The on-line detection of biological particle emissions from selected agricultural materials using the WIBS-4 (Waveband Integrated Bioaerosol Sensor) technique.

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34 **Abstract**

35 Agricultural activities have, for some time, been linked to adverse health effects such as Farmers'
36 lung, hypersensitivity pneumonitis, aspergillosis and chronic obstructive pulmonary disease (COPD)
37 This connection is known to be, at least in part, due to the numerous microbiological organisms that
38 live and grow on materials found in occupational settings such as barns, animal shelters, stables and
39 composting sites. Traditional techniques for determining biological release of fungal spores and
40 bacteria require intensive, experienced human resources and considerable time to determine ambient
41 concentrations. However more recently the fluorescence and light scattering signals obtained from
42 primary biological aerosol particles (PBAP) have been utilized for their near real-time counting and
43 characterisation abilities.

44 In the current study, data collected for the bioaerosol types released from hay and silage were counted
45 and identified using a combination of the WIBS-4 bioaerosol sensor approach and impaction/optical
46 microscopy. Particle emissions were characterised according to particle numbers, their size
47 distributions, particle asymmetry values and fluorescence characteristics. The variables obtained were
48 shown to provide potential "fingerprint" signatures for PBAP emissions emanating from two
49 important compost components, namely, silage and hay. Comparisons between the data acquired by
50 the WIBS-4 bioaerosol sensor, optical microscopy findings and also previous literature suggest that
51 the likely identification of *Aspergillus*/*Penicillium* type spores and bacterial species released from hay
52 and silage was achieved on a relatively rapid time-scale.

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68 **Introduction**

69 Primary biological aerosol particles (PBAP) are ubiquitous in the atmosphere and both their
70 dimensions and (bio)-chemical composition have led to many studies on their potential health effects
71 (Garrett et al., 1998, Mullins and Seaton, 1978, Kirkhorn and Garry, 2000).They include a range of
72 differing types of particle including pollen, fungal spores, bacteria and viruses. Due to the diameters
73 of certain PBAP and sub-PBAP entities being in the micron and less range, they are able to penetrate
74 deep toward the inner lining of the lungs into the alveoli (Mullins and Seaton, 1978, Hassim et al.,
75 1998). Larger bioaerosol particles such as pollen can get trapped in the nasopharyx and trachea; very
76 small ones such as viruses can travel much more extensively, even across the blood brain barrier
77 (Shors, 2012, Hassim et al., 1998). Once viral, bacterial or fungal components have travelled into the
78 lung they can infect and contaminate healthy lung cells and cause numerous respiratory illnesses and
79 infections (Portnoy et al., 2005).

80 Occupational activities such as those associated with agriculture have long been thought to be
81 potentially hazardous given the contact with harmful vapours/gases and “dusts”, which contain
82 significant levels of bacterial and fungal components (Kirkhorn and Garry, 2000, Rylander and
83 Jacobs, 1994). Many respiratory illnesses and infections such as Farmers’ lung, hypersensitivity
84 pneumonitis, aspergillosis and chronic obstructive pulmonary disease (COPD) have all been linked
85 with agricultural work (Weinhold, 2007, Bailey et al., 2008, Kirkhorn and Garry, 2000, Ader et al.,
86 2005). For some time fungal spores such as *Aspergillus fumigatus* and *Penicillium* have been
87 connected to the illnesses listed above not only in causative roles but also as agents to further develop
88 the conditions (Ader et al., 2005, Gregory and Lacey, 1963). Indeed aspergillosis owes its name to the
89 fungal spore associated with its adverse effect on the lungs.

90 Detection techniques for fungal spore types have been generally confined to methods such as
91 impaction of air samples onto adhesive sample substrates before analysis using optical microscopy.
92 This undertaking relies on the intrinsic skill of the identifier and is also very labour intensive because
93 careful preparation of the substrate is required for accurate analysis. Nonetheless this combination of
94 methodologies has been used previously to show that very large number concentrations of spores can
95 build up at agricultural sites. Indeed using the above approach Baruah showed that air sampled from a
96 cowshed could contain as many as 16,000,000 spores per m^3; the preponderance of these spores were
97 deemed to be *Aspergillus/Penicillium* as a grouping because they are difficult to distinguish by optical
98 microscopy (Baruah, 1961). The large magnitude of the spores that were released was ascribed to the
99 active media that hay and straw provide and upon which spores can grow readily. They are therefore
100 known to harbour large quantities of spores such as *Aspergillus* and *Penicillium*. In a similar manner,
101 the related study by Gregory *et al* showed as many as 3,000,000 spores/gram of hay could be liberated
102 from differing samples (Gregory and Lacey, 1963).

103 Other, more modern, methods of fungal spore concentration determination and species identification
104 include the use of culture-based analysis. While these techniques are more exact in their determination
105 of differing species, which cannot be attained for all species using optical microscopy, they also
106 require considerable time for the sampled fungal spore to grow on a suitable agar. Furthermore this
107 method suffers from the possibility of providing an underestimation of the total fungal content
108 because a portion of the fungal spores are generally unviable (Mandrioli et al., 2003).

109 The use of fluorescence and laser scattering to the qualitative and quantitative determination of PBAP
110 represents a relatively new approach to provide for their discrimination and also between biological
111 and chemical particulate matter (Després et al., 2012, Pöhlker et al., 2012). The methodology depends
112 upon the fact that many structural components and secondary metabolites of PBAP species fluoresce.
113 For example, amino acids such as tryptophan and tyrosine, some phenols and terpenoids are all known
114 to be fluorescent constituents of PBAP (Roshchina et al., 2004, Roshchina et al., 1995, Roshchina et
115 al., 1998, Pöhlker et al., 2012, Roshchina, 2003). Published studies since about 1999 have shown that
116 certain PBAP species can be monitored and discriminated using fluorescence spectroscopy (Pan et al.,
117 1999, Pinnick et al., 1999, Mitsumoto et al., 2009, Eversole et al., 2001, Pan et al., 2011). Initially, the
118 detection efficiency of such a direct counting technique was said to be low in comparison with the
119 methods discussed above even though few studies had been published in the area (Cartwright, 2009).
120 Hence more recently an effort has been made to properly assess the use of fluorescence for the
121 instantaneous differentiation between biological and non-biological particles (Healy et al, 2012).

122 In this regard instrumentation such as the Waveband Integrated Bioaerosol Sensor (WIBS) series and
123 the TSI UV-ASP (Ultraviolet Aerodynamic Particle Size Spectrometer) have been developed. They
124 represent novel on-line techniques for the sampling of, and discrimination between, biological and
125 non-biological particles (Hairston et al., 1997, Kaye et al., 2005, Kaye et al., 2004, Kaye et al., 2007).
126 Using UV flash lamps tuned to 280 nm and 370 nm, for the WIBS or a laser tuned to 355 nm for the
127 UV-APS, bio-fluorophores such as tryptophan and NADH/NAD(P)H, among others that are common
128 to biological entities, can be excited and the resulting emission/scattering profiles used to separate
129 them from non-biological particles. Such a non-destructive and rapid process, in theory, could be used
130 to determine and characterise individual PBAP their number counts with much greater time-resolution
131 and rapidity than any other techniques that are currently available.

132 Previously, a UV-APS system has been used within a number of controlled laboratory settings to
133 assess and quantify both *Aspergillus* and *Pencillium* cultures (Kanaani et al., 2007, Agranovski et al.,
134 2003, Agranovski et al., 2004, Kanaani et al., 2008, Agranovski and Ristovski, 2005). The study by
135 Kanaani et al investigated the percentage of fluorescent *Aspergillus* and *Pencillium* CFU over a given
136 incubation time while also measuring their size increase over the same period. Hence the UV-APS
137 method was found to be able to distinguish between both species under well controlled laboratory

138 experiments. However it was also assessed as unlikely that such a level of identification and
139 discrimination under ambient, field conditions could be achieved. Much more recently the multi-
140 wavelength WIBS instrumental approach has been used in a controlled laboratory setting, for the
141 possible classification between several differing PBAP species (Healy et al., 2012a). Furthermore
142 both types of instrumentation have been used for environmental ambient field-sampling campaigns in
143 a number of different regions and climates (Gabey et al., 2010, Stanley et al., 2011, Huffman et al.,
144 2010, 2012, Toprak et al., 2013).

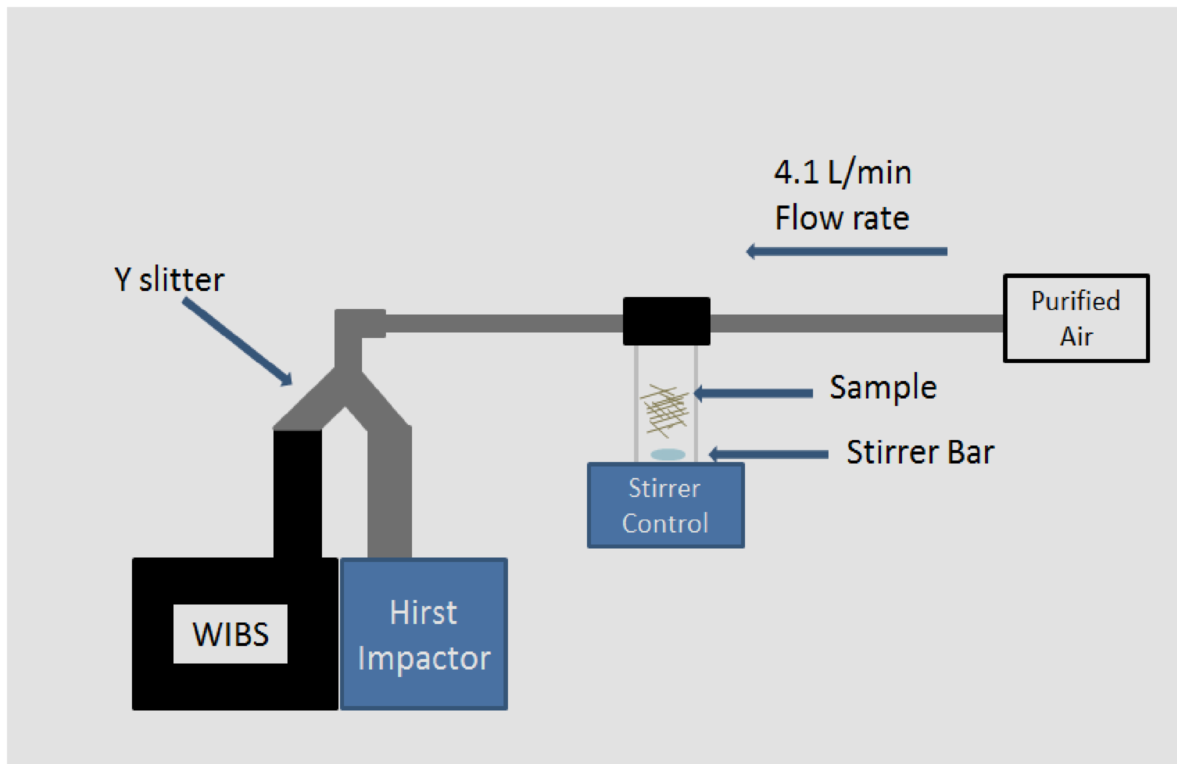
145 In the study described here, the particle emissions from hay and silage samples were characterised
146 under controlled laboratory conditions by comparing novel (WIBS-4) and traditional methods (optical
147 microscopy) for PBAP measurement. Efforts to obtain multi-parameter signatures classified according
148 to particle size, "shape" and fluorescent characteristics were subsequently undertaken. The approach
149 was built on the use of data bases comprising many individual particle signals obtained with the
150 WIBS-4 in terms of their fluorescence and physical characteristics. The "dust" releases from the hay
151 and silage were also visually characterised using traditional optical microscopy. By these means the
152 ability of the WIBS-4 to analyse both biological and non-biological particles released by composting
153 material was achieved. The results signify the potential for further application of the on-line technique
154 for monitoring composting sites and other waste facilities.

155

156 **Methods, Materials and Instrumentation**

157 *Experimental set-up*

158 A schematic of the set-up used to undertake the experiments described here is shown in Figure 1.
159 Purified air was flowed directly into the sample holder at a rate of 4.1 L/min while the WIBS-4
160 instrument sucked the flow at 2.38-2.42 L/min and the Hirst-type impactor at 2.4 L/min. The sample
161 holder lid was designed so as to force the air over the sample held within the container, in effect
162 causing a cyclonic air flow pattern to envelop the sample. This procedure coupled with extra sample
163 agitation provided by a magnetic rotating, stirrer bar, induced sample fragments and biological entities
164 *etc* to be released.



165

166 **Figure 1: Schematic of the experimental set-up used in the current study. (Not to scale).**

167 The WIBS-4 signals from hay/silage were evaluated with four replicate runs being made. The
168 measurement process involved placing 3-5 g of fresh sample into the container shown in Figure 1 and
169 allowing the air flows and stirrer bar to release particles and/or fragments. The Hirst/SporeWatch
170 impaction sampler and WIBS-4 instrument were then run until over 1000 particle measurements had
171 been made. Thus datasets consisting of 4000 particles each formed the basis of the analyses described
172 below.

173 *Materials*

174 Hay was sourced from a local dry cattle farm and is representative and comparable to other samples
175 located at similar establishments in the region. Hay is grass that has been cut during the summer
176 months and allowed to dry and then formed into bales. Subsequently it is wrapped in a protective
177 material so as to ensure that moisture does not penetrate. The hay sample used in the current study
178 was composed of dry faded yellow/golden grass blades/stalks and was less brittle in texture than fresh
179 cut grass.

180 Silage is similar to hay in that grass is again the base material. However in the samples used here the
181 grass was cut and not allowed to dry; it thus underwent anaerobic digestion and breakdown of the
182 source material, in part, by inoculant bacteria. The silage is generally covered and held in a pit rather
183 than in bales, as hay is. The material used here appeared dark coloured with a distinct odour and was
184 denser than the hay samples. It should be emphasised that at the outset it was not known whether the

185 silage or hay materials contained any particles with biological origin. Hence the analyses performed
186 were essentially “blind”.

187 Samples of “pure” *Aspergillius* and *Pencillium notatum* purchased from Allergon AB Sweden were
188 also investigated during this work. It should be noted that these fungal spore samples did however
189 contain mycelia and hyphae fragments. The samples were stored at 2 ºC in sealed containers and thus
190 had little, if any, interaction with the environment. The spores could be considered aged given that
191 they were kept in storage for over two years.

192 *Instrumentation: WIBS-4 (Waveband Integrated Bioaerosol Sensor)*

193 A WIBS-4 instrument, as described previously (Healy et al., 2012a), was used for the fluorescence
194 experiments described here. It represents the newest version of the suite of single particle instruments
195 developed by the University of Hertfordshire. Previous versions have been tested in a number of field
196 campaign environments such as a tropical forest and in urban sites (Gabey et al., 2010, Stanley et al.,
197 2011, Toprak et al., 2013).

198 The WIBS-4 instrument is based around a central optical chamber about which a continuous-wave
199 635 nm diode laser and two xenon UV flash lamps are located. The diode laser is used for individual
200 particle detection including size and “shape” information. This characterization is achieved through
201 the use of a quadrant photomultiplier tube which uses the scattering properties of a particle to
202 determine its asymmetry factor (AF) and optical size (D_0). The AF value of a particle is determined
203 through the scattered light intensity distribution detected upon the quadrant photomultiplier tube.
204 Spherical particles scatter even amounts of light in each quadrant leading to low AF. The converse is
205 seen for irregular and rod-shaped particles for which differing light intensities are scattered into each
206 quadrant dependent upon particle shape. More detailed descriptions of the origin, meaning and scale
207 of the AF value have been described previously. (Healy et al., 2012a, Gabey et al., 2010)

208 The two xenon UV flash incorporated in the WIBS-4 emit at 280 nm and 370 nm, which are centered
209 on the excitation maxima of the biologically relevant molecules tryptophan and NAD(P)H
210 (Nicotinamide adenine dinucleotide). However it should be mentioned that these are only a few of the
211 known bio-fluorophores that could be excited at these wavelengths. Furthermore some chemical
212 particulates such as diesel particles/droplets and other related organic compounds could act as
213 interferant signals in appropriate conditions as they often absorb and emit in the same regions as the
214 bio-fluorophores mentioned above. However they are generally < 1 μm in size. Upon excitation the
215 fluorescence is evaluated in three detector channels, known as FL1, FL2, and FL3. These channels
216 record the total fluorescence over a wavelength range FL1 = 310-400 nm with both FL2 and FL3 =
217 420-650 nm upon excitation at 280 nm (FL1/FL2) and 370 nm (FL3). Differences in the design of

218 WIBS-4 compared to previous versions of the instrument have been noted elsewhere (Healy et al.,
219 2012a).

220

221 *Optical microscopy analysis*

222 Biological "dust" resulting from each replicate run was sampled using a Hirst-type volumetric
223 impactor "SporeWatch" (Burkard). The particles were collected on a silicone tape (Lanzoni) before
224 being mounted on a microscope slide, which was then viewed using a VWR TR500 compound
225 microscope with magnifications of 400× and 1000×. Images of the particles collected were obtained
226 using a digital camera.

227

228 *Filtering and data analysis*

229 The size limit of detection (D_{50}), in terms of particle counts for the WIBS-4 instrument, has been
230 previously determined and reported elsewhere (Healy et al., 2012b). Therefore particles below ~0.5
231 µm in diameter were not considered for the purpose of the current study and thus were removed from
232 the overall dataset. The limits of detection for each of the three fluorescent channels FL1, FL2 and
233 FL3 of the WIBS-4 were also ascertained using a method comparable to that previously used (Gabey
234 et al., 2009). Hence the WIBS-4 instrument was placed into "forced trigger mode", which in effect
235 causes the xenon flash lamps to fire on empty space. The threshold of fluorescence was then
236 determined by using 3× the standard deviation plus the mean of the modal fluorescence values
237 attained from this "forced triggering" (Gabey et al., 2009). These threshold values for FL1, FL2 and
238 FL3 were, in turn, used to distinguish between non-fluorescent and fluorescent particles sampled
239 throughout the set of experiments.

240 Particles of analytical interest were chosen by the size criterion and the fact that they fluoresced above
241 the determined thresholds in one or more of the FL channels. The data points resulting were used in
242 the analyses described below; particles not reaching both the required size and fluorescent criteria
243 were filtered out accordingly.

244

245 **Results and Discussion**

246 Substantial numbers of fluorescent particles were released by both of the samples investigated here.
247 The signals obtained and their data analyses are summarised below.

248

Particle Size, Shape and Number Count

The number-size distributions of the fluorescent particles released during the hay and silage experiments are shown in Figure 2. An almost simple Gaussian mono-modal peak can be seen for the hay sample size distribution (Figure 2 B) with the majority of the fluorescent particles (above the threshold) exhibiting diameters (D_o) between ~1.7 and ~3.7 µm. The peak size was measured to be between 2.3 and 2.5 µm, which is, interestingly, close to the size range known fungal spores such as *Aspergillus/Penicillium*. Previous studies using the UV-APS technique indicate that the mean aerodynamic diameter for *Aspergillus* and *Penicillium* fungal particles at age one and two weeks are 2.40 ± 0.12 and 3.55 ± 0.14 µm respectively (Kanaani et al., 2007). These measurements are in good agreement with previously obtained literature data for the spores (Latgé, 1999). Therefore given the results found in the current WIBS-4 study a likely candidate responsible for the peak histogram column are *Aspergillus/Penicillium* spores.

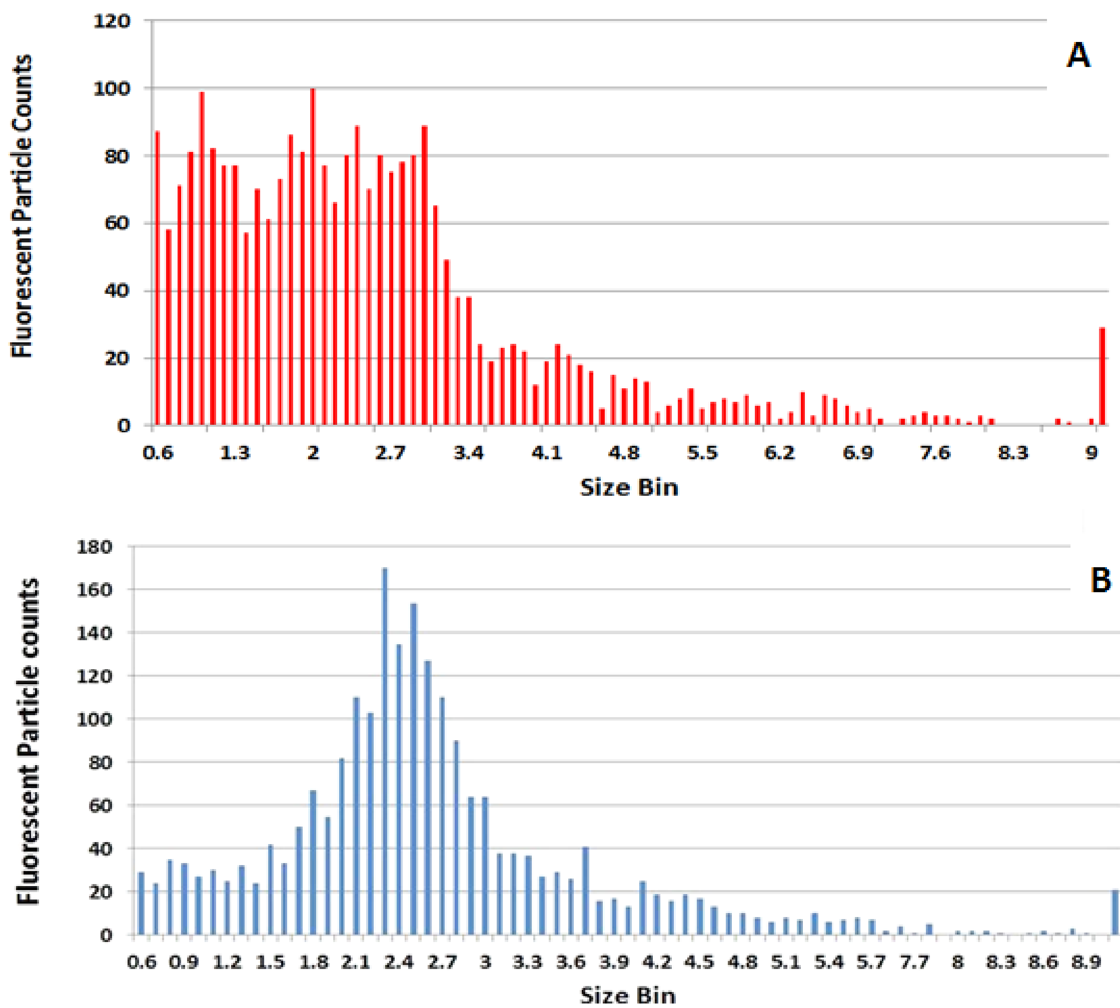
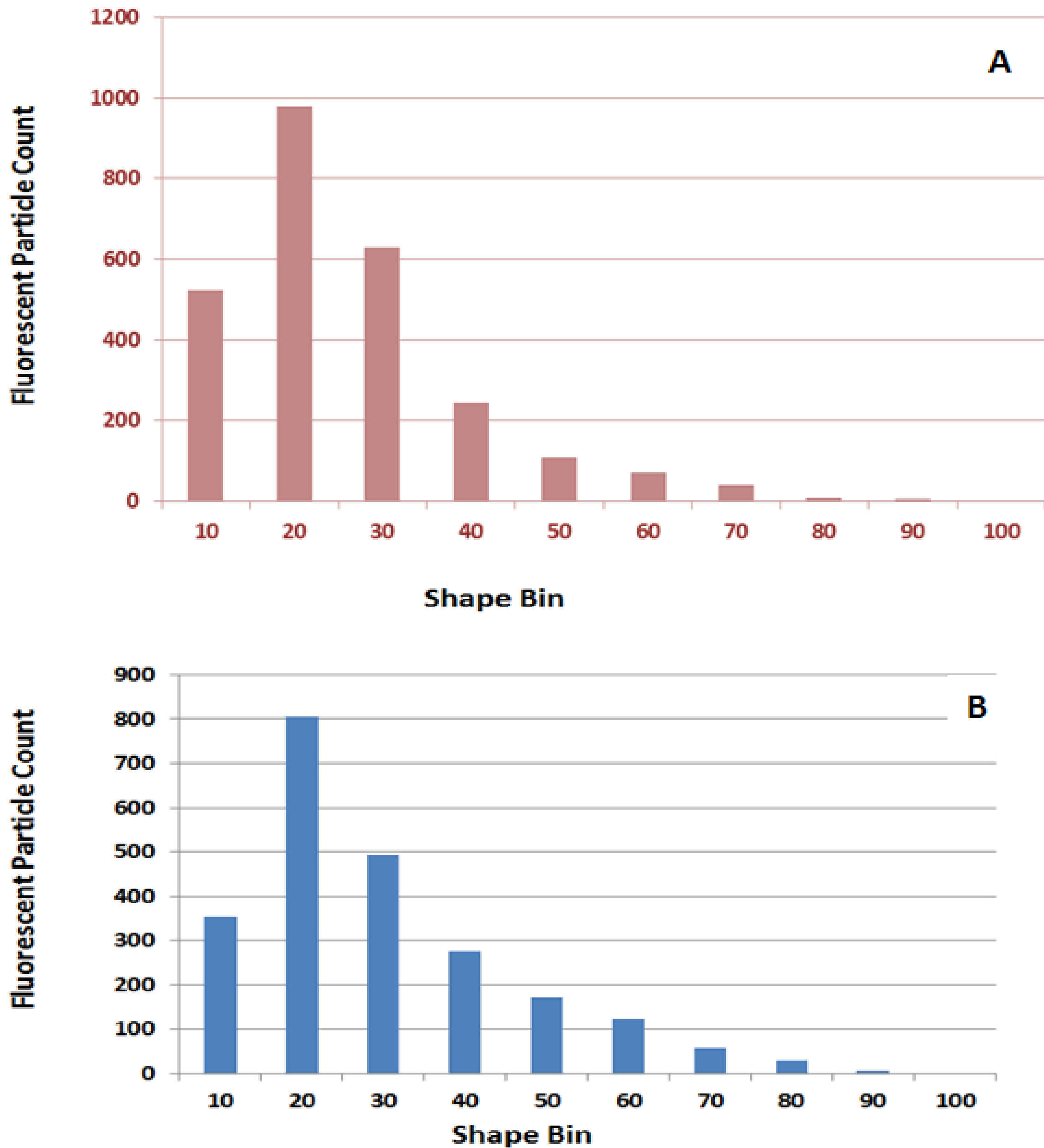


Figure 2: Number-Size histogram for the fluorescent particles released from silage (A) and hay (B) samples.

In comparison to the results shown for the hay histogram it can be seen that the silage experiments yielded a very different distribution with a great number of particles monitored between 0.5 μm and 3 μm. Furthermore peak concentrations are found below 2 μm for the silage data, an observation that is in clear contrast to the hay number-size data. However significant numbers of particles were still monitored in the size range relevant to *Aspergillus/Penicillium* spores for the silage sample but the smaller particles (< 2 μm) emitted are also suggestive of a significant bacterial content. Likely candidates for these smaller particles (based on sizing information alone) are the *Thermophilic Actinomycetes* (Busse and Holgate, 2000, Reponen et al., 1998, Reponen et al., 2001). They are also known to be fluorescent and are released from composting material (Darken, 1961, Leblanc and Dufour, 2002, Pinnick et al., 1999). Given that silage undergoes anaerobic digestion by bacteria it is not an unexpected result to sample a large number of small particles with such origins.

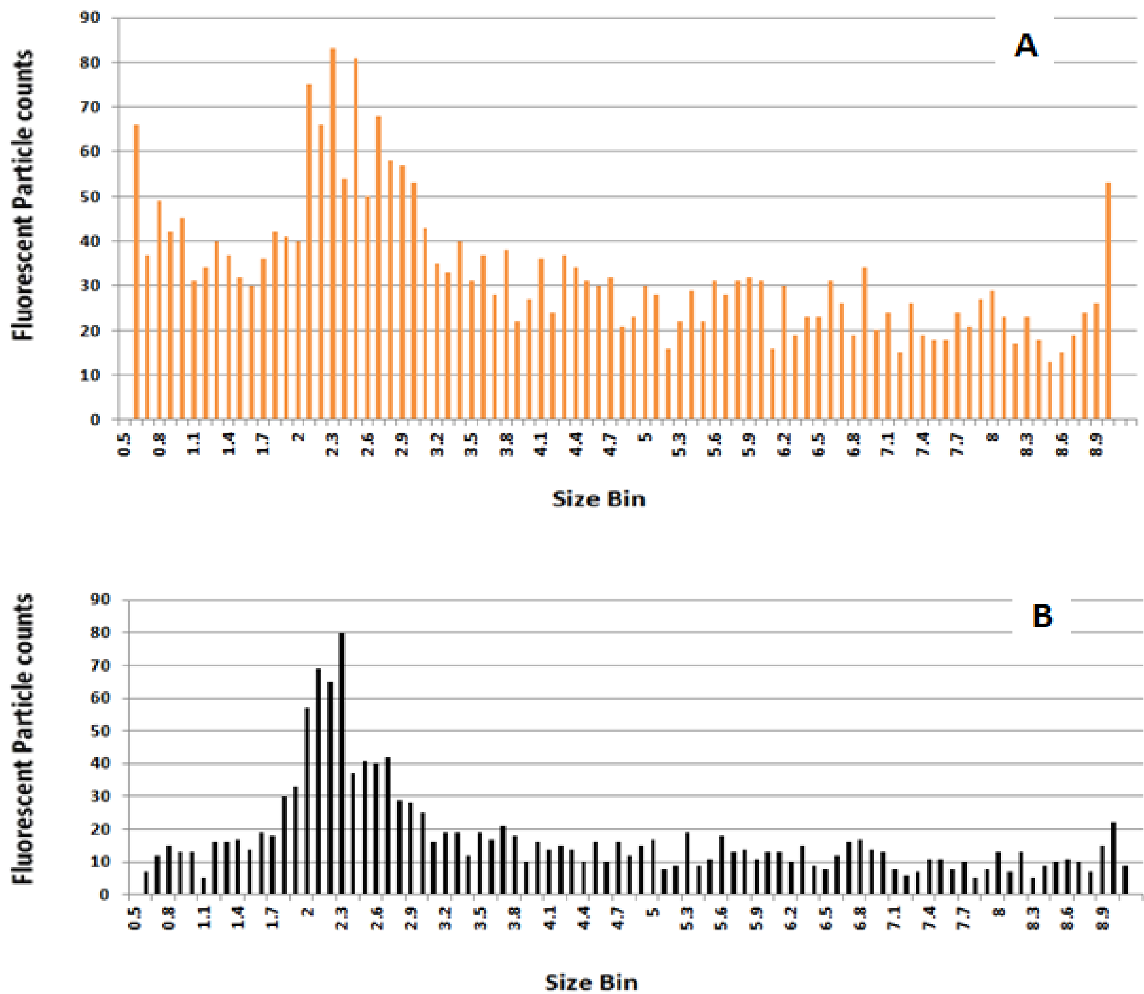


275

276 **Figure 3: Shape/AF value histogram for the fluorescent particles released from silage (A) and**
277 **hay (B) samples.**

278 The shapes of individual particles were also evaluated using the WIBS-4 instrument (Figure 3). The
279 histograms show that the majority of both data sets comprise AF values maximising at 20, which is
280 indicative of a spherical/ globular nature for most of the particles. From WIBS calibration
281 experiments AF values between 30-50 AF can be considered to have irregular shapes whilst those
282 above 50 would be more rod-like in shape. The measured spherical nature of the vast majority of the
283 particles detected for both the silage and hay samples provides further evidence that
284 *Aspergillus/Penicillium* spores are present as they are known to be of such morphologies. The results
285 further suggest that the small (<2 µm) particles emitted by silage are likely globular/rounded and thus

286 a cocci bacterial type, like *Thermophilic Actinomycetes* is also contributing. An interesting aspect of
287 the data is that the monitored *non-fluorescent* particles showed very different size ranges and shape
288 distributions to those registered for the fluorescent particles. The bulk of these particles were found to
289 be <1 μm in size and had a significantly different shape distribution from the fluorescent particles,
290 peaking between 30-50 AF units. In summary irregularly shaped, sub-micron sized, non-fluorescent
291 particles were also emitted from the hay and silage samples and therefore likely of non-biological
292 origin .



293

294 **Figure 4: Number-Size histogram for the fluorescent particles released from pure aged**
295 ***Penicillium* (A) and *Aspergillus* (B) samples.**

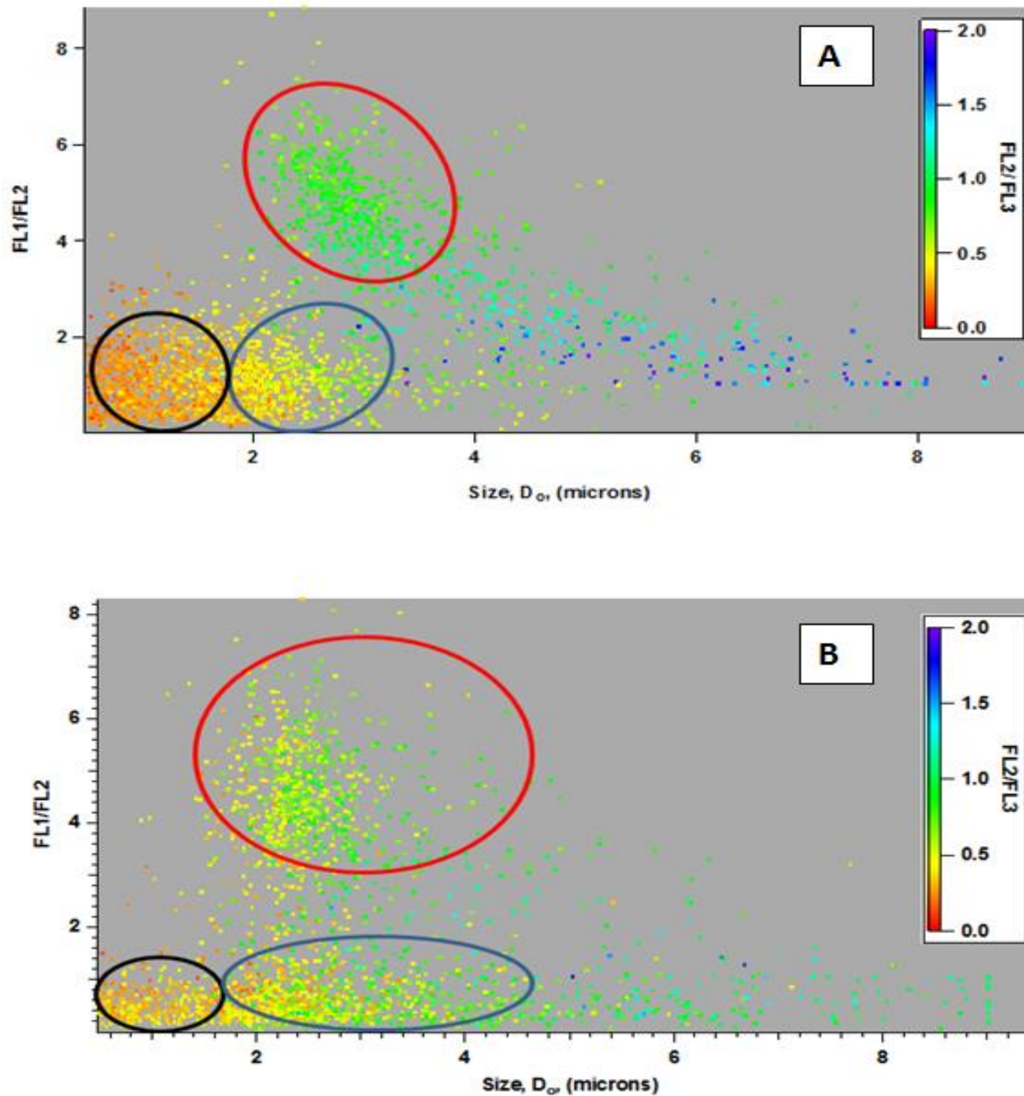
296 In order to provide a more firm basis for the suggestion that the bioaerosol content was, in part, due to
297 *Aspergillus/Penicillium* spores, pure aged samples of both fungal types and related mycelia were also
298 evaluated using the WIBS-4 technique. The results of these experiments are shown in Figure 4. Clear
299 similarities are seen between these number-size histograms and the ones obtained for the hay and
300 silage samples. These distributions also add further evidence to the suggestion that more than one

301 particle type is contributing to the size distribution associated with the silage experiment. Shape, AF, 302 distributions were also obtained for the purchased fungal spores and were again seen to maximise at 303 20 AF units indicating that significant numbers of the particles were spherical in nature. However a 304 greater contribution to the AF values >30 were also noted for the purchased spores versus the hay and 305 silage samples. This observation is most likely due to the presence of fragmented mycelia parts that 306 are associated with the "pure" fungal spore samples.

307 *Discriminatory profiles for the fluorescence signals*

308 The optical scattering data providing information on size and shape obtainable from the WIBS 309 approach is, of course, supplemented by the actual fluorescence intensities that are registered in the 310 FL1, FL2 and FL3 detection channels. Hence this information was used to further elucidate the 311 identities of the potential particle types being released by the hay and silage samples.

312 The data obtained can be viewed in a number of alternate graphical plots in order to provide further 313 ways to potentially discriminate between, and perhaps identify, the emitted particles. One such 314 treatment is shown in Figure 5 where the ratios of FL1 intensity to FL2 intensity (y-axis) *vs* size (x-315 axis) also provide discriminatory evidence relevant to the assessment of the silage and hay bioaerosol 316 emissions. Hence the particles contained in the red oval are clearly separable in terms of their 317 FL1/FL2 ratios from those highlighted inside the blue and black oval markers. The particles enclosed 318 by the blue and red ovals have the same size approximately (~2-3 μm) and display similar FL2/FL3 319 values (as shown on the colour bar) for the hay experiment. The silage data shows more variation 320 although the two 2-3 μm enclosures still exhibit FL1/FL2 intensity ratios that are distinctly different. 321 There are a number of possible reasons to explain these observations. Firstly two discrete species of 322 spore, in the same size range, could have been discriminated between by the WIBS technique: such an 323 explanation would represent a first example of this possibility using WIBS. It is not though unlikely 324 because two similarly sized spore types are known to be emitted by hay/silage: *Aspergillus* and 325 *Penicillium.* In this context it should be noted that previous fluorescence studies have shown that 326 *Aspergillus* and *Penicillium* display very similar emission spectra between 400-700 nm with 327 excitation at 370 nm, as used in the FL3 channel measurements (O'Connor et al., 2011).



328

329 **Figure 5: 3-D plots of FL1/FL2 versus Size, D_o, Microns for the fluorescent particles released**
330 **from silage (A) and hay (B) samples. Colour bar shows ratio of FL2/FL3.**

331 The second possibility is that the separation shown in Figure 5 is due to the viability of the spores.
332 While very little work has been performed before on this topic with regard to the fluorescence
333 properties of spores, the study by Wu *et al* does suggest that viability decreases with increasingly
334 intense fluorescent signals. However further work on this topic is required before its importance can
335 be fully ascertained (Wu and Warren, 1984). It is worthy of note that if this explanation does prove to
336 be the case then there would be considerable impact on compost facility evaluations because their
337 biological particle release data are based on culture methods that do not take into account non-viable
338 examples (Després et al., 2012).

339

340 An alternate study regarding viability has shown that bacterial spores with higher intrinsic
341 fluorescence are correlated with “culturability” (Laflamme et al., 2005). However the experiments
342 performed employed a much smaller fluorescence range than applied in the current study. They also
343 used an excitation wavelength different to those used here. A publication by Kanaani et al has also
344 shown that the percentage of fluorescent particles decreases with age of the sample; thus spores with
345 lower fluorescence intensities could represent aged spores (Kanaani 20007). In fact spores are
346 considered to lose their viability from the moment that they are released and the drop in observed
347 fluorescence may therefore be due to the decay of biomolecules such as NADH/NAD(P)H and the
348 flavins (Flannigan et al., 1994). Similarly results obtained using an earlier version of the UV-APS,
349 known as a FLAPS, indicated that cultivable counts correlated with fluorescent counts (Ho, 2002).
350 Thus the possibility of non-viable particles being underestimated by real-time fluorescent techniques
351 may exist.

352 Finally there is the possibility that the data split shown in Figure 5 is caused by clumps or clusters of
353 bacteria adhered to themselves or to other non-fluorescent material. The ratio of FL1/FL2 remains
354 consistent for the particles encompassed by the black and blue oval markers indicating similar
355 fluorescent components. However given the variations in FL3 values exhibited by the particles such a
356 possibility remains the least likely.

357 The particles highlighted by the black ovals in Figure 5 show different size characteristics from all
358 others. The colour bar also indicates that these particles exhibit divergent FL2/FL3 profiles again in
359 contrast to the remainder of the particles. These observations are most evident in relation to the silage
360 data where the smaller particles are obviously dissimilar to the larger particles indicated not only in
361 the blue and red ovals but also to all the other non-highlighted particles.

362 A clearer picture emerges for hay and silage samples using this type of data treatment when the same
363 approach is made for the purchased, aged *Aspergillius* and *Pencillium* samples. The results obtained
364 are shown in Figure 6 where it can be seen that both types of spore exhibit similar but not identical
365 FL1/FL2 *vs* size profiles.

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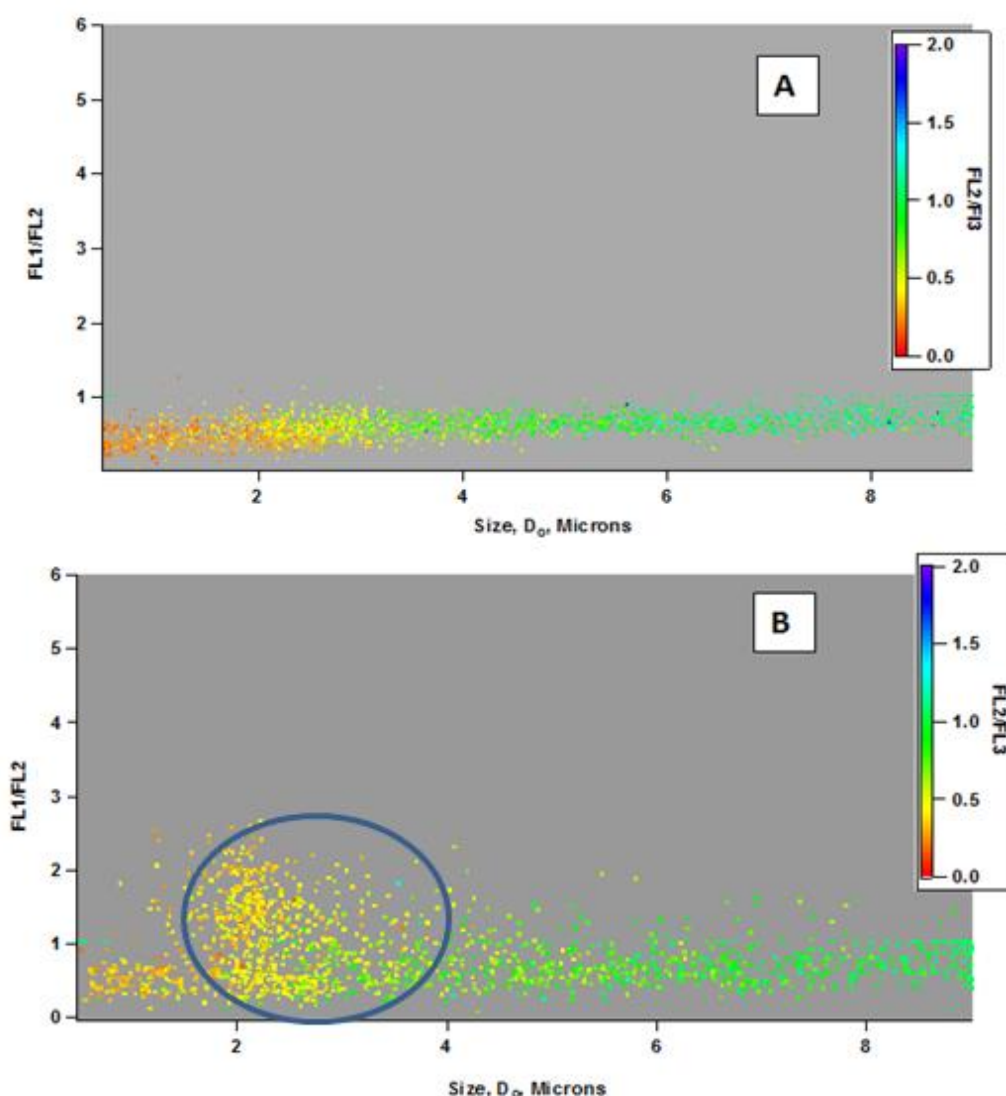
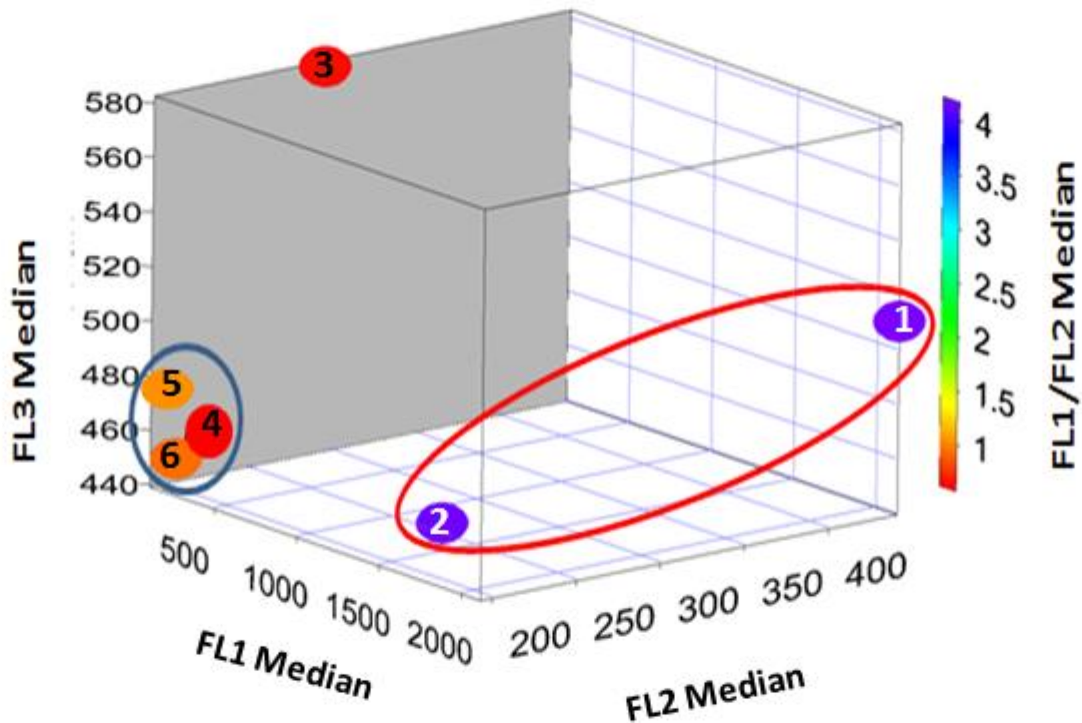


367 **Figure 6: 3-D Plots of FL1/FL2 versus Size, D_o, Microns for the fluorescent particles released**
368 **from aged *Penicillium* (A) and *Aspergillus* (B) samples. Colour bar shows ratio of FL2/FL3.**

369 Most of the particles sampled for the *Penicillium* (Figure 6A) and *Aspergillus* (Figure 6B) spores
370 display very similar FL1/FL2 values over almost the full size range as well as comparable FL2/FL3
371 values. However smaller particles (< 2 μm) for *Pencillium* do appear to provide slightly different
372 FL2/FL3 ratios from the *Aspergillus* spores as denoted by the colour bar. A second area of deviation
373 occurs in the 2-4 μm size range where the plots clearly show that there is far higher variation for the
374 FL1/FL2 ratio related to the *Aspergillus* sample in comparison with the *Penicillium* spores. Hence a
375 significant number of particles with FL1/FL2 ratios >1 are registered for *Aspergillus*. This result
376 contrasts to the *Penicillium* data for which virtually all particles exhibit ratios <1. This overall finding
377 is of interest because the aged *Aspergillus* spores show almost identical behaviour to the particles
378 highlighted in the blue ovals plotted in Figures 5A and 5B. These latter data were, of course, obtained

379 for the hay and silage samples and the results may therefore provide a basis for discriminating
380 between *Aspergillus* and *Penicillium* spores using WIBS, a process that is difficult when simply
381 employing analysis by optical microscopy.

382 Figure 7 below further highlights this discriminatory aspect of the data by focusing on the 1.5-4 μm.
383 size region. This 4-D plot shows the median values measured for the various particle clusters
384 highlighted previously in Figures 5 and 6.



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386 **Figure 7: 4-D plot showing the median values of FL1, FL2 and FL3 for fluorescent particles**
387 **with sizes between 1.5 and 4 μm for 1: Silage (high FL1/FL2 ratio), 2: Hay (high FL1/FL2 ratio),**
388 **3: (Aged *Penicillium)*, 4: (Aged *Aspergillus)*, 5: Silage (Low FL1/FL2 ratio), 6: Hay (Low**
389 **FL1/FL2 ratio). The colour bar indicates FL1/FL2 median ratios.**

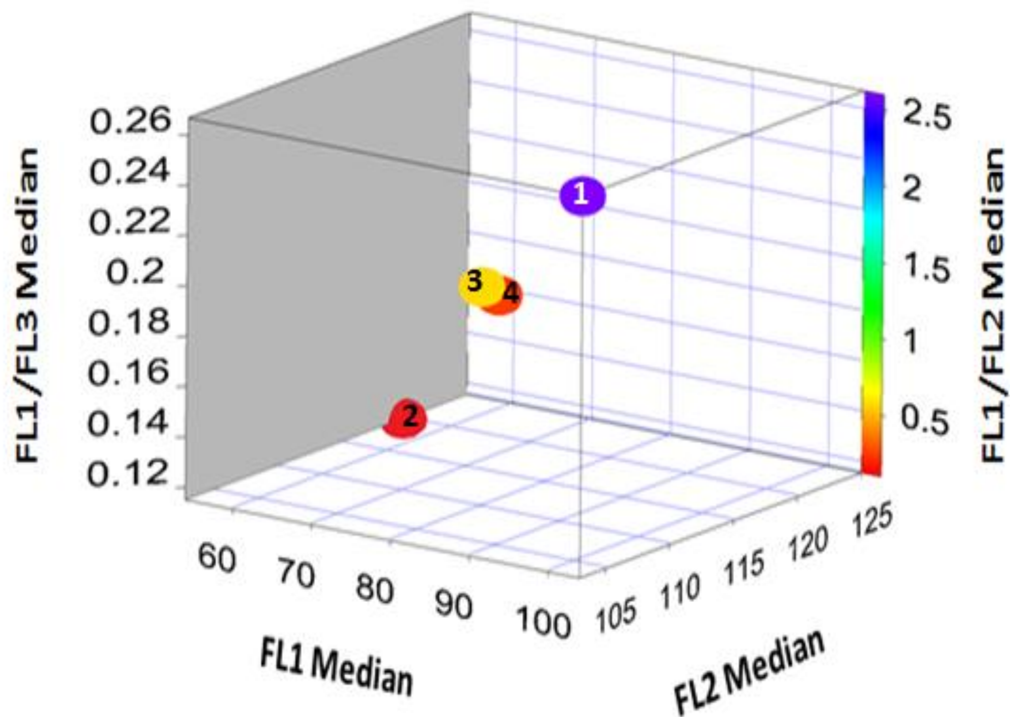
390 Distinct separations of the data can be seen for the two median value sets (1) and (2) enclosed by the
391 red oval, which are well removed from all of the other sets. These points correspond to the high
392 FL1/FL2 ratio particles highlighted by the red ovals in Figure 5 for the silage and hay experiments. It
393 is clear that these samples exhibit far higher FL1 median values than any of the other data sets
394 obtained.

395 The aged *Penicillium* and *Aspergillus* spores labelled (3) and (4) respectively in Figure 7 show similar
396 FL1/FL2 ratios and similar FL1 median values. However the species are differentiated in terms of
397 FL3 values, where *Pencillium* (3) display higher median values. Another interesting aspect of the plot

398 is related to the data labelled (4), (5) and (6) enclosed by the blue oval. Clearly the median values
399 measured in the FL1, FL2 and FL3 channels for the hay and silage experiments are very similar to
400 those obtained for the aged *Aspergillus* spores. These observations provide further evidence that
401 *Aspergillus* spores were monitored at least to some degree by WIBS from the hay and silage releases.

402 A data treatment similar to that shown in Figure 7 but for particles in the size range < 1.5 μm is given
403 in Figure 8. Here the *Penicillium* and *Aspergillus* fluorescence ratio signals, (3) and (4) respectively,
404 almost overlap. In contrast the median values associated with silage, (1) and hay (2) particles are well
405 separated from reach other as well as from the aged fungal spore samples indicating little relationship
406 in this smaller size range. Particles from the silage experiment gave the highest FL1 median values of
407 the groups investigated here, while those from hay displayed the highest FL2 values. These higher
408 fluorescence intensities might be indicative of the presence of one or more bacterial components
409 given the size range that is involved. Thus silage inoculants contain one or more strains of lactic acid
410 bacteria, the most common being *Lactobacillus plantarum*, which has been shown to possess
411 dimensions in the 0.5-1.2 x 1.0-10 μm size range and are mainly rod-like (Pedersen 1936, Viticulture
412 & Enology 2010). However other possibilities such as the *Thermophilic Actinomycetes* are spherical
413 and perhaps more likely candidates from the AF values measured here.

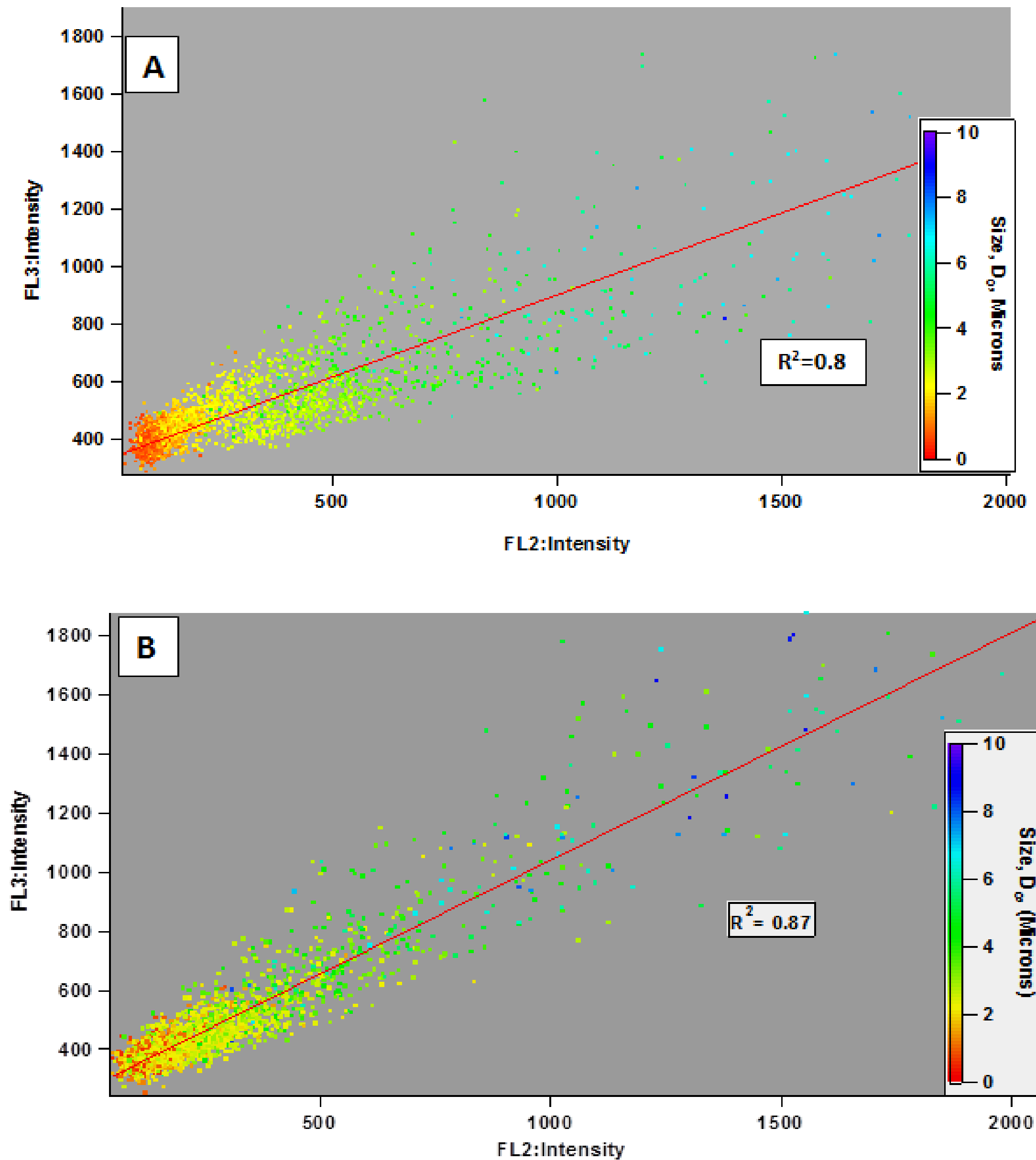
414



415

416 **Figure 8: 4-D plot showing the median values of FL1, FL2 and FL1/FL3 for fluorescent**
417 **particles <1.5 µm sampled for 1: Silage, 2: Hay, 3: Aged *Pencillium*, 4: Aged *Aspergillus*. The**
418 **colour bar indicates the FL1/FL2 ratio.**

419 Figure 9 shows the FL2 *vs* FL3 fluorescent intensities for the silage and hay data-sets. The results
420 indicate that there is a linear correlation (R^2 = 0.8-0.9) between the signals. However it should be
421 noted that some particles with high fluorescence intensities saturated the fluorescence detector for
422 both channels and so these data were filtered out in order to properly ascertain the association
423 between the two channels. Given that both channels reflect fluorescence emitted between 420 and 650
424 nm but with differing excitation wavelengths (FL2/280 nm and FL3/370 nm) such a result is not
425 unexpected. One further aspect indicated by Figure 9 is that increasing fluorescence intensities would
426 appear to be associated with increasing size, probably because larger particles would contain higher
427 concentrations of biofluorophores. Similar correlations, with even higher R^2 values, were seen for
428 both the purchased, aged *Aspergillus* and *Pencillium* samples. This finding is likely related to the fact
429 that more than one type of bio-particle is present in the hay and silage samples



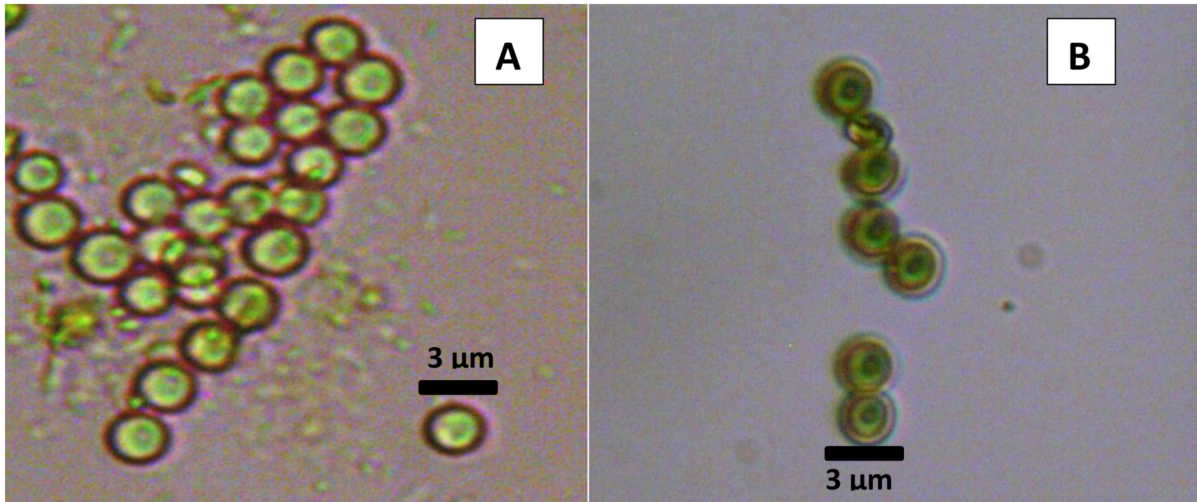
430

431 **Figure 9: 3-D plots of FL2 versus FL3 for the fluorescent particles released from the silage (A)**
432 **and hay (B) samples. The colour bar indicates size, D_o.**

433 *Optical microscopy analysis*

434 Impaction sampling using a SporeWatch apparatus was also employed for collecting particles released
435 from the silage and hay used in the hay and silage experiments. This provided a more traditional
436 approach to the visualization of the particles being emitted using optical microscopy. Therefore an
437 important degree of validation for the WIBS-4 data on size, shape and possible identity was obtained.

438



439 **Figure 10: Optical images showing the most prominent particle type sampled from Silage (A)**
440 **and Hay (B).**

441 The particles were affixed to a suitable substrate before being examined using the light microscope as
442 described above. It can be confirmed by reference to the microbiology literature that the optical
443 images shown in Figure 10 conclusively prove that *Aspergillus*/*Penicillium* spores were present and
444 detectable in significant quantities. These fungal spores were observed in greatest abundance for the
445 hay sample as compared to the other particle types that were present.

446 The optical images shown in Figures 9 A and B display the major particles in the size-range, ~2-4 µm,
447 corresponding to measurements obtained using the WIBS-4 instrument. Also it is clear that the spores
448 are effectively round/ globular in shape as the AF data measured by WIBS-4 indicate. In the case of
449 the silage sample (9A) large quantities of bacteria were also detected thus further collaborating the
450 WIBS-4 measurements.

451 Particles >4 µm were also detected by the optical microscope and can be identified as clumps of
452 *Aspergillus*/*Pencillium* fungal spores, mycelia/hyphae fragments, other spores and even pollen grains.
453 Such constituents were monitored in much less quantities than the *Aspergillus*/*Penicillium* spores for
454 both the hay and silage samples.

455

456 **Conclusions**

457 It was clear from the WIBS-4 visual display results that once sampling began substantial amounts of
458 fluorescent and therefore, potentially, biological particles were measured. The results obtained from
459 the experiments described above have shown for the first time that the sizing, "shaping" and likely
460 classification of particles released from environmentally representative sources used in composting
461 (*i.e.* hay and silage) is possible using the on-line WIBS-4 approach. This methodology requires the

462 analysis of a wide range of variables, (*i.e.* fluorescence signals, their ratios, size and "shape" data)
463 using 2-D, 3-D and 4-D graphical treatments to provide a range of complementary, visually useful
464 plots for data interpretation. There was evidence that the WIBS-4 instrument provided data which
465 could identify *Aspergillus* and *Penicillium* spores. Thus given the potentially vast quantities of spores
466 released by agricultural and compost facilities, the results obtained indicate that the WIBS technique
467 could, in principle, be of considerable use for both the on-line monitoring and enforcement activities
468 required at such sites. Other advantages for instruments such as the WIBS-4 in such a role is the rapid
469 analysis of the data in comparison to more traditional techniques and the far lower time resolutions
470 (minutes-seconds) for determining bioaerosol ambient concentrations.

471 It was shown that the WIBS instrument could also monitor the presence of particle types smaller than
472 the target fungal spores because they exhibited quite different physical and fluorescent characteristics.
473 These particles are most likely of a bacterial origin. Such a conclusion could prove to be exciting
474 given the number of possible future applications that exist for the on-line detection of ambient
475 bacteria. Possible deployment in health care facilities to ensure stable levels of background microbial
476 content or in operating theatres where microbial and fungal concentrations need to be kept low,
477 represent only two of the possible prospective applications.

478

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482

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