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

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17 **ABSTRACT:**

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

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

36

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

40 Abbreviation list

41 CFC closed-face cassette

42 (r)DNA (ribosomal)desoxyribonucleic acid

43 HTS high-throughput sequencing

44 WSP waste sorting plant

45 IR Indoor Reference

46 ITS internal transcribed spacer

47 OEL Occupational Exposure Limit values

48 OTU Operational taxonomic unit

49 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

74 Exposure Limit values (OELs) available for airborne biological agents. As the
75 health issues caused by bioaerosols are linked to the type of species and
76 compounds to which workers are exposed, one of the reasons explaining the
77 current lack of OELs may be linked to the complex composition of bioaerosols
78 in the workplace atmosphere, such as WSP. Most of the existing published
79 studies provide biodiversity data in WSP by culture and identification of
80 microorganisms on nutrient media. They revealed that predominant airborne
81 microorganisms in WSP belonged to *Aspergillus* and *Penicillium* for fungi and
82 to *Bacillus* and *Staphylococcus* for bacteria (Breum et al., 1999b; Lehtinen et
83 al., 2013; Madsen et al., 2019; Rahkonen, 1992; Viegas et al., 2014). A recent
84 study has provided new data based on high-throughput sequencing (HTS)
85 measurements, confirming previous culture-dependant findings, as well as
86 highlighting new microbial taxa such as *Wallemia*, for fungi (Degois et al.,
87 2017). Indeed, HTS has provided a new approach for environmental microbial
88 studies over the last decade. The technique was already being used to
89 characterize microbial communities in swine houses (Kumari et al., 2016),
90 composting facilities (Mbareche et al., 2017b) or wastewater treatment plants
91 (Lin et al., 2014). It provides a solution to some of the limitations of the culture
92 method but it also introduces other types of biases such as DNA extraction
93 efficiency or errors of sequencing (Duquenne, 2018; Mbareche et al., 2017a).
94 Nevertheless, the use of HTS in WSP has enabled a more complex
95 bioaerosol composition to be revealed, with more microbial taxa detected, as
96 opposed to culture (Degois et al., 2017).

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

99 temperature, relative humidity and, more generally, the seasons were found to
100 be potential determinants of biodiversity of airborne microbial communities for
101 wastewater treatment plants (Ding et al., 2016), swine houses (Kumari et al.,
102 2016) and outdoor air (Bowers et al., 2012). As the impact of each of these
103 factors on biodiversity varies from study to study, no conclusions can be
104 drawn regarding the mode of action for environmental factors. To date, such
105 an investigation has not yet been carried out for bioaerosols emitted in French
106 WSPs. Yet, a better understanding of factors affecting airborne communities
107 would improve knowledge about microbial exposure for workers and would
108 provide useful data for establishing further sampling strategies as well as for
109 managing biological risk. Biodiversity measurements were taken in the
110 working environment of a waste sorting plant (WSP) over the period of one
111 year.

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

117

118 **2 Material and methods**

119 **2.1 Description of the waste sorting plant**

120

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

123 and industrial waste collected from 44 towns (representing approximately
124 235,000 inhabitants). An overview of the general organization of the plant and
125 the sorting activities is provided in Figure 1. Household waste comprised
126 various materials (cardboard, different types of plastic packaging, cartons and
127 metals) unloaded directly onto the ground by trucks in a waste unloading area
128 (Figure 1 ❶). Waste was then selected at random to be fed into the sorting
129 chain to then be manually sorted by workers in sorting cabins (Figure
130 1 ❷and❸) before being stored in pits. Industrial waste such as furniture or
131 wood items was fed into a wood chipper (Figure 1 ❹) and sent to an
132 incineration unit adjacent to the WSP along a conveyor belt to supply the hot
133 water system of the neighbouring town. As mentioned above, incoming waste
134 was treated at random but the mode of treatment was mostly based on a “last
135 in, first out” approach. However, it was not possible to specify the storage time
136 and the nature of the waste treated on the days of sampling.

137 The company daily recorded the amount of waste delivered to the unloading
138 area, shredded or squeezed by the press. The median mass was 71,640 kg
139 (59,200-89,920 kg) in the unloading area, 16,320 kg (5,040-24,500 kg) for the
140 shredder, and 23,296 kg (18,800-39,151 kg) for the press. Even if it mainly
141 included food packaging, most of the treated waste was of varying materials
142 (between 59% and 86% of daily mass of waste) and the remaining fraction
143 consisted of large cardboard items and papers/magazines processed through
144 a separate sorting chain, which was not examined in this study.

145

146 **2.2 Sampling methods and strategy**

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

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

170 **2.3 Pre-treatment of samples and DNA extraction**

171 To elute the particles from the filter, a volume of 5 mL of a sterile solution
172 (0.01 % Tween 80 in ultra-pure water) was introduced into each CFC through
173 the inlet. Then, the cassette inlets and outlets were closed with caps and
174 shaken (Heildolph®, Multi-Reax shaker) at 2000 rpm for 20 min and at room
175 temperature. DNA extraction was performed using a commercial kit
176 (FastDNA® SPIN kit for soil, MP Biomedicals, Illkirch, France), according to
177 the manufacturer's instructions, with 500 µL of eluted particle solution. The
178 DNA samples were stored at -20°C until sequencing.

179 **2.4 DNA sequencing and sequence data analysis**

180 The DNA samples were purified, amplified and sequenced by the INRA
181 Transfert Environnement (Narbonne, France) sequencing platform. The
182 bacterial biodiversity was examined by sequencing the 16S rDNA (v3-v5
183 variable region) using primers F-5'-GTGYCAGCMGCCGCGGTA-3' and R-5'-
184 CCCCGYCAATTCMTTTRAGT-3'. The primers used to amplify the specific
185 fungal barcode ITS2 were w404 5'-
186 CTTTCCCTACACGACGCTCTTCCGATCTTGTGARTCATCGAATCTTTG-3'
187 and w40 5'-
188 GGAGTTCAGACGTGTGCTCTTCCGATCTTCCCTCCGCTTATTGATATGC-3'.
189 For the DNA amplification by PCR, the mixture contained 5 µL of DNA
190 template, 4 µL of dNTPs (25 mM), 1 µL of each primer (100 ng/µL), and 0.5
191 µL of Taq polymerase in a total volume of 50 µL. The PCR cycle was 2 min at
192 95°C (denaturation step), 1 min at 56°C or 65°C for ITS2 or 16S rDNA
193 amplification respectively (annealing step) and 1 min at 72°C (elongation
194 step). The two genetic targets were sequenced using paired-end sequencing

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

208 **2.5 Statistical analysis**

209 **2.5.1 Sample labelling**

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

215 **2.5.2 Diversity analysis**

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

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

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

232 ***2.5.3 Correlation calculation***

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

236 ***2.5.4 Bayesian networks analysis***

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

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

244 Based on this data, the idea is to automatically build a network that highlights
245 the statistical correlations between the measurements, based on the
246 frequency of shared genus between samples. Each measurement becomes a
247 node and whenever a statistical correlation is strong enough (p value $<$
248 0.0001), then a link is established between the nodes (Figure S1).

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

258 **3 Results**

259 **3.1 Sampling conditions**

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

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

278 **3.2 General sequencing data**

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

289

290 **3.3 Bacterial biodiversity and monthly variation**

291 ***3.3.1 Dominant bacterial phyla and their variations***

292 Thirty bacterial phyla were observed but 96% of the reads represented the
293 five most prominent phyla in all the samples (Figure 2A, B, and C):
294 Actinobacteria, Bacteroidetes, Cynaobacteria, Firmicutes and Proteobacteria.
295 Among them, Firmicutes and Proteobacteria were the two most dominant
296 specimens in samples collected in waste treatment areas (median=41.5% and
297 24.6% respectively) and in the IR (median= 20.0% and 48.6% respectively),
298 followed by Actinobacteria and Bacteroidetes. The variation of the main
299 bacterial phyla over the course of the study is illustrated in Figure 2A, B, and
300 C for three sampling locations: unloading waste area, in the first-sorting cabin
301 and in the IR (for the other sampling points, see Figure S4A, B, and C in the
302 supplementary materials). A high proportion of Cyanobacteria was recorded in
303 February in the unloading waste area with 65.7% of the bacterial reads
304 attributed to this phylum. This result was not observed at any other sampling
305 site or at any other time during the sampling campaign.

306 ***3.3.2 Dominant bacterial genera and their variations***

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

314 As for phyla, the description of the results is focused on three sampling points:
315 the unloading waste area, the first-sorting cabin and the IR (Figure 2D, E, and
316 F). Results from the other sampling plants are presented in Figure S4 of the
317 supplementary materials. One of the most dominant bacterial genera found at
318 each sampling point, including the IR, was *Staphylococcus* (Figure 2D, E and
319 F) with a median of approximately 9% of relative abundance in all sampling
320 points, except near the press in March. *Prevotella* and *Pseudomonas* were
321 present throughout the whole year in substantial proportion in the two waste
322 treatment locations (about 3%) and in lower proportion in the IR (less than
323 0.6%). *Streptococcus* was also found every month but in lower proportions
324 with a maximal value of 11.3%, observed in the IR in January. Other bacterial
325 genera were frequently found during the year in the bioaerosols collected in
326 the unloading area and in the first sorting cabin: *Lactobacillus* (3%), *Bacillus*
327 (1%), *Leuconostoc* (2%) which was absent in the IR, as well as *Aerococcus*,
328 *Lactococcus*, *Acinetobacter*, and *Corynebacterium*.

329 **3.4 Fungal biodiversity and its variation**

330 **3.4.1 Dominant fungal phyla and their variations over the year**

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

338 **3.4.2 Dominant fungal genera and their variations over the year**

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

343 The relative abundance of the fungal microbiome found in the unloading
344 waste area, the first-sorting cabin and the IR and its variation over the year is
345 described in Figure 3. The fungal biodiversity found in the sorting cabin, near
346 the shredder and near the press is illustrated in Figure S5 of the
347 supplementary materials. Among the core microbiome, 8 genera were found
348 in huge proportions throughout the whole year: *Cladosporium*, *Alternaria*,
349 *Debaryomyces*, *Penicillium*, *Candida*, *Wallemia*, *Cryptococcus*, and *Mucor*
350 (Figure 3 and Figure S5 of the supplementary materials). *Wallemia* was the
351 predominant genus in the two occupational sampling points (Figure 3 D, E,
352 and F). It represented 17.3% (up to 66.4% in February) and 21.4% (up to
353 54.8% in November) of the ITS2 reads in the unloading waste area and in the
354 first sorting cabin respectively. In the IR, *Wallemia* was also recorded in
355 substantial proportion (6.5%). *Cladosporium* and *Debaryomyces* were also
356 dominant genera found at the three sampling locations throughout the year.
357 *Cladosporium* was the predominant genus in the IR. In the unloading waste
358 area and in the first-sorting cabin, about 5% of fungal reads were attributed to
359 *Penicillium* and *Candida* (5.2% and 6.1% in the unloading waste area; 5.2%
360 and 4.5% in the first-sorting cabin). They only represented about 1% in the IR.

361 **3.5 Variation of alpha biodiversity**

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

366 **3.6 Season and humidity as the main factors of microbial diversity**

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

371 There was more similarity between bacterial communities in waste treatment
372 areas and IR in fall-summer than in winter-spring. A positive correlation was
373 observed between the QS values and temperature values (Spearman r_s p-
374 value=0.51, n=20). The higher the temperature was, the closer the bacterial
375 composition of bioaerosols was to that of IR.. The sampling location had an
376 impact on bacterial biodiversity regarding Sorensen-Dice coefficient (Kruskall-
377 Wallis test: p=0.003). For fungal biodiversity, the values varied from 0.12 to
378 0.64 (Figure 4B). The coefficient was minimal (about 0.15) in summer and
379 maximal in spring with values superior to 0.5. The fungal composition in
380 bioaerosols collected at the IR and at the sampling points in the WSP showed
381 closer similarities in spring and in fall. The fungal biodiversity was negatively
382 correlated with the relative humidity of the air (Spearman r_s p-value=-0.67,
383 n=20), the higher the RH, the more the composition of fungal bioaerosols
384 differed, as compared to the IR.

385 **3.7 Results from Bayesian networks statistics**

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

410 **4 Discussion**

411 **4.1 Overview of the sampling campaign**

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

418 **4.2 Dominant airborne microorganisms in the WSP**

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

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

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

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

459 (Fröhlich-Nowoisky et al., 2009; Li et al., 2019; Zeng et al., 2004). They
460 usually differ in their xerotolerance and tolerance to solutes (NaCl, MgCl₂),
461 growth rate and temperature, enzymes and metabolites production profiles as
462 well as in their size of conidia (Jančič et al., 2015; Jančič et al., 2016). The
463 predominance of *Wallemia* in bioaerosols from the WSP can be explained by
464 the presence of food packaging among waste (trays, yoghurt pots and sauce
465 jars, beverage bottles, etc.) on which food residue remains, creating favorable
466 conditions for their growth and survival. This residue may come from food
467 items with the water activity reduced initially or that has been lowered by
468 dehydration during the period prior to sorting. Similar hypotheses can be put
469 forward to explain the predominance of other fungal genera such as
470 *Cladosporium* and *Penicillium*, as several species belonging to these genera
471 were found to be able to grow in the presence of low water activity (Gunde-
472 Cimerman et al., 2003). Their growth and survival on paper and cardboard
473 have also been shown (Das et al., 1997).

474

475 The presence of *Wallemia* in bioaerosols from WSP has already been
476 demonstrated in our previous published study carried out using molecular
477 methods in the same working environment (Degois et al., 2017). This result
478 contrasts with the results of studies conducted using culture-based methods
479 for which this genus has not been identified (Tolvanen, 2001; Viegas et al.,
480 2014). Fungal species belonging to *Wallemia* have a very low growth rate on
481 usual culture media and its numeration is usually impaired due to masking by
482 faster growing species. Thus, its presence in different environments is
483 underestimated while using culture-based methods. On the other hand, the

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

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

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

507 **4.3 Reported health disorders cause by the detected microbial taxa**

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

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

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

532 health effects such as irritation of the upper airways or mucous membranes,
533 inflammation (Bønløkke et al., 2006; Lawniczek-Walczyk and Gorny, 2010)
534 and irritation of the respiratory system, fever and diarrhea (Duquenne et al.,
535 2013; Liebers et al., 2008). Exposure to these compounds was reported in
536 other plants treating solid and dry waste (Lehtinen et al., 2013; Tolvanen,
537 2001).

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

555 **4.4 Sorting activities compared to the indoor reference**

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

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

595 **4.5 Seasonal variations of microbial biodiversity in bioaerosol**

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

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

615 Several studies found a seasonal variability of microbial biodiversity in
616 bioaerosols but the impact of the season on microbial communities depended
617 on the study and the investigated place (Bowers et al., 2012; Ding et al.,
618 2016; Fröhlich-Nowoisky et al., 2009; Kumari et al., 2016; Nehme et al.,
619 2008). No general tendency was observed with regards the published data
620 and the impact of environmental parameters is difficult to predict (Liao and
621 Luo, 2005). To our knowledge, the impact of environmental parameters on
622 airborne biodiversity has not been assessed in a waste sorting plant and
623 therefore, our results cannot be compared with any other published data.

624 **4.6 Other determinants of biodiversity in bioaerosols**

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

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

641 The impact of the environment on microbial biodiversity in bioaerosols is
642 multifactorial and complex and combines not only the climatic conditions but
643 also others parameters such as the amount and nature of waste or other
644 pollutant sources. This confirms that microbial biodiversity and more
645 generally, bioaerosol composition would be multifactorial and difficult to
646 characterize. So far, other parameters have also been proven to impact
647 microbial concentrations such as human activity (Maron et al., 2006), particles
648 size and density of urban area (Fang et al., 2007), air pollution (Gao et al.,
649 2015; Gao et al., 2016), CO₂ concentrations (Chao et al., 2002), operations of
650 a process (Tolvanen, 2001), indoor air pollutants (Wang et al., 2010), the site
651 investigated (Fracchia et al., 2006) and physical, environmental and
652 ecological factors (Emerson et al., 2016).

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

656 **4.7 Limitations of the study**

657 A large number of samples were collected, at different workstations and at
658 different times of the year. This provided a satisfactory overview of the
659 airborne microbial biodiversity in the WSP. Stationary samples were adequate
660 in providing information about the exposure of workers in the cabins, but less
661 apt regarding machine operators who moved around during their shift.

662 Samples near the press, the shredder and the unloading area therefore did
663 not allow for the characterization of the bioaerosol exposure for these
664 employees. However, sampling on filters and analysis by molecular biology
665 allowed for long-term sampling (5 hours) for a better representation of
666 exposure assessment than short-term sampling with Andersen type impactors
667 used for the analyzes by culture. Molecular biology methods and particularly
668 HTS are limited due to biases for DNA extraction, PCR or sequence analysis
669 (Haugland et al., 1999; Mbareche et al., 2017b; Yamamoto et al., 2014).

670 These techniques are based on DNA analysis, which does not provide
671 information on the viability and pathogenicity of airborne microorganisms and
672 the associated health risk for workers. In addition, short sequences that are
673 generated by sequencing limit the identification of microorganisms at the
674 genus level, which may hamper a sound health risk assessment. However,
675 HTS provides an overview of microbial biodiversity in poorly characterized
676 fields. Finally, exposure to viruses was not assessed in this study.

677

678 **4.8 Further prospects**

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

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

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

704

705 **5 Conclusions**

706

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

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

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

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

729

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739 **8 References**

740

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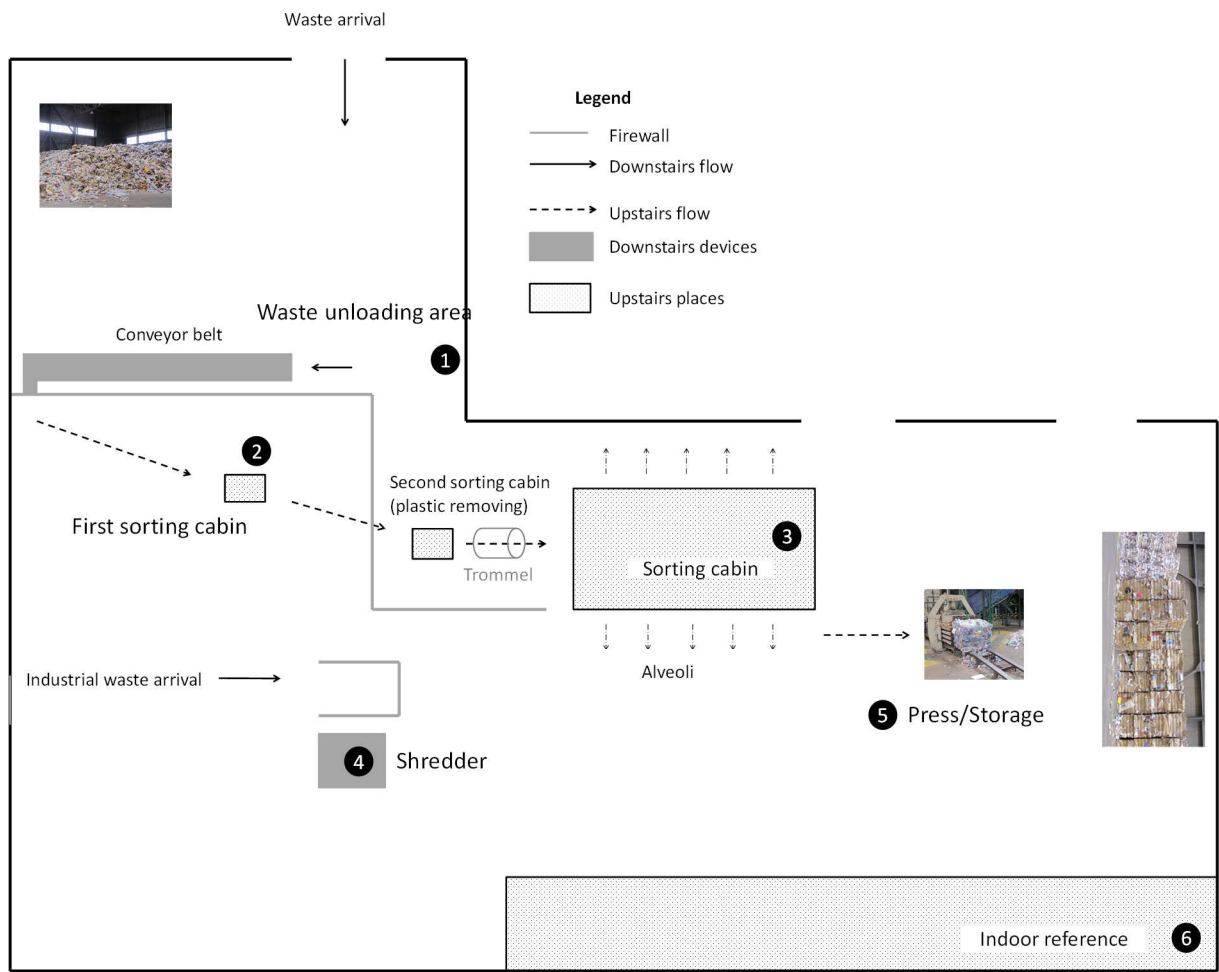
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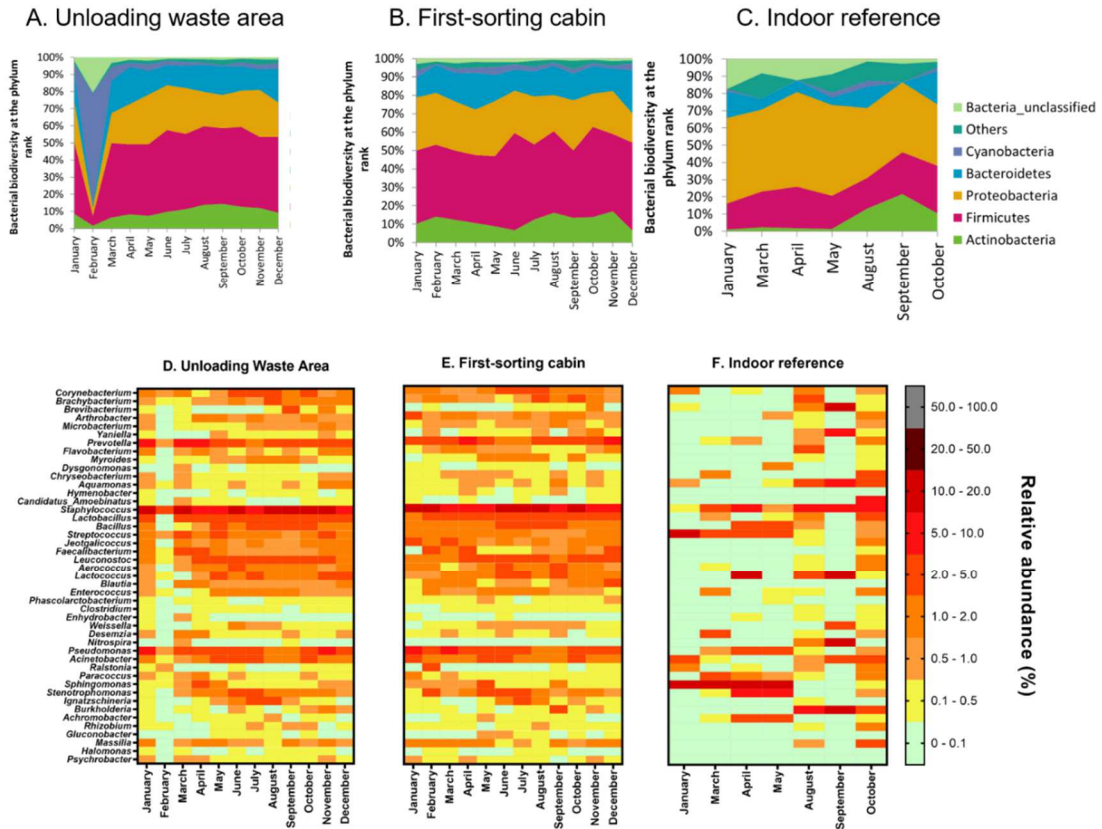


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977 **Figure 1:** Schematic organization of the household waste sorting plant and location
 978 of the sampling points.

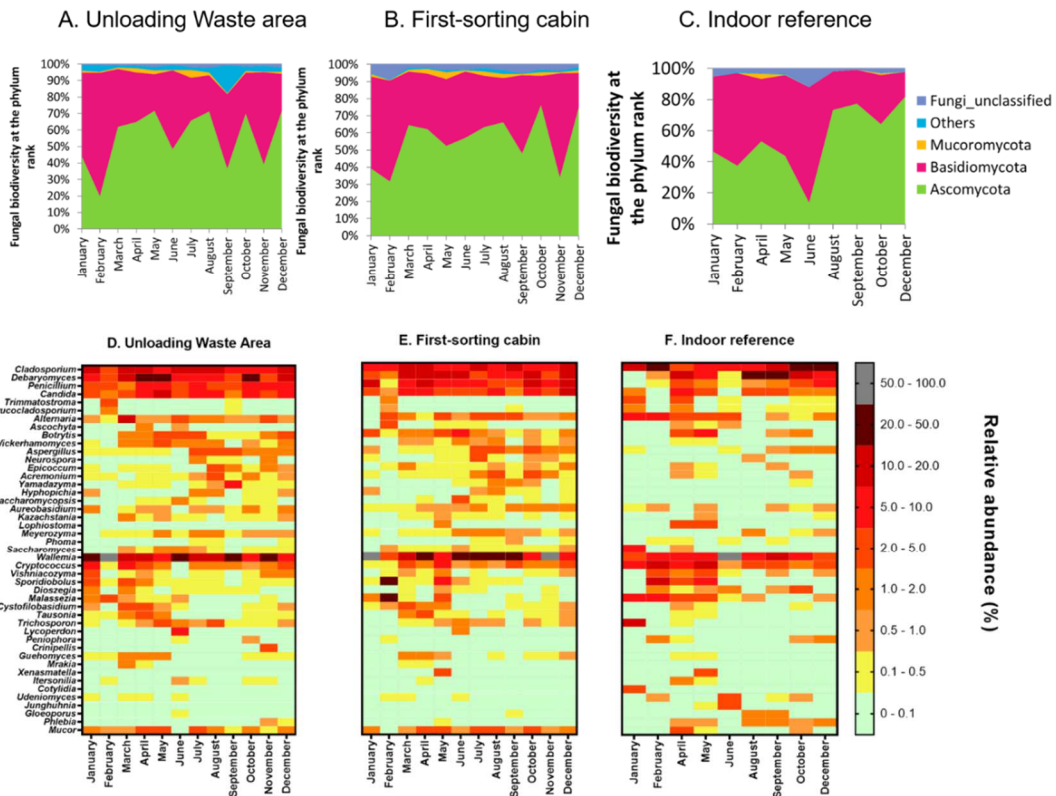
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981 **Figure 2:** Bacterial biodiversity at the phylum (A, B and C) and at the genus (D,E and
 982 F) levels in bioaerosols collected in the unloading waste area, in the first-sorting
 983 cabin and in the indoor reference. To facilitate the reading,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

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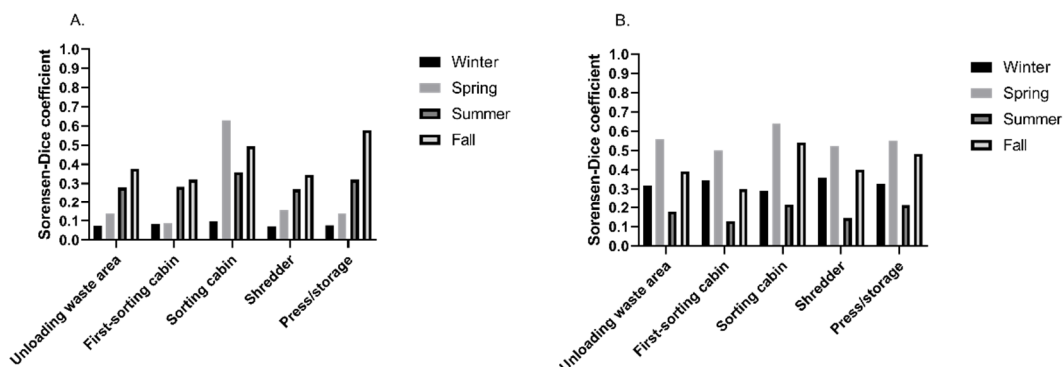


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987 **Figure 3: Fungal biodiversity at the phylum (A, B and C) and at the genus (D, E**
 988 **and F) levels in bioaerosols collected in the unloading waste area, in the first-**
 989 **sorting cabin and in the indoor reference. To facilitate the reading,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

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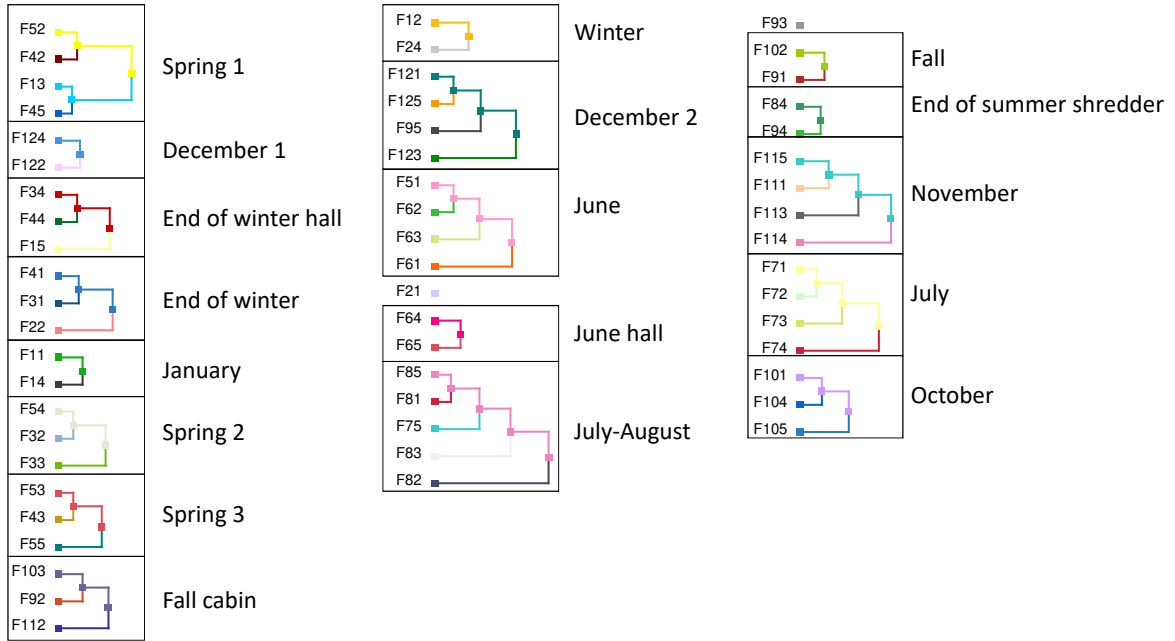


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994 **Figure 4: Sorensen-Dice coefficient for A. bacterial and B. fungal biodiversity. This**
 995 **coefficient represents a distance of community composition between a sampling**
 996 **point and the indoor reference. A coefficient near 1 means that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

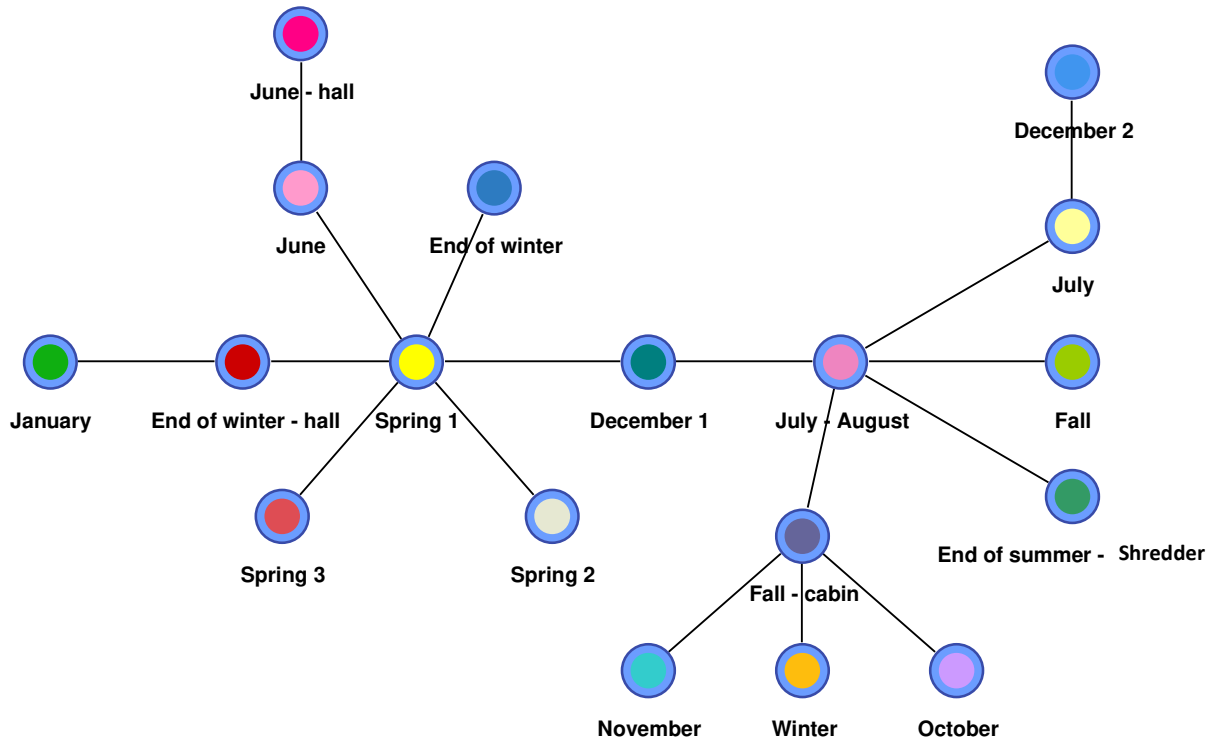
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A



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B



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1001 **Figure 5:** Bayesian network analysis of biodiversity data for fungi in bioaerosol
 1002 samples collected over one year in the WSP. **A:** Details of each cluster regrouping
 1003 samples with statistical similar composition in fungal genera; bioaerosol samples
 1004 were clustered depending on their common bacterial genera and each cluster
 1005 corresponds to a node. Identification of samples in nodes was made as followed: The
 1006 letter F reports to fungal sample, the first number to the month and the last number to
 1007 the sampling location. **B:** Links representing the statistical correlations between
 1008 nodes (p value < 0.0001).

1009

Bacteria	Unloading	31,282	15,290	35,073	12	None
	area					
	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
Fungi	Unloading	26,894	19,552	46,777	12	None
	area					
	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

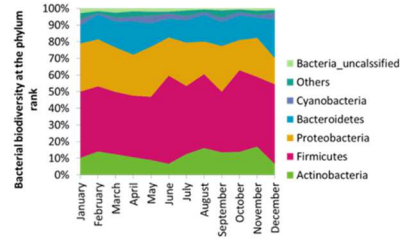
1012 **Table 1:** Overview of high-throughput sequencing data obtained from bioaerosol
1013 samples taken in the WSP during the year of sampling. Bacterial biodiversity was
1014 measured by sequencing of a region of 16S rDNA and fungal biodiversity was
1015 measured by sequencing of ITS2 region.



6 locations, 12 months



Bioaerosols sampling



Bacterial and fungal biodiversity analysis