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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

A survey of biofilms on wastewater aeration diffusers suggests bacterial community composition and function vary by substrate type and time

Peter A. Noble[1,2] · Hee-Deung Park[3,4] · Betty H. Olson[4] · Pitiporn Asvapathanagul[5] · M. Colby Hunter[2] · Manel Garrido-Baserba[4] · Sang-Hoon Lee[3] · Diego Rosso[4]

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Abstract Aeration diffusers in wastewater treatment plants generate air bubbles that promote mixing, distribution of dissolved oxygen, and microbial processing of dissolved and suspended matter in bulk solution. Biofouling of diffusers represents a significant problem to wastewater treatment plants because biofilms decrease oxygen transfer efficiency and increase backpressure on the blower. To better understand biofouling, we conducted a pilot study to survey the bacterial community composition and function of biofilms on different diffuser substrates and compare them to those in the bulk solution. DNA was extracted from the surface of ethylene–propylene-diene monomer (EPDM), polyurethane, and silicone diffusers operated for 15 months in a municipal treatment plant and sampled at 3 and 9 months. The bacterial community composition and function of the biofilms and bulk solution were determined by amplifying the 16S rRNA genes and pyrosequencing the amplicons and raw metagenomic DNA. The ordination plots and dendrograms of the 16S rRNA and functional genes showed that while the bacterial community composition and function of the bulk solution was independent of sampling time, the composition and function of the biofilms differed by diffuser type and testing time. For the EPDM and silicone diffusers, the biofilm communities were more similar in composition to the bulk solution at 3 months than 9 months. In contrast, the bacteria on the polyurethane diffusers were more dissimilar to the bulk solution at 3 months than 9 months. Taken together, the survey showed that the community composition and function of bacterial biofilms depend on the diffuser substrate and testing time, which warrants further elucidation.

Keywords Next-generation sequencing · Wastewater · Aeration diffusers · Biofouling

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✉ Betty H. Olson
bholson@uci.edu

✉ Diego Rosso
rossodr@gmail.com

[1] Department of Periodontics, University of Washington, Box 357444, Seattle, WA, USA

[2] PhD Program in Microbiology, Alabama State University, Montgomery, AL, USA

[3] School of Civil, Environmental and Architectural Engineering, Korea University, Anam-Dong, Seongbuk-Gu, Seoul 136-713, South Korea

[4] The Henry Samueli School of Engineering, University of California, Irvine, Irvine, CA, USA

[5] Civil Engineering and Construction Engineering Management, College of Engineering at California State University, Long Beach, CA, USA

Introduction

Most municipal treatment plants use aeration diffusers to transfer air to wastewater in order to facilitate mixing and microbial processing of dissolved and suspended organic/inorganic matter. Aeration diffusers generate bubbles by passing compressed air through pores in the diffuser surface. The size of the pore has a significant effect on specific energy consumption, with fine-pore diffusers having lower energy consumption than coarse-pore diffusers. The reason treatment plants use fine-pore diffusers is because aeration is the largest expense in operating a treatment plant and bubble size affects aeration efficiency (mass of oxygen transferred per unit energy required) with small bubbles offering higher efficiency

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than larger ones (Rosso et al. 2008a; Stenstrom et al. 1984; Reardon 1995). Yet, despite this improved efficiency, fine-pore diffusers are more susceptible to biofouling than coarse-pore diffusers. Biofouling greatly diminishes oxygen transfer efficiency and increases backpressure on the blower (Rosso et al. 2008b; Rosso and Shaw 2015). To mitigate these problems, regular maintenance of fine-pore diffusers is required, which varies in both complexity and cost.

Fine-pore diffusers can be composed of many different types of coating materials that serve as microbial substrates such as ethylene–propylene-diene monomer (EPDM), polyurethane, silicone, and ceramic. Although these diffuser substrates will eventually foul in wastewater treatment plants (Garrido-Baserba et al. 2016), it is not well known if different substrates affect biofilm community composition, function and diversity, and, potentially, the extent of biofouling. To fill the void, we conducted a pilot study to survey the community composition, function, and diversity of biofilms on EPDM, polyurethane, and silicone diffusers. We also surveyed the bacteria in the mixed liquor suspended solids (MLSS) (i.e., bulk solution) because bacteria in the bulk are presumably responsible for colonizing the diffuser substrates and generating the biofilms. Each diffuser was sampled twice (i.e., 3 and 9 months) to gauge if biofilm development remained constant or changed with time. This pilot study was needed (i) to provide key information for the design of an extensive study involving more sampling, substrates, and testing times and (ii) to aid in the development of broad hypotheses concerning biofilm community and function on diffuser substrates under nutrient-rich conditions.

We conjecture that the bacterial community composition and function in bulk solution and the biofilms will be more similar at 3 months than 9 months because bacteria in the bulk solution initially adhere to the diffuser substrates and participate in biofilm development (Fletcher 1996) and, with time, the bacteria in the two microhabitats (i.e., bulk solution and the biofilms) diverge in both diversity and bacterial abundance as predicted by meta-community ecology (Besemer et al. 2012).

The objectives of this study are two-fold: (i) to determine if the bacterial community composition and function differs between the diffuser substrates and the bulk solution and (ii) to determine if the composition and function of the biofilms on the diffuser substrates change with testing time.

Materials and methods

Column test tank and sample collection The diffusers were placed into a testing tank with dimensions of 1 × 1 × 1.5 m (L × W × D) that was continuously circulated with activated sludge from the first aerobic zone of the activated sludge process of the Irvine Ranch Water District's Michelson Water Reclamation Plant (MWRP) (as described in Rosso and Shaw (2015)). It should be noted that the MWRP wastewater influent: was 90 % domestic waste, had a temperature of 19 to 27 °C, and contained methanol to promote denitrification. The tank was operated in biological nutrient removal mode, and part of the activated sludge was fed from the bottom of the tank to the top and continuously circulated until it was discharged back to the main plant.

Biofilm samples were collected from tube diffusers and the MLSS from bulk solution at 3 and 9 months. Three-inch width of EPDM, silicone, and polyurethane membrane samples were taken using autoclaved scissors and placed into a sterilized container. Two hundred and fifty milliliters of MLSS was obtained and deposited into a sterilized sampling container. All samples were placed on ice and transported to the laboratory for processing.

DNA extraction The MLSS DNA extractions followed the Yu and Mohn (1999) bead beating protocol with addition of DNA purification using phenol/chloroform/isoamyl alcohol and chloroform, which was described by Asvapathanagul et al. (2012). One milliliter of each MLSS replicate was used for the DNA extraction. After DNA precipitation with isopropanol, each DNA extract for MLSS sample was suspended in 50 μl of sterile high-performance liquid chromatography (HPLC) grade water (Fisher Scientific, Fairlawn, NJ).

The biofilms were positioned on a clean cling wrap surface and a sterile cutting template placed over the diffuser. Care was taken to place the template over an area where the diffuser cover appeared uniform. An X-ACTO knife dipped in alcohol and flamed prior to each cut was used to excise a 1 cm × 1 cm piece of diffuser membrane that was placed in a 2 ml microcentrifuge tube. The entire 1.00 × 1.00 cm membrane piece with attached biofilm was included for DNA extraction. All biofilm samples were subjected to the same DNA extraction using the bead beating protocol as the MLSS samples. The original protocol specified by Asvapathanagul et al. (2012) was modified by increasing the duration of bead beating from 20 to 30 s. In the final step, 15 μl of sterile HPLC grade water (Fisher Scientific, Fairlawn, NJ) was added to elute biofilm DNAs for each cell lysis. All concentrations and purity of DNA extracts were measured at 260 and 280 nm using the Nanodrop2000 (Thermo Scientific, Wilmington, DE). All DNA samples were immediately stored until use at −80 °C.

PCR amplification Bacterial 16S rRNA genes were amplified using universal primers (27F: 5′-AGAGTT TGATCC/ ATGGCTCAG-3′ and 1492R: 5′-TACGGT/ CTACCTTGTTACGACTT-3′) using the GemTaq kit from MGQuest (Cat# EP012) as previously described (Can et al. 2014). The PCR program involved a pre-amplification step of

10 cycles with annealing temperature of 56 °C followed by 20 amplification cycles with annealing temperature of 58 °C. In each cycle, elongation time was 1 min 10 s, at 72 °C. PCR was finalized by extended elongation for 5 min. PCR products were purified with Qiagen columns (California, USA). The purity (A260/A280) and quantity of the DNA for each sample was determined using the NanoDrop (Agilent, USA).

Shotgun pyrosequencing Five hundred nanograms of purified PCR product was labeled with a Multiplex Identifier (MID) during the Roche Rapid Library preparation step. Four to 12 MID-tagged sequences, representing each of the samples, were combined in equimolar concentrations and subjected to emPCR and DNA sequencing protocols as specified by the manufacturer's recommendations for the 454 GS Junior Instrument. In samples that yielded an insufficient number of reads (e.g., <10,000), the emulsion PCR (emPCR) and DNA sequencing was repeated.

Five hundred nanograms of metagenomic DNA were used to prepare libraries. The libraries were amplified on beads using emPCR and the beads were then loaded onto a picotiter plate and inserted into the 454 sequencing platform following the manufacturer's recommendations.

DNA sequence post-processing DNA sequences were separated by their respective Multiplex Identifiers (MIDs) and uploaded to the MG-RAST server for processing (Meyer et al. 2008). The MG-RAST pipeline assesses the quality of the sequences, removes artificial replicated sequences, removes short sequences (multiplication of standard deviation for length cutoff of 2.0), and removes sequences with ambiguous bp (non-ACGT; maximum allowed number of ambiguous base pair was set to 5). The pipeline annotates the sequences and allowed the integration of the data with metagenomic and genomic samples.

Rarefaction and alpha diversity analyses were conducted using commands in Mothur Project (Schloss et al. 1984). In brief, an input file (list format) was initially generated based on the number of sequences annotated to each species. The input file was utilized to calculate rarefaction and diversity indices based on rarefaction.single (see http://www.mothur.org/wiki/Rarefaction.single) and summary.single commands in the Mothur Project (see http://www.mothur.org/wiki/Summary.single).

The M5RNA database was used as annotation source for the 16S rRNA genes, with minimum sequence identity of 97 %, maximum *e*-value cutoff at 10^{-5}, and minimum sequence length of 100 bases. The Subsystem database was used to annotate the metagenomic data with minimum sequence identity of 60 %, maximum *e*-value cutoff at 10^{-5}, and minimum sequence length of 100 bases. The output puts the annotated genes into categories by their gene function (i.e., from general (Level 1) to a specific (Level 3)). For recruitment plots, microorganisms having at least 50 hits to their genome (using maximum *e*-value cutoff at 10^{-3}) were considered present in the metagenomic sample.

Principal component and cluster analyses, ordination plots, and biplots Orthogonal transformation of the annotated genera abundances to their principal components (PC) was conducted using normalized abundances (mean of zero and standard deviation of one) as previously described (Pozhitkov et al. 2015). Specifically, abundances were increased by one, log transformed, and centered to produce relative values. In order to standardize relative values, they were divided by the standard deviation of the log values. Principal component analysis (PCA) was determined using the matrix of Euclidean distances, D. The data were graphed on a two-dimensional ordination plot. To determine the relative contribution of the microbial genera to the plot, we let X denote the 8 by 134 genera (number of samples by genera) matrix of the normalized abundance values. The matrix X was used to produce an 8 × 8 matrix D of distances between all pairs of samples. To investigate and visualize differences between the eight samples, the first two principal components, PC1 and PC2, of the distance matrix D were retained and the projection of each species was calculated onto the (PC1, PC2) plane; those species with the largest projections (correlation cutoff of ±0.7) are displayed in a biplot.

Orthogonal transformation of the annotated Level 3 Subsystem genes to their principal components (PC) was conducted using normalized abundances (mean of zero and standard deviation of one) as outlined above. The data were graphed on a two-dimensional ordination plot. To determine the relative contribution of the genes to each plot, we let X denote the 7 by 820 genes (metagenomic samples by genes) matrix of the normalized abundance values. The matrix X was used to produce a 7 × 7 matrix D of distances between all pairs of samples.

Hierarchical clustering was performed on the Pearson correlation matrix of the normalized (mean of zero and standard deviation of one) gene abundances of the samples. UPGMA was the linkage method used to produce the dendrograms.

These analyses were performed using SAS JMP and/or custom-designed programs in C++. Pearson correlation coefficient was used to measure the linear correlation between two variables. Two-tailed *t* tests with unequal variance were used to determine if there were significant differences in gene functions by diffuser substrate and testing time. The significance level for all statistical tests was set to alpha = 0.05.

DNA sequencing depository numbers

The MG-RAST files corresponding to this project are 4570968.3, 4570967.3, 4570966.3, 4570965.3, 4539081.3,

4539082.3, 4539083.3, 4561639.3, 4561640.3, 4561641.3, 4561642.3, 4561643.3, 4561644.3, 4561645.3, and 4561646.3.

Results

DNA sequence reads

The number of 16S rRNA gene amplicon and metagenomic reads by sample type and testing time is shown in Table 1. The number of reads for the 16S rRNA amplicons ranged from 15,128 to 69,545, while the number of reads for the metagenomic data ranged from 40,535 to 72,926. Note that we did not get sufficient metagenomic DNA for sequencing sample P3. Rarefaction curves of most 16S rRNA gene samples approached an asymptote indicating sufficient reads for comparisons of diffuser type and testing time (Fig. S1).

Bacterial diversity

We used three bacterial diversity indices (i.e., Chao1, ACE, Shannon) to characterize the biological samples (Table 2). The Chao1 and ACE estimate species richness while Shannon index combines species richness and abundance into a single value of evenness (Shannon and Weaver 1984). Chao1 differs from ACE because it estimates the total number of species present in a community based on the number of rare species (Chao 1987), while ACE estimates the total number of species based on all species with fewer than 10 individuals (Chao and Lee 1992). For the Shannon index, biological communities dominated by a few species have a low evenness, while those having species that are equally distributed exhibit a high evenness.

In general, the Chao1, ACE, and Shannon indices were all in agreement (Table 2). At 3 months, the diversity of the bacteria in the bulk solution was lower than that of the silicone and polyurethane diffusers but higher than that of the EPDM diffuser. At 9 months, the diversity in the bulk solution was higher than those of all diffuser substrates. Bacterial diversity in the bulk solution and those in the biofilms are therefore different, with the highest diversity occurring in the bulk solution at 9 months. The diversities of the bacterial biofilms increased with testing time (i.e., 3 to 9 months) for the EPDM and silicone diffusers but decreased for the biofilms on the polyurethane diffusers.

Phyla/class differences

At 3 months, the bulk solution and the biofilms of the EPDM and silicone diffusers consisted of mostly bacteria in the phyla *Proteobacteria* and *Bacteroidetes* (Fig. 1) with 88 % of the bacteria in the EPDM diffuser biofilm composed of an uncultured proteobacterium species. In contrast, the biofilm on the polyurethane diffuser differed from the other samples owing to 65 % of the total bacteria belonging to the genus *Rhodococcus* in the phylum *Actinobacteria*, which is a metabolically versatile bacterium, capable of degrading hydrocarbon and aromatic compounds (Alvarez 2010). At 9 months, most bacteria in the bulk solution and on the EPDM and silicone diffuser biofilms were associated with the phyla *Proteobacteria* and *Bacteroidetes* (Fig. 1), with 52 % of the bacteria in the EPDM diffuser biofilm composed of an uncultured *Gammaproteobacterium* species. In contrast to the other biofilm samples, 54 % of the bacteria in the polyurethane diffuser biofilm were associated with genus *Exiguobacterium* in the phylum *Firmicutes*, which is capable of degrading aromatic polymers in biofilms (Yang et al. 2015).

Multivariate analysis of the bacterial communities

Two axes of the ordination plot, representing the normalized abundances for 1703 bacterial species (based on amplified

Table 1 Number of DNA reads by diffuser biofilm and bulk solution samples and testing time

Sample ID	Diffuser type	Testing time (months)	16S rRNA amplicon reads	Metagenomic reads
E3	EPDM	3	15,128	63,496
M3	MLSS	3	69,111	44,227
S3	Silicone	3	29,551	40,535
P3	Polyurethane	3	34,793	–[a]
E9	EPDM	9	25,334	47,094
M9	MLSS	9	69,545	48,437
S9	Silicone	9	30,736	44,376
P9	Polyurethane	9	28,034	72,926

EPDM ethylene propylene diene monomer (M-class) rubber, *MLSS* mixed liquor suspended solids

[a] Not sufficient DNA for sequencing

Table 2 Diversity indices by sample. Averages and 95 % confidence levels are shown

Samples	Observed species	Diversity indices					
		Chao I		ACE		Shannon	
M3	396	474.5	444.4 ± 523.4	496.1	464.6 ± 542.1	3.5	3.4 ± 3.5
E3	171	225.3	199.8 ± 273.2	235.0	209.1 ± 278.5	1.6	1.6 ± 1.7
S3	437	545.0	507.7 ± 602.1	580.0	539.2 ± 636.9	4.2	4.1 ± 4.2
P3	530	629.7	593.5 ± 686.6	633.7	602.7 ± 677.9	3.6	3.6 ± 3.6
M9	597	738.3	693.3 ± 804.3	767.2	722.9 ± 826.9	4.2	4.2 ± 4.2
E9	288	379.1	341.0 ± 444.4	377.1	345.8 ± 425.4	3.0	3.0 ± 3.0
S9	477	673.3	606.6 ± 774.1	659.0	608.6 ± 728.6	4.0	3.9 ± 4.0
P9	392	486.6	451.3 ± 542.8	519.8	480.9 ± 575.7	3.4	3.4 ± 3.5

16S rRNA genes), explained 55.3 % of the variability (Fig. 2a). The 3-month EPDM and silicone biofilms were proximal to one another as well as the two bulk samples indicating similarities in bacterial composition. The dendrogram supports this interpretation since the samples were found in the same cluster at a dissimilarity of 0.93 (Fig. 2b).

To more rigorously investigate the bacterial composition of the samples, a database was constructed that consisted of common genera in the amplified 16S rRNA gene database and the unamplified metagenomic database. The rationale here is that bacteria common to both databases will provide a better representation of the bacteria in the samples than the individual databases. Henceforth, the new database is referred to as "confirmed genera."

Two axes of the ordination plot of the confirmed genera showed that 69.4 % of the variability was explained by the normalized abundances of 134 genera (Fig. 2d). Similar patterns were observed in the groupings of the samples in Fig. 2d as well as those in Fig. 2a. For example, the 3-month EPDM and silicone biofilms and two bulk solution samples all have *x*-values of >1 in both ordination plots (Fig. 2a, d). The dendrogram of the confirmed genera validates these findings since the samples were in the same cluster at a dissimilarity of 0.54 (Fig. 2c). Of note, these samples were in the same cluster at a dissimilarity of 0.93 in Fig. 2b. Hence, the results obtained from the analyses of the 134 confirmed genera are in general agreement with those obtained using the 1703 bacterial species with more variability explained in the two axes (i.e., 69.4 vs 55.3 %).

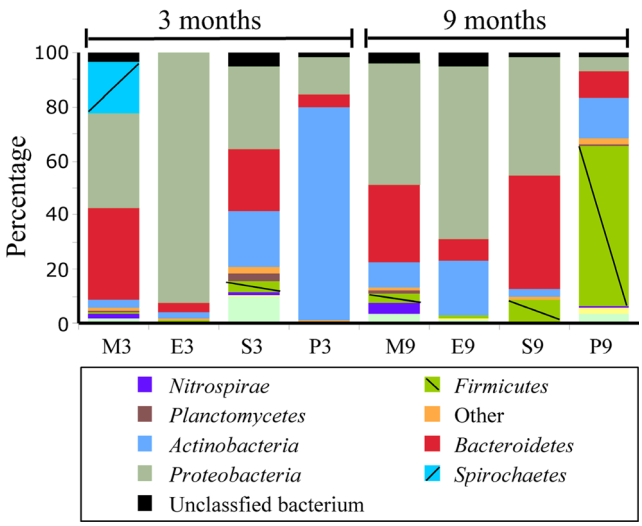

Fig 1 Major phyla/classes of bacteria by sample type and testing time. *M3* MLSS 3-month, *E3* EPDM 3-month, *S3* silicone 3-month, *P* polyurethane 3-month, *M9* MLSS 9-month, *E9* EPDM 9-month, *S3* silicone 9-month, *P* polyurethane 9-month. The "Other" category consisted of phyla/classes of bacteria of *Chlamydiae*, *Gemmatimonadetes*, *Pteridophyta*, *Ochrophyta*, *Cryptophyta*, *Cyanobacteria*, *Tenericutes*, *Fusobacteria*, *Synergistetes*, *TM7*, *Chlorobi*, *Sphingobacteria*, *Acidobacteria*, and *Deinococcus-Thermus*

Below, we compared the bacterial community compositions in the bulk solution and the biofilms by considering (i) the positions of the samples in the ordinations (Fig. 2d), (ii) the vectors of the bacterial genera in the biplots (Fig. 2e), (iii) the relative genera abundances in the samples (Fig. 3), and (iv) the correlations of the abundances of the genera by sample type and testing time. In these comparisons, only confirmed genera that significantly contributed to the ordination plot were examined (i.e., Fig. 2d).

Bacteria specific to the bulk solution and/or the biofilms

Nitrosospira in the phylum/class *Betaproteobacteria* was specific to the bulk solution (Fig. 3) and therefore does not directly contribute to the fouling of the diffusers.

Only one bacterium was found in both the bulk solution and the biofilm of a specific diffuser (i.e., silicone): *Candidatus* Accumulibacter (phylum *Betaproteobacteria*) (Fig. 3).

Bacteria found in both the bulk and in biofilms of all diffusers included genera in phyla *Actinobacteria* (*Frankia*, *Rhodococcus*), Bacteroidetes (*Cytophaga*), *Proteobacteria* (class *Betaproteobacteria* (unclassified species and unclassified Family *Comamonadaceae*) and class *Gamma proteobacteria* (*Moraxella*, *Stenotrophomonas*)), and *Planctomycetes* (*Pirellula*) (Fig. 3). These bacteria showed

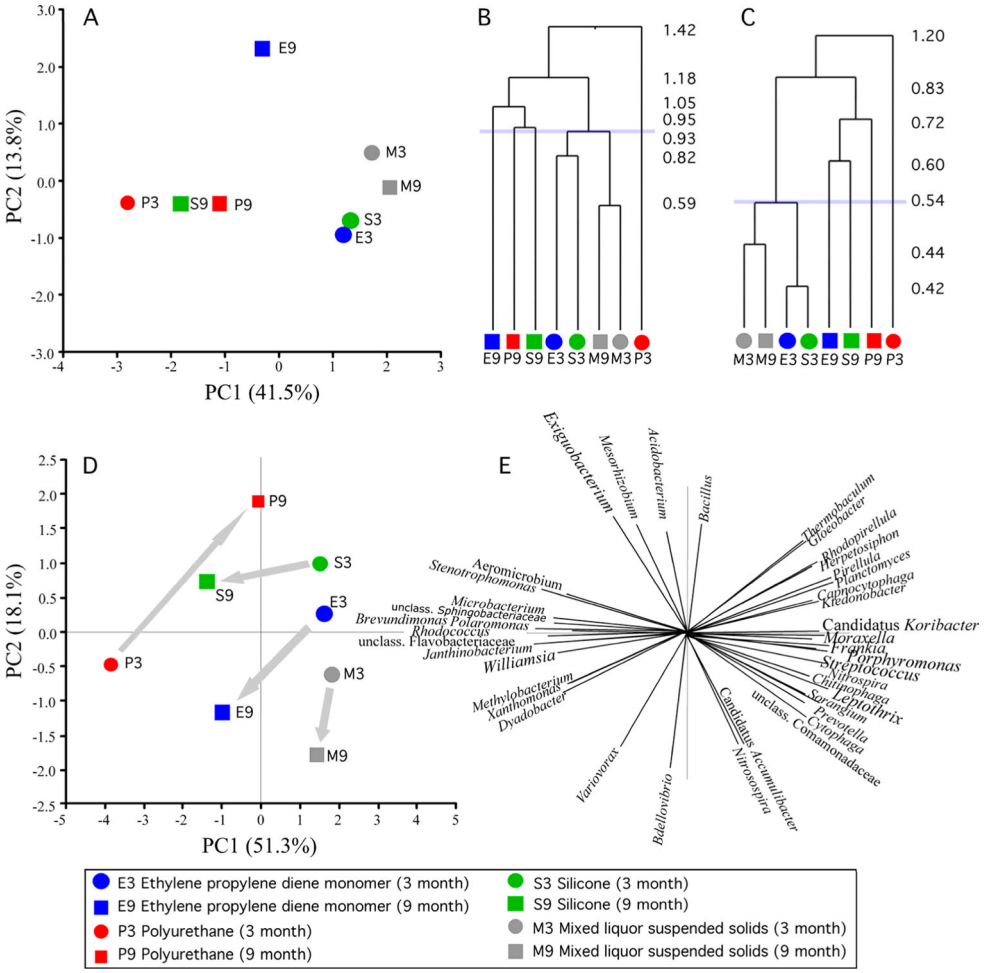

Fig 2 Distribution of bacterial taxa by sample. **a** Ordination plot of all bacterial species (n = 1703) based on relative abundances of amplified 16S rRNA genes. **b** Dendrogram of all bacterial species showing that samples E3, S3, M3, and M9 are dissimilar at 0.93 with the rest different from one another. **c** Dendrogram of confirmed bacterial genera (found in both 16S rRNA amplified gene samples and metagenomic data) showing that samples E3, S3, M3, and M9 are dissimilar at 0.54 while the other samples are different from one another. **d** Ordination plot of confirmed bacterial genera (n = 134) based on relative abundances of amplified 16S rRNA genes with *gray arrows* depicting directional change. **e** Biplot of bacteria genera significantly contributing to the ordination plot (r cutoff < −0.7 or >0.7)

no preference for a specific microhabitat (bulk solution/diffuser biofilm/specific diffuser substrate).

Bacteria not found in the bulk solution but found on a specific diffuser substrate included those in the phylum *Bacteroidetes* (unclassified *Sphingobacteriaceae*, unclassified *Flavobacteriaceae*) and phylum/class *Betaproteobacterium* (*Janthinobacterium*, *Polaromonas*). Bacteria of the *Sphingobacteriaceae* were found in the biofilms of silicone and polyurethane diffusers (Fig. 3); bacteria of the *Flavobacteriaceae* were found in the biofilms of all diffusers at 9 months; *Janthinobacterium* was found in biofilms of polyurethane diffusers at 3 months and silicone diffusers at 9 months; and *Polaromonas* was found in the biofilms of silicone diffusers at 9 months. We could not find any obvious patterns in the abundances of other bacteria shown in Fig. 3.

The correlations of the bacterial abundances were used to compare samples. The bacterial abundance of the bulk solution at 3 and 9 months was strongly correlated (r = 0.76), indicating that sampling time had no significant effects on community composition in the bulk solution. At 3 months, bacteria in the EPDM biofilm had a strong correlation to those in the bulk solution (r = 0.67), while those on the silicone biofilm had a very strong correlation (r = 0.78). The polyurethane biofilm had no correlation (r = 0.19) to the bacteria in the bulk solution. At 9 months, bacteria on both the EPDM and silicone biofilms still showed a significant but weaker association with the bacteria in the bulk solution (r = 0.51, r = 0.40, respectively), while the polyurethane biofilm showed a moderate correlation (r = 0.39). Hence, bacteria in the biofilms of certain diffusers (e.g., EPDM and silicone) have similar abundances to those in the bulk solution, but these similarities depend on testing time with higher correlations occurring at 3 months and lower correlations occurring at 9 months. The polyurethane biofilms, however, showed an opposite trend since the bacteria were more similar to those in the bulk solution at 9 months than 3 months, which indicates that biofilm development was presumably slower than other diffusers.

In summary, the results are aligned with the hypothesis that bacteria in the bulk solution seed the diffusers with cells, and some of these cells have preferences for attaching to one diffuser substrate over another, while others have no preferences. The fact that the abundances of bacteria in the bulk solution remain about the same while those in the biofilms change with

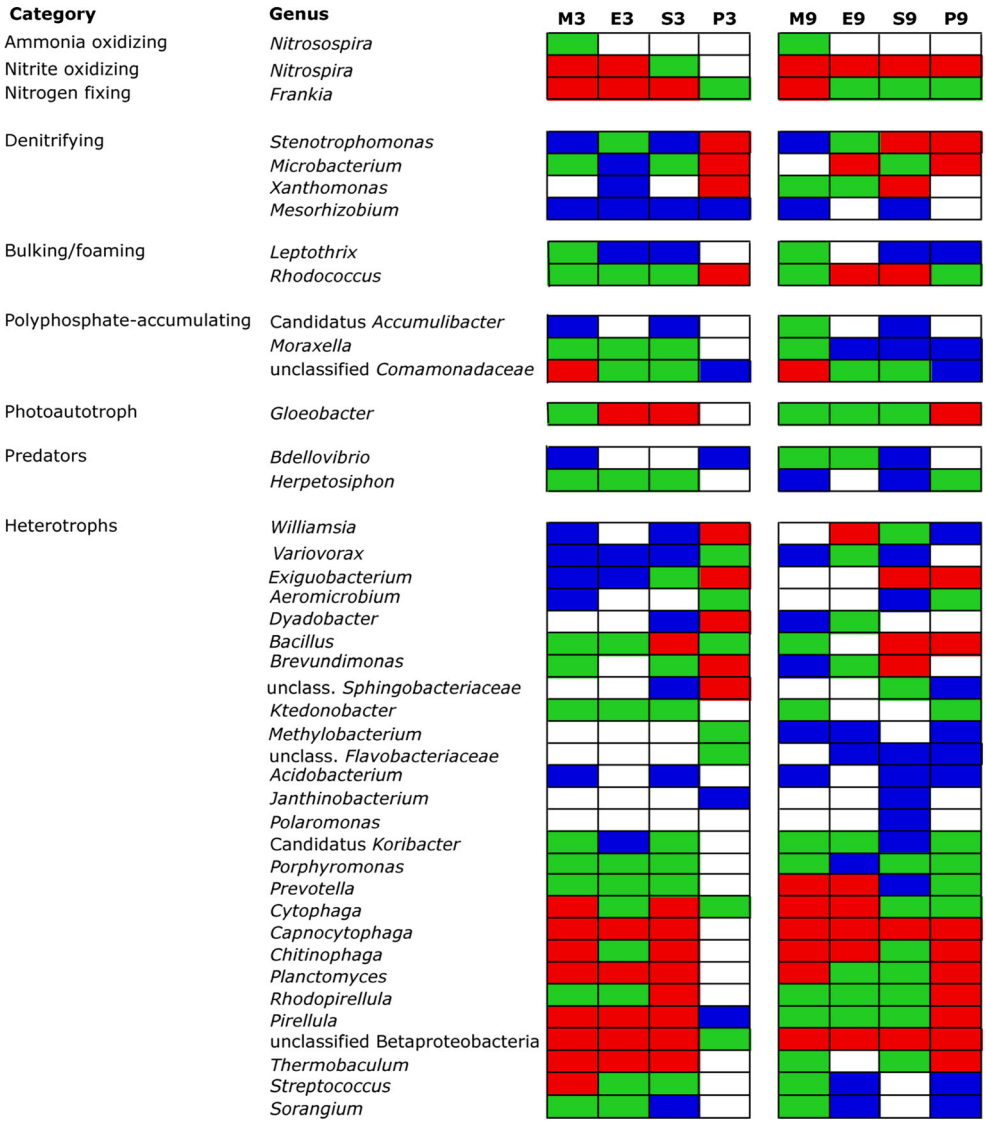

Fig 3 The relative abundance (log scale) of confirmed taxa by sample type and testing time. The relative abundance was calculated as the number of sequences that are assigned to the taxa normalized to a mean of zero and a standard deviation of one for all taxa in a sample. *Red*, high abundance; *green*, average abundance; *blue*, low abundance; *white*, not detected

testing time suggests that once bacteria attach to the diffuser, some colonize the substrate while others do not.

Biofilm genera abundances by testing time

As shown by the shift of the samples in the ordination plot (Fig. 2d), bacterial abundances of the biofilms changed with testing time (Fig. 2e). For both the EPDM and silicone biofilms, the relative abundances of genera in the phyla *Actinobacteria* (*Williamsia*, *Rhodococcus*) and Proteobacteria (*Xanthomonas*, *Brevundimonas*) increased (Fig. 3). These findings are aligned with the biplot (Fig. 2e) as vectors representing the genera *Williamsia*, *Rhodococcus*, *Xanthomonas*, and *Brevundimonas* shift from right to left. For the EPDM biofilms, the relative abundances of genera in the phyla *Bacteroidetes* (*Dyadobacter*), *Actinobacteria* (*Microbacterium*), and *Proteobacteria* (*Variovorax* and *Bdellovibrio*) increased (Fig. 3) and the vectors of these genera shift from right to left and from top to bottom (Fig. 2e). For the silicone biofilms, the relative abundances of genera in the phyla *Proteobacteria* (*Stenotrophomonas*) and *Firmicutes* (*Exiguobacterium*) increased and the vectors shift from right to left in the biplot (Fig. 2e). For the polyurethane biofilms, the relative abundances of genera in the phyla *Nitrospirae* (*Nitrospira*) and *Cyanobacteria* (*Gleobacter*), *Chloroflexi* (*Herpetosiphon*), *Firmicutes* (*Bacillus*), and *Chloroflexi* (*Ktedonobacter*) increased (Figs. 2d and 3). These findings are also aligned with the biplot (Fig. 2e) as vectors representing *Nitrospira*, *Gleobacter*, *Herpetosiphon*, and *Ktedonobacter* shift from left to right in the biplot while the vector representing *Bacillus* shifts from bottom to top. These results are consistent with the hypothesis that the abundances of certain bacteria increased in biofilms of specific diffusers over the 9-month testing time.

Multivariate analysis of gene functions

The two axes of the ordination plot of the metagenomic data explained 70.5 % of the variability in the data (Fig. 4a). The bulk solution samples (M3, M9) were proximal to one another, indicating gene annotation abundances were similar and independent of sampling time. Biplots were inconclusive and not shown. The dendrogram showed that at a dissimilarity of 0.60, samples M3 and M9 are within the same cluster (Fig. 4b); samples E3, S3, S9, and P9 are within a second cluster; and sample E9 is in a third. The presumed reason that sample E9 was different from the others is because only a few genes (n = 140 genes) were annotated by the Subsystems database. Taken together, gene functions of the bacteria in the biofilms were different than those in the bulk solution.

Temporal effects on gene functions in the bulk solution

To determine temporal effects on functional community composition in the bulk solution, we compared the average gene function abundances by testing time. Two-tailed t tests of 27 out of the 28 gene functions revealed no significant differences in the abundance of genes in the bulk solution by time (i.e., M3 vs M9). The one exception was the gene function associated with metabolism of aromatic compounds. The average abundance of this gene function in the bulk solution at 3 and 9 months was 1.15 and 0.91 arbitrary units (a.u.), respectively (Two-tailed t test, $P < 0.035$, df = 27). The elevated abundance of this gene function in the bulk solution could be due to the presence of aromatic compounds in the municipal wastewater at the 3-month sampling time. Hence, temporal effects on the functional community composition in the bulk solution were minimal.

Gene functions in the bulk solution and diffuser biofilms

To determine functional differences between the diffuser biofilms and the bulk solution, gene functions that yielded statistical differences in averaged abundances were identified. We found differences in the bulk solution and the biofilms for 9 out of the 28 gene functions (Table S1). In general, gene functions involving carbohydrates (e.g., uptake and utilization of monosaccharide, organic acids, and sugar alcohol, and fermentation) were more abundant in the bulk solution than the biofilms, which is consistent with bacterial processing of the dissolved and suspended organic matter in the bulk solution. However, at 9 months, gene functions involving the uptake and utilization of disaccharide and oligosaccharides (1.41 vs 0.72 a.u., $P < 0.02$, df = 5) and iron acquisition and metabolism (1.00 vs 0.74 a.u., $P < 0.001$, df = 20) were more abundant in the EPDM biofilm than the bulk solution. The uptake and utilization of these molecules is associated with biofilm development, as shown in previous studies (e.g., Ajdic and Chen 2013; Saha et al. 2013).

Other differences in abundances between the bulk solution and the biofilms include gene functions that varied by diffuser substrate and testing time. For the EPDM biofilms at 3 months, gene functions dealing with resistance to antibiotics and toxic compounds were more abundant in the biofilm than the bulk solution (1.23 vs 0.77 a.u., $P < 0.007$, df = 18) (Table S1). These genes encode proteins are important to bacterial growth in the biofilm because they efflux or inactivate compounds that would otherwise impair bacterial growth (Høiby et al. 2010; Harrison et al. 2007).

At 3 months, gene functions in the silicone biofilm involving cell division and cell cycle were significantly more abundant in the biofilm than the bulk solution (1.38 vs 1.18 a.u., $P < 0.004$, df = 6), while gene functions involving nitrogen metabolism (1.43 vs 2.08 a.u., $P < 0.26$, df = 7) and stress response (0.97 vs 1.33 a.u., $P < 0.31$, df = 29) were less abundant. The increase in cell division and cell cycling functions included genes encoding proteins involved in cytoskeleton and macromolecule synthesis, which suggests that the cells are proliferating in the biofilm. The decrease in abundance of nitrogen metabolism functions suggest that biofilm bacteria might be less involved in processing of nitrogen (e.g.,

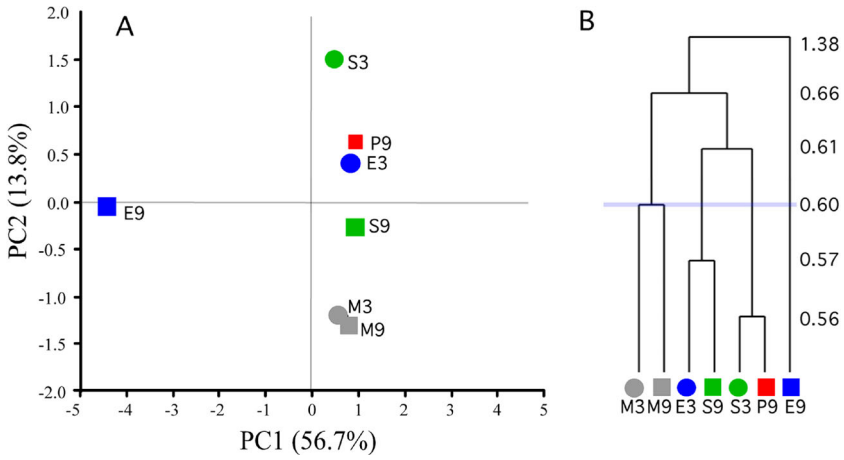

Fig 4 Distribution of samples using Subsystem gene functions. **a** Ordination plot of genes (n = 820). **b** Dendrogram of genes. At a similarity value of 0.60, the MLSS samples are within one cluster; samples E3, S3, S9, and P9 are in second cluster; and sample E9 is in third cluster

ammonia assimilation, nitrate, and nitrite ammonification) than their counterparts in the bulk solution. The decrease of stress response genes is consistent with biofilm bacteria under low oxidative stress relative to those in the bulk solution (Jabubowski and Walkowiak 2015).

At 9 months, gene functions for the polyurethane biofilm involving amino acids utilization (1.90 vs 1.51 a.u., $P < 0.004$, df = 55) were more abundant in the biofilm than the bulk solution. Similarly, for the silicone biofilm, gene functions involving cell wall and capsule biosynthesis (0.98 vs 0.71 a.u., $P < 0.023$, df = 34) were more abundant in the biofilm than the bulk solution. A higher abundance of these functions is consistent with cell growth and biofilm development, which was not observed in the EPDM diffuser. Together, the results suggest that some functional genes in the biofilms are dissimilar to those in the bulk solution with increasing testing time.

In summary, changes in the relative abundance of gene functions (Table S1) corroborate the results observed in the ordination plot and dendrogram (Fig. 4); that is, gene functions in the bacterial biofilms significantly differ from those in the bulk solution and vary by diffuser substrate and testing time.

Gene functions in diffuser biofilms by testing time

To determine if biofilm development vary by diffuser substrate, we compared gene function abundances by testing time.

For the EPDM biofilms, gene functions involving amino acids and derivatives (average abundance at 3 and 9 months, 1.65 vs 1.22 a.u., respectively, $P < 0.04$, df = 55) and fatty acids, lipids, and isoprenoids (1.53 vs 0.88 a.u., $P < 0.049$, df = 14) significantly decreased with testing time (Table S2). The decrease in amino acids and derivative genes presumably indicates that fewer bacteria were involved in nitrogen metabolism in the EPDM biofilms. This finding is supported by a similar decrease in the abundance of gene functions involving the metabolism of arginine and the urea cycle (1.82 vs 0.95 a.u., $P < 0.042$, df = 8). The decrease in fatty acids, lipids, and isoprenoids gene functions presumably indicates a slowdown in biofilm development since these components are needed to synthesize cell membranes and quinones in the electron transport chain (Lange et al. 2000). The increase in gene functions involving iron acquisition and metabolism (0.71 vs 1.00 a.u., $P < 0.001$, df = 20) and gene regulation and cell signaling (0.67 vs 1.06 a.u., $P < 0.007$, df = 21) is also consistent with biofilm development. Specifically, iron is needed as an electron acceptor and a nutrient for bacterial growth (Appenzeller et al. 2005). Hence, the increase in iron acquisition and metabolism genes (e.g., siderophores) presumably indicates that the bacteria in the biofilms are iron deficient. The increase in gene regulation and cell signaling genes included genes encoding quorum-sensing proteins, which are essential for biofilm development (Rashid et al. 2000). Taken together, the decrease in amino acid and derivatives, fatty acids, lipids, and isoprenoids and the increase in iron acquisition and cell signaling genes with testing time is consistent with bacteria colonizing the EPDM diffusers and an overall decrease in cellular growth.

For the silicone biofilms, gene functions involving carbohydrate utilization (1.25 vs 1.10 a.u., $P < 0.005$, df = 87), nitrogen metabolism (1.42 vs 0.95 a.u., $P < 0.029$, df = 7), phosphorus metabolism (1.38 vs 1.18, $P < 0.049$, df = 5), and stress response (0.95 vs 0.74 a.u., $P < 0.027$, df = 29) decreased with testing time (Table S2). One of the most significant decreases in carbohydrate utilization was the gene function involving single-carbon molecules (1.69 vs 1.03, $P < 0.014$, df = 5). This decrease could be due to the absence of *Ktedonobacter* in the silicone biofilm at 9 months. This bacterium has the potential for carbon monoxide oxidation (Weber and King 2010) and was abundant in the silicone biofilm at 3 months but was not detected at 9 months (Fig. 3). Despite the overall decrease in carbohydrate gene functions with testing time, there were increases in gene functions involving disaccharide and oligosaccharide utilization (0.76 vs 1.41 a.u., $P < 0.04$, df = 9) and fermentation (0.94 vs 2.15 a.u., $P < 0.019$, df = 5), which implies that bacteria in the biofilm are involved in fermenting more complex saccharide molecules. The decrease in nitrogen metabolism, phosphorus metabolism, and stress response genes with testing time, combined with the decreased abundance of nitrogen metabolism and stress response genes in the biofilm relative to the bulk solution, indicates that gene functions in the silicone biofilms are diverging away from those in the bulk solution.

There were distinct differences in the gene function abundances by testing time and diffuser substrate (Table S2). Particularly interesting is the lack of common gene functions by diffuser substrate. This phenomenon could be attributed to biofilms at different stages of development and/or biofilms having different bacterial community compositions.

As an aid to readers, we have provided a summary of the major results in Table 3.

Discussion

Biofouling of aeration diffusers represents a significant problem to municipal wastewater treatment plants—yet, there is a paucity of information on the bacteria that foul diffusers. As a pilot survey, we attempted to fill the void by sampling the biofilms on three different diffuser substrates with the objective of determining if the bacterial community composition and function of the biofilms differed from those of the bulk solution. We were also interested in determining if similar

Table 3 Summary of major results

Summary statement	Evidence
Microbial community composition and gene functions of the bulk solution were similar at both sampling times.	Ordination plot showed that the 3- and 9-month MLSS samples are in close proximity to one another (Figs. 2 and 4).
Microbial community composition and gene functions between biofilms and the bulk solution became more differentiated with increased time.	Ordination plots showed that the diffuser biofilms moved distally from the MLSS samples at 9 month compared to the 3-month sample (Figs. 2 and 4).
Microbial composition of the diffuser biofilms were unique indicating the diffuser substrate affects biofilm composition.	16S rRNA gene abundances of the biofilm on the different diffusers differ from each other as well as from the bulk solution (Fig. 3).
Diffuser substrate affects biofilm assembly.	Metagenomic gene functions remained virtually the same in the bulk solution with time, whereas in the diffusers, the gene functions were unique (from the bulk solution and other diffusers) and also shifted with testing time (Fig. 4).
Some gene functions were more abundant in the biofilm of certain diffusers than the bulk solution and changed with time.	Two-tailed *t* tests showed significant differences in gene abundances in Tables S1 and S2.

bacteria were involved in fouling the diffusers. We compared two testing times to examine if the bacterial community composition and function of the biofilms changed with time. Although future studies with larger sample sizes and a wider range of testing times are needed for additional elucidation, to our knowledge, this is the first study to investigate community composition and function of bacterial biofilms on aeration diffusers in a municipal wastewater treatment plant.

Chemical and physical composition of the bulk solution and diffusers

The chemical composition of the bulk solution (i.e., MLSS) was within the typical range of primary effluent in Southern California with the following measured parameters and concentrations: mean cell retention time (MCRT), 8 ± 2.7 days; biological oxygen demand, 150 ± 28; chemical oxygen demand, 295 ± 39 mg/l; ammonia concentration, 22 ± 7 mg/l; nitrate concentration, 10 ± 2 mg/l; mixed liquor suspended solids, 30 mg/l (Ross and Shaw 2015; Garrido-Baserba et al. 2016).

The physical and chemical properties of the diffuser substrates are different from one another. Specifically, the EPDM and polyurethane diffusers are composed of organic compounds such as plasticizers, while the silicone diffusers are composed of non-organic materials. The presence of organic compounds in diffusers is important to this study because bacteria use organic compounds as a carbon source to support their growth (Wagner and von Hoessle 2004; Hansen et al. 2004). Degradation of diffusers by bacteria subsequently alters the thickness (as measured by a pressure sensor) and hardness (as measured by a durometer) of the diffusers. Both the thickness and hardness of a diffuser is important to oxygen transfer and aeration efficiencies because they affect bubble size and shape. The Rosso and Shaw (2015) study showed that thickness of EPDM and polyurethane diffusers was highly variable over the year (that included the sampling time of our study), with EPDM diffusers becoming thicker than polyurethane diffusers with time, while silicone diffusers did not significantly change. In terms of hardness, polyurethane diffusers have higher hardness with time than the other diffusers (Rosso and Shaw 2015). A high hardness value indicates that pores in the diffuser have reduced stretchability (compared to new diffusers), which affects bubble size and shape. Taken together, the physical and chemical properties of diffusers do have a significant effect on the bacteria that colonize them and, in turn, the bacteria in the biofilms alter the diffusers, which ultimately affect oxygen transfer and aeration efficiencies.

Local and regional assembly of biofilms

The metacommunity concept (Leibold et al. 2004) explains species assembly of local communities in response to the source community. If regional effects emphasizing dispersal and demographic equivalence of species dominate, local communities should be similar to the source community (Langenheder and Székely 2011). On the other hand, if local effects such as abiotic factors and species interactions dominate, species should assemble differently from the source community (so-called species sorting) (Langenheder and Székely 2011). In this regard, the bulk solution could be viewed as the source community affecting the bacterial community composition in the diffuser biofilm (i.e., local community) because the bulk solution provides the source of bacterial cells that attach and colonize the diffuser. The bulk solution is a high turbulence kinetic energy ecosystem where fine-pore diffusers release hot compressed air through tiny pores that

vigorously mix the bacterial cells with nutrient-rich matter (Wagner and von Hoessle 2004). In contrast, diffuser biofilms are low turbulent kinetic energy ecosystems because the extracellular polysaccharide matrix protects the attached bacteria from physicochemical (e.g., oxidizing agents) and biological processes (e.g., viruses and predators) (Donlan 2002; Costerton et al. 1987, 1999). Previous studies (Besemer et al. 2009, 2012; Zhang et al. 2014) suggest that the bacteria in the biofilms typically have low dispersal rates and long residence times and that local abiotic and biotic factors determine the bacterial community composition. Given that residence time plays an important role in the assembly of biofilms, we rationalized that factors associated with the high and low turbulent kinetic energy might be important in shaping biofilm bacterial communities in our study.

We found that the bacterial communities in the EPDM and silicone biofilms were similar to each other at 3 months. These communities were also similar to the bacterial communities of the bulk solution at 3 months. In addition, we found that the bacterial communities in the polyurethane biofilms were similar to those of the bulk solution at 9 months. Therefore, we may infer that regional factors were important in shaping the communities of the EPDM and silicone biofilms at 3 months and the polyurethane biofilms at 9 months. These findings are in agreement with the study of Langenheder and Székely (2011) that demonstrated the importance of regional factors during early bacterial colonization in rock pool local communities. In contrast, we found that the bacterial communities in the polyurethane biofilms were dissimilar to those of the bulk solution at 3 months and the bacterial communities in the EPDM and silicone biofilms were more dissimilar to those in the bulk solution at 9 months than 3 months. Therefore, we may infer that the bacteria, different from the source community (i.e., bulk solution), were selected by local factors (e.g., chemical properties of the diffusers and bacterial interactions). In other words, the bacterial communities were assembled by a species sorting process in the polyurethane at 3 months and the EPDM and silicone biofilms at 9 months. Taken together, these results suggest that both regional and local factors play roles in bacterial assembly of the biofilms, but their effects vary by the diffuser substrate and testing time.

The influence of regional and local factors on the assembly of biofilm communities extends to the assembly of functional genes, particularly in how these factors transition through time. Results from the EPDM and silicone biofilms provide examples of what happens to functional genes in the case of weakening regional factors and strengthening local factors. Here, we found significant changes in the abundances of certain functional genes in the EPDM and silicone biofilms. For the EPDM biofilms, gene functions involving amino acids, fatty acids, lipids, and isoprenoid utilization decreased while those involving iron acquisition/metabolism and cell signaling increased (Table S2). For the silicone diffusers, gene functions involving the utilization of most carbohydrates, nitrogen and phosphorus metabolism, and stress response decreased, while gene functions involving disaccharides and oligosaccharides utilization and fermentation increased (Table S2). These changes are consistent with the hypothesis that the bacteria in the EPDM and silicone biofilms are becoming functionally different from those in the bulk solution with increasing testing time.

Biofilm development and diversity

It is important to acknowledge that biofilm development and diversity are interconnected. Specifically, early-stage biofilms have high diversity due to the random assortment of many species from the bulk solution, mid-stage biofilms have moderate to low diversity due to many competitive species dominating the biofilms, and late-stage biofilms have moderate to high diversity due to many species embedded in the extracellular matrix that increases nutrient availability (Jackson et al. 2001). We found that bacterial diversity in the biofilms increased with testing time for EPDM and silicone diffusers but decreased for polyurethane diffusers. The increase in diversity for the EPDM and silicone diffusers presumably reflects a transition from mid- to late-stage biofilm development. The fact that the abundance of genera in the bulk solution and those in the EPDM and silicone biofilms are strongly correlated suggests that some of the increased diversity could be attributed to the recruitment of secondary colonizers from the bulk solution. On the other hand, the decrease in bacterial diversity for the polyurethane biofilms presumably reflects a transition from early to mid-stage biofilm development, which suggests slower biofilm development on polyurethane diffusers than the other substrates. Hence, the comparison of the diversities on the three different substrates indicates that diversity was dependent on both the substrate and the testing time.

Garrido-Baserba et al. (2016) determined the total biomass (i.e., DNA/cm^2, cells/cm^2, EPS concentration, and total suspended solids (TSS) concentration) of biofilms on each of the diffuser substrates (membrane surface coating materials) used in our study for the same testing times. All diffusers had approximately the same total biomass by testing time, except polyurethane, and the overall biomass increased from 3 to 9 months. The reason this is important to our study is that it shows that, although biofilm biomass on the diffusers increased with time, the diversity of the bacteria in the biofilms was dependent on the diffuser substrate.

It should be noted that Kwon et al. (2010) reported that the composition of the bacteria biofilms on a synthetic mesh (made of acryl and polyether) was significantly different from those in the bulk solution in a wastewater treatment plant. A comparable difference was also found in our study but only for the polyurethane diffusers at 3 months. As stated above,

the presumed reason for this difference is local factors are shaping the bacterial community composition of the polyurethane biofilm during early to mid-stage development.

The effects of different substrates on community composition have been reported in several aquatic studies. Both Dang and Lovell (2000) and Zhang et al. (2014) showed that community composition of the bacteria in marine biofilms differed by substrate and suggested that the properties of the substrate such as surface roughness, hydrophilicity/hydrophobicity, and chemical functional groups affect biofilm development. Chung et al. (2010) also reported differences in community composition over 20 days in marine biofilms on polystyrene and granite rocks. After 10 days, Meier et al. (2013) showed that biofilms on artificial substrates (glass, copper, Delrin, and poly-methyl methacrylate) were compositionally different, while after 155 days of incubation, Bellou et al. (2012) showed that regional factors, such as depth and local factors such as substrate type (e.g., titanium aluminum, limestone, shale, and glass) were both important in shaping biofilm community composition. Lee et al. (2014) showed that bacterial biofilm communities in the polyethersulfone and polytetrafluoroethylene substrates were more similar to each other than those in the polyvinylidene fluoride after 15 days. Collectively, these studies point to substrate and time as key factors in determining biofilm community composition and suggest that different substrates have both short and long-term effects on bacterial community composition in different aquatic environments.

Although bacteria in the diffuser biofilms originate from the bulk solution, the composition of the biofilm communities on the diffusers was found to be dependent on the substrate and testing time. For some diffusers, there was significant correlation in the bacteria found in the bulk solution and the biofilm, indicating that factors associated with the bulk solution are mostly determining biofilm community composition. However, for other substrates, no correlation was found at 3 months, indicating that local factors, such as species sorting, are probably determining biofilm community composition. In terms of gene functions, there were significant differences in the abundances of genes between the bulk solution and the diffuser biofilms and between the substrate and testing time.

Although our study did not identify the diffuser substrate that was optimal for minimizing biofouling in wastewater treatment plants, it does provide a framework for future investigations. Since only three substrates were examined at 3 and 9 months, future studies could increase the number of different diffuser substrates as well as the frequency and number of the testing times, in order to get a better idea of microbial population dynamics and to determine if an optimal substrate exists. This is important because it could help identify the best substrate for diffusers and might help determine the optimal times for cleaning maintenance. It is possible that biofilms on some substrates might be more susceptible to removal from the diffuser surface at certain times than others. Such studies will also provide a fundamental link between microbial population dynamics and oxygen transfer and aeration efficiencies, thus allowing operators at wastewater treatment plants to set conditions that minimize the fouling of diffusers.

Author contributions PA collected the samples; BHO and DR developed the experimental design; PA and MGB extracted the DNA; MCH and PAN amplified and sequenced the DNA; HDP and PAN analyzed the DNA sequences and carried out the statistical analyses; and BHO, HDP, and PAN wrote the manuscript and all authors agree with its content.

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by the authors.

Conflict of interest The authors declare that they have no competing interests.

References

Ajdic D, Chen Z (2013) A novel phosphotransferase system of *Streptococcus mutans* is responsible for transport of carbohydrates with α-1,3 linkage. Mol Oral Microbiol 28:114–128. doi:10.1111/omi.12009

Alvarez H (2010) Biology of *Rhodococcus*. Springer-Verlag, Berlin ISBN: 978-3-642-12937-7

Appenzeller BM, Yañez C, Jorand F, Block JC (2005) Advantage provided by iron for *Escherichia coli* growth and cultivability in drinking water. Appl Environ Microbiol 71:5621–5623

Asvapathanagul P, Huang Z, Gedalanga PB, Baylor A, Olson BH (2012) Interaction of operational and physicochemical factors leading to *Gordonia amarae*-like foaming in an incompletely nitrifying activated sludge plant. Appl Environ Microbiol 78:8165–8175. doi:10.1128/AEM.00404-12

Bellou N, Papathanassiou E, Dobretsov S, Lykousis V, Colijn F (2012) The effect of substratum type, orientation and depth on the development of bacterial deep-sea biofilm communities grown on artificial substrata deployed in the Eastern Mediterranean. Biofouling 28:199–213. doi:10.1080/08927014.2012.662675

Besemer K, Hödl I, Singer G, Battin TJ (2009) Architectural differentiation reflects bacterial community structure in stream biofilms. ISME J 3:1318–1324. doi:10.1038/ismej.2009.73

Besemer K, Peter H, Logue JB, Langenheder S, Lindström ES, Tranvik LJ, Battin TJ (2012) Unraveling assembly of stream biofilm communities. ISME J 6:1459–1468. doi:10.1038/ismej.2011.205

Can I, Javan GT, Pozhitkov AE, Noble PA (2014) Distinctive thanatomicrobiome signatures found in the blood and internal organs of humans. J Microbiol Methods 106:1–7. doi:10.1016/j.mimet.2014.07.026

Chao A (1987) Estimating the population size for capture-recapture data with unequal catchability. Biometrics 43:783–791

Chao A, Lee S-M (1992) Estimating the number of classes via sample coverage. J Am Stat Assoc 87:210–217

Chung HC, Lee OO, Huang YL, Mok SY, Kolter R, Qian PY (2010) Bacterial community succession and chemical profiles of subtidal biofilms in relation to larval settlement of the polychaete *Hydroides elegans*. ISME J 4:817–828. doi:10.1038/ismej.2009.157

Costerton JW, Cheng KJ, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322

Dang H, Lovell CR (2000) Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. Appl Environ Microbiol 66:467–475

Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8:881–890

Fletcher M (1996) Bacterial adhesion: molecular and ecological diversity, Series in ecological and applied microbiology series, #19. Wiley, Hoboken, NJ, p. 361 ISBN: 978-0-471-02185-8

Garrido-Baserba M, Asvapathanagul P, McCarthy GW, Gocke TE, Olson BH, Park HD, Al-Omari A, Murthy S, Bott CB, Wett B, Smeraldi JD, Shaw AR, Rosso D (2016) Linking biofilm growth to fouling and aeration performance of fine-pore diffuser in activated sludge. Water Res 90:317–328. doi:10.1016/j.watres.2015.12.011

Hansen G, Estevez MA, Es-Said OS (2004) On the shrinking and hardening of EPDM rubber membranes in water sanitation filtration tanks. Eng Fail Anal 11:361–367. doi:10.1016/j.engfailanal.2003.06.003

Harrison JJ, Ceri H, Turner RJ (2007) Multimetal resistance and tolerance in microbial biofilms. Nat Rev Microbiol 5:928–938. doi:10.1038/nrmicro1774

Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. Int J Antimicrob Agents 35:322–332. doi:10.1016/j.ijantimicag.2009.12.011

Jabubowski W, Walkowiak B (2015) Resistance to oxidative stress in biofilm and planktonic cells. Braz Arch Biol Technol 58:300–308. doi:10.1590/S1516-8913201500356

Jackson CR, Churchill PF, Roden EA (2001) Successional changes in bacterial assemblage structure during epilithic biofilm development. Ecology 82:555–566. doi:10.1890/0012-9658(2001)082[0555:SCIBAS]2.0.CO;2 ISSN: 0012-9658

Kwon S, Kim TS, Yu GH, Jung JH, Park HD (2010) Bacterial community composition and diversity of a full-scale integrated fixed-film activated sludge system as investigated by pyrosequencing. J Microbiol Biotechnol 20:1717–1723

Lange BM, Rujan T, Martin W, Croteau R (2000) Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. Proc Natl Acad Sci U S A 97:13172–13177

Langenheder S, Székely AJ (2011) Species sorting and neutral processes are both important during the initial assembly of bacterial communities. ISME J 5:1086–1094. doi:10.1038/ismej.2010.207

Lee SH, Hong TI, Kim B, Hong S, Park HD (2014) Comparison of bacterial communities of biofilms formed on different membrane surfaces. World J Microbiol Biotechnol 30:777–782. doi:10.1007/s11274-013-1460-8

Leibold MA, Holyoak M, Mouquet N, Amarasekare P, Chase JM, Hoopes MF, Holt RD, Shurin JB, Law R, Tilman D, Loreau M, Gonzalex A (2004) The metacommunity concept: a framework for multi-scale community ecology. Ecol Lett 7:601–613. doi:10.1111/j.1461-0248.2004.00608.x

Meier A, Tsaloglou NM, Mowlem MC, Keevil CW, Connelly DP (2013) Hyperbaric biofilms on engineering surfaces formed in the deep sea. Biofouling 29:1029–1042. doi:10.1080/08927014.2013.824967

Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA (2008) The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinform 9:386. doi:10.1186/1471-2105-9-386

Pozhitkov AE, Leroux BG, Randolph TW, Beikler T, Flemmig TF, Noble PA (2015) Towards microbiome transplant as a therapy for periodontitis: as an exploratory study of periodontitis microbial signature contrasted by oral health, caries and edentulism. BMC Oral Health 15:125. doi:10.1186/s12903-015-0109-4

Rashid MH, Rumbaugh K, Passador L, Davies DG, Hamood AN, Iglewski BH, Kornberg A (2000) Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A 97:9636–9641

Reardon DJ (1995) Turning down the power. Civ Eng 65:54–56

Rosso D, Shaw AR. (2015) Framework for Energy Neutral Treatment for the 21st Century through Energy Efficient Aeration. Water Intelligence Online 2015. IWA Publishing. ISSN Online: 1476–1777. ISBN13: 9781780406794, eISBN: 9781780406794

Rosso D, Stenstrom MK, Larson LE (2008a) Aeration of large-scale municipal wastewater treatment plants: state of the art. Water Sci Technol 57:973–978. doi:10.2166/wst.2008.218

Rosso D, Libra JA, Wiehe W, Stenstrom MK (2008b) Membrane properties change in fine-pore aeration diffusers: full-scale variations of transfer efficiency and headloss. Water Res 42:2640–2648. doi:10.1016/j.watres.2008.01.014

Saha R, Saha N, Donofrio RS, Bestervelt LL (2013) Microbial siderophores: a mini review. J. Basic Microbiol 53:303–317. doi:10.1002/jobm.201100552

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541. doi:10.1128/AEM.01541-09

Shannon CE, Weaver W (1984) A mathematical theory of communication. Bell Syst Tech J 27:379–423 and 27:623-656

Stenstrom MK, Vazirinejad HO, Ng A. (1984) Economic evaluation of upgrading aeration systems. J. Water Pollut Control Fed 56:20–26. http://www.jstor.org/stable/25042152

Wagner M, von Hoessle R (2004) Biological coating of EPDM-membranes of fine bubble diffusers. Water Sci Technol 50:79–85

Weber CF, King GM (2010) Distribution and diversity of carbon monoxide-oxidizing bacteria and bulk bacterial communities across a succession gradient on a Hawaiian volcanic deposit. Environ Microbiol 12:1855–1867. doi:10.1111/j.1462-2920.2010.02190.x

Yang Y, Yang J, Wu W, Zhao J, Song Y, Gao L, Yang R, Jiang L (2015) Biodegradation and mineralization of polystyrene by plastic-eating mealworms. 2. Role of gut microorganisms. Environ Sci Technol 49:12087–12093

Yu Z, Mohn WW (1999) Killing two birds with one stone: simultaneous extraction of DNA and RNA from activated sludge biomass. Can J Microbiol 45:269–272. doi:10.1139/w98-211

Zhang WP, Wang Y, Tian RM, Bougouffa S, Yang B, Cao HL, Zhang G, Wong YH, Xu W, Batang Z, Al-Suwailem A, Zhang QPY (2014) Species sorting during biofilm assembly by artificial substrates deployed in a cold seep system. Sci Rep 17:6647. doi:10.1038/srep06647