Contribution of bacterial biodiversity on the operational performance of a styrene biotrickling filter

Kevin J. Portune, M. Carmen Pérez, Javier Álvarez-Hornos, and Carmen Gabaldón[#]

Research Group GI^{2}AM, Department of Chemical Engineering, Universitat de València, Burjassot, Spain.

#Address correspondence to Carmen Gabaldón, carmen.gabaldon@uv.es

Research Group GI^{2}AM, Department of Chemical Engineering, Universitat de València, Av. De la Universitat S/N, 46100, Burjassot, Spain.

Phone: +34 96 354 34 37

Abstract

Long-term operational stability of biotrickling filters (BTFs) degrading volatile organic compounds (VOCs) is dependent on both physicochemical as well as biological properties. Effects of increasingly stressful levels of air pollutants on the microbial structure of biofilms within BTFs are not well understood, especially for VOCs such as styrene. To investigate the relationship between biofilm biodiversity and operational stability, the temporal dynamics of a biofilm from a biotrickling filter subjected to stepwise increasing levels of air polluted with styrene was investigated using 16S rDNA pyrosequencing and PCR-denaturing gradient gel electrophoresis (PCR-DGGE). As styrene contaminant loads were increased, microbial community composition was distinctly altered and diversity was initially reduced in early stages but gradually stabilized and increased diversity in later stages, suggesting a recovery and acclimatization period within the microbial community during incremental exposure of the pollutant. Although temporary reductions in known styrene-degrading bacterial genera (*Pseudomonas* and *Rhodococcus*) occurred under increased styrene loads, stable BTF performance was maintained due to functional redundancy. New candidate genera for styrene degradation (*Azoarcus*, *Dokdonella*) were identified in conditions of high styrene loads, and may have supported the observed stable BTF performance throughout the experiment. Styrene inlet load was found to be important modulator of community composition and may have been partly responsible for the observed temporary reductions of *Pseudomonas*. Notable differences between dominant genera detected via pyrosequencing compared to species detected by PCR-DGGE suggests that simultaneous implementation of both techniques is valuable for fully characterizing dynamic microbial communities.

Keywords:

biotrickling filter, microbial community, pyrosequencing, qPCR, styrene

1 Introduction

Increasing awareness of potential health and environmental hazards through the release of industrially produced volatile organic compounds (VOCs) has led to tighter regulations in VOC-producing factories in recent years. To offset the rising costs of efficient disposal methods of VOCs, industries are increasingly exploring bioreactors as cheaper and cost-effective alternatives. Air purifying bioreactors can vary in design (biofilters, biotrickling filters, among others) (Iranpour et al., 2005) but most consist of a reactor vessel filled with a solid packing material to provide support for the growth of biofilms, which are responsible for the degradation and elimination of pollutant feed sources. Biotrickling filters contain inorganic materials to which an organic inoculum source must be added to provide the initial biotic component. In biotrickling filters, water is uniformly sprayed over the packing material to form a liquid phase, which is then recirculated over the column at continual or intermittent periods. Gaseous pollutants are transferred to the liquid phase where they are then oxidized by microorganisms that are present within the biofilms.

The central objective in the optimization of air biofiltration is identifying the principal factors that can influence the stability of operational performance. The efficiency of air biofiltration can be affected by operational factors, including inlet load, empty bed residence time (EBRT), pH, temperature and nutrients, all of which can affect microorganism survival and growth. Fully understanding the complex interactions between microbial community dynamics and their responses to changes in engineered ecosystems is essential for establishing and maintaining ecosystem stability (Briones and Raskin, 2003). Acclimatization of the resident microbial populations to the pollutant is highly dynamic, bioreactors operating conditions and contaminant loading concentrations have been shown to heavily influence this acclimatization period (Cabrol

et al., 2012a). Also, shifts on microbial diversity in the initial operation resulted in a significant different microbial community structure when purifying toluene than when treating other seven sequentially VOCs in a lab-scale biofilter (Lu et al., 2018). By other side, a comparative study under continuous or discontinuous conditions from the beginning of the operation treating five aromatic compounds revealed that those genes involved in biofilm formation were less active under discontinuous operation (Feng et al., 2019); and differences in robustness levels of microbial communities among different compounds with varying recalcitrance have also been observed in studies using transient substrate pulses (Cabrol et al., 2012b) or by sequentially using different aromatic pollutant combinations (Liao et al, 2018) . Although discontinuous operation and changes or transient pulses of pollutants may reflect fluctuations commonly found in industrial facilities, an extensive examination of how biofilms can respond and adapt to gradually increasing stressful conditions needs to be evaluated. Therefore, stepwise increases of inlet loads in carefully controlled laboratory settings allow the examination of microbial community adaptation and evolution under increasingly stressful physicochemical conditions.

Styrene is an organic compound and precursor to polystyrene, which is an important component used in many industrial goods such as packaging materials and plastic containers. Due to styrene's highly volatile and potentially toxic nature, release of styrene gas to the environment requires proper management and disposal methods. Although many bacterial isolates have demonstrated a capacity to utilize styrene as an energy and carbon source (Hartmans et al., 1990), most research on styrene-degrading microorganisms has focused primarily on a few bacterial genera including *Pseudomonas* spp. (Beltrametti et al., 1997; Iwanade et al., 2005; Jang et al., 2005; Jang et al., 2006; Okamoto et al., 2003), *Rhodococcus* spp. (Jung and Park, 2005; Warhurst et

al., 1994), *Xanthobacter* (Hartmans et al., 1989), and *Brevibacillus* (Hwang et al., 2008) as well as several yeast strains (Cox et al., 1997; Qi et al., 2002; Rene et al., 2011). Among studies examining the application of bacteria to degrade styrene emissions by air biofiltration, most have employed monocultures or only several selected bacterial groups as the inoculum for the biological component of bioreactors (Hwang et al., 2008; Iwanade et al., 2005; Jang et al., 2004; Jang et al., 2005; Jang et al., 2006; Jung and Park, 2005; Okamoto et al., 2003) or examined the effects of a complex community inoculum (i.e. activated sludge) source in bioreactors degrading styrene (Pérez et al., 2015). However, systematic increases in styrene levels and subsequent acclimation responses of the biofilms still remain to be determined in biofiltration studies.

This study measured the temporal dynamics and acclimatization of microbial biofilms from a biotrickling filter degrading styrene air emissions undergoing stepwise increases of the concentration pollutant during a long-term operation period. In-depth analysis of the bacterial community and composition was carried out using pyrosequencing, and complementarily molecular tools such as denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) were applied for comparative purpose. In addition, the relationship between the operational parameters (inlet load, nutrient content, EBRT and pH) and the microbial ecology was investigated.

2 Materials and methods

2.1 Biotrickling filter operation conditions and sample collection

A biotrickling filter (BTF) was constructed with a 20 L methacrylate column (14.4 cm internal diameter, 123 cm bed length) packed with polypropylene rings (25 mm nominal diameter, specific surface area of 207 m^2 m^{-3}, 71 kg m^{-3} bulk density, 92% void fraction) (Flexiring, Koch-Glitsch B.V.B.A., Belgium). Along the length of the

column, two gas sampling ports at 0, and 123 cm from the inlet port located at the bottom of the BTF facilitated measurements of styrene gas concentrations, while a sampling port located at 20 cm from the inlet port were used for biofilm collection using sterile spatulas. Compressed air was pumped through a mass flow controller (Bronkhorst Hi-Tec, The Netherlands) to adjust the EBRT. The air then mixed with styrene supplied through a syringe pump (New Era, infusion/withdraw NE 1000 model, New York, USA). The styrene/air mixture entered the BTF at the bottom of the column and exited the cylinder through an outlet gas line at the top of the column. The BTF was equipped with a recirculation tank that was used to intermittently feed the recirculation solution into the bioreactor via countercurrent mode with respect to the air flow using a centrifugal pump (2.5-3 L min^{-1} for 15 min every 2 h). A nutrient solution (9.7 g NH_4Cl L^{-1}, 0.9 g $MgSO_4 \cdot 7H_2O$ L^{-1}, 2.2 g $(NH_4)_2HPO_4$ L^{-1}, 0.5 g $NaHCO_3$ L^{-1}, 0.4 g NaOH L^{-1}, 0.6 g KCl L^{-1} and Ca, Fe, Zn, Co, Mn, Na, Ni, B, I, Se, Cr, Cu and vitamins at trace doses, buffered at pH 8) was supplied to the recirculation tank using a peristaltic pump. The flow rate was maintained at a mass ratio of carbon to nitrogen of 25 (corresponding to a flow rate of nutrients between 32 and 160 mL d^{-1}) in order to assure that the nitrogen in the recirculation solution was not limiting.

At the start of the experiment, the BTF was inoculated with 1 L of activated sludge from a municipal wastewater treatment plant (located in Quart-Benager, Valencia, Spain) and was continuously recirculated over the bed for 24 h. The BTF was operated at 22°C under continuous styrene loading for >5 months. A start-up period was conducted for 20 days at the beginning of the experiment using styrene inlet loads of 13 g m^{-3} h^{-1} and an EBRT of 60 s. After, six different stages with inlet loads within 17–29 g m^{-3} h^{-1} and EBRTs within 30–60 s were applied.

151 **2.2 Analytical methods**

152 Styrene gas concentrations were measured daily at the inlet and outlet sampling
153 ports using a total hydrocarbon analyzer (Nira Mercury 901, Spirax Sarco, Spain). The
154 response factor of the total hydrocarbon analyzer was determined by a gas
155 chromatograph (model 7890, Agilent Technologies, Santa Clara, USA) equipped with a
156 1 mL automated gas valve injection system and a flame ionization detector and a Rtx®-
157 VMS capillary column (30 m x 0.25 mm x 1.4 µm). Helium was used as the carrier gas
158 at a flow rate of 1.3 mL min^{-1}. Temperatures of the injector, oven and detector were
159 250ºC, 100ºC and 240ºC, respectively.

160 The pH of the recirculating solution was monitored daily using a pH/Cond 340i
161 multimeter (WTW, Germany). Twice per week, volatile suspended solids (VSS), anions
162 (nitrate, phosphate, sulfate and chloride) and cations (ammonium, sodium, potassium,
163 calcium and magnesium) were analyzed using an 883 Basic IC Plus Ionic
164 Chromatograph (Metrohm, Gomensoro S.A., Madrid, Spain).

165

166 **2.3 Nucleic acid isolation and 16S rDNA 454 pyrosequencing**

167 Samples from biofilms for microbial analysis were taken on the last day of the
168 start up period and each of the 6 stages (days 20, 45, 67, 87, 109, 133, and 155) before
169 the change of operational conditions for each respective stage. DNA extraction, 16S
170 rDNA amplification, and 454 pyrosequencing and analysis using the software program
171 mothur (Schloss et al., 2009) were carried out as described in Portune et al. (2015).
172 Samples were multiplexed using barcoded primers and all samples from each of the 7
173 time points were run in a single pyrosequencing run. A dendrogram for assessing
174 similarity between communities of different time points was created via the tree.shared
175 command within mothur using a distance matrix that was calculated using the Bray–

Curtis dissimilarity on a shared OTU file and then used in hierarchical clustering by the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

2.4 Statistical analysis

In order to visualize variation in community structure with distance, non-metric multidimensional scaling (NMDS) was conducted in R, version 3.31 (Team, 2016). Using a shared OTU file, a distance matrix was produced by the veg.dist command in the R package vegan version 2.0-10 (Oksanen et al., 2013) using the Bray-Curtis dissimilarity. The NMDS was created by the isoMDS function in the R package MASS (Venables et al., 2002) using 2 selected dimensions.

To examine the relationship between microbial community structure and different BTF physicochemical parameters, a canonical correspondence analysis (CCA) was conducted using the R package vegan (Oksanen et al., 2013). Inlet load, nitrate, phosphate, sulfate and magnesium were chosen as the BTF physicochemical parameters to include in the model since the average values of these parameters varied across time points and thus could have had an impact on bacterial community structure and composition. Average values for environmental variables for each time point and abundances of bacterial genera were $\log10_{(x+1)}$ transformed prior to analysis with CCA. Significance of the CCA model and explanatory variables was tested using permutation tests (999 permutations) from the vegan package.

2.5 Quantitative Real-Time PCR (qPCR)

In order to validate the results of the relative abundances of *Pseudomonas* spp. detected by pyrosequencing, quantitative PCR (qPCR) was employed using *Pseudomonas* genus-specific primers Pse435F (5´-ACTTTAAGTTGGGAGGAAGGG-

3´) and Pse686R (5´-ACACAGGAAATTCCACCACCC-3´) (Bergmark et al., 2012). Plasmid standards containing the target region were first generated for the primer sets Pse435F/Pse686R and Eub338F/Eub518R (general Eubacterial primers) using genomic DNA extracted from cultures of a strain of the bacteria *Pseudomonas putida* isolated from the BTF. PCR was carried out in a MyGene L Series Peltier thermal cycler (LongGene Scientific Instruments, Hangzhou) where each respective primer pair was used in separate 20 µL reactions consisting of final concentrations of 1X ExTaq buffer (Takara), 0.2mM dNTPs, 0.5µM each respective primer pair, 0.5 units of ExTaq (Takara), and the remaining volume with nuclease free molecular biology grade water (Fisher). Final concentrations of each primer pair were experimentally optimized to obtain the highest specificity of the amplification. PCR cycling conditions for the *Pseudomonas* genus-specific gene consisted of 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. Cycling conditions using the Eub338F/Eub518R primer set were the same except an annealing temperature of 55°C was used instead. PCR amplicons were examined by gel electrophoresis to confirm the specificity of the reaction by observing a single amplified band with the correct expected size. Amplicons were purified using a High Pure PCR Product Purification kit (Roche) and cloned using a TOPO TA cloning kit (Life Technologies). Plasmids were isolated using a PureLink Quick Plasmid Miniprep kit (Life Technologies S. A., Alcobendas, Spain) and the DNA concentrations were determined by PicoGreen fluorometry (Molecular Probes, Life Technologies). qPCR was then carried out using a StepOne™ Real-Time PCR System (Applied Biosystems) using SYBR™ Green PCR Master Mix and primer sets Pse435F/Pse686R and Eub338F/Eub518R (final concentrations of 0.1 µM and 0.25 µM, respectively) for detecting *Pseudomonas* spp. and general eubacteria, respectively. Cycling conditions

for the Pse435F/Pse686R primer set consisted of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, followed by a melting curve analysis. Similar conditions were used for the Eub338F/Eub518R primer set except the annealing temperature was changed to 55°C. Standard curves were generated using triplicate 10-fold serial dilutions of each respective plasmid DNA and calculations of target copy number were performed as described in Fierer et al. (2005). Relative fractional abundances of *Pseudomonas* spp. were calculated as the ratio between the *Pseudomonas*-specific copy numbers versus the general Eubacterial copy numbers.

2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

Extracted DNA from samples from each of the 7 time points described above was amplified by PCR using the primers 341F with a 40 bp GC clamp (5´-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3´) (Muzyer et al., 1993) and 907R (5´-CCGTCAATTCMTTTGAGTTT-3´) (Muzyer et al., 1998) targeting the 16S rRNA gene. Reactions consisted of 50 µL volumes with final concentrations of 1X ExTaq PCR buffer, 0.2 mM dNTPs, 0.5 µM forward and reverse primers, and 0.75 units of ExTaq DNA polymerase (Takara Bio, Ōtsu, Shiga, Japan). PCR cycling conditions consisted of 94°C for 5 min, followed by 20 cycles of 94°C for 60 s, a touchdown annealing step of 0.5°C decreasing increments for every cycle starting at 65°C for 30 s, and an extension of 72°C for 60 s, followed by 10 cycles of 94°C for 60 s, 65°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 10 min. Separation of PCR amplicons by DGGE was carried out using a KuroGEL 2020Y-DGGE system (VWR International Eurolab S. L.) using 6% (v/v) polyacrylamide gels and a denaturant gradient of 30-55%. The definition of a 100% denaturing solution consists of 7 M urea and 40% formamide. Electrophoresis was

251 performed at 100 V for 15 hours in 1X TAE buffer at 60°C. DGGE gels were stained
252 with the nucleic acid-binding reagent EuroSafe (EuroClone, Milan, Italy) and visualized
253 using a MiniBIS Pro transilluminator (DNR Bio-Imaging Systems Ltd., Jerusalem,
254 Israel). DGGE bands were excised with sterile scalpels and placed in 30 µl sterilized
255 Milli-Q water, then the polyacrylamide gels for each band were physically crushed
256 using a sterile pipet tip. Solutions were frozen overnight, centrifuged at 13,000 rpm for
257 2 minutes, then the supernatant was removed and 2 µL was used in a new PCR
258 according to the conditions previously described above. Isolated DGGE bands were
259 compared to the original BTF samples in order to confirm correct positioning and
260 appearance of a single band. Remaining PCR products were purified using a High Pure
261 PCR Product Purification Kit (Roche, Basel, Switzerland) and bi-directionally
262 sequenced on a 3730X DNA Analyzer (Applied Biosystems, Life Technologies S.A.).
263 Taxonomic classification was carried out using the RDP Classifier (Wang et al., 2007)
264 and NCBI´s Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990).
265 Accession number PRJEB22918 contains all sequences of the pyrosequencing runs that
266 were deposited in the European Nucleotide Archive (ENA) database
267 (https://www.ebi.ac.uk/ena/data/view/PRJEB22918).

268 **3 Results**

269 **3.1 Biotrickling filter performance**

270 Full evaluation of the BTF performance was described elsewhere (Pérez et al.,
271 2015). In the work presented here, average values (mean ± standard deviation) of BTF
272 performance and actual operational conditions were calculated for the BTF start-up
273 period and six experimental stages (Table 1). After an initial start-up period of 20 days
274 where the BTF inoculum was acclimated to a low styrene inlet load and concentration
275 (13.0 ± 0.8 g styrene m^{-3} h^{-1}, 216 ± 14 mg Nm^{-3}, respectively), the styrene inlet load was

incrementally increased over three stages to 26.6 ± 2.3 g styrene m^{-3} h^{-1}, while maintaining an empty bed residence time (EBRT) of 60 seconds. During stage four, the EBRT was decreased to 45 seconds while maintaining the same styrene inlet load, whereas the load was increased during stage 5 to yield the highest styrene inlet concentration (512 mg Nm^{-3}). During the sixth and final stage, the EBRT was reduced to 30 seconds and the inlet load was reduced to values similar to stage 2. Removal efficiencies during the start-up period reached >90%, but dropped to average stable values between 74.7 ± 6.4% and 82.0 ± 4.2% during stage 2 through stage 6. Elimination capacities ranged from 11.8 ± 1.4 g styrene m^{-3} h^{-1} at the start of the experiment to 31.8 ± 2.1g styrene m^{-3} h^{-1} during stage 5. The dissolved nitrate (NO_3^-), calcium (Ca^{2+}) and magnesium (Mg^{2+}) were detected in concentrations of 99.1, 23.4 and 7.0 mg L^{-1}, respectively, during the startup period; but were substantially reduced during the remaining time points of the experiment. The high average concentration of these elements during first weeks could be attributed to their presence in the inoculum (concentrations on day 0 of 42.6, 44.0 and 12.9 mg L^{-1} of NH_4^+, Ca^{2+}, Mg^{2+} in the recirculation solution, respectively). Ammonium (NH_4^+), phosphate (PO_4^{3-}), sodium (Na^+) and chloride (Cl^-) were maintained at relatively high stable levels during the course of the experimental stages. The percentage of volatile suspended solids (VSS) was maintained in stables values within 76.9–88.1%.

3.2 Bacterial community analysis by pyrosequencing and qPCR

The pyrosequencing run yielded between 10,990 and 15,708 raw reads for the 7 sampling time points (Table 2). Using the lowest number of normalized sequences for all samples (10,464), a range from 376 to 378 OTUs were detected in the early experimental stages up to 67 days, but this range dropped to 257 at the end of stage 5

when styrene inlet concentrations were at their highest levels. Bacterial diversity indices Inverse Simpson and Shannon's diversity revealed that moderate diversity measures were found during the beginning (20 days) and end (133, 155 days) of the experiment, while the lowest diversity measurements were detected during days 67, 87 and 109. The highest diversity measures were detected at 45 days. Evenness measures were consistent across all sampling time points except for an elevated value detected at 45 days.

Dendrograms generated by the Bray-Curtis dissimilarity revealed a clear change in bacterial composition over time as samples from the early time points (days 20, 45) clustered together while the middle (days 67, 87 and 109) and late time points (days 133 and 155) demonstrated higher similarity to each other, respectively (Fig. 1). NMDS analysis revealed a large separation between the first and last 2 time points compared to the intermediate time points (i.e. 67, 87, 109 days) along the first ordination axis, while separations between the first four time points compared to later time points was observed along the second ordination axis (Fig. S1).

The highest percentages of bacterial sequences that could be classified to known genera across all time points were assigned to *Hydrogenophaga* (Fig. 2). Among the top 25 classified genera, *Brevundimonas*, *Pseudoxanthomonas* and *Stenotrophomonas* were detected in high relative abundances during the start of the experiment (26.2%, 7.4% and 4.1% of total sequences, respectively), but steadily decreased over the course of the experimental stages, while the reverse trend was observed for the genus *Azoarcus*. Both *Rhodococcus* and *Pseudomonas* were detected in high percentages of total bacteria at the start (6.6% and 15.6% of total sequences, respectively) and end of the experimental stages (10.7% and 11.1% of total sequences, respectively), while *Pseudomonas* decreased substantially during time points at 67, 87 and 109 days. *Achromobacter* abundance also declined from the first stages (up to 2.1% of total sequences) from time

point 67 days on (<0.4% of total sequences). High percentages of unclassified bacteria at the genera level were detected at 67 and 87 days. The majority of these unclassified sequences displayed sequence similarity to the *Xanthomonadaceae* family at bootstrap confidence estimates below 50% using the RDP classifier, with up to 23.2% and 22.7% of total sequences found at 67 and 87 days, respectively (data not shown). A substantial percentage of unclassified sequences also showed sequence similarity to the family *Comamonadaceae* at bootstrap confidence estimates below 50%, ranging from 0.9% to 5.7% of the total sequences among different time points (data not shown). At the family level, the *Comamonadaceae* and *Xanthomonadaceae* families made up the dominant members, followed by *Caulobacteraceae*, *Nocardiaceae* and *Pseudomonadacea* (Fig. S2). The majority of total bacterial sequences that could be assigned to a known phylum were classified as belonging to Proteobacteria, with percentages ranging from 67% to 81% across the seven time points (Fig. S3). Other dominant phyla that were detected in abundances of up to 10% of total sequences during the course of the treatment period include *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Spirochaetes*.

In order to confirm the observed trend of the sharp reduction of the genus *Pseudomonas* from time points at 67, 87 and 109 days, qPCR was employed using *Pseudomonas* genus-specific primers. The average percentage of the total eubacterial copies that could be attributed to *Pseudomonas* was then calculated. qPCR results confirmed the observed pyrosequencing results of a sharp reduction of *Pseudomonas* to levels less than 1% of the total observed bacteria during stages 2-4 (day 67 to day 109) with subsequent increases during stages 5 and 6 (day 133 and day 155) achieving average percentages up to 15% (Fig. 3).

3.3 Bacterial community by DGGE

Numerous bacterial species belonging to the phylum Bacteriodetes were detected, with *Chitinophaga pinensis, Galbibacter mesophilus, Cytophaga xylanolytica, Solitalea koreensis* and *Leadbetterella byssophila* among dominant members found in mid- to late experimental stages with high styrene levels (Table S1, Fig. 4, bands 1, 6, 7, 9, 10). *Hydrogenophaga sp.* (*defluvii* or *atypica,* band 15) was detected in high abundances at each experimental stage, particularly during late stages with high styrene levels. Other prominent bacteria belonging to the phylum Proteobacteria detected in high abundances during late experimental stages with high styrene levels include *Azoarcus communis, Xenophilus azovorans* and *Paucibacter toxinivorans* (bands 12, 13, 14, respectively). Similar trends between bacterial species detected by DGGE and pyrosequencing profiles of respective bacterial genera over the experimental stages include *Hydrogenophaga*, *Azoarcus, Paracoccus, Spirochaeta* and *Rhodococcus* (Figs 2 and 4, bands 15, 12, 16, 18, 19, 20, respectively). Furthermore, similar trends observed in DGGE profiles for the species *Dokdonella immobilis* (Fig. 4, band 17) were found between a large portion of sequences in the unclassified genera at day 67 from pyrosequencing that were found to be taxonomically similar to *Dokdonella* (family *Xanthomonadaceae*) although they couldn't be assigned to this genus at the designated 50% bootstrap cutoff (data not shown).

3.4 Relationship between operational conditions and taxonomy

Canonical correspondence analysis (CCA) was used to examine the relationships between microbial community structure and BTF physicochemical parameters. Permutation analysis revealed that the overall CCA model and explanatory variable

styrene inlet load was significant ($p<0.05$), and highly correlated with CCA1 (Fig. 5). The first two axes of the CCA accounted for 51% of the constrained inertia. Several bacterial genera from the top 25 most abundant groups (from Fig. 2) were correlated with increasing styrene inlet load including *Hydrogenophaga*, *Spirochaeta*, *Shinella*, *Aquimonas* and *Azoarcus*. Several bacterial genera, including *Azoarcus*, *Luteimonas*, *Desulfovibrio*, *Stappia* and *Spirochaeta* were correlated with high phosphate concentration, while *Achromobacter* showed opposite effect. Few bacterial genera including *Brevundimonas*, *Pseudoxanthomonas* and *Stenotrophomonas* were correlated with increasing concentrations of sulfate and nitrate while *Pseudomonas* and *Gp4* were correlated with increasing concentrations of magnesium and nitrate.

4 Discussion

Under steady-state conditions in bioreactors where operational parameters are held constant, microbial diversity and composition of biofilms has been found to be highly stable (Falk et al., 2009). In contrast, other studies observed substantial shifts in microbial community composition in biofilters undergoing steady-state operation, whereas no differences in bacterial diversity were observed (Cabrol et al., 2012a; Nadarajah et al., 2007). When operational parameters of bioreactors (i.e. contaminant load, temperature, nutrients) are altered, microbial community structure and diversity has been observed to be highly dynamic (Miura et al., 2007; Nadarajah et al., 2007; Portune et al., 2015). In this study, microbial community composition and diversity was stable during both the start-up phase and stage 1, but radically changed during stage 2 as averaged styrene inlet loads and inlet concentrations were subsequently increased to 23.5 ± 0.8 g styrene m^{-3} h^{-1} and 391 ± 13 mg Nm^{-3}, respectively. Clustering and NMDs analysis further revealed a gradual evolution of community composition after stage 2, with mid- and later time points grouping together, during which operational conditions

were continually more stressful (i.e. higher styrene inlet loads, inlet concentrations). Microbial diversity also sharply dropped during stage 2 and remained low during stages 3 and 4, after which diversity began to increase again in the later stages. These patterns suggest a recovery and acclimatization period in the microbial community structure as bacterial composition and diversity were initially largely affected by the rising styrene contaminant concentrations but eventually became stable after stage 4, even after styrene inlet concentrations/loads were increased to the highest levels during stage 5. In studies where bacterial communities were exposed to repeated shock loads of contaminants over time, similar results were observed in which biofilms that were close to the inlet contaminant sources became accustomed to high concentrations of contaminants and microbial communities were thus relatively unaffected by pulses of high contaminant loads (Cabrol et al., 2012b). Interestingly, even as styrene inlet loads/concentrations in this study were substantially reduced along with a reduction in EBRT during the final stage 6, bacterial composition and diversity were largely similar to that observed in stage 5. This indicates that the bacterial composition within the biofilm had reached a stable equilibrium and was relatively unaffected by varying styrene levels.

Changes in individual bacterial groups were observed and followed very different trends depending on the phylotype. Pyrosequencing and DGGE analysis revealed the bacterial genera *Hydrogenophaga*, *Azoarcus*, *Spirochaeta* and the well-known styrene-degrading genus *Rhodococcus* to be prominent genera that increased in abundance as styrene inlet load and inlet concentrations were increased. *Hydrogenophaga* spp. have been previously isolated from activated sludge and wastewater (Kampfer et al., 2005; Yoon et al., 2008) and *Azoarcus* has been identified as a dominant member in coking wastewater treatment plants (Ma et al., 2015).

Recently, our group identified both *Azoarcus* and *Hydrogenophaga* as dominant members of the bacterial community in biofilters treating high styrene emissions (Portune et al., 2015). Genes for styrene monooxygenase, which encodes the first enzyme in the pathway for styrene degradation, have been identified in ENSEMBL (Kersey et al., 2016) for both *Hydrogenopha* sp. T4 and several *Azoarcus* sp., thus supporting their probable role as instrumental bacterial groups responsible for styrene degradation in this study. The large percentages of unclassified sequences at the genera level assigned to the *Xanthomonadaceae* family at days 67 and 87 in this experiment suggest a prominent role in styrene degradation for specific groups from this family. According to our DGGE results, *Dokdonella immobilis*, from the family *Xanthomonadaceae*, may in fact be the bacterial species that is represented by these unclassified sequences as this species was found to be a dominant bacterial species at both days 67 and 87. As *Dokdonella immobilis* sp. has been previously isolated from a batch reactor treating other aromatic compounds such as triphenylmethane (Liu et al., 2013), this species is a likely new candidate for styrene degradation. In a previous study, Arnold et al. (1997) identified several bacterial isolates including the genera *Tsukamurella*, *Sphingomonas*, *Xanthomonas* and *Pseudomonas* with styrene degradation capability. In this study, both *Tsukamurella* and *Sphingomonas* were found in low abundances but did not appear to positively respond to increasing styrene concentrations. The substantial decrease of *Pseudomonas* bacteria only on stage 2 to 4 cannot be explained by the increase of styrene concentrations; for example, *Pseudomonas putida* has been shown capable of growing in media culture containing more than 50% (v/v) toluene and high concentrations of cyclohexane, xylene, styrene and heptanol (Inoue and Horikoshi, 1989). According to the canonical analysis (Fig. 5) *Pseudomonas* abundance was correlated with increasing nitrate and magnesium

concentration, a possible explanation for the sharp decrease of its abundance on stages 1 to 4 could be the development of anoxic/anaerobic microenvironments within the biofilm. The activity of the sulfate-reducing *Desulfovibrio* species (a well-known anaerobic aerotolerant species) along with the fast reduction of nitrate from stage 2 points to some limitation of oxygen availability within the biofilm. Furthermore, the bacterial genera *Brevundimonas* and *Achromobacter*, which appeared to be important degraders of styrene (Portune et al., 2015; Liao et al, 2018) were substantially reduced under increasing concentrations of styrene in this study. However, it is unclear whether other factors may have influenced the decline of these bacterial genera. From the canonical analysis (Fig. 5) the progressive decline of *Achromobacter* genus abundance seems to be correlated to the lowest phosphate concentrations observed at the beginning of the experiment (stages 1 and 2). Whereas the decline of *Brevundimonas* genus can be related not only to the low nitrate concentrations from stage 1 on (as in the case of *Pseudomonas*), but also to the concomitant reduction of sulfate, as its abundance was correlated with increasing concentrations of nitrate and sulfate (Fig 5). Other novel bacterial genera identified through pyrosequencing and DGGE analysis that are potentially associated with styrene degradation include *Aquimonas*, *Spirochaeta*, *Shinella*, *Fusibacter*, *Roseateles*, *Stappia*, *Xenophilus, Chitinophaga* and *Solitalea*. However, as no genetic information is currently known regarding styrene degradation pathways in these organisms, confirmation of styrene degradation ability is still needed in cultured experiments.

Depending on their responses to environmental disturbances in the context of ecosystem processes, microbial communities can be characterized as resistant, resilient or exhibiting functional redundancy (Allison and Martiny, 2008). Resistance occurs when microbial community composition remains unchanged in the face of a

475 disturbance; resilience is when the microbial composition is altered by the disturbance
476 but returns to the original composition over time; and functional redundancy is
477 characterized by a microbial composition that remains altered but performs ecosystem
478 processes similar to the original community. The continual changes in microbial
479 composition observed throughout the experimental stages in this study suggest that the
480 microbial population was neither resistant nor resilient to the disturbances of stepwise
481 increasing styrene loads. Poor structural resistance capacity for microbial populations is
482 well-described in gas biofiltration communities as well as wastewater ecosystems and
483 soils (reviewed in Cabrol and Malhautier, 2011). However, BTF performance in this
484 study (i.e. RE and EC) remained relatively stable from stage 2 until the end of the
485 experiment, despite reductions in well-known styrene degrading bacterial genera
486 including *Pseudomonas* and to some extent *Brevundimonas* and *Rhodococcus* during
487 the stages 2-4, suggesting some degree of functional redundancy from the population
488 after stage 1. Functional redundancy is a common feature found in numerous types of
489 bioreactor and wastewater environments (Ayarza et al., 2010; Cabrol et al., 2012a;
490 Fernandez et al., 1999; Franklin and Mills, 2006; Zumstein et al., 2000). Although
491 styrene catabolism has been demonstrated only in a few select bacterial species, it is
492 probable that uncharacterized bacterial groups that have the ability to oxidize styrene
493 were instrumental in maintaining operational stability observed here. New bacterial
494 candidates for styrene degradation have been proposed (see above) and new styrene-
495 degrading species are likely to be identified in the future as studies with high throughput
496 sequencing techniques become more common. Furthermore, in addition to the recovery
497 of high abundances of the genus *Pseudomonas* at the end of the experiment, the
498 recovery of increased microbial diversity in stage 4 following a reduction in earlier
499 stages indicates a level of resilience in select bacterial groups to elevated styrene loads.

500 It is possible that the lower/milder stepwise styrene loads may have selected for more
501 tolerant or resistant microbes that were able to withstand the greater loads at later
502 stages, similar to results observed in previous studies in gas biofilters and anaerobic
503 digestors degrading other contaminants (Cabrol et al., 2016; De Vrieze et al., 2013;
504 Irvine and Moe, 2001; McMahon et al., 2004).

505 Nutrient limitation is a potential problem in all bioreactor applications during
506 long-scale operation, particularly if the packing material is made of an inorganic
507 substance. Nutrient supplementation is well known to increase bioreactor operational
508 performance, such as increasing removal efficiency and elimination capacity (Chan and
509 Lin, 2006; Morgenroth et al., 1996; Son et al., 2005; Weckhuysen et al., 1993; Yu et al.,
510 2003). Although literature on the effects of nutrient supplementation on both microbial
511 community structure and bioreactor performance is limited, addition of specific
512 nutrients has been shown to affect microbial community diversity while simultaneously
513 increasing removal efficiency (Chen et al., 2009). Nitrogen limitation in particular has
514 been demonstrated to play an important role in biofilm growth. Nitrogen concentrations
515 have been shown to have a greater influence on the mineralization of VOCs such as
516 toluene compared to other nutrients like phosphorus and potassium (Delhoménie et al.,
517 2001). Furthermore, additions of ammonia or nitrate in a biofilter degrading toluene
518 increased the elimination capacity by more than a factor of 10 (Beuger and Gostomski,
519 2009), thus showing a specific beneficial role for nutrients in reactor operational
520 performance. High levels of ammonium detected throughout the experimental stages
521 may have provided a suitable nitrogen source for most bacteria, since the assimilation of
522 ammonia is known to be easier than nitrate in some conventional and trickle-bed air
523 biofilters (Jorio et al., 2000; Song et al., 2003; Wang et al., 2009; Yang et al., 2002) In

fact, Sempere et al. (2011) showed that addition of ammonia gave better performance results than addition of nitrate for styrene removal in biotrickling filters.

Next-generation sequencing (NGS) methods have a distinct advantage over older traditional molecular methods in the characterization of bacterial communities in that a greater amount of information on species abundance can be obtained, especially for non-dominant members. Combinations of pyrosequencing and PCR-DGGE have been simultaneously applied in a recent study examining shifts in bacterial community diversity and composition in response to front aeration in the horizontal subsurface flow constructed wetlands, and similar bacterial diversity was found using both methods (Zhong et al., 2015). Comparison of results from pyrosequencing and DGGE in this study revealed similarities in several dominant bacterial groups. Curiously, no *Pseudomonas* spp. were detected using DGGE, despite a high relative abundance found using pyrosequencing. Furthermore, DGGE analysis further revealed many dominant bacterial species belonging to the phylum Bacteroidetes that were not detected by pyrosequencing. The use of different primers for pyrosequencing and DGGE likely played a role in at least some of the differences in detected taxonomic groups by each respective method. After examination of the reverse primer 907R from Muyzer et al. (1998) used for DGGE analysis in this study, we found a single-base mismatch between the primer and classified sequences assigned to the genus *Pseudomonas* through pyrosequencing (data not shown), thus explaining why *Pseudomonas* may not have been detected using DGGE. These results stress the necessity for careful selection of PCR primers for use in any molecular method for examining microbial communities within bioreactors.

5 Conclusions

Gradual increases on styrene contaminant loads appear to shape and condition microbial communities to withstand increasing levels of contaminants. In addition, anoxic microenvironments within the heterogeneous biofilm impacted on the relative abundance of some identified styrene degraders such *Brevundimonas* and *Pseudomonas*. It is likely important that a compositionally- and functionally-diverse initial microbial community is provided as the inoculum source since functional redundancy appears to play a major role in maintaining bioreactor performance. Therefore, the use of activated sludge as an inoculum source, which typically contains highly diverse microbial populations, appears to satisfy these requirements and confers several advantages over inoculations with mono- or mixed cultures of bacteria and/or fungi in in addition to being less expensive compared to these latter options.

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804 **Figure Captions**

805 **Figure 1.** Dendrogram using Bray-Curtis dissimilarity to show the relationship between
806 microbial composition among sampling time points.

807

808 **Figure 2.** Percentage of total bacterial sequences (mean of replicates) assigned to the
809 top 25 most abundant bacterial genera in the 7 sampling time points. Combined
810 percentages of remaining classified genera are labeled as Remaining Genera. Sequences
811 labeled as Unclassified (Phyla), Unclassified (Class), Unclassified (Order), Unclassified
812 (Family) could not be assigned to any classified taxon at each respective taxonomic
813 level using the Ribosomal Database Project Classifier at the selected bootstrap
814 confidence estimate threshold of 50%.

815

816 **Figure 3.** Relative abundances of *Pseudomonas* spp. at each of the 7 sampling time
817 points as represented by the average percent of eubacterial copy number that is assigned
818 to *Pseudomonas* using *Pseudomonas*-specific PCR primers.

819

820 **Figure 4.** DGGE profiles for bacterial 16S rRNA genes at each of the 7 sampling time
821 points. Numbers represent DGGE bands found at determined heights of DNA fragment
822 separation that correspond to dominant species found across time points.

823

824 **Figure 5.** Canonical correspondence analysis (CCA) of bacterial community
825 composition with operational conditions. Arrows represent the styrene inlet load and
826 nitrate, magnesium, phosphate and sulfate concentrations of the recirculation solution.
827 Percentages within parentheses on each axis represent the percent of total inertia
828 explained by each axis. Samples at each of the 7 sampling time points are represented

by gray squares. Numbers associated with circles represent the top 25 most abundant genera detected over the 7 sampling time points found in Fig. 2.

832 **Table 1** Average biotrickling filter performance and operational conditions (mean ± S.D.). Samples for microbial analysis were taken on days 20,

833 45, 67, 87, 109, 133, and 155.

Parameter	**Start-up Days 0-20**	**Stage 1 Days 20-45**	**Stage 2 Days 45-67**	**Stage 3 Days 67-87**	**Stage 4 Days 87-109**	**Stage 5 Days 109-133**	**Stage 6 Days 133-155**
BTF Performance							
EBRT (s)	60	60	60	60	45	45	30
Styrene inlet load (g styrene m^{-3} h^{-1})	13.0 ± 0.8	17.4 ± 0.7	23.5 ± 0.8	26.6 ± 2.3	28.4 ± 4.0	41.0 ± 1.2	23.8 ± 1.2
Styrene inlet concentrations (mg Nm^{-3})	216 ± 14	290 ± 12	391 ± 13	444 ± 39	355 ± 50	512 ± 15	198 ± 10
Styrene outlet concentrations (mg Nm^{-3})	19 ± 19	31 ± 22	99 ± 28	109 ± 40	64 ± 10	115 ± 25	45 ± 11
Removal Efficiency (%)	91.2 ± 8.9	89.3 ± 7.7	74.7 ± 6.4	75.5 ± 8.0	82.0 ± 4.2	77.5 ± 4.8	77.3± 5.8
Styrene Elimination Capacity (g styrene m^{-3} h^{-1})	11.8 ± 1.4	15.5 ± 1.6	17.5 ± 1.2	20.1 ± 2.3	23.3 ± 4.3	31.8 ± 2.1	18.4 ± 1.8
Recirculation Solution							
pH	8.28 ± 0.62	8.25 ± 0.32	8.12 ± 0.37	7.97 ± 0.45	8.06 ± 0.51	7.51 ± 0.70	8.09 ± 0.22
NO_3^- (mg L^{-1})	99.1 ± 34.1	2.6 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	1.1 ± 0.3	1.8 ± 0.5	1.9 ± 0.3
NH_4^+ (mg L^{-1})	20.7 ± 4.1	31.3 ± 4.4	67.4 ± 12.5	24.0 ± 6.0	30.0 ± 7.3	60.8 ± 12.7	69.9 ± 7.8
PO_4^{3-} (mg L^{-1})	65.4 ± 15.8	75.8 ± 15.5	107.0 ± 17.8	90.4± 23.4	112.5 ± 25.5	147.3 ± 36.3	319.9 ± 15.2
SO_4^{2-} (mg L^{-1})	68.6 ± 20.0	50.6 ± 17.6	28.0 ± 8.7	7.9 ± 2.7	4.7 ± 0.6	24.9 ± 6.1	58.4 ± 13.4

Na^{+} (mg L^{-1})	771 ± 170.2	1232 ± 110.4	1205 ± 49.9	1310 ± 47.3	1683 ± 79.2	1793 ± 127.6	2338 ± 168.6
Cl^{-} (mg L^{-1})	859 ± 194.8	1588 ± 119.1	1323 ± 95.3	1756 ± 170.9	2170 ± 151.9	2683 ± 117.5	3088 ± 68.0
K^{+} (mg L^{-1})	8.7 ± 5.7	31.3 ± 4.63	64.1 ± 6.7	75.7 ± 18.5	107.5 ± 22.3	102.6 ± 9.3	174.7 ± 35.2
Ca^{2+} (mg L^{-1})	23.4 ± 13.4	3.5 ± 0.8	2.0 ± 0.8	2.1 ± 0.9	0.7 ± 0.3	0.9 ± 0.4	1.3 ± 0.7
Mg^{2+} (mg L^{-1})	7.0 ± 3.5	0.2 ± 0.2	0.1 ± 0.1	0.4 ± 0.3	0.9 ± 0.2	2.0 ± 0.8	3.0 ± 0.6
%VSS[a]	76.9 ± 1.8	79.3 ± 1.1	80.7 ± 2.4	88.1 ± 2.5	85.7 ± 3.9	83.6 ± 1.6	86.7 ± 2.3

[a](Volatile suspended solids)

845 **Table 2** Summary of raw and processed sequences from 454 pyrosequencing, including richness estimators (Observed OTUs), species diversity

846 indices (Inverse Simpson and Shannon's Diversity) and evenness from samples for each of the 7 sampling time points.

Operational Stage	Sample	Raw reads	Sequences post-processing	Normalized sequences	OTUs[a]	Inverse Simpson (1/D)	Shannon Diversity (H´)	Evenness (J´)
-	20 days	12,032	11,778	10,464	377	11.8 (11.4-12.2)[b]	3.44 (3.40-3.47)[b]	0.580
1	45 days	14,932	14,119	10,464	376	33.7 (32.7-34.8)[b]	4.17 (4.14-4.19)[b]	0.703
2	67 days	15,708	14,197	10,464	378	5.6 (5.4-5.8)[b]	3.11 (3.06-3.15)[b]	0.524
3	87 days	11,468	10,531	10,464	272	7.7 (7.4-7.9)[b]	2.99 (2.95-3.02)[b]	0.533
4	109 days	11,854	11,105	10,464	268	8.1 (7.8-8.5)[b]	3.07 (3.03-3.10)[b]	0.548
5	133 days	10,990	10,464	10,464	257	13.5 (13.1-13.8)[b]	3.28 (3.24-3.31)[b]	0.590
6	155 days	11,261	10,876	10,464	293	15.1 (14.7-15.5)[b]	3.36 (3.33-3.39)[b]	0.591

847

848 [a](Operational taxonomic units observed after normalization)

849 [b](95% confidence intervals of respective estimators shown in parentheses)

850

Figure1

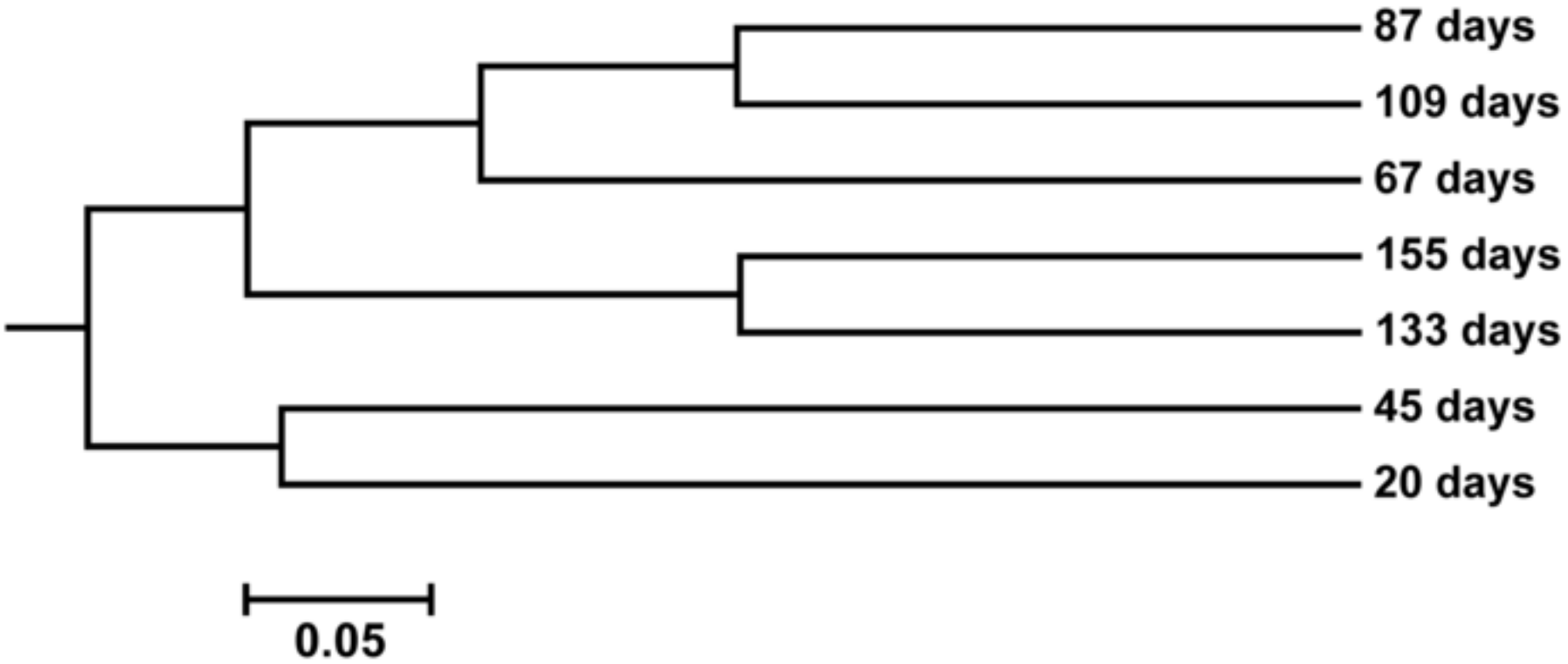


Figure2

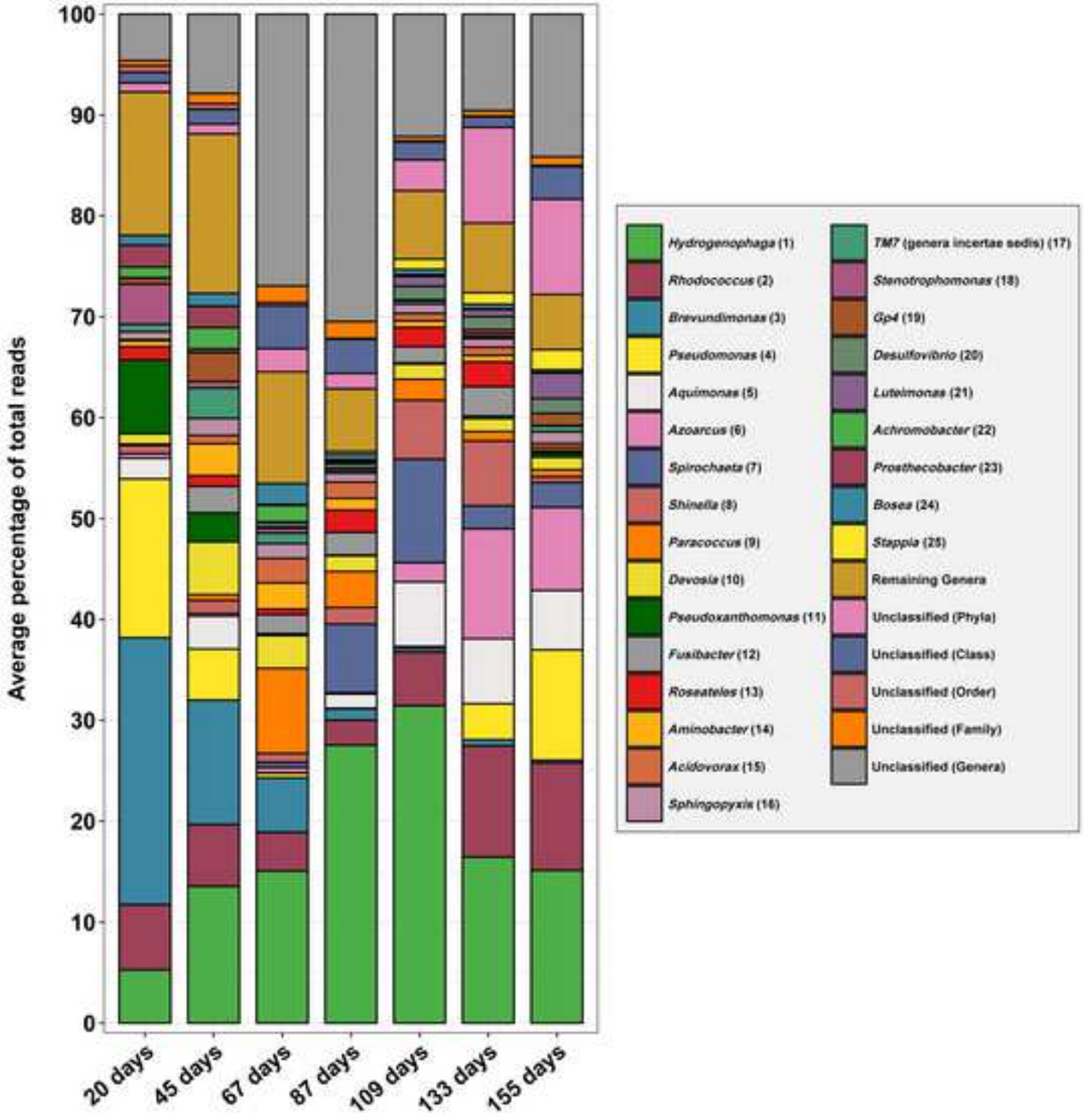

Average percentage of total reads
100
90
80
70
60
50
40
30
20
10
0
20 days
45 days
67 days
87 days
109 days
133 days
155 days
Hydrogenophaga (1)
Rhodococcus (2)
Brevundimonas (3)
Pseudomonas (4)
Aquimonas (5)
Azoarcus (6)
Spirochaeta (7)
Shinella (8)
Paracoccus (9)
Devosia (10)
Pseudoxanthomonas (11)
Fusibacter (12)
Roseateles (13)
Aminobacter (14)
Acidovorax (15)
Sphingopyxis (16)
TM7 (genera incertae sedis) (17)
Stenotrophomonas (18)
Gp4 (19)
Desulfovibrio (20)
Luteimonas (21)
Achromobacter (22)
Prosthecobacter (23)
Bosea (24)
Stappia (25)
Remaining Genera
Unclassified (Phyla)
Unclassified (Class)
Unclassified (Order)
Unclassified (Family)
Unclassified (Genera)

Figure3

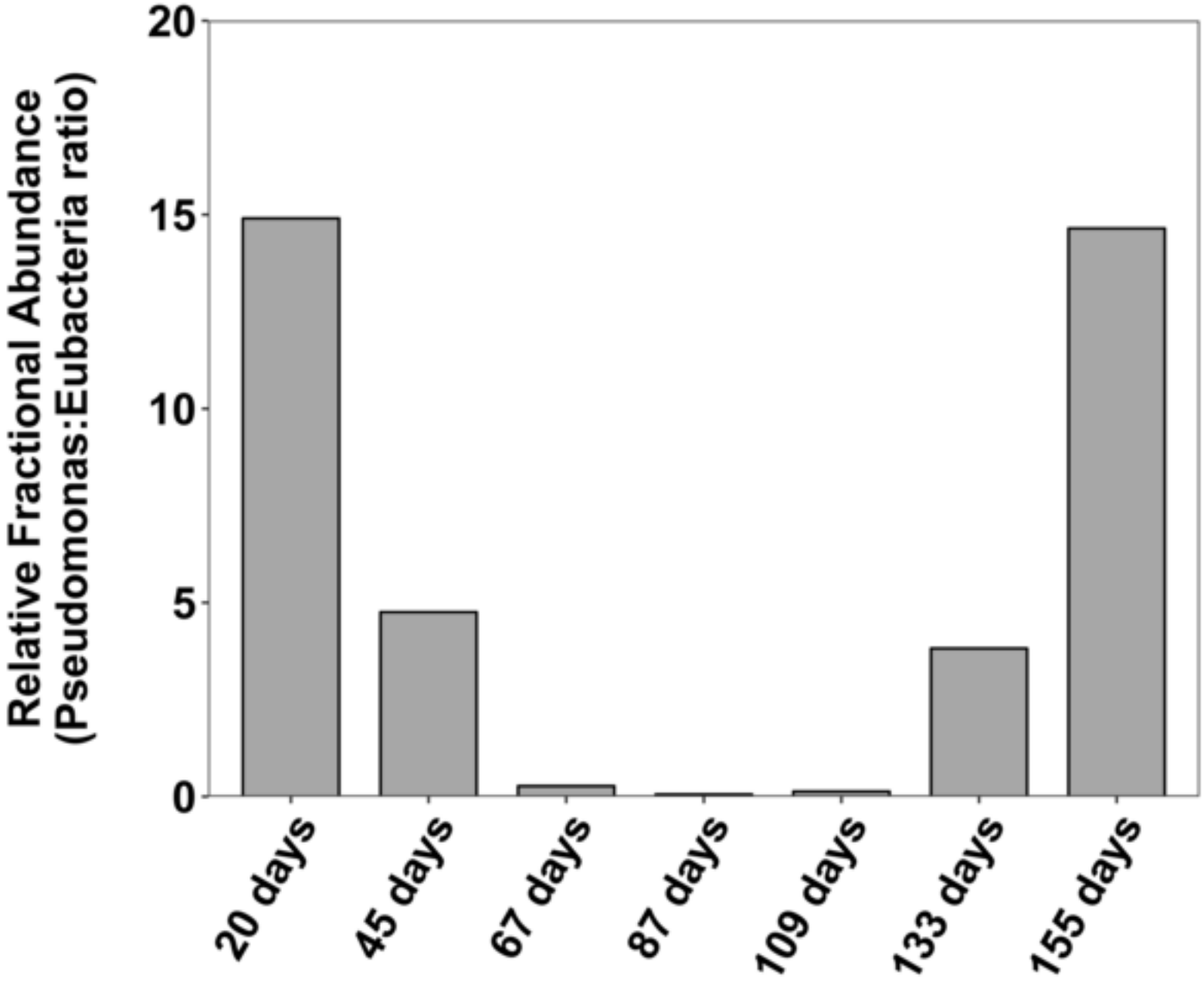

Relative Fractional Abundance
(Pseudomonas:Eubacteria ratio)
20
15
10
5
0
20 days
45 days
67 days
87 days
109 days
133 days
155 days

Figure4

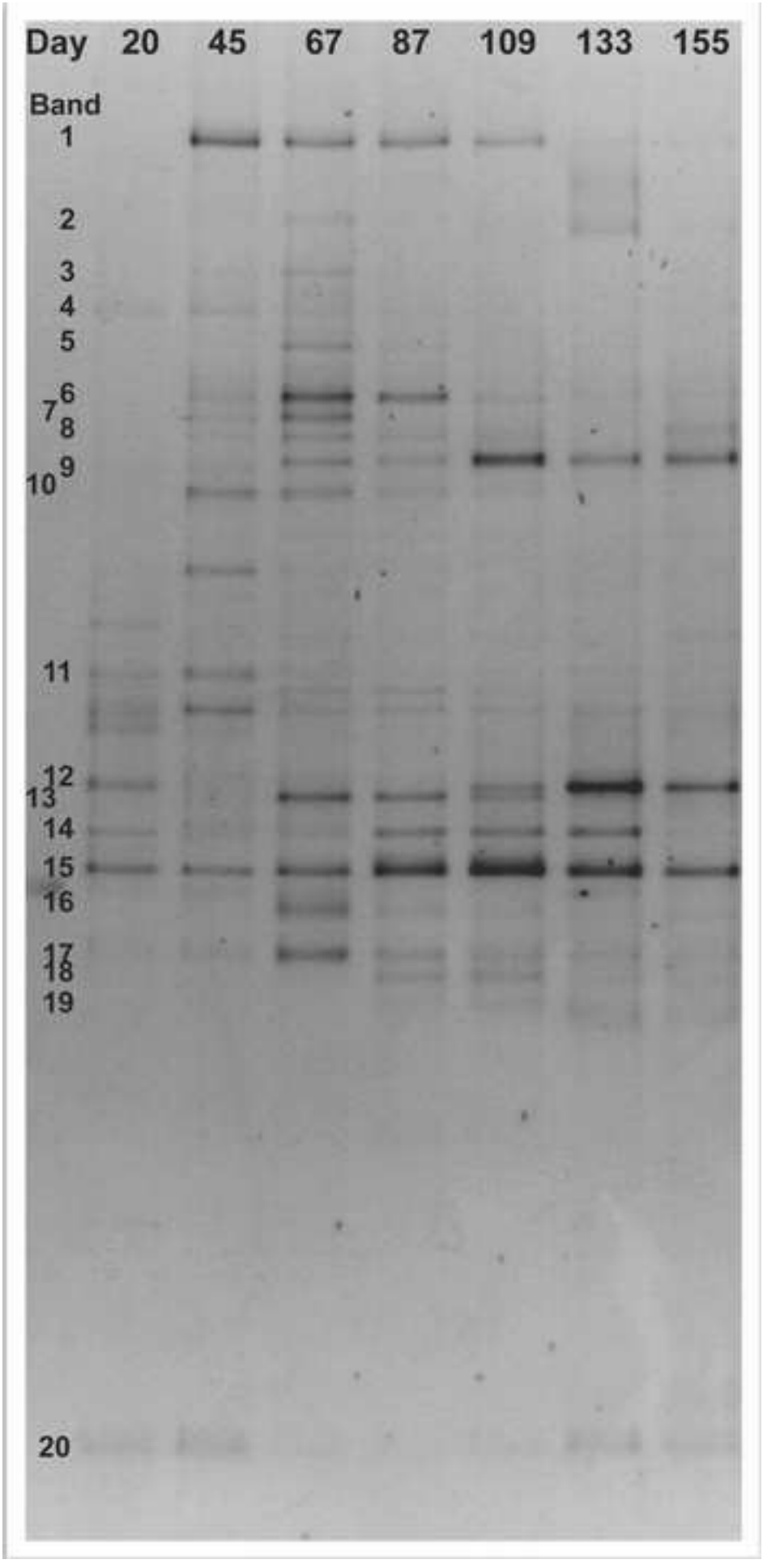
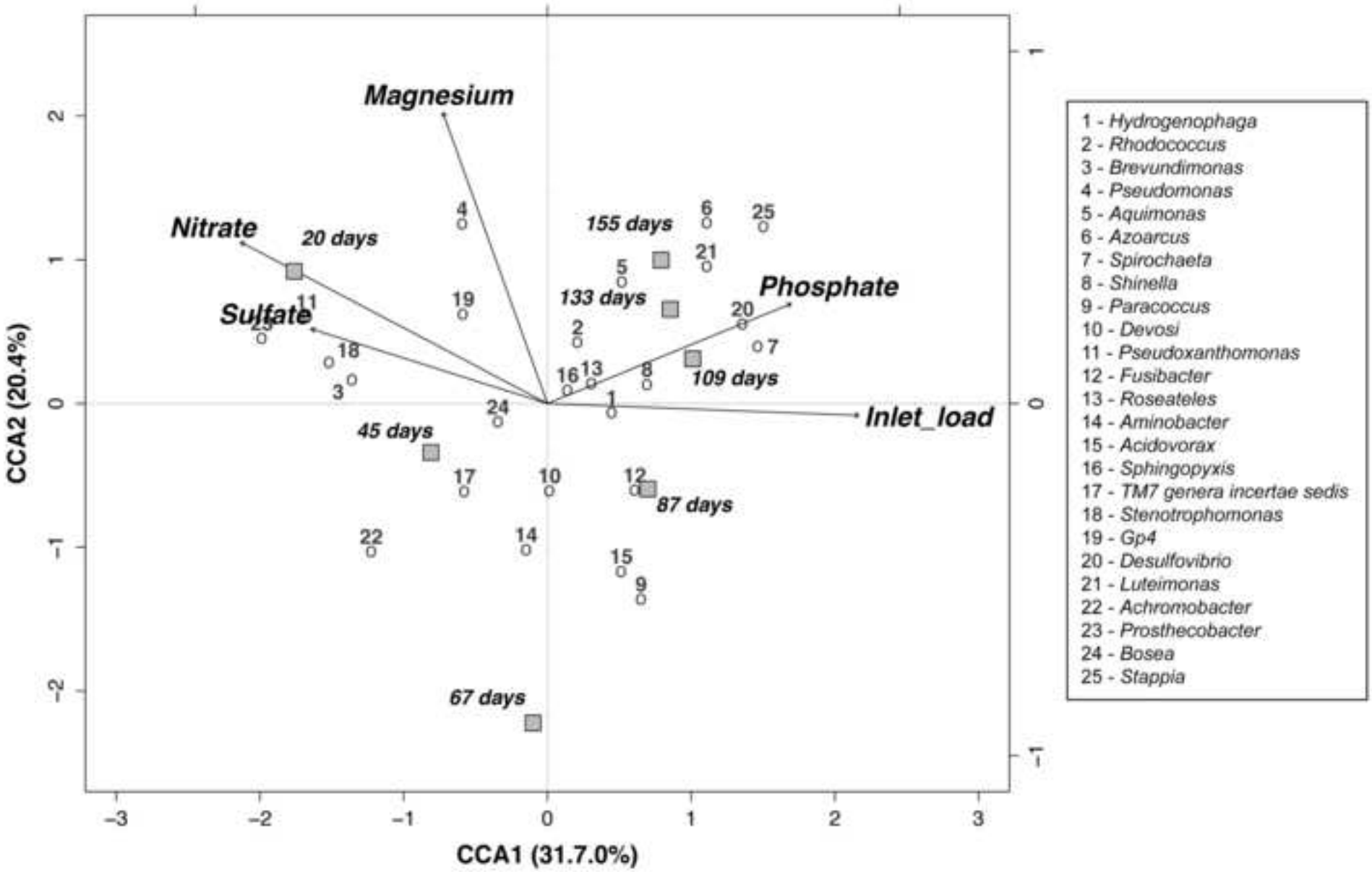

Day 20 45 67 87 109 133 155
Band
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Figure5


Magnesium
Nitrate
20 days
Sulfate
155 days
133 days
Phosphate
109 days
Inlet_load
45 days
87 days
67 days
CCA1 (31.7.0%)
CCA2 (20.4%)
1 - Hydrogenophaga
2 - Rhodococcus
3 - Brevundimonas
4 - Pseudomonas
5 - Aquimonas
6 - Azoarcus
7 - Spirochaeta
8 - Shinella
9 - Paracoccus
10 - Devosi
11 - Pseudoxanthomonas
12 - Fusibacter
13 - Roseateles
14 - Aminobacter
15 - Acidovorax
16 - Sphingopyxis
17 - TM7 genera incertae sedis
18 - Stenotrophomonas
19 - Gp4
20 - Desulfovibrio
21 - Luteimonas
22 - Achromobacter
23 - Prosthecobacter
24 - Bosea
25 - Stappia