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Changes in bacterial and eukaryotic communities during sewage decomposition in Mississippi river water

Asja Korajkic [a], Laura Wegener Parfrey [b,1], Brian R. McMinn [a], Yoshiki Vazquez Baeza [b], Will VanTeuren [b], Rob Knight [b,c], Orin C. Shanks [d,*]

[a] National Exposure Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, USA
[b] Biofrontiers Institute, University of Colorado, Boulder, CO, USA
[c] Howard Hughes Medical Institute, Boulder, CO, USA
[d] National Risk Management Research Laboratory, US. Environmental Protection Agency, Cincinnati, USA

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ABSTRACT

Microbial decay processes are one of the mechanisms whereby sewage contamination is reduced in the environment. This decomposition process involves a highly complex array of bacterial and eukaryotic communities from both sewage and ambient waters. However, relatively little is known about how these communities change due to mixing and subsequent decomposition of the sewage contaminant. We investigated decay of sewage in upper Mississippi River using Illumina sequencing of 16S and 18S rRNA gene hypervariable regions and qPCR for human-associated and general fecal Bacteroidales indicators. Mixtures of primary treated sewage and river water were placed in dialysis bags and incubated *in situ* under ambient conditions for seven days. We assessed changes in microbial community composition under two treatments in a replicated factorial design: sunlight exposure versus shaded and presence versus absence of native river microbiota. Initial diversity was higher in sewage compared to river water for 16S sequences, but the reverse was observed for 18S sequences. Both treatments significantly shifted community composition for eukaryotes and bacteria ($P < 0.05$). Data indicated that the presence of native river microbiota, rather than exposure to sunlight, accounted for the majority of variation between treatments for both 16S ($R = 0.50$; $P > 0.001$) and 18S ($R = 0.91$; $P = 0.001$) communities. A comparison of 16S sequence data and fecal indicator qPCR measurements indicated that the latter was a good predictor of overall bacterial community change over time (rho: 0.804–0.814, $P = 0.001$). These findings suggest that biotic interactions, such as predation by bacterivorous protozoa, can be critical factors in the decomposition of sewage in freshwater habitats and support the use of Bacteroidales genetic markers as indicators of fecal pollution.

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* *Corresponding author*. Tel.: +1 513 569 7314.
E-mail address: Shanks.orin@epa.gov (O.C. Shanks).
[1] Current address: University of British Columbia, Vancouver, British Columbia, Canada.
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1. Introduction

The advent of high-throughput DNA sequencing technologies makes it feasible to characterize the composition of microbial communities of both fecal pollution sources and indigenous aquatic communities. High-throughput sequencing data is available for human sewage (McLellan et al., 2013; Shanks et al., 2013), fecal microbiota from a variety of human, agricultural, and wildlife animal species (Unno et al., 2012, 2010) as well as various natural environments such as marine and freshwater systems (Humbert et al., 2009; Staley et al., 2013), groundwater (Lin et al., 2012) and intertidal/marine sediments (Lemke et al., 2009; Wang et al., 2012). Due to large differences between fecal-derived and indigenous aquatic microbial population structures, it may be possible to discriminate between these different populations when mixed in an environmental system (Cao et al., 2013; Unno et al., 2012, 2010). This strategy has been employed to characterize human fecal pollution in Lake Michigan during wet and dry weather events (Newton et al., 2013), to identify riverine intrusion to underground aquifers (Lin et al., 2012), to characterize wastewater impacted riparian buffer zones (Ducey et al., 2013), and to quantify sources of contamination by estimating the proportion of an invading community in a set of indoor environments (neonatal intensive care units, offices and molecular biology laboratories) (Knights et al., 2011).

To date, most of the information available about sewage decomposition in natural environments is based on the decay of specific fecal indicators such as *Escherichia coli*, enterococci, and host-associated Bacteroidales, a common choice for fecal source identification applications due to high concentrations in mammalian feces and evidence of coevolution with animal hosts (Harwood et al., 2014). These studies suggest that the decay of sewage in ambient waters is influenced by environmental factors such as the water type (marine or freshwater) (Green et al., 2011; Korajkic et al., 2013) and temperature (Okabe and Shimazu, 2007). Exposure to ambient sunlight has yielded somewhat conflicting results and there is a lack of concurrence on whether it has an impact on decay (Bae and Wuertz, 2009; Green et al., 2011; Korajkic et al., 2013, 2014). The effect of predation, competition and viral lysis is often overlooked, although recent studies suggest that these biotic interactions are important factors in decay (Dick et al., 2010; Korajkic et al., 2013, 2014; Wanjugi and Harwood, 2013, 2014). Furthermore, the extent of influence of any environmental factor can vary from one indicator to another and it remains unclear which member(s) of the aquatic microbial community play the most important role in sewage decomposition.

To address this research gap, we conducted an *in situ* experiment in the upper Mississippi River to characterize temporal changes in microbial communities associated with the decomposition of primary treated sewage over seven-days. We characterized bacteria and microbial eukaryotes using high-throughput DNA sequencing of partial 16S and 18S rRNA genes at depths of millions of sequences per sample using the Illumina HiSeq platform. These data combined with real-time quantitative PCR (qPCR) measurements of two Bacteroidales fecal indicators allowed us to characterize changes in microbial communities over time, evaluate the influence of solar radiation and biotic factors on decomposition, and examine the utility of high-throughput DNA sequencing for predicting levels of sewage pollution in a riverine environment.

2. Materials and methods

2.1. Field experiment and treatments

Mixtures of primary treated sewage and river water (final volume 200 mL) were placed in dialysis bags at a ratio of 1:1 and incubated *in situ* over a seven day period in the Upper Mississippi River, as previously described (Korajkic et al., 2014). A 1:1 ratio was selected to allow monitoring of decomposition for less abundant community members. Experimental treatments were designed to isolate the effects of ambient sunlight exposure (~3.08 kW h m^{-2} day^{-1} from http://eosweb.larc.nasa.gov) and indigenous riverine microbiota. Briefly, treatments included: A) exposure to both sunlight and river microbiota, B) exposure to sunlight while biotic interactions were reduced (river water filter-sterilized through 0.45 μm, 0.22 μm pore size nitrocellulose filters and a positively charged NanoCeram cartridge filter), C) exposure to river microbiota and reduced sunlight (shading), and D) reduced biotic interactions and shading. Sunlight exposed treatments were ensured by incubating dialysis bags approximately 1–2 cm below the surface of the water, while shaded treatments were incubated under a black tarp covering. The background changes (control samples: river only) in bacterial and eukaryotic communities were captured by incubating river water only under sunlight and shaded conditions. In addition, the primary treated sewage used to seed treatment samples was also characterized (control: sewage only). Triplicate dialysis bags were harvested per treatment at the beginning of experiment (T_{0h}), after 72 h (T_{72h}), and approximately every other day (T_{120h} and T_{168h}) for seven days. The potential blockage of the sunlight by the dialysis bag material was evaluated and found to be minimal (i.e. <10%) (Korajkic et al., 2014).

2.2. Sample processing

Fifty milliliters from each dialysis bag was filtered through a polycarbonate (0.40 μm pore size, 47 mm diameter) and nitrocellulose (0.45 μm pore size, 47 mm diameter) membrane filters for sequencing and qPCR analyses (Korajkic et al., 2014), respectively. Samples were stored at −80 °C until further processing (<6 months). Nucleic acids were extracted from each filter type using PowerSoil® DNA Isolation Kit (MoBio laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions except for the following: 1) additional 10 min incubation of bead beating tube containing filter and C1 reagent at 65 °C followed by: 2) utilization of FastPrep® homogenizer (MP Biomedicals, Santa Ana, CA) for 1 min at 60 ms^{-1} instead of vortexing.

2.3. 16S and 18S rRNA sample preparation, barcoding and Illumina sequencing

Briefly, samples were prepared for sequencing according to the earth microbiome project (EMP) standard protocols

(Caporaso et al., 2011) available on the EMP webpage (http://www.earthmicrobiome.org/emp-standard-protocols/). The V4–V5 fragment of the 16S rRNA gene was amplified with 515f-806r primers and the V9 region of the 18S was amplified with 1391f-EukBr primers with the addition of mammal blocking primer. All primers and protocols for amplification and sequencing are accessible at EMP webpage. The only deviation from the standard EMP protocol was the cycle number for the 16S amplification; 20 cycles was used instead of the recommended 35. Prior to amending the protocol, amplicons resulting from different cycle numbers (15, 20, 25, 30 and 35) were visualized by agarose gel electrophoresis to identify the lowest cycle number that still yielded consistent PCR products. Amplification where cycle number exceeds 25 may introduce bias (Ahn et al., 2012; Lee et al., 2012; Pinto and Raskin, 2012; Wu et al., 2010). Negative controls included extraction blanks for DNA purification and no template controls for PCR amplification. Following amplification, PCR triplicates were pooled into a single sample and successful amplification was verified by agarose gel electrophoresis as described in the standard EMP protocols. The purity (determined by ratio of absorbance readings at 260 nm and 280 nm) and concentration of DNA in samples containing visible bands was assessed using NanoDrop ND-1000 (Thermo-Fisher Scientific, Wilmington, DE). Amplified DNA was purified with QIAquick96 PCR purification kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. Purified amplicons were normalized to either 120 ng/μl (16S) or 240 ng/μl (18S) by pooling equal amounts from each sample into a new low-retention microtube prior to sequencing. Pools were sequenced on one lane each of an Illumina HiSeq platform at the BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado (Caporaso et al., 2012). In both cases, filtering to remove short and poor quality sequences, as well as demultiplexing were done with split_libraries_fastq.py in QIIME (version 1.5.0) using default settings. For eukaryotes (18S) there were 101 million raw sequence reads with 83 million passing quality control filters (82.2%). The bacterial (16S) dataset consisted of 73 million raw sequence reads with 42 million passed by filters (57.5%). A total of 40.8 million 16S reads and 45.5 million 18S reads representing 3546 and 1485 operational taxonomic units (OTUs) after filtering, respectively were available for diversity analyses. MiMARKS (Yilmaz et al., 2011) compliant metadata and sequence data are available on the QIIME database (http://www.microbio.me/qiime; 16S: study 989 and 18S: study 1485).

2.4. Operational taxonomic unit assignments

Bacterial and eukaryotic reads were each processed using QIIME version 1.5.0 (Caporaso et al., 2010). Quality filtered sequences were clustered into OTUs according to sequence similarity using a 97% similarity threshold against the respective reference databases. The February 2011 release of the Greengenes reference database (DeSantis et al., 2006) was used for 16S data. A curated version of the SILVA 108 (Pruesse et al., 2007) was used as the reference database for 18S data. The database was curated to reflect eukaryotic taxonomy and phylogeny (Parfrey et al., 2014; Yilmaz et al., 2014). Both databases are available for download at (http://qiime.org/home_static/dataFiles.html). The two-step open reference protocol was used for both 16S and 18S data. Briefly, sequences were initially clustered against the reference database and those that were within 97% similarity were assigned to reference OTUs and these inherited the reference database taxonomy. The remaining sequences were clustered into *de novo* OTUs with UCLUST (Edgar, 2010) within QIIME. Taxonomy was assigned to these sequences using the RDP classifier in QIIME with 80% confidence threshold (Cole et al., 2009). Taxonomy assignments for 18S OTUs were further refined according to phylogeny. Datasets were filtered to remove low abundance OTUs making up <0.0005% of reads in the total dataset as recommended for Illumina generated data (Bokulich et al., 2013). Both datasets were further filtered to remove non 16S and 18S sequences, respectively. The 16S dataset was filtered to remove samples with fewer than 100,000 sequences per sample for data analysis (one sample failed to pass this filter) and data was rarefied to 100,000 sequences per sample prior to diversity analyses. The 18S dataset was filtered to remove samples with fewer than 15,000 sequences per sample and data was rarefied to 15,000 sequences per sample for all diversity analyses. Four samples had fewer than 15,000 sequences for 18S, including all three replicates of the T_{0h} reduced biota treatment. To account for this, relative taxon abundance in Fig. 1, panel B was conducted on all samples with greater than 1000 sequences per sample.

2.5. Fecal indicator abundance qPCR data and 16S community shifts

To determine if HF183 and GenBac3 fecal indicator qPCR measurements, two commonly used methods for estimating fecal pollution levels in recreational waters, were good predictors of overall 16S community change over the course of the study, Mantel tests between the Unifrac distance matrix and qPCR data were calculated within QIIME. Experimental protocols, calibration models, concentration estimates, and controls (no template, extraction blanks, sample processing controls, and inhibition tests) for GenBac3 and HF183 qPCR assays are reported elsewhere (Korajkic et al., 2014).

2.6. Data analyses

Phylogenetic trees were constructed by placing representative sequences for each *de novo* OTU into the respective reference tree with the RAxML EPA algorithm (Berger et al., 2011). The unweighted UniFrac metric, a beta-diversity metric that takes phylogeny into account (Lozupone and Knight, 2005), was used to assess shifts in community composition, which were visualized with principal coordinate plots. We calculated the Shannon diversity index, which takes into account richness and evenness, using QIIME software to assess changes in alpha diversity. To characterize the trajectory of dominant taxa (at the order level) influencing 16S community structure over the course of the study, OTUs with a relative abundance $\geq$ 5% at any time point and their abundance at each time point were plotted. Because the difference between the sunlight exposed and shaded treatments was minimal compared to riverine microbiota treatments (PERMANOVA Pseudo-F 3.19 for sterilization treatment versus 2.36 for

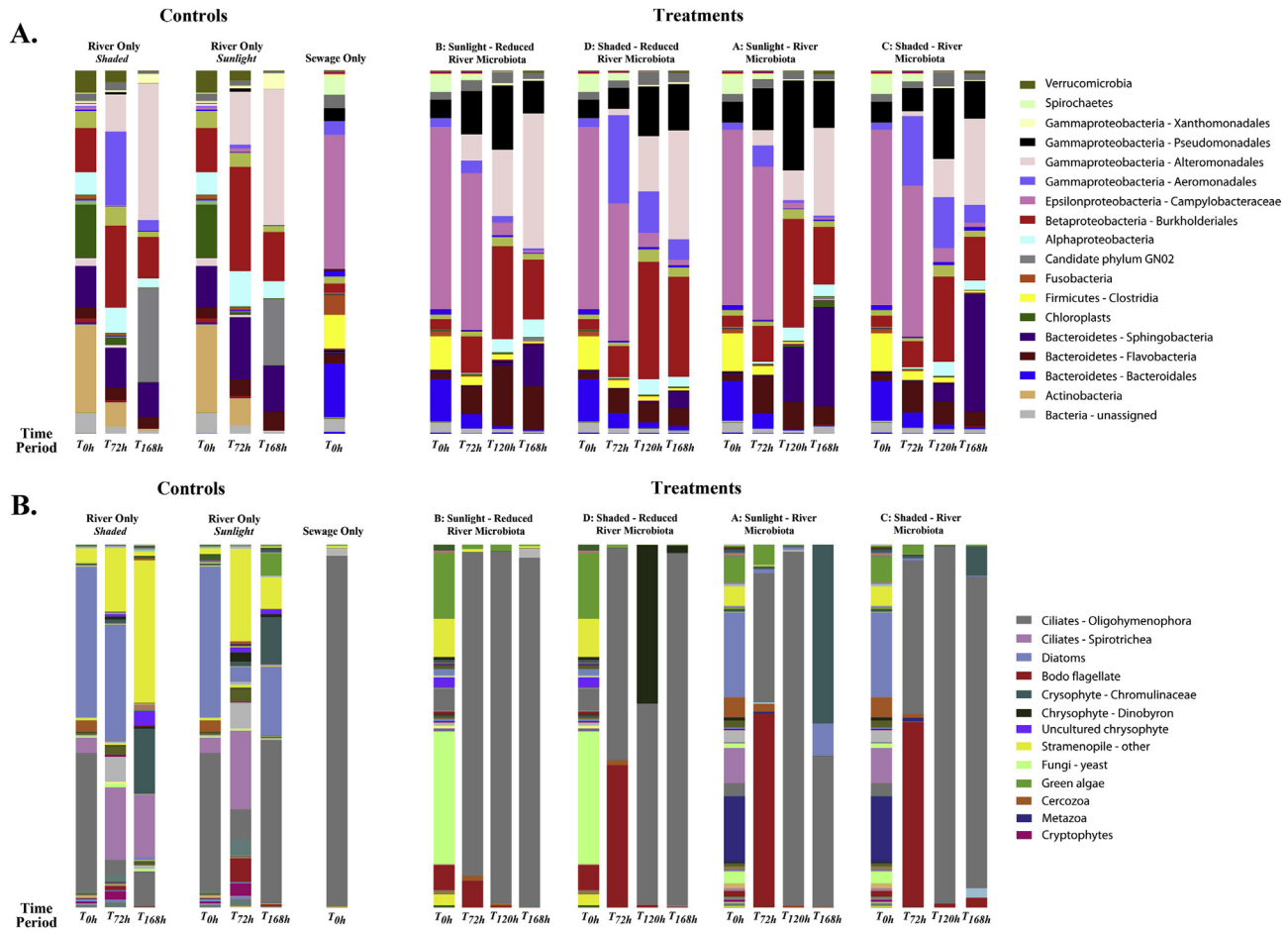

Fig. 1 – Relative abundance histograms for 16S rRNA (panel A) and 18S rRNA (panel B) sequences across different treatments and time points. Data are average of three replicate filters. Only taxa with at least 0.05% abundance in the total dataset are identified in the legend.

sunlight treatment), sunlight and shaded datasets were pooled for trajectory plotting. The impact of treatment on community composition was analyzed by a PERMANOVA on the unweighted UniFrac distance matrix using a model with time point as a random factor and treatment variables nested under time point as fixed factors in the PRIMER 6 package (Clarke and Gorley, 2006). Potential correlations between bacterial and eukaryotic communities were assessed by Mantel tests and Procrustes analysis of the UniFrac distances matrices and principal coordinate plots, respectively.

3. Results

3.1. *Community diversity of 16S and 18S rRNA sequences*

The relative abundance of taxonomic assignments for all samples and time points for 16S (panel A) and 18S (panel B) are shown in Fig. 1. Irrespective of the treatment, alpha diversity of 16S sequences was relatively stable over the course of the experiment with Shannon diversity index values ranging from 3.95 ± 0.6 to 6.34 ± 0.2 (Fig. 2). In contrast, alpha diversity of eukaryotic (18S) sequences was initially high (Shannon diversity index value of 5.56 ± 0.4), but then rapidly decreased by T_{72h} to values ranging from 1.38 ± 0.9 to 1.78 ± 0.3 (Fig. 2). There was a clear shift in the beta diversity of 16S bacterial communities over time and by treatment (Fig. 3, panel A). A similar trend was observed for 18S eukaryotic communities in the river only controls, but clustering of treatment samples was less obvious compared to 16S findings (Fig. 3, panel B).

3.2. *Changes in the 16S community structure over time*

At T_{0h} irrespective of treatment, the relative abundance of 16S taxa closely resembled primary sewage effluent (Fig. 1, panel A). Primary sewage effluent harbored diverse bacteria at high abundances that overwhelmed the riverine bacteria. To visualize the change in dominant sewage and river taxa (those with ≥5% relative abundance in any sample), abundance trajectories were plotted after pooling sunlight and shaded treatments (Fig. 4). Dominant taxa present in sewage at T_{0h} included Campylobacterales (36.8%), Bacteroidales (14.0%), Clostridiales (6.06%), Aeromondales (3.74%), Pseudomonadales (3.36%), Flavobacteriales (2.51%), other (3.52%) and

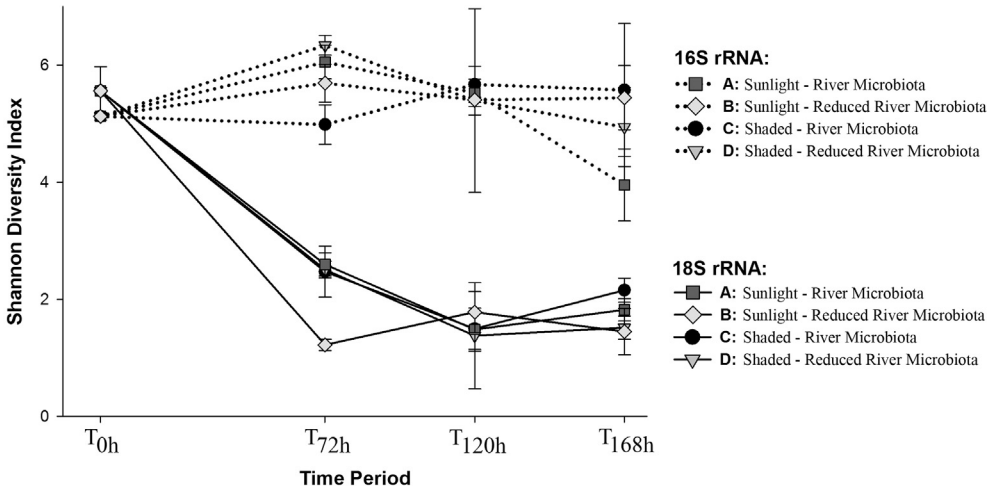

Fig. 2 – Plot of Shannon diversity index for 16S rRNA (dotted lines) and 18S rRNA (solid lined) microbial communities over course of study. Error bars indicate one standard deviation from the mean Shannon diversity index.

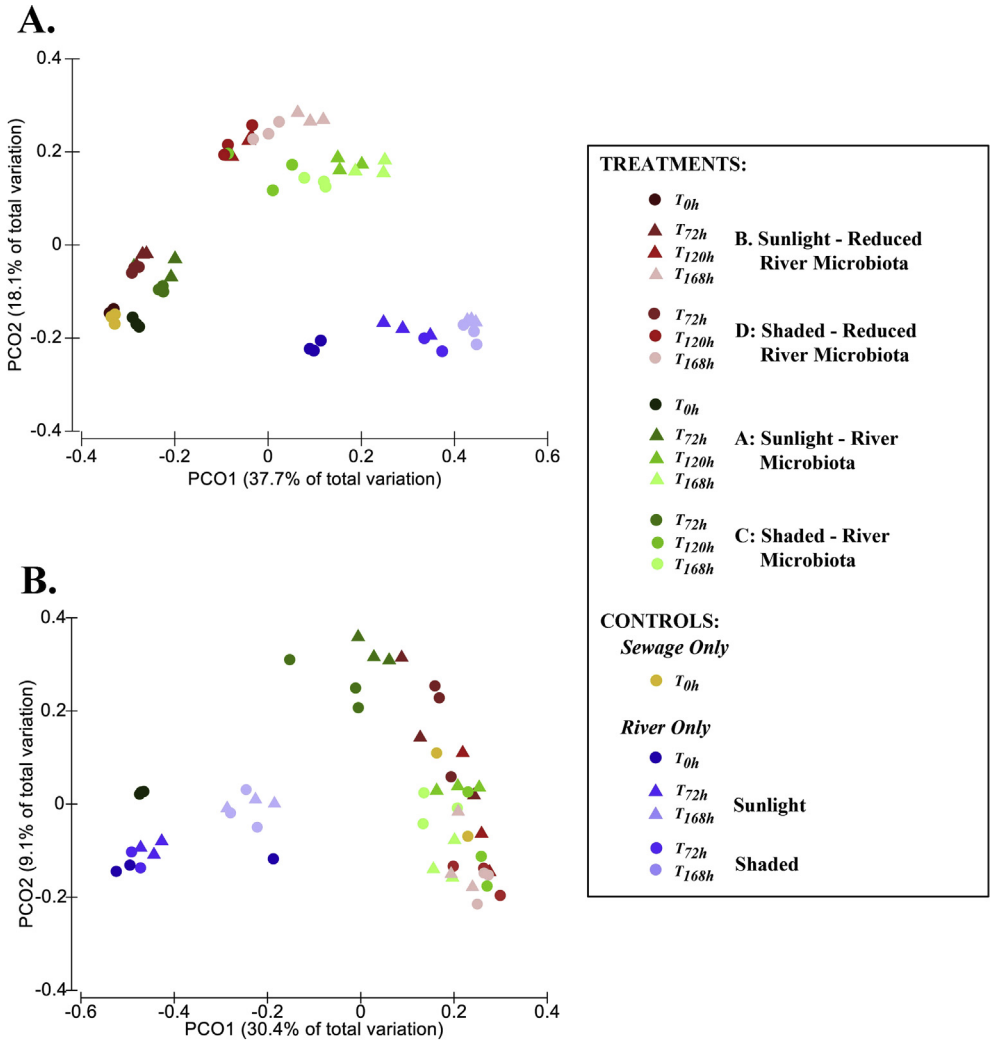

Fig. 3 – Principal component analysis (PCOA) plots for 16S rRNA (panel A) and 18S rRNA (panel B) microbial communities.

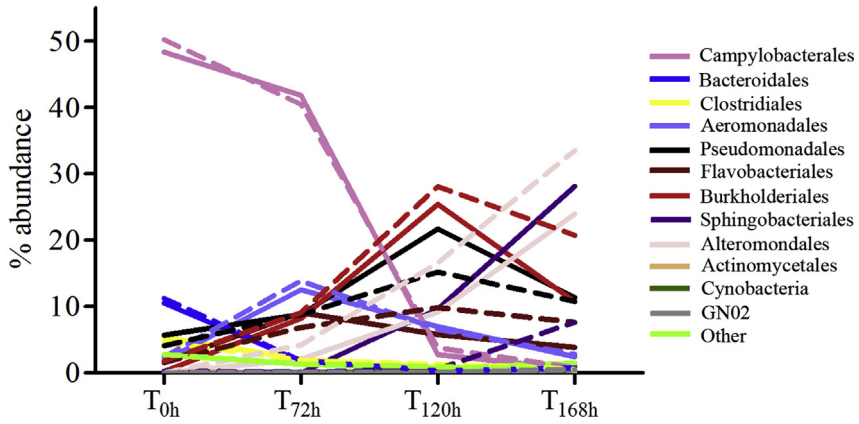

Fig. 4 – Trajectory plot showing the change in relative abundance of dominant taxa at each time point. Solid lines refer to samples from reduced river microbiota treatments (B and D). Dashed lines indicate samples from treatments containing the full complement of river microbiota (A and C).

Burkholderiales (2.59%) while Sphingobacteriales, Alteromonadales, Actinomycetales, Cyanobacteria and GN02 were minor contributors (each contributed < 1% of the total abundance). These taxa comprised 73.2% of the 16S sequences. Dominant taxa in the control river sample at T_{0h} totaled 66.1% and included Actinomycetales (19.3%), Cyanobacteria (15.6%), Burkholderiales (11.6%), Sphingobacteriales (9.3%), other (5.67%) and Flavobacteriales (2.27%) while Campylobacterales, Bacteroidales, Clostridiales, Aeromonadales, Pseudomonadales, Alteromonadales and GN02 each contributed less than 1%.

As the experiment progressed, there was an overall decrease in taxonomic groups associated with fecal communities (Campylobacterales, Bacteroidales and Clostridiales) and a concomitant increase in some taxa characteristic of river water (e.g. Burkholderiales and Sphingobacteria) (Fig. 4). The relative abundance of Campylobacterales and Bacteroidales in sewage (T_{0h}) was nearly 100-fold higher compared to the river water (T_{0h}) and regardless of the treatment both groups decreased sharply and made up < 1% of the total community at T_{168h} (Figs. 1 and 4). The Clostridiales group was present in sewage (T_{0h}) at a lower abundance (~6%), but it followed a similar trend decreasing approximately 10-fold over the course of the experiment (Figs. 1 and 4). Aeromonadales and Pseudomonadales were also approximately 10-fold higher in sewage (T_{0h}) compared to the river water (T_{0h}), but exhibited different trends through the experiment. Aeromonadales bloomed in both the river controls and experimental treatments at T_{72h}, especially in the shaded treatments, and then declined. In contrast, Pseudomonadales rose to make up a steady 10–20% of relative abundance in all treatments. Burkholderiales and Alteromonadales, two taxa initially higher in relative abundance in river water (T_{0h}) compared to sewage (T_{0h}) (Fig. 4) both exhibited similar trends indicating a steady bloom through T_{120h} followed by a more rapid die-off by T_{168h}, when the majority of river microbiota was absent (Fig. 4). Overall percent abundance of Sphingobacteriales was initially more than 10-fold higher in the river water (T_{0h}) compared to sewage (T_{0h}) and exhibited a steady increase in abundance for all treatments. Actinomycetales and Cyanobacteria were nearly exclusive to river water (>1000-fold higher relative abundance compared to sewage T_{0h}) with levels remaining relatively stable over the course of the study (Figs. 1 and 4).

Comparisons of qPCR fecal indicator abundance and 16S data indicate that the former are good predictors of overall 16S sequence community change (Mantel test; HF183 rho = 0.814, $p = 0.001$; GenBac3 rho = 0.804, $p = 0.001$).

3.3. *Changes in the 18S community structure over time*

Generally, the changes in 18S communities over time were less pronounced compared to 16S, with fewer eukaryotic taxa present overall. Control samples containing only river water were characterized mostly by diatoms, ciliates (e.g. Oligohymenophora and Spirotrichea) and algae (e.g. Chrysophytes, Stramenopiles) (Fig. 1, panel B). Primary sewage effluent on the other hand was less diverse and dominated almost exclusively by Oligohymenophora ciliates, predominately *Tetrahymena*, which are a common inhabitant of sewage (Curds and Vandyke, 1966; Martin-Cereceda et al., 2002; Moreno et al., 2010). We also detected low amounts of fungi, *Blastocystis* and other taxa characteristic of the mammalian gut in sewage and in the reduced microbiota treatment, though this treatment amplified very poorly (less than 4000 sequences per sample compared to an average of 100,000 sequences per sample in other treatments) and was therefore excluded from diversity analyses. Poor amplification was expected due to extensive filtration to reduce indigenous river microbiota. At T_{0h}, the treatments were composed primarily of diverse microbes resembling the riverine community and representative taxa included green algae, yeast, diatoms, ciliates and flagellates (Fig. 1, panel B). As the time progressed, there was a dramatic drop in eukaryotic diversity (Fig. 2). Just a few taxa, primarily Oligohymenophora (ciliates) and the flagellate *Bodo* (Fig. 1B), became dominant for the remainder of the experiment, irrespective of treatment.

3.4. Treatment effects

For both bacteria (16S) and eukaryotes (18S) all treatments were significant ($p \leq 0.022$), with time point explaining most of the variation. The eukaryotic and bacterial community changes were significantly correlated overall (All samples Mantel: R = 0.55 and P = 0.001; Procrustes P = 0.001; M2 = 0.397; Treatments only Mantel $R = 0.49963$ $P = 0.001$; Procrustes $P = 0.001$; $M2 = 0.427$). For bacterial sequences, control samples containing river water only clustered separately from samples containing sewage and there was an apparent temporal grouping of sequences where samples from T_{0h} were clustered separately from T_{72h} and T_{168h} (Fig. 3, panel A). The remainder of the samples all contained primary sewage effluent and T_{0h} samples clustered relatively closely with sequences from sewage (Fig. 3, panel A). Separation based on some of the treatments (i.e. level of indigenous river microbiota) become evident after T_{72h} and this trend continued in the subsequent samples collected at T_{120h} and T_{168h} (Fig. 3, panel A). The temporal trend in eukaryotic communities was similar, although not as pronounced (Fig. 3, panel B). Control samples containing river water only and T_{0h} samples clustered closely together and the earlier time point (T_{72h}) exhibited separation based on the amount of indigenous river microbiota present (Fig. 3, panel B). However, this trend was not as clear for subsequent time points.

4. Discussion

4.1. Trends in 16S communities

Distribution of 16S sequences in primary sewage effluent resembled those previously described for sewage infrastructure and for a core human fecal microbiome (McLellan et al., 2013; Shanks et al., 2013). The high abundance of Campylobacterales sequences was not surprising as they are frequently detected in sewage and sewage impacted waters (Hellein et al., 2011; Hokajarvi et al., 2013; Jokinen et al., 2011; Rechenburg and Kistemann, 2009). Furthermore, their close association with birds and waterfowl (Abulreesh et al., 2006; Antilles et al., 2013; Rutledge et al., 2013; Van Dyke et al., 2010) suggests that at least some of these sequences may originate from non-human sources. Bacterial communities associated with the Mississippi river water were largely represented by Alpha, Beta and Gamma proteobacteria, Actinobacteria, Bacteroidetes (Sphingobacteria and Flavobacteria) and Verrucomicrobia, a finding that is consistent with an earlier study of the Mississippi river (Staley et al., 2013) and other freshwater systems (Garcia-Armisen et al., 2014; Ghai et al., 2011; Newton and McMahon, 2011; Oh et al., 2011).

Decomposition of primary sewage effluent in the Mississippi river water was characterized by a rapid decrease in Campylobacterales and other sewage related sequences including Bacteroidales and Clostridiales. As time elapsed, there was a noticeable increase in the riverine community sequences, especially those belonging to Sphingobacteriales and Alteromonadales. The latter are a part of gamma proteobacteria, a phylum known to comprise transient members of freshwater bacterial communities (Newton et al., 2006) that appear to be most prevalent during fall months (Newton and McMahon, 2011). The bloom of Sphingobacteriales (Bacteroidetes) after exposure to nutrient rich sewage is consistent with an earlier report that showed an increase in abundance of this group following the addition of nutrients to lake water mesocosms (Newton and McMahon, 2011; Pernthaler et al., 2004). Over time, bacterial community composition in our treatments became more similar to those of Mississippi river than the primary sewage effluent indicating that the riverine ecosystem may be able to recover following a single severe pollution event. An earlier study indicated that natural river communities are able to recover after major perturbations such as wastewater discharges (Garcia-Armisen et al., 2014), however that study was investigating spatial rather than temporal patterns.

4.2. Trends in 18S communities

There is considerably less known about eukaryotic communities associated with sewage, but studies conducted to date parallel our findings in that bacterivorous freshwater ciliates are often detected (Curds and Vandyke, 1966; Drzewicki and Kulikowska, 2011; Dubber and Gray, 2011; Hu et al., 2012; Moreno et al., 2010). A few eukaryotes typically associated with the human gut or feces, including *Blastocystis* and yeasts *Candida* and *Saccharomyces*, were detected at very low levels (less than 0.01%) in the original sewage samples, but other taxa typical of the gut such as *Entamoeba* species (Hamad et al., 2012; Parfrey et al., 2014; Scanlan and Marchesi, 2008) were absent. This may be explained by the lower 18S community diversity in the human gut compared to bacterial diversity (Nam et al., 2008; Scanlan and Marchesi, 2008), low prevalence of gut protists and worms in United States populations (Parfrey et al., 2014), and/or lower rates of environmental proliferation of gut eukaryotes. Eukaryotic communities from both river water and sewage were dominated by ciliates from the class Oligohymenophorea consisting of free-living, bacterivorous species typically distributed in freshwater systems (Pernthaler, 2005) and previously detected in sewage (Curds and Vandyke, 1966; Drzewicki and Kulikowska, 2011; Dubber and Gray, 2011; Hu et al., 2012; Martin-Cereceda et al., 2002; Moreno et al., 2010). The high abundance of these protists in sewage supports the notion that untreated sewage is a mixture of microorganisms originating not only from the human gastrointestinal tract, but also from freshwater environmental sources such as rainwater infiltration that likely bloom in the high nutrient sewage (McLellan et al., 2013; Shanks et al., 2013).

The mixing of primary sewage effluent with river water caused dramatic changes in eukaryotic communities. Initially (T_{72h}), there was an increase in flagellate abundance, primarily in bacterivorous *Bodo* spp., which were not present in primary sewage effluent at detectable levels, but did comprise a minor fraction of the riverine community. In addition to *Bodo* spp., members of Oligohymenophorea (ciliates) also increased initially and at T_{120h} were the dominant members irrespective of the treatment. These results suggest that only a few generalist bacterivorous taxa played a key role in decomposition of sewage over the course of the study. This trend

corresponds well with the estimated time required for freshwater protozoan communities to adjust to the influx of prey organisms (Pirlot et al., 2007; Servais et al., 2007; Zingel et al., 2007). The role of bacterivorous protists in many decay studies is often overlooked in favor of other environmental variables such as sunlight exposure, salinity, and temperature (Bae and Wuertz, 2009; Green et al., 2011; Okabe and Shimazu, 2007). Our findings suggest that ciliates and flagellates from freshwater environments and from the pollution source itself play an important role in the removal of sewage-associated taxa once discharged into an ambient freshwater source. Additional research is warranted to continue the development of methods to more accurately characterize 18S communities in ambient water and describe their role in the maintenance of ecological health.

4.3. *Implications for water quality research*

Characterization of the change in microbial community structure over the course of a sewage pollution event provides several new insights for water quality research. For example, the apparent ability of an indigenous river microbial community to return to a near pre-disturbance state following a pollution event suggests that natural water communities can readily recover after a single, high concentration pollution exposure. However, it remains unclear how an indigenous community would respond to a continuous pollution source and at different initial seeding concentrations. It is likely that different seeding concentrations of sewage will alter the predation and competition influences described in this study and it has been suggested that resilience may be a function of seasonality (Garcia-Armisen et al., 2014). These additional considerations offer new challenges for water quality monitoring applications that rely on estimating the age or degree of decomposition of a pollution source. It is evident that future studies must continue to recognize and further describe the role that indigenous and pollution source microbiota play in the decay of fecal pollution in natural waters.

5. Conclusions

- Biotic interactions are a critical factor in the decomposition of sewage fecal bacteria when mixed with ambient freshwater.
- Human-associated (HF183) and general Bacteroidales (GenBac3) qPCR measurements are good predictors of sewage 16S bacterial community decomposition.
- Changes in community diversity are associated with several bacterial and eukaryotic groups suggesting that simultaneous characterization of 16S and 18S communities is necessary to describe mechanisms of fecal pollution decomposition in natural waters.

6. Disclaimer

The United States Environmental Protection Agency through its Office of Research and Development funded and managed the research described here. It has been subjected to Agency's *administrative review and approved for publication*. The views expressed in this article are those of the author(s) and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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