Their growth and survival on paper and cardboard have also been shown (Das et al., 1997).

The presence of *Wallemia* in bioaerosols from WSP has already been demonstrated in our previous published study carried out using molecular methods in the same working environment (Degois et al., 2017). This result contrasts with the results of studies conducted using culture-based methods for which this genus has not been identified (Tolvanen, 2001; Viegas et al., 2014). Fungal species belonging to *Wallemia* have a very low growth rate on usual culture media and its numeration is usually impaired due to masking by faster growing species. Thus, its presence in different environments is underestimated while using culture-based methods. On the other hand, the

*Aspergillus* genus was poorly detected in the present study as compared to previous published studies (Degois et al., 2017; Tolvanen, 2001). This under-representation may be due to specific factors at our site (undetermined environmental or ecological factors) or due to a methodological bias. Indeed, we have already shown that *Aspergillus* might be under-represented in HTS analyses compared to culture methods, probably due to an inefficient DNA extraction (Degois et al., 2019). These specific examples refer to the complementarity of the two approaches whose respective advantages and limitations have already been widely discussed (Degois et al., 2017; Degois et al., 2019; Duquenne, 2018; Mbareche et al., 2017).

The biodiversity of airborne bacterial communities emitted in waste sorting centers is different, compared to that recorded at composting or anaerobic digestion facilities where bacteria belonging to *Saccharopolyspora*, *Saccharomonospora*, *Thermomonospora*, *Thermobifida*, or *Legionella* can be found. These bacteria are thermophilic and/or are involved in the degradation process of organic matter, as seen in composting or anaerobic digestion processes and related to the type of waste treated in this sort of facility (Dubuis et al., 2017; Wéry, 2014). The airborne fungal core microbiome found in the WSP was similar, as described before, as other facilities treating dry and solid waste.

The present study provides additional biodiversity data about bioaerosols emitted in WSP and reveals their actual complex composition throughout the whole year.

### 4.3 Reported health disorders cause by the detected microbial taxa

Several of the dominant microbial genera found in the present study include species that are involved in health disorders among general and occupational populations. For example, some *Prevotella* species have been involved in human health disorders such as pulmonary diseases and chronic otitis and sinusitis (Tanaka et al., 2008). The effects of airborne fungal particles are well documented in the literature and the risk associated with exposure to airborne fungal particles is better documented than the bacterial risk. Fungi can induce different types of health issues including respiratory allergies (asthma, rhinitis or hypersensitivity pneumonitis), toxicity, and irritation or infection. Exposure to airborne fungal species belonging to *Cladosporium*, *Aspergillus* and *Penicillium* has been associated with allergic skin diseases such as contact dermatitis (Kallawicha et al., 2016). In addition, these fungi produce volatile organic compounds that may cause several symptoms such as a dry cough, pharyngitis or headache. Furthermore, the wall cell of *Aspergillus* and *Candida ssp.* are composed of mannan, which is a known allergic compound (Dyląg, 2017). In addition, *Wallemia sebi* is involved in allergic disorders such as farmer's lung disease and produces a toxic metabolite, wallemiol, which has the same effect as the mycotoxin penicillic acid (Wood et al., 1990).

Because of the high number of genus, only the main genera are represented in the present paper. However, as the exposure-response relationship was not established, the minor and rare taxa could also affect worker health.

Furthermore, the pathogenic property is not the only aspect to consider and some airborne bacteria present in minority proportions can carry endotoxins or  $\beta$ -D-glucans. These components have been reported to induce several

health effects such as irritation of the upper airways or mucous membranes, inflammation (Bønløkke et al., 2006; Lawniczek-Walczyk and Gorny, 2010) and irritation of the respiratory system, fever and diarrhea (Duquenne et al., 2013; Liebers et al., 2008). Exposure to these compounds was reported in other plants treating solid and dry waste (Lehtinen et al., 2013; Tolvanen, 2001).

On the other hand, the biodiversity data provided by the present study needs to be expanded for an eligible risk assessment in the WSP. Firstly, the relationship between airborne microorganism exposure and human health disorders must be specified (Walser et al., 2015). Secondly, personal samplings are required for the assessment of occupational exposure. Indeed, stationary sampling does not take into account the movement of workers during their shift, nor the professional gestures that can affect exposure. Finally, the HTS technologies do not allow the identification at the species level due to the short reads provided by Illumina sequencing, the sequencing depth, the sequenced regions and the errors of sequence annotations in databases (Nilsson et al., 2006; Yamamoto and Bibby, 2014a). The genus level does not allow for a precise risk assessment. Indeed, a genus can gather a large number of species (e.g. more than a hundred for the genus *Bacillus*) and within a genus, only some species can be pathogenic (Kwok et al., 1999). Nevertheless, the HTS constitutes a powerful screening and characterization tool at the genus level, enabling to focus on potential pathogenic species, in a second and more targeted process.



#### 4.4 Sorting activities compared to the indoor reference

The Sorensen-Dice coefficient is a good index to measure a difference in the composition of two samples. In the present study, a significant difference (index value  $<0.5$ ) was found between samples collected in areas with ongoing sorting activities and the IR, and this was the case for all seasons. This result suggests specific bioaerosols exposure for workers handling waste compared to the administrative staff. For airborne bacterial communities, the difference in biodiversity between waste-exposed sampling points and the IR was higher in winter and in spring when the highest differences in temperature between the two sampling locations were taken into consideration. The cold climatic conditions were apparently less favorable to bacterial growth of most bacteria encountered during warmer seasons. Despite the low temperatures measured, the specific richness was higher at waste-exposed locations than in IR. This suggested that the waste contributes significantly to bacterial biodiversity. For fungal communities, the difference in composition between waste-exposed sampling points and the reference was less significant, with higher differences observed when the temperatures were extreme (in winter and in summer). The IR was heated during the winter and cooled during the summer with an air conditioning system (air drying). Moreover, the air in the administrative building was renewed with the outdoor air that surrounded the waste treatment building. As a consequence, the indoor air may have been polluted by the outdoor air. Such a phenomenon has already been described (Adams et al., 2013) and could partly explain the similarities in fungal biodiversity between the two locations in winter and in summer. In the present study, no differences in microbial biodiversity were observed between the

sampling points inside the waste treatment buildings, whatever statistical method was used (Bayesian network, Shannon index). This could be explained by the temperature and the relative humidity, which were similar between sampling points, especially in the hall (sampling points 1, 4 and 5). Waste represented the main source of bioaerosol emissions. With the exception of sampling point number 4, the type of waste was identical for all sampling points, and the only difference was the duration of storage at each point (several days, weeks or months at the locations 1 and 5 versus just a few minutes at locations 2 and 3). In addition, the workers opened the two doors located on either side of the box in order to cool the first-sorting cabin during the hot season. However, the first-sorting cabin was located in front of the shredder which produced large amounts of airborne dust and which may have contaminated the air of the first-sorting cabin. All these observations could explain the similarities of microbial composition detected between the waste-exposed locations.

#### **4.5 Seasonal variations of microbial biodiversity in bioaerosol**

The Bayesian network approach was used for clustering the fungal bioaerosol samples depending on their composition similarities. The results showed correlation according to the month or the season of sampling (consecutive months) regardless of the locations of the waste-exposed samples. The period of sampling did not explain correlation of the airborne bacterial samples. No other parameters (number of workers, sampling locations, amount of waste) could provide an explanation for how samples were clustered by the Bayesian network analysis for both bacterial and fungal communities.

Seasonal variations in the concentration of airborne microorganisms were investigated in studies carried out on outdoor air from different countries (Bezerra et al., 2014; Bowers et al., 2013; Crandall and Gilbert, 2017) and on indoor air (Chao et al., 2002; Frankel et al., 2012; Wang et al., 2010). The seasonal variations were also investigated in occupational environments such as a wastewater treatment plant (Ding et al., 2016; Oppliger et al., 2005), swine confinement buildings (Nehme et al., 2008), composting plants (Fracchia et al., 2006) and biowaste collection plants (Nielsen et al., 2000). The corresponding studies reported contrasting results regarding to the seasonal effect on concentrations of airborne microorganisms.

Several studies found a seasonal variability of microbial biodiversity in bioaerosols but the impact of the season on microbial communities depended on the study and the investigated place (Bowers et al., 2012; Ding et al., 2016; Fröhlich-Nowoisky et al., 2009; Kumari et al., 2016; Nehme et al., 2008). No general tendency was observed with regards the published data and the impact of environmental parameters is difficult to predict (Liao and Luo, 2005). To our knowledge, the impact of environmental parameters on airborne biodiversity has not been assessed in a waste sorting plant and therefore, our results cannot be compared with any other published data.

#### **4.6 Other determinants of biodiversity in bioaerosols**

Temperature and relative humidity of the air may partly explain our results, as these parameters were shown to greatly influence microbial growth (Brown and Mohr, 2016), as well as the aerosolization of fungal spores from contaminated matters (Madsen, 2012). In the present study, the meteorological conditions for sampling were very different throughout the

year. However, for a given sampling day, the temperature and humidity conditions were different between the hall and the two cabins, and showed correlation in the same cluster. Thus, temperature and relative humidity were not the only parameters impacting the airborne fungal biodiversity in the WSP. This hypothesis was confirmed by an extended statistical analysis performed on the Shannon index values, combining multilinear regression analysis and general linear models. This analysis considered the temperature and the relative humidity at the sampling point but also the temperature, the relative humidity and the rainfall during the day of the sampling and three days before. The best-fit model took into account all the previous parameters however it could explain only 40% of the Shannon index values (data not shown).

The impact of the environment on microbial biodiversity in bioaerosols is multifactorial and complex and combines not only the climatic conditions but also others parameters such as the amount and nature of waste or other pollutant sources. This confirms that microbial biodiversity and more generally, bioaerosol composition would be multifactorial and difficult to characterize. So far, other parameters have also been proven to impact microbial concentrations such as human activity (Maron et al., 2006), particles size and density of urban area (Fang et al., 2007), air pollution (Gao et al., 2015; Gao et al., 2016), CO<sub>2</sub> concentrations (Chao et al., 2002), operations of a process (Tolvanen, 2001), indoor air pollutants (Wang et al., 2010), the site investigated (Fracchia et al., 2006) and physical, environmental and ecological factors (Emerson et al., 2016).

The age of the stored waste has also been shown to have an impact on both airborne bacteria and fungi exposure levels (Gladding and Gwyther, 2017; Schlosser et al., 2015).

#### **4.7 Limitations of the study**

A large number of samples were collected, at different workstations and at different times of the year. This provided a satisfactory overview of the airborne microbial biodiversity in the WSP. Stationary samples were adequate in providing information about the exposure of workers in the cabins, but less apt regarding machine operators who moved around during their shift. Samples near the press, the shredder and the unloading area therefore did not allow for the characterization of the bioaerosol exposure for these employees. However, sampling on filters and analysis by molecular biology allowed for long-term sampling (5 hours) for a better representation of exposure assessment than short-term sampling with Andersen type impactors used for the analyzes by culture. Molecular biology methods and particularly HTS are limited due to biases for DNA extraction, PCR or sequence analysis (Haugland et al., 1999; Mbareche et al., 2017b; Yamamoto et al., 2014). These techniques are based on DNA analysis, which does not provide information on the viability and pathogenicity of airborne microorganisms and the associated health risk for workers. In addition, short sequences that are generated by sequencing limit the identification of microorganisms at the genus level, which may hamper a sound health risk assessment. However, HTS provides an overview of microbial biodiversity in poorly characterized fields. Finally, exposure to viruses was not assessed in this study.

#### 4.8 Further prospects

Most studies in waste treatment plants focus on fungi. Since bacteria were proven to be present in higher abundance than fungi, and to be the main colonizers of household waste bins (Gladding and Gwyther, 2017), more investigations of bacterial biodiversity in WSP would add to knowledge about risk assessment in this field. To improve this knowledge, it would be relevant to couple HTS sequencing with other techniques such as sequencing longer sequences or an analysis by culture followed by an identification by MALDITOF-MS, which would provide identification at the species level and/or allow the viability of microorganisms to be preserved. An analysis of the viral risk in WSP would also be relevant. Thanks to the present study, airborne microbial biodiversity was measured over a period of one year in one WSP. It would be interesting to compare with other WSPs with a similar or different organization.

Evaluating the impact of environmental factors on airborne biodiversity in workplaces is not easy especially due to the significant amount of unknown or uncontrolled factors. A pilot study would be an accurate way of assessing all the potential parameters (in particular the nature of waste) that could have an effect on biodiversity and evaluating how these parameters modify the composition of airborne microorganisms. Even if such studies describe more simple conditions as compared to the occupational environment, they would contribute to a more accurate understanding of such a complex phenomenon. The biodiversity results obtained at the genus level also represent a good basis for the definition of taxa to be monitored in this sector for occupational

health purposes, if similar measurements confirm their presence and abundance in other WSPs.

## 5 Conclusions

The study investigated the microbial diversity in bioaerosols emitted over one year in different representative areas of a French WSP.

The observed biodiversity was variable and dependant on the season, and was also found to be more complex than in studies using culture-based methods. Among the large number of genera observed, the genera *Staphylococcus*, *Streptococcus*, *Prevotella*, *Pseudomonas*, *Cladosporium*, *Wallemia*, *Penicillium*, and *Candida* were recorded in high proportions throughout the year. The season seemed to have an impact on fungal biodiversity. The parameters affecting bacterial biodiversity were probably multifactorial and are yet to be identified. Workers handling waste were exposed to specific bioaerosols, different from those emitted in the offices. Because of the high bioaerosol complexity in WSP, a long-term monitoring survey would be more appropriate in providing an accurate assessment of bioaerosol composition.

Analysis of the composition of bioaerosols by HTS provided a good overview of the microorganisms workers may be exposed to. This has improved knowledge in terms of exposure measurements in this occupational environment. This technique should be coupled with methods of identification at the species level and/or conservation of viability for better risk assessment.

Even if the study is not exhaustive, it has produced an overview of airborne biodiversity over a period of one year for a standard facility, which will assist us in making future studies more relevant.

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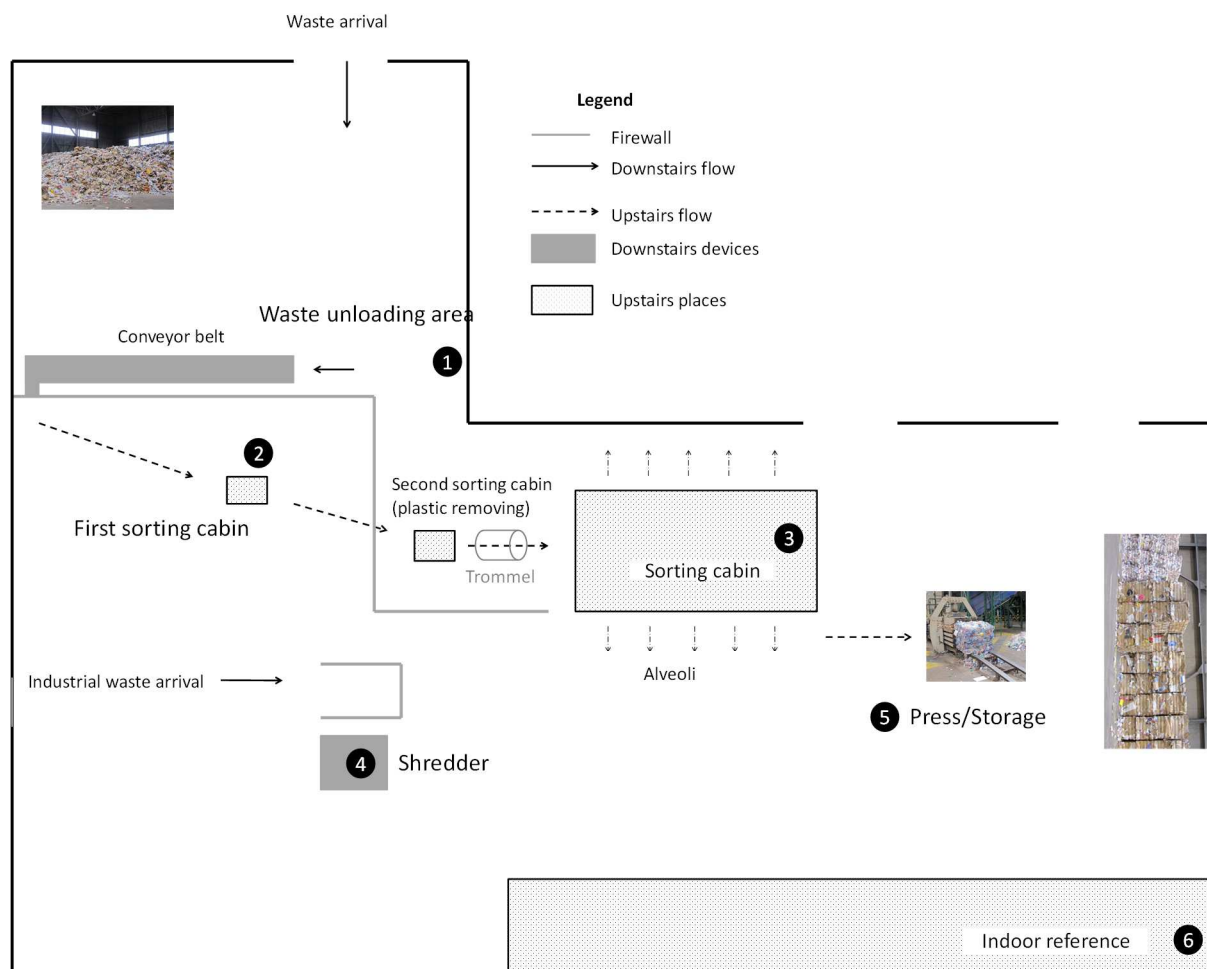
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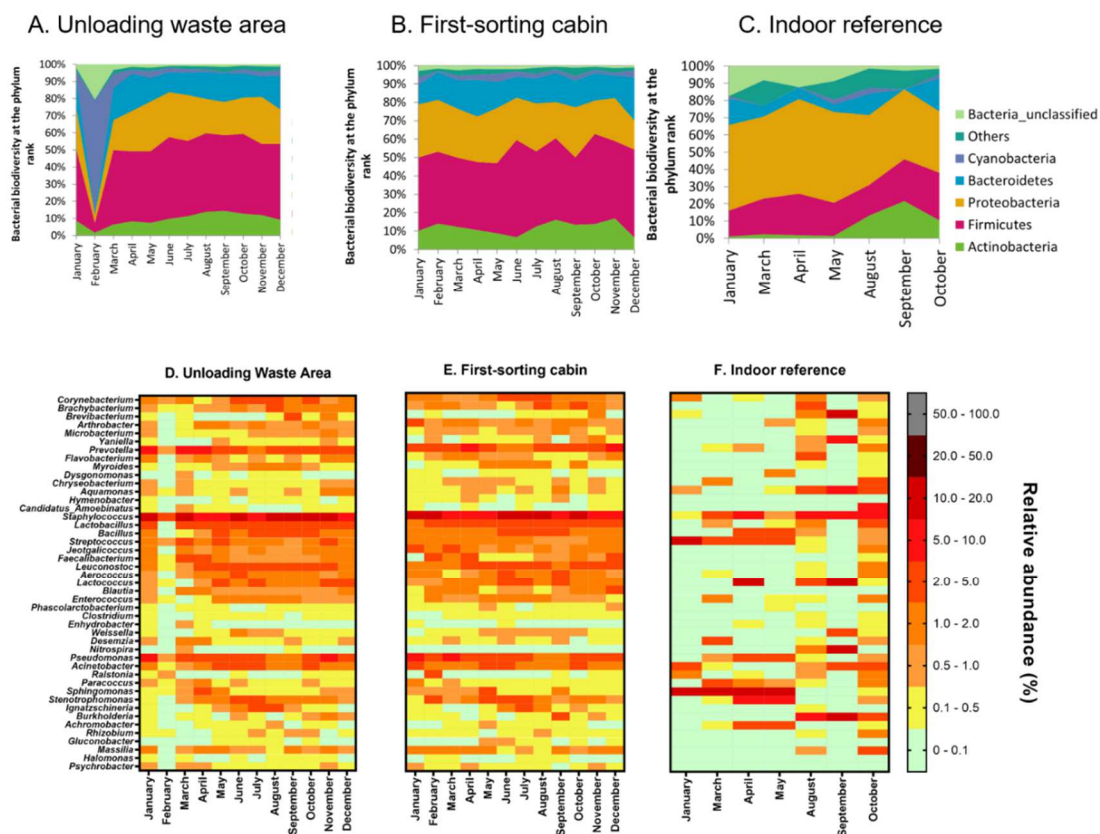
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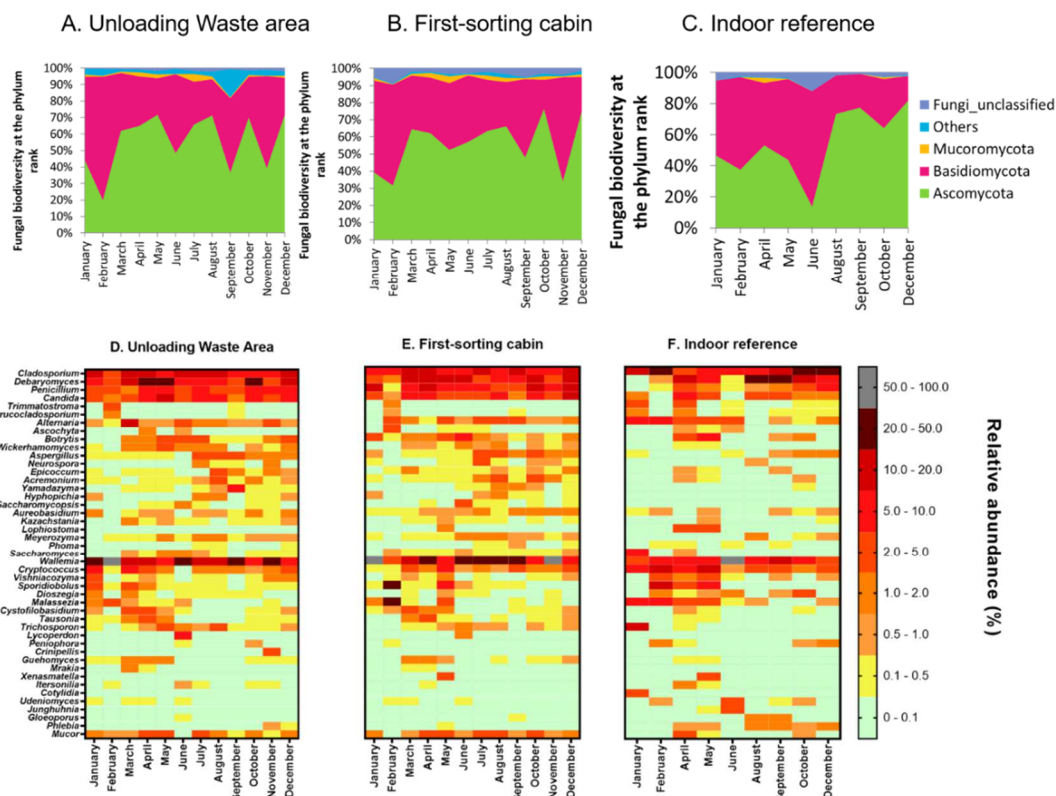
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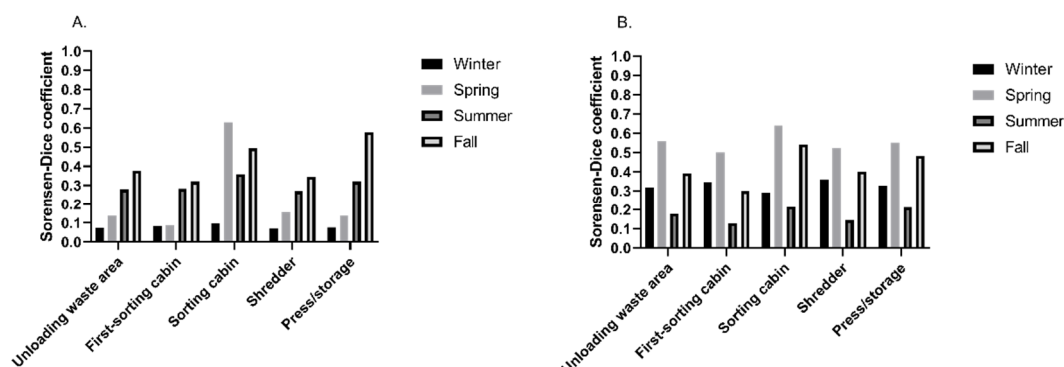
**Figure 1:** Schematic organization of the household waste sorting plant and location of the sampling points.



**Figure 2:** Bacterial biodiversity at the phylum (A, B and C) and at the genus (D,E and F) levels in bioaerosols collected in the unloading waste area, in the first-sorting cabin and in the indoor reference. To facilitate the reading, only the 45 main genera found in a proportion superior than 0.3% were represented.

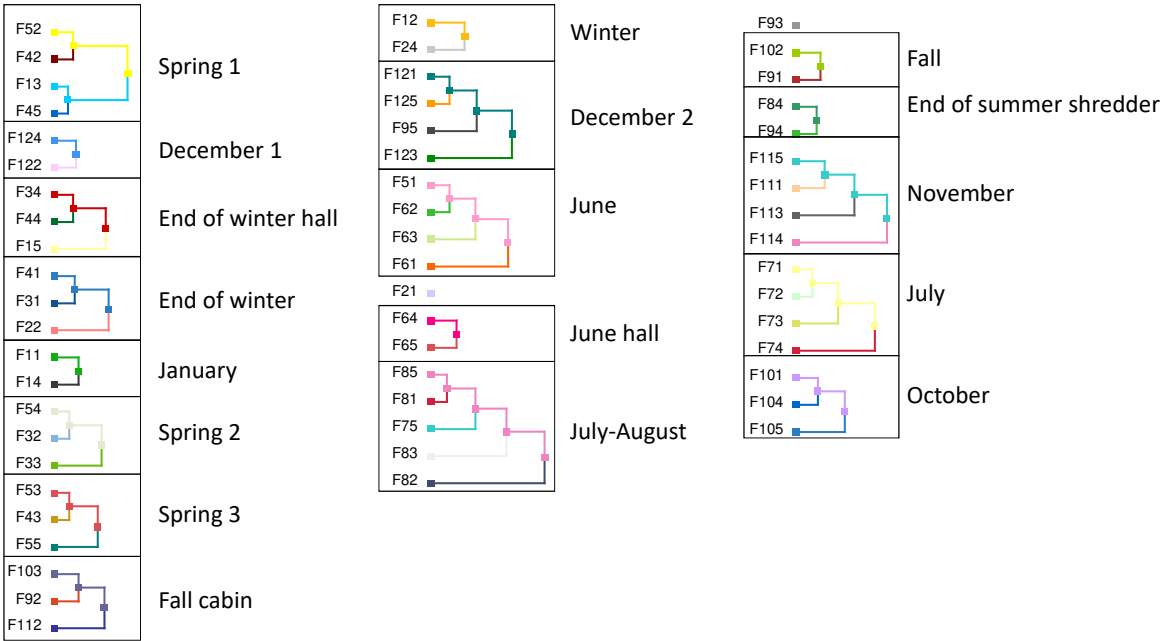


**Figure 3: Fungal biodiversity at the phylum (A, B and C) and at the genus (D, E and F) levels in bioaerosols collected in the unloading waste area, in the first-sorting cabin and in the indoor reference. To facilitate the reading, only the 45 main genera found in a proportion superior than 0.3% were represented.**

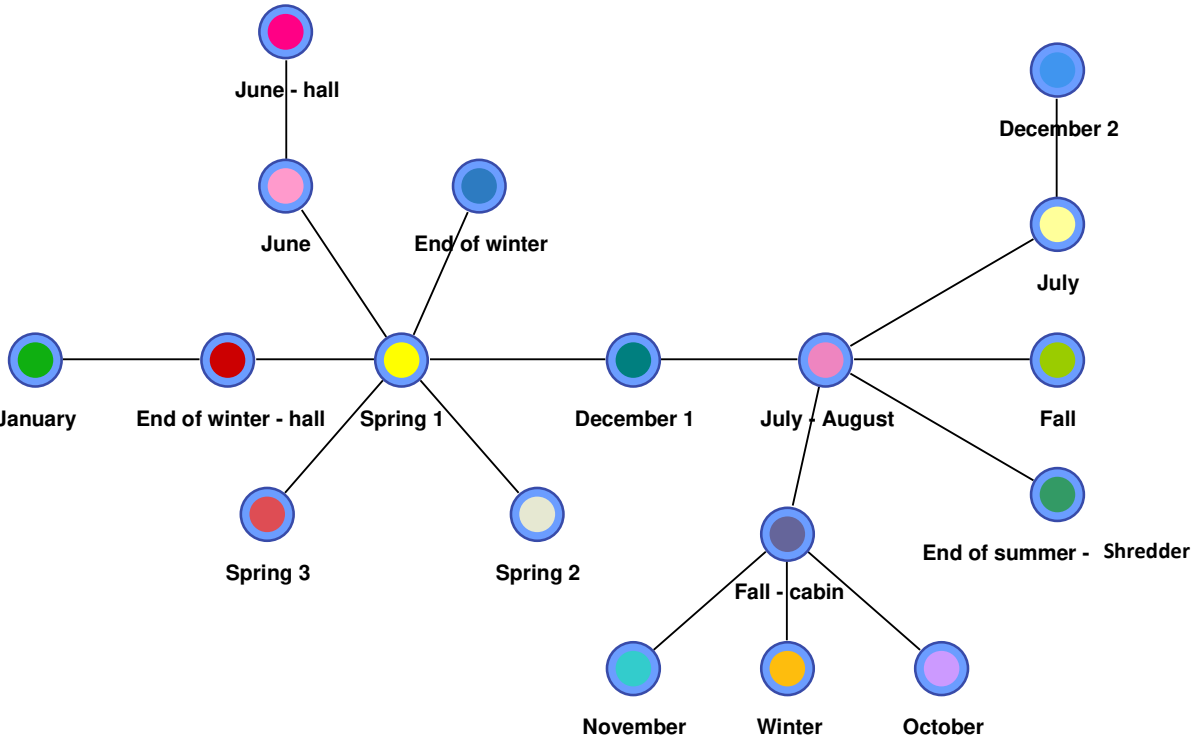


**Figure 4: Sorensen-Dice coefficient for A. bacterial and B. fungal biodiversity. This coefficient represents a distance of community composition between a sampling point and the indoor reference. A coefficient near 1 means that the two samples had a similar composition.**

A



B



**Figure 5:** Bayesian network analysis of biodiversity data for fungi in bioaerosol samples collected over one year in the WSP. **A:** Details of each cluster regrouping samples with statistical similar composition in fungal genera; bioaerosol samples were clustered depending on their common bacterial genera and each cluster corresponds to a node. Identification of samples in nodes was made as followed: The letter F reports to fungal sample, the first number to the month and the last number to the sampling location. **B:** Links representing the statistical correlations between nodes ( $p$  value < 0.0001).



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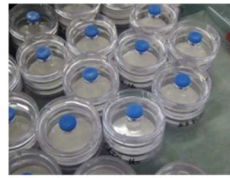
<b>Bacteria</b>	<b>Unloading area</b>	<b>31,282</b>	<b>15,290</b>	<b>35,073</b>	<b>12</b>	<b>None</b>
	First-sorting cabin	30,393.5	4,918	42,222	12	None
	Sorting cabin	33,875	1,581	37,463	10	February, November
	Shredder	33,467	2,660	39,888	11	November
	Press/storage	28,075	-	40,098	11	February
	Indoor reference	2,078	-	22,105	7	February, June, July, November, December
<b>Fungi</b>	<b>Unloading area</b>	<b>26,894</b>	<b>19,552</b>	<b>46,777</b>	<b>12</b>	<b>None</b>
	First-sorting cabin	30,346	16,681	44,581	12	None
	Sorting cabin	23,309.5	12,199	49,119	12	None
	Shredder	25,825.5	12,583	47,207	12	None
	Press/storage	26,144	12,934	43,240	11	March
	Indoor reference	23,477	12,325	52,283	8	February, March, July, October

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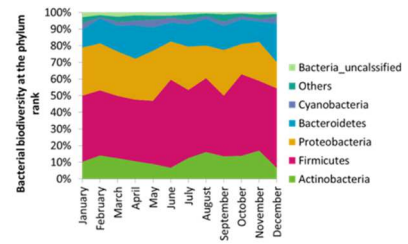
1012 **Table 1:** Overview of high-throughput sequencing data obtained from bioaerosol  
1013 samples taken in the WSP during the year of sampling. Bacterial biodiversity was  
1014 measured by sequencing of a region of 16S rDNA and fungal biodiversity was  
1015 measured by sequencing of ITS2 region.



6 locations, 12 months



Bioaerosols sampling



Bacterial and fungal biodiversity analysis