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Microbial Community Assembly and Succession on Lake Sturgeon Egg Surfaces as a Function of Simulated Spawning Stream Flow Rate

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Abstract We investigated microbial succession on lake sturgeon (*Acipenser fulvescens*) egg surfaces over the course of their incubation period as a function of simulated stream flow rate. The primary objective was to characterize the microbial community assembly during succession and to examine how simulated stream flow rate affect the successional process. Sturgeon eggs were reared under three flow regimes; high (0.55 m/s), low (0.18 m/s), and variable (0.35 and 0.11 m/s alternating 12 h intervals). Eggs were collected from each flow regime at different egg developmental stages. Microbial community DNA was extracted from egg surface and the communities were examined using 16S rRNA gene-based terminal restriction fragment length polymorphism and 454 pyrosequencing. Analysis of these datasets using principal component analysis revealed that microbial communities were clustered by egg developmental stages (early, middle, and late) regardless of flow regimes. 454 pyrosequencing data suggested that 90–98 % of the microbial communities were composed of the phyla Proteobacteria and Bacteroidetes throughout succession. β-Protebacteria was more dominant in the early stage, Bacteroidetes became more dominant in the middle stage, and α-Proteobacteria became dominant in the late stage. A total of 360 genera and 5,826 OTUs at 97 % similarity cutoff were associated with the eggs. Midway through egg development, the egg-associated communities of the low flow regime had a higher diversity than those communities developed under high or variable flow regimes. Results show that microbial community turnover occurred during embryogenesis, and stream flow rate influenced the microbial succession processes on the sturgeon egg surfaces.

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Introduction

The process of succession involves the colonization of open space and subsequent sequential changes in community composition. This process has been studied primarily using plant systems [1]. Microbial succession has been recently studied in conjunction with various host animals [2–4], host plants [5], and natural [6, 7] and artificial [8–10] environments. Microbes serve as ideal targets for studying succession because the microbial succession can be effectively controlled and observed in an experimental setting, thus allowing for novel information to be learned about factors affecting the succession process [11].

Results from a number of studies suggest that microbial succession is a complex process [4–6, 11]. For example,

Redford and Fierer [5] studied microbial community assembly on the newly synthesized cottonwood leaf surface. The authors found a strong temporal effect whereby microbial community compositions on leaves from different trees sampled on the same day were more similar to each other than those from the same tree sampled on different dates. In contrast, Palmer and colleagues studied microbial succession in the human infant gut and found that inter-individual variations dominated over temporal variation [4]. Previous studies have also shown that microbial species richness varies with time during succession. Redford and Fierer [5] found a positive linear relationship between microbial species richness on the cottonwood leaf surfaces and time. In contrast, Jackson and colleagues found that species richness of microbial communities on glass slides in aquatic environments fluctuated over time, with high diversity in the beginning due to stochastic colonization, subsequent decline in diversity due to species sorting, followed by an increase due to maturation [6]. These differences across different studies could be attributed to differences in the rate of dispersal, host characteristics, environmental conditions, disturbances, and temporal scales of their studies [11].

Although numerous studies have demonstrated that environmental conditions are keys in explaining microbial community assembly [12–15], few studies have examined the effect of environmental conditions on microbial succession [7, 16]. Besemer and colleagues studied the effect of stream flow velocity on microbial succession on ceramic coupon surfaces in streams [16]. They found that midway through the successional process, community assemblages developed under turbulent flow were different from those developed under other flows. Lyautey and colleagues studied the effect of environmental factors such as light and water temperature on microbial succession on pebble surfaces in natural streams and found that both affected the successional process [7]. This small collection of studies suggests that microbial succession is dependent on environmental factors, and further research may be needed to better understand such effects.

In addition, despite the solid foundation of research on microbial succession on various hosts, no studies have yet investigated microbial succession on fish eggs. Fish eggs serve as a good model for studying microbial succession, since egg surfaces provide an open niche for aquatic microbes. Aquatic microbes start colonizing the egg surfaces as soon as eggs are deposited in a stream, and the microbial community subsequently develops on the egg surface during embryogenesis. Microbial community turnovers are expected to occur as microbes on the egg surfaces compete for egg nutrients [17], metabolites excreted by eggs change during embryogenesis [18, 19], and microbes on the egg surfaces are selected against by host innate immunity including lysozyme secretion [20, 21]. Studies about microbial succession on egg surfaces in streams will provide insights about the effect of live hosts on microbial succession by providing a comparison to previous microbial succession studies performed using natural [7] and artificial substrates [6, 16] in streams. Moreover, despite a multitude of studies conducted on various terrestrial hosts such as plant surfaces and the human gut, few studies have been conducted on microbial succession in aquatic environments, places where microbes are found in significantly higher densities and thus may adopt novel successional processes not observed elsewhere in nature.

We present here a study on microbial community succession on the lake sturgeon (*Acipenser fulvescens*) egg surface. Lake sturgeon populations have decreased drastically over the past 100 years due to anthropogenic activities such as overfishing and dam construction [22]. One such population is the Black Lake population, Michigan [23, 24]. Spawning habitats have been altered since the construction of Kleber dam in 1949 on the Upper Black River, which is the sole spawning stream for the Black Lake population. Despite recent restoration efforts, natural recruitment is limited, which is likely attributed to high egg mortality [25]. Our primary objective was to understand how microbial community assembly changes throughout the fish embryonic development and to acquire fundamental knowledge about microbial community assembly on the egg surface during succession. Characterizing microbial community assembly on the egg surface during incubation will help illuminate the potential causal relationship between microbes and egg mortality. We were also interested in investigating how changes in stream flow rate affected microbial community assembly during microbial succession. Flow rate is an important environmental factor to consider in this system because construction of dams has altered steam flow rate [26–29] and in turn potentially affected downstream ecosystems in many ways, including altering the interactions between microbes and fish eggs.

Methods

Experimental Design

This experiment was conducted at a lake sturgeon streamside rearing facility (details in [30]) located on the Upper Black River system in Michigan during May 2007 in the midst of the lake sturgeon spawning season. Incoming river water was filtered using a sand filtration system to remove large particulate matter before being gravity-fed in a flow-through design to experimental flumes. We tested the effects of three different flow regimes on microbial community succession on the egg surface over time. A total of six flume channels were used with two replicates for each flow regime. The first flow regime consisted of a constant high flow velocity (0.55±0.01 m/s) representing a fast current section of a natural stream which embryos experience in the natural river

setting [26]. The second flow regime was a low flow velocity (0.18±0.01 m/s) that represented both slower areas in the river and minimum flow that eggs naturally experience during dry spring seasons. A third flow regime was a variable flow. This variable flow regime was set to be high for 12 h and low for 12 h (0.35±0.01 and 0.11±0.01 m/s, respectively), which is typical of many hydro electric dams that operate during periods of peak electrical demand [26]. Gametes used in this study were collected from adult lake sturgeon captured in the act of spawning in the Upper Black River, Michigan. Eggs were obtained from one female and were fertilized with milt from two males that were selected randomly from a pool of candidates. Immediately upon fertilization, approximately 100 eggs were placed on plexi-glass plates (7.6×5 cm). Eggs were allowed to adhere to plates in standing stream water. Thus, the conditions of initial colonization were standardized across the eggs to be exposed to the three different flow regimes. Plates were then placed within experimental flume channels constructed from a 3" PVC pipe cut lengthwise. The plates were set at a constant depth (2 cm from the bottom of the pipe) across all six flume channels. A single water source was used for all six experimental flumes so infusion of stream microbes was constant across the flow treatments. Six to ten eggs were randomly collected from each flume channel at five embryonic developmental stages (day 2, day 3, day 5, day 6, and day 7) and were immediately preserved in 70 % ethanol. Over 90 % of all eggs that hatched did so between day 9 and day 11. The total number of samples collected in this study was 30 (6 flume channels × 5 developmental stages). Water temperature (degrees Celsius) was also measured at hourly intervals throughout the experiment. Dissolved oxygen concentration of the source water was above 9 mg/L throughout the experiment.

DNA Extraction and Terminal Restriction Fragment Length Polymorphism

The 30 microbial community samples were subjected to DNA extraction. Microbial community genomic DNA was extracted from the surfaces of 4 or 5 eggs per sample using the PowerSoil™ Kit (MO BIO Laboratories Inc., CA) following bead beating according to the manufacturer's protocol. We elected to pool four to five eggs from each time point/flume to provide an averaged representative view of the community and ensure sufficient DNA for analyses. 16S rRNA gene-based terminal restriction fragment length polymorphism (TRFLP) [31, 32] was performed to compare microbial community assembly across the 30 samples. TRFLP technique has been used to compare microbial community composition across environmental gradients [12, 33, 34], locations [12, 35, 36], and times [8, 10, 37]. The detailed procedures are as follows. 16S rDNA amplification was performed using the universal bacterial primers 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') (5' FAM-labelled) and 1389R (5'-ACG GGC GGT GTG TAC AAG-3') (unlabelled) [38, 39]. PCR reaction was conducted in a 100-μL reaction volume, containing 20 to 40 μL template DNA (1 to 4 ng μL^{-1}), 5.0 U of *Taq* DNA polymerase (Invitrogen Corp., Carlsbad, CA), in a final concentration of 0.2 μM of each primer, 0.25 mM of each deoxynucleoside triphosphate, 10 mM Tris–HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was performed under the following cycle conditions: an initial denaturation step at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 30s, and extension at 72 °C for 110 s. A final extension step at 72 °C for 7 min was then performed. The PCR product was purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The purified PCR products were subjected to enzyme digestion with either *Hha*I or *Msp*I (Gibco BRL). The reaction mixture contained 2.0 μL of 10× reaction buffer (Gibco BRL), 0.3 μL of enzyme (20U/μL, Gibco BRL), about 200 ng of purified PCR product, and pure water to a final volume of 20 μL. The enzyme digestion was carried out for 2 h at 37 °C. Two technical replicates (10 μL each) of the digested DNA samples were sent to Michigan State University's sequencing facility, and the DNA fragments were separated on an ABI 3100 Genetic Analyzer automated sequencer (Applied Biosystems Instruments, Foster City, CA) in GeneScan mode. The 5' terminal restriction fragments (TRFs) were detected by excitation of the 6-FAM molecule attached to the forward primer. The sizes and abundance (peak height) of the terminal fragments were calculated using GeneScan 3.7. The resultant peak heights were filtered to eliminate peaks with a height below the background noise threshold (set at 50 fluorescence units). Each terminal fragment corresponds to a phylotype, and peak height indicates relative abundance of a phylotype. In order to align TRF peaks across the 30 samples, the TRFLP profiles were processed with T-Align (http://inismor.ucd.ie/~talign/index.html), and the output of T-Align was used for the microbial community analysis. A total of 123 phylotypes and 130 phylotypes were detected from the 30 samples using endonuclease *Hha*I and *Msp*I, respectively. On average, 26.6 and 25.6 phylotypes were detected per sample for *Hha*I and *Msp*I, respectively.

Microbial Community Analysis using TRFLP Data

We employed the Bray–Curtis dissimilarity index [40] to compare microbial community composition among samples from different flow treatments and egg developmental stages. We employed general linear models with the dissimilarity index as the dependent variable to assess the effect of time and flow treatments on the index. Principle component analysis (PCA) was performed using TRFLP data from the 30 samples to elucidate underlying patterns across samples. The data consisted of 25 columns which represented 25 major

phylotypes that had 3 % or higher relative abundance for at least one of the 30 samples, and 30 rows which represented relative abundance of each major phylotype in the 30 samples. The scores of principle component 1 (PC1) and 2 (PC2) were used to elucidate the temporal similarity of the microbial community composition, and the loadings of PC1 and PC2 were used to elucidate the distribution patterns of the 25 major phylotypes. Phylotype richness *S* (*S*=the number of distinct terminal restriction fragments in each sample) was determined from the TRFLP profiles. All distinct TRFs, including both major and minor phylotypes, were included in this analysis. Prior to analyzing the relationship between phylotype richness and time, extremely high or low total fluorescence signals caused by over or under loading of digested DNA samples were removed to eliminate non-biological effects on phylotype richness. A general linear model was used to investigate the relationship between the microbial phylotype richness and time. A quadratic term for time was also included in the model. We conducted all analyses using both *Hha*I and *Msp*I, but both showed similar results (data not shown); therefore, we present only the *Hha*I results. The general linear model and PCA were conducted using R version 2.10.0 [41]. The Bray–Curtis dissimilarity matrix was generated in R using the "ecodist" package.

454 Pyrosequencing

To characterize the microbial community at different time points and under different flow regimes, nine samples representing three flow regimes (high, low, and variable) at three time points (day 2, day 5, and day 7) were assigned for pyrosequencing. Since the TRFLP data suggested that variation among two replicated flows within the same flow regime at the same time point was small, the extracted genomic DNAs of the two replicated flows within the same flow regime were pooled. To evaluate the reproducibility of samples, technical replicates were included for the three day 5 samples (high, low, and variable at day 5). Hypervariable region V3–V5 in 16S rRNA gene was amplified using a forward primer 357F (5'-CCTACGGGAGGCAGCAG-3') and a reverse primer 926R (5'-CCGTCAATTCMTTTRAGT-3') as previously described [42–44]. 454 "A" adapter and tag sequences were contained in the reverse primer, and "B" adapter was contained in the forward primer. PCR amplification was performed in 75 μL reaction, using 3 U of AccuPrime Taq HiFi (Invitrogen, Grand Island, NY), 7.5 μL of supplied 10× buffer II, 1.5 μL of 10 μM primers, and approximately 30 ng of template DNA measured by Nanodrop ND1000 (Thermo Scientific Inc). The PCR cycle condition was as follows: denaturation at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 30 s, extension at 72 °C for 5 min [44]. The PCR amplicons were cleaned using Agencourt AmPure XP Beads (Beckman Coulter, Inc., Brea, CA), and pyrosequencing of the amplicons was performed using 454 GS FLX titanium platform (454 Life Science, Branford, CT) at the research technology support facility at Michigan State University.

Pyrosequencing Data Processing and Analysis

Raw sequence reads were processed using Ribosomal Database Project (RDP) pipeline [45] to sort the data by tag sequence, to trim tag and primer sequences, and to filter out low quality sequences with minimum quality score of 20 (probability threshold of 0.01) and minimum read length of 300 bp. The taxonomy of the filtered reads was assigned using RDP Classifier at a bootstrap threshold of 80 % [46]. Bray–Curtis dissimilarity among the nine samples was determined using the classifier output at the genus level. OTUs of the filtered reads at 97 % similarity cutoff were determined using RDP complete-linkage clustering algorithms with 0.03 maximum cluster distances. The underlying patterns of the nine samples were analyzed by PCA using OTUs at the 97 % similarity cutoff. To construct the principal component plot, the linkage cluster output was transformed to relative abundance to minimize the variation in the total number of reads across samples. OTUs that had a relative abundance of 0.03 % or greater in at least one of the samples were included for PCA analysis. This cutoff removed all singletons from the analysis. The relative abundance matrix was then square root transformed to minimize skewness in the dataset prior to PCA analysis (i.e., Hellinger transformation [47]). Jaccard index tree of OTUs at the 97 % similarity level was constructed using RDP pipeline. The diversity index including Shannon species evenness (*E*) for each sample was determined by RDP pipeline diversity index estimator using the 97 % OTUs. Rarefaction curves of the 97 % OTUs for the samples were generated using RDP pipeline. To assess the relationship between the number of OTUs and time, the 97 % OTUs were rarefied at 6,000 reads per sample.

Results

Analysis of Microbial Community Succession Using TRFLP

Using TRFLP analysis, we detected considerable temporal variation in microbial community composition on lake sturgeon eggs across sequential embryonic developmental stages (Fig. 1). The temporal variation in microbial community composition was consistently observed in all six flume channels (two replicates for each of the three flow regimes). Microbial community compositions estimated from samples collected from the same developmental stage were more similar to each other than those from different developmental

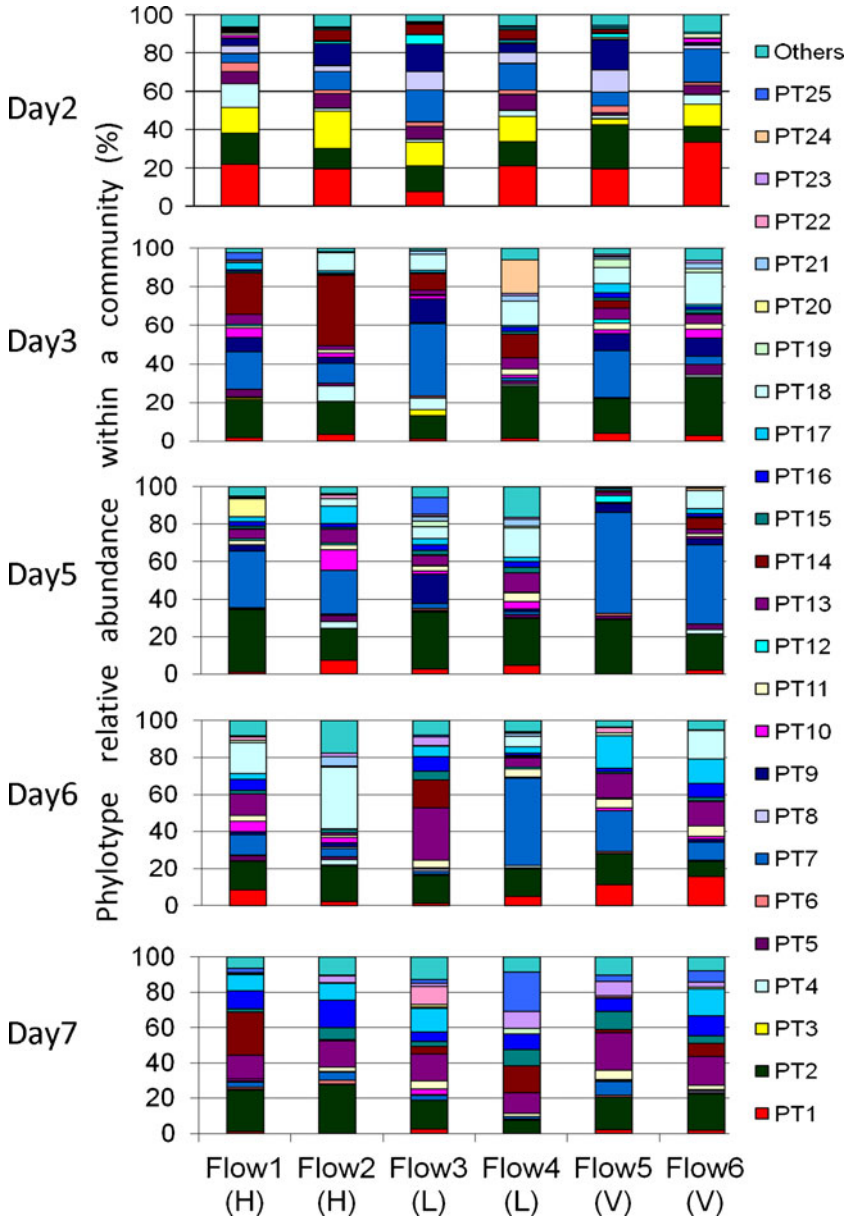

Fig. 1 Temporal and flow rate effects on microbial community composition during microbial succession using *Hha*I. 25 major phylotypes that had 3 % or higher relative abundance for at least one community sample were included. The rest were grouped into the category of "others". Each color/pattern represents a unique terminal fragment (phylotype, PT). The experiment consists of three flow regimes (high: flows 1 and 2, low: flows 3 and 4, and variable: flows 5 and 6) sampled at days 2, 3, 5, 6, and 7 during incubation in stream water

stages, regardless of flow regimes (Table 1). For example, the mean Bray–Curtis dissimilarities within day 2 samples and between day 2 and day 3 samples were 0.301 and 0.594, respectively (Table 1). These two means were statistically significantly different ($F_{1,49}=135$, $p<0.001$). The dissimilarity between the initial microbial community composition and subsequent microbial community compositions increased during the course of embryogenesis as the community departed from the initial community (Table 1, left column).

Stream flow rate had a more subtle effect on the composition of the microbial communities than egg developmental stage (Fig. 1). Microbial communities were similar at the early (day 2) and late (day 7) developmental stages, regardless of flow regimes. However, the microbial composition of the low flow regime departed from that in the high and variable flow regimes during mid-developmental stages. This is evident in the diagonal array of the dissimilarity index in Table 1 that shows an increasing variance of dissimilarity (which we attribute to differences in flow). At day 5, the microbial community composition differed among the three flow regimes (Fig. 1: day 5). The mean dissimilarity within the same flow regime (high–high, low–low, and variable–variable) was 0.339, whereas the mean dissimilarity between high and low flow regimes was 0.478 (Online Resource 1). Microbial communities reared in the variable flow regime were more similar to that in the high flow regime (dissimilarity=0.380) than to the low flow regime (dissimilarity=0.554), suggesting that the high flow portion of the variable flow regime had a more dominant effect on microbial community structure than the low flow.

Table 1 Bray–Curtis dissimilarity index matrix using TRFLP data summarized by day

	Day 2 Mean[a] (sd)	Day 3 Mean (sd)	Day 5 Mean (sd)	Day 6 Mean (sd)	Day 7 Mean (sd)
Day 2	0.301 (0.083)				
Day 3	0.594 (0.082)	0.426 (0.082)			
Day 5	0.624 (0.073)	0.443 (0.114)	0.444 (0.104)		
Day 6	0.639 (0.068)	0.510 (0.103)	0.497 (0.141)	0.490 (0.134)	
Day 7	0.747 (0.064)	0.592 (0.092)	0.585 (0.110)	0.518 (0.140)	0.369 (0.095)

[a] Mean Bray–Curtis dissimilarity across all flow regimes

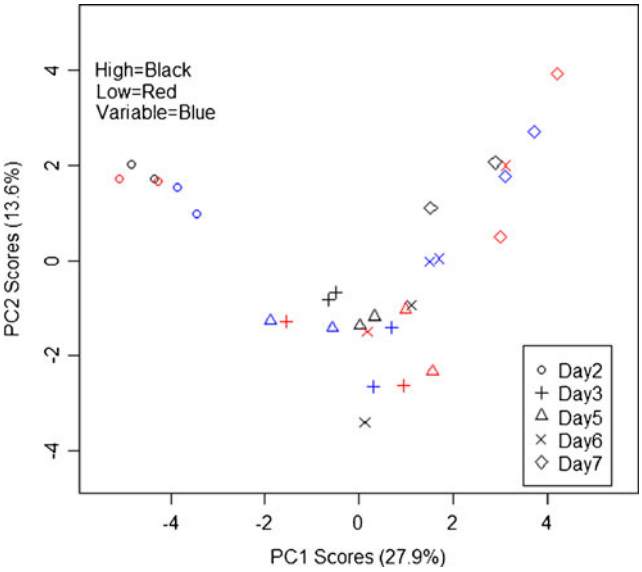

Fig. 2 Principle component score plots revealing the temporal clustering of microbial community assemblages on egg surfaces. PC1 and PC2 account for 27.9 % and 13.6 % of the data variations, respectively. *Hha*I-digested TRFLP data were used for the analysis

Principle component plots also showed the strong temporal clustering of microbial communities on the egg surfaces (Fig. 2). Microbial communities sampled from the same or close developmental stages were clustered together regardless of flow regime. Microbial communities were clustered into three different embryonic developmental stages; the early (day 2), the middle (day 3, day 5, and day 6), and the late (day 7), respectively. This PCA plot shows that the temporal effect was more dominant in explaining microbial community assembly than flow rate effects. We also found that certain microbial phylotypes were more strongly associated with certain egg developmental stages (Online Resource 2). Out of the 25 dominant microbial phylotypes we detected using *Hha*I, we found that eight phylotypes were predominantly associated with the early stage, seven phylotypes peaked in the middle, and nine phylotypes were preferentially associated with the late stages of egg development. This trend was also depicted in a loading principle component plot (Online Resource 3).

There was a significant positive linear relationship between microbial phylotype richness on egg surfaces and time (Fig. 3 solid line: $F_{1,25}=4.73$, $p=0.039$). In contrast, there was no significant main effect of flow regime on microbial phylotype richness according to a linear regression model ($F_{2,24}=0.658$, $p=0.53$). However, the relationship between microbial phylotype richness and time was dependent on flow regime. At the low flow regime, the relationship between microbial phylotype richness and time was negative quadratic (Fig. 3 dotted line: $F_{2,5}=10.26$, $p=0.017$) with the highest richness at the middle developmental stage. In contrast, under the high and variable flow regimes, there was a moderate positive quadratic relationship between microbial phylotype richness and time (Fig. 3 breaking line (high): $F_{2,7}=1.543$, $p=0.28$; dotted-dash line (variable): $F_{2,6}=13.1$, $p=0.006$) with lower phylotype richness at the middle developmental stage. In other words, at day 5, eggs reared in the low flow regime were associated with a relatively large number of microbial phylotypes, while eggs reared in high or variable flow regimes were associated with a fewer number of phylotypes.

Microbial Community Analysis Using 454 Pyrosequencing

To characterize the microbial community assembly at different egg developmental stages and under different flow

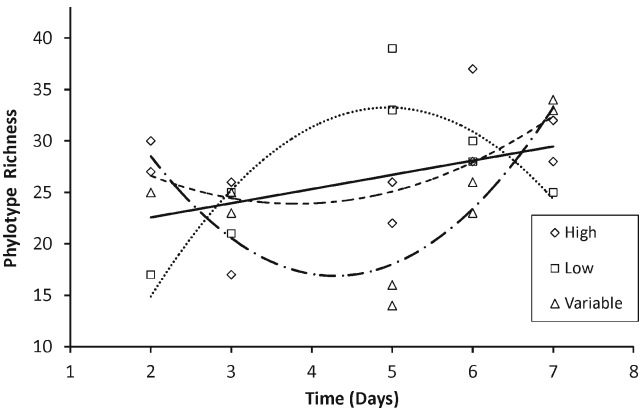

Fig. 3 Microbial phylotype richness and time relationships during succession across flow regimes (high (*empty diamond*), low (*empty square*), and variable (*empty triangle*)). The *straight solid line* is a linear regression line for all data points ($F_{1,25}=4.73$, $p=0.039$, $R^2=0.16$). The *dotted line* is a positive quadratic regression for the low flow regime ($F_{2,5}=10.26$, $p=0.017$, $R^2=0.80$). The *breaking line* is a negative quadratic regression for the high flow regime ($F_{2,7}=1.543$, $p=0.28$, $R^2=0.31$). The *dotted-dash line* is a negative quadratic regression for variable flow regime ($F_{2,6}=13.1$, $p=0.006$, $R^2=0.81$). The phylotype richness determined by *Hha*I-digested TRFLP was used for this analysis

regimes, nine samples representing three flow regimes (high, low, and variable) at three time points (day 2, day 5, and day 7) were subjected to pyrosequencing. We obtained an average number of 13,468±7,779 reads with an average length of 450.08±1.97 bp per sample. These reads were classified using RDP classifier into 23 phyla, 45 classes, and 360 genera across the nine samples. The average numbers of phyla, classes, and genera per sample were 14.5±2.6, 25.3±4.5, and 138.7±20.2, respectively. Two technical replicates for day 5 samples showed an almost identical community composition at each taxonomic level, indicating that the pyrosequencing data were reproducible.

A temporal compositional trend during microbial succession was detected at the phylum/class level (Fig. 4). Two phyla, Proteobacteria and Bacteroidetes, comprised 90 % to 98 % of the egg surface microbial community throughout egg development. Proteobacteria was more dominant in the early (58 % to 74 %) and late (71 % to 82 %) egg developmental stages, while Bacteroidetes was dominant in the middle stage (63 % for high and 81 % for variable flow regime) except under the low flow regime (36 %). The dominant classes of the phylum Proteobacteria included β, α, and γ-Proteobacteria. β-Proteobacteria were more dominant in the early egg developmental stage and α-Proteobacteria became more dominant by the late stage.

The temporal trend was also found at the genus level of analysis (Fig. 5). *Flavobacterium* was one of the most dominant genera in the egg surface microbial community during succession and showed a strong temporal trend (Fig. 5). Genus *Flavobacterium* accounted for a large proportion of the egg surface microbial community in the early (20 % to 40 %), middle (25 to 80 %), and late (9 to 13 %) egg developmental stages. The large variation in *Flavobacterium* relative abundance during the middle stage can be attributed to flow treatment. At day 5, *Flavobacterium* accounted for 55 % to 80 % of the egg surface microbial community under high and variable flow regimes but only 25 % under the low flow regime. Other genera also exhibited a temporal trend in abundance during succession. Genera such as *Brevundimonas*, *Undibacterium*, *Massilia*, *Acidovorax*, and *Rheinheimera* decreased as egg development progressed, *Flectobacillus* and *Fluviicola* peaked at intermediate periods, and *Rhodobacter*, *Catellibacterium*, and *Devosia* (all α-Proteobacteria) were dominant towards the end of development. Although almost all sequences were classified at phylum/class level, 12 % to 47 % of the egg surface microbial communities per sample were unclassified at genus level, and the proportion of the unclassified genera increased as egg development progressed. We characterized a total of 360 genera in this analysis, but only two, *Flavobacterium* and *Albidiferax*, maintained a relative abundance over 2 % throughout the entire incubation period. More detailed genus information is provided in Online Resource 4.

Bray–Curtis dissimilarity analysis conducted at the genus level showed that microbial communities taken from the same time points were more similar to each other than to those from other time points (Table 2). The dissimilarity between the initial and subsequent communities increased during the course of embryogenesis as the later communities departed from the initial community (Table 2, left column). At day 5, the microbial community under low flow regime differed from those under high and variable flow, similar to the results seen with TRFLP.

RDP complete cluster linkage algorithms revealed the existence of 5,826 distinct OTUs across the samples at 97 % similarity cutoff with the average OTUs 1,216.8±201.0 per sample. Rarefaction curves of OTUs at the 97 % similarity for the nine samples including technical replicates are shown in Online Resource 5. The rarefaction curves show that our level of sampling did not cover the complete diversity of the communities. To assess the underlying patterns across different time points and flow regimes, the data matrix of the 97 % OTUs was subjected to

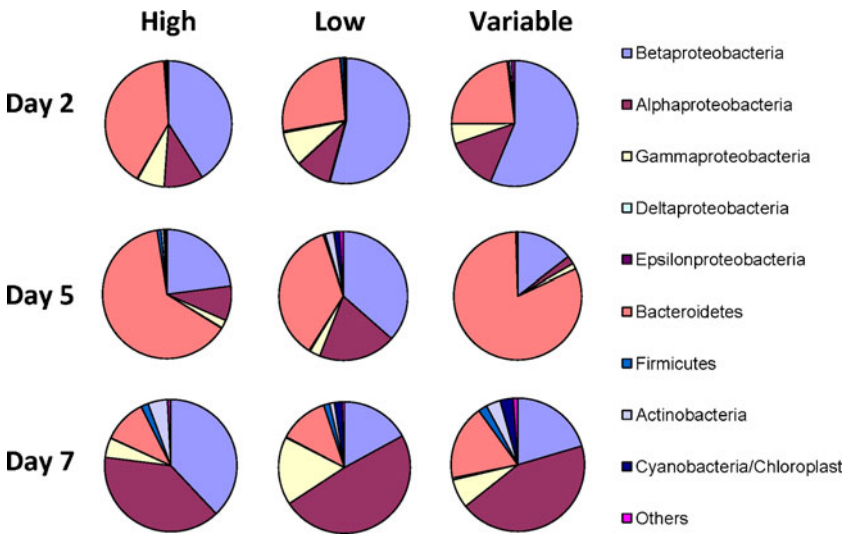

Fig. 4 Characterization of the egg surface microbial community assembly using the RDP classifier output at the phylum level (and class level for Proteobacteria). An average number of 13,468±7,779 reads with an average length of 450.08±1.97 bp per sample were obtained

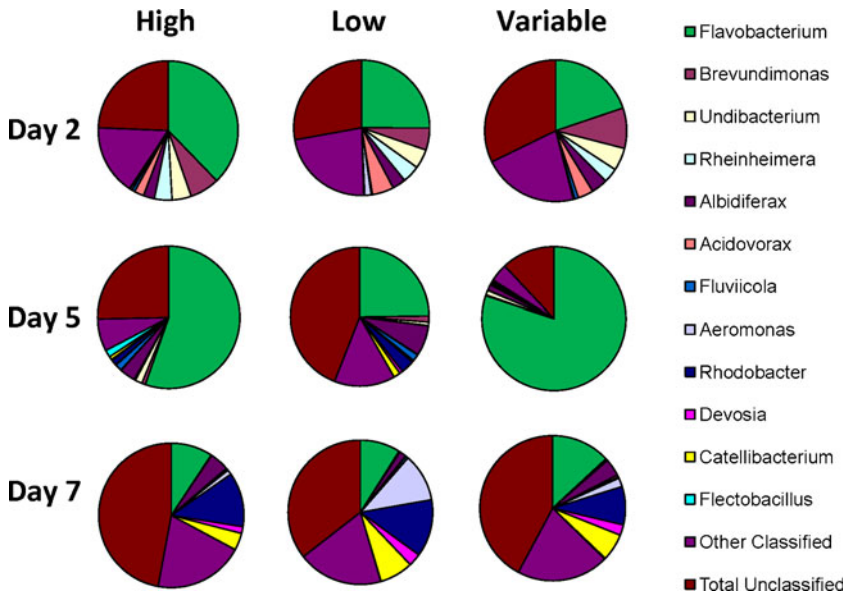

Fig. 5 Characterization of the egg surface microbial community assembly using the RDP classifier output at the genus level. An average number of 13,468±7,779 reads with an average length of 450.08±1.97 bp per sample were obtained. Across the nine samples, 360 genera were identified using RDP classifier with an average of 138.7±20.2 genera per sample

PCA. The first two principal components captured the temporal trend in microbial community structures, while PC3 accounted for differences between low flow regime and both high and variable flow regimes at day 5 (Fig. 6). The Jaccard distance matrices using the 97 % OTUs also grouped samples collected at the same time point together (Online Resource 6) while separating low flow regime from high and variable flow regimes within the day 5 group. The 97 % OTUs rarefied at 6,000 reads and time relationship showed a similar trend to what was found using TRFLP (Fig. 7). The Shannon species evenness (*E*) calculated using the 97 % OTUs revealed that microbial communities developed at day 5 under high and variable flow were unevenly distributed (Fig. 7), which is congruent with the dominance of genus *Flavobacterium* in these communities.

Discussion

To our knowledge, this is the first study that documents microbial succession on fish egg surfaces using pyrosequencing. Although we obtained the average of 13,400 reads per sample using the pyrosequencing, our rarefaction curves indicate that microbial community diversity had incomplete coverage. However, our integrative approach of combining analysis of microbial community structures using TRFLP and subsequent characterization of microbial community assembly using pyrosequencing was effective in comprehensively identifying microbial community succession patterns. Both our TRFLP and pyrosequencing results clearly demonstrated that microbial species replacement was occurring on fish egg surfaces. The fish egg microbial communities were clustered into three different embryonic developmental stages: the early, the middle, and the late, which is similar to what Besemer and colleagues found with microbial succession on ceramic coupons in streams [16], although they observed this trend over a longer time frame. The fact that we detected a temporal trend during microbial succession at the phylum/class level suggests that the change in microbial community assembly is not subtle, but rather drastic and occurs rapidly over the short period of embryological development. This is particularly evident from our assessment that only two genera out of 360 detected genera

Table 2 Bray–Curtis dissimilarity matrix using the RDP classifier output at genus level

	High D2	Low D2	Var D2	High D5	Low D5	Var D5	High D7	Low D7
Low D2	0.199							
Var D2	0.261	0.226						
High D5	0.366	0.511	0.561					
Low D5	0.384	0.465	0.512	0.352				
Var D5	0.429	0.581	0.645	0.185	0.481			
High D7	0.684	0.633	0.652	0.688	0.513	0.772		
Low D7	0.754	0.731	0.750	0.740	0.601	0.805	0.323	
Var D7	0.642	0.597	0.606	0.618	0.442	0.712	0.285	0.293

Unclassified genera were not included in this analysis

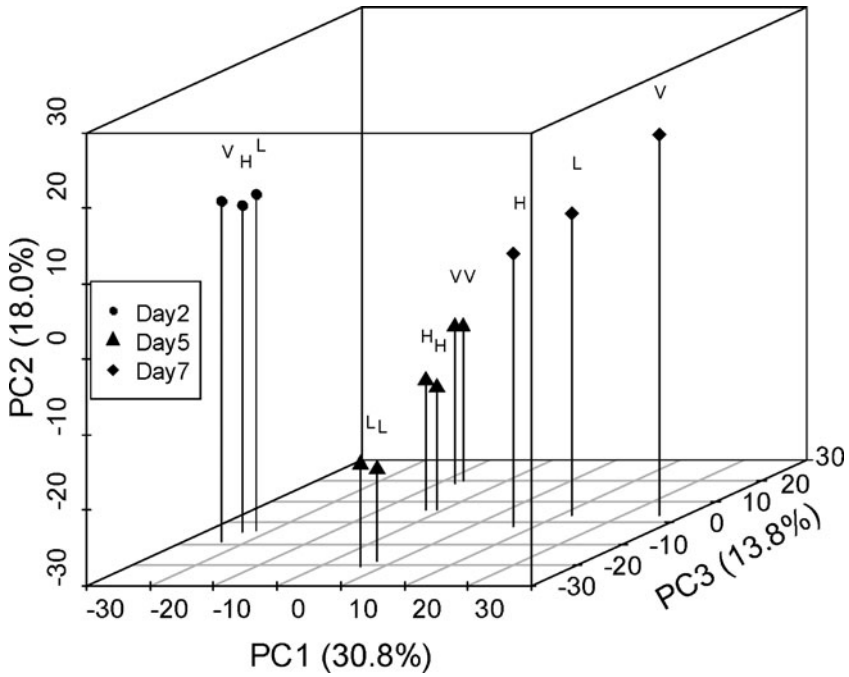

Fig. 6 Principle component score plots using OTUs defined at 97 % similarity cutoff. PC1, PC2, and PC3 accounted for 30.8 %, 18.0 %, and 13.8 % of the data variations, respectively. *H*, *L*, *V* in the figure denotes high, low, and variable flow, respectively. Two technical replicates of day 5 samples were included

maintained a relative abundance of over 2 % throughout the entire incubation period.

One possible factor that may have contributed to the observed microbial community shifts during embryogenesis is the change in the chemistry at the egg surface. Previous studies suggest that chemistry at fish egg surfaces changes during egg development [18–21]. Chadwick and Wright studied the nitrogen excretion patterns from Atlantic Cod eggs and found that the excretion of ammonia increased linearly while urea excretion decreased as the eggs developed [18]. Although nitrogen excretion patterns have never been studied in sturgeon, this type of metabolite excretion could contribute to a change in the microbial community on the egg surface. Fish eggs are also known to secrete antimicrobial substances such as lysozyme [20, 21], thus bactericidal activities might select for or against certain microbial species. One study on a relationship between egg innate immunity of small freshwater metazoan and egg microbial communities demonstrated that microbial communities on egg surfaces were altered when the type of antimicrobial peptides on eggs changed from maternally provisioned to that secreted by eggs [48, 49]. Because over 90 % of the egg surface microbial communities we found were composed of gram-negative phyla Proteobacteria and Bacteroidetes, it is possible that microbial community assembly on the egg surface was shaped by egg-excreted lysozyme, which acts on peptidoglycan layers of gram-positive bacteria [50].

The observed microbial community turnover also could be attributed to changes in the microbial community composition of the surrounding water during the experiment. The microbial community in freshwater streams varies temporally and spatially

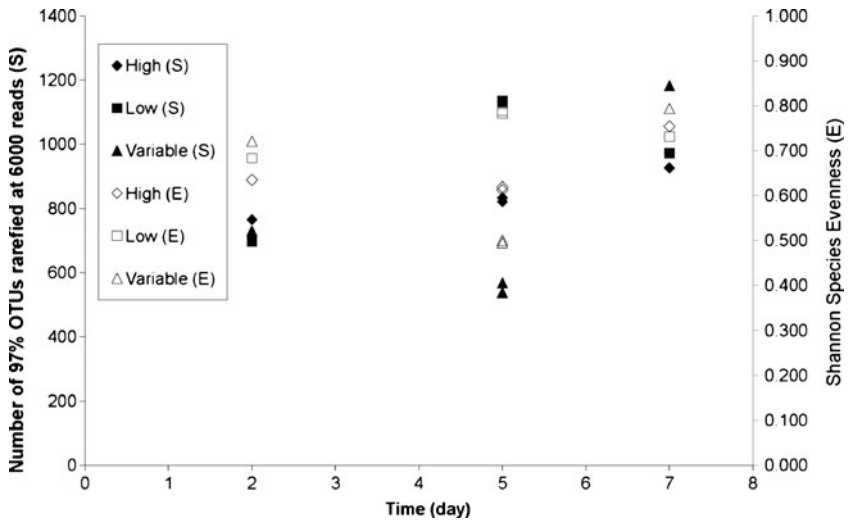

Fig. 7 Microbial species richness (97 % OTUs rarefied at 6,000 reads), microbial species evenness, and time relationships during succession on egg surfaces across flow regimes. (*S*) denotes species richness displayed using *filled symbols* and (*E*) denotes species evenness displayed using *open symbols*

[37, 51, 52]. We may have observed a microbial community that stochastically dispersed onto the egg surface from the water column [53, 54]. However, the structure of the egg-associated microbial community characterized in this study differed from the structure of the typical aquatic microbial community described previously [55]. Despite having a similar high prevalence of β, α, and γ-Proteobacteria and Bacteroidetes as commonly seen in freshwater communities, our observed egg surface microbial community assemblages had almost undetected levels of the phylum Actinobacteria, a microbe typically dominant in freshwater environments. We collected water samples from our stream during the spawning season of a different year (Fujimoto et al. unpublished study) and detected substantial numbers of the phylum Actinobacteria but greatly diminished representation on the egg surfaces reared in the same water (Online Resource 7). In addition, the dominance of over 50 % relative abundance of *Flavobacterium* in the high and variable flow regimes at day 5 deviated strongly from freshwater microbial communities. These findings suggest that dispersal alone is unlikely to explain the egg-associated microbial community assembly.

The microbial community turnover we observed on fish egg surfaces occurred during a 7-day period, which is significantly shorter than the time frame in which microbial succession is typically observed on non-living substrate surfaces [6, 7, 16, 51]. Our results suggest that microbial species sorting on the egg surface can occur in a short time frame, perhaps due to the living egg-related effects such as host innate immunity [21], secretion of metabolites [18], and existence of three dimensional structures on the egg surface, unlike the comparably long time required for such sorting to be observed on abiotic substrate surfaces where species sorting after initial colonization is limited by resource competition and microbe–microbe interaction [51].

Another finding of our study was the effect of flow rate on microbial community assembly. Despite the lack of flow effect on the early and late egg surface microbial communities, the differences in phylotype diversity observed between eggs reared under low versus high and variable flow regimes at day 5 were revealing. These findings suggest that the microbial community compositions were uniform at the early stage, diverged toward the middle stage dependent on the flow rate experienced by the eggs, and converged at the late stage of embryogenesis. A similar trend was found by Besemer and colleagues [16] who observed a significant effect of the flow velocity on ceramic surface microbial communities midway through the succession process but not in initial and mature microbial communities.

One possible explanation for the flow effect could be that high flow rate selected for certain microbial species that have the ability to adhere to the egg surface under greater shear force. One previous study tested the effect of flow velocity on microbial community diversity in water pipes found that increasing flow velocity in pipes lowered microbial community diversity [56]. At low flow rate, the selection pressure was presumably relaxed; therefore, many different microbial species with lower adhesive capabilities could stay on the egg surface. The dominance of *Flavobacterium* in the high and variable flow regimes could be related to the fact that this genus contains particularly adhesive species [57, 58]. However, we do not know why the effect of flow on community assembly was observed in the middle of the embryogenesis, but not observed at the early and late time points.

It is important to consider the potential implications of the effect of flow rate on microbe–egg interactions for host life history. Another study by our group [25] showed that over 80 % of egg mortality occurs during the first half of the incubation period, which is from day 0 to day 5 for our experimental conditions. *Flavobacterium*, the dominant genus in both high and variable flow at day 5, encompasses ranges of species including known fish pathogens such as *F. columnare* [59], *F. psychrophilum* [60], and *F. branchiophilum* [61]. On the other hand, under low flow regime at day 5, phylum Proteobacteria, which also includes some fish pathogens such as genus *Aeromonas* [62], was dominant. The net effect of flow velocity on microbe–host interaction is difficult to determine. However, it is important to note that fish eggs deposited in natural streams may not receive the high flow velocity used in this experiment, since the flow at the bottom of streams is lower than that in middle of water column due to the effect of the boundary layer [63].

In this study, we focused on flow rate as a key environmental variable that could influence microbe–host interactions. However, water temperature is also an important environmental covariate that may affect the microbial community assembly in aquatic systems [7, 64]. In this experiment, all eggs reared in the six flume channels were exposed to water from the same source and thus experienced the same water temperature throughout the experiment. However, the daily mean ambient water temperature gradually increased during the course of the experiment (Online Resource 8), which may have contributed to the temporal variation of microbial community composition that we observed during embryogenesis.

One implication of this study is that natural stream systems could create variation in microbial community succession due to differences in natural flow rate observed in streams during the spawning season. Streams may have significantly less precipitation during one spawning season than other average seasons simply by chance, which could lead to significantly lower stream flow rates during spawning events. Our analysis suggests that we should expect more microbial phylotypes adhering to egg surfaces during the critical period of embryogenesis in such dry spawning seasons.

The variable flow regime, which mimicked dam-manipulated flow, resulted in similar successional patterns to what we

observed under the high flow regime. Although the high and low flows were alternated in the variable flow regime, the high flow operation period of the variable flow might have had a dominant effect on microbial community composition. High flow rate of the simulated stream could have acted as a disturbance to microbial community formation on the egg surface. Since naturally occurring eggs potentially experience high flow during embryogenesis, our result implies that dam operations that include periods of high flow would not alter the natural course of successional patterns on egg surfaces. However, not all dams are operated in the same way as this experiment (alternating 12 h intervals), thus we cannot generalize our findings to all dam systems. Furthermore, a dam could potentially alter water chemistry, temperature, nutrients, and water microbial community composition in a spawning stream [65], which in turn could influence the microbial communities during succession.

In conclusion, this is the first microbial succession study conducted on fish eggs. Although previous studies have documented succession patterns on abiotic substrates in streams, our study breaks new ground in demonstrating that microbial community shifts can occur in a remarkably short period on a biotic surface. We hope this study will stimulate additional studies in this area and encourage other researchers to delve deeper into understanding the underlying mechanisms behind the patterns we observed.

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