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Abstract: The succession of bacterial and fungal populations was assessed in an activated sludge (AS) diffusion bioreactor treating a synthetic malodorous emission containing H2S, toluene, butanone and alpha-pinene. Microbial community characteristics (bacterial and fungal diversity, richness, evenness and composition) and bioreactor function relationships were evaluated at different empty bed residence times (EBRTs) and after process fluctuations and operational failures (robustness test). For H2S, butanone and toluene, the bioreactor showed a stable and efficient abatement performance regardless of the EBRT and fluctuations applied, while low alpha-pinene removals were observed. While no clear positive or negative relationship between community characteristics and bioreactor functions was observed, ecological parameters such as evenness and community dynamics seemed to be of importance for maintaining reactor stability. The optimal degree of evenness of the inoculum likely contributed to the high robustness of the system towards the fluctuations imposed. Actinobacteria, Proteobacteria and Fungi (Hypocreales, Chaeatothyriales) were the most abundant groups retrieved from the AS system with a putative key role in the degradation of butanone and toluene. Typical H2S and alpha-pinene degraders were not retrieved from the system. The inoculation of P. fluorescens, a known alpha-pinene degrader, to the system did not result in the enhancement of the degradation of this compound. This strain was likely outcompeted by the microorganisms already adapted to the AS environment.

Response to Reviewers: Dear Editor,

We highly appreciate the opportunity to resubmit our manuscript "Microbial community changes during different empty bed residence times and operational fluctuations in an air diffusion reactor for odor abatement". Enclosed is the current version of our paper which includes the comment of Reviewer 4. This modification has been highlighted in red.

We hope that this modification satisfies the request of the reviewer. Please do not hesitate to contact us at your convenience if you need further information.

Your sincerely

Reviewer 1:

The paper was improved and all the suggestions were incorporated. The paper now should be accepted.

Reviewer 3:

Authors did a great job answering the reviewers.

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I'm happy that my comments have been addressed. I would recommend this for publishing now. My only minor comment is in line 98 where 'pH/mV/°C' should be changed to 'pH/conductivity/temperature'

The authors changed 'pH/mV/°C' (former line 98) to 'pH/conductivity/temperature' (current line 98) according to the Reviewer 4's recommendation.

Maria Jesús García Galán
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Dear Editor,

Please find enclosed our paper "Microbial community changes during different empty bed residence times and operational fluctuations in an air diffusion reactor for odor abatement" co-authored by Elisa Rodríguez Rodríguez, Pedro A. García-Encina, Raúl Muñoz and Raquel Lebrero. The paper is submitted for publication in the special issue ISEB2016 of Science of the Total Environment.

Innovative biological techniques for odor treatment in wastewater treatment plants (WWTP) are necessary to overcome the typical limitations of conventional biofilters such as their high footprint and the difficulties associated to the control of environmental parameters. Activated sludge diffusion systems (AS), - where malodorous emissions are directly sparged into the aeration tank of a wastewater treatment plant (WWTP) -, constitute a promising alternative, but the lack of knowledge about its performance when treating volatile organic compounds and the microbiology underlying the biodegradation processes still limit their widespread application.

This work presents a systematic evaluation of the succession of the microbial communities (bacteria and fungi) developed in an AS system treating butanone, H_2S, toluene and alpha-pinene. The relationships between the bacterial/fungal community structure and the functionalities of the system were evaluated under steady state at different empty bed residence times and under transient conditions during operational failures and process fluctuations (robustness test). Key ecological parameters (evenness, richness, diversity, dynamics) as well as bacterial and fungal composition were assessed by means of a double-denaturing gradient gel electrophoresis approach.

This work fits within the subject area of "Waste and water treatment "of the journal and possess a multidisciplinary character combining results of biosphere, atmosphere and hydrosphere.

We look forward to your evaluation.

Yours sincerely,

Elisa Rodríguez Rodríguez

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RESPONSE TO REVIEWERS

Ref. No.: STOTEN-D-16-05536R1
Manuscript title: "MICROBIAL COMMUNITY CHANGES DURING DIFFERENT EMPTY BED RESIDENCE TIMES AND OPERATIONAL FLUCTUATIONS IN AN AIR DIFFUSION REACTOR FOR ODOR ABATEMENT"

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*Graphical Abstract
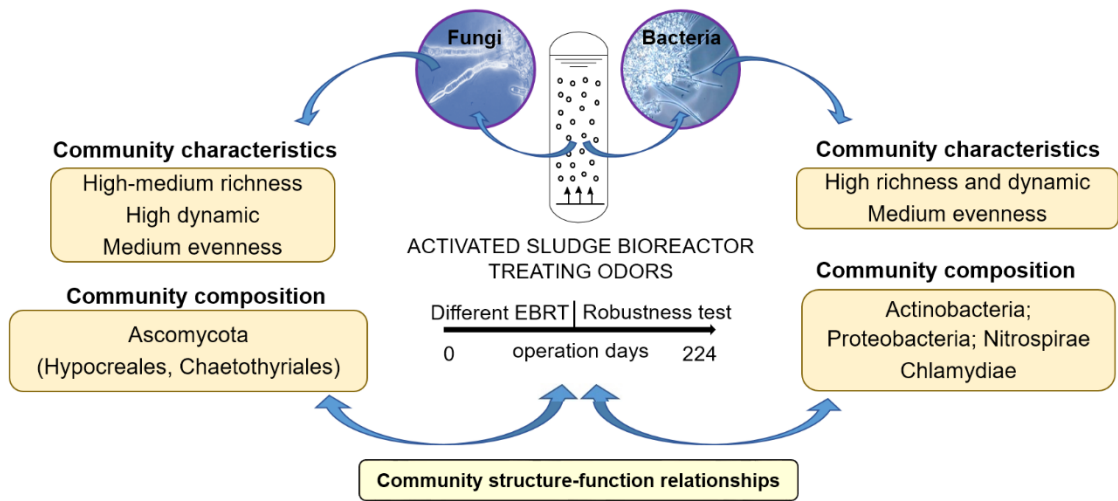
1 - Microbial structure-function relationships evaluated in an odor treating

2   bioreactor

3 - Evenness and dynamics supported functional stability of the bioreactor

4 - Actinobacteria and Proteobacteria were highly represented

5 - Fungi within Hypocreales and Chaetothyriales orders played a role in the

6   process

7 - Typical H_2S and alpha-pinene degraders were not detected.

Microbial community changes during different empty bed residence times and operational fluctuations in an air diffusion reactor for odor abatement

Elisa Rodríguez[a*], Pedro A. García-Encina[a], Raúl Muñoz[a], Raquel Lebrero[a]

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Abstract

The succession of bacterial and fungal populations was assessed in an activated sludge (AS) diffusion bioreactor treating a synthetic malodorous emission containing H_2S, toluene, butanone and alpha-pinene. Microbial community characteristics (bacterial and fungal diversity, richness, evenness and composition) and bioreactor function relationships were evaluated at different empty bed residence times (EBRTs) and after process fluctuations and operational failures (robustness test). For H_2S, butanone and toluene, the bioreactor showed a stable and efficient abatement performance regardless of the EBRT and fluctuations applied, while low alpha-pinene removals were observed. While no clear positive or negative relationship between community characteristics and bioreactor functions was observed, ecological parameters such as evenness and community dynamics seemed to be of importance for maintaining reactor stability. The optimal degree of evenness of the inoculum likely contributed to the high robustness of the system towards the fluctuations imposed. Actinobacteria, Proteobacteria and Fungi (Hypocreales, Chaeatothyriales) were the most abundant groups retrieved from the AS system with a putative key role in the degradation of butanone and toluene. Typical H_2S and alpha-pinene degraders were not retrieved from the system. The inoculation of *P. fluorescens,* a known alpha-pinene degrader, to the system did not result in the enhancement of the degradation of this compound. This strain was likely outcompeted by the microorganisms already adapted to the AS environment.

Keywords: activated sludge; volatile organic compound; bacteria; fungi; evenness; fluctuation

1. Introduction

Changes of microbial communities (microbial succession) in natural and engineered ecosystems (bioreactors) in response to variations in operating or environmental conditions constitute nowadays a main issue in microbial ecology to predict system or ecosystem behaviour (Pholchan et al., 2013). Several characteristics of the microbial community structure seem to play a key role in maintaining functional stability under these variations. Among them, richness, evenness, dynamics, functional redundancy, microbial composition and microbial interactions seem to be of utmost importance for controlling reactor and ecosystem functioning (Bell et al., 2005; Cabrol et al., 2012; Wittebolle et al., 2009). For example, communities exhibiting intermediate evenness values (some species are dominant but most of them are present in decreasing lower amounts) have been shown to better deal with changing environmental conditions, since they have a pool of less dominant species able to replace the leading ones under operating or environmental fluctuations (Marzorati et al., 2008). Species richness effects on ecosystem functioning can decrease when functionally redundant species exist in the community (Bell et al., 2005).

The number of studies addressing this topic in bioreactors treating gas pollutants and malodorous emissions (with harmful effects on both human health and natural ecosystems) has increased during the last decade. Since biofilters and biotrickling filters are by far the most commonly implemented technologies for odor abatement, microbial-based studies have primarily focused on these systems (Cabrol et al., 2012; Lebrero et al., 2012, 2013; Prenafeta-Boldú et al., 2012). In addition to bacteria, fungi have been also investigated in biofilters (Prenafeta-Boldú et al., 2012), due to their ability to degrade complex organic pollutants and their superior performance compared to

60 bacteria when present in media-based odor treatment bioreactors (Estrada et al., 2013;

61 Harms et al., 2011).

62 However, emerging odor treatment technologies such as activated sludge (AS) diffusion

63 bioreactors (based on the direct sparging of the malodorous emission into the aeration

64 tank in wastewater treatment plants (WWTPs)) have been less studied from both a

65 microbiological and engineering point of view. This technology represents a cost-

66 effective alternative to biofilters and biotrickling filters due to it avoids problems related

67 to packing media compaction, moisture control or accumulation of toxic metabolites.

68 Nevertheless, the lack of reliable data concerning wastewater treatment performance

69 during the abatement of volatile organic compounds (VOCs) from malodorous streams,

70 and the lack of knowledge on the ability of AS systems to cope with process

71 fluctuations and operational failures still limit its widespread application (Bowker,

72 2000). In this context, unraveling the structure and dynamics of the microbial

73 communities (both bacteria and fungi) governing AS systems treating malodorous

74 emissions under steady or transient operating conditions can contribute to prevent

75 undesirable malfunction events.

76 This study was thus conducted to assess the temporal variation in the structure and

77 composition of the microbial communities (bacteria and fungi) in an AS diffusion

78 bioreactor treating a mixture of VOCs (toluene, butanone and alpha-pinene) and H_2S at

79 low inlet concentrations (mg m^{-3}), which simulated a simplified odorous emission from

80 a WWTP. The evolution of the microbial populations and their associated ecological

81 parameters were correlated with bioreactor performance during the operation of the

82 system at different empty bed residence times (EBRTs). The system was also evaluated

83 during the analysis of AS robustness versus typical operational fluctuations.

2. Material and methods

2.1. Bioreactor set-up and physical-chemical analysis

The configuration of the AS diffusion bioreactor and the analytical procedures employed to monitor the system were described in detail in Lebrero et al. (2010, 2011). Briefly, the AS system consisted of a jacketed column with a working volume of 8.5 liters operated at 20 ºC. The reactor was inoculated with 1 liter of concentrated (17 g L$^-$^{1}) return municipal activated sludge from Valladolid WWTP (Spain) resuspended in a SO_4^{2-}-free mineral salt medium (MSM) to a volume of 7.5 liters. The pH was maintained at 6.3 ± 0.3 by daily addition of a $NaOH-Na_2CO_3$ solution.

The inlet and outlet concentration of CO_2 and H_2S were analyzed using a GC-TCD (Varian CP-3800) and an electrochemical sensor (Dräger X-am 5000) calibrated in the 0-40 ppm range, respectively. Gas samples for VOC analysis were collected in 250mL calibrated glass bulbs (SUPELCO) and pre-concentrated by SPME. VOC inlet and outlet concentrations were then determined by GC-MS according to Lebrero et al. (2010). The pH was measured using a pH/conductivity/temperature meter (pH 510 Eutech Instruments, Nijkerk, The Netherlands). Biomass concentration in the AS unit was estimated via culture absorbance measurements (optical density at 600 nm) in a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) and as total solids concentration (Lebrero et al., 2010). Sulfate concentration was determined by HPLC-IC using an IC-Pak Anion HC (150mm×4.6mm) column. Finally, dissolved total organic carbon (DOC), dissolved inorganic carbon (DIC) and dissolved total nitrogen (DTN) were periodically recorded in the AS system using a TOC-V$_{CSH}$ analyzer (Shimadzu, Tokyo, Japan) coupled with a TN module based on chemiluminesce detection (TNM-1, Shimadzu).

2.2. Bioreactor operation: effect of different EBRTs and alpha-pinene addition

Details about the operation of the AS system under steady conditions can be found in Lebrero et al. (2011). A schematic representation of the different conditions applied to the reactor is shown in Fig. 1. Briefly, a mixture of H_2S, toluene and butanone with pre-humidified and filtered ambient air was fed to the bioreactor at concentrations of 16.9-23.8 $mg.m^{-3}$, 0.40-0.60 $mg.m^{-3}$ and 4.3-6.3 $mg.m^{-3}$, respectively. During the first 121 days, the degradation rate of these compounds in the system was evaluated at different EBRTs (day 0 to 29: 94 s; day 29 to 43: 74 s; day 43 to 59: 55 s; day 59 to 95: 48 s; day 95 to 162: 32 s) in the absence of any additional carbon source and at infinite sludge retention time (SRT) (no biomass withdrawal). At day 95 (EBRT of 48 s), after observing a biomass aggregation and compaction in the reactor, glucose was added to the AS system, and the SRT was set-up at 25 days (340 ml of mixed liquor were daily withdrawn and replaced with fresh MSM containing 2 g L^{-1} of glucose) in order to simulate real WWTPs operation. At day 121 (32 s EBRT), alpha-pinene was supplemented to the previous gas mixture to evaluate the degradation rate of a hydrophobic odorant in the bioreactor. To enhance alpha-pinene biodegradation, a culture of *Pseudomonas fluorescens* NCIMB 11671, purchased from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland), was inoculated to the bioreactor at day 132. The system was finally maintained at 32 s of EBRT until the end of the experiment (day 162).

The VOCs butanone, toluene and α-pinene were selected as model VOCs representing soluble, moderately soluble and hydrophobic compounds commonly found in WWTP emissions (Lehtinen and Veijanen, 2011; Zarra et al., 2008). H_2S was selected as model sulfur odorant, widely present in malodorous emissions from WWTPs and sewage works.

6

2.3. Bioreactor operation: robustness analysis

To systematically evaluate the effect of the operational failures and fluctuations in the robustness analysis, the EBRT was set up at 50 s to ensure this parameter was not a limiting factor for bioreactor performance. The robustness test consisted of process fluctuations and simulated operational failures imposed to the AS system from day 163 onwards (Fig. 1). Process response to fluctuations in odorant loading was evaluated by applying two sequential 3-h step increases (3-fold and 6-fold increases) in the inlet H_2S and VOCs loading, with a 20 h recovery period between them. After system stabilization, the robustness of the AS bioreactor towards a three-day starvation period (only humidified air was supplied) and a five-day shutdown period (neither polluted air supply nor glucose addition) was investigated. Finally, the response of the AS bioreactor to a 3-day interruption of pH control (pH decreased to 2.8) was assessed.

Each operational condition during the testing of different EBRTs and during robustness analysis was maintained for at least 3 weeks to ensure stable steady states. Details concerning robustness analysis can be found in Lebrero et al. (2010).

2.4. Sampling, DNA extraction and 16S / 18S rRNA gene amplification

Biomass samples of each operation condition were collected in sterile polypropylene tubes and immediately stored at -20 ºC for subsequent molecular analysis. Samples were named with the operation day at which they were collected. Their corresponding operational conditions were as follows: 0 (inoculum), 13 and 29 (EBRT: 94 s), 43 (EBRT: 74 s), 59 (EBRT: 55 s), 79, 90, 95 (EBRT: 48 s), 108, 115, 127, 144 and 162 (EBRT: 32 s), 175 (after fluctuations in odorant loading), 182 (after starvation period), 197 (after process shutdown), and 224 (after interruption in pH control) (Fig. 1).

156 DNA extraction was carried out according to Rodríguez et al. (2012). The quality of the

157 extracted DNA was checked by agarose gel (1.2 % w/v) electrophoresis and DNA was

158 stored at -20 ℃. Amplification of bacterial 16S rRNA gene fragments was performed

159 using the primer pair 1401R and 968F-GC (SIGMA-Aldrich, USA) (Nübel et al., 1996).

160 For the amplification of fungal 18S rRNA gene, a nested PCR approach was used with

161 primer pairs nu-SSU-0817F and nu-SSU-1536R (first round) and nu-SSU-0817F and

162 nu-SSU-0817R (second round) (SIGMA-Aldrich, USA) (Borneman and Hartin, 2000).

163 The PCR mixture included 1-2 µL of each primer, 25 µL of PCR Mastermix (Bioline,

164 Ecogen-Spain) (containing *Taq* DNA polymerase, PCR reaction buffer and

165 deoxynucleotides (dNTPs)), 1-2 µL of DNA template and Milli−Q water up to a final

166 volume of 50 µL. PCR was performed in an iCycler Thermal Cycler (BioRad

167 Laboratories, USA) with the following thermo-cycling program for bacterial

168 amplification: 2 min of pre-denaturation at 95 °C, 35 cycles of denaturation at 95 °C for

169 30 s, annealing at 56 °C for 45 s, and elongation at 72 °C for 1 min, with a final 5 min

170 elongation at 72 °C. For fungal amplification, the thermo-cycling program in the nested

171 PCR reactions included 5 min of pre-denaturation at 94 °C, 35 cycles of denaturation at

172 94 °C for 45 s, annealing at 50 °C for 45 s, and elongation at 72 °C for 1 min, and a

173 final 5 min elongation at 72 °C. Size and yield of PCR products were verified in 1.8 %

174 (w/v) agarose gels and subsequent SYBR Green I staining (SIGMA-Aldrich, USA).

175 **2.5. Resolution of 16S / 18S rRNA amplicons in gradient polyacrylamide gels**

176 PCR amplicons were resolved by double denaturing gradient gel electrophoresis

177 (DGGE) using a D-Code Universal Mutation Detection System (BioRad Laboratories,

178 USA). Polyacrylamide gels with a porous gradient of acrylamide/bisacrylamide of 6-

179 10%, and a urea/formamide denaturant gradient of 45−65 % and 23−45 % for bacterial

180 and fungal communities, respectively, were used. Electrophoresis was performed at 60

181 °C, with TAE 0.5-X as running buffer, at 64 V for 18 h for bacterial amplicons, and at

182 70 V for 18 h for fungal PCR products. The gels were stained with SYBR Green I for 1

183 h. Individual bands were excised from the DGGE gels with a sterile blade, resuspended

184 in 50 µl of ultrapure water, and maintained at 60 °C for 1 h to allow DNA extraction

185 from the gel. A volume of 5 µL of the supernatant was used for reamplification with the

186 original primer sets and PCR programs. Before sequencing at Secugen S.L., PCR

187 products were purified with the GenElute PCR DNA Purification Kit (Sigma-Aldrich,

188 USA).

189 **2.6. DGGE profile analysis for sample clustering and ecological parameters**
190   **determination**

191 Range-weighted richness (Rr), evenness/functional organization (Fo) and temporal

192 dynamics (UPGMA clustering) of the bacterial and fungal communities were calculated

193 based on the bacterial and fungal DGGE profiles. DGGE profiles were analyzed using

194 the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

195 Dendrograms (reflecting community dynamics), were constructed according to Lebrero

196 et al. (2012). Briefly, they were created by UPGMA clustering (500 resampling

197 experiments), based on the similarity indices of the DGGE profiles calculated using the

198 Pearson product–moment correlation coefficient (Fig. 3 and Fig. 4). Shannon–Wiener

199 diversity index was also calculated according to Lebrero et al. (2012).

200 Richness and evenness parameters were calculated according to Marzorati et al. (2008).

201 Ranged weighted richness (Rr), which indicates the richness and genetic diversity

202 within a bacterial community, was calculated based on the total number of bands (N)

203 and the denaturing gradient between the first and the last band of each lane (Dg),

204 according to the equation:

205 $$Rr = N^2 \times Dg \qquad (Eq.\ 1)$$

206 Community evenness was graphically represented by using Pareto-Lorenz evenness

207 distribution curves, i.e. for each DGGE lane, the bands were ranked from high to low

208 based on their intensities. Consecutively, the cumulative normalized number of bands

209 was used as x-axis, and their respective cumulative normalized band intensities were

210 used as y-axis (data not shown). For the interpretation of these curves, the functional

211 organization (Fo) of the community was calculated by scoring the y-axis projection of

212 their intercepts with the vertical 20 % x-axis. Results of Rr and evenness parameters

213 were evaluated according to the values proposed by Marzorati et al. (2008).

214 **2.7. Analysis of bacterial and fungal nucleotide sequences**

215 Chimeric sequences were detected and then removed by using DECIPHER (Wright et

216 al., 2012) and UCHIME v8.1 (Edgar et al., 2011) for bacterial and fungal nucleotide

217 sequences, respectively. Classification of bacterial sequences into taxonomic ranks was

218 performed using different bioinformatic tools to observe differences in taxonomic

219 assignments. The RDP Classifier (50 %, 80 %, 95 % bootstrap value) (Wang et al.,

220 2007), the UTAX Algorithm within the Usearch Sequence Analysis Tool (0.9 cut-off)

221 (Edgar, 2010) using Bio-Linux 8 (Field et al., 2006) and the Ez-Taxon-e Database (Kim

222 et al., 2012) (using the taxonomic thresholds proposed by Yarza et al. (2014) to

223 manually classify microorganisms into the different taxonomic ranks) were used.

224 Fungal nucleotide sequences were classified using SINA alignment web service based

225 on the SILVA database (Quast et al., 2013). For both bacterial and fungal sequences,

226 their closest relatives in GenBank (Blastn) were retrieved to evaluate the presence of the

227 AS microorganisms in similar environments.

228 Nucleotide sequences obtained in this study were deposited at GenBank under accession

229 numbers HQ147605 to HQ147612 and KX893872 to KX893882 (bacteria) and

230 KX907435 to KX907449 (fungi).

3. Results and Discussion

3.1. Bioreactor operation: effect of different EBRTs and alpha-pinene addition

233 The macroscopic performance of the AS bioreactor at different EBRTs was reported in

234 detail in Lebrero et al. (2011). Briefly, the removal efficiencies (REs) of H_2S, butanone

235 and toluene remained high regardless of the EBRT applied (94 s, 74 s, 55 s, 48 s and 32

236 s) (Table 1 and Fig. S1). At day 95, a rapid decrease in the biomass concentration and in

237 butanone and toluene REs was observed concomitantly with a biomass compaction

238 phenomenon at the bottom of the reactor. The addition of glucose to the AS unit and

239 process operation at a sludge retention time (SRT) of 25 days led to the rapid

240 resuspension of the biomass, the increase in biomass concentration and the recovery of

241 the preceding elimination performance (Fig. S1). Alpha-pinene, which was

242 supplemented from day 121 until day 162, was initially removed at approximately 21

243 %, but its RE decreased to 6.8 ± 1.9 % after two days of operation, and remained

244 constant for the following 40 days (Table 1). The addition of 250 ml of a *Pseudomonas*

245 *fluorescens* culture (alpha-pinene degrading species) after 11 days of alpha-pinene

246 feeding did not result in a significant enhancement of the removal of this terpene.

3.2. Bioreactor operation: robustness analysis

248 In general, the results obtained from robustness assays (Lebrero et al., 2010) indicated a

249 high capacity of the AS diffusion system to rapidly recover from negative events.

250 Hydrogen sulfide REs remained unaffected despite the operational fluctuations and

251 failures applied. Butanone and toluene REs remained stable at 99.7 ± 0.0 % and $98.4 \pm$

252   0.1 %, respectively, regardless of the odorant inlet concentration. After a three-day

253   starvation period, no loss in the pollutant abatement performance or long-term damage

254   was observed. Thus, steady state REs were rapidly recovered within the first 30 min

255   after the resumption of pollutants supply. After a five-day process shutdown, the

256   previous butanone and toluene abatement performance was rapidly restored within 3

257   and 6.5 hours, respectively. Failure in pH control led to a minimum RE of 39 % for

258   toluene and the reactor was not able to recover the previous steady state REs (98.6 ± 0.2

259   %) for this compound. A new steady state RE of 95.5 ± 0.1 % was achieved seven days

260   after pH control restoration. Conversely, the decrease in pH did not affect butanone RE,

261   which remained constant at steady state values of 99.8 ± 0.0 % (Table 2).

262   The RE of alpha-pinene increased from steady state values of 5.7 ± 0.8 % to a

263   maximum value of 23.5 % during the surges in the odorant inlet load. The RE of this

264   terpene also increased immediately after process start-up following the shutdown period

265   (maximum RE of 19.9 %), returning to steady values after 3 h. Finally, alpha-pinene

266   REs increased up to 50.8 ± 1.1 % 12 days after the first induced failure in pH control

267   (Table 2).

268   **3.3. Community structure at different EBRTs and in response to alpha-pinene**
269   **addition**

270   In general, this operational period resulted in highly dynamic bacterial and fungal

271   communities as observed by the Pearson similarities within the samples (Fig. 2 and Fig.

272   3). Changes in bacterial and fungal populations also can be observed in their respective

273   DGGE profiles (Fig 4. and Fig. 5). Balanced populations with medium Fo values

274   (ranging from 33 to 40 % for bacteria and from 28 to 41 % for fungi) (excluding sample

275   29 of fungal populations which showed a low Fo value) were observed (Fig. 6). Fo

276   indicates the ability of the community to organize in an adequate distribution of

277 dominant and non-dominant microorganisms that should assure the potentiality of

278 counteracting the effect of a sudden stress exposure. The ranged weighted richness was

279 high for bacterial populations, while medium to high Rr values were recorded for fungi

280 (excluding sample 79 which showed a low value). Shannon diversity, which generally

281 ranges from 1.5 to 3.5 (low and high evenness and richness, respectively), showed high

282 values for bacterial communities (between 3.0 and 3.7), and slightly lower values for

283 fungal populations (2.3 to 3.0) (Fig. 6).

284 These characteristics are generally associated with microbial communities able to

285 rapidly respond to changing conditions. For example, high Rr values - which were

286 particularly high for bacteria- represent populations with a high flexibility. This means

287 that a high number of species in the system offer multiple pathways for the degradation

288 of the different organic compounds, increasing functional redundancy and contributing

289 to a better adaptation to changes (De Vrieze et al., 2013). Similarly, a dynamic

290 microbial community, along with an optimal degree of evenness (medium Fo values

291 (Marzorati et al., 2008)), are considered of key importance to guarantee functional

292 stability in microbial communities (De Vrieze et al., 2013).

293 In accordance with these results, the AS system showed a stable performance for VOCs

294 and H_2S removal under the different EBRTs tested (Table 1 and Fig. S1). Only between

295 days 90 and 95 (48 s EBRT), a period of instability and low butanone and toluene REs

296 was recorded concomitant with biomass compaction and sedimentation and with a

297 decrease in suspended solids concentration (from 1 to 0.12 g L^{-1}) (Lebrero et al., 2011).

298 This unstable operation matched with the lowest Rr values observed for fungal

299 communities (Fig. 6), suggesting that the absence of sludge renewal and the lack of a

300 readily available organic carbon source mainly impacted fungal populations in terms of

301 richness. It is also important to note that a high degree of bacterial and fungal

13

community dynamics was observed at 48 s EBRT, indicating a period clearly marked by abrupt changes in the populations present in the system (Fig. 2 and Fig. 3). In this sense, sample 79 (the first collected at 48 s EBRT) clustered separately from sample 59 (collected at 55 s EBRT), showing a similarity of 30 % and 47 % between them for bacterial and fungal communities, respectively. At day 90, the structure of the bacterial and fungal communities was more similar to the communities present at day 59. However, at day 95, bacterial and fungal populations had undergone again important shifts, with a similarity of 36 and 56 % between samples from days 90 and 95 for bacterial and fungal communities, respectively. Despite microbial populations and reactor performance clearly being affected at this period, the system rapidly recovered butanone and toluene REs after glucose addition and process operation with a periodic sludge withdrawal (Fig. S1). The high dynamics and flexibility (Rr), and the optimum organization of the communities (Fo), likely contributed to maintain reactor stability under operational variations, as previously observed in other research works (Cabrol et al., 2012; De Vrieze et al., 2013; Firmino et al., 2015).

The application of the lowest EBRT (32 s) did not result in a detrimental effect in reactor performance, which maintained high REs for butanone, toluene and H_2S (Table 1 and Fig. S1). Fo and Shannon diversity did not significantly change and fungal communities recovered high Rr values (except for sample at day 162, which showed a medium value) at this stage (Fig. 6). The addition of alpha-pinene at day 121 did not trigger important changes in the ecological parameters measured in this study, likely due to the lack of an active microbial community capable of degrading alpha-pinene at the low concentrations present in the mixed liquor (0.10-0.13 mg L^{-1}) (Lebrero et al., 2011).

3.4. Community structure under process fluctuations and operational failures

The organization of the bacterial and fungal communities (Fo) remained at its medium values regardless of the operational failure or fluctuations applied (Fig. 6), highlighting the relevance of this parameter in maintaining functional stability. As well as for the results obtained during steady state operation, the operational changes imposed mainly affected bacterial richness (Rr) rather than community evenness. Bacterial Rr gradually decreased from high values (fluctuations in odorant loading: 105, starvation period: 77, process shutdown: 62) to medium values after pH control failure (25). Likewise, fungal Rr decreased from high values (fluctuations in odorant loading: 43, starvation period: 37) to a medium value following process shutdown (21) and to a final low value (3) after pH control failure (Fig. 6).

The lack of pH control significantly influenced the bacterial community structure as demonstrated by the low similarity (49 %) between samples drawn at days 197 and 224 (before and after pH failure). Fungal populations also experienced significant changes both after the five-day process shutdown and following the pH control failure, with Pearson similarity values of 30 % between samples 182 and 197 (collected after process shutdown), and of 46 % between samples 197 and 224 (collected after pH failure) (Fig. 2 and Fig. 3). The higher degree of dynamics and the loss of richness in fungal and bacterial populations after process shut-down and/or pH control failure demonstrated a stronger effect of these events compared to fluctuations in inlet load and process starvation.

In terms of AS performance, while functional responses depended on the compound analyzed (H_2S REs remained high regardless of the event applied and alpha-pinene removal increased during robustness tests), the lowest butanone and toluene REs together with the highest recovery times were observed after process-shut down and pH

351 control failure (Table 2). The different response patterns observed for the different

352 functions (individual pollutant removal) in the AS system, and the weak correlations

353 between the ecological parameters and the AS performance, could be related to certain

354 community traits (growth rates, competition, functional redundancy, etc.) influencing

355 the community-function relationships.

356 **3.5. Taxonomic assignment of bacterial and fungal populations from the AS**
357 **system**

358 After chimera checking, nineteen valid sequences belonging to bacteria were retrieved

359 from the AS system. Similar results in classification were obtained using the RDP

360 Classifier at 95 % bootstrap, the UTAX algorithm and the Ez-Taxon-e database.

361 However, at confidence thresholds of 50 % (recommended threshold for gene fragments

362 of length between 50 and 250 nucleotides) and 80 % (original recommended threshold

363 of the RDP Classifier (Wang et al., 2007), the RDP Classifier resulted in a higher

364 prediction mainly at the genus level (Table S1). These results support previous

365 observations indicating that RDP naïve Bayesian classifier could tend to "overclassify"

366 (i.e. to give high confidence values to predictions when in fact the sequence belongs to a

367 novel taxon (http://drive5.com/usearch/manual/rdp_case.html).

368 Among the most similar classifications, Ez-Taxon-e-based results seemed to be a

369 compromise between the results obtained by the RDP tool (95 % bootstrap) and the

370 UTAX algorithm in terms of taxonomic resolution. This means that UTAX classified

371 less sequences to lower taxonomic levels, while the RDP classifier assigned more

372 sequences to lower taxonomic ranks (Table S1), compared to Ez-Taxon-e. This could

373 reflect an RDP over classification or an UTAX under classification of some bacterial

374 sequences.

At higher taxonomic ranks (phylum and class), Ez-Taxon-e, UTAX and RDP (95 % bootstrap) showed almost consistent results. Proteobacteria phylum included bands 2, 3, 6, 13, 14, 15, 19; Actinobacteria phylum contained bands 4, 5, 10, 11, 17 (also including band 9 when using Ez-taxon-e). Band 7 was associated to the phylum Nitrospirae, and band 1 to the phylum "*Candidatus* Saccharibacteria" (remaining unclassified by using the Ez-taxon-e). The three classifications provided 4 unclassified bands (Table S1).

Fifteen valid fungal sequences were obtained from the AS diffusion reactor. The taxonomic classification of these sequences by means of SINA web tool showed four unclassified sequences. The rest of the nucleotide fragments were classified within the Ascomycota phylum (subphylum Pezizomycotina) (Table S3). Only the DGGE fragments 10 and 15 were classified to the genus level (*Fusarium*). All other bands were classified to the "order" taxonomic rank: bands 3, 5, 7, 8, 9, 12, 13 belonged to Hypocreales, while bands 6 and 14 to Eurotiales and Chaetothyriales, respectively (Table S3).

3.6. Correlation of sequencing information with potential roles of microorganisms

3.6.1 Bacteria

Phyla Proteobacteria and Actinobacteria, which have been commonly found as predominant groups in other bioreactors treating malodorous emissions (Estrada et al., 2012; Kristiansen et al., 2011; Lebrero et al., 2013; Muñoz et al., 2013), were found overrepresented.

Certain Gamma-Proteobacteria had a limited role on VOC and H_2S degradation, since their corresponding bands (2, 13, 14, 15) appeared mainly at day 95 (Fig. 4). On the contrary, band 6, affiliated to the genus *Rhodanobacter* (Xanthomonadaceae family)

399 (Table S1 and Table S2), was present from the AS start-up until day 162 (Fig. 4).

400 Although not known to be able to degrade the compounds in the feed, other

401 Xanthomonadaceae-like microorganisms have been detected in a biofilter fed with

402 methyl-mercaptan, toluene, alpha-pinene and hexane at trace level concentrations

403 (Lebrero et al., 2012).

404 Alpha-Proteobacteria (band 3) and Beta-Proteobacteria (band 19) had a key role in the

405 AS system based on its almost continuous presence along the whole reactor run (Fig 4).

406 Microorganisms of the Rhizobiales order (band 3) and of the Comamonadaceae family

407 (band 19), have been previously detected in other gas-treatment systems (Table S2)

408 (Kristiansen et al., 2011; Lebrero et al., 2013). Some Rhizobiales are able to utilize

409 linear and polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs)

410 and aromatic heterocycles (Teng et al., 2015). *Comamonas* species are able to degrade

411 toluene (Jiang et al., 2015).

412 Actinobacteria phyla, represented by six nucleotide sequences within the Actinobacteria

413 class (bands 4, 5, 9,10, 11 and 17) (Table S1), showed high band intensities during the

414 operational failures and fluctuations applied (Fig. 4), suggesting the key role of these

415 microorganisms in maintaining robustness in the AS system. Actinobacteria-like

416 microorganisms could be involved in butanone or toluene degradation in the reactor

417 since they are able to degrade aromatic hydrocarbons and ketones (Silini et al., 2016;

418 Thorenoor et al., 2009).

419 The *Nitrospira* genus (band 7) within the Nitrospirae phylum was also detected (Table

420 S1 and Fig. 4). Nitrifying bacteria can oxidize a broad range of aromatic and non-

421 aromatic hydrocarbons (Silva et al., 2009). This band showed 99% identity at

422   GeneBank to a sequence obtained from stirred-tank reactors treating toluene at different

423   concentrations (Estrada et al., 2012) (Table S2).

424   The "*Candidatus* Saccharibacteria" phylum (Candidate Division TM7) was represented

425   by DGGE fragment 1 at EBRTs of 55 s and 48 s (Table S1). A TM7 toluene-degrading

426   bacteria has been identified by stable isotope probing in agricultural soil (Luo et al.,

427   2009).

428   3.6.2   *Fungi*

429   Only the Ascomycota phylum (Pezizomycotina subphylum), considered a fungal group

430   with the ability to transform a large range of organic pollutants (Harms et al., 2011),

431   was found in the AS reactor (Table S3). Most DGGE bands, were assigned to the

432   Hypocreales order, while Eurotiales and Chaetothyriales were represented by one band

433   each (Table S3). The same groups were observed from three gas biofilters during the

434   degradation of toluene, ethylbenzene and p-xylene, respectively (Prenafeta-Boldú et al.,

435   2012). However, despite the ability of Eurotiales to degrade aliphatic hydrocarbons,

436   chlorophenols and polycyclic aromatic hydrocarbons (Harms et al., 2011), this group

437   did not have a role in the degradation of the target gas pollutants, since band 6 was only

438   present in the inoculum.

439   Most of the Hypocreales (bands 5, 7, 8, 9), were present from the beginning of reactor

440   operation until the operation at an EBRT of 32 s, excluding bands 3 and 13 which

441   appeared exclusively at 94 s and 48 s EBRT, respectively, and band 12, which remained

442   along the whole reactor operation but decreased in intensity from the first disturbance

443   onwards (Fig. 5). Bands 10 and 15 within this order belonged to *Fusarium* (Table S3), a

444   fungus capable of degrading aromatic hydrocarbons or compounds that contain aromatic

445   rings (Chulalaksananukul et al., 2006). Qi et al. (2005) observed the development of

446   *Fusarium oxysporum* and *Fusarium nygama* in a fungal biofilter initially inoculated

447   with *Cladosporium sphaerospermum*, treating toluene among others compounds. Band

448   10 was present along the whole reactor operation; its intensity being solely affected by

449   pH failure. Band 15 was present at 32 s EBRT and during robustness analysis, and was

450   also negatively affected by pH failure in terms of band intensity (Fig. 5).

451   Chaetothyriales (band 14), which are able to assimilate toluene (*Cladophialophora*,

452   *Exophiala)* (Harms et al., 2011), were also present at an EBRT of 32 s and during

453   robustness analysis (Fig. 5). Likely, *Fusarium-* and Chaetothyriales-like

454   microorganisms played a key role in maintaining functional performance of the system

455   during fluctuations.

4. Conclusions

457   A partial correlation between ecological parameters (Rr, Shannon diversity, evenness,

458   dynamics, composition) and AS reactor functions (H_2S, butanone, toluene and alpha-

459   pinene removal) was observed, suggesting that multiple ecosystem properties (evenness,

460   stress tolerance, evolutionary adaptation, growth rate, diversity, etc.) can shape

461   microbial responses under fluctuations. Despite the great complexity associated to

462   understand the drivers of functional stability, evenness and dynamic parameters seemed

463   to play a role to maintain the stability of the reactor.

464   Proteobacteria and Actinobacteria were the most abundant bacterial groups retrieved

465   from the AS system. The fungal orders detected (Hypocreales, Eurotiales,

466   Chaetothyriales), are commonly encountered in off-gas treatment bioreactors. While

467   most of bacteria and fungi retrieved from the bioreactor have been previously classified

468   as toluene or butanone degraders, typical H_2S oxidizers were not detected, despite high

469   H_2S REs observed. Finally, neither known fungal or bacterial alpha-pinene degraders

470   nor *Pseudomonas fluorescens* were found, their absence resulting in low alpha-pinene

471   abatement efficiencies. *P. fluorescens* was likely outcompeted by the microorganisms

472   already adapted to the characteristics of the AS environment.

Acknowledgements

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613

Figure Captions

614

615 **Figure 1.** A schematic representation of the operational changes applied to the activated

616 sludge bioreactor in this work.

617 **Figure 2.** Bacterial dendrogram showing clustering of samples along AS reactor

618 operation. Nodes with a bootstrap support value of 90% or higher are indicated by a

619 black dot. Samples (indicated by the collection day) and their corresponding EBRT

620 appear at the bottom of the figure. Samples collected before glucose addition at day 95

621 are in boldface. Samples subjected to robustness tests (175: inlet load fluctuations, 182:

622 starvation, 197: shut-down, 224: pH failure) are underlined. Samples collected between

623 these two periods are in normal font.

624 **Figure 3.** Fungal dendrogram showing clustering of samples along AS reactor

625 operation. Nodes with a bootstrap support value of 90% or higher are indicated by a

626 black dot. Samples (indicated by the collection day) and their corresponding EBRT

627 appear at the bottom of the figure. Samples collected before glucose addition at day 95

628 are in boldface. Samples subjected to robustness tests (175: inlet load fluctuations, 182:

629 starvation, 197: shut-down, 224: pH failure) are underlined. Samples collected between

630 these two periods are in normal font.

631 **Figure 4.** DGGE profile showing changes in bacterial population due to variations in

632 EBRT (lane lower numbers), glucose addition and establishment of a 25 d SRT at day

633 95 (A), alpha-pinene addition at day 121 (B), *P. fluorescens* addition at day 132 (C),

634 fluctuations in odorant loading (D), starvation conditions (E), process shut-down (F)

635 and pH failure (G). Lane upper labels indicate the operation day at which samples were

636 collected. Bands sequenced are indicated by "b" followed by the corresponding number

637 of each band.

638   **Figure 5.** DGGE profile showing changes in fungal population due to variations in

639   EBRT (lane lower numbers), glucose addition and establishment of a 25 d SRT at day

640   95 (A), alpha-pinene addition at day 121 (B), *P. fluorescens* addition at day 132 (C),

641   fluctuations in odorant loading (D), starvation conditions (E), process shut-down (F)

642   and pH failure (G). Lane upper labels indicate the operation day at which samples were

643   collected. Bands sequenced are indicated by "b" followed by the corresponding number

644   of each band.

645   **Figure 6.** Shannon diversity index (H), ranged weighted richness (Rr) and functional

646   organization (Fo) of bacterial and fungal communities calculated from the DGGE

647   patterns at days 0, 13, 29, 43, 59, 79, 90, 95, 108, 115, 127, 144, 162 (steady operation),

648   175, 182, 197 and 224 (robustness test). The EBRTs tested are indicated in the upper

649   part of each graph and by vertical dotted lines. The continuous line represents the

650   beginning of AS operation at 25 d of sludge retention time and with glucose addition.

651   Vertical dashed lines represent fluctuations and operational failures applied. Vertical

652   dashed and dot line indicate the addition of alpha-pinene at day 121.

653

654

1   # Microbial community changes during different empty

2   # bed residence times and operational fluctuations in an

3   # air diffusion reactor for odor abatement

4

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Abstract

The succession of bacterial and fungal populations was assessed in an activated sludge (AS) diffusion bioreactor treating a synthetic malodorous emission containing H_2S, toluene, butanone and alpha-pinene. Microbial community characteristics (bacterial and fungal diversity, richness, evenness and composition) and bioreactor function relationships were evaluated at different empty bed residence times (EBRTs) and after process fluctuations and operational failures (robustness test). For H_2S, butanone and toluene, the bioreactor showed a stable and efficient abatement performance regardless of the EBRT and fluctuations applied, while low alpha-pinene removals were observed. While no clear positive or negative relationship between community characteristics and bioreactor functions was observed, ecological parameters such as evenness and community dynamics seemed to be of importance for maintaining reactor stability. The optimal degree of evenness of the inoculum likely contributed to the high robustness of the system towards the fluctuations imposed. Actinobacteria, Proteobacteria and Fungi (Hypocreales, Chaeatothyriales) were the most abundant groups retrieved from the AS system with a putative key role in the degradation of butanone and toluene. Typical H_2S and alpha-pinene degraders were not retrieved from the system. The inoculation of *P. fluorescens,* a known alpha-pinene degrader, to the system did not result in the enhancement of the degradation of this compound. This strain was likely outcompeted by the microorganisms already adapted to the AS environment.

Keywords: activated sludge; volatile organic compound; bacteria; fungi; evenness; fluctuation

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1. Introduction

Changes of microbial communities (microbial succession) in natural and engineered ecosystems (bioreactors) in response to variations in operating or environmental conditions constitute nowadays a main issue in microbial ecology to predict system or ecosystem behaviour (Pholchan et al., 2013). Several characteristics of the microbial community structure seem to play a key role in maintaining functional stability under these variations. Among them, richness, evenness, dynamics, functional redundancy, microbial composition and microbial interactions seem to be of utmost importance for controlling reactor and ecosystem functioning (Bell et al., 2005; Cabrol et al., 2012; Wittebolle et al., 2009). For example, communities exhibiting intermediate evenness values (some species are dominant but most of them are present in decreasing lower amounts) have been shown to better deal with changing environmental conditions, since they have a pool of less dominant species able to replace the leading ones under operating or environmental fluctuations (Marzorati et al., 2008). Species richness effects on ecosystem functioning can decrease when functionally redundant species exist in the community (Bell et al., 2005).

The number of studies addressing this topic in bioreactors treating gas pollutants and malodorous emissions (with harmful effects on both human health and natural ecosystems) has increased during the last decade. Since biofilters and biotrickling filters are by far the most commonly implemented technologies for odor abatement, microbial-based studies have primarily focused on these systems (Cabrol et al., 2012; Lebrero et al., 2012, 2013; Prenafeta-Boldú et al., 2012). In addition to bacteria, fungi have been also investigated in biofilters (Prenafeta-Boldú et al., 2012), due to their ability to degrade complex organic pollutants and their superior performance compared to

60 bacteria when present in media-based odor treatment bioreactors (Estrada et al., 2013;

61 Harms et al., 2011).

62 However, emerging odor treatment technologies such as activated sludge (AS) diffusion

63 bioreactors (based on the direct sparging of the malodorous emission into the aeration

64 tank in wastewater treatment plants (WWTPs)) have been less studied from both a

65 microbiological and engineering point of view. This technology represents a cost-

66 effective alternative to biofilters and biotrickling filters due to it avoids problems related

67 to packing media compaction, moisture control or accumulation of toxic metabolites.

68 Nevertheless, the lack of reliable data concerning wastewater treatment performance

69 during the abatement of volatile organic compounds (VOCs) from malodorous streams,

70 and the lack of knowledge on the ability of AS systems to cope with process

71 fluctuations and operational failures still limit its widespread application (Bowker,

72 2000). In this context, unraveling the structure and dynamics of the microbial

73 communities (both bacteria and fungi) governing AS systems treating malodorous

74 emissions under steady or transient operating conditions can contribute to prevent

75 undesirable malfunction events.

76 This study was thus conducted to assess the temporal variation in the structure and

77 composition of the microbial communities (bacteria and fungi) in an AS diffusion

78 bioreactor treating a mixture of VOCs (toluene, butanone and alpha-pinene) and H_2S at

79 low inlet concentrations (mg m^{-3}), which simulated a simplified odorous emission from

80 a WWTP. The evolution of the microbial populations and their associated ecological

81 parameters were correlated with bioreactor performance during the operation of the

82 system at different empty bed residence times (EBRTs). The system was also evaluated

83 during the analysis of AS robustness versus typical operational fluctuations.

2. Material and methods

2.1. Bioreactor set-up and physical-chemical analysis

The configuration of the AS diffusion bioreactor and the analytical procedures employed to monitor the system were described in detail in Lebrero et al. (2010, 2011). Briefly, the AS system consisted of a jacketed column with a working volume of 8.5 liters operated at 20 ºC. The reactor was inoculated with 1 liter of concentrated (17 g L$^-$^{1}) return municipal activated sludge from Valladolid WWTP (Spain) resuspended in a SO_4^{2-}-free mineral salt medium (MSM) to a volume of 7.5 liters. The pH was maintained at 6.3 ± 0.3 by daily addition of a NaOH-Na$_2$CO$_3$ solution.

The inlet and outlet concentration of CO_2 and H_2S were analyzed using a GC-TCD (Varian CP-3800) and an electrochemical sensor (Dräger X-am 5000) calibrated in the 0-40 ppm range, respectively. Gas samples for VOC analysis were collected in 250mL calibrated glass bulbs (SUPELCO) and pre-concentrated by SPME. VOC inlet and outlet concentrations were then determined by GC-MS according to Lebrero et al. (2010). The pH was measured using a pH/conductivity/temperature meter (pH 510 Eutech Instruments, Nijkerk, The Netherlands). Biomass concentration in the AS unit was estimated via culture absorbance measurements (optical density at 600 nm) in a Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) and as total solids concentration (Lebrero et al., 2010). Sulfate concentration was determined by HPLC-IC using an IC-Pak Anion HC (150mm×4.6mm) column. Finally, dissolved total organic carbon (DOC), dissolved inorganic carbon (DIC) and dissolved total nitrogen (DTN) were periodically recorded in the AS system using a TOC-V$_{CSH}$ analyzer (Shimadzu, Tokyo, Japan) coupled with a TN module based on chemiluminesce detection (TNM-1, Shimadzu).

2.2. Bioreactor operation: effect of different EBRTs and alpha-pinene addition

Details about the operation of the AS system under steady conditions can be found in Lebrero et al. (2011). A schematic representation of the different conditions applied to the reactor is shown in Fig. 1. Briefly, a mixture of H_2S, toluene and butanone with pre-humidified and filtered ambient air was fed to the bioreactor at concentrations of 16.9-23.8 $mg.m^{-3}$, 0.40-0.60 $mg.m^{-3}$ and 4.3-6.3 $mg.m^{-3}$, respectively. During the first 121 days, the degradation rate of these compounds in the system was evaluated at different EBRTs (day 0 to 29: 94 s; day 29 to 43: 74 s; day 43 to 59: 55 s; day 59 to 95: 48 s; day 95 to 162: 32 s) in the absence of any additional carbon source and at infinite sludge retention time (SRT) (no biomass withdrawal). At day 95 (EBRT of 48 s), after observing a biomass aggregation and compaction in the reactor, glucose was added to the AS system, and the SRT was set-up at 25 days (340 ml of mixed liquor were daily withdrawn and replaced with fresh MSM containing 2 g L^{-1} of glucose) in order to simulate real WWTPs operation. At day 121 (32 s EBRT), alpha-pinene was supplemented to the previous gas mixture to evaluate the degradation rate of a hydrophobic odorant in the bioreactor. To enhance alpha-pinene biodegradation, a culture of *Pseudomonas fluorescens* NCIMB 11671, purchased from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland), was inoculated to the bioreactor at day 132. The system was finally maintained at 32 s of EBRT until the end of the experiment (day 162).

The VOCs butanone, toluene and α-pinene were selected as model VOCs representing soluble, moderately soluble and hydrophobic compounds commonly found in WWTP emissions (Lehtinen and Veijanen, 2011; Zarra et al., 2008). H_2S was selected as model sulfur odorant, widely present in malodorous emissions from WWTPs and sewage works.

2.3. Bioreactor operation: robustness analysis

To systematically evaluate the effect of the operational failures and fluctuations in the robustness analysis, the EBRT was set up at 50 s to ensure this parameter was not a limiting factor for bioreactor performance. The robustness test consisted of process fluctuations and simulated operational failures imposed to the AS system from day 163 onwards (Fig. 1). Process response to fluctuations in odorant loading was evaluated by applying two sequential 3-h step increases (3-fold and 6-fold increases) in the inlet H_2S and VOCs loading, with a 20 h recovery period between them. After system stabilization, the robustness of the AS bioreactor towards a three-day starvation period (only humidified air was supplied) and a five-day shutdown period (neither polluted air supply nor glucose addition) was investigated. Finally, the response of the AS bioreactor to a 3-day interruption of pH control (pH decreased to 2.8) was assessed.

Each operational condition during the testing of different EBRTs and during robustness analysis was maintained for at least 3 weeks to ensure stable steady states. Details concerning robustness analysis can be found in Lebrero et al. (2010).

2.4. Sampling, DNA extraction and 16S / 18S rRNA gene amplification

Biomass samples of each operation condition were collected in sterile polypropylene tubes and immediately stored at -20 ºC for subsequent molecular analysis. Samples were named with the operation day at which they were collected. Their corresponding operational conditions were as follows: 0 (inoculum), 13 and 29 (EBRT: 94 s), 43 (EBRT: 74 s), 59 (EBRT: 55 s), 79, 90, 95 (EBRT: 48 s), 108, 115, 127, 144 and 162 (EBRT: 32 s), 175 (after fluctuations in odorant loading), 182 (after starvation period), 197 (after process shutdown), and 224 (after interruption in pH control) (Fig. 1).

7

156 DNA extraction was carried out according to Rodríguez et al. (2012). The quality of the

157 extracted DNA was checked by agarose gel (1.2 % w/v) electrophoresis and DNA was

158 stored at -20 ºC. Amplification of bacterial 16S rRNA gene fragments was performed

159 using the primer pair 1401R and 968F-GC (SIGMA-Aldrich, USA) (Nübel et al., 1996).

160 For the amplification of fungal 18S rRNA gene, a nested PCR approach was used with

161 primer pairs nu-SSU-0817F and nu-SSU-1536R (first round) and nu-SSU-0817F and

162 nu-SSU-0817R (second round) (SIGMA-Aldrich, USA) (Borneman and Hartin, 2000).

163 The PCR mixture included 1-2 µL of each primer, 25 µL of PCR Mastermix (Bioline,

164 Ecogen-Spain) (containing *Taq* DNA polymerase, PCR reaction buffer and

165 deoxynucleotides (dNTPs)), 1-2 µL of DNA template and Milli−Q water up to a final

166 volume of 50 µL. PCR was performed in an iCycler Thermal Cycler (BioRad

167 Laboratories, USA) with the following thermo-cycling program for bacterial

168 amplification: 2 min of pre-denaturation at 95 °C, 35 cycles of denaturation at 95 °C for

169 30 s, annealing at 56 °C for 45 s, and elongation at 72 °C for 1 min, with a final 5 min

170 elongation at 72 °C. For fungal amplification, the thermo-cycling program in the nested

171 PCR reactions included 5 min of pre-denaturation at 94 °C, 35 cycles of denaturation at

172 94 °C for 45 s, annealing at 50 °C for 45 s, and elongation at 72 °C for 1 min, and a

173 final 5 min elongation at 72 °C. Size and yield of PCR products were verified in 1.8 %

174 (w/v) agarose gels and subsequent SYBR Green I staining (SIGMA-Aldrich, USA).

175 **2.5. Resolution of 16S / 18S rRNA amplicons in gradient polyacrylamide gels**

176 PCR amplicons were resolved by double denaturing gradient gel electrophoresis

177 (DGGE) using a D-Code Universal Mutation Detection System (BioRad Laboratories,

178 USA). Polyacrylamide gels with a porous gradient of acrylamide/bisacrylamide of 6-

179 10%, and a urea/formamide denaturant gradient of 45−65 % and 23−45 % for bacterial

180 and fungal communities, respectively, were used. Electrophoresis was performed at 60

181 °C, with TAE 0.5-X as running buffer, at 64 V for 18 h for bacterial amplicons, and at

182 70 V for 18 h for fungal PCR products. The gels were stained with SYBR Green I for 1

183 h. Individual bands were excised from the DGGE gels with a sterile blade, resuspended

184 in 50 µl of ultrapure water, and maintained at 60 °C for 1 h to allow DNA extraction

185 from the gel. A volume of 5 µL of the supernatant was used for reamplification with the

186 original primer sets and PCR programs. Before sequencing at Secugen S.L., PCR

187 products were purified with the GenElute PCR DNA Purification Kit (Sigma-Aldrich,

188 USA).

189 **2.6. DGGE profile analysis for sample clustering and ecological parameters**
190 **determination**

191 Range-weighted richness (Rr), evenness/functional organization (Fo) and temporal

192 dynamics (UPGMA clustering) of the bacterial and fungal communities were calculated

193 based on the bacterial and fungal DGGE profiles. DGGE profiles were analyzed using

194 the GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

195 Dendrograms (reflecting community dynamics), were constructed according to Lebrero

196 et al. (2012). Briefly, they were created by UPGMA clustering (500 resampling

197 experiments), based on the similarity indices of the DGGE profiles calculated using the

198 Pearson product–moment correlation coefficient (Fig. 3 and Fig. 4). Shannon–Wiener

199 diversity index was also calculated according to Lebrero et al. (2012).

200 Richness and evenness parameters were calculated according to Marzorati et al. (2008).

201 Ranged weighted richness (Rr), which indicates the richness and genetic diversity

202 within a bacterial community, was calculated based on the total number of bands (N)

203 and the denaturing gradient between the first and the last band of each lane (Dg),

204 according to the equation:

205 $$Rr = N^2 \times Dg \qquad (Eq. \; 1)$$

206 Community evenness was graphically represented by using Pareto-Lorenz evenness

207 distribution curves, i.e. for each DGGE lane, the bands were ranked from high to low

208 based on their intensities. Consecutively, the cumulative normalized number of bands

209 was used as x-axis, and their respective cumulative normalized band intensities were

210 used as y-axis (data not shown). For the interpretation of these curves, the functional

211 organization (Fo) of the community was calculated by scoring the y-axis projection of

212 their intercepts with the vertical 20 % x-axis. Results of Rr and evenness parameters

213 were evaluated according to the values proposed by Marzorati et al. (2008).

214 **2.7. Analysis of bacterial and fungal nucleotide sequences**

215 Chimeric sequences were detected and then removed by using DECIPHER (Wright et

216 al., 2012) and UCHIME v8.1 (Edgar et al., 2011) for bacterial and fungal nucleotide

217 sequences, respectively. Classification of bacterial sequences into taxonomic ranks was

218 performed using different bioinformatic tools to observe differences in taxonomic

219 assignments. The RDP Classifier (50 %, 80 %, 95 % bootstrap value) (Wang et al.,

220 2007), the UTAX Algorithm within the Usearch Sequence Analysis Tool (0.9 cut-off)

221 (Edgar, 2010) using Bio-Linux 8 (Field et al., 2006) and the Ez-Taxon-e Database (Kim

222 et al., 2012) (using the taxonomic thresholds proposed by Yarza et al. (2014) to

223 manually classify microorganisms into the different taxonomic ranks) were used.

224 Fungal nucleotide sequences were classified using SINA alignment web service based

225 on the SILVA database (Quast et al., 2013). For both bacterial and fungal sequences,

226 their closest relatives in GenBank (Blastn) were retrieved to evaluate the presence of the

227 AS microorganisms in similar environments.

228 Nucleotide sequences obtained in this study were deposited at GenBank under accession

229 numbers HQ147605 to HQ147612 and KX893872 to KX893882 (bacteria) and

230 KX907435 to KX907449 (fungi).

3. Results and Discussion

3.1. Bioreactor operation: effect of different EBRTs and alpha-pinene addition

233 The macroscopic performance of the AS bioreactor at different EBRTs was reported in

234 detail in Lebrero et al. (2011). Briefly, the removal efficiencies (REs) of H_2S, butanone

235 and toluene remained high regardless of the EBRT applied (94 s, 74 s, 55 s, 48 s and 32

236 s) (Table 1 and Fig. S1). At day 95, a rapid decrease in the biomass concentration and in

237 butanone and toluene REs was observed concomitantly with a biomass compaction

238 phenomenon at the bottom of the reactor. The addition of glucose to the AS unit and

239 process operation at a sludge retention time (SRT) of 25 days led to the rapid

240 resuspension of the biomass, the increase in biomass concentration and the recovery of

241 the preceding elimination performance (Fig. S1). Alpha-pinene, which was

242 supplemented from day 121 until day 162, was initially removed at approximately 21

243 %, but its RE decreased to 6.8 ± 1.9 % after two days of operation, and remained

244 constant for the following 40 days (Table 1). The addition of 250 ml of a *Pseudomonas*

245 *fluorescens* culture (alpha-pinene degrading species) after 11 days of alpha-pinene

246 feeding did not result in a significant enhancement of the removal of this terpene.

3.2. Bioreactor operation: robustness analysis

248 In general, the results obtained from robustness assays (Lebrero et al., 2010) indicated a

249 high capacity of the AS diffusion system to rapidly recover from negative events.

250 Hydrogen sulfide REs remained unaffected despite the operational fluctuations and

251 failures applied. Butanone and toluene REs remained stable at 99.7 ± 0.0 % and $98.4 \pm$

252 0.1 %, respectively, regardless of the odorant inlet concentration. After a three-day

253 starvation period, no loss in the pollutant abatement performance or long-term damage

254 was observed. Thus, steady state REs were rapidly recovered within the first 30 min

255 after the resumption of pollutants supply. After a five-day process shutdown, the

256 previous butanone and toluene abatement performance was rapidly restored within 3

257 and 6.5 hours, respectively. Failure in pH control led to a minimum RE of 39 % for

258 toluene and the reactor was not able to recover the previous steady state REs (98.6 ± 0.2

259 %) for this compound. A new steady state RE of 95.5 ± 0.1 % was achieved seven days

260 after pH control restoration. Conversely, the decrease in pH did not affect butanone RE,

261 which remained constant at steady state values of 99.8 ± 0.0 % (Table 2).

262 The RE of alpha-pinene increased from steady state values of 5.7 ± 0.8 % to a

263 maximum value of 23.5 % during the surges in the odorant inlet load. The RE of this

264 terpene also increased immediately after process start-up following the shutdown period

265 (maximum RE of 19.9 %), returning to steady values after 3 h. Finally, alpha-pinene

266 REs increased up to 50.8 ± 1.1 % 12 days after the first induced failure in pH control

267 (Table 2).

268 **3.3. Community structure at different EBRTs and in response to alpha-pinene**
269 **addition**

270 In general, this operational period resulted in highly dynamic bacterial and fungal

271 communities as observed by the Pearson similarities within the samples (Fig. 2 and Fig.

272 3). Changes in bacterial and fungal populations also can be observed in their respective

273 DGGE profiles (Fig 4. and Fig. 5). Balanced populations with medium Fo values

274 (ranging from 33 to 40 % for bacteria and from 28 to 41 % for fungi) (excluding sample

275 29 of fungal populations which showed a low Fo value) were observed (Fig. 6). Fo

276 indicates the ability of the community to organize in an adequate distribution of

277 dominant and non-dominant microorganisms that should assure the potentiality of

278 counteracting the effect of a sudden stress exposure. The ranged weighted richness was

279 high for bacterial populations, while medium to high Rr values were recorded for fungi

280 (excluding sample 79 which showed a low value). Shannon diversity, which generally

281 ranges from 1.5 to 3.5 (low and high evenness and richness, respectively), showed high

282 values for bacterial communities (between 3.0 and 3.7), and slightly lower values for

283 fungal populations (2.3 to 3.0) (Fig. 6).

284 These characteristics are generally associated with microbial communities able to

285 rapidly respond to changing conditions. For example, high Rr values - which were

286 particularly high for bacteria- represent populations with a high flexibility. This means

287 that a high number of species in the system offer multiple pathways for the degradation

288 of the different organic compounds, increasing functional redundancy and contributing

289 to a better adaptation to changes (De Vrieze et al., 2013). Similarly, a dynamic

290 microbial community, along with an optimal degree of evenness (medium Fo values

291 (Marzorati et al., 2008)), are considered of key importance to guarantee functional

292 stability in microbial communities (De Vrieze et al., 2013).

293 In accordance with these results, the AS system showed a stable performance for VOCs

294 and H_2S removal under the different EBRTs tested (Table 1 and Fig. S1). Only between

295 days 90 and 95 (48 s EBRT), a period of instability and low butanone and toluene REs

296 was recorded concomitant with biomass compaction and sedimentation and with a

297 decrease in suspended solids concentration (from 1 to 0.12 g L^{-1}) (Lebrero et al., 2011).

298 This unstable operation matched with the lowest Rr values observed for fungal

299 communities (Fig. 6), suggesting that the absence of sludge renewal and the lack of a

300 readily available organic carbon source mainly impacted fungal populations in terms of

301 richness. It is also important to note that a high degree of bacterial and fungal

community dynamics was observed at 48 s EBRT, indicating a period clearly marked by abrupt changes in the populations present in the system (Fig. 2 and Fig. 3). In this sense, sample 79 (the first collected at 48 s EBRT) clustered separately from sample 59 (collected at 55 s EBRT), showing a similarity of 30 % and 47 % between them for bacterial and fungal communities, respectively. At day 90, the structure of the bacterial and fungal communities was more similar to the communities present at day 59. However, at day 95, bacterial and fungal populations had undergone again important shifts, with a similarity of 36 and 56 % between samples from days 90 and 95 for bacterial and fungal communities, respectively. Despite microbial populations and reactor performance clearly being affected at this period, the system rapidly recovered butanone and toluene REs after glucose addition and process operation with a periodic sludge withdrawal (Fig. S1). The high dynamics and flexibility (Rr), and the optimum organization of the communities (Fo), likely contributed to maintain reactor stability under operational variations, as previously observed in other research works (Cabrol et al., 2012; De Vrieze et al., 2013; Firmino et al., 2015).

The application of the lowest EBRT (32 s) did not result in a detrimental effect in reactor performance, which maintained high REs for butanone, toluene and H_2S (Table 1 and Fig. S1). Fo and Shannon diversity did not significantly change and fungal communities recovered high Rr values (except for sample at day 162, which showed a medium value) at this stage (Fig. 6). The addition of alpha-pinene at day 121 did not trigger important changes in the ecological parameters measured in this study, likely due to the lack of an active microbial community capable of degrading alpha-pinene at the low concentrations present in the mixed liquor (0.10-0.13 mg L^{-1}) (Lebrero et al., 2011).

3.4. Community structure under process fluctuations and operational failures

The organization of the bacterial and fungal communities (Fo) remained at its medium values regardless of the operational failure or fluctuations applied (Fig. 6), highlighting the relevance of this parameter in maintaining functional stability. As well as for the results obtained during steady state operation, the operational changes imposed mainly affected bacterial richness (Rr) rather than community evenness. Bacterial Rr gradually decreased from high values (fluctuations in odorant loading: 105, starvation period: 77, process shutdown: 62) to medium values after pH control failure (25). Likewise, fungal Rr decreased from high values (fluctuations in odorant loading: 43, starvation period: 37) to a medium value following process shutdown (21) and to a final low value (3) after pH control failure (Fig. 6).

The lack of pH control significantly influenced the bacterial community structure as demonstrated by the low similarity (49 %) between samples drawn at days 197 and 224 (before and after pH failure). Fungal populations also experienced significant changes both after the five-day process shutdown and following the pH control failure, with Pearson similarity values of 30 % between samples 182 and 197 (collected after process shutdown), and of 46 % between samples 197 and 224 (collected after pH failure) (Fig. 2 and Fig. 3). The higher degree of dynamics and the loss of richness in fungal and bacterial populations after process shut-down and/or pH control failure demonstrated a stronger effect of these events compared to fluctuations in inlet load and process starvation.

In terms of AS performance, while functional responses depended on the compound analyzed (H_2S REs remained high regardless of the event applied and alpha-pinene removal increased during robustness tests), the lowest butanone and toluene REs together with the highest recovery times were observed after process-shut down and pH

351 control failure (Table 2). The different response patterns observed for the different

352 functions (individual pollutant removal) in the AS system, and the weak correlations

353 between the ecological parameters and the AS performance, could be related to certain

354 community traits (growth rates, competition, functional redundancy, etc.) influencing

355 the community-function relationships.

3.5. Taxonomic assignment of bacterial and fungal populations from the AS
356
357 **system**

358 After chimera checking, nineteen valid sequences belonging to bacteria were retrieved

359 from the AS system. Similar results in classification were obtained using the RDP

360 Classifier at 95 % bootstrap, the UTAX algorithm and the Ez-Taxon-e database.

361 However, at confidence thresholds of 50 % (recommended threshold for gene fragments

362 of length between 50 and 250 nucleotides) and 80 % (original recommended threshold

363 of the RDP Classifier (Wang et al., 2007), the RDP Classifier resulted in a higher

364 prediction mainly at the genus level (Table S1). These results support previous

365 observations indicating that RDP naïve Bayesian classifier could tend to "overclassify"

366 (i.e. to give high confidence values to predictions when in fact the sequence belongs to a

367 novel taxon (http://drive5.com/usearch/manual/rdp_case.html).

368 Among the most similar classifications, Ez-Taxon-e-based results seemed to be a

369 compromise between the results obtained by the RDP tool (95 % bootstrap) and the

370 UTAX algorithm in terms of taxonomic resolution. This means that UTAX classified

371 less sequences to lower taxonomic levels, while the RDP classifier assigned more

372 sequences to lower taxonomic ranks (Table S1), compared to Ez-Taxon-e. This could

373 reflect an RDP over classification or an UTAX under classification of some bacterial

374 sequences.

At higher taxonomic ranks (phylum and class), Ez-Taxon-e, UTAX and RDP (95 %
bootstrap) showed almost consistent results. Proteobacteria phylum included bands 2, 3,
6, 13, 14, 15, 19; Actinobacteria phylum contained bands 4, 5, 10, 11, 17 (also including
band 9 when using Ez-taxon-e). Band 7 was associated to the phylum Nitrospirae, and
band 1 to the phylum "*Candidatus* Saccharibacteria" (remaining unclassified by using
the Ez-taxon-e). The three classifications provided 4 unclassified bands (Table S1).

Fifteen valid fungal sequences were obtained from the AS diffusion reactor. The
taxonomic classification of these sequences by means of SINA web tool showed four
unclassified sequences. The rest of the nucleotide fragments were classified within the
Ascomycota phylum (subphylum Pezizomycotina) (Table S3). Only the DGGE
fragments 10 and 15 were classified to the genus level (*Fusarium*). All other bands were
classified to the "order" taxonomic rank: bands 3, 5, 7, 8, 9, 12, 13 belonged to
Hypocreales, while bands 6 and 14 to Eurotiales and Chaetothyriales, respectively
(Table S3).

3.6. Correlation of sequencing information with potential roles of microorganisms

3.6.1   Bacteria

Phyla Proteobacteria and Actinobacteria, which have been commonly found as
predominant groups in other bioreactors treating malodorous emissions (Estrada et al.,
2012; Kristiansen et al., 2011; Lebrero et al., 2013; Muñoz et al., 2013), were found
overrepresented.

Certain Gamma-Proteobacteria had a limited role on VOC and H_2S degradation, since
their corresponding bands (2, 13, 14, 15) appeared mainly at day 95 (Fig. 4). On the
contrary, band 6, affiliated to the genus *Rhodanobacter* (Xanthomonadaceae family)

399 (Table S1 and Table S2), was present from the AS start-up until day 162 (Fig. 4).

400 Although not known to be able to degrade the compounds in the feed, other

401 Xanthomonadaceae-like microorganisms have been detected in a biofilter fed with

402 methyl-mercaptan, toluene, alpha-pinene and hexane at trace level concentrations

403 (Lebrero et al., 2012).

404 Alpha-Proteobacteria (band 3) and Beta-Proteobacteria (band 19) had a key role in the

405 AS system based on its almost continuous presence along the whole reactor run (Fig 4).

406 Microorganisms of the Rhizobiales order (band 3) and of the Comamonadaceae family

407 (band 19), have been previously detected in other gas-treatment systems (Table S2)

408 (Kristiansen et al., 2011; Lebrero et al., 2013). Some Rhizobiales are able to utilize

409 linear and polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs)

410 and aromatic heterocycles (Teng et al., 2015). *Comamonas* species are able to degrade

411 toluene (Jiang et al., 2015).

412 Actinobacteria phyla, represented by six nucleotide sequences within the Actinobacteria

413 class (bands 4, 5, 9,10, 11 and 17) (Table S1), showed high band intensities during the

414 operational failures and fluctuations applied (Fig. 4), suggesting the key role of these

415 microorganisms in maintaining robustness in the AS system. Actinobacteria-like

416 microorganisms could be involved in butanone or toluene degradation in the reactor

417 since they are able to degrade aromatic hydrocarbons and ketones (Silini et al., 2016;

418 Thorenoor et al., 2009).

419 The *Nitrospira* genus (band 7) within the Nitrospirae phylum was also detected (Table

420 S1 and Fig. 4). Nitrifying bacteria can oxidize a broad range of aromatic and non-

421 aromatic hydrocarbons (Silva et al., 2009). This band showed 99% identity at

422   GeneBank to a sequence obtained from stirred-tank reactors treating toluene at different

423   concentrations (Estrada et al., 2012) (Table S2).

424   The "*Candidatus* Saccharibacteria" phylum (Candidate Division TM7) was represented

425   by DGGE fragment 1 at EBRTs of 55 s and 48 s (Table S1). A TM7 toluene-degrading

426   bacteria has been identified by stable isotope probing in agricultural soil (Luo et al.,

427   2009).

428   3.6.2   *Fungi*

429   Only the Ascomycota phylum (Pezizomycotina subphylum), considered a fungal group

430   with the ability to transform a large range of organic pollutants (Harms et al., 2011),

431   was found in the AS reactor (Table S3). Most DGGE bands, were assigned to the

432   Hypocreales order, while Eurotiales and Chaetothyriales were represented by one band

433   each (Table S3). The same groups were observed from three gas biofilters during the

434   degradation of toluene, ethylbenzene and p-xylene, respectively (Prenafeta-Boldú et al.,

435   2012). However, despite the ability of Eurotiales to degrade aliphatic hydrocarbons,

436   chlorophenols and polycyclic aromatic hydrocarbons (Harms et al., 2011), this group

437   did not have a role in the degradation of the target gas pollutants, since band 6 was only

438   present in the inoculum.

439   Most of the Hypocreales (bands 5, 7, 8, 9), were present from the beginning of reactor

440   operation until the operation at an EBRT of 32 s, excluding bands 3 and 13 which

441   appeared exclusively at 94 s and 48 s EBRT, respectively, and band 12, which remained

442   along the whole reactor operation but decreased in intensity from the first disturbance

443   onwards (Fig. 5). Bands 10 and 15 within this order belonged to *Fusarium* (Table S3), a

444   fungus capable of degrading aromatic hydrocarbons or compounds that contain aromatic

445   rings (Chulalaksananukul et al., 2006). Qi et al. (2005) observed the development of

446   *Fusarium oxysporum* and *Fusarium nygama* in a fungal biofilter initially inoculated

447   with *Cladosporium sphaerospermum*, treating toluene among others compounds. Band

448   10 was present along the whole reactor operation; its intensity being solely affected by

449   pH failure. Band 15 was present at 32 s EBRT and during robustness analysis, and was

450   also negatively affected by pH failure in terms of band intensity (Fig. 5).

451   Chaetothyriales (band 14), which are able to assimilate toluene (*Cladophialophora*,

452   *Exophiala)* (Harms et al., 2011), were also present at an EBRT of 32 s and during

453   robustness analysis (Fig. 5). Likely, *Fusarium-* and Chaetothyriales-like

454   microorganisms played a key role in maintaining functional performance of the system

455   during fluctuations.

456   **4. Conclusions**

457   A partial correlation between ecological parameters (Rr, Shannon diversity, evenness,

458   dynamics, composition) and AS reactor functions (H_2S, butanone, toluene and alpha-

459   pinene removal) was observed, suggesting that multiple ecosystem properties (evenness,

460   stress tolerance, evolutionary adaptation, growth rate, diversity, etc.) can shape

461   microbial responses under fluctuations. Despite the great complexity associated to

462   understand the drivers of functional stability, evenness and dynamic parameters seemed

463   to play a role to maintain the stability of the reactor.

464   Proteobacteria and Actinobacteria were the most abundant bacterial groups retrieved

465   from the AS system. The fungal orders detected (Hypocreales, Eurotiales,

466   Chaetothyriales), are commonly encountered in off-gas treatment bioreactors. While

467   most of bacteria and fungi retrieved from the bioreactor have been previously classified

468   as toluene or butanone degraders, typical H_2S oxidizers were not detected, despite high

469   H_2S REs observed. Finally, neither known fungal or bacterial alpha-pinene degraders

470   nor *Pseudomonas fluorescens* were found, their absence resulting in low alpha-pinene

471   abatement efficiencies. *P. fluorescens* was likely outcompeted by the microorganisms

472   already adapted to the characteristics of the AS environment.

Acknowledgements

22

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613

Figure Captions

614

615 **Figure 1.** A schematic representation of the operational changes applied to the activated

616 sludge bioreactor in this work.

617 **Figure 2.** Bacterial dendrogram showing clustering of samples along AS reactor

618 operation. Nodes with a bootstrap support value of 90% or higher are indicated by a

619 black dot. Samples (indicated by the collection day) and their corresponding EBRT

620 appear at the bottom of the figure. Samples collected before glucose addition at day 95

621 are in boldface. Samples subjected to robustness tests (175: inlet load fluctuations, 182:

622 starvation, 197: shut-down, 224: pH failure) are underlined. Samples collected between

623 these two periods are in normal font.

624 **Figure 3.** Fungal dendrogram showing clustering of samples along AS reactor

625 operation. Nodes with a bootstrap support value of 90% or higher are indicated by a

626 black dot. Samples (indicated by the collection day) and their corresponding EBRT

627 appear at the bottom of the figure. Samples collected before glucose addition at day 95

628 are in boldface. Samples subjected to robustness tests (175: inlet load fluctuations, 182:

629 starvation, 197: shut-down, 224: pH failure) are underlined. Samples collected between

630 these two periods are in normal font.

631 **Figure 4.** DGGE profile showing changes in bacterial population due to variations in

632 EBRT (lane lower numbers), glucose addition and establishment of a 25 d SRT at day

633 95 (A), alpha-pinene addition at day 121 (B), *P. fluorescens* addition at day 132 (C),

634 fluctuations in odorant loading (D), starvation conditions (E), process shut-down (F)

635 and pH failure (G). Lane upper labels indicate the operation day at which samples were

636 collected. Bands sequenced are indicated by "b" followed by the corresponding number

637 of each band.

638   **Figure 5.** DGGE profile showing changes in fungal population due to variations in

639   EBRT (lane lower numbers), glucose addition and establishment of a 25 d SRT at day

640   95 (A), alpha-pinene addition at day 121 (B), *P. fluorescens* addition at day 132 (C),

641   fluctuations in odorant loading (D), starvation conditions (E), process shut-down (F)

642   and pH failure (G). Lane upper labels indicate the operation day at which samples were

643   collected. Bands sequenced are indicated by "b" followed by the corresponding number

644   of each band.

645   **Figure 6.** Shannon diversity index (H), ranged weighted richness (Rr) and functional

646   organization (Fo) of bacterial and fungal communities calculated from the DGGE

647   patterns at days 0, 13, 29, 43, 59, 79, 90, 95, 108, 115, 127, 144, 162 (steady operation),

648   175, 182, 197 and 224 (robustness test). The EBRTs tested are indicated in the upper

649   part of each graph and by vertical dotted lines. The continuous line represents the

650   beginning of AS operation at 25 d of sludge retention time and with glucose addition.

651   Vertical dashed lines represent fluctuations and operational failures applied. Vertical

652   dashed and dot line indicate the addition of alpha-pinene at day 121.

653

654

Table 1. Steady state removal efficiencies (REs) of H_2S and VOCs in the AS diffusion system under different EBRTs

Steady performance				
EBRT	**Removal Efficiencies (%)**			
	H_2S	**Butanone**	**Toluene**	**α-pinene**
95	100%	100%	98.4 ± 2.8	
74	100%	98.4 ± 0.6	97.9 ± 0.3	
55	100%	99.3 ± 0.1	96.6 ± 1.0	
49	100%	99.3 ± 0.3	95.0 ± 1.4	
32	100%	99.7 ± 0.1	96.2 ± 1.2	6.8 ± 1.9

Table 2
Click here to download Table: Table 2.docx

Table 2. VOCs removal efficiencies (RE) during the perturbation and the corresponding recovery time (Rt) of the AS diffusion system during the robustness tests

Robustness analysis						
	Butanone		Toluene		α-pinene	
Perturbation	RE (%)	Rt (h)	RE (%)	Rt (h)	RE (%)	Rt (h)
Inlet load fluctuation	99.7 ± 0.0	n.e.	98.4 ± 0.1	n.e.	23.5 [b]	0.5
Starvation period	99.8 ± 0.0	n.e.	98.5 ± 0.3	n.e.	5.7 ± 3.5	n.e.
Process shutdown	83.0 [a]	3	14.0 [a]	6.5	19.9 [b]	3
pH failure	99.8 ± 0.0	n.e.	39.0 [a]	145	50.8 [b]	-

n.e.: no effect, previous RE steady values were maintained during the perturbation or recovered immediately after restoration of prior conditions

[a] RE decreased during perturbation; the minimum RE value is shown

[b] RE increased during perturbation; the maximum RE value is shown
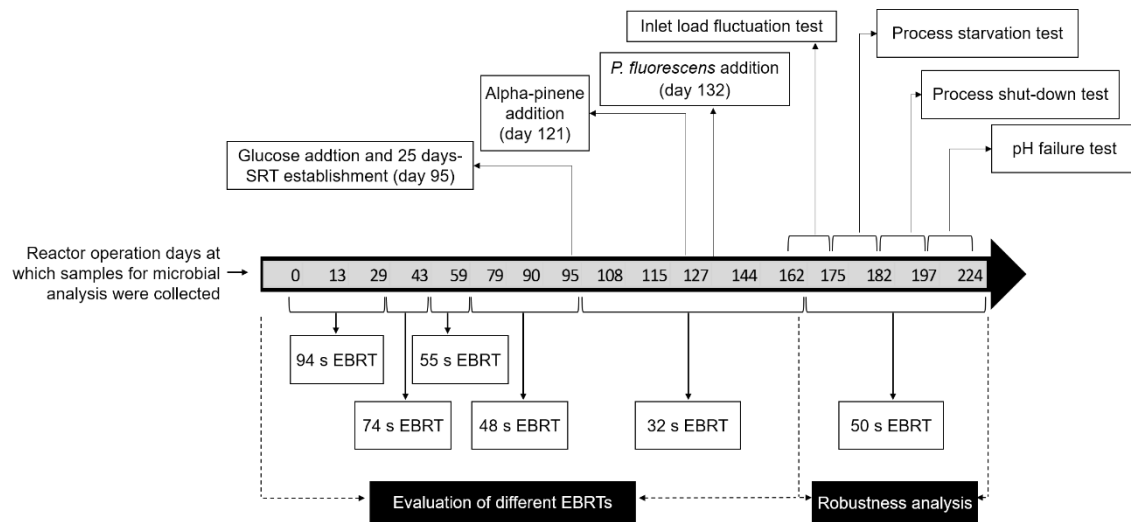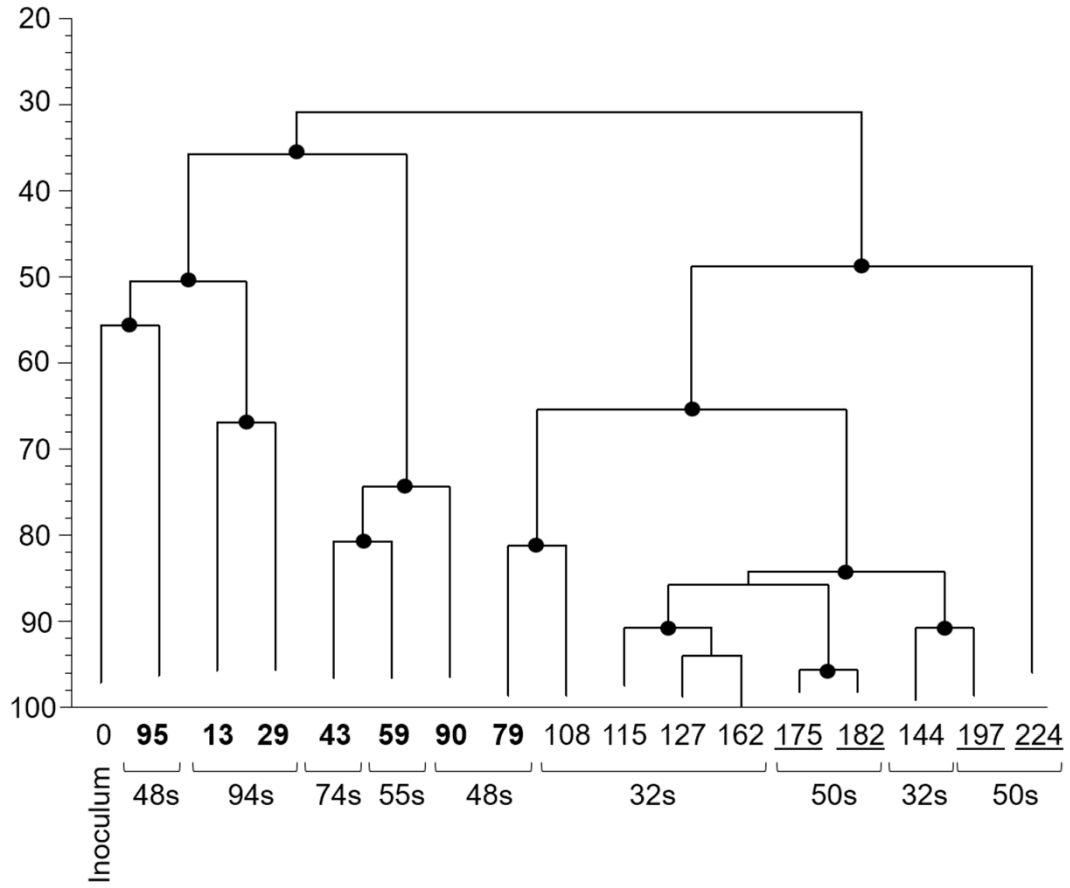
Figure 1

Figure 2

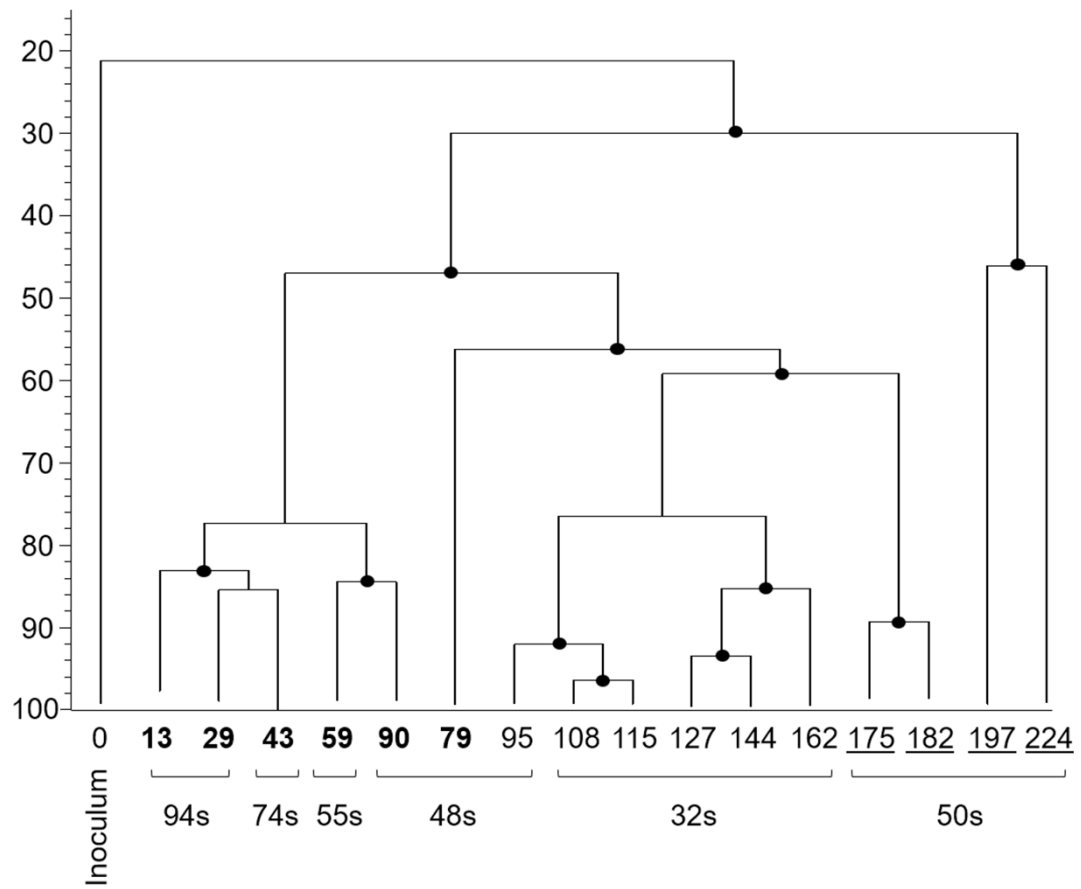**Figure 3**

Figure 4
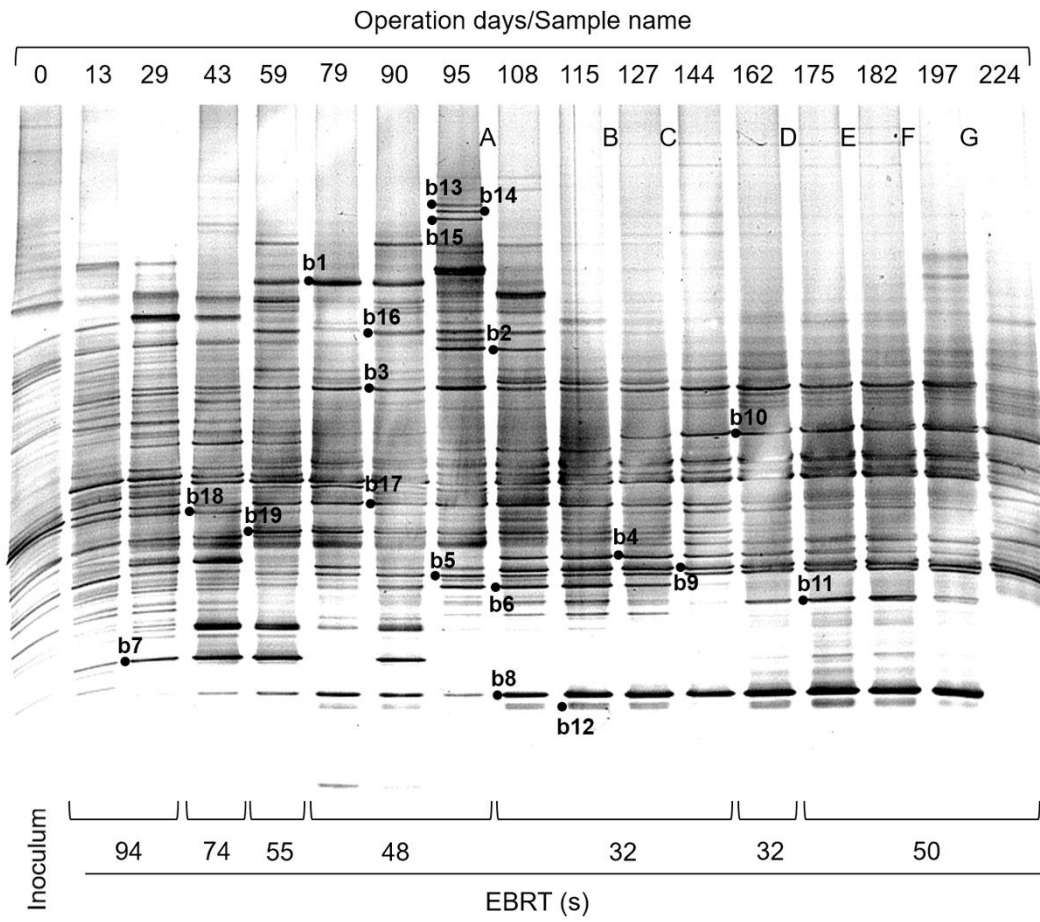**Click here to download Figure: FIG4.DOCX.docx**
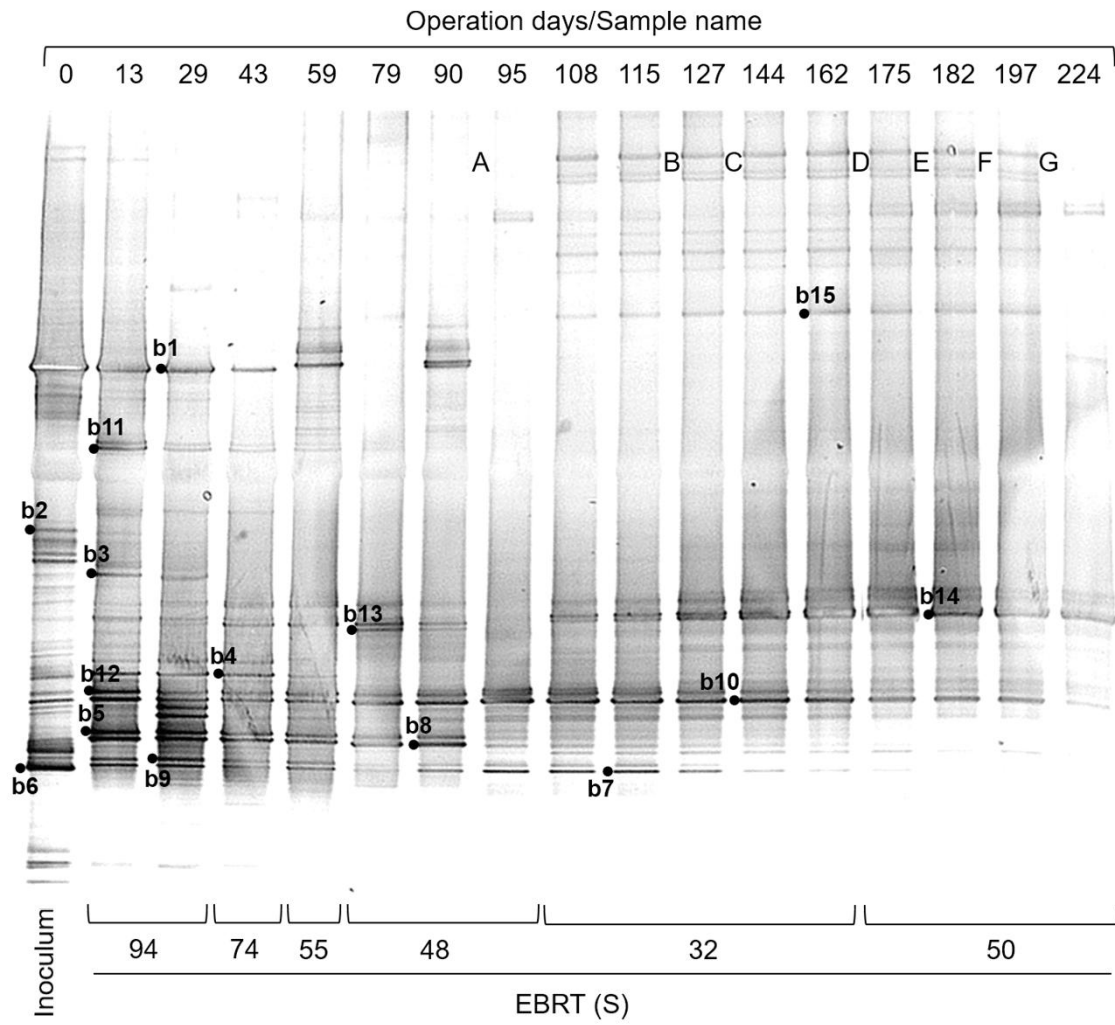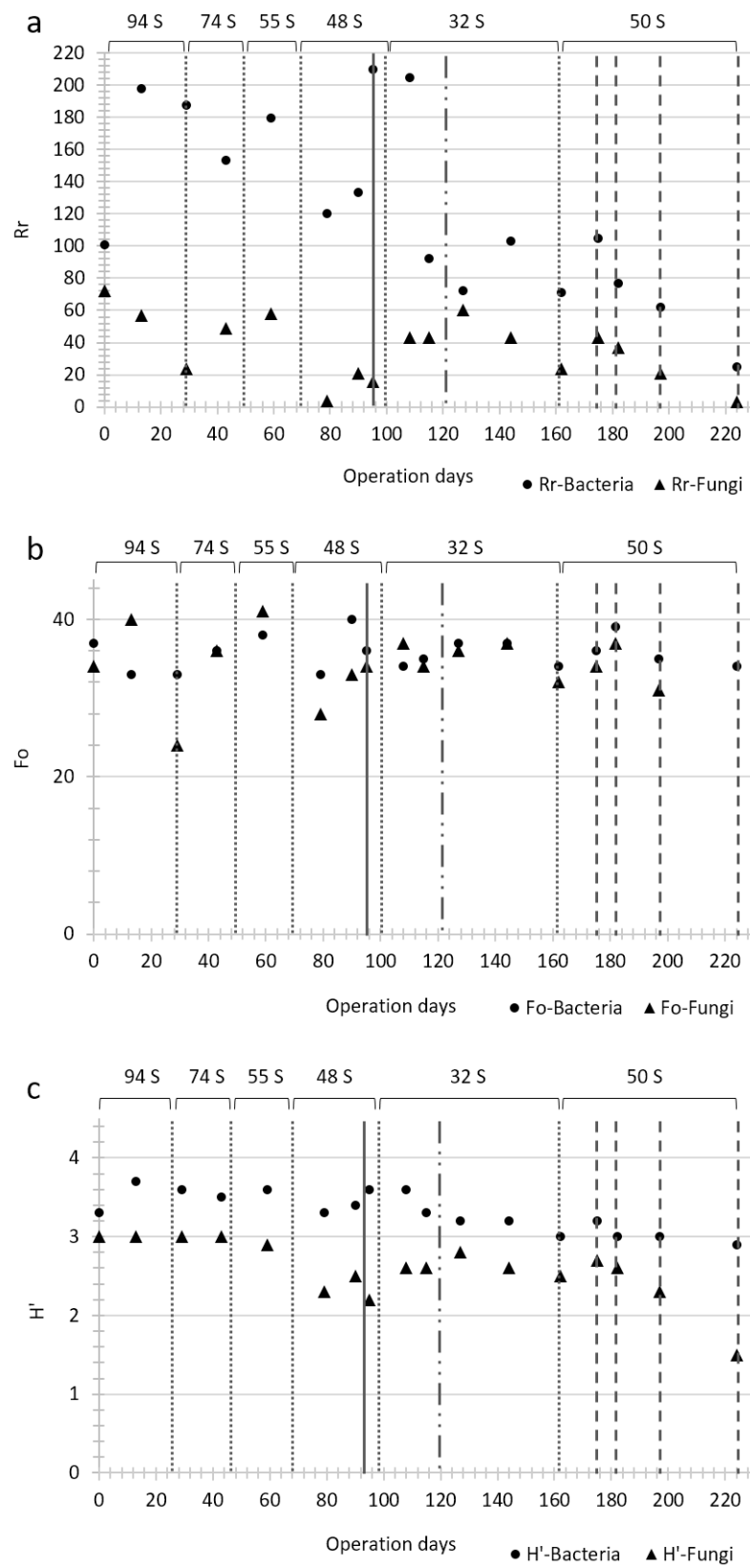
Figure 4

Figure 5

Figure 6

Supplementary material for on-line publication only
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