BACTERIAL COMMUNITY ASSEMBLY AND STABILITY ON THE SURFACE OF THE LAKE STURGEON (*ACIPENSER FULVESCENS)* EGGS

By

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ABSTRACT

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High egg and larvae mortality represents a significant problem in aquaculture. Scientific evidence for a variety of fish, shrimp and shellfish species support the hypothesis that fish–microbe interactions are the major factors determining high levels of egg mortality. As such, in order to address this problem systematically, it is crucial to extend the knowledge of egg-associated microbial communities and develop a detailed understanding of their potential probiotic effects on the wellbeing of fish eggs. In this dissertation we present the results of several studies related to development of bacterial communities on the surface of Lake Sturgeon eggs. Throughout this work our perspective on bacterial species associated with Sturgeon eggs is to treat them as an integral part of the egg-bacteria symbiotic relationship with the focus on manipulating bacterial communities in order to decrease egg mortality.

First, we discuss the results of *in vitro* biofilm formation assays for six bacterial species previously isolated from the surface of healthy Lake Sturgeon (*Acipebser fulvesens*) eggs. The goal of this study was to understand how these bacterial species interact with each other when present in the same environment. We used a crystal violet assay, resazurin assay and Terminal Restriction Fragment Length Polymorphisms (T-RFLP) to analyze biofilm biomass formation, biofilm metabolic activity, and changes in the abundance of each isolate in double-species biofilms, respectively. Our results indicated that one of these isolates, *Brevundimonas F16*, produces a robust biofilm *in*

vitro. Furthermore, biofilm formation increases significantly in mixed cultures of *Brevundimonas-Hydrogenophaga* and *Brevundimonas-Acidovorax*. However, biofilm formation decreased in mixed cultures that included *Pseudomonas C22*.

Next, we describe the results of the study on how established biofilms of egg isolates interact with the river water microbiome. Our goal in this experiment was to measure the susceptibility to secondary colonization of a preexisting biofilm due to exposure to the river water. We measured the compositional stability of biofilms and identified specific river genera that invade or are recruited by preexisting biofilms. In this study, we were able to detect both highly resilient and weak biofilms, specific exclusions and recruitments of river populations by established biofilms of egg isolates, and apparent enhancements of biofilm development.

Finally, we present the results of *in vivo* studies in the hatchery. Here we investigated the effect of early use of monosaccharaides and potential probiotics on assembly and stability of Lake Sturgeon egg-associated bacterial communities. Our results indicate that both monosaccharaides and bacterial treatments during early developmental stages of the egg could change the early egg-associated bacterial communities. Moreover, some members of *Pseudomonas* and *Rheinheimera* genera could be egg mutualists, protecting the eggs against pathogenic fungi and bacteria such as certain species from the genera *Aeromonas* and *Flavobacterium.*

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KEY TO SYMBOLS AND ABBREVIATIONS

16s rRNA	a component of the 30S small subunit of prokaryotic ribosomes
EPS	Extracellular Polymeric Substance
EPSs	Extracellular polysaccharides
MiCA	Microbial Community Analysis III
NMDS	non-metric multidimensional scaling
OTUs	Operational Taxonomic Units
PCR	polymerase chain reaction
PCR-DGGE	polymerase chain reaction–denaturing gradient gel electrophoresis
RDP	Ribosomal Database Project
rRNA	ribosomal Ribonucleic acid
SIMPER	Similarity-Percentage
TRFLP	terminal restriction fragment length polymorphism

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

ABSTRACT

A major challenge in aquaculture is high fish egg mortality due to adverse microbial activities. Thus far, use of antibiotics and disinfectants not only have had limited success in decreasing high egg mortality of different fish species, but also are associated with environmental problems such as negative effects on fish and water quality, and on the development of antibiotic-resistant fish pathogens. Therefore, there is an increasing need for alternative “green” techniques to manipulate fish eggs microbial communities such as use of probiotics. To date, there are very few studies that have investigated potential applications of probiotic bacteria to decrease high fish egg mortality. As such, a better understanding of egg-associated bacterial communities and their interactions with eggs in different fish species is essential for identifying probiotic bacteria. This knowledge will help to beneficially manipulate the microbial communities of fish eggs to increase the eggs survivability. In the following chapter, we provide a literature overview of the existing knowledge on the symbiotic relationships between single bacterial species, as well as bacterial communities, and their hosts, with the emphasis on aquatic organisms. Additionally, we discuss implications of bacterial communities that assemble on fish eggs and describe egg associated bacterial communities of the Lake Sturgeon. Finally, we provide an overview of the present dissertation.

INTRODUCTION

In nature, different taxa living in the same environment can use symbiotic associations to establish a fitness advantage. Symbiosis was first defined by Heinrich Anton de Bary in 1879 as "the living together of unlike organisms" [1]. Symbiotic relationships are divided to three groups based on how the organisms involved, and how they affect each other: 1) mutualism (both organisms benefit), 2) commensalism (one benefits while the other is neither harmed nor benefits), and 3) parasitism (one benefits and the other is harmed) [1, 2]. Bacteria can form such symbiotic relationships with other bacteria as well as other organisms. Symbiotic bacteria are transmitted to their hosts in two ways: symbionts that are transmitted vertically and those that are transmitted horizontally [3].

Vertical transmission, which is the direct transmission from adult host to its offspring [3], ensures an efficient recolonization of the host. A well-known example of vertical transmission is the pea aphid *Acyrthosiphon pisum* and its obligatory bacterial endosymbiont, *Buchnera aphidicola* [4, 5]. The genome of *Buchnera aphidicola* is much smaller than most other bacteria (0.64 MB), and approximately 30% of its metabolic products are dedicated to synthesis of amino acids and vitamins that aphids require [3, 6]. In this mutualistic relationship, neither pea aphid nor *Buchnera aphidicola* can live without each other. Not in all symbiotic relationships organisms involved lose their autonomy however. Another endosymbiont of the pea aphid that is transmitted vertically s the bacterium *Hamiltonella defense. Hamiltonella defense* has a genome that is three times larger than *Buchnera aphidicola*. *Hamiltonella defense* benefits aphids by killing the eggs and the larvae of parasitoid wasps. Nevertheless, in the absence of parasitoid

wasps, aphids do not need *Hamiltonella defense* and stop transmitting it to their offspring [3, 7]. When the parasitoid wasps reappear in the environment, *Hamiltonella defense* recolonizes the aphids and is again transmitted vertically [3, 7].

Vertical transmission is not the most common form of transmitting symbiotic bacteria to their hosts. Many symbiotic relationships between bacteria and their hosts are considered horizontal transmission, which is often seen when the host attracts symbionts from its environment. Bobtail squids (*Euprymna scolopes*) and their mutualistic bioluminescent bacteria (*Vibrio fischeri*) is a well-known example of horizontal transmission [4, 8-11]. *V. fischeri* help the squid camouflage from both its predators and prey [12]. This mutualism begins within hours of hatching, when the squids' length is about 2mm. At this stage, bobtail squids must attract their *V. fischeri* symbionts from seawater where these bacteria are sparse [3, 10]. These squids use blind-ended crypts to keep their *Vibrio* symbionts. Beating flagella-like structures found around pores that lead to these crypts result in formation of bacterial aggregates. In response to bacterial aggregates and the peptidoglycans in their cell walls the squid produce mucus near the pores. Although initially different species of bacteria are found in this mucus, for some yet unknown reason, *V. fischeri* become dominant after a few hours. Soon after, the *Vibrio* travels through the pores and into the crypts by using their flagella [3, 10, 13]. To establish a successful symbiosis, *V. fischeri* must possess bioluminescence genes and functional regulators. In fact, lightless *V. fischeri* are outcompeted by their luminescent counterparts [3, 10, 14, 15]. Furthermore, the presence and growth of *V. fischeri* in the crypts result in production of lipopolysaccharides and tracheal cytotoxin. These compounds induce cell death, and

change the crypts and ducts in squid [3, 16]. At this stage, the squid and its *V. fischeri* are bound to each other, despite the fact that squids release the majority of their *Vibrio* symbionts into the water every day [3, 17].

Bacterial communities and their hosts

With development of culture-independent methods to study bacterial communities we now understand that in most environments, many species of bacteria are frequently present simultaneously, so it is natural to expect these bacteria constantly interact with one another and, thus, affect each other in a variety of ways. These bacteria-bacteria interactions, however, make studying bacteria in their natural habitat substantially more challenging. As such, bacteria have been traditionally studied *in vitro* as single-species planktonic cells. Advancements in imaging and metagenomics in recent years made analysis of bacteria-bacteria interactions in bacterial communities more feasible.

The ability to study these interactions has become even more important because recent studies suggest that the majority of bacteria exist as multispecies biofilms, assemblages of surface associated bacterial communities embedded within an extracellular polymeric substance (EPS). These multispecies biofilms are far more than a passive assemblage of cells. In fact, they are shown to be structurally and ecologically complex communities that can form and develop on a range of abiotic and biotic surfaces [18-25].

Assembly of multispecies biofilms on a given surface is regulated by taxa present in the environment, availability of resources, environmental conditions, and interactions between the present taxa [24]. Interactions between different species of bacteria within

multispecies biofilms can be antagonistic, mutualistic or commensalistic, and may lead to improved survivability of these bacteria [24]. Recent studies have identified antagonistic interactions, including the production of bacteriotoxins and the lowering of pH, as well as many mutualistic and commensalistic interactions such as coaggregation, conjugation, protection from antibiotics, enzyme complementation and organized spatial niche-partitioning within multispecies biofilm [26-35].

In aquatic environments abiotic as well as biotic surfaces such as aquatic organisms are colonized by a variety of bacteria that form multispecies biofilm communities. Moreover, these multispecies biofilm communities can have symbiotic interactions with their hosts. One of the well-studied examples of such mutualism in aquatic environments is the coral hosts and their mutualistic bacteria. Corals are marine invertebrates that have a symbiotic relationship with an algae (zooxanthellae) in which the algae provide the corals with photosynthesis products that corals can use for energy, and the corals provide the algae with a safe environment, nitrogenous waste and carbon dioxide that the algae can use. Corals do not have an antibody-mediated immune response to pathogens including bacteria and fungi, however some corals are protected against different pathogens by shifting mutualistic bacterial communities that provide temporally immunity-like services [36, 37]. The potential to manipulate the bacterial communities of corals for disease management has been investigated and the results are promising [36, 38, 39].

Another well-studied example is chytridiomycosis, a widespread infectious disease that is common among amphibians and is caused by *Batrachochytrium dendrobatidis* (Bd) a fungal pathogen. Studies have shown that the growth of Bd can be

strongly moderated or completely inhibited by antimicrobial compounds secreted by some members of the bacterial communities of amphibians skin such as *Janthinobacterium lividum* and *Lysobacter gummosus* [36, 40-42]. Use of these bacteria as probiotics may be a plausible conservation solution to the chytridiomycosis pandemic.

Effects of bacterial communities on fish eggs

Mortality during early oncogenic stages exceeds 95% for many fish species [43-46]. Scientific evidence as well as experience in hatcheries for a variety of fish species supports the hypothesis that fish egg-microbe interactions in general and fish egg-bacteria interactions specifically, play a significant role in the eggs well-being and mortality [47]. However, fish egg associated bacterial communities are still poorly characterized and thus require more comprehensive investigation. Expanding the knowledge of egg associated bacterial communities is the main focus of the present dissertation.

Dannevig was the first person to describe bacterial growth on the eggs of cod off the Canadian coast early in the twentieth century [48]. He showed a positive correlation between bacterial growth on these fish eggs and egg mortality. More recent work by a number of investigators has confirmed that eggs released into the aquatic environment are rapidly colonized [47, 49-51]. One major reason for this colonization is related to the structure of the egg surface. Chorion, the outer layer of the fertilized fish eggs, is rich in glycoproteins [52-55]. Such proteins are well-known determinants for adhesion of microbes [55-57]. Indeed, receptors existing on the surface of eggs as well as maternal

Organism	Identified phylotypes found on the egg	Characterization of bacterial isolates	Reference
O. kisutch	*A. hydrophila*, *Cytophaga* and *Pseudomonas*.	Culture dependent	[63]
O. keta	*A. hydrophila*, *Cytophaga* and *Pseudomonas*.	Culture dependent	[63]
S. gairdneri	*A. hydrophila*, *Cytophaga* and *Pseudomonas*.	Culture dependent	[63]
O. smasou and O. keta	*Flavobacterium*, *Cytophaga*, and *Pseudomonas*.	Culture dependent	[62]
C. auratus	*Flavobacterilum spp*., *A. hydrophila*, *Pseudomonas spp*., *Micrococcus spp*., and *A. piunctata*.	Culture dependent	[60]
G. morhua	*Pseudomonas*, *Alteromonas*, *Aeromonas*, *Flavobacterium*, *L. mucor*, *V. fischeri*, *Caulobacter*, and *Seliberia spp.*	Culture dependent	[55]
H. hippoglossus	*Pseudomonas*, *Alteromonas*, *Aeromonas*, and *Flavobacterium*, *L. mucor*, *Moraxella*, and *Alcaligenes*	Culture dependent	[55]
S. gairdneri - Richardson	*Pseudomonas sp.*, *A. hydrophila*, and *Cvtophaga* species.	Culture dependent	[64]
S. trutta	*Pseudomonas sp.* and *A. hydrophila*	Culture dependent	[64]
S. pilchardus	*Vibrio*, *Pseudoalteromonas*, *Pseudomonas* and *Moraxella*, *Aeromonas*, *Tenacibaculum* (*Flexibacter*), *Flavobacterium*, *Cytophaga spp*., *V. anguillarum*, *V. fischeri*, and *T. ovolyticum*	Culture dependent	[65]
O. kisutch	β-Proteobacteria (*Janthinobacterium* and *Rhodoferax*).	Culture independent- PCR–DGGE of 16S rRNA gene	[32]
O. mykiss	*Flavobacterium-Cytophaga*, *Aeromonas*, *Acinetobacter*, *Moraxella*, *Coryneforms*, Gr (+) Coccus, *Enterobactericeae* constituted groups.	Culture dependent	[66]
A. fulvescens	360 genera were associated with the eggs. 90–98 % of the bacterial communities were composed of the phyla Proteobacteria and Bacteroidetes throughout egg development.	Culture independent- 16S rRNA gene- TRFLP and 454 pyrosequencing	[59]
Coregonus spp.	*Moraxellaceae, Oxalobacteraceae, Comamonadaceae, Leuconostocaceae*, and *Streptococcaceae*.	Culture Independent- 454 pyrosequencing of 16s rRNA gene	[61]
S. trutta	Most common families were *Rhodobacteraceae*, *Xanthomonadaceae*, *Oxalobacteraceae*, *Enterobacteriaceae*, *Streptococcaceae*, *Moraxellaceae*, *Comamonadaceae*, and *Flavobacteriaceae*.	Culture Independent- 454 pyrosequencing of 16s rRNA gene	[67]

Table 1.1. Summary of prior investigations that described the structure of the microbial communities on fish eggs.

factors can selectively favor colonization of certain bacteria [58]. This fact manifests itself in the species-specific differences that were found between microbial groups that populate egg surfaces of different fish species [55, 59-62] (Table 1).

In addition to eggs exposed to stream water, bacterial species have also been detected on aseptically harvested (directly from the ovaries) unfertilized eggs of cod [55]. In some fish like the Lake Sturgeon, eggs are extruded through parts of the lower gastrointestinal (GI) tract where eggs can come into contact with GI microbiome.

Bacterial colonization can affect both eggs [68] and developing embryos [69, 70] in different ways. For example, excessive biofilm formation on the eggs surface can lead to hypoxia in developing embryos and elevate egg mortality [49, 55, 71, 72]. Also, an inverse correlation was observed between the presence of certain bacteria and physical strength of fish eggs [73]. Some pathogens such as *Flexibacter ovolyticus* produce exoproteolytic enzymes that can damage the chorion, and result in high mortality of the egg [68]. Additionally, toxins produced by certain members of the egg associated bacterial community can harm the developing embryo [64]. On the other hand, the presence and/or abundance of certain bacterial species on the egg surface can also be beneficial for eggs and/or developing embryos [49, 71]. Colonization of the egg surface with a diverse community of egg mutualists can be a barrier against adhesion and/or harmful activities of various pathogens. Also, some egg associated bacterial species benefit the eggs by producing antimicrobial compounds against other bacterial or fungal fish pathogens [74, 75]. Identification of natural populations of symbiotic bacteria or prebiotics that restrain the adhesion or growth of harmful microbes could provide better control of egg colonization and potentially decrease high mortality.

Lake Sturgeon (*Acipenser fluvescens*) is an ancient family of fresh water fish that is sensitive to habitat destruction and overharvest [59, 76-78]. In fact, in the past century the population of this fish has reduced significantly worldwide and in the United States due to overfishing and dam construction to the point that now this fish is considered an endangered species. Restoring the population of Lake Sturgeon worldwide has proven to be challenging due to several factors including delayed maturity, infrequent reproduction and high egg mortality [76-78]. The main contributor to high egg mortality in Lake Sturgeon is developmental arrest [76]. Developmental arrest can be caused by several factors such as poor egg and/or sperm quality, polyspermy, extreme fluctuations in water temperature, low water flow rates and egg-associated microbial communities. As mentioned above, egg-associated microbial communities in fish in general and in Lake Sturgeon specifically are not well understood. For example, previous analysis with 16s rRNA clone libraries showed aseptically harvested unfertilized eggs may not always be sterile [77]. However, electron micrographs of aseptically harvested unfertilized eggs show no microbial presence on the egg surface just before females release their eggs in the water [78]. This led to the hypothesis that microbial communities that are found on the egg surface originate from river water. This is an important fact as it allows us to exclude maternal effects from consideration.

One day after fertilization and exposure to river water, however, egg surfaces are colonized by a variety of microbial species including fungi and bacteria. Former studies showed a significant correlation between bacterial communities on the Lake Sturgeon egg surfaces and egg mortality [59, 77, 78]. In these studies 360 bacterial genera associated with the egg surface were identified using culture-independent methods.

Fujimoto (2012) also found that egg communities are distinct from the source water bacterial community, are shaped within 60 minutes of exposure to river water, change through egg developmental stages, and depend on environmental conditions.

There are a few key results that support the hypothesis that egg-associated bacterial communities affect the egg mortality. First, Fujimoto (2012) has shown reducing the bacterial load on the egg surface by fertilizing and rearing eggs in filtered and UV-treated stream water can decrease the egg mortality significantly. The reason behind is that presence of a larger microbial population on the egg surface prevents oxygen diffusion to the egg and, thus, results in the egg suffocation. The second result was that exposure of the eggs to a bacterial species isolated from the surface of healthy eggs belonging to the genus *Acidovorax* during egg fertilization, changed the eggs early bacterial community and reduced eggs mortality. This result suggested that early intervention into bacterial community allows one to alter the fate of fish eggs. Further analysis showed certain bacterial isolates exhibit strong biofilm forming capabilities and are capable of producing antimicrobial agents that affect the growth of other isolates and certain fish pathogens [59, 77, 78].

Objectives and thesis overview

In this study we consider three topics related to bacterial communities associated with the Lake Sturgeon eggs. In Chapter 2 we discuss the results of *in vitro* biofilm formation experiments. The main objective of this study was to analyze bacteria-bacteria interactions during biofilm formation between bacterial species isolated from the surface of healthy Lake Sturgeon eggs. After initial screening among egg isolates using a crystal violet assay, four egg isolates from the genera *Pseudomonas*,

Hydrogenophaga, *Brevundimonas*, and *Acidovorax* were chosen for further assessment of their biofilm forming capabilities in single- and double-species mixtures. We used crystal violet assay, resazurin assay and terminal restriction fragment length polymorphism (TRFLP) for measuring biomass, metabolic activity and relative abundance of these species in double-species biofilms. Our results indicate that *Brevundimonas* had the highest biofilm biomass while *Acidovorax* had the highest metabolic activity. Also, double-species biofilms of these species produced higher levels of biomass and metabolic activity as compared to their single species counterparts. Based on these results we chose *Hydrogenophaga*, *Brevundimonas*, and *Acidovorax* as potential probiotics for further experiments on live eggs in the hatchery. The methods used in this chapter can be applied for *in vitro* screening of effective probiotics for other fish and amphibian species.

Next, we discuss interactions between members of the river water microbiome with established biofilms of egg bacterial isolates in Chapter 3. In this study we hypothesized that assembly of a mixed species biofilm can be influenced by specific attributes of early colonizers that select for subsequent colonizers. We used crystal violet assays to measure the biofilm biomass of initial colonizers. Then, we analyzed bacterial communities 4, 8, and 24 hours after exposure to river water using next generation (Illumina) sequencing of the 16S ribosomal RNA gene. We observed that river water bacterial communities were significantly different from the community formed after 24 hours on replicated experimental plates. Also, founding populations showed a broad range of biofilm stability after 24 hours. Secondary colonizers from the river community were unique for all pre-established biofilms at all time points. Moreover, at

early time points (4 and 8 hours), established single-species biofilms of *Hydrogenophaga*, *Brevundimonas*, and *Acidovorax* selected for the other two genera from the river water community, suggesting that there is mutualism between these three bacterial species in early stages of biofilm formation in aquatic environments. Results suggest that manipulating the early bacterial communities of aquatic surfaces including fish eggs, may lead to substantial changes in the succession of these communities, which in turn may change the fate of the hosts.

In Chapter 4 we discuss results of *in vivo* studies that were focused on colonization of Lake Sturgeon eggs by bacteria from the stream water microbiome. Specifically, our objective was to identify early bacterial colonizers and evaluated the possibility of intervening in early bacterial communities using competitors to binding of early colonizers in the form of a monosaccharide or a bacterial species. To determine taxa of early colonizers, aseptically harvested unfertilized eggs were exposed to stream water supplemented with glucose, galactose or mannose for short periods of time, up to 135 minutes. These monosaccharides were chosen based on a previous monosaccharide analysis of the Lake Sturgeon egg casing. For early intervention experiment using bacterial species we exposed Lake Sturgeon eggs during fertilization to *Hydrogenophaga*, *Brevundimonas*, *Acidovorax* or a double-mixture of these bacterial isolates. After 45 minutes these eggs were transferred to and reared in stream water. Egg samples were collected immediately after fertilization (before exposure to stream water), one day, and five days after fertilization and exposure to stream water. Since it was easy to differentiate between healthy and unhealthy eggs five days after fertilization and exposure to stream water, both healthy and unhealthy egg samples were collected

at this time point. Generally in these experiments, we extended pervious studies by analyzing a larger number of both healthy and unhealthy eggs, and performing next generation (Illumina) sequencing of 16S ribosomal RNA gene instead of a limited number of healthy eggs and clone libraries. Results of these experiments indicate that *Rheinheimera* was associated with healthy eggs five days after fertilization and exposure to stream water, suggesting this species could be a potential mutualist of the Lake Sturgeon egg. Furthermore, these results suggest that specific chemistry of the egg (defined by the female fish) affects the early bacterial communities on egg surfaces, and assembly of a community can be influenced by specific attributes of early colonizers, that select for subsequent colonizers. Importantly, early intervention with sugars and bacterial isolates can change the early bacterial communities of the egg surface, which can potentially change the fate of the eggs.

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CHAPTER 2. INVESTIGATING BIOFILM FORMATION IN LAKE STURGEON BACTERIAL ISOLATES

ABSTRACT

Biofilms are rarely composed of a single species. In natural environments biofilms are multispecies constructs comprising all possible complex interactions between species. In this chapter we investigated multispecies biofilms formed by six bacterial species (*Massilia B13, Pseudomonas C22, Pseudomonas D2, Acidovorax F19, Hydrogenophaga F14*, and *Brevundimonas F16*) isolated from healthy Lake Sturgeon eggs. The main objective of these studies is to identify bacterial species that show mutualism/antagonism during biofilm formation *in vitro*. Better understanding of species interactions could lead to intervention strategies effective in protecting the egg by reducing egg mortality. The choice of these species was dictated by their relative abundance on the surface of healthy eggs and our ability to culture them under laboratory conditions. First, we investigated biofilm formation of these isolates individually and in mixed cultures, where the emphasis was on bacteria-bacteria interactions between selected species. In order to investigate interactions between pairs we measured both total biofilm biomass using crystal violet and biofilm metabolic activity using resazurin assays. Moreover, we measured changes in the abundance of each isolate in double-species biofilms using Terminal Restriction Fragment Length Polymorphism (T-RFLP). Results indicate that interactions between certain bacterial species can promote or suppress biofilm formation in their mixed cultures, suggesting that the egg well being is determined not only by individual bacteria present in the same environment but whether these species act as mutualists or antagonists to each other. Next, we studied previously observed phenomenon of *Pseudomonas C22* exhibiting substantial antimicrobial activity against many of the isolates as well as some common

fish pathogens when tested with a simple agar overlay. To determine how this activity might affect biofilm formation, we quantified biofilm biomass and metabolic activity in mixed cultures of *Pseudomonas C22* and several isolates known to exhibit good biofilm forming abilities in single culture. In particular, our experiment results of mixtures of *Pseudomonas C22* with *Flavobacterium columnare* demonstrate that, in addition to inter-species interactions, the order in which these species appear in the environment can play a significant role in the development of egg associated bacterial communities. Separately, we tested how environmental stresses such as certain metals (Nickel, Calcium and Magnesium), and an antibiotic (tobramycin) affected biofilm development of *Pseudomonas C22, Hydrogenophaga F14, Brevundimonas F16*, and *Acidovorax F19*. The results indicate that Nickel has the most appreciable and inhibitory effect on biofilm formation of selected bacterial isolates and their mixtures, suggesting that Nickel contamination of aquatic environments can have a significant effect on microbial communities associated with Lake Sturgeon eggs. Finally, our results with tobramycin show that this antibiotic has an inhibitory effect on fish pathogen *F. columnare* without having noticeable detrimental effects on the biofilm formation of fish egg bacterial mutualists and commensals.

INTRODUCTION

In aquatic environments many bacteria are enclosed within multi-species biofilms, which are complex heterogeneous aggregations of microorganisms [1, 2]. The term “biofilm” was formalized for these communities by Mack and colleagues in 1975, and is now considered to refer to populations of microbes attached to a surface and surrounded by an extracellular polymeric substance (EPS) [3, 4]. EPS is made of polysaccharides, lipids, proteins, and nucleic acids that form a hydrogel and removes and protects the cell collective from the surrounding environment [1, 5-9]. Studies show that not only are biofilms the principal microbial life form under most conditions, but also they are structurally and ecologically complex communities [7, 10-17].

Attachment to a surface is the initial step of microbial biofilm formation. A variety of factors such as surface charge, stage of growth, and nutrient concentrations affect the initial attachment of microbes to biotic and abiotic surfaces [18-20]. Bivalent cations such as Mg^{2+} and Ca^{2+} affect biofilm formation in bacteria both directly (by affecting electrostatic interactions) and indirectly (as cofactors to enzymes that influence bacterial attachments) [18-20]. Studies also suggest that microbes are more resistant to environmental stress such as antimicrobial substances and high concentrations of metals in their biofilm mode of growth [21-24]. Although the mechanisms that cause this resistance to environmental stresses are not yet fully understood, the presence of dormant cells with low metabolic activity contained within extracellular polymeric substances in biofilms are mentioned in literature as factors that cause such resistance [18, 19].

In recent years, use of high-throughput sequencing and high-resolution imaging methods have enabled scientists to examine the complexity of and interactions in multi-species biofilms. As a result there has been a shift towards studying multi-species biofilms and the inter-species interactions that affect the development, structure and functions of these biofilms both in nature, and industrial and medical environments [25].

Unlike air, water is a more ideal environment for microbial growth. Like all other cells, microbial cells are made largely of water, and water is necessary for them to dissolve their food, get it into their cells, and for voiding of the waste products made during chemical reactions necessary for their maintenance and growth. Essentially all aquatic ecosystems contain both pelagic and biofilm-bound populations of microbes. As a result, aquatic organisms like fish and amphibians have co-evolved over millions of years with the aquatic microbial community. Adult aquatic vertebrates and their eggs face an environment with up to 10^7 microbes per milliliter [63]. Understanding the nature of these interactions may be critical to the conservation of communities inhabiting aquatic ecosystems. These interactions begin when a sterile egg is extruded from a female and is rapidly colonized by aquatic bacteria and fungi.

One of the challenges many species of fish face is the effect of microbes on their eggs mortality. In fact, mortality during early oncogenic stages can exceed 95% for many fish species [26-29]. An endangered ancient family of fish that is affected significantly by high egg mortality caused by microbial communities, is the Lake Sturgeon [12].

A native of the North Amercian Great Lakes, Lake Sturgeon (*Acipenser fluvescens*) is one of 26 freshwater Sturgeon species. This ancient family of fish first

appeared in the fossil record in the Upper Cretaceous period (136 million years ago). Lake Sturgeons are characterized by a long life span (55 years for males and up to 150 for females) and delayed maturity (24-26 years for females and 12-17 years for males) [30]. The population of this fish declined dramatically during the 20th century due to excessive fishing and dam construction. As a result, Lake Sturgeon is now listed as threatened or endangered throughout its original range in the United States [31].

A mature female Lake Sturgeon lays ~100,000-800,000 eggs during each spawning season, however, natural recruitment is limited due in part to delayed maturity and low frequency of reproduction, but mostly because of high mortality during early oncogenic stages such as egg and free-embryo [32-38]. Recent studies have shown one of the main determinants of mortality is the egg's associated bacterial community [32, 37, 38]. Electron micrographs of the surface of aseptically harvested Lake Sturgeon eggs show that eggs are essentially sterile when extruded into river water during spawning [39]. However, within minutes of exposure to stream water egg surfaces develop an adhesive quality, and the egg surface microbial community develops [37, 40]. The egg's adhesive properties may aid in the initial attachment of planktonic bacteria in the stream water, an effect that appears to be selective for certain bacterial species [37, 38].

Within 24 hours of exposure, different taxa of bacteria are found on the egg surface in the form of multi-species biofilms and in total, 360 bacterial genera have been identified in the Lake Sturgeon's egg-associated community [38-40]. Previous studies, using cultivation independent approaches, have shown that the egg associated bacterial community is dynamic with α-Proteobacteria most frequent during early stage of egg

development, Bacteroides during the middle stage, and ß-Proteobacteria during the late stage [38]. These studies have also showed that there is a significant difference between the river water and the egg associated bacterial communities [37, 38]. For example, healthy Lake Sturgeon egg surfaces are enriched in a variety of genera, including *Acidovorax*, *Hydrogenophaga*, *Brevundimonas, Rheinheimera,* and *Massilia*, relative to source waters, while *Flavobacterium* are relatively less abundant [37]. In these studies genus *Pseudomonas* had relatively the same abundance in both egg and water communities whereas *Flavobacterium, Polynucleobacter* and *Limnohabitans* were the most abundant genera in the stream water. Fujimoto also reported that eggs fertilized and reared in UV treated/filtered water have significantly lower egg mortality (~30%) compared to those fertilized and reared in stream water [37]. Further analysis showed that the bacterial community of the eggs fertilized and reared in UV treated/filtered water contained larger populations of *Massilia, Acidovorax*, *Sphingobium*, *Pseudomonas, Pseudorhodoferax, Aquabacterium* and *Pelomonas*, compared to eggs fertilized and reared in stream water, suggesting these genera may have a mutualistic relationship with Lake Sturgeon eggs. Also, fertilizing the eggs in the presence of *Acidovorax F19,* isolated from the healthy Lake Sturgeon egg surfaces, not only changed the early bacterial community of the eggs but also reduced the overall egg mortality by 18%, suggesting this isolate could be used as a probiotic [37]. Such studies not only highlight the significance of microbe-microbe interactions on the formation and succession of microbial communities but also show the importance of these interactions on the wellbeing of their hosts and disease management.

In this study we investigated bacteria-bacteria interactions during biofilm formation of six bacterial species isolated from healthy Lake Sturgeon eggs, *in vitro*. These species were chosen for two reasons: 1) based on previous studies, they belonged to the same genera that were highly abundant on healthy Lake Sturgeon egg surfaces, and 2) the practical issue that they were isolated and grew well in the laboratory. First, we investigated *in vitro* biofilm formation in these isolates individually, and in mix cultures by measuring the total biofilm biomass using crystal violet assay [40]. Second, we pursued Fujimoto's observation that *Pseudomonas C22* had substantial antimicrobial activity against many of the isolates as well as some common fish pathogens when tested with a simple agar overlay [39]. To determine how this activity might affect biofilm formation we measured biofilm formation in mixed cultures of *Pseudomonas* C22 and several isolates with good biofilm forming abilities in single culture. Separately, we tested how environmental stress such as certain metals (Nickel, Calcium and Magnesium), and an antibiotic (tobramycin) affect biofilm development of *Pseudomonas C22, Hydrogenophaga F14, Brevundimonas F16*, and *Acidovorax F19*. Based on the results of these experiments, three pairs of bacterial species were chosen for further investigation due to their potential mutualism during biofilm formation: *Hydrogenophaga F14-Brevundimonas F16, Brevundimonas F16-Acidovorax F19,* and *Pseudomonas C22-Flavobacterium columnare*. In order to investigate interactions within these pairs we measured the total biofilm biomass using crystal violet and we assessed biofilm metabolic activity using resazurin assay [41, 42]. Moreover, we measured changes in the abundance of each isolate in double-species biofilms using Terminal Restriction Fragment Length Polymorphism (T-RFLP) [43].

MATERIAL AND METHODS

Bacterial species. In this chapter we study biofilm formation of the following Lake Strugeon egg bacterial isolates: *Massilia* B13*, Pseudomonas* C22, *Pseudomonas* D2, *Acidovorax* F19, *Hydrogenophaga* F14, and *Brevundimonas* F16. These bacterial species were isolated from the surface of healthy fertilized eggs of Lake Sturgeon as described in detail by Fujimoto [39]. These isolates were kept in the refrigerator at -80°C in 20% glycerol solution. Upon performing our experiments, we revived and maintained these bacterial isolates on BA Difco R2A medium. The medium was composed of 0.5g proteose peptone, 0.5g casamino acids, 0.5g yeast extract, 0.5g dextrose, 0.5g soluble starch, 0.3g dipotassium phosphate, 0.3g sodium pyruvate, 0.05g Magnesium sulfate and 15mg agar in 1L MiliQ Water. As these bacterial species were originally isolated on R2A, they grew better on R2Broth compared to other liquid media tested. Therefore, we tested all bacterial isolates for biofilm development using R2Broth.

Dr. Michael Bagdasarian of Michigan State University provided a control species *Pseudomonas aeruginosa* PA01. This species was used as a positive control for biofilm formation in crystal violet and resazurin assays performed in this study.

Phylogenetic analysis: 16S rRNA gene sequencing. Four isolates (*Pseudomonas* C22, *Hydrogenophaga* F14, *Brevundimonas* F16, and *Acidovorax* F19) were cultivated in 10mL of R2-Broth overnight and cells were collected them by centrifugation (10,000 RPM at 4°C in SS34 rotor). Genomic DNA was extracted from cell pellets using a MoBio UltraClean Microbial Kit. Following standard protocol, we amplified 16S rRNA gene using “universal” primers 27F (5’- AGA GTT TGA TCM TGG CTC AG - 3’), and 1389R (5’-ACG GGC GGT GTG TAC AAG - 3’). Amplicons were

purified using Qiagen PCR Cleanup columns according to the vendor's protocol and sequenced these PCR products at the Michigan State University Research Technology Support Facility using an ABI 3730xl capillary electrophoresis system. Species were sequenced with three primers to cover nearly the entire gene (27F (5'- AGA GTT TGA TCM TGG CTC AG - 3'), 1100R (5'-GGG TTG CGC TCG TTG – 3'), and 1389R (5'-ACG GGC GGT GTG TAC AAG - 3'). The three separate reads were assembled using ARB (version arb-5.5) [51]. These sequences are available at the NCBI database [GenBank accession numbers are as follows: *Pseudomonas* C22: MH465524, *Hydrogenophaga* F14: MH465525, *Brevundimonas* F16: MH465526, and *Acidovorax* F19: MH465527]. 16s rRNA gene sequences were compared with rRNA sequences in the Ribosomal Database using Ribosomal Database Project's Seqmatch algorithm (http://rdp.cme.msu.edu/seqmatch/), which was used to find the closest 16S rRNAs of both the isolated and uncultured, type and non-type species deposited in the RDP database [52].

Formation of single- and mixed-species biofilms. In order to study *in vitro* biofilm formation of single bacterial isolates [*Massilia B13* (B13)*, Pseudomonas C22* (C22)*, Hydrogenophaga F14* (F14)*, Brevundimonas F16* (F16)*,* and *Acidovorax F19* (F19)], we added 50μL of bacterial culture, grown overnight in R2Broth, to 100μL of sterile R2Broth in one well of a 96-well plate (4-12 replicates per condition based on the experiment), for each trial. We used *Pseudomonas aeruginosa* as a positive control and 12 wells with un-inoculated sterile R2Broth as negative control. We incubated all plates in ambient temperature on a shaker (100 RPM) for 48 or 96 hours, depending on the timeline of the experiment.

To investigate biofilm formation in double-species mixed-cultures (B13+C22, B13+D2, B13+F14, B13+F16, B13+F19, C22+D2, C22+F14, C22+F16, C22+F19, D2+F14, D2+F16, D2+F19, F14+F16, F14+F19, F16+F19), we combined 25µL of overnight culture of each Lake Sturgeon egg isolate, grown in R2Broth, with 100µL of sterile R2Broth in one well of a 96-well plate (4-12 replicates per condition based on the experiment). Where we had more than two egg isolate combinations (3-, 4-, 5-, and 6-species combinations) we mixed the overnight bacterial cultures in a way that the total would sum up to 50µL of bacterial culture, added to 100µL of sterile R2Broth in one well of a 96-well plate (4 replicates). These plates were also incubated in the similar conditions mentioned above. In all experiments, pre- and post-incubation optical density at 600 nm was measured to quantify starting concentrations and further growth within the broth.

In addition to single- and mixed-species biofilm formation, we studied the effect of one isolate on the established biofilm of the other isolate by performing “invasion” tests. In these experiments, for each trial we added 50µL of overnight culture of the first species to 100µL of sterile R2Broth in one well of a 96-well plate. After 48 hours of incubation in ambient temperature on a shaker (100 RPM), we removed pelagic cells and washed the wells three times using sterile physiological saline. Then, we added 50µL of overnight culture of the second species, plus extra 100µL of sterile R2Broth. Finally, we continued incubating plates for another 48 hours in the same conditions.

***Brevundimonas* titration assays.** We measured biofilm formation of *Hydrogenophaga F14* and *Acidovorax F19* in co-culture with different concentrations of *Brevundimonas F16*, essentially titrating these cultures with *Brevundimonas F16* (Live

titration). *Brevundimonas F16* is closely related to the genus *Caulobacter* and both genera have representatives that form asymmetrical rod-shaped cells with holdfasts at one end that attach readily to surfaces. We observed this holdfast in microscopic images of *Brevundimonas* and posited that this holdfast provides interactions with not only surfaces but with other species in solution as well, and that this may impact biofilm formation.

For this experiment, R2A plates were streaked with *Brevundimonas F16* and incubated in ambient conditions for a week. These plates were then scrapped with 5 mL sterile distilled water. The tubes containing the mixture of *Brevundimonas F16* and sterile water were then mixed with a vortex at maximum speed for 2 minutes and then centrifuged (10,000 RPM) for 30 minutes at 4^{o}C. Cell pellets were then re-suspended in 5 mL fresh sterile R2Broth. Next, the re-suspended cell pellet was diluted to the following dilutions using fresh R2Broth: 0.3x, 0.2x, 0.1x, 0.05x, and 0.01x. The biofilms were further established using the procedure above, using different concentrations of *Brevundimonas F16* cells instead of its overnight culture. Biofilm biomass and metabolic activity was measured after 48 hours using crystal violet and resazurin assays.

Additionally, we tested the biofilm formation of above mixed cultures using dead *Brevundimonas F16* cells, to investigate if active culture of *Brevundimonas* was required for biofilm formation (Dead titration). This experiment was performed like the live titration assay except that after centrifuging (10,000 RPM) for 30 minutes at 4^{o}C the cell pellets were re-suspended in 5 mL of 80% ethanol and were kept at 4^{o}C for two weeks. After two weeks, 1 mL of the dead *Brevundimonas F16*/ethanol solution was centrifuged (10,000 RPM) for 10 minutes. The resulting dead cell pellet was then re-suspended in 1

mL fresh R2Broth. This step was repeated one more time, after which the re-suspended dead cell pellet was diluted to the following dilutions: 0.3x, 0.2x, 0.1x, 0.05x, and 0.01x. The biofilm formation assay was then performed as described above using the original dilution (1x) as well as the mentioned dilutions of dead *Brevundimonas F16* cells. Biofilm biomass was measured after 48 hours using crystal violet assay.

Metal assays. As mentioned before, metals are known to play an important part in bacterial biofilm formation [18-20]. In particular, Mg^{2+} and Ca^{2+} are thought in some cases to provide structural support and Ni^{2+} is a stimulus for biofilm formation in several systems. In these set of experiments, double-species mutualistic biofilm formation of *Brevundimonas F16-Hydrogenophaga F14* and *Brevundimonas F16-Acidovorax F19* as well as antagonistic biofilm formation of *Brevundimonas F16-Pseudomonas C22* were tested in R2Broth supplemented with $CaCl_2$ (10μM, 20μM, 50μM, and 100μM), $MgCl_2$ (100μM, 200μM, 500μM, and 1mM), or $NiCl_2$ (100μM, 200μM, 300μM, and 400μM). Biofilm biomass was measured in these biofilms using crystal violet assay after 48 hours of incubation in ambient temperature on a shaker (100 RPM).

Tobramycin and milk protein assay. To determine if milk protein or tobramycin influences biofilm formation by *Pseudomonas C22, F. columnare, Hydrogenophaga F14, Brevundimonas F16,* and *Acidovorax F19*, wells of 96-well microtier plates containing R2Broth supplemented with 2.5% milk protein with or without tobramycin (5 μg/mL) were inoculated with single- or double-species mixtures of these species and then incubated at ambient temperature on a shaker (100 RPM) for 48 hours. After 48 hours, biofilm biomass was measured using crystal violet assay.

Measuring biofilms. The strength of biofilm-forming capabilities of bacterial species was assessed using several metrics. The first metric was biomass, which represents the mass of the entire biofilm including both live and dead cells as well as extracellular polymeric substance. Estimating biomass is equally important in both single- and double-species biofilms as it allows us to deduce whether combining certain bacterial species promotes or inhibits biofilm growth. We used a crystal violet assay [41, 44] to measure biofilm biomass of Lake Sturgeon bacterial isolates and their mixtures. A next criterion that was used to evaluate biofilm-forming potential of a bacterial species and/or species combination was metabolic activity of cells in the biofilm. Note that we could not identify the actual processes that cause a particular species to be metabolically active. However, comparisons of metabolic activity between single- and double-species biofilms allowed us to infer changes in metabolic activity that might result from mutualism, commensalism or antagonism between species. In this experiment, we used a resazurin assay [42, 45, 46] to quantify metabolic activity of biofilms. Finally, measuring presence/relative abundance of individual isolates in mixed biofilms was of interest as a method for determining the composition of the biofilm. Similarly to metabolic activity measurement, results for relative abundance provided information pertaining to the nature of bacteria-bacteria interactions within the species mixture. We used Terminal Restriction Fragment Length Polymorphism (TRFLP) to determine the presence of particular bacterial species in their double-species mixtures. These three methods are described in more detail below.

Crystal Violet assay. We estimated biofilm-forming strength of Lake Strugeon egg isolates by measuring the overall biofilm biomass using the crystal violet assay [41,

44]. Crystal violet is a triarylmethane dye that forms a bond with negatively charged molecules and polysaccharides on the surface of bacterial cells within the biofilm and/or the extracellular matrix [42]. As such, both cells, including living and dead, as well as biofilm extracellular polymeric substance are stained by crystal violet.

In our experiments, both immediately after inoculating the 96-well microtiter plates, and 48 or 96 hours after incubating the plates with single- or double-species mixtures (see the section above), we measured absorbance at 600 nm wavelength to assess the planktonic growth in R2Broth. In 48 or 96 hours cultures we transferred the pelagic cells to a new microtiter plate before measuring the absorbance. Then, we washed wells of the old plate carefully using distilled water three times and stained established biofilms with 200μL of crystal violet dye (0.1% concentration). After staining for 15 minutes, plates were rinsed twice in distilled water and then inverted in order to dry completely. To extract crystal violet stain from biofilms, we added 200μL of acetic acid (30% concentration) to each well for 15 minutes. Finally, we transferred resulting dye solution from each well to a new set of plates and estimated biofilm biomass by measuring the absorbance of each solution using a spectrophotometer at 600 nm wavelength.

Resazurin Assay. To measure cell viability within formed biofilms we used resazurin assay [42, 45, 46]. Resazurin, also known as CellTiter-Blue (CTB) or AlamarBlue, is a blue non-fluorescent dye that reduces to the pink highly fluorescent resorufin by cellular metabolic activity. This effect increases proportionally with the number of cells that are metabolically active and, thus, serves as an indicator of cell viability. In this work, we implemented the following protocol. First, we washed the wells

of 96-well microtiter plates with established biofilms three times using sterile physiological saline. Next, we added 100µl of physiological saline and 20µl of commercially available resazurin solution (CellTiter-Blue, CTB, Promega) to each well [42]. We determined the end point of the reaction by measuring fluorescence (λ_{ex}: 560 nm and λ_{em}: 590 nm) after 60, 120, 180, 240, and 300 minutes of incubation (25°C) [42]. We observed that fluorescence strength approaches its steady-state value by 180 minutes, suggesting this time span as the optimal incubation period for all chosen bacterial isolates. Extended incubation time (240 and 300 minutes) did not result in an increase of the fluorescence signal.

Terminal Restriction Fragment Length Polymorphism (T-RFLP). DNA was extracted from biofilms by adding 100µl of Alkaline-PEG (pH=13.3) (REF) to each well after washing them three times with physiological saline [47]. Microtiter plates were incubated for 15 minutes at 25°C temperature to extract biofilm. Extracted biofilm was used as template in 16S rDNA PCR amplifications using universal bacterial primers with a FAM labelled forward primer [48, 49]:

- 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') (5íFAM-labelled);
- 1389R (5'-ACG GGC GGT GTG TAC AAG-3') (unlabelled).

PCR reactions were performed in a total reaction volume of 50µL using the following protocol and conditions: 1) initial denaturation step at 94°C for 5 min; 2) 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 110s; and 3) extension step at 72°C for 7 min. Finally, PCR products were purified using QIAquick PCR purification kit (Qiagen) following the protocol supplied by the manufacturer.

Purified PCR products were digested with HhaI (Gibco BRL). The reaction mixture was comprised of 2.0 µL of 10X reaction buffer (Gibco BRL), 0.3µL of enzyme (20U/µL, Gibco BRL), approximately 200ng of purified PCR product and sterile water to 20 µl total. Digestion was for 2 hours at 37^{o}C. Two replicates (10µL each) of each digested DNA sample, along with un-cut samples serving as a control, were sent to to Michigan State University's sequencing facility for terminal fragment analysis. The DNA fragments were separated on an ABI Genetic Analyzer 3130XL (Applied Biosystem) and ROX 1000 size standard was incorporated into each sample. 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 Peak-Scanner^{TM} Software v2.0 (Applied Biosystem). Fragments smaller than 50 bases and less than 50 florescence units were removed from the analysis. The T-RFLP profiles were binned with T-Align, (http://inismor.ucd.ie/~talign/index.html) and the relative amounts of specific ribotypes were determined by calculating the peak areas. 16S rDNA sequences of the Lake Sturgeon egg isolates were used to determine expected fragment length produced after restriction digestion using HhaI. We used the intensity of fluorescence signal (peak height) associated with each of these fragment sizes to estimate relative abundance of each species in a mixed culture biofilm. Essentially, the peak height of the fluorescence signal is larger for more abundant bacterial species in double-species biofilms.

Statistical analysis. To test whether the difference in means is statistically significant an analysis of variance was performed (One-way ANOVA) using R version 3.2.3 [50]. If the null hypothesis of equal means were rejected, then Tukey's Honest

Significant Differences test (95% confidence intervals) was performed for pair-wise comparisons between group means with correction for multiple testing in order to determine what is driving the difference in means. Results for Tukey HSD were reported as p adj. values (p-value after adjustment for the multiple comparisons).

RESULTS

To identify bacterial species among egg isolates that show mutualism or antagonism during biofilm formation *in vitro,* we chose six Lake Sturgeon egg isolates: *Massilia* B13*, Pseudomonas* C22, *Pseudomonas* D2, *Acidovorax* F19, *Hydrogenophaga* F14, and *Brevundimonas* F16. These isolates were in high abundance on healthy egg surfaces.

Phylogenetic analysis: 16S rRNA gene sequencing. Taxonomy results based on Sanger sequencing of 16s rRNA gene of four Lake Sturgeon egg isolates (*Pseudomonas C22*, *Hydrogenophaga F14*, *Brevundimonas F16*, and *Acidovorax F19*) confirmed the sequences to the genus level but not to the species level (Table 1). *Pseudomonas C22*, *Hydrogenophaga F14, Brevundimonas F16*, and *Acidovorax F19* were most closely related to the following type species respectively: *Pseudomonas fluorescens, Hydrogenophaga atypica*, *Brevundimonas subvibrioides*, and *Acidovorax radicis*. These results were also confirmed by pairwise average nucleotide identity (ANI) analysis between the egg isolates genomes and the existing sequenced genomes of the species from the same genus (Appendix A).

Egg Isolate	F14	F16	F19	C22
Domain	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria	Proteobacteria	Proteobacteria
Class	Betaproteobacteria	Alphaproteobacteria	Betaproteobacteria	Gammaproteobacteria
Order	Burkholderiales	Caulobacterales	Burkholderiales	Pseudomonadales
Family	Comamonadaceae	Caulobacteraceae	Comamonadaceae	Pseudomonadaceae
Genus	Hydrogenophaga	Brevundimonas	Acidovorax	Pseudomonas
Most closely related type species	*Hydrogenophaga atypica* (S_ab score: 0.926; unique common oligomers: 1425)	*Brevundimonas subvibrioides* (S_ab score: 0.929; unique common oligomers: 1347)	*Acidovorax radicis* (S_ab score: 0.963; unique common oligomers: 1405)	*Pseudomonas fluorescens* (S_ab score: 0.942; unique common oligomers: 1445)

Table 2.1. Taxonomy results based on Sanger sequencing of 16S rRNA gene of the four Lake Sturgeon egg isolates S_ab score: This is the number of unique 7-base oligomers shared between the egg isolate sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences. An S_ab score of 1.000 indicates a perfect match. An S_ab score of ≤ 0.975 suggests that the sequences belong to two different bacterial species [53].

Formation of single- and mixed-species biofilms. Biofilm studies included *Pseudomonas aeruginosa PA01* as a positive control for biofilm formation. This species produces large amounts of biofilm (from 1.5-3.5 A600nm) depending on the media and length of incubation. The standard crystal violet assay was used to measure biofilm formation (48 hrs) in single and mixed cultures. In single species cultures, *Massilia B13*, *Pseudomonas C22*, *Pseudomonas D2*, and *Hydrogenophaga F14* formed modest levels of biofilm biomass, while *Acidovorax F19* showed stronger biofilm forming capability and *Brevundimonas F16* formed a robust biofilm (Fig. 1).

In mixed cultures of two species, all tested species formed more biofilm biomass compared to their single-species counterparts when paired with *Massilia B13*, with the

exception of *Acidovorax F19*. Although not statistically significant (p adj.= 0.99), even *Pseudomonas C22* had a modest increase in biofilm biomass when paired with *Massilia B13*. When *Pseudomonas C22* was paired with *Massilia B13, Hydrogenophaga F14* (p adj.= 0.72), or *Acidovorax F19* (p adj.= 0.0000033), more biofilm was formed compared to *Pseudomonas C22* alone (Fig. 1). Co-culturing *Brevundimonas F16* with *Pseudomonas C22* (p adj.= 0) or *Pseudomonas D2* (p adj.= 0) had a strong negative effect on biofilm production. *Acidovorax F19* also appeared antagonistic to *Hydrogenophaga F14*. When the 3-, 4-, 5- and 6-member cultures were measured for biofilm production after 48 hours, the only weakly positive biofilm producing mixtures contained *Brevundimonas F16* in the absence of *Pseudomonas C22*.

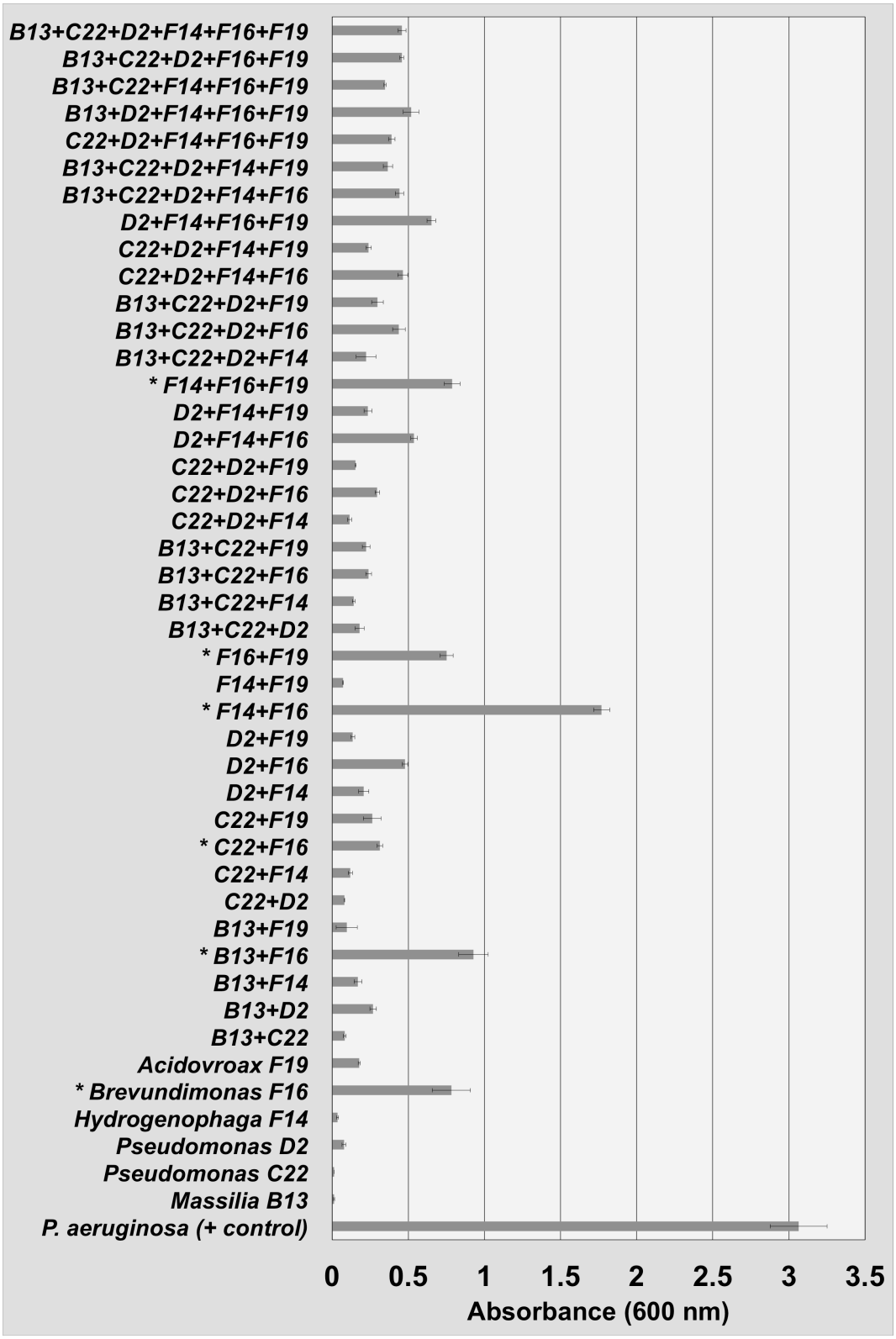

Figure 2.1. *In vitro* biofilm formation after 48 hours in single and mix cultures of six selected healthy Lake Sturgeon egg isolates (3 replicates). Overall ANOVA results: F(44, 90) = 356.4, p = 2e-16.

Pseudomonas C22 exhibited antimicrobial activity against many of the Lake Sturgeon egg isolates as well as six common fish pathogens [39]. To investigate how this activity might affect biofilm formation of *Massilia* B13*, Acidovorax* F19, *Hydrogenophaga* F14, and *Brevundimonas* F16, we measured biofilm formation of these species after 48 hours of co-culture with *Pseudomonas C22* (Fig. 2). Furthermore, we investigated the effect of *Pseudomonas C22* antimicrobial activity on established biofilms of these species. All mixed cultures with *Pseudomonas C22* produced biofilm biomass lower than their single-species counterparts. Furthermore, the biomass of established 48 hr. biofilm of *Brevundimonas F16* decreased significantly when exposed to *Pseudomonas C22* culture for 48 hours (p adj.= 0). These results were consistent with Fujimoto's finding of this species expressing antimicrobial activity in soft agar overlays [39].

To distinguish effects of adding fresh media and introducing *Pseudomonas C22* on development of established biofilms, we supplemented established 48 hr. biofilms of above species with either fresh overnight culture of the same species or fresh R2Broth medium and continued the incubation under the same conditions for another 48 hours (Fig. 2). *Acidovorax F19* had a fairly low level of biofilm biomass and showed little change as a consequence of longer incubation times or supplementation at two days. *Brevundimonas F16* showed high biofilm formation at 2 and 4 days, which was substantially (but not significantly, p adj.= 0.0605) boosted by supplementation with fresh media at 2 days and significantly increase by the addition of overnight culture (p adj.= 0).

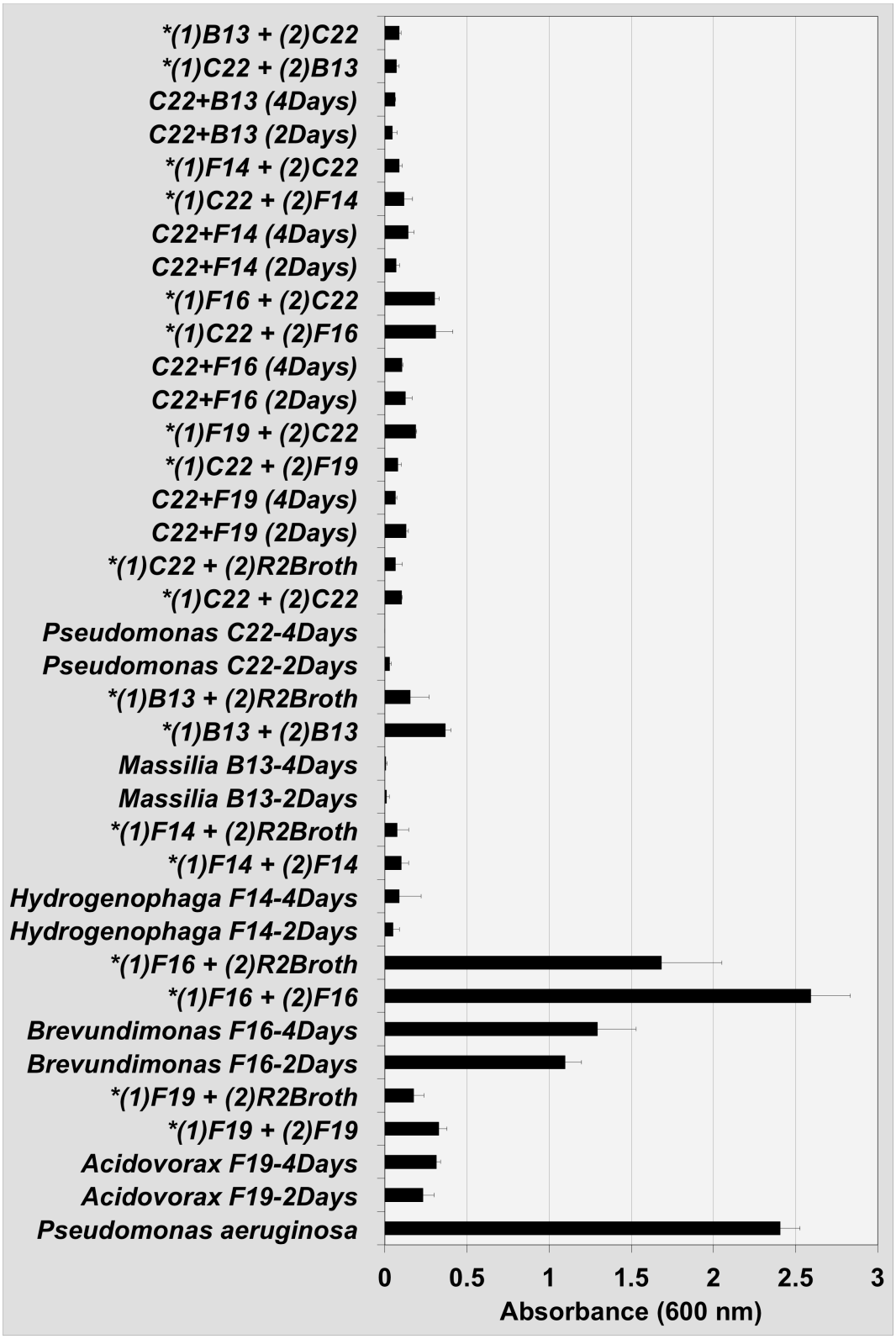

Figure 2.2. Effects of *Pseudomonas C22* antimicrobial activity on biofilm formation (co-cultured) or established biofilms of other Lake Sturgeon egg isolates (4 replicates). Overall ANOVA results: $F(36, 111) = 170.4$, $p = 2e\text{-}16$.

Hydrogenophaga F14, *Pseudomonas C22* and *Massilia B13* had only modest biofilm formation at 2 and 4 days with no change in *Hydrogenophaga F14* or *Pseudomonas C22* with supplementation of fresh media or new culture at 48 hours. *Massilia B13* showed a modest but statistically significant increase in biofilm after supplementation with fresh culture (p adj.= 0.00031) but not with fresh media (p adj.= 0.94).

While *Masillia B13* appeared to be an interesting isolate in terms of its biofilm forming abilities, and association with healthy eggs, we excluded this species from studies because of difficulties with growth (frequently forming clumps). In the experiments that followed we focused on *Pseudomonas C22* (antimicrobial activity), *Acidovorax F19* (indication of probiotic effect) [37], and *Brevundimonas F16* and *Hydrogenophaga F14* (apparent strong mutualism in the formation of biofilms).

***Brevundimonas* titration assays.** Data suggested *Brevundimonas F16* is an interesting isolate in how it forms biofilm and interacts with other species. To further explore these interactions we titrated an inoculum of *Hydrogenophaga F14* or *Acidovorax F19* with increasing amounts of live *Brevundimonas F16*. In both cases there was a proportional increase in the amount of biofilm biomass produced with increasing amounts of *Brevundimonas F16* (Fig. 3).

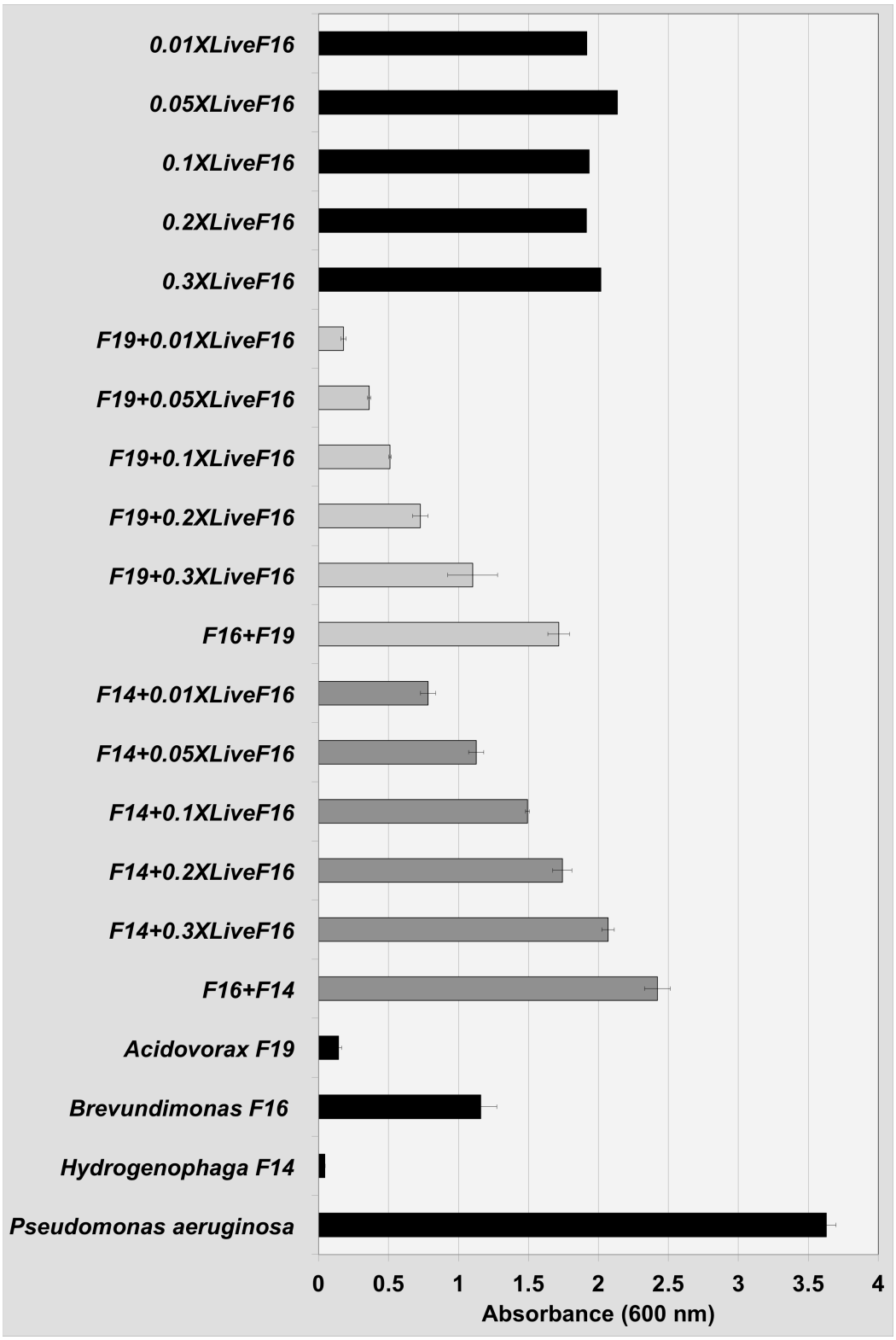

Figure 2.3. Changes in the total biomass produced in 48 hr. biofilms when initially an inoculum of *Hydrogenophaga F14* or *Acidovorax F19* was titrated with increasing amounts of *Brevundimonas F16* (Live titration) (12 replicates). Overall ANOVA results: $F(20, 231) = 71.47$, $p = 2e\text{-}16$.

When an inoculum of *Hydrogenophaga F14* was titrated with increasing amounts of ethanol-killed *Brevundimonas F16*, the biofilm biomass was approximately 20% of the live mixed culture, but 4x larger than *Hydrogenophaga F14* alone (Fig. 4). When *Acidovorax F19* was titrated with dead *Brevundimonas F16*, the biofilm biomass at the highest *Brevundimonas* F16 concentration was 25% of the live mixture (2.5x *Acidovorax* pure culture biofilm). At lower concentrations biofilm was approximately 10% of the live mixture and close to what was observed with *Acidovorax F19* alone. The amount of biofilm produced with all concentrations of ethanol-killed *Brevundimonas F16* alone was negligible.

The effect of metals on biofilm formation. Metals are important in biofilm formation. Figures 5, 6, and 7 show how three of these metals (Mg^{2+}, Ca^{2+}, and Ni^{2+}) influenced formation of single- and double-species biofilms. We used *Brevundimonas F16* as the isolate with the highest biofilm forming capabilities *in vitro*, its double-species biofilms with potential mutualists *Hydrogenophaga F14* and *Acidovorax F19*, and its double-species biofilm with the antagonist *Pseudomonas C22*. Biofilm biomass was measured after 48 hours of inoculation and exposure to different concentrations of these metals using the crystal violet assay.

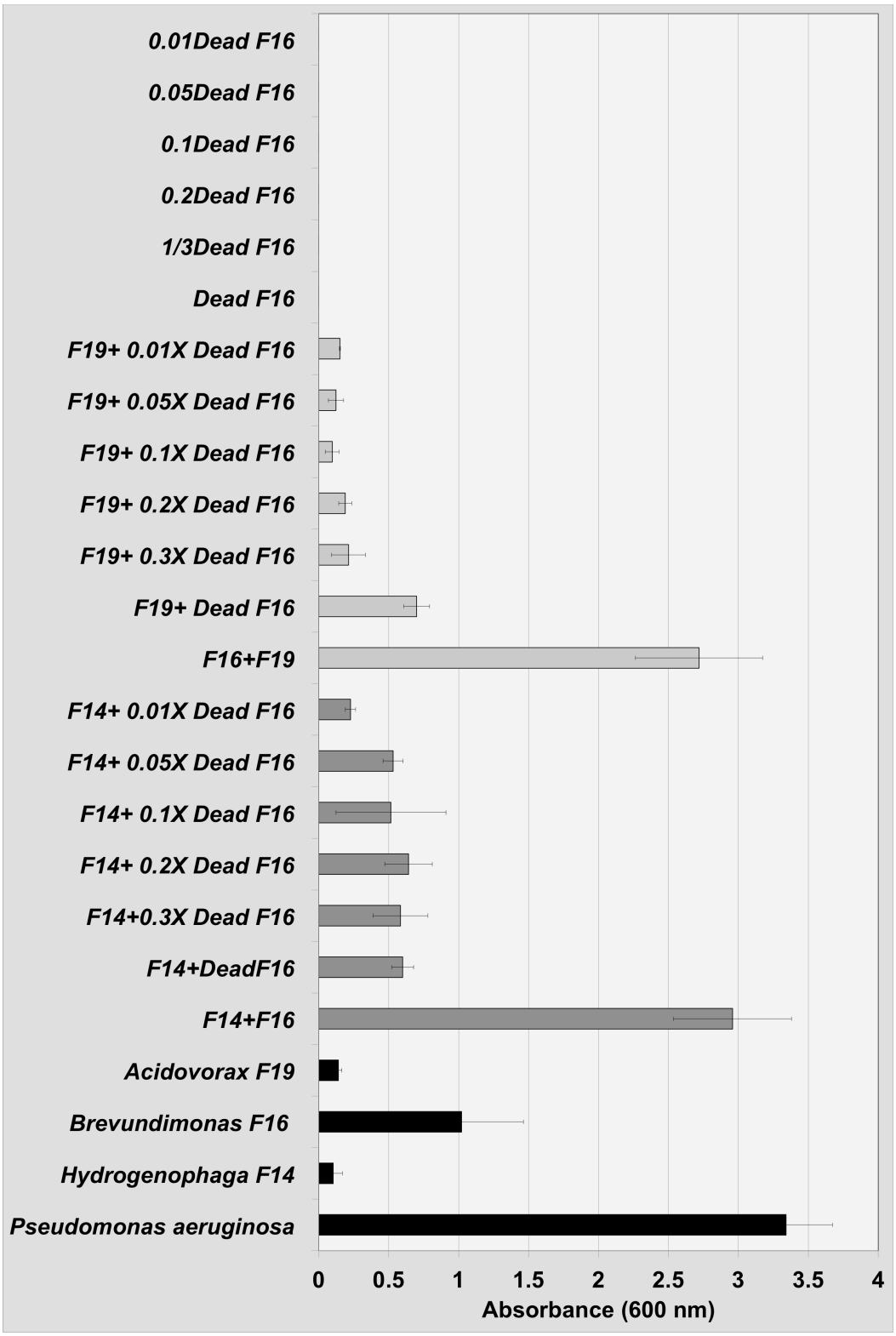

Figure 2.4. Changes in total biomass produced in 48 hr. biofilms when initially an inoculum of *Hydrogenophaga F14* or *Acidovorax F19* was titrated with increasing amounts of ethanol killed *Brevundimonas F16* (Dead titration) (12 replicates). Overall ANOVA results: $F(20, 231) = 296.8$, $p = 2e\text{-}16$.

Increasing concentrations of Mg^{2+} had no effect on biofilm production of single- and double-species cultures with the exception of *Hydrogenophaga F14,* which exhibited a substantial increase in biofilm biomass as Mg^{2+} concentration went up (Fig. 5).

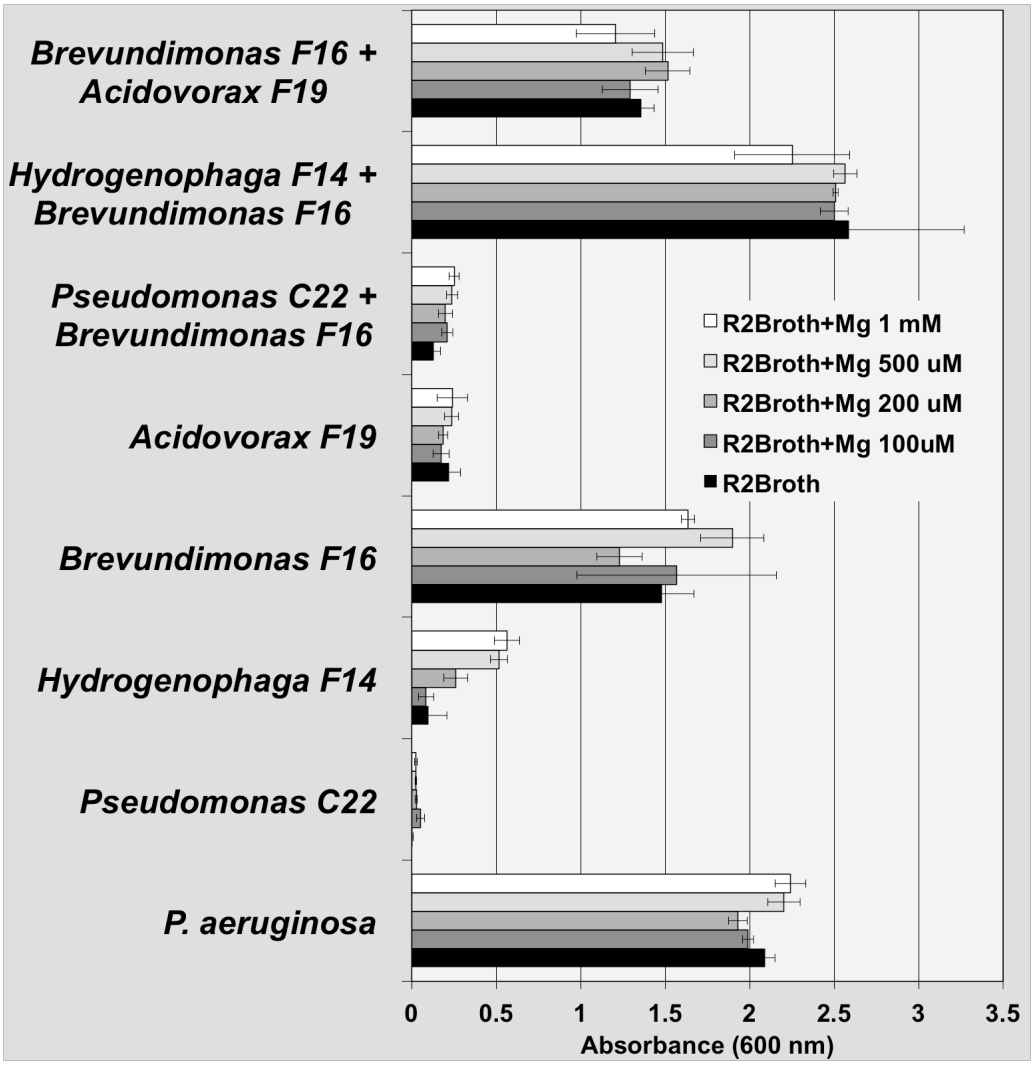

Figure 2.5. Biofilm formation in *Brevundimonas* F16, and its double-species mixes with *Hydrogenophaga* F14, *Acidovorax* F19 or *Pseudomonas* C22 in the presence of different concentrations of Mg^{2+} (4 replicates). Biofilm biomass was measured 48 hours after inoculation and exposure to the metals.

Elevated Ca^{2+} concentrations (50μM and 100μM) increased biofilm biomass formation in *P. aeruginosa* [F(4, 15)= 150.6, p= 6.47e-12] and to a lesser degree in

Brevundimonas F16 [F(4,15)= 2.68, p=0.0993]. However, these concentrations of Ca^{2+} decreased the amount of biofilm biomass detected in the mixed culture samples (*Hydrogenophaga F14-Brevundimonas F16* and *Brevundimonas F16-Acidovorax F19*) [$F^{F14+F16}(4, 15)= 9.267$, p= 0.00056, and $F^{F16+F19}(4 , 15)= 89.58$, p= 2.78e-10, respectively].

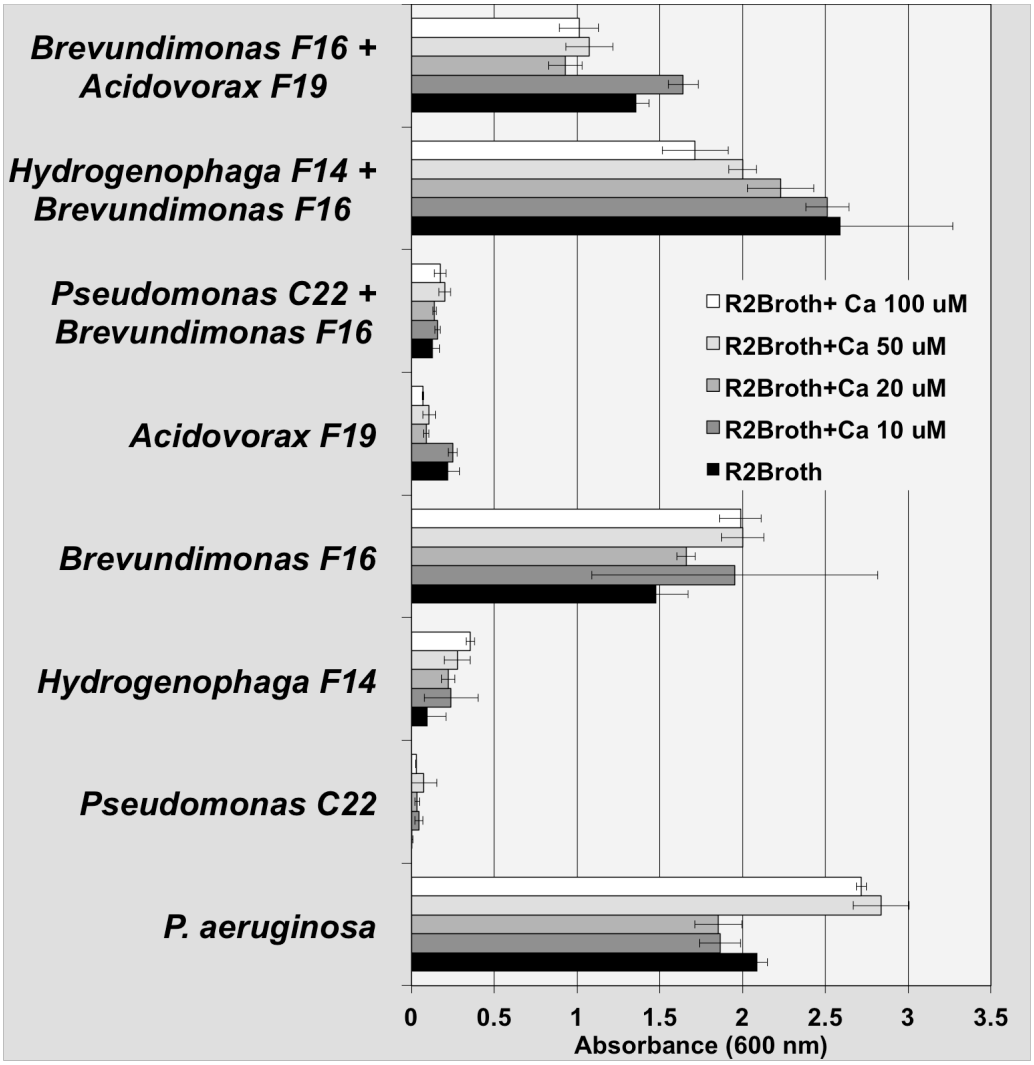

Figure 2.6. Biofilm formation in *Brevundimonas* F16, and its double-species mixes with *Hydrogenophaga* F14, *Acidovorax* F19 or *Pseudomonas* C22 in the presence of different concentrations of Ca^{2+} (4 replicates). Biofilm biomass was measured 48 hours after inoculation and exposure to the metals.

Increasing concentrations of Ni^{2+} decreased the biofilm biomass in *P.aeruginosa* [F(4, 15)= 4483, p= 2e-16], *Brevundimonas F16* [F(4, 15)= 1179, p= 2e-16], *Brevundimonas F16-Hydrogenophaga F14* [F(4, 15)= 87.2, p= 3.37e-10], and *Brevundimonas F16-Acidovorax F19* [F(4, 15)= 366.4, p= 9.29e-15] samples. *Brevundimonas F16* seemed particularly susceptible to Ni^{2+}. Ni^{2+} had little or no effect on *Hydrogenophaga F14*, *Acidovorax F19*, *Pseudomonas C22* and the *F16-C22* mixture.

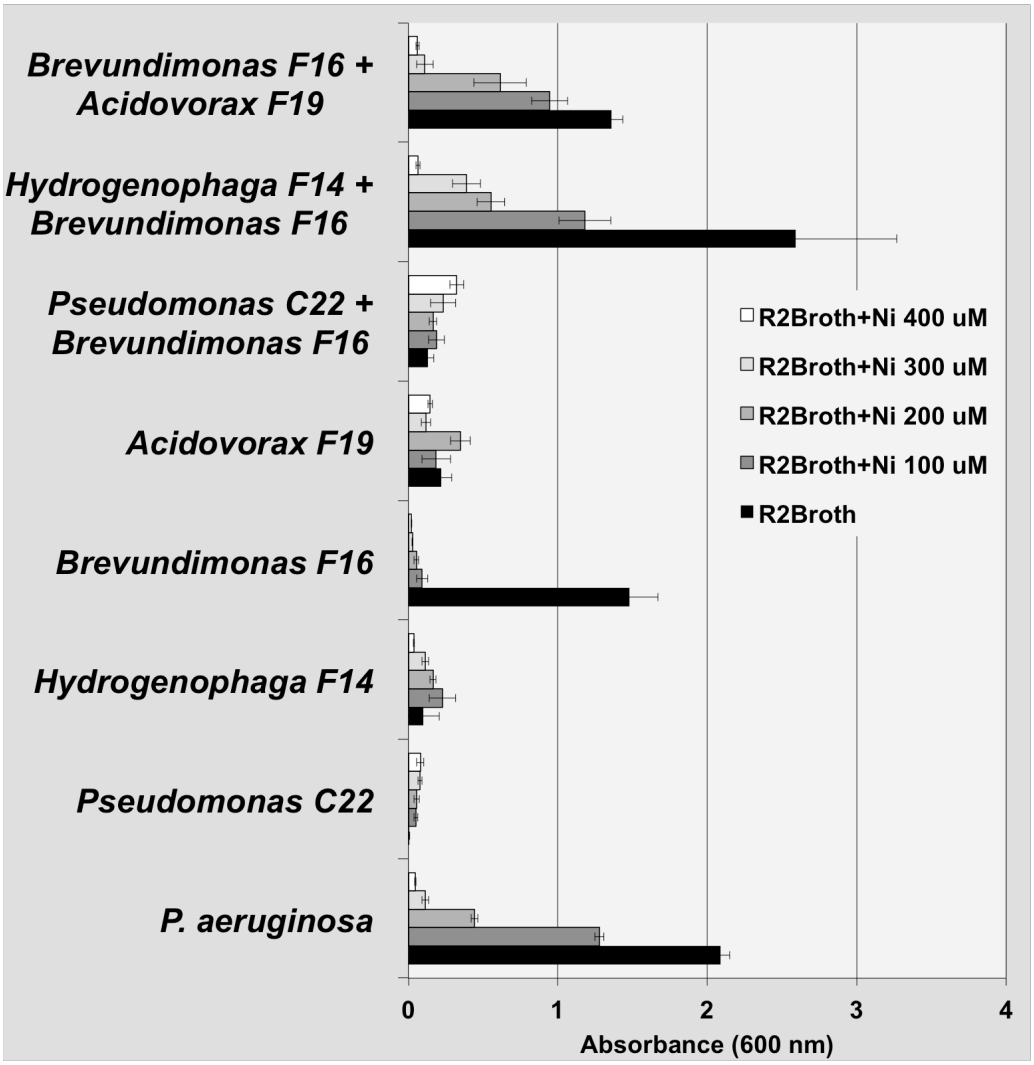

Figure 2.7. Biofilm formation in *Brevundimonas* F16, and its double-species mixes with *Hydrogenophaga* F14, *Acidovorax* F19 or *Pseudomonas* C22 in the presence of different concentrations of Ni^{2+} (4 replicates). Biofilm biomass was measured 48 hours after inoculation and exposure to the metals.

Metabolic activity of biofilms measured with resazurin. In addition to crystal violet to measure biofilm biomass, we used resazurin assay to measure metabolic activity. To further extend our biofilm analyses we included *Pseudomonas* C22-*Flavobacterium columnare* mixed culture biofilm to our double-species biofilms (*Hydrogenophaga F14-Brevundimonas F16* and *Brevundimonas F16-Acidovorax F19*) because Fujimoto's previous results showed that although *Pseudomonas C22* had a negative effect on the growth of some fish pathogens, when co-cultured with *Flavobacterium columnare,* it appeared to enhance biofilm formation [39]. Furthermore, to determine the phylogenetic composition of mixed culture biofilms we established double species cultures and then determined the fraction of each population in the biofilm using T-RFLP. The results are presented in figures 8-12.

Figure 8 illustrates biofilm biomass changes in single and double species cultures. In addition, it also summarizes the results of "invasion" tests where we examined changes in established biofilm when a different bacterial species is introduced. Again, we focus here on the three double species mixes mentioned above (*Pseudomonas C22-F. columnare, Hydrogenophaga F14-Brevundimonas F16,* and *Brevundimonas F16-Acidovorax F19*).

P. aeruginosa (positive control) produced a robust biofilm biomass at both 48 and 96 hours. Single species biofilms were similar to levels presented in previous figures. Specifically, *Brevundimonas F16* produced substantially more biomass compared to *Hydrogenophaga F14* (p adj.= 0) *and Acidovorax F19* (p adj.= 0).

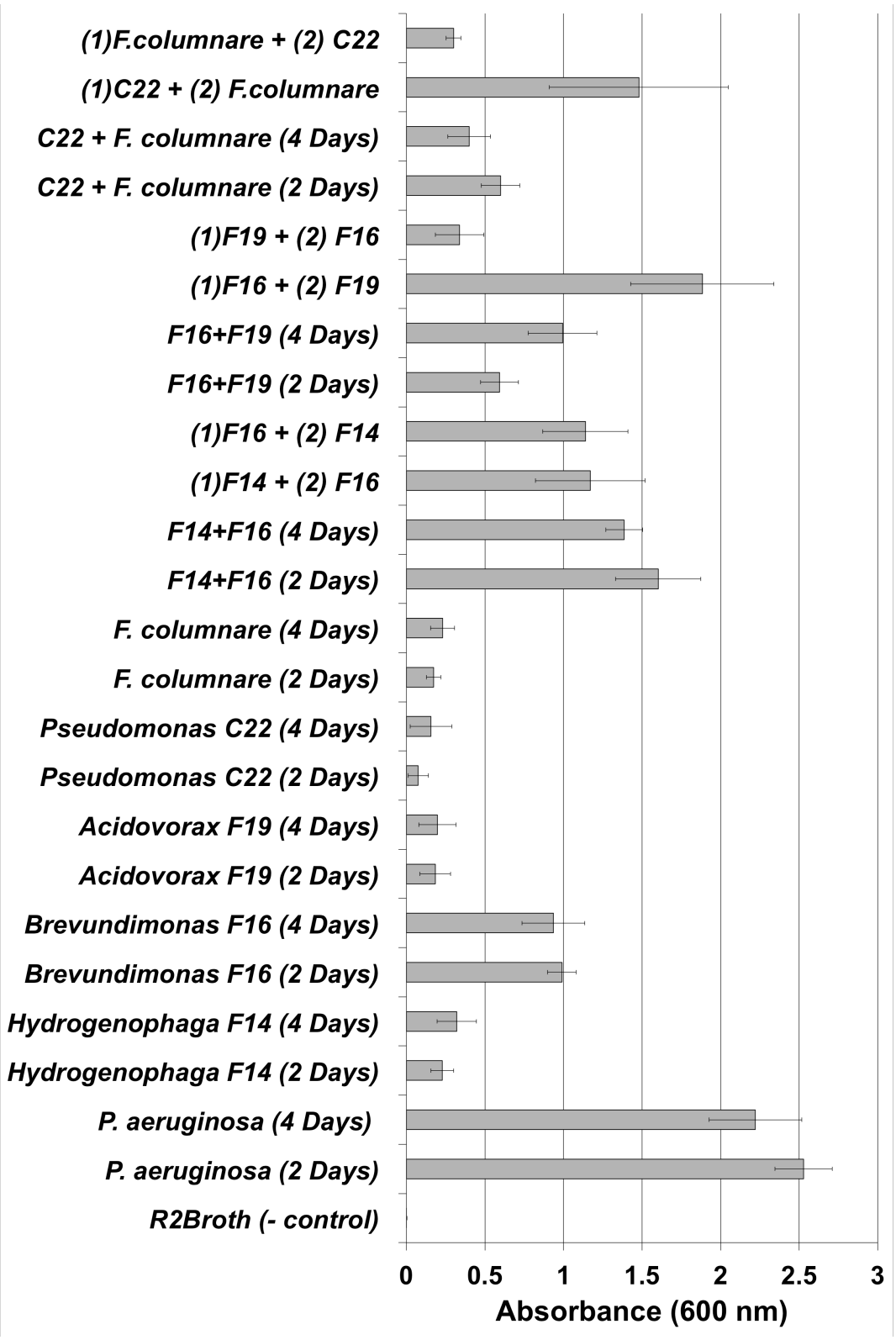

Figure 2.8. Changes in biofilm biomass in single and mix cultures, as well as "invasion" tests as measured by crystal violet assay (12 replicates). Overall ANOVA results: F(29, 342) = 124.6, p = 2e-16.

As expected from previous experiments, biomass of mixed culture biofilms was close to the combined biomass of their single species. The biomass of "invasion" biofilms was not statistically different from 2 and 4 day mixed culture biofilms of *Brevundimonas F16-Hydrogenophaga F14* (p adj.^{F14F16}= 0.79 and p adj.^{F16F14} = 0.51 respectively). In "invasion" tests of the mixed culture biofilms of *Brevundimonas F16-Acidovorax F19* however, 48-hour biofilms of *Brevundimonas F16* appeared capable of recruiting *Acidovorax F19* whereas the reverse was not true as measured by crystal violet assay (Fig. 8). In mixed culture biofilms of *Pseudomonas C22-F. columnare* when *Pseudomonas C22* was allowed to establish a biofilm, subsequent challenge with *F. columnare* culture stimulated biofilm biomass production significantly (p adj.= 0) (Fig. 8). This was not the case in the reverse order (p adj.= 1).

Metabolic activities as measured by resazurin assays did not parallel the biomass amounts measured with crystal violet assay (Fig. 9). The metabolic activity of the positive control, *P. aeruginosa*, was less than *Brevundimonas F16* even though *P. aeruginosa* had twice as much biofilm biomass as measured with crystal violet. Furthermore, the metabolic activity of *Hydrogenophaga F14* was less than *Brevundimonas F16*, and roughly paralleled with measured biomass amounts. Metabolic activity of mixed culture biofilms of *Brevundimonas F16-Hydrogenophaga F14* tended to be slightly greater than the additive of single-species metabolic activity measurements, although this increase was not statistically significant. The metabolic activity of mixed culture biofilms of *F. columnare-Pseudomonas C22* was higher than combined single-species measurements (Fig. 9). Also, in the "invasion" tests the metabolic activity of the biofilm followed the biomass levels.

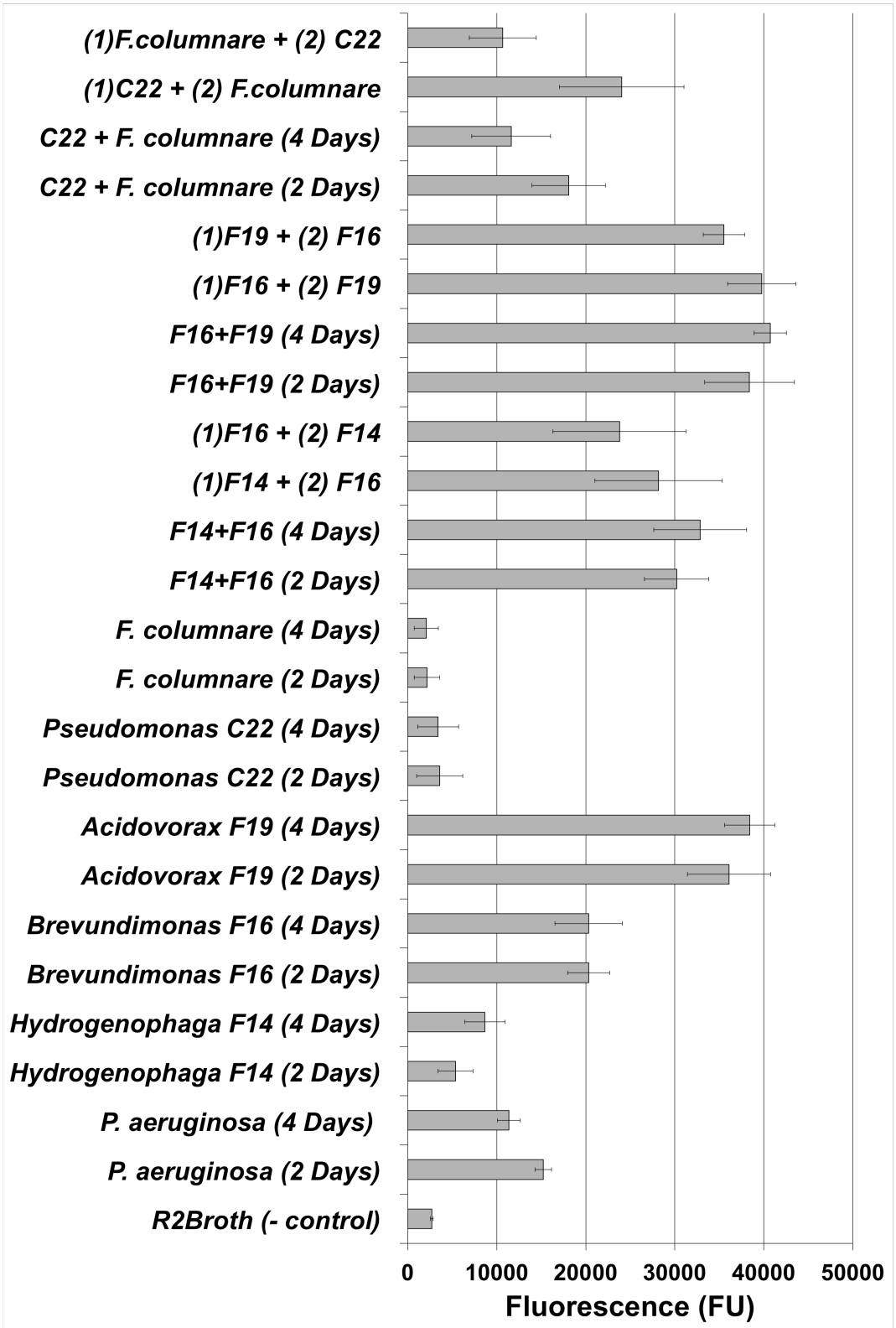

Figure 2.9. Changes in biofilm metabolic activity in single and mix cultures, as well as "invasion" tests as measured by resazurin assay (8 replicates). Overall ANOVA results: $F(29, 258) = 90.91$, $p = 2e\text{-}16$.

In spite of significantly lower biomass (~30% of *Brevundimonas F16*, and ~13% of *P. aeruginosa*), *Acidovorax F19* had the highest metabolic activity of all four Lake Sturgeon egg isolates. The metabolic activity of this isolate was twice as much as *P. aeruginosa* (p adj.= 0) and ~30% more than *Brevundimonas F16* (p adj.= 0) as measured by resazurin assay. Moreover, the activities of mixed and "invasion" biofilms of *Brevundimonas F16* and *Acidovorax F19*, reflected the high activity of *Acidovorax F19* (p adj.= 1), undiminished by the co-cultured species.

The species demographics as measured by T-RFLP indicated that in all *Brevundimonas F16-Hydrogenophaga F14* mixed-culture biofilms, *Brevundimonas F16* was the dominant population at approximately 95% - even in the 2- and 4-day double-species biofilms with higher biomass and metabolic activity (Fig. 10). In *Brevundimonas F16-Acidovorax F19* mixed-culture biofilms, although *Brevundimonas F16* was the dominant population in these biofilms, *Acidovorax F19* maintained a substantial fraction, up to 30% in some cases, of these biofilms. Interestingly, the demographics estimated by T-RFLP suggested that *F. columnare* was a minor component of the double-species biofilms (no more than 5%) (Fig. 11).

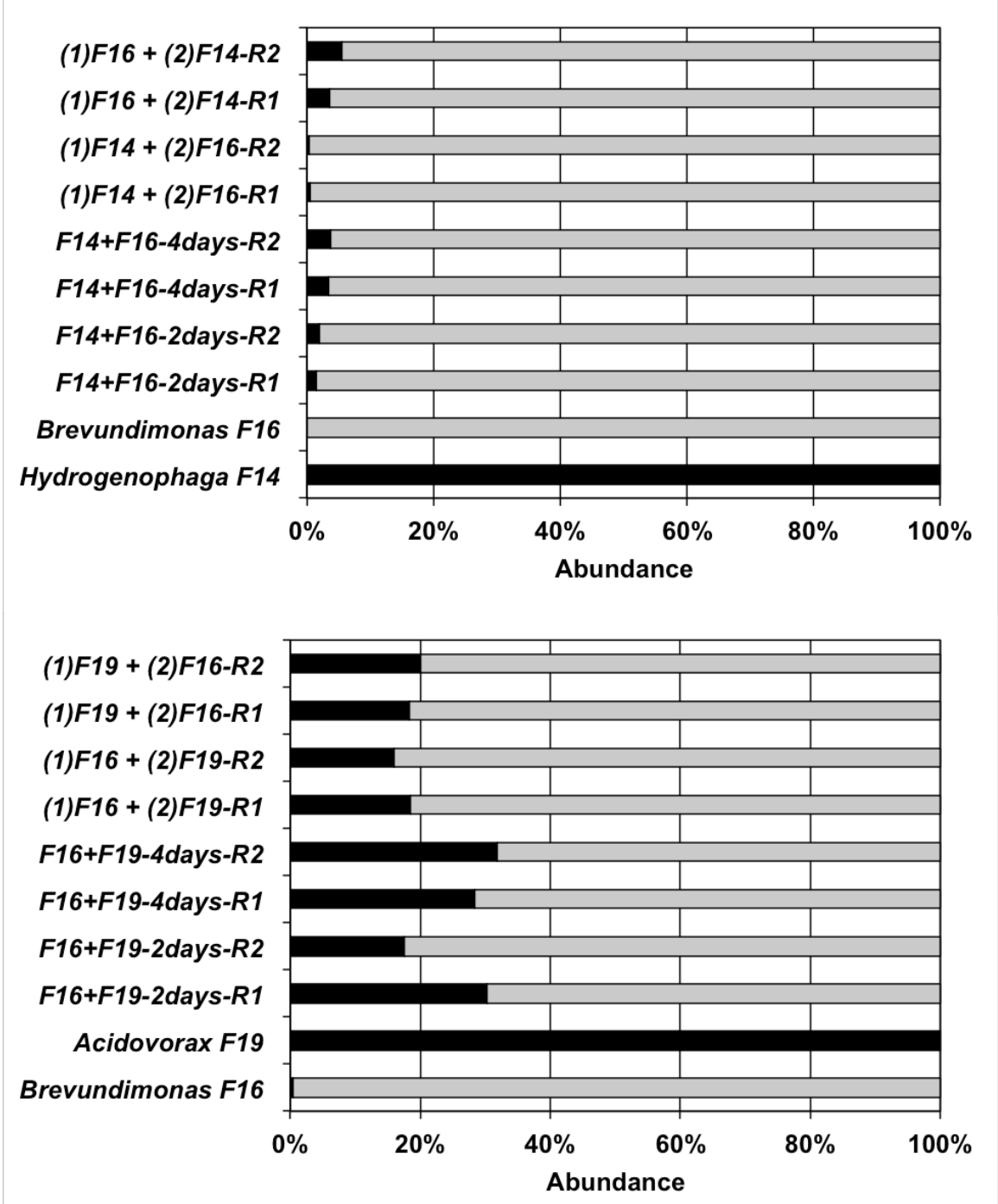

Figure 2.10. The presence/relative abundance of *Hydrogenophaga* F14, *Brevundimonas* F16, and Acidovorax F19 in 2 and 4 days single- and double-species biofilms, as well as "invasion" biofilms as measured using T-RFLP (based on the abundance of expected forward terminal fragments after digestion with HhaI) R1= biological replicate 1, R2= biological replicate 2.

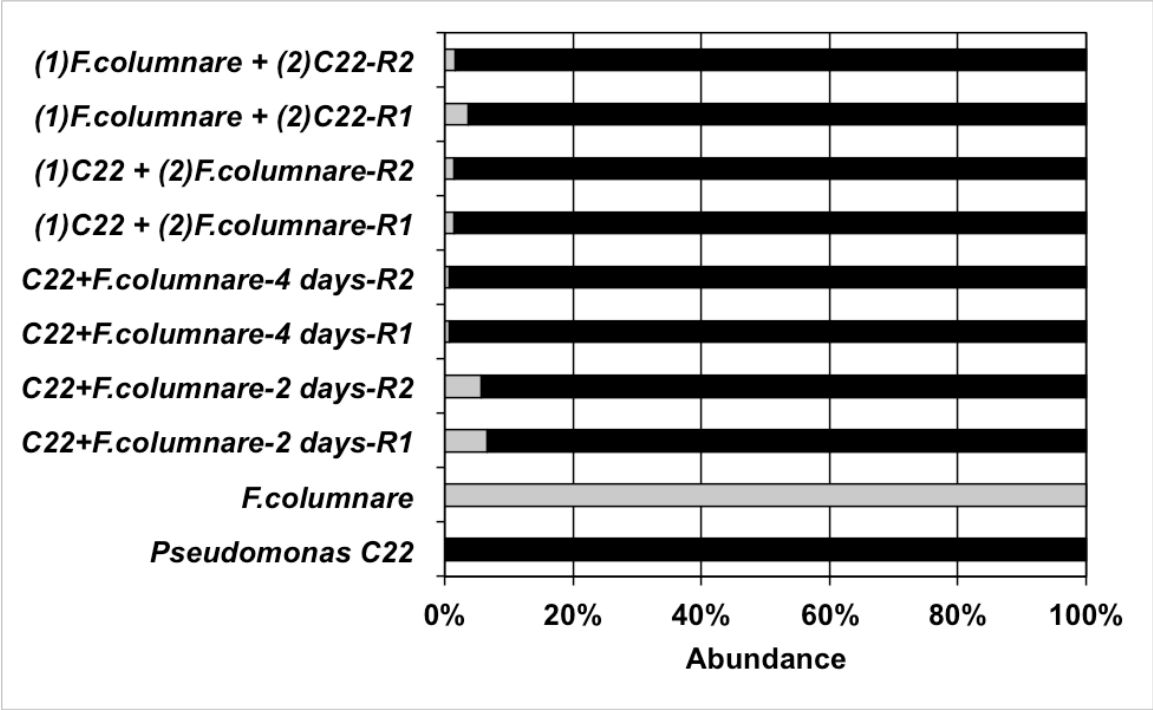

Figure 2.11. The presence/relative abundance of *Pseudomonas* (C22) and *Flavobacterium columnare* in 2- and 4-days single- and double-species biofilms, as well as "invasion" biofilms as measured using T-RFLP (based on the abundance of expected forward terminal fragments after digestion with HhaI) R1= biological replicate 1, R2= biological replicate 2.

Tobramycin and milk protein assay. Previous work in our lab (Ye et al. submitted) has shown that the addition of milk protein (MP) to cultures of selected species can greatly promote biofilm formation. In addition, although traditional antibiotics (eg. tobramycin) used against bacterial infections have been shown to decrease biofilm formation in various single-species bacterial biofilms, these antibiotics were not effective when the same species were found in mixed species biofilms [62]. We further tested the effect of milk protein and tobramycin on biofilm formation of *P. aeruginosa* as well as single and mixed species cultures of *Pseudomonas C22, F. columnare, Hydrogenophaga F14, Brevundimonas F16,* and *Acidovorax F19*. Single species and mixed culture biofilm biomass were as expected from results of previous

experiments (Fig. 12). *P. aeruginosa* biofilms was not enhanced by milk protein and was diminished by tobramycin, however, was not affected by tobramycin when milk protein was present. Regarding *Pseudomonas C22*, no statistical difference was observed between biofilm biomass formed in the presence or absence of tobramycin (p adj.= 1) but the biofilm level was low. However, the addition of 2.5% milk protein enhanced the biofilm formation of *Pseudomonas C22* (p adj.= 0.0064) and the addition of tobramycin to *Pseudomonas C22* cultures supplemented with milk protein decreased the amount of biofilm biomass (p adj.= 0.75). *F. columnare* produced good biofilm under these conditions that was significantly inhibited by tobramycin and greatly enhanced when 2.5% milk protein was added (p adj.= 0). The addition of tobramycin to *F. columnare* cultures supplemented with milk protein had no effect on the high biofilm biomass (p adj.= 0.98).

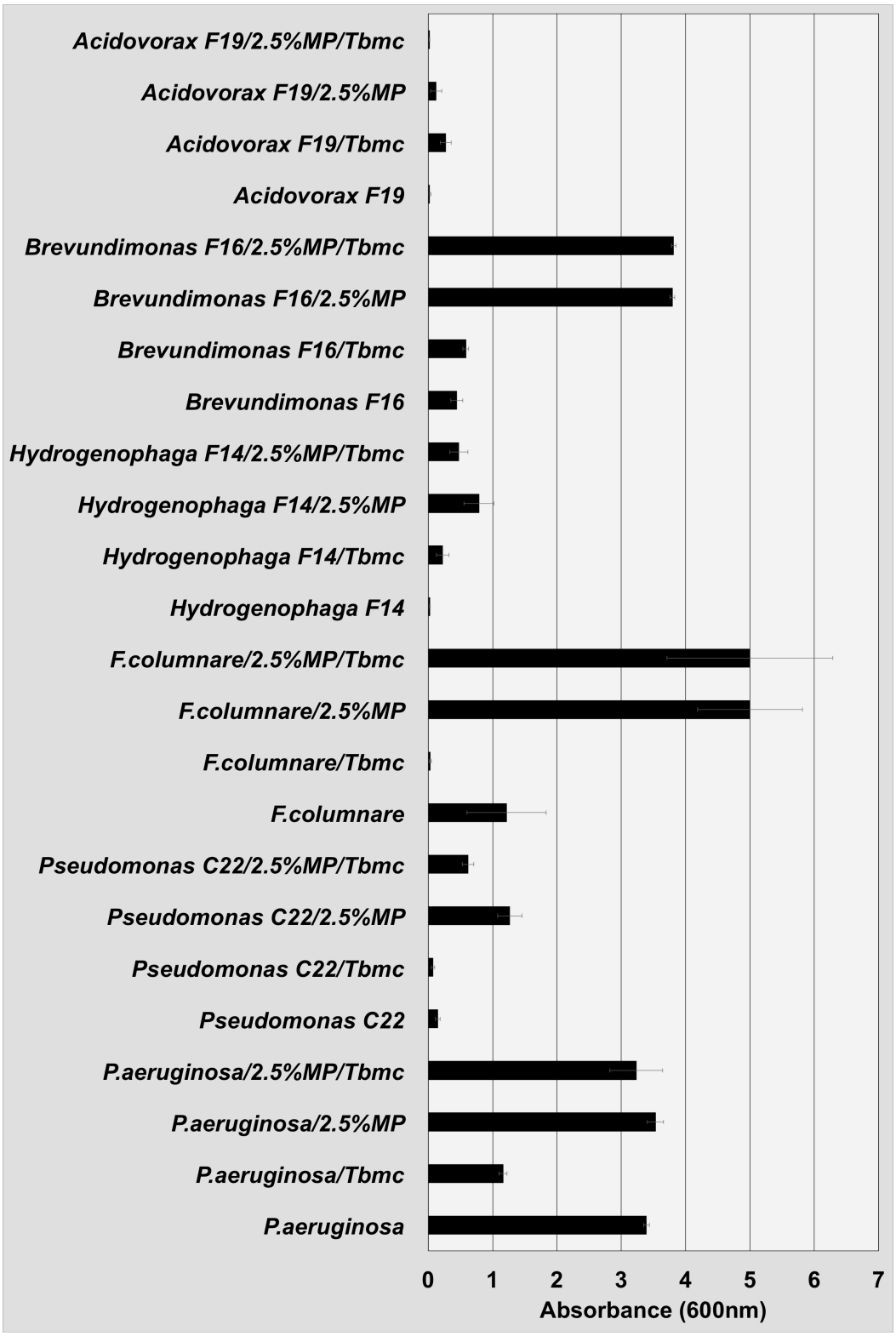

Figure 2.12. Biofilm formation of four Lake Sturgeon isolates and *F. columnare* in the presence of 2.5% milk protein (MP) and/or tobramycin (Tbmc) as measured by crystal violet (4 replicates). Overall ANOVA results for data presented on figures 12 and 13: $F(35, 108) = 801.8$, $p = 2e\text{-}16$.

No statistical difference was observed between biofilm biomass of *Hydrogenophaga F14* (p adj.= 1), and *Brevundimonas F16* (p adj.=1) with or without tobramycin. When the cultures were supplemented with milk protein, *Hydrogenophaga F14* had a modest increase in biofilm production, which was diminished when tobramycin was added (p adj.= 0.41). Supplementing *Brevundimonas F16* cultures with milk protein enhanced the biofilm biomass of this species substantially (p adj.= 0). Addition of tobramycin to these cultures had no effect on the high biofilm biomass of these cultures (p adj.= 1). *Acidovorax F19* produced modest level of biofilm with or without tobramycin and milk protein.

The mixed culture of *Pseudomonas C22* and *F. columnare* produced modest biofilm biomass that was decreased with addition of tobramycin (Fig. 13), although this decrease was not statistically significant. The mixed culture of *Pseudomonas C22-F. columnare* produced robust biofilm biomass with the addition of milk protein (p adj.= 0) although only about 60% as much as the single species biofilm of *F. columnare* alone in these conditions. The addition of tobramycin had no effect on the mixed biofilm of *Pseudomonas C22-F. columnare* when milk protein was present. Mixed cultures of *Hydrogenophaga F14-Brevundimonas F16* produced more biofilm than either alone (p adj.= 1), while addition of milk protein resulted in further increase in biomass (p adj.= 0). In both cases, the biofilms were resistant to tobramycin. The modest level of biofilm produced by mixed cultures of *Brevundimonas F16-Acidovorax F19* was enhanced slightly by tobramycin (p adj.= 0.99) and more by milk protein (p adj.= 0.087).

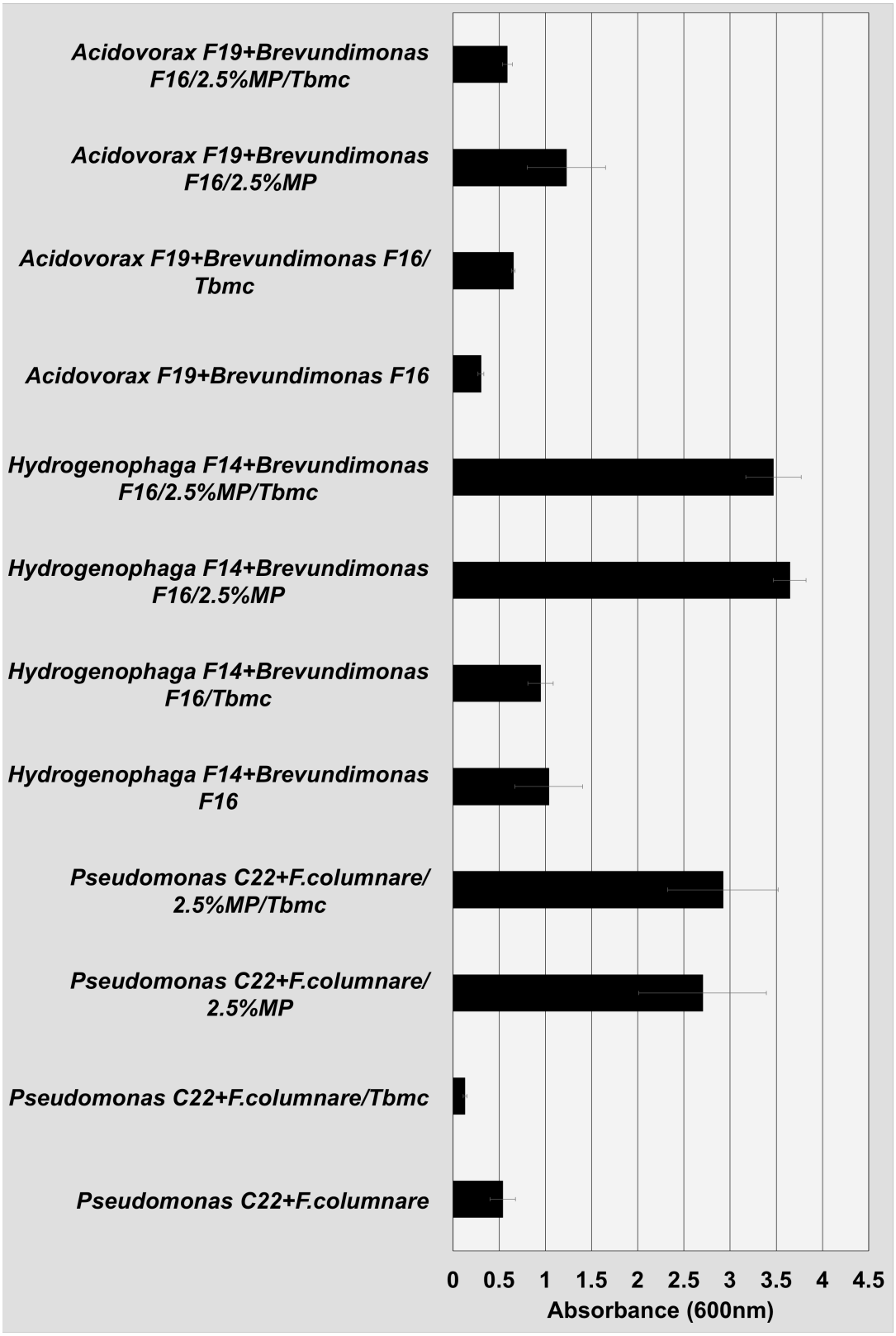

Figure 2.13. Biofilm formation of mixed cultures in the presence of 2.5% milk protein (MP) and/or tobramycin (Tbmc) as measured by crystal violet (4 replicates).

DISCUSSION

In search of probiotics to reduce the high egg mortality of the Lake Sturgeon, six bacterial species previously isolated from healthy egg surfaces belonging to the genera *Pseudomonas, Massilia, Acidovorax*, *Hydrogenophaga*, and *Brevundimonas* were chosen for *in vitro* biofilm formation studies. Fertilizing the eggs in the presence of one of these species, *Acidovorax F19*, had shown to reduce the overall egg mortality by 18%, suggesting this isolate could be used as a probiotic [37]. The main objective of this study was to identify bacterial species among healthy Lake Sturgeon egg isolates that show mutualism or antagonism during biofilm formation *in vitro*. Our hypothesis is that using these species together on live eggs would be more effective in protecting the egg from pathogens and reducing high Lake Sturgeon egg mortality. This hypothesis will be tested further in chapter 4.

In this study, we drew our conclusions on mutualistic/antagonistic nature of bacteria-bacteria interactions based on the results of three independent assays: crystal violet assay, resazurin assay and T-RFLP. As mentioned previously, crystal violet assay measures total biofilm biomass. As such, if total biomass of mixed culture biofilm is greater than the sum of the biomasses produced by individual single-species biofilms in the same conditions, one plausible explanaition would be that additional biomass is due to mutualistic interactions between the two species. The opposite result would indicate antagonistic interactions. Resazurin assays allowed us to examine biofilm formation in a different way by measuring metabolic activity of cells. While an increase in total biomass of mixed culture biofilms can be attributed to an increase in the amount of extracellular matrix, resazurin assays uncover bacteria-bacteria interactions in the form of increased

metabolic activity. In case of an interaction, either antagonistic or mututalilstic, we expect to see a higher metabolic activity in mixed biofilm. Finally, T-RFLP measures presence and relative abundance of individual isolates in double-species biofilms. Thus, criteria for mutualistic interactions are as follows: increased total biomass, elevated levels of metabolic activity and presence of both bacterial isolates in the biofilm. When only first two criteria are met, we can conclude that the species that is absent (or greatly reduced) from the biofilm promotes biofilm formation of the other species at its own expense. As for antagonistic interactions, we hypothesize that they would be observed as reduced total biomass, even in the presence of increased levels of metabolic activity.

Among the species examined, *Brevundimonas F16* had the strongest *in vitro* biofilm formation (biomass) individually. This result indicates that it has the highest adhesive property when it comes to initial attachment to polystyrene compared to other species in this study. Species belonging to *Brevundimonas* genus are known to have strong adhesive structures called holdfast, similar to their close relatives *Caulobacter*. This might be one possible explanation for their strong biofilm forming capabilities in microtiter plates. Furthermore, biofilm formation of *Brevundimonas F16* was substantially boosted by supplementation with fresh overnight culture of itself or *Acidovorax F19*. This observation suggests that established biofilms of *Brevundimonas F16* can recruit not only pelagic cells of the same species but also other select bacterial species. Interestingly, when combined with the results of biofilm formation of individual species, this result indicates that although *Acidovorax F19* has much worse adhesive properties with respect to polystyrene, it is capable of attaching to established biofilm of *Brevundimonas F16* as a secondary colonizer. This can be due to the presence of

holdfast on *Brevundimonas* cells that promotes interaction with *Acidovorax* specifically or because of metabolic cross-feeding. While the molecular nature of the interaction between *Brevundimonas F16* and *Acidovorax F19* remains unknown, it is important to note that these two bacterial species appear to exhibit mutualistic interactions and thus are of interest from a probiotic perspective. It is possible that *Acidovorax F19*, in the pelagic phase, contributes nutrients to *Brevundimonas F16* biofilms, and this hypothesis should be examined more closely in the future.

Our titration experiments have also revealed an interesting pattern of biofilm formation in mixed cultures where *Brevundimonas F16* was present. Specifically, presence of *Brevundimonas F16*, live or dead, resulted in increase of the total biofilm biomass. Similar to previous experiment, this change in the biofilm biomass can be attributed to the holdfast (highly adhesive structure) of *Brevundimonas* cells that influences early attachment, or metabolic linkages with other bacterial species. Our observation of increased biofilm biomass in the presence of dead *Brevundimonas* points towards the former hypothesis. At the same time, the fact that the titration with live *Brevundimonas* resulted in even larger biofilm growth is suggestive that metabolic interactions also play an important role in this mutualism.

We also studied the effect of three bivalent ions (Mg^{2+}, Ca^{2+} and Ni^{2+}) on biofilm formation of selected Lake Sturgeon egg isolates. Although there are no reported studies on the effect of these metals on biofilm formation of *Hydrogenophaga*, *Brevundimonas* and *Acidovorax*, these ions are known to impact biofilm formation in other bacterial species in two ways: (i) influencing adhesion to surfaces by affecting the electrostatic interactions between the bacterial cell envelope and abiotic or biotic

surfaces, and (ii) altering the expression of genes related to biofilm formation such as extracellular polymeric matrix biosynthesis genes [18-20]. In our experiments, magnesium slightly enhanced biofilm formation only in *Hydrogenophaga F14*. Calcium had no effect on biofilm formation of any of the tested species, and nickel notably decreased biofilm formation in *Brevundimonas F16* and any mixed cultures with *Brevundimonas*. However, it should be noted that mixed biofilms showed higher level of resistance to nickel as compared to single-species biofilm of *Brevundimonas*. These results are not surprising due to the fact that effects of these metals on biofilm formation were found to be species specific [18]. For example, magnesium has shown to enhance initial adhesion to surfaces in *Staphylococcus epidermidis, Pseudomonas fluorescens* and some *Pseudomonas aeruginosa* species, but has no effect on the adhesion of *Streptococci* or some *P. aeruginosa* species [18, 54-56].

While nickel concentrations used in this study were much higher than those found in highly contaminated waters [57], it is important to account for its potential presence and effect on microbial communities when conducting studies on aquatic biofilms as our data suggests that nickel contamination of freshwater may substantially impact biofilm formation. Additionally, our results with Nickel suggest that bacteria-bacteria interactions during biofilm formation can play an important role in protecting Nickel-sensitive bacterial species.

Crystal violet assay showed that all mixed cultures exhibit changes in their total biofilm biomass as compared to their single-culture counterparts. As we discussed above, these changes can be a result of increase or decrease in the amount of biofilm's extracellular matrix and/or number of the biofilm's live and dead cells. In order to get

additional insight about bacteria-bacteria interactions during biofilm formation, we further measured biofilm metabolic activity using resazurin assay, which focuses on the viable cells in biofilm. It is important to mention that bacterial biofilms are known to contain higher numbers of slow- or non-growing presister cells with low metabolic activity [58]. However, this is a temporary state and we hypothesized that mutualistic and antagonistic bacteria-bacteria interactions will increase the number of cells with higher metabolic activity in the biofilms. In fact, we observed double-species biofilms of *Brevundimonas F16-Hydrogenophga F14,* and *Pseudomonas C22-F. columnare* showed higher metabolic activity compared to their single-species counterparts. Per our hypothesis, this increase in the metabolic activity of mixed biofilms compared to their single counterparts can be an indication of mutualistic or antagonistic interactions between the two species due to activities such as production of nutrients needed for the mutualistic partner or production of antimicrobial compounds against the other species.

As mentioned above, mixed cultures of *Pseudomonas C22* and *F. columnare,* a common fish pathogen, produced higher biomass as compared to *Pseudomonas C22* or *F. columnare* alone. Furthermore, these biofilms also showed higher metabolic activity as compared to their single-species counterparts. T-RFLP studies showed *Pseudomonas C22* dominated these mixed biofilms (more than 95%). These results indicate an interaction between these bacteria in their pelagic form; at the same time, interaction of *F. columnare* in its pelagic form with established *Pseudomonas C22* biofilms boosts the biofilm forming capabilities of the latter even further (Figs. 9 and 11). Although the underlying mechanism remains unknown, this result suggests that *F. columnare* stimulates biofilm formation in *Pseudomonas C22*. Importantly, this effect

can impact the fish eggs microbial community composition and, thus, its subsequent fate, as we will discuss further in chapters 3 and 4. Finally, taxonomy results based on Sanger sequencing of 16s rRNA gene of *Pseudomonas C22* showed its closest type species is *Pseudomonas fluorescens*. In general, we expect that *Pseudomonas C22* might be a probiotic because of its antimicrobial and antifungal activity. Previous observations support this hypothesis; certain *P. fluorescens* species are known to have mutualistic relationships with specific plant species, protecting them from pathogenic fungi such as *Pythium* and *Fusarium* [60, 61]. These findings on biofilm formation in the presence of *F. columnare* and taxonomy results suggest that *Pseudomonas C22* can be a potential Lake Sturgeon egg mutualist, protecting eggs from pathogenic bacteria and fungi by producing antimicrobial compounds. This hypothesis should be examined further in future studies on live fish eggs.

In our experiment with tobramycin, *F. columnare* exhibited high level of sensitivity to this antibiotic, whereas *Pseudomonas C22, Hydrogenophaga F14, Brevundimonas F16,* and *Acidovorax F19* showed significant resistance. These data are important for the task of decreasing egg mortality of Lake Sturgeon fish eggs as it allows us to conclude that some antibiotics could be used against known fish pathogens without affecting mutualistic or commensal bacterial populations associated with fish eggs. On the other hand, one has to be aware of the fact that prolonged exposure to antibiotics can result in development of antibiotic-resistant bacterial species, which imposes limitations on the use of antibiotics in hatcheries. This, however, does not eliminate the need to study the effect of antibitocis further. Specifically, our results need to be varified on live fish eggs, as presence of certain exogenous proteins, as was observed in the

case of milk proteins, can have significant effect on susceptibility of bacterial species to antibiotics including tobramycin. Our results indicated that exogenous protein ameliorates the effect of tobramycin on *P. aeruginosa* and *F. columnare*. Therefore when these pathogens are in the presence of substantial exogenous protein their biofilm formation is protected from tobramycin.

To conclude, results of our experiments outlined in the present chapter suggest that *Pseudomonas C22, Hydrogenophaga* F14, *Brevundimonas* F16 and *Acidovorax* F19 can affect Lake Sturgeon egg's wellbeing either directly by producing antimicrobial compounds against pathogenic bacteria and fungi or indirectly by helping an egg mutualist attach to the egg surface. In our studies (Chapter 4), relative abundance of the genera *Hydrogenophaga*, *Brevundimonas* and *Acidovorax* are generally lower in river water as compared to *Pseudomonas.* Given our observations of antagonistic interactions between *Pseudomonas C22* and other isolates, we can expect low amounts of biofilm formation on the egg surface by these species. However, these speculations are subject to debate as (i) egg surface is chemically different from polystyrene, and (ii) presence of other microbial species in river water can affect the attachment process of mentioned isolates in different ways by either promoting or inhibiting it further. While in hatcheries we can control, to some extent, microbial composition of water, chemistry of the egg surface remains a parameter that, as we expect, can affect bacterial biofilm formation on the egg surface.

APPENDIX

APPENDIX A

Some bacterial species share a high level of 16s rRNA gene sequence similarity (>99%) although they clearly do not belong to the same species [64, 65]. As a result in some cases differentiating between two bacterial species using 16s rRNA gene sequences alone, is impossible. Since the genome sequence of a bacterial species is the ultimate information for microbial taxonomy, ANI, the pairwise comparison of the genome sequences of two species, is one of the most robust measurements of genomic relatedness between species. An ANI threshold range of 95-96% has been suggested for species demarcation [64, 66-69].

To isolate genomic DNA, four lake sturgeon egg isolates (*Pseudomonas* C22, *Hydrogenophaga* F14, *Brevundimonas* F16, and *Acidovorax* F19) were grown in 50 ml R2Broth at 25°C with shaking at 120 rpm. Cells were harvested in exponential growth phase by centrifugation at 10,000 RPM in an SS34 rotor at 4°C for 40 minutes. Genomic DNA was extracted using a MoBio UltraClean Microbial Kit. Whole-genome sequencing of these species was carried out on an Illumina Miseq platform (Michigan State University, East Lansing, MI). Genome assemblies were done using SPAdes (version: 3.6.2) through Pathosystems Resource Integration Center (PATRIC) website [70, 71]. All genomic assemblies and annotations were done through PATRIC website (https://www.patricbrc.org/) [8]. For assemblies SPAdes genome assembly algorithm (version 3.6.2) was used through PATRIC website (Table A-1). The resulting contigs (unpublished data) were then used for pairwise ANI analysis between the egg isolates and the existing sequenced genomes of the species from the same genus through Integrated Microbial Genomes (IMG) system website (https://img.jgi.doe.gov/cgi-

bin/m/main.cgi). The results suggest that the Lake Sturgeon egg isolates belong to the genera *Pseudomonas, Hydrogenophaga, Brevundimonas* and *Acidovorax* but are previously un-described species (Table A-2).

	Pseudomonas C22	***Hydrogenophaga F14***	***Brevundimonas F16***	***Acidovorax F19***
Genome size (bp)	6389875	4732947	3504343	6928800
Contigs	46	80	26	156
Largest contig (bp)	936531	640853	550594	262464
N50	736130	237902	237384	81179
GC (%)	60.05	65.06	67.94	67.30
Coding region (CDS)	5758	4611	3444	6775
Repeat region	119	142	44	275
tRNA	63	43	29	57
rRNA	7	6	3	3
Proteins with functional assignments	4540	3167	2224	4750
Hypothetical proteins	1218	1444	1220	2025

Table A-1. Genome statistics of four Lake Sturgeon egg isolates.

Lake Sturgeon egg isolates	Closest species	Closest species ID	ANI (%)
***Pseudomonas* C22**	*Pseudomonas fluorescens*	2713897148	87.38
	Pseudomonas putida	2568526024	83.26
	Pseudomonas fluorescens	2506210029	82.88
Hydrogenophaga F14	*Hydrogenophaga pseudoflava*	2731957687	82.8
	Hydrogenophaga palleronii	2681813002	82.53
	Hydrogenophaga taeniospiralis	2731957688	82.27
Brevundimonas F16	*Brevundimonas subvibrioides*	648028010	87.89
	Brevundimonas bacteroides	2561511096	83.87
	Brevundimonas diminuta	651285001	82.11
Acidovorax F19	*Acidovorax radicis*	2547132374	85.7
	Acidovorax radicis	2547132375	85.61
	Acidovorax temperans	2627854134	83.25

Table A-2. Top three closest bacterial species to four egg isolates based on Average nucleotide identity (ANI).

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CHAPTER 3. BIOFILM STABILITY AND ASSEMBLY IN LAKE STURGEON EGG ISOLATES: THE ROLE OF SPECIES-SPECIFIC SELECTION

ABSTRACT

Bacteria frequently live in mixed biofilms on biotic or abiotic surfaces. Our work describes inter-species cooperation and competition and how these affect biofilm development and stability. The bacteria selected were isolates from Lake Sturgeon eggs that had been characterized previously. Ten single or double species biofilms were established in 24-well microtiter plates for 2 days. After 2 days of incubation, the established biofilms were washed aseptically and then exposed to river water for 24 hours. We used the crystal violet assay to measure biofilm biomass. Founding population biofilm stability as well as river populations recruited by established biofilms were evaluated by amplicon sequencing (Illumina). Cluster and ordination analyses were used to compare all biofilm communities. Community analysis showed that only a fraction of river bacterial populations attached to microtiter plates. The river biofilm community had greatly increased populations of *Aeromonas*, *Rheinheimera*, *Arcobacter*, *Shewanella*, and *Acinetobacter* compared to river water. We observed a broad range of founding population biofilm stability in response to river water. *P. aeruginosa* biofilm was highly resistant to river water and comprised 93.5% of the detectable community after 24 hours of incubation. River populations that joined *P. aeruginosa* were *Rheinheimera*, *Shewanella* and *Flavobacterium*. Biofilms formed by *Deinococcus aquaticus F4* were fragile when exposed to river water and lost an order of magnitude of crystal violet staining material, while biofilms formed by *Brevundimonas F16* increased in biomass after 24 hours of exposure to river water, however only 49% of the community was *Brevundimonas*. River populations that joined *Brevundimonas F16* were *Rheinheimera*, *Aeromonas*, *Arcobacter*, *Flavobacterium*, *Fluviicola*, and

Hydrogenophaga. Some genera like *Fluviicola*, and *Arcicella* were found at very low levels in river water and were not a part of the biofilm community established by the river water alone on polystyrene, but were found in elevated levels in some of the established biofilms, suggesting that these species were secondary colonizers selected in some fashion by the primary biofilm population. Although *Aeromonas* was significantly abundant in the river biofilm community, its abundance decreased significantly in the established biofilm communities, suggesting a selection against this genus once there is an established biofilm. This work identified specific bacterial species isolated from the Lake Sturgeon egg surfaces with either robust or fragile biofilms upon challenge with river water microbiome. Established biofilm communities selected for a unique collection of secondary colonizing populations from river water, indicating that historical contingency defines the structure of biofilms. The uniqueness of all communities that were selected by pre-established biofilm populations was statistically supported. Knowing the freshwater bacterial populations that are selected by specific established biofilms will provide greater understanding in how mixed biofilms assemble. Details of the assembly process could then assist in the development of probiotics that can direct fish egg-associated community assembly and reduce egg mortality.

INTRODUCTION

In nature bacteria frequently live in multi-species biofilms attached to a range of biotic and abiotic surfaces. Historically, most of the research on biofilms has been conducted on single-species biofilms due primarily to the technical challenges posed by multi-species biofilms [1-10]. In recent years, however, advancements in high-throughput sequencing techniques and fine-resolution imaging methods have enabled scientists to examine the complexity of multi-species biofilms and microbial interactions within these bacterial assemblages which, in turn, modulate the growth, composition and functions of these biofilms [11].

Bacterial species within multispecies biofilm communities can participate in different symbiotic interactions [3, 12-15]. Recent studies have identified antagonistic interactions, including the production of bacteriotoxins and the lowering of pH, as well as many mutualistic and commensalistic interactions such as coaggregation, conjugation, protection from antibiotics, enzyme complementation and organized spatial niche-partitioning within the biofilm [1, 7, 16-20]. The significance of these symbiotic interactions on multispecies biofilm development was first described with respect to oral cavities and later in various multispecies biofilms found in non-host environments such as food processing equipment and portable water supply systems [3-5, 7-9, 21].

The oral cavity is perhaps the most well studied example of a bacterial multi-species biofilm [3, 21-23]. Generally, oral ecosystems are characterized by high diversity that can experience successional waves and is impacted by food, oral hygiene, location in the mouth, and interactions with host tissues and their secretions [23]. Bacterial diversity in tooth cavities seems to be higher than a healthy mouth; in fact,

several surveys that utilized pyrosequencing methods indicated that around 500 species are found in an individual tooth cavity whereas, between 100-200 different microbial species were found in the healthy mouth of human individuals [23, 24]. Interestingly, the majority of oral bacteria are capable of adhering to teeth, epithelial tissue or other bacterial species that have already attached to these surfaces. At early stages of plaque formation, Streptococci and gram-positive rods such as *Actinomyces naeslundii* as well as their coaggregation partners are among first bacteria that colonize the tooth surface [23, 25-38]. After 24 hours, the plaque is composed of bacteria belonging to different morphological types [23, 39]. In fact, the major periodontal pathogen *Porphyromonas gingivalis* is an example of dental plaque secondary colonizers that coaggregates with initial colonizers such as *Streptococcus gordonii* [40]. Bacterium *Fusobacterium nucleatum* exhibits more partnerships than any other bacteria in the dental plaque community [23]. This bacterium described as a “bridge organism” referring to its ability to co-aggregate with both initial and tertiary colonizers, thus linking them together [12, 30, 41]. Studies showed that if *F. nucleatum* is absent, many subsequent colonizers are present in reduced numbers in the dental plaque community [42].

Aquatic environments offer a greater diversity of surfaces for microbes to colonize compared to the oral cavity. These include surfaces of inorganic matter, aquatic plants and animals, and already established microbial biofilms on abiotic or biotic surfaces. By colonizing these surfaces and forming or joining other biofilms, microbes substantially extend their evolutionary strategy from the dynamic pelagic life to one with more positional permanence, perhaps a reduced metabolism with continued access to nutrients and a certain degree of protection environmental stresses [43].

Perhaps the vast majority of microbes in aquatic environments live in biofilms, making aquatic biofilms important active sites for both heterotrophy and phototrophy (depending on location) and key sites for carbon and nitrogen cycles [44]. Moreover, unlike *in vitro* conditions, microbial biofilms in aquatic environments are constantly exposed to a diverse inoculum of microbes from the water column, making them unique models for studying microbial interactions during biofilm formation and development. In recent years with developments in next-generation sequencing it has become possible to gain a better insight into the biodiversity and phylogenetic composition of aquatic microbial biofilms [44]. However our understanding of how these biofilms interact with aquatic animals and how they affect the wellbeing of their hosts is still very limited.

In this chapter, our work focused on the bacterial community associated with Lake Sturgeon eggs [11, 45, 46]. Lake Sturgeon is an endangered species of fresh water fish with significantly high egg mortality. Electron micrographs from the surface of Lake Sturgeon eggs exposed to river water for 24 hours show morphologically diverse bacteria in biofilm-like structures, although aseptically harvested eggs show no trace of microbial presence on their surface [46]. Additional studies revealed the phylogenetic composition of microbial communities found on recently extruded eggs (within 24 hours of exposure to river water) as well as the microbial load that accumulates on egg surfaces and the impact this has on egg mortality [45]. To extend our understanding of community assembly on the egg we have isolated several hundred different bacterial species from the surface of healthy Lake Sturgeon eggs and investigated their capacity to form biofilm *in vitro* [45, 46]. We focus on the influence selected isolates from healthy Lake Sturgeon eggs have on early community assembly by establishing biofilms on

these isolates *in vitro* followed by a challenge with river water. Six Lake Sturgeon egg isolates that were found to be abundant on healthy eggs based on previous studies [45] were chosen for these experiments: *Acidovorax F19* (indication of probiotic effect based on Fujimoto's work), *Hydrogenophaga F14, Brevundimonas F16* (best biofilm formation *in vitro,* also strong mutualism during biofilm formation with *Acidovorax F19* and *Hydrogenophaga F14*), *Deinococcus aquaticus F4* (a potential Lake Sturgeon egg commensal based on Fujimoto's study), *Bacillus C20*, and *Serratia D14* (both exhibiting antimicrobial activity based on Fujimoto's study). The stability of single- or double-species biofilms upon exposure to river water was measured by crystal violet assay and the specific river populations that invade or are recruited by these established biofilms were identified using amplicon-based sequencing (Illumina) of 16s rRNA gene. With this approach, we can establish unique fate maps for community assembly in which specific bacterial populations appear to select for secondary colonizers. Knowledge of the river water populations that are selected by specific established biofilms can assist us in the development of probiotics that can direct assembly of fish egg-associated microbial community and reduce high levels of egg mortality.

MATERIAL AND METHODS

Biofilm formation. Six Lake Sturgeon egg bacterial isolates as well as a strong biofilm-forming and opportunistic pathogen (*Pseudomonas aeruginosa PA01*), and a soil isolate (*Serratia RL10*) were chosen for biofilm formation. The Lake Sturgeon isolates included: *Bacillus* C20, *Deinococcus aquaticus* F4, *Serratia* D14, *Hydrogenophaga* F14, *Brevundimonas* F16, and *Acidovorax* F19. Isolates were chosen

based on their high abundance on healthy Lake Sturgeon egg isolates, their *in vitro* biofilm forming capabilities, mutualism during biofilm formation or antimicrobial activity. Bacteria were isolated on R2A agar (Difco) from the Black Lake Sturgeon population in Onaway, Michigan during 2009 and 2010 spawning season [45].

Biofilm formation. Single-species biofilms of the above Lake Sturgeon egg bacterial isolates as well as double-species biofilms (*Hydrogenophaga F14-Brevundimonas F16*, and *Brevundimonas F16-Acidovorax F19*) were established in 24-well microtiter plates for 48 hours. For establishing single-species biofilms 500μL of overnight broth culture of each bacterial species in R2Broth was added to 1mL of fresh R2Broth in 24-well microtiter plates. In double-species biofilms, 250μL of overnight broth culture of each bacterial species in R2Broth was used with 1 ml of fresh R2Broth. Double-species mixtures were selected based on their apparent mutualism in biofilm formation (Chapter 2). The plates were incubated at 25°C on a shaker (100 RPM) for 48 hours. Each unique biofilm had four replicates.

After 48 hours, the single- and double-species biofilms were washed with sterile Physiological Saline (PS) twice and then exposed to 1.5 mL filtered (0.22 μm filter, filtered twice) or unfiltered Red Cedar river water for an additional 24 hours (same incubation conditions as before). *Pseudomonas aeruginosa PA01* provided by Dr. Michael Bagdasarian of Michigan State University, was used as a positive control and four wells with sterile R2Broth were used as negative control. River water was added to untreated wells as served as a positive control for river biofilm.

Measuring biofilm biomass. To assess planktonic growth in established 48 hours biofilm cultures before exposure to river water, we transferred the broth culture to

new plates post incubation and measured absorbance at 600 nm wavelength. Four replicates of each sample in the original microtiter plates were washed twice with sterile PS after 48 hours of biofilm formation before exposure to Red Cedar river water, or in samples that had been exposed to filtered or unfiltered river water for 24 hours. Total biofilm biomass was measured using a crystal violet assay [47].

Biofilm biomass formed in 24 well-microtiter plates was stained with 1.5 mL of crystal violet dye (0.1%) for 15 minutes. Then the plates were rinsed with distilled water three times and inverted in order to dry completely. To extract crystal violet stain, 1.2 mL of acetic acid (30%) was added to each well for 15 minutes. Finally, all the dye solution within each well was transferred into new 24-well microtiter plates and the absorbance was measured at 600 nm wavelength using a Biotek Epoch plate reader.

DNA extraction. After establishment of 48 hours biofilms, samples were removed at 4, 8 and 24 hours to monitor the change in community structure over time. Because sampling for communities is destructive, three replicates of four reactions were established for each condition and one set was sampled at each time point. Replicates of each biofilm community were washed twice with PS after 48 hours of biofilm formation, and 4, 8 and 24 hours after exposure to filtered or unfiltered river water. Cells in the biofilm were lysed with 1.2 mL of Alkaline PEG (pH=13.3) added to each well [48]. Plates were then incubated at room temperature for 15 minutes before transferring to -20^{o}C freezer. To determine the phylogenetic structure of the bacterial community of the Red Cedar, replicates of river water (500 ml) were filtered through 0.22 µm filter membranes (Steritech) using a vacuum pump. The filters bearing the retentate were transferred to 50 ml Corning ® centrifuge tubes containing 80% ethanol and stored at 4

^{o}C. Just prior to extraction, the filters and solutions were vortexed vigorously and the cells were pelleted by centrifugation (20 minutes in a Fiberlite F18 rotor at 12,000 RPM. After decanting, the pellet was extracted with Alkaline PEG [48].

Illumina sequencing and bacterial community analysis. For each replicate of each sample as well as Red Cedar river water samples, 40 μL of genomic DNA extracted with Alkaline PEG were sent to the Michigan State University Research Technology Support Facility (RTSF) for further processing using an Illumina MiSeq platform (total of 182 samples). For each sample, uniquely indexed primers were used to amplify the V4 region of 16S rRNA gene of the bacterial community DNA [49]. Amplification products were then normalized (Invitrogen SequalPrep normalization plate). After pooling normalized samples, PCR reaction products were cleaned with AMPure XP beads. The pool was then loaded on an Illumina MiSeq v2 flow cell and sequenced with a 500-cycle v2 reagent kit (PE250 reads). Base calling was performed by Illumina Real Time Analysis Software (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ files with Illumina Bcl2fastq v1.8.4.

We used Mothur version v.1.35.1 (http://www.mothur.org) to further analysis the bacterial community of the samples [50]. Mothur's standard operating procedure was used for processing of the raw sequencing data (http://www.mothur.org/wiki/MiSeq_SOP) [51]. Mothur-formatted version 123 of Silva 16S rRNA gene database was used to achieve alignment [52]. Any sequences classified as Mitochondria, unknown, Archaea, or Eukaryota were then removed from the data set. The samples were then subsampled at 15,000 sequences per sample. Sequences were preclustered and chimeric sequences were removed using a Mothur-

formatted version of the Ribosomal Database Project (RDP) training set version 14 and uchime, based on the Mothur protocol. Finally, sequences were classified into Operational Taxonomic Units (OTUs) of ≥97% sequence identity.

Statistical analysis. To analyze crystal violet assay results we used analysis of variance (One-way ANOVA) using R version 3.2.3 [53]. If the null hypothesis of equal means were rejected, then Tukey's Honest Significant Differences test (95% confidence intervals) was performed for pair-wise comparisons between group means with correction for multiple testing in order to determine what is driving the difference in means. Results for Tukey HSD were reported as p adj. values (p-value after adjustment for the multiple comparisons).

To analyze diversity of the bacterial communities in the biofilm samples, the shared and taxonomy output files of Mothur were used. Paleontological Statistics Software Package For Education and Data Analysis (PAST) software was used for further analysis of the bacterial communities with the alpha diversity indices (Simpson, Shannon, and Chao-1), multivariate tests, and Analysis Of Similarities (ANOSIM) [41]. The Bray-Curtis dissimilarity was used to quantify the differences in the abundance of different OUTs between samples. To visualize the level of similarity between samples Nonmetric Multidimensional Scaling (NMDS) plots were generated based on microbial abundance and composition using the Bray-Curtis similarity index. NMDS uses an algorithm that takes the multidimensional data and presents it in a two- or three-dimensional space. This "goodness of fit" is measured by stress value. Stress values <0.05 are considered excellent (the two- or three-dimensional visualization is a valid representation of the differences between the samples) and stress values <0.1

correspond were considered good ordination [54]. To measure statistical significance between the samples, Analysis Of Similarities (ANOSIM, a distribution free analysis of similarity) was used. The number of permutations used in this test was 9999. Based on ANOSIM analysis, statistically the most dissimilar samples were the ones that not only had a P value less than 0.03 (the null hypothesis was rejected, meaning the average rank similarity between objects within a group was not the same as the average rank similarity between objects between groups), but also had R-values that were close to 1.

Clustering analysis was performed in PAST and the resulting trees were edited in FigTree v.1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/). The genera responsible for dissimilarities between the biofilm communities were identified by Similarity Percentages (SIMPER) based on the Bray-Curtis dissimilarity matrix [41].

Availability of supporting data. Raw sequence data is available at the NCBI database (SRA accession number: SRP150750) and the code for the Mothur analysis is available at (https://doi.org/10.6084/m9.figshare.6555755.v1).

RESULTS

Our goal in this study was 1.) to determine the resilience of the established biofilm of our sturgeon egg isolates when challenged with a freshwater community, and 2.) to determine river populations that might be specifically excluded by or attracted to the established biofilms. We established single- or double-species biofilms over 48 hours, gently washed them with sterile saline buffer and then challenged the biofilms with natural river water for 24 hours. The crystal violet assay was used to measure biofilm biomass before and 24 hours after exposure to filtered and unfiltered river water.

Unfiltered river water contains all of the microbial community normally present while the filtered (0.22 μm) water has most of the microbial populations removed but retains the same small molecule and viral composition of the unfiltered water. Filtered and unfiltered river water was used to separate the effects of the filterable components of river water (most bacterial, archael and fungal species) from the effects of viruses and water chemistry. We recognize the potential confounding influence of unfilterable pico-eukayotes and prokaryotes.

Figure 1 presents the consequences of challenging established biofilms in microtiter plates with unfiltered or filtered river water. A broad range of changes in biomass was observed in response to exposure to river water in the established biofilm communities. As measured by crystal violet assay, after 48 hours and prior to challenge with river water, there was a diversity of biofilm biomass formed by tested bacterial isolates. We detect robust biofilms in *P. aeruginosa*, *Dinococcus aquaticus* F4, *Brevundimonas* F16, and in the mixed communities of *Hydrogenophaga F14-Brevundimonas F16* and *Acidovorax F19-Brevundimonas F16* at this time point. The remaining species have shown modest levels of biofilm formation under these conditions. The biofilm formed by the river water (Water Biofilm) was low and difficult to measure with crystal violet as compared with the positive control, *P. aeruginosa*.

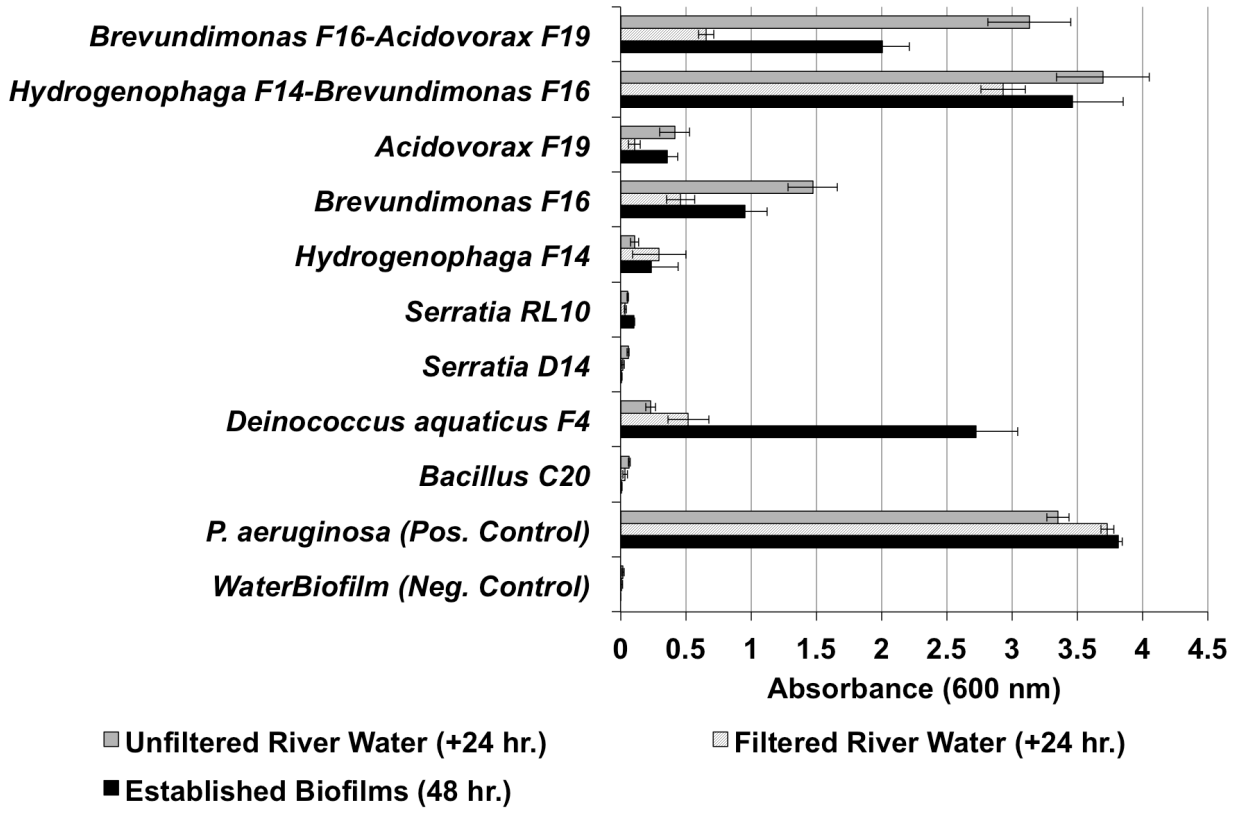


Figure 3.1. Biofilm biomass in single- and double-species biofilms before and after exposure to filtered and unfiltered river water (replicated 4 times). Overall ANOVA results: F(28, 87) = 257.9, p = 2e-16.

The addition of filtered river water was found to either have no effect, or to diminish the amount of biofilm biomass detected at 48hrs. For example, with *P. aeruginosa* and *Hydrogenophaga F14* there was no appreciable change in the amount of biofilm after incubation with filtered river water. In contrast, *Dinococcus aquaticus F4* (p adj.= 0), *Brevundimonas F16* (p adj.= 0.012), and the mixed *Brevundimonas F16-Acidovorax F19* (p adj.= 0) biofilms were found with diminished biomass when challenged with filtered river water. When biofilms were challenged with unfiltered river water several species showed statistically insignificant changes as compared to unchallenged biofilms. However, *Dinococcus aquaticus F4* (p adj.= 0) showed reduced biofilm biomass while *Brevundimonas F16* (p adj.= 0.006) and the mixed biofilm of

Brevundimonas F16-Acidovorax F19 (p adj.= 0) had significantly increased biofilm biomass levels by at least 30%.

16s-rDNA amplicon Illumina sequencing was used to establish the demographics of early biofilm assembly at 4, 8 and 24 hours (4 replicates at each time point). The river community was quite complex with over 500 detected bacterial operational taxonomic units (OTUs) in our samples (Table 1). All of the established biofilm communities had one detected phylotype (two in the case of two-member biofilms) after 48 hrs of incubation (data not shown). The biofilm formed from river water had an average of 401 OTUs after 4 hours of incubation. After four hours of exposure to river water, anywhere from 61-435 OTUs were detected on the established biofilms. In most cases the founding populations at 4 hours and occasionally at 8 hours (post river water exposure) dominated biofilm communities. The richness and diversity of river water was higher than river biofilm after 24 hour incubation as measured by the Simpson, Shannon, and Chao1 indices; in most cases diversity of each community decreased over time (Table 1). The highest decline in diversity was observed in the biofilm communities of river water, *Acidovorax F19*, and the mixed biofilm of *Hydrogenophaga F14-Brevundimonas F16*. In the latter case, Simpson index decreased from 0.98 at T4 (4 hours after exposure to river water) to 0.86 at T24 (24 hours after exposure to river water).

In biofilm communities of *Bacillus* C20, *P. aeruginosa*, and the mixed biofilm of *Brevundimonas F16-Acidovorax F19*, diversity increased with time. For example, in the double biofilm of *Brevundimonas F16-Acidovorax F19*, Simpson index increased from 0.39, 4 hours after exposure to river water, to 0.96, 24 hours after exposure to river water (Table 1). Also, Chao1 estimated the bacterial communities of double species

biofilms to have significantly lower number of OTUs 24 hours after exposure to river microbiome, compared to their single-species counterparts.

Sample	OTUs	Total Seq.	NFP Seq.	Simpson	Shannon	Chao1	Sample	OTUs	Total Seq.	NFP Seq.	Simpson	Shannon	Chao1
River Water	525	18387	18387	0.98	4.83	574.9	**T4-D14**	435	36514	5094	0.98	4.66	542
T4-RB	401	24305	24305	0.97	4.47	432.4	**T8-D14**	289	16938	8584	0.98	4.76	301.2
T8-RB	481	21885	21885	0.98	4.96	548.4	**T24-D14**	183	29855	8590	0.95	4.03	198
T24-RB	389	27595	27595	0.86	3.28	443.2	**T4-RL10**	139	43569	577	0.98	4.24	382.8
T4-F14	380	36668	1633	0.96	4.63	668.8	**T8-RL10**	240	30437	2439	0.99	4.93	255.8
T8-F14	172	32365	1233	0.97	4.42	207.9	**T24-RL10**	200	30213	9649	0.96	4.17	229.3
T24-F14	402	32501	10769	0.93	3.83	499.8	**T4-C20**	494	22952	14077	0.95	4.21	536.2
T4-F14F16	108	40998	240	0.98	4.25	315.8	**T8-C20**	415	21740	13163	0.95	4.07	481.5
T8-F14F16	112	48345	284	0.97	4.14	250.3	**T24-C20**	370	27978	22274	0.97	4.27	404.2
T24-F14F16	182	34584	4514	0.86	2.98	278.1	**T4-F4**	552	45985	7272	0.97	4.53	667.4
T4-F16	219	47222	656	0.97	4.51	551.1	**T8-F4**	356	25841	12657	0.93	3.95	387.9
T8-F16	147	48439	786	0.76	2.95	317.6	**T24-F4**	157	22464	20174	0.96	3.77	178.1
T24-F16	431	38681	16950	0.91	3.48	562.1	**T4-PA**	61	28354	346	0.73	2.12	222.1
T4-F16F19	96	42554	1771	0.39	1.29	384	**T8-PA**	333	54697	1048	0.98	5.03	617
T8-F16F19	193	31032	661	0.99	4.86	282.5	**T24-PA**	169	37604	2959	0.86	2.81	242
T24-F16F19	244	31260	5849	0.96	4.23	287.3							
T4-F19	70	24967	109	0.96	3.82	424							
T8-F19	149	33015	496	0.97	4.25	249.1							
T24-F19	315	31271	4663	0.85	3.27	456.4							

Table 3.1. Bacterial alpha-diversity in all biofilm samples at 4 (T4), 8 (T8), and 24 (T24) hours after exposure to unfiltered river water. This analysis was done after removing the sequences of the founding populations in the biofilm communities (Total Seq.= Total number of sequences, NFP Seq.= Non-founding population sequences). All calculations were done based on the average number of sequences in four replicates. RB= River Biofilm, F14= *Hydrogenophaga F14,* F16= *Brevundimonas F16*, F19= *Acidovorax F19*, D14= *Serratia D14*, RL10= *Serratia RL10*, C20= *Bacillus C20*, F4= *Deinococcus aquaticus F4,* PA= *Pseudomonas aeruginosa.*

Many changes in founding population taxonomic composition and relative abundance were observed in response to exposure to river water in the established biofilm communities (Figure 2, A-D). In the established biofilms of *Bacillus C20* and *D. aquaticus F4,* the founding population began to diminish immediately after exposure to river microbiota (Fig. 2A). Biofilm communities of *Bacillus C20* and *D. aquaticus F4*, shifted significantly after 24 hours of exposure to river microbiota and was mostly (95%) composed of members of river water bacterial community. The most resilient of the established biofilm communities to river water microbiota were *Acidovorax F19* and *P. aeruginosa* (Fig. 2B). These species were the most dominant population in the biofilm community even after 24 hours of exposure to river microbiome. After challenge with river microbiota for 24 hours, 20% of the biofilm communities of *Hydrogenophaga F14*, *Serratia D14*, and *Serratia RL10* were derived from the river water community (Fig. 2A and 2B). In *Serratia D14,* although the founding population decreased at 4 and 8 hours, it increased slightly after 24 hours (Fig. 2A). The population of *Brevundimonas F16* decreased to 50% of the total biofilm community after 24 hours of exposure to the river microbiome (Fig. 2B).

In double-species biofilms (*Hydrogenophaga F14-Brevundimonas F16*, and *Brevundimonas F16-Acidovorax F19*), all three species followed a trajectory different from their single-species biofilms (Fig. 2C and 2D). No significant changes were observed in the ratio of *Hydrogenophaga F14*, and *Brevundimonas F16* 24 hours after challenge with river microbiota. However, *Acidovorax F19* share of the mixed biofilm decreased significantly over 24 hours (Fig. 2D).

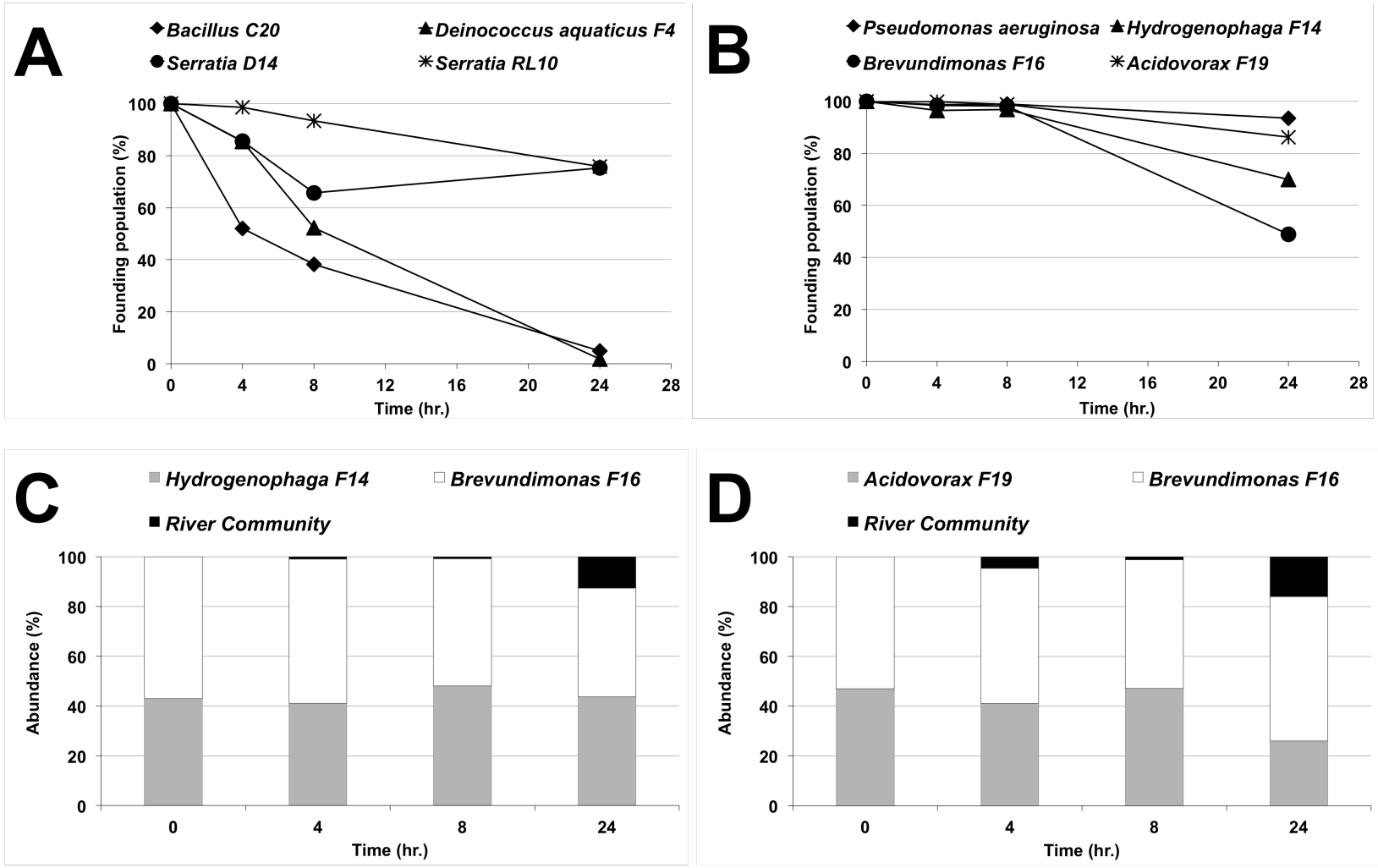


Figure 3.2. Founding population stability in single-species (A and B) and double-species biofilms (C and D). Here we focused on the fraction of the founding populations that remain in biofilm as a function of time. The plotted values are the fraction of the founding population sequences in the biofilm community at four different time points after exposure to unfiltered river water [T=0 (established 48 hr. biofilm of founding populations before challenge with river water), T=4 hr., T=8 hr., and T=24 hr.]. Four replicates of each sample were used for this calculation.

Analysis of the 179 biofilm communities revealed significant differences in the abundance and composition of taxa based on the Bray-Curtis dissimilarity index (ANOSIM P < 0.0001, R= 0.92). All biofilm communities changed significantly during the 24-hour exposure to river microbiota (Appendix B: Tables S-1 and S-2, Fig. S-2A to S-2G). Based on Nonmetric Multidimensional Scaling (NMDS) and cluster analyses, we documented the dissimilarity between river biofilm and river water taxonomic composition even after 4 hours (Fig. 3, Appendix B: Fig. S-1A to S-1D).

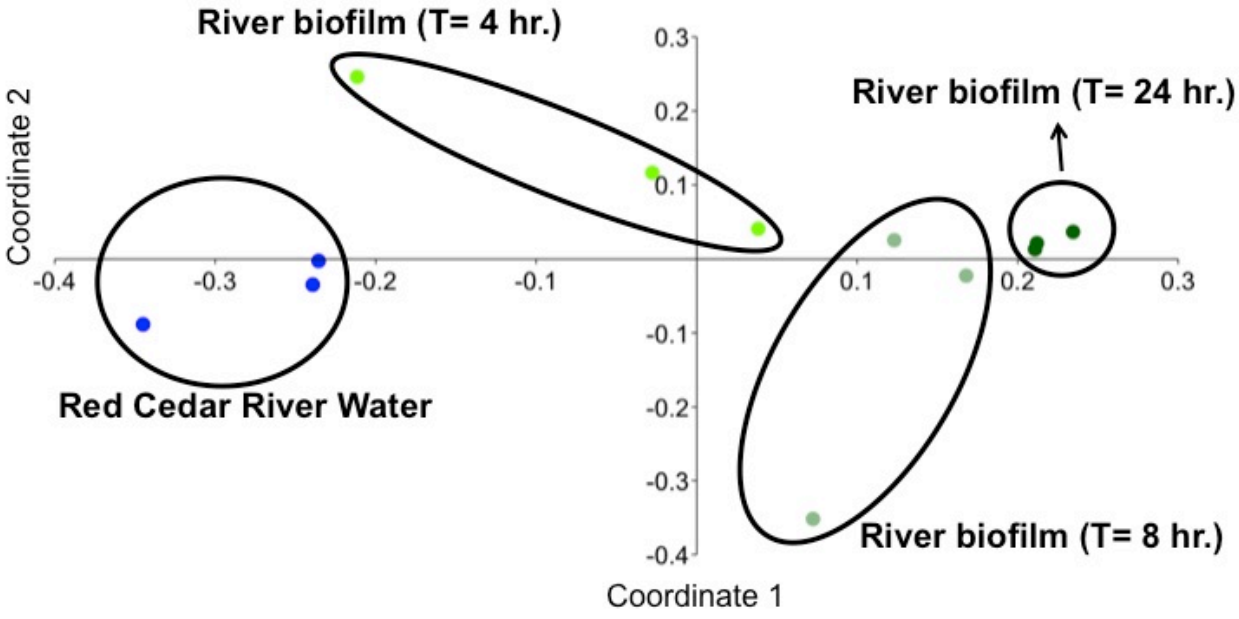


Figure 3.3. Three-dimensional non-metric multidimensional scaling (NMDS) analysis of river water, and river biofilm samples (stress value = 0.067). Bacterial communities formed 4, 8 and 24 hours after exposure to the river water on the polystyrene wells are significantly different from the river water bacterial community.

ANOSIM results, however, suggested that the community formed 4 hours after exposure to the river water on the polystyrene wells was not significantly different from the river water community (ANOSIM P=0.198), whereas the taxonomical composition of communities formed 8 (ANOSIM P=0.027, R= 1) and 24 hours (ANOSIM P=0.026, R= 1) after exposure were significantly different from the river water community (Appendix B: Tables S-1 and S-2). The most abundant members of the river water bacterial community were *Polynucleobacter, Comamonadacea, Acidovorax, Cytophagaceae* and *Flavobacterium* (Fig. 4). In the river biofilm community 4 hours after exposure to river water, the top five populations were *Arcobacter, Shewanella, Aeromonas, Rhodobacteraceae* and *Rheinheimera.* 8 hours after exposure, *Arcobacter* and *Aeromonas* were still the top genera in these samples, whereas *Comamonadaceae* and *Acidovorax* became more abundant. 24 hours after exposure to river microbiota the most abundant genera were *Aeromonas, Rheinheimera, Acidovorax, Shewanella, Flavobacterium,* and *Acinetobacter*.

After 24 hours of incubation, the genera *Aeromonas*, *Rheinheimera*, and *Polynucleobacter* contributed most to the dissimilarity between the river water and river biofilm communities (24.6%, 7.1%, and 4.5% contribution to dissimilarity, respectively). On average, river biofilm was characterized by significantly higher abundance of *Aeromonas*, and *Rheinheimera* (35% and 15% of total biofilm community, respectively) compared to river water (0.6% and 0.3% of total water community, respectively) (Fig. 4). However, river water had higher abundance of *Polynucleobacter* as compared to river biofilm (9.8% compared to 0.01%) (Fig. 4).

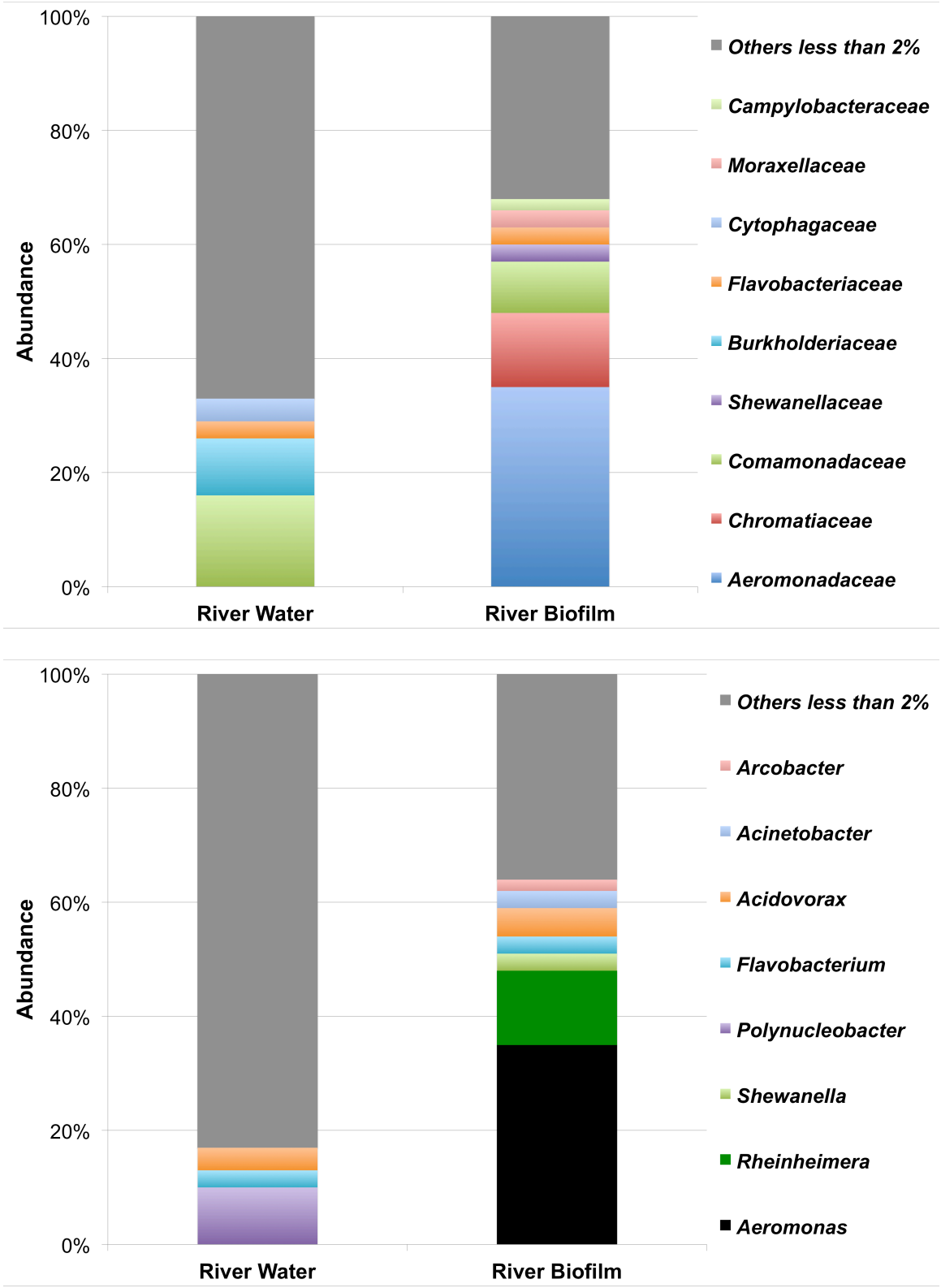


Figure 3.4. Most abundant bacterial families (top) and genera (bottom) in river water and 24 hour river biofilm communities.

24 hours after exposure to river water, taxonomic composition of communities formed on established biofilms were different from both river biofilm and river water communities (Fig. 5, Appendix B: Tables S-1 and S-2). ANOSIM results suggested that river biofilm community was significantly different from the majority of other established biofilm communities at all the three different tested time points (P-values bellow 0.03 for all communities and R-values close to 1). *Bacillus C20* and *Serratia RL10* were the only exceptions, their communities were not too different and they did not show large biofilm forming capabilities based on the crystal violet results (Appendix B: Tables S-1 and S-2). The overall average dissimilarity (%) between river biofilm and other biofilm communities 24 hours after exposure to river microbiome were as followed: *P. aeruginosa* (86.99), *Deinococcus aquaticus F4* (71.73), *Serratia D14* (88.75), *Hydrogenophaga F14* (65.99), *Brevundimonas F16* (76.71), *Acidovorax F19* (76.95), *Hydrogenophaga-Brevundimonas* mixed biofilm (92.39), and *Brevundimonas-Acidovorax* mixed biofilm (93.07) (Appendix B: Table S-3).

Aeromonas was the genus that contributed most to the dissimilarity between river biofilm and other established biofilm communities. It accounted for 35% of the river biofilm community but its abundance in the established biofilm communities of bacterial isolates 24 hours after exposure to river water ranged from 7% in *Deinococcus aquaticus F4* to 0% in *P. aeruginosa* biofilm communities (Fig. 6).

After exposure to river microbiota, in most biofilm communities, significant differences between the taxonomical composition of established biofilms of tested bacterial isolates was observed (Appendix B: Tables S-1 and S-2, Fig. S2-A to G).

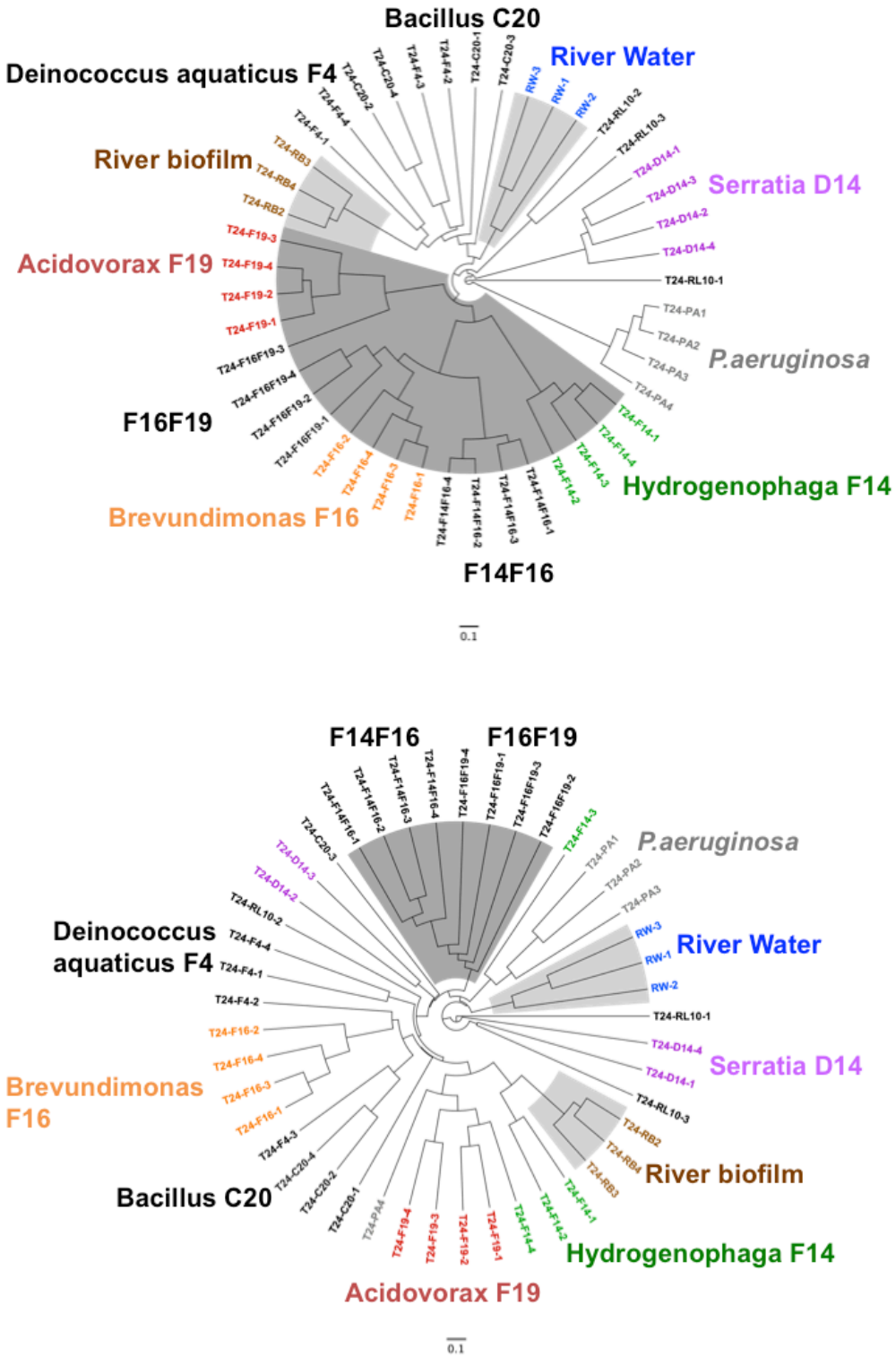


Figure 3.5. Cluster analysis of biofilm communities of tested bacterial isolates 24 hours after exposure to river microbiota (Bray-Curtis). (Top) Founding populations included in the calculation, and (Bottom) founding populations removed prior to clustering.

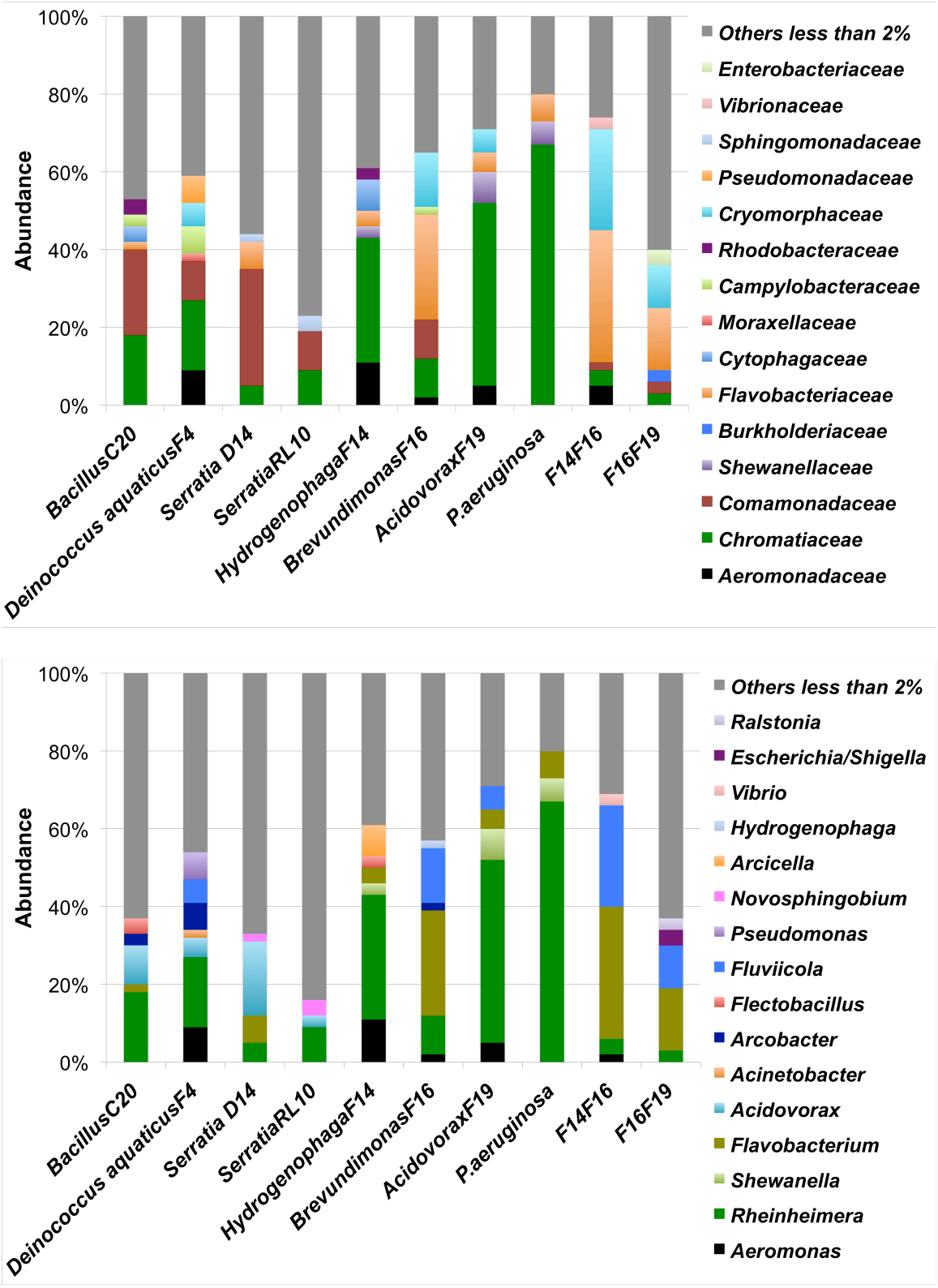


Figure 3.6. Most abundant bacterial families and genera in 24 hour established biofilm communities. Founding populations were removed prior to analysis.

At early time points (4 and 8 hours), each of the established single-species biofilms of *Hydrogenophaga F14*, *Brevundimonas F16*, and *Acidovorax F19* selected for the other two genera from the river water community (Table 2).

	T= 4 hr.	T= 8 hr.	T= 24 hr.
Hydrogenophaga F14	***Brevundimonas, Rheinheimera, Comamondaceae, Fluviicola, Rhodobacteraceae***	***Rheinheimera, Acinetobacter, Fluviicola, Comamonadaceae, Fluviicola***	***Rheinheimera, Aeromonas, Arcicella, Flavobacterium, Rheinheimera***
Brevundimonas F16	***Hydrogenophaga, Acidovorax, Comamondaceae, Tolumonas, Arcobacter***	***Acidovorax, Streptococcus, Acidovorax, Fluviicola, Comamonadaceae***	***Flavobacterium, Fluviicola, Rheinheimera, Comamondaceae, Flavobacterium***
Acidovorax F19	***Hydrogenophaga, Brevundimonas, Serratia, Novosphingobium, Fluviicola***	***Rheinheimera, Fluviicola, Flavobacterium, Brevundimonas, Hydrogenophaga***	***Rheinheimera, Shewanella, Fluviicola, Aeromonas, Rheinheimera***
F16 + F14	***Fluviicola, Fluviicola, Comamondaceae, Acidovorax, Acidovorax***	***Fluviicola, Flectobacillus, Acidovorax, Pseudomonas, Fluviicola***	***Flavobacterium, Fluviicola, Rheinheimera, Vibrio, Aeromonas***
F16 + F19	***Pseudomonas, Pseudomonas, Pseudomonas, Hydrogenophaga, Comamonadaceae***	***Fluviicola, Rheinheimera, Flavobacterium, Arcobacter, Vibrio***	***Flavobacterium, Fluviicola, Escherichia/Shigella, Rheinheimera, Pseudomonas***

Table 3.2. Top five bacterial members of the river microbiota found in double-species biofilm communities and their single-species counterparts at different time points after exposure to river water.

Some genera like *Flavobacterium*, and *Rheinheimera* were found in all studied communities (Fig. 7). Additionally, 24 hours after exposure to river microbiome *Bacillus C20, Hydrogenophaga F14* and *Acidovorax F19* biofilms had the lowest abundance of

potential fish pathogens, *Aeromonas* and *Flavobacterium,* while having the highest levels of the potential Lake Sturgeon fish egg mutualist *Rheinheimera* (Fig. 7).

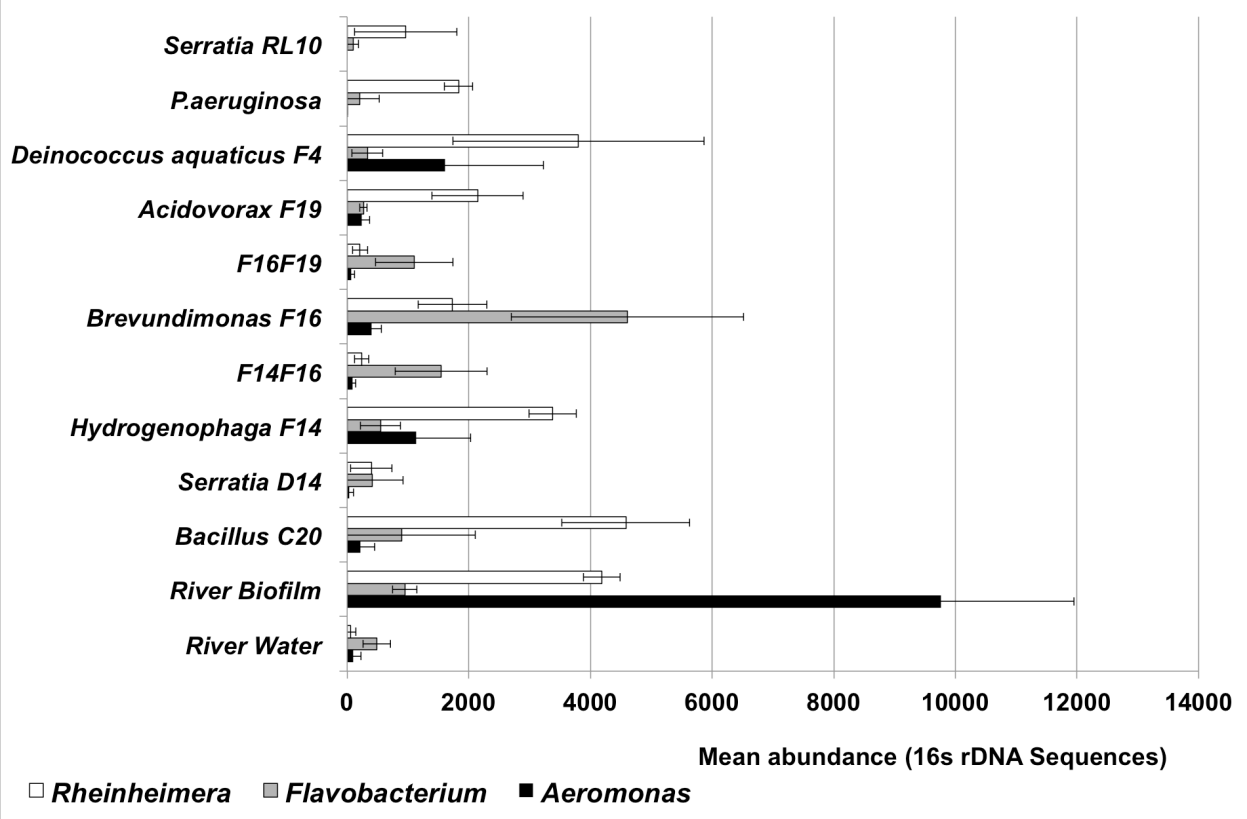


Figure 3.7. Comparing sequence numbers of the genera *Rheinheimera*, *Aeromonas* and *Flavobacterium* in different 24 hour biofilm communities. For this figure the mean abundance of 16s rDNA sequences of four replicates for each genus was calculated.

Brevundimonas F16 and the mixed biofilm communities of *Brevundimonas F16-Hydrogenophaga F14* and *Brevundimonas F16-Acidovorax F19* had the highest abundance of *Flavobacterium* in their biofilm communities 24 hours after exposure to river microbiome. Interestingly, when viewed at the OTU level (0.03 cutoff), each of these biofilm communities attracted specific OTUs of the genera *Flavobacterium* and *Rheinheimera* that were unique to that community (Table 3).

	T = 4 hr.			T = 8 hr.			T = 24 hr.		
	Aero	***Flavo***	***Rhe***	***Aero***	***Flavo***	***Rhe***	***Aero***	***Flavo***	***Rhe***
River Biofilm	-	22, 42	10	11	22, 52	10	11	22, 60, 70	10, 17, 41, 45
Bacillus C20	-	22, 42	10	-	22	10	11	33, 42	10, 17, 41
Deinococcus aquaticus F4	11	22, 52	10	11	22	10, 17, 41, 45, 48	11, 84	15	10, 17, 41
Serratia D14	-	67, 22, 52, 111, 94	10	-	22	10	-	15, 89, 42, 11	10, 45
Serratia RL10	-	67, 111,120	-	11	67, 22, 89	10	-	60	10, 45
P. aeruginosa	-	22	-	-	22	-	-	33, 479, 573, 1374, 294, 1181, 15	10, 17, 117, 145, 48, 1171, 1123, 46, 1504
Hydrogenophaga F14	11	22, 52	10, 17	11	22, 42, 52	10	11	22, 38	10, 17, 41, 45, 48
Brevundimonas F16	11	22, 42	10	11	22, 15, 33, 52	10	11	15, 33, 96, 112, 22, 205	10
Acidovorax F19	-	42, 146	10	11	22, 33, 15, 70	10, 17	11	22, 15, 33, 70, 42	10, 41, 17, 45, 46, 48, 805, 787
F14 + F16	-	22, 42	10	-	22, 15	10	11	15, 33, 112, 52, 238, 22	10, 41
F16 + F19	-	22	10	-	15, 22	10	11	15, 33, 22, 112, 301, 238	10

Table 3.3. Comparing different OTUs that belong to genera *Rheinheimera* (Rhe), *Aeromonas* (Aero) and *Flavobacterium* (Flavo) in different established biofilm communities at different time points (T=4 hr., T=8 hr., and T=24 hr.). In this table numbers represent OTU numbers, for example “11” means “OTU11”.

DISCUSSION

In search for potential probiotics to reduce the high egg mortality in Lake Sturgeon eggs, we studied the influence of established biofilms of selected egg isolates on the community assembly when these biofilms were challenged with the river water. To quantify changes in community composition, we measured the formation of biofilms in wells lacking any previously established biofilm (river biofilm) as a comparative control for natural biofilm formation in microtiter plates. We reasoned that any deviation between this pure river biofilm and river biofilm formed in the presence of established biofilm would be caused initially by changes in surface chemistry available to river populations. As mentioned above, this approach allowed us to measure the stability or susceptibility to secondary colonization by river populations. Secondary colonization can be a result of aggression, collaboration, or even an absence of interactions, other than binding. However, without any knowledge of the changes in the founding population over time, we cannot identify whether a river population is invasive in an aggressive manner or is collaborative with the founding population. As a result, by determining the fraction of the founding population that is lost over 24 hours, we can distinguish between mutualistic and invasive interactions.

We assumed there were two aspects to these “invasions” or “mutualisms”. The first aspect simply involved the binding of members of the river community to the established biofilm, which we assumed could occur early (within 4 hours) after introducing river water. The second aspect involves more subtle inter-species interactions that we assumed would take longer to develop and detect. For example, the expression of antimicrobials, the consequences of starvation as the founding

populations shifted from weak broth to river water, or effects of bacteriophages are all ecological attributes that we assumed would require additional time to become expressed (24 hours). Using a replicated experimental approach, we were able to detect development of highly resilient and weak biofilms, specific exclusions and recruitments of members of river water bacterial community by tested isolates and apparent enhancements of biofilm development.

There were detectable differences between Lake Sturgeon egg isolates in their ability to form biofilms in R2Broth. This is attributable to genetic differences between these bacterial species. We also detected differences in biofilm stability after exposure to filtered or unfiltered river water in the tested bacterial isolates. While *P. aeruginosa* biofilm biomass was only slightly diminished by river water, *Deinococcus aquaticus F4* was dramatically reduced by both filtered and unfiltered river water, suggesting that the shift from weak broth to more oligotrophic river water caused starvation and then abandonment of the biofilm. This pattern was in contrast to those seen in *Brevundimonas F16*, *Acidovorax F19* and the mixed biofilm of *Brevundimonas-Acidovorax*, where the filtered river water significantly reduced the biofilm biomass but the unfiltered river water increased the biofilm biomass. These observations suggest that certain river populations, those that can be removed by the filtration process, appear to contribute to and promote the existing biofilms of the tested species. This result indicates that as biofilms attached to the Lake Sturgeon egg surface, these bacterial isolates indeed interact with members of the river microbiota and are able to recruit some members of the river water community that, in turn, can affect the wellbeing of the Lake Sturgeon eggs.

The results from Illumina sequencing revealed the phylogenetic composition of bacterial communities and confirmed shifts in demographics as a result of immigration of river populations into existing biofilm. The Gram-positive species, *Bacillus C20* and *Deinococcus aquaticus F4* both abandoned the biofilm within 24 hours. The remaining species and combinations were resilient and accounted for 50-95% of biofilm communities. Of particular interest to us were the mixed communities of *Brevundimonas F16* with either *Acidovorax F19* or *Hydrogenophaga F14*. These two combinations were selected because they showed increased biofilm biomass when mixed (Chapter 2). If we were to predict their demographics after challenge with river water then a drop of 30-40% would be expected, based on the results from single-species biofilms. However, as mixed biofilms they appeared more resilient, losing only 10-15% of the total population. This strongly suggests that their alliance produces a biofilm that is more resilient to invasion by natural river populations.

Additional studies on the Lake Sturgeon eggs suggested that colonization is a rapid phenomenon that can start as early as 15 minutes after exposure to river water. The present study also suggests that river biofilm community moves away from the pelagic community in as little as 4 hours after exposure to river microbiome (Fig. 3). Different patterns of OTU numbers were detected in the biofilm communities that were challenged with river microbiota (Table 1). In river biofilm community (RB) the number of OTUs was high after 4 hours and remained high during the course of challenge with river water. In other biofilm communities it either increased (*Acidovorax F19* and the mixed biofilm of *Brevundimonas-Acidovorax*) or decreased (*Serratia D14*, *Deinococcus aquaticus F4*, and *Bacillus C20*) over time. One clearly detectable trend was the

diminution of OTUs by established biofilms at early time points, best observed in *Brevundimonas F16*, *Acidovorax F19*, *Serratia RL10*, *P. aeruginosa* and the mixed biofilm of *Hydrogenophaga-Brevundimonas* communities. This data suggests that the initial binding of populations from river microbiota was strongly influenced by the established biofilms. One might propose that the initial binding is somewhat immune from the effect of more subtle community interactions such as aggressive or mutualistic behaviors that require longer time.

When it comes to the number of OTUs belonging to river water bacterial community detected in the established biofilms of tested isolates, the numbers increased with the increase of exposure time to river microbiota. This is consistent with invasion of the established biofilms, rapidly in some cases but gradually in most, by river water populations. The river water biofilm community is distinct indicating a non-neutral selection process that changes and evolves over time. Major populations from the river are excluded from this process.

The genera *Aeromonas* (e.g. *A. hydrophila* and *A. salmonicida*) and *Flavobacterium* (e.g. *F. columnare*, *F. johnsonae*, *F. psychrophilum*, and *F. branchiophilum*) are among the major bacterial pathogens that affect fish [53, 55-68]. *Aeromonas* seemed quite accepting of a charged and hydrophobic surface (tissue culture microtiter plates) but not as accepting of the same surface preconditioned with other biofilms, which is an indication that these bacterial species may be probiotics in their exclusion of *Aeromonas* from their biofilm. However, the abundance of *Flavobacterium* increased significantly in the biofilm community of *Brevundimonas F16* after exposure to river water (Figures 6 and 7). Furthermore, each established biofilm

seemed to attract a specific set of OTUs associated with the genus *Flavobacterium* (Table 3). We know the egg selects for some species of *Flavobacterium* and not others but we do not know if it selects for pathogenic *Flavobacterium* species. While current study does not address this distinction, it is important to identify which isolates attract pathogenic *Flavobacterium* species.

Rheinheimera has been shown to be a producer of antimicrobial compounds, making it a potential genus that can provide protection against pathogens [69, 70]. In our experiments, although higher abundance of *Rheinheimera* appeared to have little to no effect on *Aeromonas* population, it was in correlation with lower *Flavobacterium* abundance (Fig. 7). Based on this result *Rheinheimera* appears to be a potential probiotic of Lake Sturgeon eggs against *Flavobacterium* and, thus, its effect on the eggs wellbeing needs to be investigated *in vivo* in the future.

To summarize, in this work we have identified bacterial populations that formed robust and resilient biofilm communities (e.g. *Brevundimonas F16* and *Pseudomonas aeruginosa*) when challenged with river water, and those that were considerably more sensitive to invading populations (e.g. *Deinococcus aquaticus F4*). Our results indicate that established biofilm communities selected for and/or excluded populations from river water, resulting in a biofilm community quite distinct from a community formed in the absence of prior colonization. These data indicate that historical contingency can play a significant role in the structuring of biofilms. The uniqueness of all communities that were selected by pre-established biofilm populations was statistically supported, suggesting that significant diversity in biofilm assembly exists in nature. Finally, our study has shown that using this *in vitro* biofilm formation assay has the capacity to

reveal unique features of multispecies bacterial biofilms and it can be used as a rapid screening method for putative probiotics that may direct egg-associated community assembly and reduce egg mortality in fish.

APPENDIX

P-value, ANOSIM	River Water	T4-RiverBiofilm	T4-C20	T4-D14	T4-F14	T4-F14F16
River Water						
T4-RiverBiofilm	0.198					
T4-C20	0.026	0.028				
T4-D14	0.029	0.027	0.031			
T4-F14	0.031	0.028	0.028	0.031		
T4-F14F16	0.031	0.027	0.027	0.032	0.028	
T4-F16	0.028	0.034	0.029	0.029	0.028	0.031
T4-F16F19	0.027	0.029	0.030	0.029	0.026	0.028
T4-F19	0.028	0.033	0.029	0.032	0.030	0.029
T4-F4	0.026	0.029	0.029	0.028	0.028	0.027
T4-PA	0.028	0.029	0.026	0.027	0.026	0.029
T4-RL10	0.096	0.101	0.030	0.029	0.030	0.027
T8-RiverBiofilm	0.027	0.303	0.028	0.027	0.031	0.029
T8-C20	0.031	0.119	0.057	0.029	0.026	0.028
T8-D14	0.028	0.024	0.029	0.028	0.029	0.032
T8-F14	0.033	0.027	0.026	0.027	0.910	0.030
T8-F14F16	0.029	0.028	0.030	0.030	0.031	0.413
T8-F16	0.029	0.027	0.032	0.028	0.028	0.029
T8-F16F19	0.027	0.029	0.029	0.028	0.027	0.028
T8-F19	0.029	0.027	0.030	0.028	0.030	0.029
T8-F4	0.029	0.027	0.029	0.030	0.027	0.027
T8-PA	0.026	0.030	0.027	0.029	0.031	0.026
T8-RL10	0.098	0.095	0.027	0.030	0.028	0.030
T24-RiverBiofilm	0.026	0.101	0.031	0.028	0.032	0.029
T24-C20	0.031	0.196	0.030	0.025	0.032	0.028
T24-D14	0.028	0.029	0.029	0.056	0.029	0.029
T24-F14	0.029	0.024	0.030	0.028	0.032	0.027
T24-F14F16	0.029	0.029	0.028	0.030	0.028	0.029
T24-F16	0.027	0.030	0.029	0.028	0.031	0.028
T24-F16F19	0.031	0.028	0.026	0.030	0.029	0.029
T24-F19	0.029	0.027	0.031	0.028	0.028	0.029
T24-F4	0.029	0.027	0.027	0.026	0.029	0.028
T24-PA	0.028	0.028	0.029	0.029	0.027	0.029
T24-RL10	0.103	0.098	0.029	0.031	0.029	0.030

Table S-1. Results of pairwise Analysis Of Similarities (ANOSIM) between different communities. Founding populations were removed prior to analysis (P- values).

Table S-1 (cont'd)

P-value, ANOSIM	T4-F16	T4-F16F19	T4-F19	T4-F4	T4-PA	T4-RL10
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19	0.029					
T4-F19	0.030	0.028				
T4-F4	0.030	0.031	0.026			
T4-PA	0.030	0.029	0.031	0.028		
T4-RL10	0.027	0.029	0.032	0.027	0.029	
T8-RiverBiofilm	0.030	0.028	0.029	0.026	0.033	0.097
T8-C20	0.028	0.029	0.025	0.030	0.031	0.027
T8-D14	0.029	0.030	0.029	0.030	0.028	0.028
T8-F14	0.028	0.027	0.029	0.028	0.031	0.030
T8-F14F16	0.027	0.031	0.029	0.027	0.028	0.027
T8-F16	0.867	0.029	0.029	0.031	0.029	0.027
T8-F16F19	0.033	0.058	0.029	0.031	0.029	0.026
T8-F19	0.027	0.029	0.202	0.027	0.033	0.032
T8-F4	0.029	0.029	0.027	0.029	0.031	0.028
T8-PA	0.026	0.029	0.028	0.032	0.058	0.029
T8-RL10	0.028	0.028	0.028	0.028	0.027	0.102
T24-RiverBiofilm	0.031	0.030	0.030	0.031	0.027	0.105
T24-C20	0.028	0.030	0.031	0.027	0.027	0.027
T24-D14	0.028	0.030	0.028	0.028	0.028	0.034
T24-F14	0.029	0.027	0.028	0.027	0.029	0.031
T24-F14F16	0.027	0.027	0.029	0.029	0.027	0.031
T24-F16	0.027	0.028	0.028	0.027	0.028	0.031
T24-F16F19	0.027	0.029	0.032	0.026	0.031	0.027
T24-F19	0.030	0.028	0.028	0.031	0.031	0.027
T24-F4	0.027	0.032	0.028	0.029	0.024	0.028
T24-PA	0.030	0.025	0.029	0.030	0.261	0.030
T24-RL10	0.027	0.027	0.028	0.026	0.032	0.104

Table S-1 (cont'd)

P-value, ANOSIM	T8-RiverBiofilm	T8-C20	T8-D14	T8-F14	T8-F14F16	T8-F16
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19						
T4-F19						
T4-F4						
T4-PA						
T4-RL10						
T8-RiverBiofilm						
T8-C20	0.058					
T8-D14	0.030	0.030				
T8-F14	0.028	0.027	0.026			
T8-F14F16	0.028	0.029	0.030	0.028		
T8-F16	0.028	0.031	0.028	0.030	0.030	
T8-F16F19	0.027	0.031	0.030	0.028	0.030	0.028
T8-F19	0.028	0.028	0.034	0.028	0.032	0.029
T8-F4	0.026	0.029	0.026	0.026	0.026	0.028
T8-PA	0.028	0.030	0.029	0.027	0.029	0.030
T8-RL10	0.101	0.028	0.029	0.029	0.032	0.026
T24-RiverBiofilm	0.098	0.030	0.029	0.027	0.027	0.028
T24-C20	0.057	0.030	0.030	0.030	0.028	0.028
T24-D14	0.031	0.029	0.054	0.027	0.030	0.026
T24-F14	0.027	0.028	0.028	0.055	0.027	0.028
T24-F14F16	0.025	0.031	0.033	0.029	0.029	0.030
T24-F16	0.027	0.027	0.030	0.030	0.029	0.029
T24-F16F19	0.027	0.029	0.028	0.029	0.029	0.030
T24-F19	0.030	0.028	0.031	0.031	0.027	0.030
T24-F4	0.029	0.028	0.027	0.030	0.029	0.027
T24-PA	0.026	0.033	0.030	0.026	0.029	0.028
T24-RL10	0.098	0.032	0.029	0.030	0.028	0.030

Table S-1 (cont'd)

P-value, ANOSIM	T8-F16F19	T8-F19	T8-F4	T8-PA	T8-RL10
River Water					
T4-RiverBiofilm					
T4-C20					
T4-D14					
T4-F14					
T4-F14F16					
T4-F16					
T4-F16F19					
T4-F19					
T4-F4					
T4-PA					
T4-RL10					
T8-RiverBiofilm					
T8-C20					
T8-D14					
T8-F14					
T8-F14F16					
T8-F16					
T8-F16F19					
T8-F19	0.030				
T8-F4	0.029	0.028			
T8-PA	0.029	0.030	0.031		
T8-RL10	0.031	0.029	0.028	0.028	
T24-RiverBiofilm	0.026	0.026	0.031	0.028	0.101
T24-C20	0.029	0.029	0.031	0.032	0.028
T24-D14	0.029	0.026	0.030	0.030	0.030
T24-F14	0.030	0.026	0.028	0.032	0.029
T24-F14F16	0.028	0.028	0.027	0.029	0.028
T24-F16	0.026	0.030	0.027	0.030	0.029
T24-F16F19	0.059	0.028	0.027	0.030	0.031
T24-F19	0.032	0.264	0.028	0.028	0.029
T24-F4	0.026	0.028	0.030	0.028	0.028
T24-PA	0.028	0.029	0.027	0.055	0.027
T24-RL10	0.027	0.029	0.026	0.028	0.202

Table S-1 (cont'd)

P-value, ANOSIM	T24-RiverBiofilm	T24-C20	T24-D14	T24-F14	T24-F14F16	T24-F16
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19						
T4-F19						
T4-F4						
T4-PA						
T4-RL10						
T8-RiverBiofilm						
T8-C20						
T8-D14						
T8-F14						
T8-F14F16						
T8-F16						
T8-F16F19						
T8-F19						
T8-F4						
T8-PA						
T8-RL10						
T24-RiverBiofilm						
T24-C20	0.084					
T24-D14	0.031	0.026				
T24-F14	0.027	0.028	0.027			
T24-F14F16	0.028	0.027	0.029	0.029		
T24-F16	0.031	0.030	0.029	0.031	0.028	
T24-F16F19	0.028	0.028	0.028	0.030	0.028	0.030
T24-F19	0.026	0.031	0.029	0.027	0.032	0.028
T24-F4	0.030	0.029	0.027	0.031	0.031	0.029
T24-PA	0.030	0.027	0.029	0.030	0.025	0.029
T24-RL10	0.097	0.028	0.028	0.028	0.031	0.031

Table S-1 (cont'd)

P-value, ANOSIM	T24-F16F19	T24-F19	T24-F4	T24-PA	T24-RL10
River Water					
T4-RiverBiofilm					
T4-C20					
T4-D14					
T4-F14					
T4-F14F16					
T4-F16					
T4-F16F19					
T4-F19					
T4-F4					
T4-PA					
T4-RL10					
T8-RiverBiofilm					
T8-C20					
T8-D14					
T8-F14					
T8-F14F16					
T8-F16					
T8-F16F19					
T8-F19					
T8-F4					
T8-PA					
T8-RL10					
T24-RiverBiofilm					
T24-C20					
T24-D14					
T24-F14					
T24-F14F16					
T24-F16					
T24-F16F19					
T24-F19	0.032				
T24-F4	0.032	0.028			
T24-PA	0.027	0.028	0.029		
T24-RL10	0.031	0.030	0.027	0.030	

R-value, ANOSIM	River Water	T4-RiverBiofilm	T4-C20	T4-D14	T4-F14	T4-F14F16
River Water						
T4-RiverBiofilm	0.333					
T4-C20	1	0.704				
T4-D14	1	0.907	1			
T4-F14	1	1	1	1		
T4-F14F16	1	1	1	1	1	
T4-F16	1	1	1	1	1	1
T4-F16F19	1	1	1	1	1	1
T4-F19	1	1	1	1	1	1
T4-F4	1	0.778	1	1	1	1
T4-PA	1	1	1	1	1	1
T4-RL10	1	1	1	1	1	1
T8-RiverBiofilm	1	0.148	0.704	1	1	1
T8-C20	0.629	0.333	0.344	0.802	1	1
T8-D14	1	0.926	1	0.625	1	1
T8-F14	1	1	1	1	0.135	1
T8-F14F16	1	1	1	1	1	0.0104
T8-F16	1	1	1	1	1	1
T8-F16F19	1	1	1	1	1	1
T8-F19	1	1	1	1	1	1
T8-F4	1	0.704	1	1	1	1
T8-PA	1	1	1	1	1	1
T8-RL10	1	1	1	1	1	1
T24-RiverBiofilm	1	0.407	1	1	1	1
T24-C20	0.889	0.167	0.583	1	1	1
T24-D14	1	0.926	1	0.427	1	1
T24-F14	1	0.833	1	1	0.729	1
T24-F14F16	1	1	1	1	1	0.635
T24-F16	1	0.722	1	1	1	1
T24-F16F19	1	1	1	1	1	0.875
T24-F19	1	0.981	1	1	1	1
T24-F4	1	0.685	0.979	1	1	1
T24-PA	1	1	1	1	1	1
T24-RL10	0.704	0.444	0.759	0.87	1	1

Table S-2. Results of pairwise Analysis Of Similarities (ANOSIM) between different communities. Founding populations were removed prior to analysis (R-values).

Table S-2 (cont'd)

R-value, ANOSIM	T4-F16	T4-F16F19	T4-F19	T4-F4	T4-PA	T4-RL10
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19	1					
T4-F19	1	1				
T4-F4	1	1	1			
T4-PA	1	1	1	1		
T4-RL10	1	1	1	1	1	
T8-RiverBiofilm	1	1	1	1	1	1
T8-C20	1	1	1	0.719	1	1
T8-D14	1	1	1	1	1	1
T8-F14	1	1	1	1	1	1
T8-F14F16	1	1	1	1	1	1
T8-F16	0.198	1	1	1	1	1
T8-F16F19	1	0.281	1	1	1	1
T8-F19	1	1	0.187	1	1	1
T8-F4	1	1	1	1	1	1
T8-PA	1	1	1	1	0.542	1
T8-RL10	1	1	1	1	1	0.852
T24-RiverBiofilm	1	1	1	1	1	1
T24-C20	1	1	1	1	1	1
T24-D14	1	1	1	1	1	1
T24-F14	1	1	1	1	1	1
T24-F14F16	1	1	1	1	1	1
T24-F16	1	1	1	1	1	1
T24-F16F19	0.792	0.469	0.979	1	1	1
T24-F19	1	1	0.354	1	1	1
T24-F4	1	1	1	1	1	1
T24-PA	1	1	1	1	0.167	1
T24-RL10	1	1	1	0.833	1	0.444

Table S-2 (cont'd)

R-value, ANOSIM	T8-RiverBiofilm	T8-C20	T8-D14	T8-F14	T8-F14F16	T8-F16
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19						
T4-F19						
T4-F4						
T4-PA						
T4-RL10						
T8-RiverBiofilm						
T8-C20	0.352					
T8-D14	1	0.8438				
T8-F14	1	1	1			
T8-F14F16	1	1	1	1		
T8-F16	1	1	1	1	1	
T8-F16F19	1	1	1	1	1	1
T8-F19	1	1	1	1	1	1
T8-F4	0.833	0.625	1	1	1	1
T8-PA	1	1	1	1	1	1
T8-RL10	1	1	1	1	1	1
T24-RiverBiofilm	0.556	0.629	1	1	1	1
T24-C20	0.37	0.417	1	1	1	1
T24-D14	1	1	0.292	1	1	1
T24-F14	0.889	0.823	1	0.489	0.979	1
T24-F14F16	1	0.989	1	1	0.739	1
T24-F16	0.944	0.677	1	1	1	1
T24-F16F19	1	1	1	1	0.937	0.781
T24-F19	1	0.989	1	1	1	1
T24-F4	0.778	0.75	1	1	1	1
T24-PA	1	1	1	1	1	1
T24-RL10	0.556	0.648	0.889	1	1	1

Table S-2 (cont'd)

R-value, ANOSIM	T8-F16F19	T8-F19	T8-F4	T8-PA	T8-RL10
River Water					
T4-RiverBiofilm					
T4-C20					
T4-D14					
T4-F14					
T4-F14F16					
T4-F16					
T4-F16F19					
T4-F19					
T4-F4					
T4-PA					
T4-RL10					
T8-RiverBiofilm					
T8-C20					
T8-D14					
T8-F14					
T8-F14F16					
T8-F16					
T8-F16F19					
T8-F19	1				
T8-F4	1	1			
T8-PA	1	1	1		
T8-RL10	1	1	1	1	
T24-RiverBiofilm	1	1	1	1	1
T24-C20	1	1	0.802	1	1
T24-D14	1	1	1	1	1
T24-F14	1	1	1	1	1
T24-F14F16	1	1	1	1	1
T24-F16	1	1	1	1	1
T24-F16F19	0.312	1	1	1	1
T24-F19	0.969	0.135	1	1	1
T24-F4	1	1	0.917	1	1
T24-PA	1	1	1	0.344	1
T24-RL10	1	1	0.778	1	0.148

Table S-2 (cont'd)

R-value, ANOSIM	T24-RiverBiofilm	T24-C20	T24-D14	T24-F14	T24-F14F16	T24-F16
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19						
T4-F19						
T4-F4						
T4-PA						
T4-RL10						
T8-RiverBiofilm						
T8-C20						
T8-D14						
T8-F14						
T8-F14F16						
T8-F16						
T8-F16F19						
T8-F19						
T8-F4						
T8-PA						
T8-RL10						
T24-RiverBiofilm						
T24-C20	0.444					
T24-D14	1	1				
T24-F14	1	0.875	1			
T24-F14F16	1	1	1	1		
T24-F16	1	0.875	1	1	1	
T24-F16F19	1	1	1	1	0.917	0.792
T24-F19	1	1	1	1	1	1
T24-F4	0.796	0.552	1	1	1	0.979
T24-PA	1	1	1	1	1	1
T24-RL10	0.556	0.667	0.852	0.833	1	0.759

Table S-2 (cont'd)

R-value, ANOSIM	T24-F16F19	T24-F19	T24-F4	T24-PA	T24-RL10
River Water					
T4-RiverBiofilm					
T4-C20					
T4-D14					
T4-F14					
T4-F14F16					
T4-F16					
T4-F16F19					
T4-F19					
T4-F4					
T4-PA					
T4-RL10					
T8-RiverBiofilm					
T8-C20					
T8-D14					
T8-F14					
T8-F14F16					
T8-F16					
T8-F16F19					
T8-F19					
T8-F4					
T8-PA					
T8-RL10					
T24-RiverBiofilm					
T24-C20					
T24-D14					
T24-F14					
T24-F14F16					
T24-F16					
T24-F16F19					
T24-F19	0.979				
T24-F4	1	1			
T24-PA	1	1	1		
T24-RL10	1	0.815	0.741	0.963	

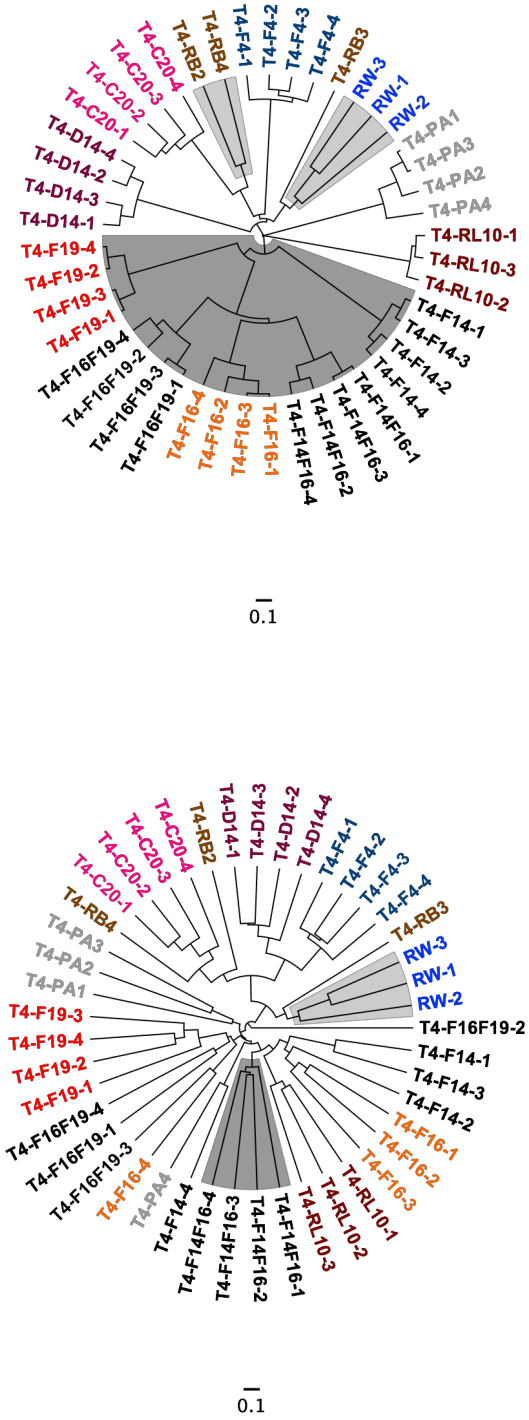
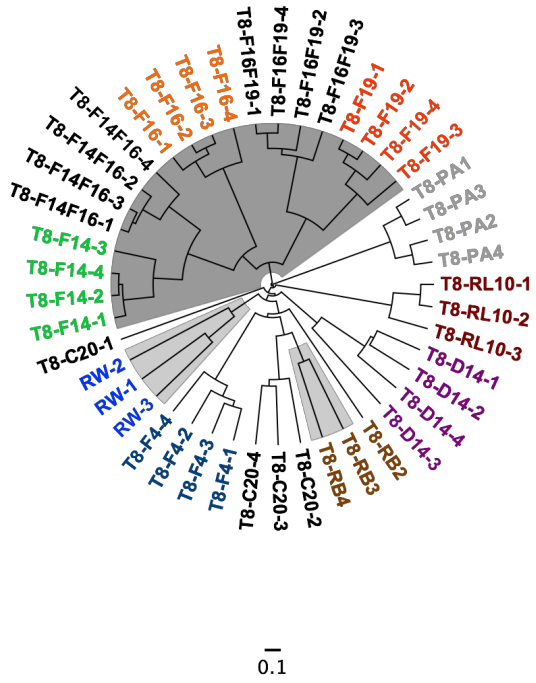
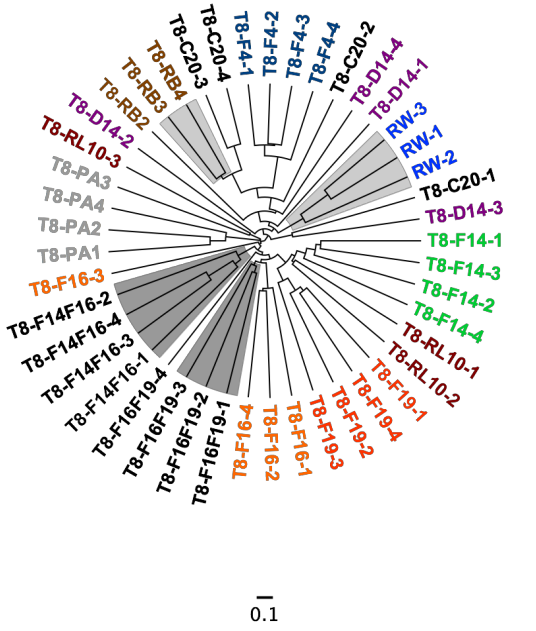


Figure S-1. Cluster analysis of the biofilm communities 4 (page 202) and 8 (page 203) hours after exposure to river water. (Top) founding populations included in the analysis, and (Bottom) founding populations removed prior to clustering. The Neighbor-Joining tree is constructed based on the Bray-Curtis dissimilarity index.

Figure S-1 (cont'd)





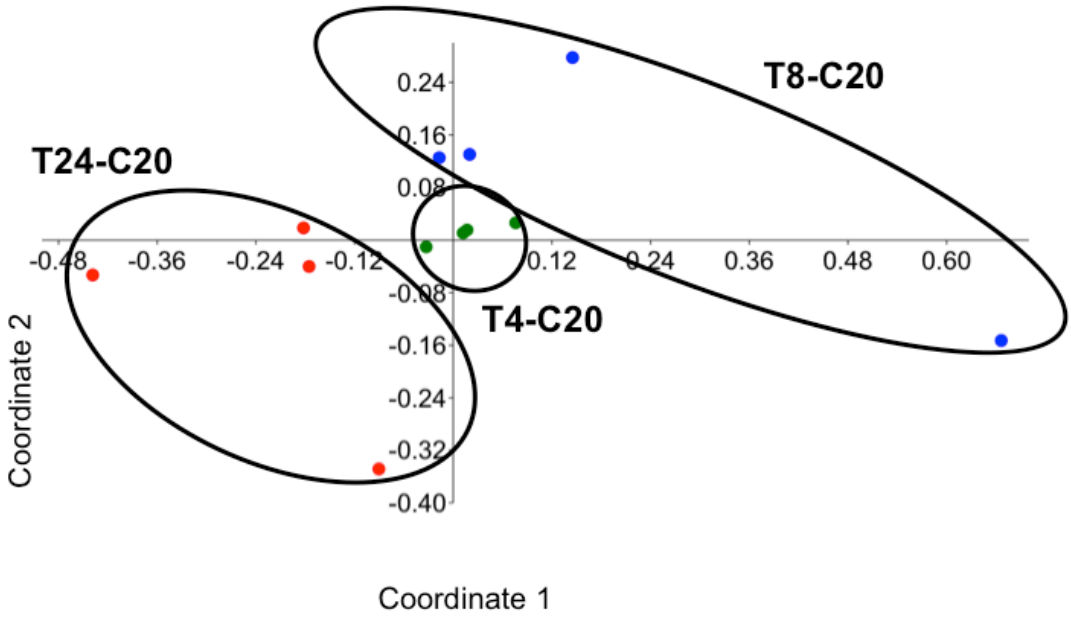


Figure S-2. Comparison of biofilm communities 4, 8, and 24 hours after exposure to river microbiome using non-metric multidimensional scaling (NMDS) analysis (stress values: C20=0.06, D14=0.09, F4=0.07, RL10=0.05, and *P. aeruginosa*=0.17, F14F16=0.1, F16F19=0.1). Founding populations were removed prior to analysis.

Figure S-2 (cont'd)

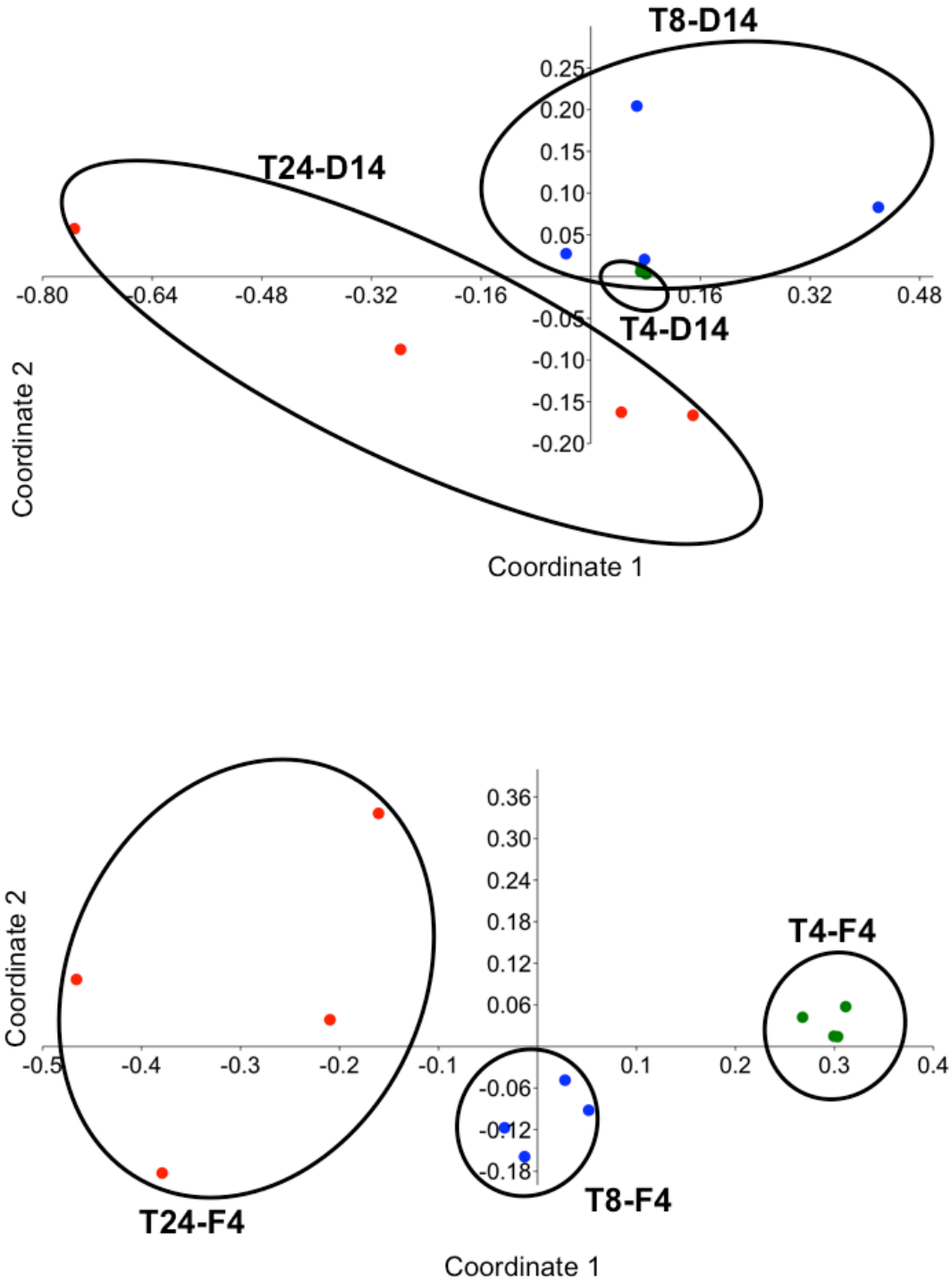
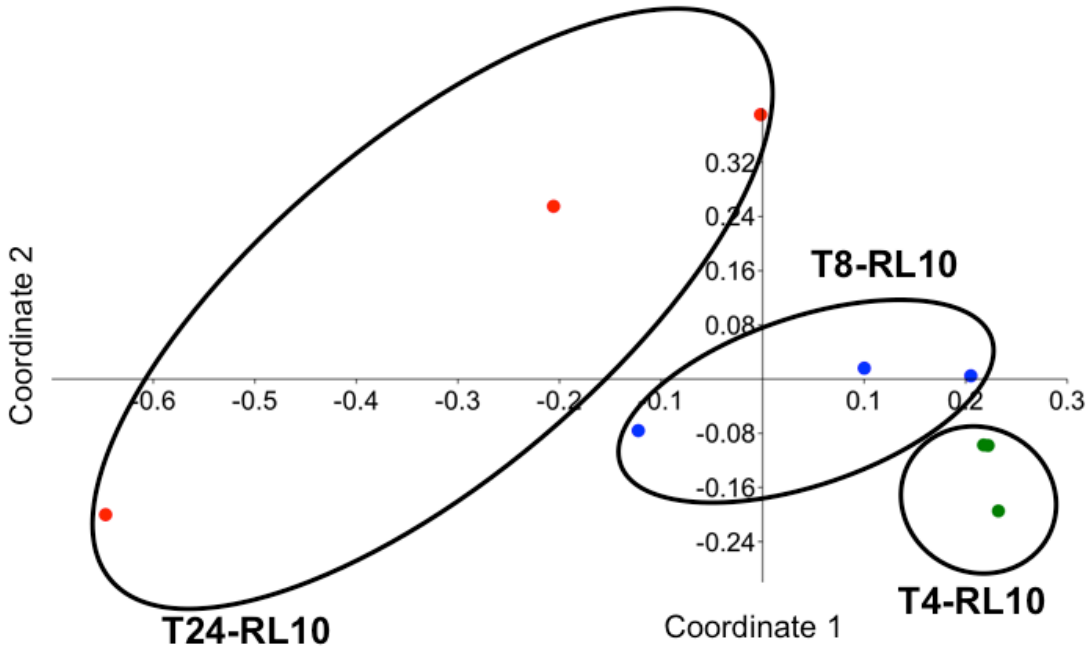


Figure S-2 (cont'd)



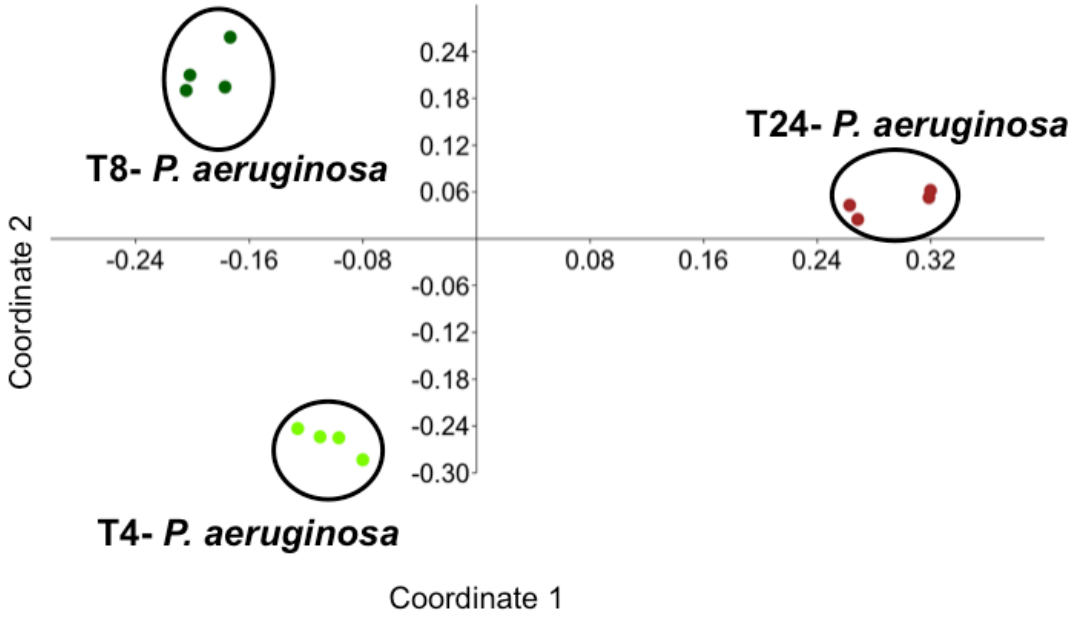
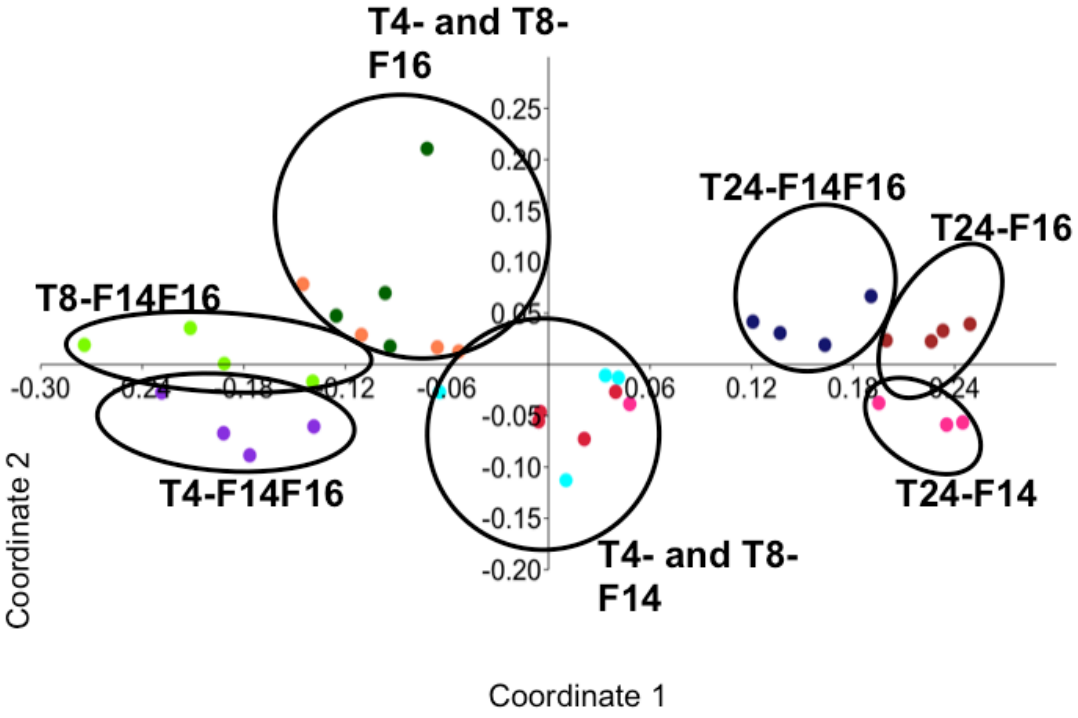
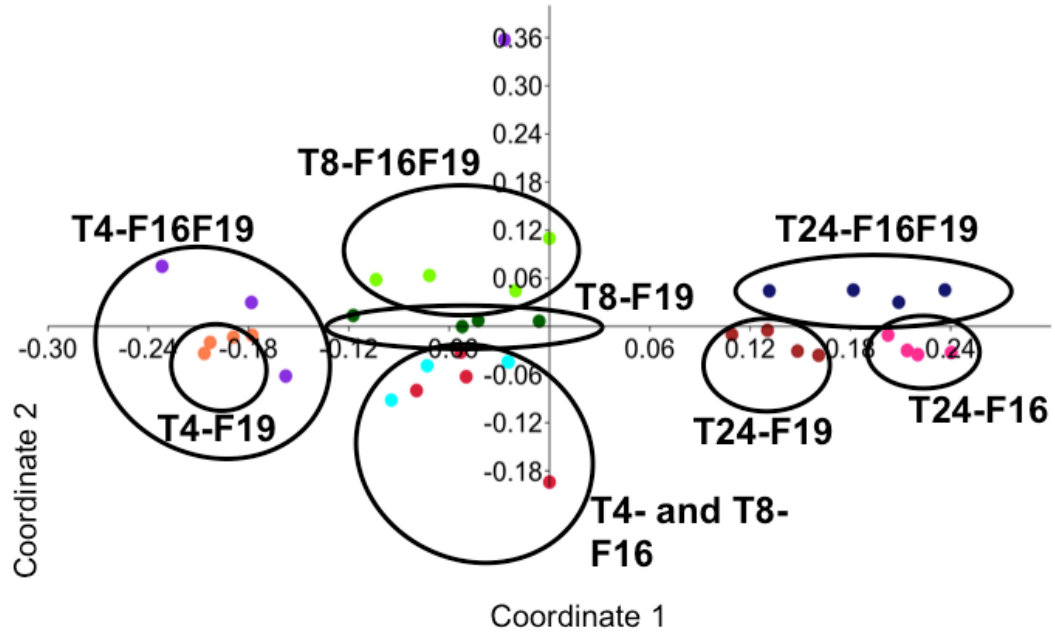


Figure S-2 (cont'd)





Overall average dissimilarity (%); SIMPER	River Water	T4-RiverBiofilm	T4-C20	T4-D14	T4-F14	T4-F14F16
River Water						
T4-RiverBiofilm	80.13					
T4-C20	81.32	68.49				
T4-D14	80.51	83.38	68.36			
T4-F14	93.85	95.98	89.68	77.85		
T4-F14F16	98.81	99.39	98.4	95.78	88.82	
T4-F16	96.09	97.42	94.53	86.66	79.49	82.59
T4-F16F19	99.42	99.56	99.1	97.51	95.47	92.89
T4-F19	99.45	99.66	99.27	97.68	92.96	92.76
T4-F4	79.59	79.2	55.01	52.48	80.4	96.54
T4-PA	99.66	99.81	99.52	98.38	93.08	91.3
T4-RL10	97.08	98.12	95.72	88.51	84.18	83.01
T8-RiverBiofilm	86.31	74.3	63.07	80.79	93.6	99.15
T8-C20	88.08	82.53	63.66	76.35	91.49	98.68
T8-D14	89.34	89.02	82.97	78.49	91.21	98.26
T8-F14	94.89	96	90.6	81.47	70.32	90.46
T8-F14F16	98.93	99.42	98.44	96.2	90.1	79.88
T8-F16	97.46	98.07	96.65	92.05	85.57	84.86
T8-F16F19	98.46	98.9	97.26	93.79	87.7	90.94
T8-F19	97.76	98.58	96.05	90.31	78.92	90.08
T8-F4	87.72	79	58.89	76.82	91.13	98.67
T8-PA	98.34	99.03	98.67	96.56	94.31	95.38
T8-RL10	94.13	95.93	91.61	82.98	80.31	93.16
T24-RiverBiofilm	85.84	80.74	77.97	84.48	94.72	99.24
T24-C20	83.14	76.67	69.78	83.59	95.15	99.29
T24-D14	90.25	90.36	90.62	86.19	94.81	98.86
T24-F14	90.29	87.87	80.73	79.75	84.77	96.77
T24-F14F16	94.57	95.08	88.53	81.08	85.84	96.76
T24-F16	89.81	83.71	70.42	79.03	92	98.68
T24-F16F19	93.77	94.28	89.28	86.52	91.42	98.4
T24-F19	94.27	89.85	80.17	79.78	83.86	96.98
T24-F4	90.86	85.32	77.86	88.34	96.35	99.4
T24-PA	98.12	95.91	91.5	90.14	88.03	96.34
T24-RL10	94	91.33	86.25	90.28	95.08	99.01

Table S-3. Overall average dissimilarity between different communities as calculated by SIMPER. This analysis was done based on just the OTUs attached to each biofilm from the river microbiome and without considering the founding populations of each biofilm community.

Table S-3 (cont'd)

Overall average dissimilarity (%); SIMPER	T4-F16	T4-F16F19	T4-F19	T4-F4	T4-PA	T4-RL10
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19	90.31					
T4-F19	88.74	87.47				
T4-F4	88.32	97.96	98.09			
T4-PA	92.17	95.79	90.45	98.34		
T4-RL10	79.62	94.17	90.51	92.23	90.85	
T8-RiverBiofilm	96.47	99.32	99.51	72.26	99.76	97.95
T8-C20	95.08	99.13	99.33	66.88	99.56	96.66
T8-D14	95.3	99.2	99.25	79.71	99.49	95.37
T8-F14	83.05	96.3	94.47	82.85	95.69	86.07
T8-F14F16	82.4	93.79	93.8	96.73	92.9	87.78
T8-F16	76.52	93.33	91	93.31	94.57	85.01
T8-F16F19	86.55	93.23	92.05	94.79	96.87	88.2
T8-F19	81.6	91.42	82.78	91.55	91.64	85.47
T8-F4	94.94	99.24	99.37	62.44	99.49	96.63
T8-PA	94.27	97.42	95.13	96.24	94.99	94.89
T8-RL10	83.91	95.95	94.98	85.38	95.96	82.3
T24-RiverBiofilm	97.31	99.57	99.66	79.29	99.79	98.22
T24-C20	97.43	99.57	99.66	77.5	99.77	98.29
T24-D14	97.48	99.46	99.36	88.49	99.47	97.39
T24-F14	94.05	98.75	98.25	77.64	98.58	93.03
T24-F14F16	93.17	98.87	99.16	82.64	99.28	94.48
T24-F16	95.43	99.29	99.49	71.84	99.63	96.69
T24-F16F19	96.24	98.56	99.01	85.59	99.42	97.34
T24-F19	92.57	98.1	97.47	76.73	98.65	93.08
T24-F4	98.2	99.79	99.79	81.61	99.74	98.67
T24-PA	93.55	97.97	97.36	87.84	95.92	94.99
T24-RL10	97.62	99.56	99.47	88.17	99.55	98.25

Table S-3 (cont'd)

Overall average dissimilarity (%); SIMPER	T8-RiverBiofilm	T8-C20	T8-D14	T8-F14	T8-F14F16	T8-F16
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19						
T4-F19						
T4-F4						
T4-PA						
T4-RL10						
T8-RiverBiofilm						
T8-C20	72.16					
T8-D14	86.85	84.34				
T8-F14	93.85	91.44	90.98			
T8-F14F16	99.12	98.54	98.18	90.88		
T8-F16	97.83	96.68	96.26	87.31	86.29	
T8-F16F19	98.53	97.49	97.14	88.19	91.07	86.82
T8-F19	97.53	96.37	95.83	76.86	89.12	84.97
T8-F4	67.64	74.12	86.72	90.79	98.64	96.98
T8-PA	99.06	98.55	97.53	95.01	95.64	89.8
T8-RL10	94.61	92.64	91.54	80.34	93.08	88.95
T24-RiverBiofilm	71.03	86.18	91.17	94.81	99.21	98.28
T24-C20	72.92	81.83	89.87	95.25	99.33	98.35
T24-D14	91.72	92.69	90.9	93.62	98.97	97.66
T24-F14	76.61	86.82	88.57	84.37	96.88	95.26
T24-F14F16	92.51	90.45	90.91	86.21	96.11	95.35
T24-F16	72.5	78.99	87.74	93.03	98.51	97.19
T24-F16F19	92.15	90.26	90.25	90.03	97.96	96.51
T24-F19	80.22	85.9	90.08	83.46	96.39	94.71
T24-F4	80.18	86.81	90.84	95.53	99.34	98.43
T24-PA	91.54	93.42	95.75	85.41	96.16	95.08
T24-RL10	88.27	90.89	93.04	94.92	99.05	98.52

Table S-3 (cont'd)

Overall average dissimilarity (%); SIMPER	T8-F16F19	T8-F19	T8-F4	T8-PA	T8-RL10
River Water					
T4-RiverBiofilm					
T4-C20					
T4-D14					
T4-F14					
T4-F14F16					
T4-F16					
T4-F16F19					
T4-F19					
T4-F4					
T4-PA					
T4-RL10					
T8-RiverBiofilm					
T8-C20					
T8-D14					
T8-F14					
T8-F14F16					
T8-F16					
T8-F16F19					
T8-F19	83.3				
T8-F4	97.58	96.45			
T8-PA	94.42	93.1	98.94		
T8-RL10	89.95	84.63	92.68	94.03	
T24-RiverBiofilm	98.78	98.1	66.66	99.26	95.76
T24-C20	98.78	98.1	71.35	99.12	95.79
T24-D14	98.31	97.25	89.22	98.15	95.28
T24-F14	95.43	91.47	68.49	97.45	90.66
T24-F14F16	95.04	92.31	88.15	98.08	90.25
T24-F16	97.91	96.8	69.01	99.01	93.59
T24-F16F19	95.45	93.53	89.63	97.88	93.03
T24-F19	93.3	89.31	66.32	97.19	90
T24-F4	98.88	98.35	68.97	99.15	96.61
T24-PA	95.75	89.53	84.48	97.02	94.47
T24-RL10	98.2	97.58	87.38	98.13	95.89

Table S-3 (cont'd)

Overall average dissimilarity (%); SIMPER	T24-RiverBiofilm	T24-C20	T24-D14	T24-F14	T24-F14F16	T24-F16
River Water						
T4-RiverBiofilm						
T4-C20						
T4-D14						
T4-F14						
T4-F14F16						
T4-F16						
T4-F16F19						
T4-F19						
T4-F4						
T4-PA						
T4-RL10						
T8-RiverBiofilm						
T8-C20						
T8-D14						
T8-F14						
T8-F14F16						
T8-F16						
T8-F16F19						
T8-F19						
T8-F4						
T8-PA						
T8-RL10						
T24-RiverBiofilm						
T24-C20	72.78					
T24-D14	88.75	88.74				
T24-F14	65.99	79.14	89.4			
T24-F14F16	92.39	91.97	91.66	83.15		
T24-F16	76.71	75.15	90.46	76.44	68.25	
T24-F16F19	93.07	91.12	91.86	88.73	67.29	78.23
T24-F19	76.95	82.12	88.03	63.34	78.18	73.93
T24-F4	71.73	76.03	91.43	80.61	85.78	72.75
T24-PA	86.99	91.17	91.96	79.32	90.21	89.78
T24-RL10	90.94	88.92	93.07	89.16	95.47	89.21

Table S-3 (cont'd)

Overall average dissimilarity (%); SIMPER	T24-F16F19	T24-F19	T24-F4	T24-PA	T24-RL10
River Water					
T4-RiverBiofilm					
T4-C20					
T4-D14					
T4-F14					
T4-F14F16					
T4-F16					
T4-F16F19					
T4-F19					
T4-F4					
T4-PA					
T4-RL10					
T8-RiverBiofilm					
T8-C20					
T8-D14					
T8-F14					
T8-F14F16					
T8-F16					
T8-F16F19					
T8-F19					
T8-F4					
T8-PA					
T8-RL10					
T24-RiverBiofilm					
T24-C20					
T24-D14					
T24-F14					
T24-F14F16					
T24-F16					
T24-F16F19					
T24-F19	82.62				
T24-F4	86.62	83.07			
T24-PA	92.15	72.23	90.42		
T24-RL10	95.25	87.34	91.29	93.42	

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CHAPTER 4. EFFECT OF EARLY USE OF POTENTIAL PROBIOTICS ON LAKE STURGEION EGGS COMMUNITY ASSEMBLY AND STABILITY DURING EGG DEVELOPMENT

ABSTRACT

Lake Sturgeon (*Acipenser fulvescens*) is a threatened species of fresh water fish with considerably high egg mortality where egg-associated bacterial communities play a significant role. In order to better understand egg mortality caused by bacterial activities, we study bacterial communities on the egg surface through their development, and how potential probiotics can direct the assembly and succession of egg-associated communities. To investigate initial bacterial attachments to the egg surface, unfertilized eggs of two females were exposed to stream water and sampled at 15, 45, 90 and 135 minutes for DNA extraction of the egg-associated community. In addition, to determine if stream bacteria were targeting glycan-containing motifs on the egg's surface, we exposed eggs to stream water supplemented with glucose, galactose or mannose. Our results indicate that the taxonomic composition of egg-associated bacterial communities changed over time. Eggs exposed to stream water for as little at 15 minutes had a diverse community attached to the surface. Furthermore, the addition of glucose and galactose shifted the egg surface bacterial communities in statistically significant ways, diminishing the attachment of selected members of water bacterial communities. There were considerable differences between egg bacterial communities of the two females both before and after exposure to stream water. To study how potential probiotics can direct egg bacterial community assembly and succession, eggs of each of the two females were fertilized with the milt of a male in the presence of single- or double-species cultures of *Hydrogenophaga F14, Brevundimonas F16*, and *Acidovorax F19.* These bacterial species were previously isolated from fertilized healthy Lake Sturgeon egg surfaces and shown to have mutualistic interactions during *in vitro* biofilm formation.

Healthy egg samples were collected immediately after fertilization, one-day post fertilization, and five-days post fertilization. Five days post fertilization moribund egg samples were also collected. We observed that *Brevundimonas* treatment resulted in nearly 100% egg mortality while the presence of *Hydrogenophaga* had the highest survival rate (~20%) among all samples. Although there were differences in egg bacterial communities from different treatments immediately after fertilization, no differences between egg communities from different treatments were observed 24 hours after fertilization. Furthermore, five days after fertilization there was no difference between bacterial communities of healthy eggs from different treatments. However, genera *Rheinheimera* and *Aeromonas* contributed most to the difference between healthy and moribund egg communities at this time point. These investigations point to possible interventions during the assembly of egg-associated microbial communities that will reduce mortality and use of harmful chemicals in hatcheries.

INTRODUCTION

Recent literature suggests that the vast majority of aquatic microbes exist as biofilms that are defined as surface-associated complex heterogeneous aggregations of microorganisms [1]. From this perspective, all body surfaces of aquatic animals and their eggs are colonized by diverse microbial populations that provide both challenges and opportunities during all stages of their development. From an evolutionary point of view, aquatic animals have been negotiating successfully with the microbial world for millions of years. However, shifts in the aquatic microbial community can lead to altered interactions between animal and microbe that shift the balance. One of the major challenges that many species of fish face is the negative effect of microbes on their early ontogenetic stages of their offspring. Mortality during the egg and larvae stages exceeds 95% for many fish species [2-5]. Scientific evidence as well as experience in hatcheries for a variety of fish species supports the hypothesis that fish egg–microbe interactions in general and fish egg-bacteria interactions specifically, play a significant role in the egg's wellbeing and mortality [6].

Recent studies have confirmed that eggs released into the aquatic environment are rapidly (within 1 hour) colonized by a variety of bacteria [6-9]. One major reason for this colonization is due to the chemical composition and structure of the chorion, the outer layer of the fertilized fish eggs, which is rich in glycoproteins [10-13]. These proteins are well-known determinants for adhesion of bacteria to biotic surfaces [13-15]. Indeed, structures existing on the surface of eggs as well as maternal factors can selectively favor colonization of certain bacteria [16]. This fact manifests itself in the species-specific differences that have been found between microbial groups that

populate egg surfaces of different fish species [13, 17-20]. Nevertheless, fish egg associated bacterial communities are still poorly characterized and, thus, require more comprehensive investigation.

Not all bacteria affect aquatic organisms in a harmful way. In fact, some bacterial species are found to have probiotic effects on aquatic organisms. A probiotic in an aquatic environment according to Verschuere et al. [21] is "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". There are a number of successful demonstrations of probiotic use in aquaculture performed on fish larvae. For example, in Halibut (*Hippoglossus hippoglossus L*.) feeding the larvae with the symbiotic isolates (*Vibrio sp*., *P. elyakovii* and *Vibrio splendidus*) decreased larvae mortality significantly [22]. In another study, African Catfish (*Clarias gariepinus*, Burchell 1822) larvae fed with crude protein and lipids supplemented with *Lactobacillus acidophilus* (probiotic) showed greater health, enhanced growth and increased survival rates [23]. In yet another study, Nile tilapia (*Oreochromis niloticus L*.) juveniles whose diet included commercial feed and Dry Oil supplemented with *Bacillus* and lactic acid bacteria exhibited higher survival rates and exhibited faster growth rates [24]. Additionally, feeding Persian Sturgeon (*Acipenser persicus*) and Beluga (*Huso huso*) fry with *Lactobacillus curvatus* and *Leuconostoc mesenteroides* led to higher survival and growth rates as well as improved intestinal enzyme activity in these fish [25]. When fed with three species from *Bacillus* genus (*subtilis*, *licheniformis* and *pumilus*), gilthead sea bream (*Sparus aurata*) larvae showed

significant increase in growth as a result of higher expression of Insulin-like growth factor I and lower levels of myostain (genes that control muscle growth and are strictly involved in fish myogenesis) [26]. Furthermore, these larvae had a higher tolerance to farming conditions.

Probiotics have also been used to control specific fish pathogens. For example, a range of probiotics has been used successfully against the pathogen (*Flavobacterium psychrophilum*) that causes coldwater disease in rainbow trout (*Oncorhynchus mykiss*) [27, 28]. Specifically, in one case, the probiotic treatment (*Rhodococcus sp.*) enhanced fish survival by decreasing the abundance of this pathogen in the treated tank water. While this probiotic species was not found on the brook charr's (*Salvelinus fontinalis*) skin mucus or in the water itself, its biofilms were detected on the surface of the tank [29].

Nearly all probiotics used in aquaculture have targeted the gut or skin microbiome [22-24, 26, 30]. In fact, although most commercial egg production techniques require disinfection, there are no described probiotics for fish egg cultivation and, as a result, there is a strong need for further studies with the goal of identification of bacterial species that can reduce egg mortality in endangered fish species such as Lake Sturgeon. Recent study on Lake Sturgeon eggs had shown that presence of *Acidovorax* F19, a bacterial species isolated from the healthy Lake Sturgeon egg surfaces, during egg fertilization reduced egg mortality by 18% [31]. Although bacterial community compositional differences associated with treatment and control groups differed during early stages of incubation, they converged after approximately 48 hours of incubation in the stream water. This result suggests that intervention during periods

of microbial community assembly at early incubation stages can influence the mortality of the eggs.

Physiologically, adhesion is determined by the presence of lectins on the surface of adhering organisms. These lectins are capable of binding to complementary carbohydrates associated with host tissues and, thus, provide means for bacterial attachment [32-34]. *In vitro* experiments showed that bacterial adhesion to animal cells could be inhibited using soluble carbohydrates when they are recognized by the surface lectins of bacteria [33, 35, 36]. Chen et al. [37] conducted a comprehensive study of bacterial adhesion in the presence of monosaccharaides. These authors reported that bacterial adhesion of *V. alginolyticus* to all fish slime under investigation has been reduced dramatically in the presence of fructose. Furthermore, mannose showed inhibitory effect on bacterial adhesion to gill and hindgut mucus, while galactose only reduced adhesion to the gill mucus. In contrast, glucose did not affect adhesion of bacteria to any fish slime. As a result, this feature of affinity of some bacterial species to specific types of fish slime (gill, skin or intestinal) could be useful in developing cost-effective strategies for reducing pathogen binding to eggs and larvae [33]. Research literature indicates that many bacterial species can adhere and colonize the slime covering casings of fish eggs [38-40]. Staining the Lake Sturgeon eggs using Periodic Acid-Schiff (PAS) method showed the outer layer of the eggs contains high proportion of carbohydrates [unpublished data]. Further monosaccharide analysis of the Lake Sturgeon casing showed that the following monosaccharaides are present on the egg surfaces: glucosamine (GlcNH2), galactosamine (GalNH2), glucose (Glc), mannose (Man), and galactose (Gal) [unpublished data].

In this chapter, we focused on the assembly of the microbial communities on Lake Sturgeon eggs [17, 31, 41]. As we mentioned above, egg-associated microbial communities can significantly influence egg mortality levels, while intervening in the community assembly at an early stage can reduce high mortality. There are two basic approaches to altering the pathways of microbial community assembly; the first approach is to add a known probiotic at relatively high concentrations to establish a robust population on the eggs surface that, in turn, will alter the subsequent assembly development. An alternative method is to alter binding patterns of bacterial populations by introducing a competitive inhibitor of binding, a specific glycan that will block binding of specific bacterial populations. In this investigation we studied both techniques. First, we investigated initial attachments of bacterial populations to the egg surface in the absence and presence of glycans. We anticipated that this experiment would determine which, if any, bacterial populations in the stream water are inhibited from binding to eggs in the presence of a glycan. If a particular species is absent or greatly reduced when one or several glycans are present during colonization, we could infer that this species uses introduced glycan(s) as a binding motif. Next, we investigated the influence that selected isolates from Sturgeon eggs have on egg mortality levels when they are included as probiotics during the eggs fertilization. In this experiment, we analyzed the communities of not only healthy eggs but also unhealthy ones using the same treatment in order to get a more comprehensive understanding of microbial populations associated with each group of eggs. In order to get a statistically robust dataset we used a larger number of eggs and Illumina sequencing of 16S ribosomal RNA gene instead of a limited number of eggs and clone libraries used in previous

studies. If successful, this approach can be used in the development of probiotics that can direct fish egg-associated bacterial community assembly in order to reduce high egg mortality.

MATERIALS AND METHODS

Study site. Our studies were conducted in a streamside hatchery on Upper Black River in Michigan during May of 2015. This river is the only spawning stream for the Black Lake Sturgeon population. Aseptically harvested gametes and milt collected from two females and two males were used for the experiments described below. Two females were biomarked as 208 (female 1) and 218 (female 2). Female 208 had a total length of 161 cm, fork length of 151 cm, girth of 72 cm and weighted 35.8 kg. Female 218 had a total length of 192 cm, fork length of 181 cm, girth of 68 cm and weighted 47.2 kg.

Early egg attachments. Staining Lake Sturgeon eggs using Periodic Acid-Schiff method showed saccharides are significantly abundant on the egg surface [unpublished data]. Monosaccharide analysis of the egg casing showed majority of these saccharides were in the form of glucose, galactose, and mannose [unpublished data]. In this experiment, we characterized the early communities of the egg surface, and also studied the possibility of manipulating the early community composition using glucose, galactose or mannose titrated in the stream water within the incubation. We hypothesized that since bacteria use glycans to attach to biotic surfaces, adding monosaccharaides to the stream water can prevent some bacterial populations from attaching to the egg surface by blocking their glycan receptors.

To this end, we used unfertilized gametes from both females for the early attachment study. For each replicate of a treatment, approximately 40 gametes aseptically harvested from one of the two females were incubated in a Sterile Specimen Cup (total volume of 100 mL) filled with 35 mL of stream water alone or stream water supplemented with a specific concentration (0.1%, 0.2%, 1% and 5%) of glucose, galactose or mannose. Stream water in this study refers to the water that was used from the hatchery stream water line from the upper Black River. This water can be slightly different from "true" stream water in that particulates were filtered from the water using 50 and 100 micron sock filters. Ten eggs were collected at each time point (T=15, T=45, T=90 and T=135 minutes after time zero) for DNA extraction and further analysis of bacterial communities. Gametes were rinsed with autoclaved distilled water and put in 80% ethanol in a sterile 2 mL Eppendorf Tube. The tubes were kept at 4^{o}C until DNA extraction.

Probiotic study. The potential probiotics used in the present work were previously isolated from the surface of healthy Lake Sturgeon eggs and were as follows: *Hydrogenophaga F14*, *Brevundimonas F16*, *Acidovorax F19*, *Hydrogenophaga-Brevundimonas* mixture, *Hydrogenophaga-Acidovorax* mixture, and *Brevundimonas-Acidovorax* mixture. We chose these isolates based on our previous *in vitro* studies (Chapter 2).

We fertilized eggs in eight different groups: six groups of potential probiotic treatments and two groups were used as controls. The control groups included eggs fertilized either in unfiltered or filtered (0.22 µm) stream water alone. For each of the two replicates of six treatments or controls, 150 eggs were fertilized in 250 mL of filtered

stream water containing 1.2 mL of milt and putative probiotic(s) with the concentration of 10^6 cfu/mL. This bacterial concentration is on par with the average concentrations of bacterial cells in the fresh water [16]. In order to measure the concentration of overnight culture, we constructed a graph for each bacterial isolate using data generated from viable plate counts (R2Agar medium) and spectrophotometric analysis (absorbance of R2Broth culture at 600 nm using a spectrometer).

Double-species combinations were mixed prior to adding them to filtered stream water for an hour. This part of experiment was motivated by our *in vitro* studies; we hypothesized that selected bacterial species can co-aggregate and, thus, result in an increase of their attachment to egg surfaces significantly [unpublished data]. Gametes were kept for 45 minutes until fertilization was complete and eggs attached to the polyethylene mesh screen of each coupling that we also used as substrates for fish embryos during the incubation process. These treatments and controls were replicated twice with each of the two different female/male combinations. Next, eggs were reared in the river water at the temperature of 18-19^{o}C at which temperature eggs developed to hatch in 6-7 days. We collected 10 healthy eggs from each replicate at three different time points: 1) after 45 minutes of fertilization and before the subsequent transfer of the eggs to river water (we will call this “immediately after fertilization”), 2) 1 day after transfer to river water (we will call this “1 day after fertilization”), and 3) 5 days after transfer to river water (we will call this “5 days after fertilization”)] for DNA extraction and further analysis of bacterial communities. Five days after fertilization, 10 unhealthy eggs from each replicate were also collected for further community analysis. Collected eggs were rinsed with autoclaved distilled water and put in 80% ethanol in sterile 2mL

Eppendorf tubes that were kept at 4°C until they were transferred to the lab and before DNA extraction.

Egg mortality. The number of dead eggs was recorded daily for each treatment with subsequent removal of dead eggs upon detection. Given this record, cumulative (final) egg mortality was found as:

$$m = n_{dead}/n_{total},$$

where m is cumulative egg mortality, n_{dead} is the total number of dead eggs and n_{total} is the total number eggs in the beginning of incubation. Cumulative egg mortality was used to compare effectiveness of different treatments.

DNA extraction. For all egg samples, in order to extract genomic DNA we used the PowerSoil™ Kit (MO BIO Laboratories Inc., CA) following bead beating per the manufacturer's protocol. Each sample was a composite of 8 eggs. For stream water samples collected during each time point (fertilization, 1 day after fertilization, 5 days after fertilization and 7 days after fertilization) we extracted genomic DNA from the filtered material [42, 43]. For each of the two replicates of the river water samples, 500 mL of river water was filtered through a 0.22 μm filter membrane (Sterlitech®) using a vacuum pump to collect the aquatic bacterial communities on the filter papers. Filters containing water bacterial communities were then transferred to 50 ml Corning ® centrifuge tubes containing 80% ethanol and stored at 4 °C until bacterial DNA extraction was performed using PowerSoil™ Kit (MO BIO Laboratories Inc., CA).

Illumina sequencing and bacterial community analysis. For each replicate of each sample as well as stream water samples, 40 μL of genomic DNA extracted with PowerSoil™ Kit (MO BIO Laboratories Inc., CA) were sent to the Michigan State

University Research Technology Support Facility (RTSF) for further processing using an Illumina MiSeq platform (total of 327 samples). For each sample, uniquely indexed primers were used to amplify the V4 region of 16S rRNA gene of the bacterial community DNA [44]. Amplification products were then normalized (Invitrogen SequalPrep normalization plate). After pooling normalized samples, PCR reaction was cleaned up (AMPure XP beads). The pool was then loaded on an Illumina MiSeq v2 flow cell and sequenced with a 500-cycle v2 reagent kit (PE250 reads). Base calling was performed by Illumina Real Time Analysis Software (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ files with Illumina Bcl2fastq v1.8.4.

We used Mothur version v.1.35.1 (http://www.mothur.org) to further analysis the bacterial community of the samples [50]. Mothur's standard operating procedure was used for processing of the raw sequencing data (http://www.mothur.org/wiki/MiSeq_SOP) [45]. Mothur-formatted version 123 of Silva 16S rRNA gene database was used to achieve alignment [46]. Any sequences classified as Mitochondria, unknown, Archaea, or Eukaryota were then removed from the data set. For early attachments and monosaccharaides study, subsampling at 11500 sequences per sample was performed. For probiotic experiment, subsampling at 9000 sequences per sample was performed. Sequences were preclustered and chimeric sequences were removed using a mothur-formatted version of the Ribosomal Database Project (RDP) training set version 14 and uchime, based on the Mothur protocol. Finally, sequences were classified into Operational Taxonomic Units (OTUs) of ≥97% sequence identity.

Statistical analysis. To analyze diversity of the bacterial communities in the samples, the shared and taxonomy output files of Mothur were used. Paleontological Statistics Software Package For Education and Data Analysis (PAST) software was used for further analysis of the bacterial communities with the multivariate tests, and Analysis Of Similarities (ANOSIM) [47]. The Bray-Curtis dissimilarity was used to quantify the differences in the abundance of different OUTs between samples. To visualize the level of similarity between samples Nonmetric Multidimensional Scaling (NMDS) plots were generated based on microbial abundance and composition using the Bray-Curtis similarity index. NMDS uses an algorithm that takes the multidimensional data and presents it in a two- or three-dimensional space. This "goodness of fit" is measured by stress value. Stress values <0.05 are considered excellent (the two- or three-dimensional visualization is a valid representation of the differences between the samples) and stress values <0.1 correspond were considered good ordination [48]. To measure statistical significance between the samples, Analysis Of Similarities (ANOSIM, a distribution free analysis of similarity) was used. The number of permutations used in this test was 9999. Based on ANOSIM analysis, statistically the most dissimilar samples were the ones that not only had a P value less than 0.03 (the null hypothesis was rejected, meaning the average rank similarity between objects within a group was not the same as the average rank similarity between objects between groups), but also had R values that were close to 1.

Clustering analysis was performed in PAST and the resulting trees were edited in FigTree v.1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/). The genera responsible for

dissimilarities between the stream water and egg communities were identified by Similarity Percentages (SIMPER) based on the Bray-Curtis dissimilarity matrix [47].

Availability of supporting data. Raw sequence data is available at the NCBI database (SRA accession number: SRP150884) and the code for the mothur analysis is available at (https://doi.org/10.6084/m9.figshare.6555737.v1 and https://doi.org/10.6084/m9.figshare.6555752.v1).

RESULTS

We used Illumina sequencing of 16s rDNA to study the assembly of bacterial communities on the Lake Sturgeon egg surfaces during different developmental stages of the egg. In order to investigate early events in community assembly on the egg surface we exposed unfertilized eggs of two females to stream water for short periods of time (15-135 minutes). Furthermore, we investigated if the pathways of community assembly could be altered using three bacterial species and three monosaccharaides early on, when the egg is released in the stream water and during fertilization. The bacterial species were previously isolated from healthy Lake Sturgeon egg surfaces [41]. Moreover, we showed that when they are co-cultured as double-species mixed cultures *in vitro* they form significantly better biofilms (Chapter 2) and are more resistant to invasion from river microbiome (Chapter 3). As a result, in this study we used both single- and double-species mixed cultures of these isolates.

Though harvested aseptically, the unfertilized eggs of both females tested in this study had a bacterial community of relatively low diversity, presumably derived from the

lower gastrointestinal tract through which the eggs are expelled (Table 1). The community was significantly different from water-exposed eggs (Fig. 1).

Sample	OTUs	Seq. per sample	Simpson	Shannon	Chao1
Stream Water	**828**	**9867**	**0.974**	**4.98**	**1105**
Eggs before exposure to stream water- Female 1	**39**	**10061**	**0.146**	**0.506**	**66.5**
Eggs before exposure to stream water - Female 2	**79**	**10056**	**0.879**	**2.817**	**82.33**
Eggs-Female 1-15 Minutes	**462**	**10065**	**0.746**	**2.635**	**528.7**
Eggs-Female 2-15 Minutes	**242**	**10059**	**0.655**	**2.438**	**260**
Eggs-Female 1-45 Minutes	**325**	**9966**	**0.892**	**4.062**	**330.6**
Eggs-Female 2-45 Minutes	**94**	**10084**	**0.527**	**1.679**	**112.3**
Eggs-Female 1-90 Minutes	**102**	**10099**	**0.747**	**2.32**	**107.1**
Eggs-Female 2-90 Minutes	**130**	**10094**	**0.775**	**2.842**	**134.7**
Eggs-Female 1-135 Minutes	**107**	**10091**	**0.728**	**2.063**	**112.6**
Eggs-Female 2-135 Minutes	**114**	**10081**	**0.75**	**2.655**	**123.3**

Table 4.1. Alpha-diversity of stream water, and unfertilized egg communities before and after exposure to stream water for 15 to 135 minutes.

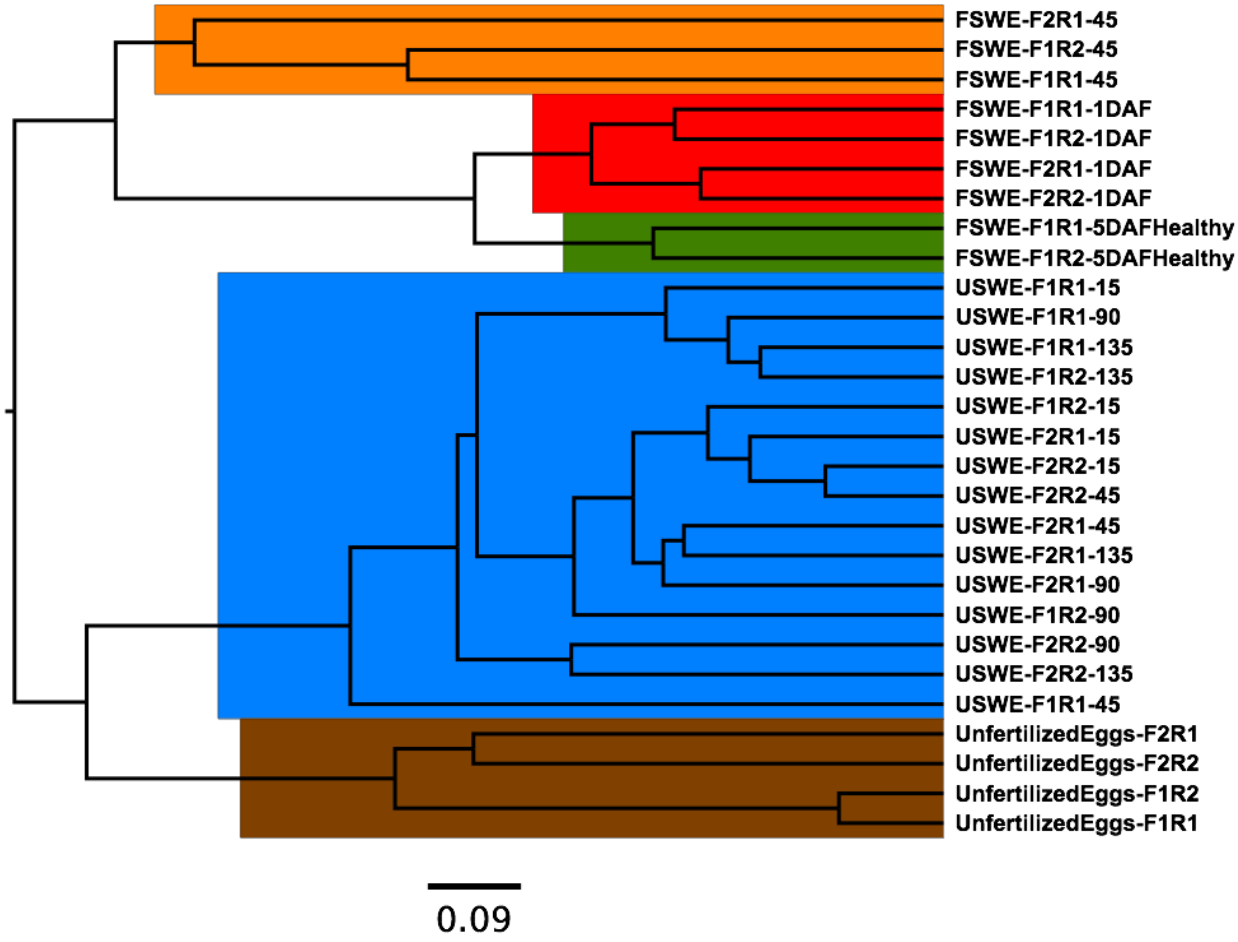

Figure 4.1. Bray-Curtis dendrogram of bacterial communities from eggs before and after exposure to stream water (Brown: aseptically harvested unfertilized eggs before exposure to stream water, Blue: unfertilized eggs exposed to stream water for 15-135 minutes, Orange: eggs fertilized in stream water for 45 minutes, Red: eggs fertilized in stream water after one day of exposure to stream water, Green: eggs fertilized in stream water after five days of exposure to stream water). USWE: unfertilized eggs exposed to stream water, FSWE: eggs fertilized in stream water.

Furthermore, there was no statistical compositional difference between bacterial communities of eggs exposed to stream water for 15 to 135 minutes. However, cluster analysis, as well as ANOSIM (ANOSIM P value= 0.0001, ANOSIM R value= 0.994) revealed three clusters with significant differences: stream water samples, eggs prior to exposure to stream water and eggs after exposure to stream water. Both cluster analysis and non-metric multidimensional scaling (NMDS) analysis showed no

difference between the stream water samples collected at different time points. Also, Analysis of Similarities (ANOSIM) did not show any significant difference between these water samples.

There was a clear difference between unfertilized egg communities before and after exposure to stream water (ANOSIM P value= 0.0001, ANOSIM R value= 0.961). The difference between these two egg communities (pre- and post-exposure to stream water) can be observed even after only 15 minutes of exposure to stream water (ANOSIM P value= 0.028, ANOSIM R value= 0.979). *Pseudomonas, Sphingorhabdus,* and *Polynucleobacter* were the most abundant genera of bacteria in stream water (Fig. 2). The most abundant bacterial genus in the egg bacterial communities of female 1 pre-exposure to stream water was *Aeromonas*. It accounted for 92% of the bacterial population found in this community. Unfertilized eggs of female 2 had a more diverse bacterial community before exposure. The most abundant genera found in this community were *Aeromonas* (28%), *Pseudomonas* (25%), *Streptococcus* (6%), and *Staphylococcus* (4%). After exposure to stream water for 15-135 minutes, the abundance of *Pseudomonas* and *Geobacillus* increased significantly in unfertilized egg communities of both females.

There was a significant difference between fertilized and unfertilized eggs exposed to stream water (Fig. 1, ANOSIM P value= 0.0001, ANOSIM R value= 1). When fertilized in stream water for 45 minutes (D0), the top five most abundant populations on the eggs of the first mating pair (Female 1 eggs x Male 1 milt) was *Aeromonas*, *Comamonadaceae*, *Streptophyta*, *Polynucleobacter* and *Microbacteriaceae* whereas the top five populations found on the fertilized egg surfaces of the second

mating pair (Female 2 eggs x Male 2 milt) were *Comamonadaceae, Pseudomonas*, *Microbacteriaceae, Sphingobacterium* and *Rhodobacteraceae.*

The difference between early-unfertilized egg communities exposed to stream water for 15-135 minutes and fertilized egg communities exposed to stream water for 1 and 5 days were also significant (Figures 1 and 3). Remarkably, as mentioned before exposure to stream water, even for 15 minutes, changed the bacterial community of eggs significantly. Specifically, at this time point there is a diverse collection of bacteria colonizing the surface dominated by *Pseudomonas, Aeromonas*, *Geobacillus* and *Bacillariophyta*. At 135 minutes of exposure, the community shifted modestly and was dominated by *Pseudomonas*, *Aeromonas*, *Geobacillus*, *Comamondaceae* and *Burkholderia* (although this shift from the 15 minutes post stream water exposure egg communities was not statistically significant). After 24 hours, the community shifted even further and was dominated by *Comamonadaceae*, *Rheinheimera*, *Undibacterium*, *Bacillariophyta*, *Rhodobacteraceae* and *Methylophilus*. After five days of exposure to stream water *Comamonadaceae, Rheinheimera, Undibacterium, Fusobacteriaceae* and *Aquaspirillum* were the top five populations found on the surfaces of healthy eggs of first mating pair. Eggs of second mating pair exposed to stream water during fertilization were not viable five days after exposure to stream water.

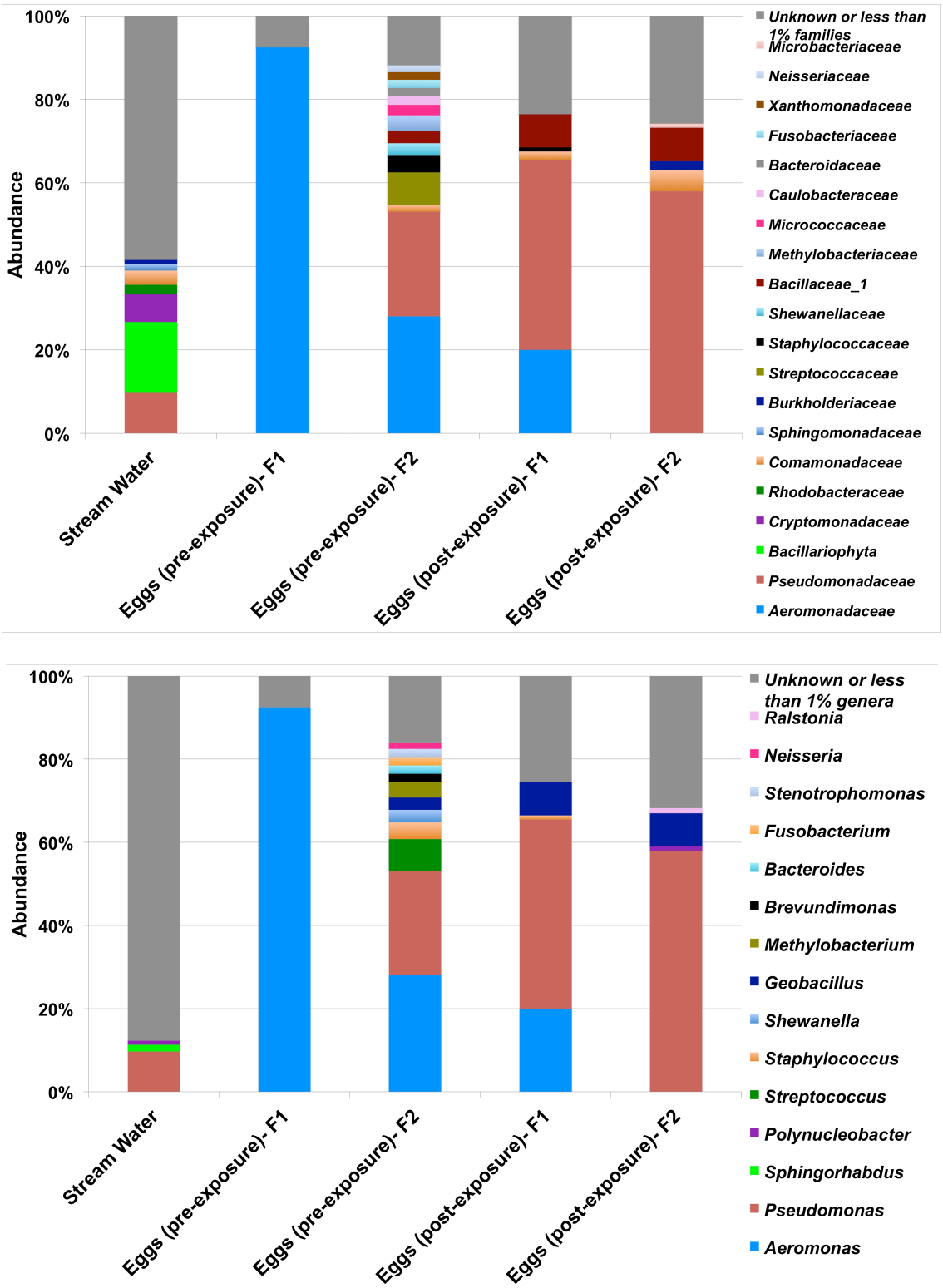

Figure 4.2. Most abundant bacterial families (top) and genera (bottom) in stream water and unfertilized egg samples before and after exposure to stream water (15-135 minutes). Unfertilized eggs exposed to stream water and stream water samples

collected at different time points were pooled together and the average was used for these calculations.

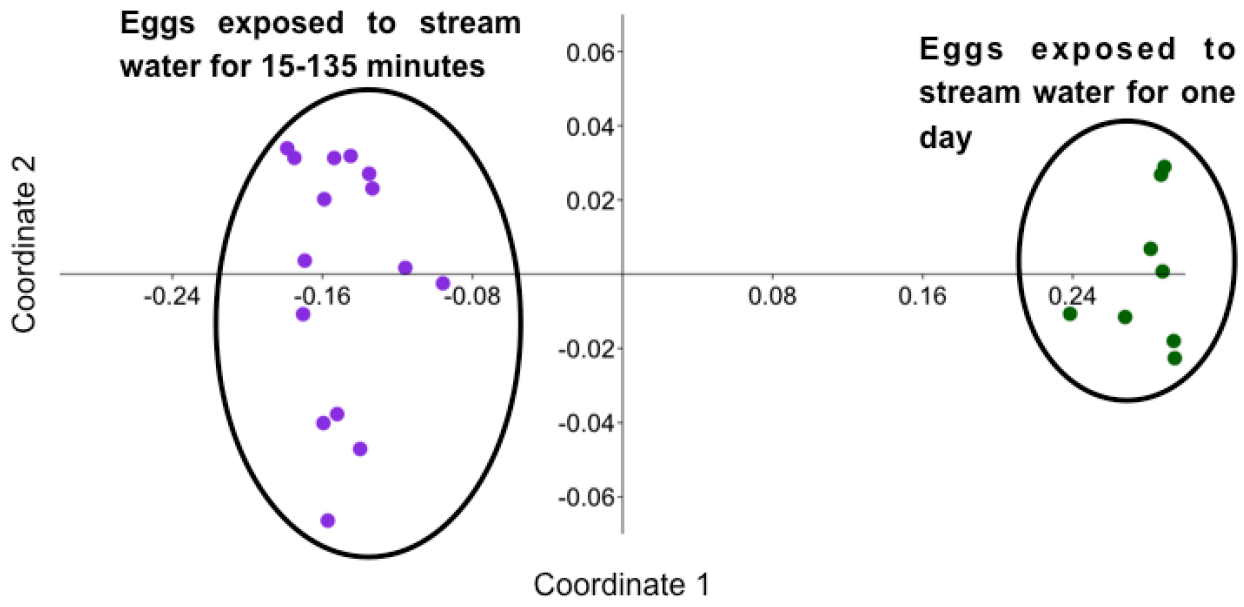

Figure 4.3. Non-metric multidimensional scaling (NMDS) analysis of eggs exposed to stream water for 15, 45, 90 and 135 minutes, or for 1 day (Stress value= 0.068, ANOSIM P value: 0.0001, ANOSIM R value: 0.891).

Exposure to monosaccharaides. Diversity of bacterial communities of unfertilized egg surfaces of female 1 did not change or slightly decreased over the course of 135 minutes of exposure to stream water when mannose, glucose or galactose were present as compared to unfertilized eggs that were exposed to stream water alone (Appendix C: Table C-1). Mannose had the same effect on egg-associated bacterial communities of the second female, however diversity of bacterial communities increased when either glucose or galactose were present.

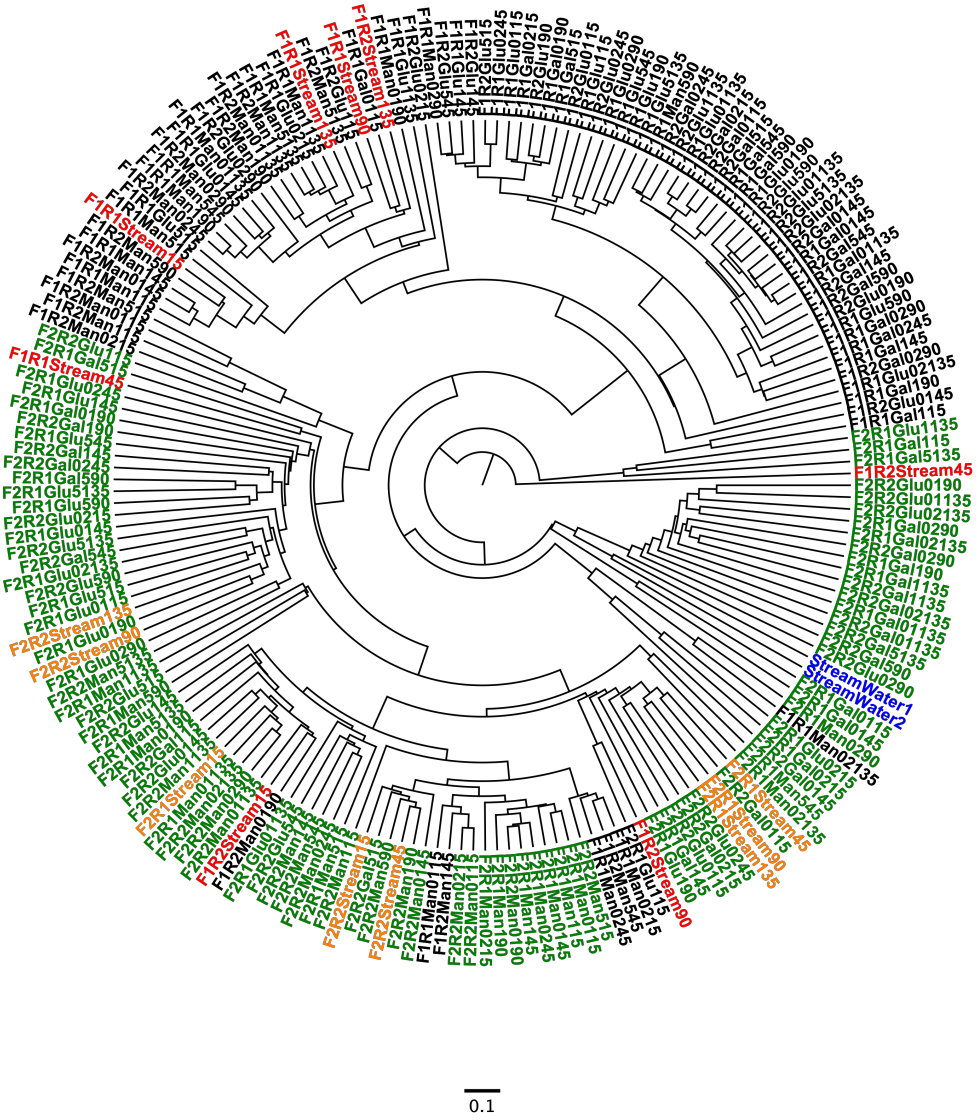

Figure 4.4. Cluster analysis (Bray-Curtis) of stream water and unfertilized egg samples exposed to stream water or stream water supplemented with one of three sugars (mannose, glucose or galactose at 0.1, 0.2, 1, or 5%) for 15, 45, 90 or 135 minutes (Blue= stream water samples, Black= female 1 gametes exposed to stream water supplemented with sugars, Green= female 2 gametes exposed to stream water supplemented with sugars, Red= female 1 gametes exposed to stream water alone, Orange= female 2 gametes exposed to stream water alone).

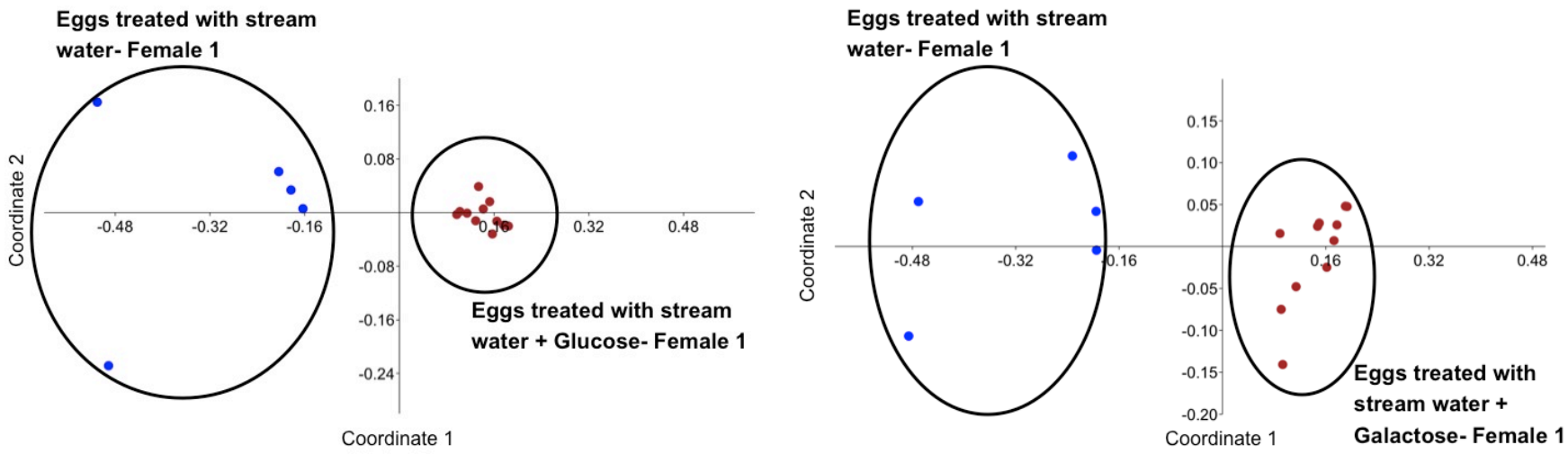

Figure 4.5. Non-metric multidimensional scaling (NMDS) analysis of unfertilized egg samples exposed to stream water alone or a mixture of stream water and glucose or galactose (1 and 5%) for 45, 90 or 135 minutes [stress values were as followed: Glucose-Female 1 (0.035), Galactose-Female 1 (0.039), and Galactose-Female 2 (0.081); ANOSIM P values were as followed: Glucose-Female 1 (0.0004), Galactose-Female 1 (0.0003), and Galactose-Female 2 (0.007); ANOSIM R values were as followed: Glucose-Female 1 (0.911), Galactose-Female 1 (0.953), and Galactose-Female 2 (0.391)]. Outliers were removed from these calculations.

Sample	Top populations that diminished on sugar treated eggs	Top populations that increased on sugar treated eggs
Mannose- Female 1	Not significant	Not significant
Mannose- Female 2	Not significant	Not significant
Glucose- Female 1	*Pseudomonas*, *Geobacillus, Staphylococcus, and Turicella*	*Aeromonas*
Glucose- Female 2	Not significant	Not significant
Galactose- Female 1	*Pseudomonas, Geobacillus and Staphylococcus*	*Aeromonas*
Galactose- Female 2	*Pseudomonas* and *Geobacillus*	*Comamonadaceae, Flavobacterium*

Table 4.2. Top bacterial populations whose relative abundance decreased or increased significantly on eggs exposed to stream water supplemented with glucose, galactose or mannose compared to eggs exposed to stream water alone (SIMPER analysis).

Overall, analysis of 208 unfertilized egg communities post-exposure to stream water supplemented with monosaccharaides revealed significant differences in the abundance and composition of taxa based on the Bray-Curtis dissimilarity index (ANOSIM P value= 0.0001, ANOSIM R value= 0.533). Generally, there was a clear difference between the stream water community and the egg surface communities (Fig. 4). Also, there was a significant distinction between unfertilized egg surface communities of female 1 exposed to mannose, glucose, and galactose and their counterparts in female 2.

There was a substantial difference between bacterial communities of the eggs exposed to stream water alone or stream water supplemented with glucose or galactose (Fig. 5). In both females, especially female 1, the abundance of *Pseudomonas* degreased significantly in egg communities that were exposed to higher concentrations

of glucose or galactose (1 or 5%) but not in the egg communities that were exposed to mannose (Table 2). In female 1, the abundance of *Aeromonas* increased significantly in the eggs that were exposed to either glucose or galactose. *Aeromonas* was either absent or at very low abundance on unfertilized egg surfaces of female 2 that were exposed to stream water alone or either glucose or galactose. There was no difference between bacterial communities of unfertilized eggs exposed to stream water alone or stream water supplemented with mannose in either of the females.

Exposure to potential probiotics. As expected, immediately after fertilization for 45 minutes and before transfer to stream water (D0), the richness and diversity of egg samples fertilized in stream water (FSWE) was significantly higher than their counterparts that were fertilized in filtered water (FFEW) (Table 3). At this time point, the highest diverstiy was found in bacterial communities of eggs fertilized in a mixutre of filtered stream water and overnight culture of *Brevundimonas F16.* One day after fertilization (1DAF), richness and diversity of egg and stream water communities were not different (Tables 1 and 3). After five days of exposure to stream water (5DAF) diversity reduced slightly in healthy egg communities compared to one day (1DAF) counterparts. Diversity reduced significnatly in moribund eggs five days post-exposure to stream water (5DAF) compared to one day post-exposure egg communities (1DAF).

Overall, analysis of 105 stream water and fertilized egg communities revealed significant differences in the abundance and composition of taxa based on the Bray-Curtis dissimilarity index (ANOSIM P value= 0.0001, ANOSIM R value= 0.943). There was a clear difference between stream water and fertilized egg bacterial communities (Fig. 6).

Sample	OUTs	Seq. per sample	Simpson	Shannon	Chao1
FSWE- F1D0	190	8948	0.97	4.24	298.3
FSWE- F2D0	68	8919	0.96	3.53	89
FFWE- F1D0	89	8955	0.83	2.65	213
FFWE-F2D0	40	8928	0.77	1.98	41.5
***Hydrogenophaga F14*-F1D0**	64	8940	0.79	2.24	83.5
***Hydrogenophaga F14*-F2D0**	42	8949	0.74	2.09	49.5
***Brevundimonas F16*-F1D0**	79	8973	0.90	3.01	139
***Brevundimonas F16*-F2D0**	86	8984	0.93	3.21	87.2
***Acidovorax F19*-F1D0**	105	8969	0.88	2.74	222.1
***Acidovorax F19*-F2D0**	57	8978	0.72	2.02	79.75
F14F16-F1D0	82	8959	0.86	2.88	97
F14F16-F2D0	43	8965	0.77	2.26	55
F16F19-F1D0	63	8966	0.60	1.70	101
F16F19-F2D0	24	8960	0.57	1.42	34
F14F19-F1D0	79	8952	0.91	2.94	113.2
F14F19-F2D0	64	8977	0.77	2.28	68.67
ALL Eggs-1DAF	1162	8424	0.96	5.04	2598
ALL Eggs-Healthy-5DAF	461	8654	0.91	3.54	901.2
ALL Eggs-Unhealthy-5DAF	221	8742	0.85	2.65	443.3

Table 4.3. Bacterial alpha-diversity in Lake Sturgeon eggs fertilized in stream water (FSWE), or filtered stream water alone (FFWE) or a mixture of filtered stream water and the overnight culture of the Lake Sturgeon egg bacterial isolates for 45 minutes. Egg samples were collected at three different time points: D0 (45 minutes after fertilization), 1DAF (one day after fertilization), and 5DAF (five days after fertilization). 45 minutes after fertilization all eggs were transferred to stream water. Five days after fertilization both healthy and unhealthy egg samples were collected. Further analysis showed that despite different treatments during fertilization all egg bacterial communities cluster together one day after fertilization (1DAF) (Figures 6 and 10). Five days after fertilization healthy and unhealthy eggs clustered separately. Thus, all the egg communities one day after fertilization, healthy egg communities five days after fertilization, and unhealthy egg communities five days after fertilization were pooled together and the average was used for calculating the alpha diversity.

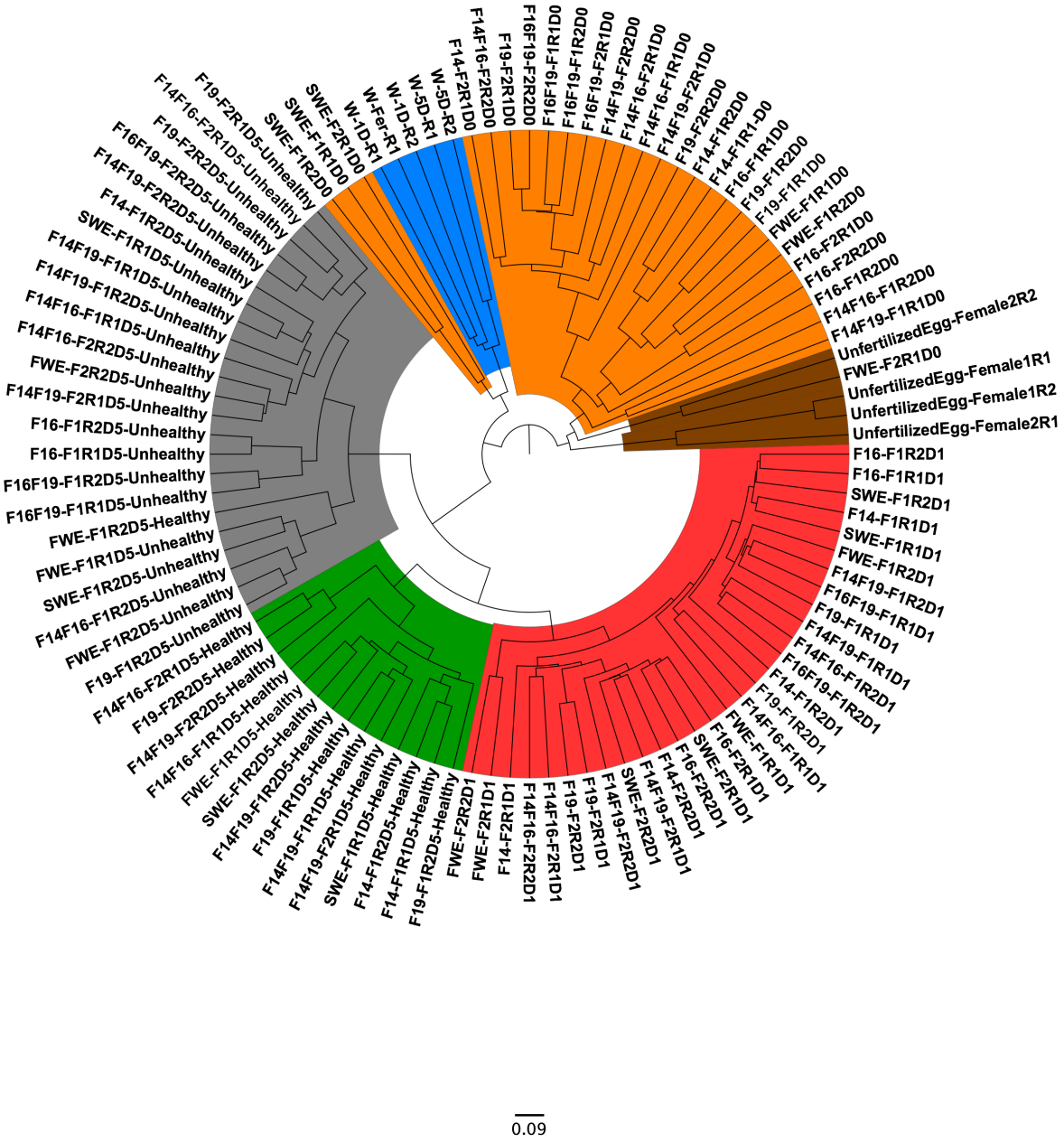

Figure 4.6. Cluster analysis (Bray-Curtis) of eggs fertilized in stream water alone (SWE), filtered stream water alone (FWE) or a mixture of filtered stream water and overnight culture of potential probiotics. After 45 minutes of fertilization in different conditions (D0) eggs were transferred to stream water for the rest of their development (Orange: D0, Red: D1, Green: D5-healthy eggs, Gray: D5-moribund eggs, Brown: unfertilized eggs before exposure to stream water, and Blue: stream water samples).

Immediately after 45 minutes of fertilization and before further transfer of eggs to stream water (D0), there was a significant distinction between bacterial communities of the eggs that were exposed to stream water and the eggs that were exposed to filtered water alone (Figures 7-9). Moreover, except eggs that were exposed to mixed culture of *Hydrogenophaga-Acidovorax* during fertilization (Fig. 9, Table 4), there was a considerable dissimilarity between egg communities that were fertilized in the presence of bacterial isolates and the eggs that were fertilized in stream water or filtered water alone (Table 4). Interestingly, exposure of eggs to double-species mixed isolates of *Hydrogenophaga F14-Brevundimonas F16* and *Brevundimonas F16- Acidovorax F19* during fertilization increased the dissimilarity between bacterial treated egg communities and controls compared to their single-species counterparts (Table 4, lower ANOSIM P values and higher ANOSIM R values).

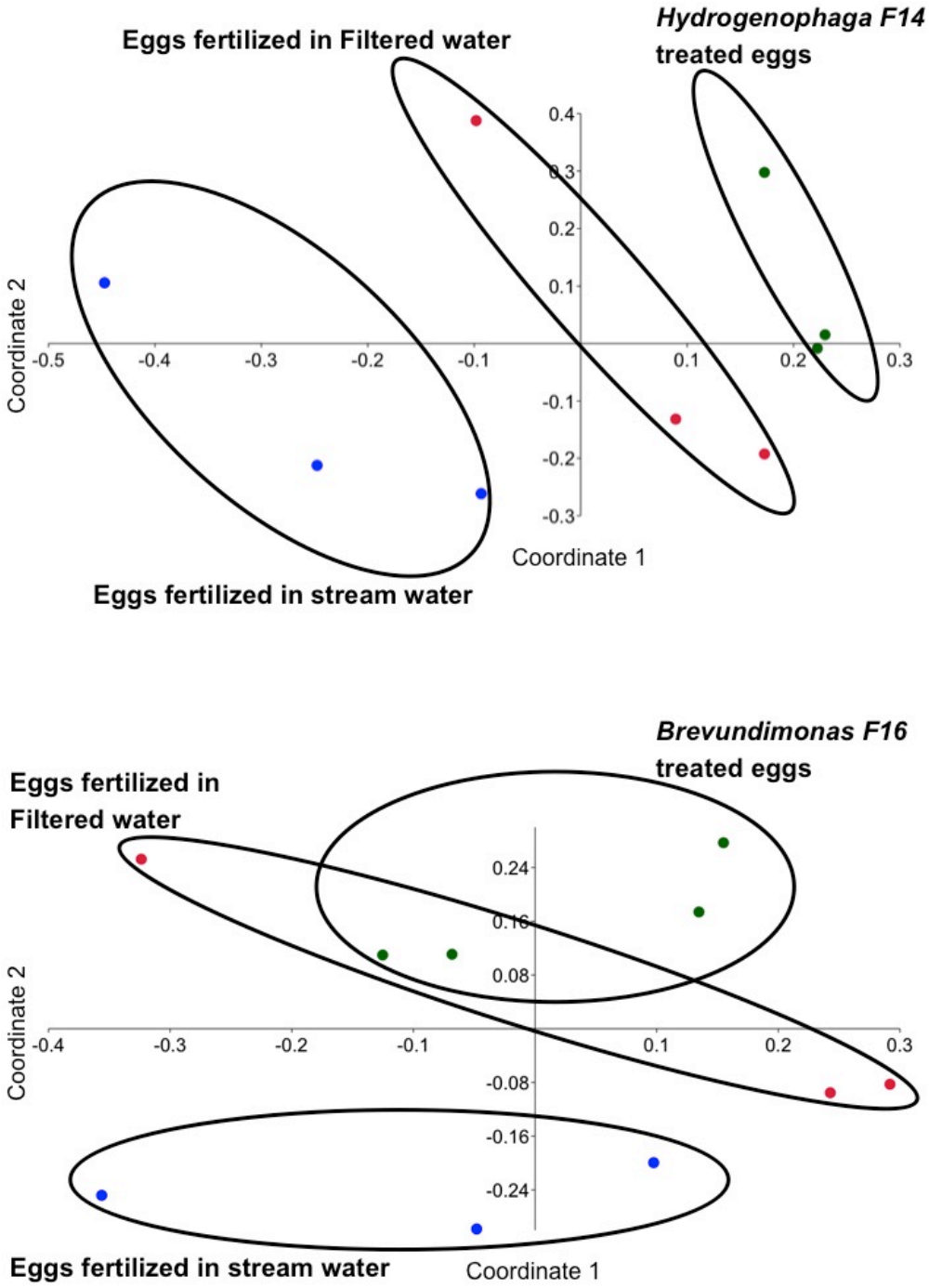

Figure 4.7. Non-metric multidimensional scaling (NMDS) analysis of Lake Sturgeon egg communities immediately after fertilization in stream water, or filtered stream water or a mixture of filtered stream water and the overnight broth culture of *Hydrogenophaga F14* (top) or *Brevundimonas F16* (bottom). Stress values were as followed: *Hydrogenophaga F14*= 0.077, and *Brevundimonas F16*= 0.06.

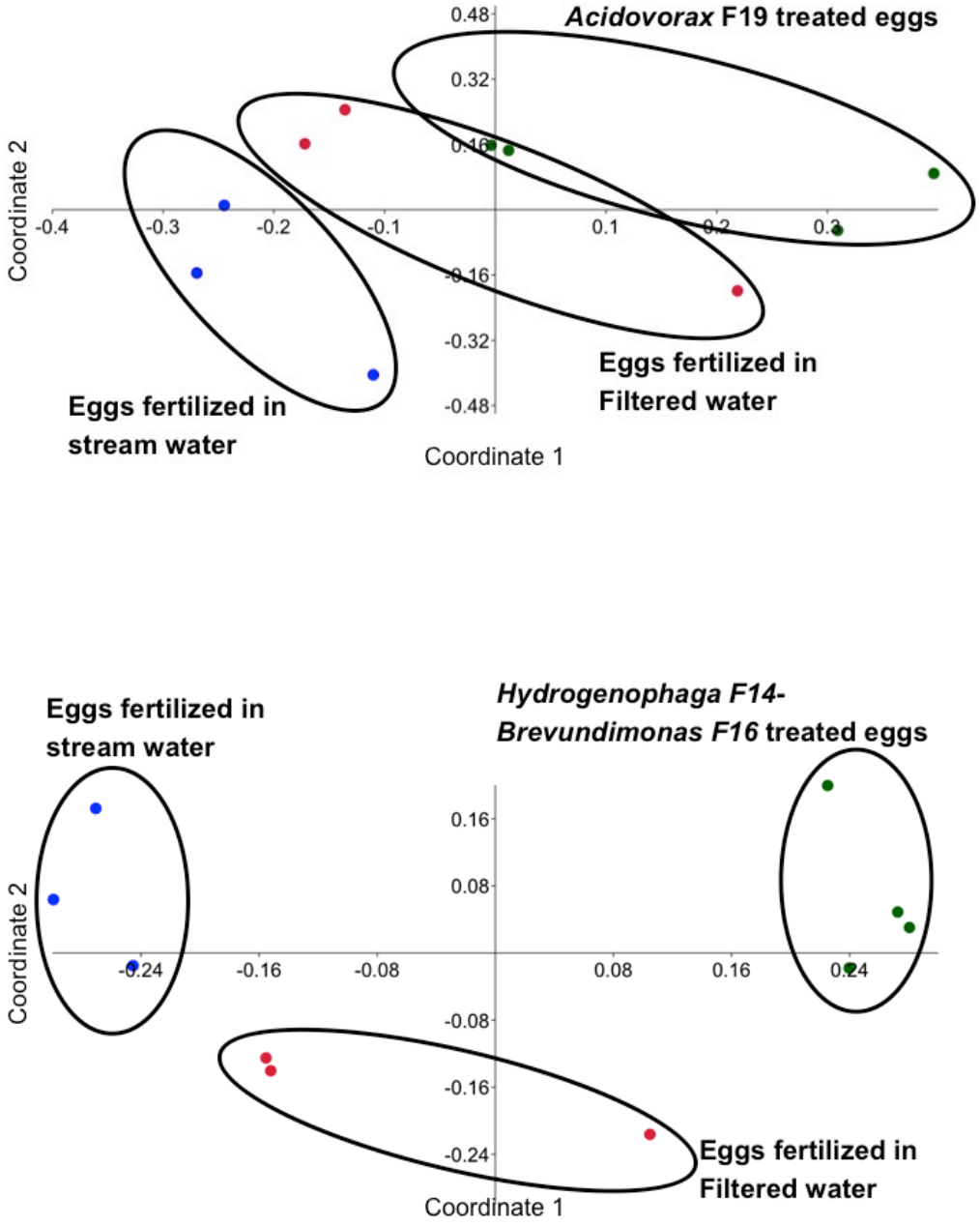

Figure 4.8. NMDS analysis of Lake Sturgeon egg communities immediately after fertilization in stream water, or filtered stream water or a mixture of filtered stream water and the overnight broth culture of *Acidovorax F19* (top) or *Brevundimonas-Hydrogenophaga* (bottom). Stress values were as followed: *Acidovorax F19*= 0.037, and *Brevundimonas- Hydrogenophaga*= 0.105.

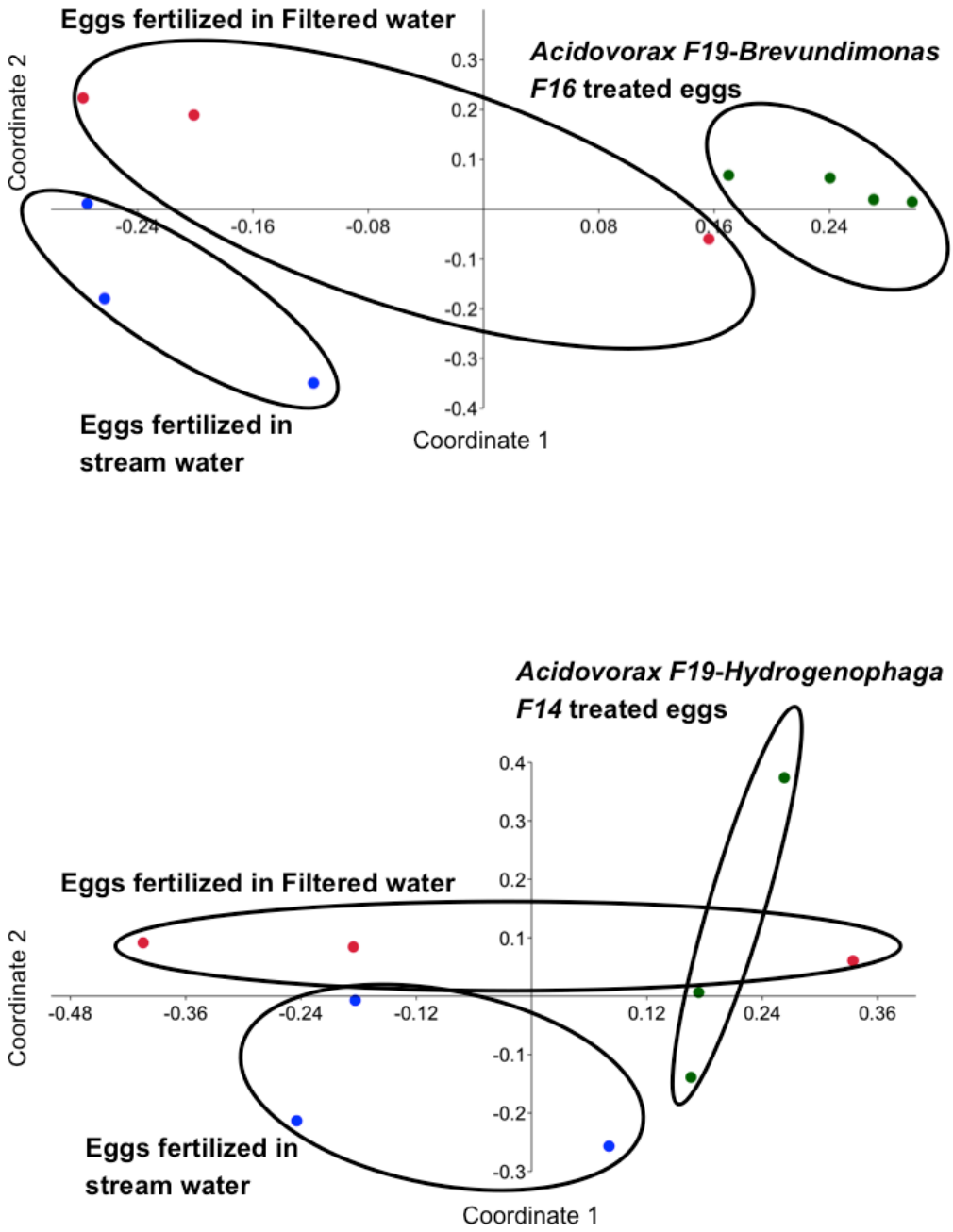

Figure 4.9. NMDS analysis of Lake Sturgeon egg communities immediately after fertilization in stream water, or filtered stream water or a mixture of filtered stream water and the overnight broth culture of *Brevundimonas-Acidovorax* (top) or *Acidovorax-Hydrogenophaga* (bottom). Stress values were as followed: *Brevundimonas-Acidovorax* = 0.057, and *Acidovorax-Hydrogenophaga* = 0.086.

Samples used	ANOSIM P value	ANOSIM R value
Filtered alone, Stream alone and *Hydrogenophaga F14*	0.025	0.407
Filtered alone, Stream alone and *Brevundimonas F16*	0.019	0.388
Filtered alone, Stream alone and *Acidovorax F19*	0.015	0.419
Filtered alone, Stream alone and *F14 + F16*	0.0029	0.697
Filtered alone, Stream alone and *F16 + F19*	0.0031	0.712
Filtered alone, Stream alone and *F14 + F19*	0.0461	0.457

Table 4.4. ANOSIM results for comparing bacterial communities of Lake Sturgeon eggs exposed to stream water alone, filtered water alone or fitered water containing an overnight culture of above bacterial isolates [immediately after fertilization egg samples before further transfer to stream water (D0)].

Immediately after fertilization and before transferring to the stream water (D0), there was a significant difference between bacterial communities of eggs exposed to different bacterial treatments (ANOSIM P value= 0.0001, ANOSIM R value= 0.837). There was also a clear distinction between the bacterial communities of the eggs immediately after fertilization (D0), and their one day (D1) and five days (D5) counterparts (Fig. 6). There was no significant difference between the 1-day after fertilization (D1) egg communities based on bacterial treatments during fertilization. Furthermore, there was a substantial difference between the healthy and unhealthy egg communities five days after fertilization (Figures 6 and 10). However, there was no difference between the healthy or unhealthy egg communities five days after fertilization based on bacterial treatment during fertilization.

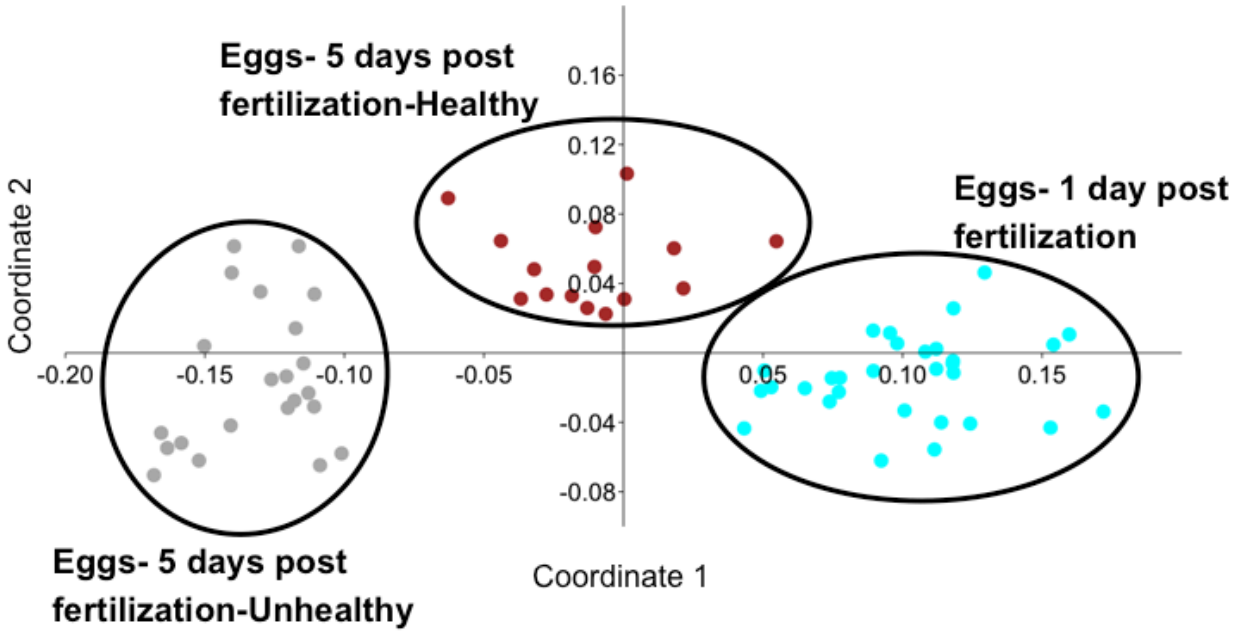

Figure 4.10. NMDS analysis of eggs one and five days post fertilization (Stress value= 0.072, ANOSIM P value= 0.0001, and ANOSIM R value= 0.931).

Although one day after fertilization there was no significant difference between the egg bacterial communities based on bacterial treatment during fertilization, there were clear differences between egg communities based on the mating pair at this time point (Fig. 11). Also, five days after fertilization the majority of surviving eggs belonged to the first mating pair (Fig. 6).

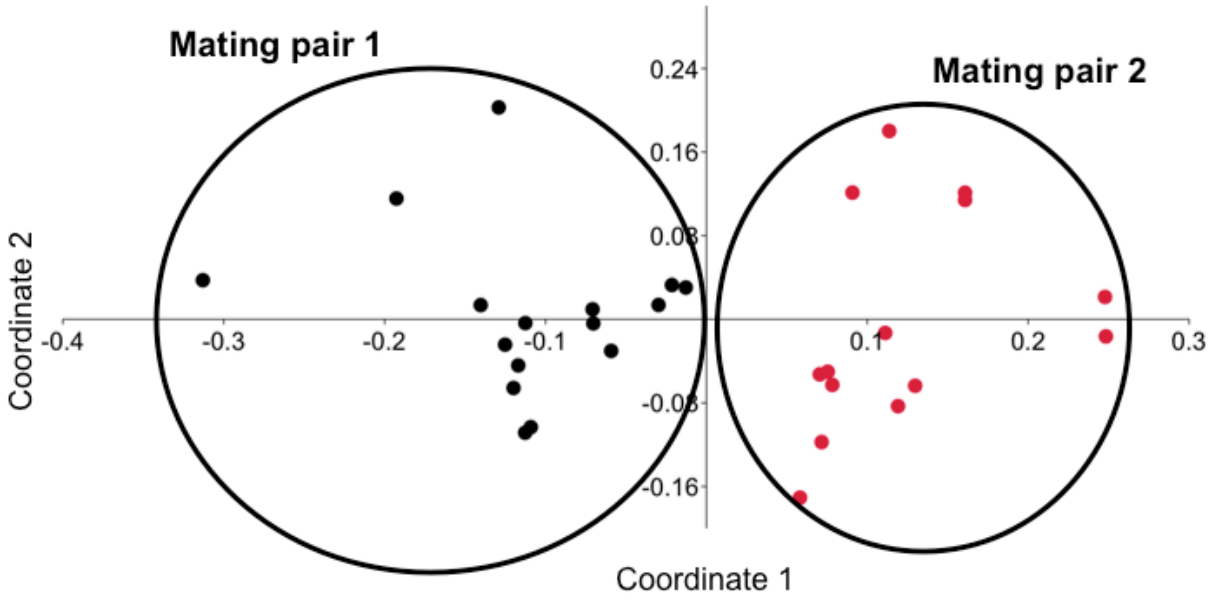

Figure 4.11. NMDS analysis of eggs one day post fertilization (Stress value= 0.122, ANOSIM P value= 0.0001, and ANOSIM R value= 0.573).

Rheinheimera, Pseudomonas and *Aeromonas* contributed most to dissimilarity between healthy and unhealthy eggs five days after fertilization (Fig. 12). At this time point, healthy eggs had higher levels of *Rheinheimera* and *Pseudomonas* whereas unhealthy eggs had significantly higher levels of *Aeromonas*.

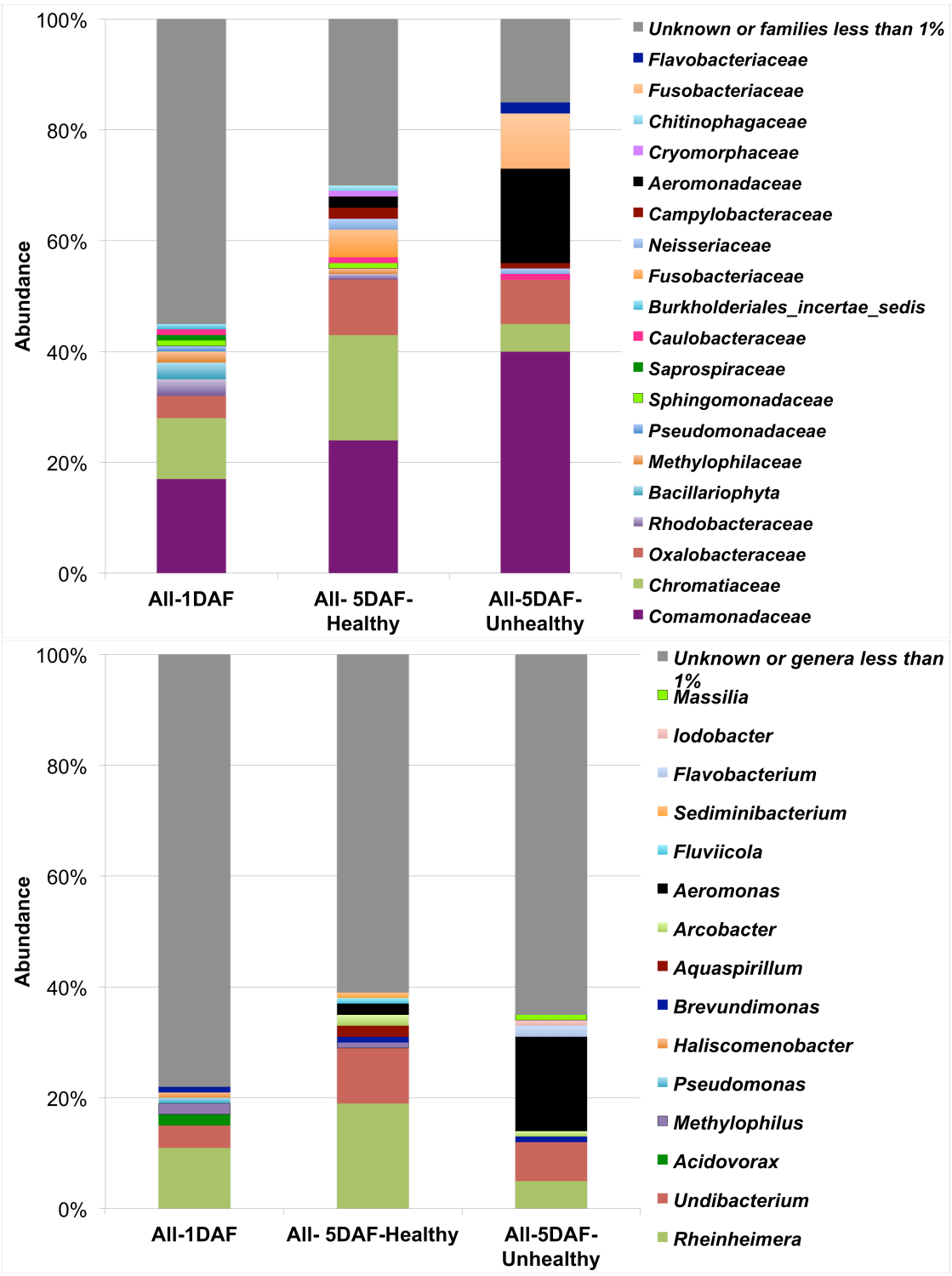

Figure 4.12. Most abundant bacterial families (top) and genera (bottom) in fertilized egg communities one day after fertilization (1DAF), and five days after fertilization (5DAF) in healthy and unhealthy eggs collected at this time point.

Egg mortality. Generally, egg mortality of eggs from both mating pairs was high (Fig. 13). Eggs exposed to *Brevundimonas F16* during fertilization had the highest egg mortality (100%), whereas eggs exposed to filtered water alone, *Hydrogenophaga F14*, *Acidovorax F19*, Brevundimonas-Hydrogenophaga, and Hydrogenophaga-Acidovorax had lower egg mortalities (~80%-90%).

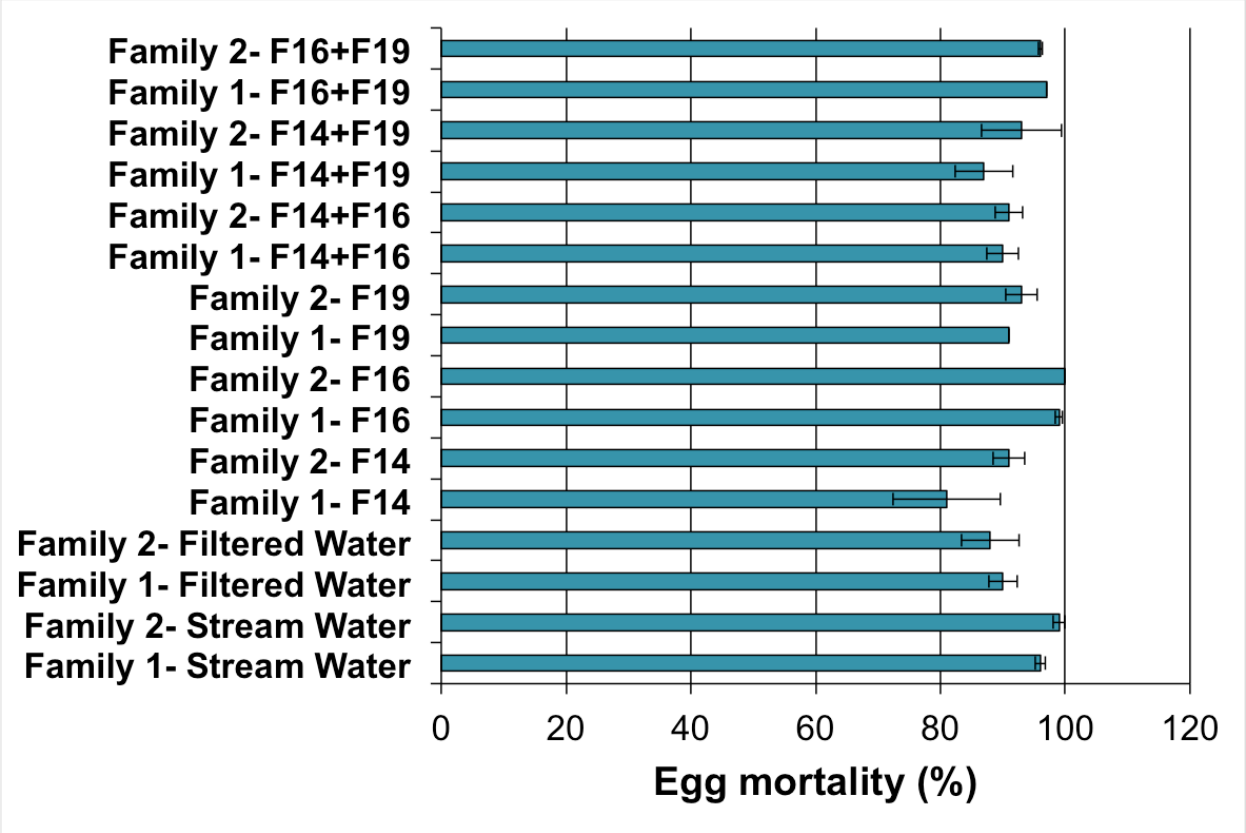

Figure 4.13. Egg mortality of two different families exposed to stream water, filtered water or filtered stream water supplemented with overnight cultures of different bacterial isolates (*Hydrogenophaga F14*, *Brevundimonas F16*, and *Acidovorax F19*). Overall ANOVA results: $F(15, 16) = 5.134$, $p = 0.00117$.

DISCUSSION

In this study we investigated the assembly of bacterial communities on the Lake Sturgeon egg surfaces during different developmental stages of the egg with and

without synthetic egg-surface glycans and putative probiotic bacteria added at the onset of incubation.

First, it is important to note that unfertilized eggs from both females used in this study were found to be not sterile although they were aseptically harvested. Fujimoto's studies [31] support this observation; although electron micrographs did not show any microbial presence on the surface of aseptically harvested eggs, 16S rRNA clone libraries derived from aseptically harvested eggs suggested they were not sterile. This can be due to the presence of bacteria in coelomic fluid that surrounds the eggs and/or egg surfaces. In this experiment, coelomic fluid was washed off the eggs before collection and DNA extraction, suggesting these bacteria were associated with the eggs. This observation implies that these microbes can colonize the eggs prior to their exposure to stream water presumably when the eggs are expelled through the lower gastrointestinal tract and, thus, affect the development of the egg associated communities in different ways.

Our results indicate that egg associated bacterial communities continue to evolve during the egg development. Exposure to stream water for 15 minutes changed the bacterial community of eggs significantly. At this time point there is a diverse collection of bacteria colonizing the surface. 135 minutes after exposure, the community shifted modestly and after 24 hours, the community shifted even further. This comparison suggests that as early as two hours post-exposure to stream water most of the egg-associated bacterial community were composed of significantly less abundant bacterial members of stream water community. While it is not clear what exactly governs this sorting process, it is expected that the trajectory of the community development plays

an important role in eggs survival and, thus, the reasons behind this selection requires further investigation.

We predicted that glycans played an important role in the binding of bacterial populations to the surface. The results from our test of this hypothesis by adding glucose, galactose or mannose to the fertilization bath, suggested that this is the case and that through chemical manipulation of the fertilization bath we could direct the community that was assembling on the egg. Next important result indicated that early intervention with monosaccharaides could change bacterial community composition on the egg surfaces. This change however, may not necessarily be beneficial for the eggs. For example, we illustrated that presence of glucose or galactose in the stream water decreased the abundance of *Pseudomonas* in the early communities (15-135 minutes) and significantly increased the abundance of *Aeromonas* on the egg surfaces. These results support the initial hypothesis that monosaccharaides affect bacterial attachment to the egg surfaces, since *Pseudomonas* was the most abundant genus in the early communities of egg surfaces that has been exposed to stream water alone. This outcome is expected to have negative effect on the egg's wellbeing because some major fish pathogens such as *Aeromonas salmonicida* and *Aeromonas hydrophila* belong to this genus. Furthermore, since the increase in abundance of *Aeromonas* happened at an early time point, it is less likely that the increase in *Aeromonas* population is due to increased growth rate or antagonistic inter-species interactions between microbes. In contrast, the increase in *Aeromonas* may be linked to a concomitant decrease in the abundance of *Pseudomonas*, as observed in the experiment. As such, use of monosaccharaides requires further investigation since

these molecules can inhibit the attachment of egg mutualists while having no effect on the attachment of fish pathogens. Therefore, adding probiotic species, such as *Pseudomonas*, during early stages of egg development could be preferable to using certain monosaccharaides like glucose and galactose.

There was a large distinction between the bacterial communities of healthy and unhealthy eggs. Healthy egg communities had considerably higher levels of *Rheinheimera* whereas unhealthy egg communities had higher levels of *Aeromonas*, *Flavobacterium*, and *Massilia*. These data suggest that there is a higher chance of finding a Lake Sturgeon egg probiotic in the genus *Rheinheimera* as some members of this genus possess a broad spectrum of antimicrobial activity including gram-positive and gram-negative bacteria, yeast and algae. This antimicrobial activity is due to the production of hydrogen peroxide through an enzymatic activity of L-lysine oxidase [49, 50]. While our results do not address how *Rheinheimera* attaches to the egg surface, our results suggest that this species is a secondary colonizer that appears on the egg surface 24 hours after exposure to stream water. This result indicates that the presence of certain initial colonizers may promote the attachment of *Rheinheimera* and thus affect the eggs fate. However, this hypothesis requires further exploration.

Another difference that we observed through the course of this work was between the bacterial communities of different females. This result indicates that variation in egg surface features can select for different bacterial populations, i.e. specific chemistry of the egg (defined by the female fish) affects early bacterial communities of egg surfaces. The chemistry of the egg surface can be affected by female's genetics and since Lake Sturgeon is a polyploid species, the genetic diversity

is expected to be high. However, to confirm this hypothesis, future studies with a higher number of females are needed.

In Lake Sturgeon, the average egg mortality reported was 91% [5]. In our study, the egg mortality was generally high in both mating pairs used, ranging from 80% to 100%. One explanation can be that eggs and/or sperm were of low quality. Additionally, high abundance of *Aeromonas* on aseptically harvested eggs from both females could contribute to observed high egg mortality. Bacterial treatment can also be the reason for high mortality numbers. For example, the highest egg mortality was observed in the eggs that were treated with *Brevundimonas F16* (~100%) during fertilization. Specifically, eggs that were exposed to *Brevundimonas F16* had a high richness and diversity in their bacterial community among the samples collected immediately after fertilization. The high richness and diversity observed in these samples might be due to the presence of holdfast, strong adhesive structures on *Brevundimonas* cells. Interestingly, fungi contamination was also higher in *Brevundimonas F16* treated eggs. Given that high egg mortality due to fungi contamination is a significant challenge in hatcheries, further investigation of fungi community of Lake Sturgeon egg surfaces and how it interacts with the bacterial community of the egg surfaces would be of interest. For instance, we observed that *Pseudomonas* species isolated from healthy Lake Sturgeon eggs not only inhibited the growth of bacterial fish pathogens but also restricted the growth of fungi suggesting some members of this genus could be egg mutualists. On the bright side, the lowest egg mortality was observed in the eggs that were treated with *Hydrogenophaga F14* (80-90%) during fertilization. As we showed in Chapter 3, *Hydrogenophaga F14* biofilms attract bacteria from *Rheinheimera* genus

from river water. At the same time, as we discussed above, *Rheinheimera* is known for its antimicrobial properties. Thus, presence of *Hydrogenophaga F14* can explain the reduction in Lake Sturgeon egg mortality.

To conclude, results of our experiments outlined in this chapter suggest that some members of *Rheinheimera* and *Pseudomonas* genera are potential Lake Sturgeon egg mutualists that can protect eggs from pathogenic bacteria and fungi. As such, focusing on isolating these two genera from the surface of healthy Lake Sturgeon eggs and investigating the ways they interact with river microbiome and eggs can lead to more effective methods for protecting eggs from pathogens and decrease high egg mortality.

APPENDIX

Sample	OTUs	Seq. per Sample	Simpson	Shannon	Chao1
F1R1-Stream-15minutes	35	11459	0.6039	1.435	35
F1R2-Stream-15minutes	53	11473	0.5776	1.647	56.75
F2R1-Stream-15minutes	54	11475	0.5062	1.477	57.33
F2R2-Stream-15minutes	43	11417	0.3659	1.068	46
F1R1-Stream-45minutes	240	10067	0.8618	3.657	253
F1R2-Stream-45minutes	75	11290	0.8517	2.532	82
F2R1-Stream-45minutes	50	11427	0.6414	1.978	50.5
F2R2-Stream-45minutes	57	11437	0.3778	1.076	59
F1R1-Stream-90minutes	50	11463	0.7367	1.986	56
F1R2-Stream-90minutes	66	11405	0.7002	2.157	69.33
F2R1-Stream-90minutes	47	11449	0.6566	1.989	47
F2R2-Stream-90minutes	94	11345	0.8541	3.154	95.2
F1R1-Stream-135minutes	50	11415	0.6944	1.734	50
F1R2-Stream-135minutes	72	11403	0.7506	2.163	77
F2R1-Stream-135minutes	55	11402	0.6599	2.009	70
F2R2-Stream-135minutes	74	11378	0.8117	2.849	77
F1R1-Man01-15minutes	192	11318	0.4161	1.489	225.7
F1R2-Man01-15minutes	86	11304	0.8643	2.614	97.25
F1R1-Man02-15minutes	128	11409	0.3913	1.249	151.1
F1R2-Man02-15minutes	99	11223	0.7537	2.139	113
F1R1-Man1-15minutes	50	11465	0.5471	1.234	51
F1R2-Man1-15minutes	80	11412	0.8041	2.623	80
F1R1-Man5-15minutes	98	11379	0.6258	1.601	102.5
F1R2-Man5-15minutes	109	11186	0.8354	2.572	115.4
F2R1-Man01-15minutes	68	11456	0.2529	0.873	70.14
F2R2-Man01-15minutes	138	11314	0.3178	1.167	140.1
F2R1-Man02-15minutes	44	11471	0.3197	1.047	51.5
F2R2-Man02-15minutes	157	11368	0.3356	1.223	171.4
F2R1-Man1-15minutes	41	11481	0.2742	0.8784	41.25
F2R2-Man1-15minutes	118	11440	0.5002	1.788	119.4
F2R1-Man5-15minutes	58	11447	0.5485	1.771	72
F2R2-Man5-15minutes	89	11392	0.4362	1.327	93.67
F1R2-Man01-45minutes	65	11434	0.5449	1.315	67.5
F1R1-Man02-45minutes	89	11443	0.4204	1.178	115

Table C-1. Bacterial alpha-diversity in unfertilized egg communities of female 1 (F1) or female 2 (F2) exposed to stream water alone (stream) or stream water supplemented with mannose (Man), glucose (Glu) or galactose (Gal) [concentrations= 0.1 (01), 0.2 (02), 1 and 5%] for 15, 45, 90 or 135 minutes.

Table C-1 (cont'd)

Sample	OTUs	Seq. per Sample	Simpson	Shannon	Chao1
F1R2-Man02-45minutes	62	11445	0.5592	1.199	67.25
F1R1-Man1-45minutes	74	11419	0.5064	1.128	87.75
F1R2-Man1-45minutes	103	11426	0.2843	0.9555	104.7
F1R1-Man5-45minutes	69	11382	0.6058	1.465	84
F1R2-Man5-45minutes	113	11407	0.4136	1.327	114.9
F2R1-Man01-45minutes	69	11477	0.2823	0.9943	72
F2R2-Man01-45minutes	50	11443	0.3471	1.101	50.33
F2R1-Man02-45minutes	45	11458	0.2204	0.7461	46
F2R2-Man02-45minutes	51	11457	0.5129	1.679	52
F2R1-Man1-45minutes	59	11464	0.2234	0.7382	62.33
F2R2-Man1-45minutes	80	11428	0.5784	2.002	81.5
F2R2-Man5-45minutes	86	11375	0.5498	1.89	87.5
F2R1-Man5-45minutes	204	10899	0.6476	1.989	227.6
F1R1-Man01-90minutes	173	11010	0.7072	2.344	189.5
F1R2-Man01-90minutes	125	11400	0.5395	1.375	135.7
F1R1-Man02-90minutes	53	11420	0.4949	1.002	56
F1R2-Man02-90minutes	49	11420	0.5842	1.243	50.5
F1R1-Man1-90minutes	72	11376	0.572	1.306	90.33
F1R2-Man1-90minutes	80	11412	0.6217	1.391	87
F1R1-Man5-90minutes	78	11364	0.3964	1.175	80
F1R2-Man5-90minutes	69	11429	0.5549	1.349	72.33
F2R1-Man01-90minutes	91	11369	0.5456	1.864	91
F2R2-Man01-90minutes	55	11452	0.2345	0.7984	56.5
F2R1-Man02-90minutes	73	11364	0.8386	2.358	76.33
F2R2-Man02-90minutes	59	11443	0.4776	1.289	62
F2R1-Man1-90minutes	69	11435	0.3023	0.9986	72.75
F2R2-Man1-90minutes	41	11470	0.3873	1.118	47
F2R1-Man5-90minutes	116	11292	0.6889	1.848	120.1
F2R2-Man5-90minutes	60	11398	0.3913	1.215	67.5
F1R1-Man01-135minutes	62	11379	0.6165	1.247	64.14
F1R2-Man01-135minutes	47	11404	0.6395	1.368	48
F1R1-Man02-135minutes	88	11196	0.8496	2.293	104.2
F1R2-Man1-135minutes	57	11372	0.6378	1.419	59
F1R1-Man1-135minutes	76	11399	0.6538	1.412	78.55
F1R1-Man5-135minutes	78	11412	0.6371	1.402	94.5
F1R2-Man5-135minutes	104	11328	0.7137	1.964	106.1
F2R1-Man01-135minutes	51	11408	0.5019	1.498	63
F2R2-Man01-135minutes	78	11422	0.5503	1.651	79.2
F2R1-Man02-135minutes	162	11198	0.7299	2.921	169.5
F2R2-Man02-135minutes	74	11390	0.5149	1.524	77
F2R1-Man1-135minutes	94	11415	0.7653	2.332	95
F2R2-Man1-135minutes	65	11413	0.5683	1.717	66.5

Table C-1 (cont'd)

Sample	OTUs	Seq. per Sample	Simpson	Shannon	Chao1
F2R1-Man5-135minutes	110	11396	0.5955	2.091	119.3
F2R2-Man5-135minutes	61	11415	0.859	2.914	61.33
F1R1-Glu01-15minutes	49	11446	0.483	1.303	52
F1R2-Glu01-15minutes	58	11425	0.6506	1.828	58
F1R1-Glu02-15minutes	45	11438	0.6449	1.429	46
F1R2-Glu02-15minutes	70	11224	0.7737	2.307	73.33
F1R1-Glu1-15minutes	49	11445	0.6225	1.695	77
F1R2-Glu1-15minutes	75	11356	0.6933	1.815	75.38
F1R1-Glu5-15minutes	34	11460	0.6391	1.567	37.33
F1R2-Glu5-15minutes	54	11354	0.5713	1.376	56
F2R1-Glu01-15minutes	100	11428	0.7811	2.782	101.5
F2R2-Glu01-15minutes	70	11417	0.7402	2.409	106
F2R1-Glu02-15minutes	121	11355	0.906	3.333	128
F2R2-Glu02-15minutes	77	11425	0.8846	3.191	78
F2R1-Glu1-15minutes	70	11413	0.7368	2.243	75.6
F2R2-Glu1-15minutes	62	11459	0.8942	2.991	90
F2R1-Glu5-15minutes	54	11408	0.7882	2.52	61
F2R2-Glu5-15minutes	58	11401	0.7344	2.006	63.25
F1R1-Glu01-45minutes	50	11408	0.6046	1.344	57
F1R2-Glu01-45minutes	135	11174	0.8266	2.798	170
F1R1-Glu02-45minutes	52	11410	0.5691	1.364	55.75
F1R2-Glu02-45minutes	47	11427	0.6295	1.756	53
F1R1-Glu1-45minutes	63	11398	0.5205	1.31	68
F1R2-Glu1-45minutes	63	11407	0.5022	1.343	64
F1R1-Glu5-45minutes	84	11355	0.6171	1.948	84.5
F1R2-Glu5-45minutes	63	11393	0.5163	1.274	63.43
F2R1-Glu01-45minutes	85	11392	0.8619	3.055	92.5
F2R2-Glu01-45minutes	95	11294	0.5646	1.836	95.17
F2R1-Glu02-45minutes	96	11467	0.8764	3.178	105
F2R2-Glu02-45minutes	68	11465	0.7134	2.357	75
F2R1-Glu1-45minutes	100	11455	0.8908	3.437	110
F2R2-Glu1-45minutes	41	11450	0.6011	1.758	41
F2R1-Glu5-45minutes	66	11419	0.8716	3.142	66
F2R2-Glu5-45minutes	91	11393	0.5419	1.873	91.25
F1R1-Glu01-90minutes	79	11248	0.2899	0.9783	82.33
F1R2-Glu01-90minutes	96	11203	0.4339	1.505	99.33
F1R1-Glu02-90minutes	116	11303	0.6669	2.133	125
F1R2-Glu02-90minutes	121	11317	0.6663	1.83	125
F1R1-Glu1-90minutes	114	11240	0.6176	2.045	123.8
F1R2-Glu1-90minutes	83	11285	0.4657	1.292	85.5
F1R1-Glu5-90minutes	81	11410	0.485	1.592	86
F1R2-Glu5-90minutes	72	11364	0.2686	0.9001	74

Table C-1 (cont'd)

Sample	OTUs	Seq. per Sample	Simpson	Shannon	Chao1
F2R1-Glu01-90minutes	121	11371	0.8428	3.156	124
F2R2-Glu01-90minutes	123	11381	0.9559	3.886	125.5
F2R1-Glu02-90minutes	73	11430	0.8517	3.028	73.5
F2R2-Glu02-90minutes	92	11434	0.9703	3.949	99.5
F2R1-Glu1-90minutes	64	11442	0.705	2.382	67.33
F2R1-Glu5-90minutes	64	11419	0.9224	3.319	64.33
F2R2-Glu5-90minutes	119	11356	0.818	3.119	128.3
F1R1-Glu01-135minutes	75	11375	0.3536	0.9472	78.75
F1R2-Glu01-135minutes	72	11397	0.2779	0.9622	73.2
F1R1-Glu02-135minutes	173	11092	0.731	2.803	182.2
F1R2-Glu02-135minutes	71	11336	0.2327	0.7926	80
F1R1-Glu1-135minutes	205	11065	0.7696	2.719	207.6
F1R2-Glu1-135minutes	105	11351	0.4316	1.413	108.1
F1R1-Glu5-135minutes	136	11184	0.6321	2.069	142
F1R2-Glu5-135minutes	86	11337	0.2863	0.9682	87.88
F2R1-Glu01-135minutes	109	11226	0.5433	1.96	109.8
F2R2-Glu01-135minutes	132	11279	0.9535	3.876	137
F2R1-Glu02-135minutes	110	11311	0.8426	3.179	125
F2R2-Glu02-135minutes	149	11263	0.968	4.19	149.8
F2R1-Glu1-135minutes	331	10247	0.9864	5.179	340.1
F2R1-Glu5-135minutes	145	11287	0.8816	3.472	146.9
F2R2-Glu5-135minutes	79	11447	0.8507	3.076	79.6
F1R1-Gal01-15minutes	66	11391	0.7127	1.833	68
F1R2-Gal01-15minutes	44	11433	0.2892	0.7729	50
F1R1-Gal02-15minutes	63	11412	0.5073	1.364	68.25
F1R2-Gal02-15minutes	34	11435	0.3452	0.8354	34.5
F1R1-Gal1-15minutes	256	10444	0.8715	3.484	284.5
F1R1-Gal5-15minutes	74	11337	0.464	1.317	77.75
F1R2-Gal5-15minutes	41	11427	0.3075	0.8146	44
F2R1-Gal01-15minutes	45	11449	0.8418	2.498	45
F2R2-Gal01-15minutes	56	11426	0.6493	1.997	70
F2R1-Gal02-15minutes	86	11373	0.7783	2.224	90
F2R2-Gal02-15minutes	61	11425	0.7167	2.253	71
F2R1-Gal1-15minutes	395	7981	0.9847	5.032	404.7
F2R2-Gal1-15minutes	75	11428	0.5744	1.92	76
F2R1-Gal5-15minutes	190	11208	0.8569	3.641	190.6
F2R2-Gal5-15minutes	45	11462	0.4183	1.156	48
F1R1-Gal01-45minutes	47	11460	0.2256	0.672	47
F1R2-Gal01-45minutes	62	11424	0.2477	0.8051	71
F1R1-Gal02-45minutes	102	11333	0.5223	1.802	116
F1R2-Gal02-45minutes	60	11319	0.4195	1.25	65
F1R1-Gal1-45minutes	116	11332	0.5449	1.838	138.5

Table C-1 (cont'd)

Sample	OTUs	Seq. per Sample	Simpson	Shannon	Chao1
F1R2-Gal1-45minutes	71	11353	0.3439	1.192	73
F1R1-Gal5-45minutes	63	11393	0.3724	1.184	63.5
F1R2-Gal5-45minutes	56	11385	0.2219	0.758	66
F2R1-Gal01-45minutes	46	11444	0.754	2.192	47
F2R2-Gal01-45minutes	101	11255	0.6417	1.593	109.7
F2R2-Gal02-45minutes	106	11356	0.9024	3.47	111.3
F2R1-Gal1-45minutes	76	11417	0.7774	2.732	79.33
F2R2-Gal1-45minutes	69	11439	0.8952	3.24	69
F2R2-Gal5-45minutes	77	11454	0.8267	2.962	80
F1R1-Gal01-90minutes	76	11411	0.455	1.218	77.2
F1R1-Gal02-90minutes	128	11214	0.475	1.737	129.7
F1R2-Gal02-90minutes	110	11307	0.5698	1.739	112.6
F1R1-Gal1-90minutes	88	11436	0.7519	2.588	109.4
F1R1-Gal5-90minutes	89	11408	0.3934	1.312	94
F1R2-Gal5-90minutes	81	11400	0.4077	1.39	81.75
F2R1-Gal01-90minutes	75	11395	0.9146	3.4	82.5
F2R1-Gal02-90minutes	101	11327	0.9619	3.848	101
F2R2-Gal02-90minutes	112	11360	0.9687	4.008	117
F2R1-Gal1-90minutes	159	11233	0.9765	4.316	160
F2R2-Gal1-90minutes	72	11461	0.8659	3.168	100
F2R1-Gal5-90minutes	118	11283	0.9074	3.474	118.2
F2R2-Gal5-90minutes	96	11376	0.9498	3.744	96.75
F1R1-Gal01-135minutes	75	11382	0.1992	0.6992	90
F2R2-Gal01-135 minutes	129	11285	0.9648	3.983	129
F2R1-Gal01-135minutes	170	11122	0.9721	4.282	172
F2R1-Gal02-135minutes	138	11312	0.9489	3.87	142.2
F2R2-Gal02-135minutes	117	11410	0.9726	4.095	117.3
F2R1-Gal1-135minutes	104	11343	0.9703	3.956	107
F2R2-Gal1-135minutes	131	11222	0.9718	4.086	131.9
F2R1-Gal5-135minutes	300	10261	0.9843	4.972	319
F2R2-Gal5-135minutes	68	11457	0.9705	3.776	68

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CHAPTER 5. CONCLUDING REMARKS

In this dissertation we have presented the results of three studies related to assembly of bacterial communities on the surface of Lake Sturgeon eggs while focusing on developing green methods for controlling microbial populations on the egg surface in order to reduce high egg mortality. Throughout this work our perspective on bacterial species associated with Sturgeon eggs was to treat them as a natural part of the egg-bacteria symbiotic relationship with the emphasis on manipulating bacterial communities in order to increase egg survivability. To recapitulate, our specific objectives were (i) to gain a comprehensive understanding of how certain bacterial species found in high abundance on the surface of healthy Lake Sturgeon eggs interact with each other, (ii) to study how these isolates interact with river microbiome after forming biofilms on the egg surface, and (iii) to study the effect of these bacterial isolates on the development of egg-associated bacterial communities and eggs' wellbeing when the eggs are treated with these isolates in their early developmental stages.

First, we tested biofilm formation capabilities of bacterial species previously isolated from healthy Lake Sturgeon eggs *in vitro.* The main reason for choosing biofilm assays was the fact that electron micrographs of Lake Sturgeon egg surfaces showed a variety of microbes on the egg surface in multi-species biofilm-like structures one day after exposure to stream water (Chapter 2) [1]. For this experiment we chose six bacterial species belonging to genera *Acidovorax*, *Hydrogenophaga*, *Pseudomonas*, *Massilia,* and *Brevundimonas* as these genera were found to be highly abundant on healthy egg surfaces [2]. The main goal of this study was to understand how these bacterial species interact in biofilm formation, to identify the species that have mutualism or potential probioses during biofilm formation, and investigate how they

respond to environmental stress. In order to determine whether certain bacteria are mutualists or antagonists we have measured biofilm biomass of mixed species biofilms and compared it with biomasses of biofilms produced by participating species in single-species cultures (Chapter 2). Specifically, if the biomass of the mixed-species biofilm was found to be smaller than the cumulative biomass of two separate biofilms, we concluded that these species exhibit antagonistic interactions. In the opposite case, when the biomass of a multi-species biofilm was found to be larger than the sum of biomasses of biofilms produced by these species individually, we also used the results of resazurin assay to identify whether increase in the biomass is accompanied by increase in the metabolic activity within the biofilm. If estimated metabolic activity is higher in multi-species biofilms as compared with their single-species counterparts (in addition to higher total biomass), interactions between participating species are likely mutualistic. Our hypothesis was that species that show mutualism during biofilm formation would be more resistant to environmental stress through mechanisms such as reducing permeability of extracellular matrix or emergence of persister cells and, thus, more effective in protecting eggs against pathogenic microbes either by preventing their initial attachment to the egg surface or producing antimicrobial compounds [3-9].

Results of our experiments revealed that *Brevundimonas F16* had the strongest *in vitro* biofilm formation capability individually, indicating that this species has the highest adhesive property when it comes to initial attachment to polystyrene when compared to other species in this study (Chapter 2). Note that *Brevundimonas* genus is known to have strong adhesive structures called holdfasts that can explain their high biofilm forming capabilities in microtiter plates. Additionally, these holdfasts appear to

have positive effect on biofilm formation in multi-species cultures containing *Brevundimonas* as one of participants. In fact, all double- and even triple-species cultures with *Brevundimonas* exhibited stronger biofilm forming capabilities as compared with their single species counterparts (Chapter 2). Following our estimations of biofilm biomass and biofilm metabolic activity, we concluded that *Brevundimonas F16* and *Hydrogenophaga F14* might be mutualists since their combined biofilm biomass was nearly 75% larger than the sum of biomasses produced by individual biofilms accompanied by almost 50% increase in the metabolic activity. In all other mixed-species cultures, biofilm biomass was not statistically different from biomasses of *Brevundimonas* and other participating species, suggesting that these species exhibit commensalistic and/or weak mutualistic interactions.

In our "invasion" tests, after establishing its initial biofilm, *Brevundimonas* exhibited an ability to recruit more members of its own pelagic population suggesting that holdfasts can promote not only initial attachment to biotic or abiotic surfaces but also inter-cellular connections between individual cells in the biofilm. However, the presence of holdfasts does not imply that biofilm formation of established *Brevundimonas F16* biofilms can be boosted by any other species. In fact, biomass of established *Brevundimonas* biofilms was boosted only with addition of *Acidovorax F19*. This observation, combined with previously mentioned mutualism with *Hydrogenophaga* in their double-species mixture, suggests that while some bacterial species may not be able to attach directly to the egg surface (like *Acidovorax*), they can still affect eggs wellbeing by being present as secondary colonizers. Our experiments on interactions of established biofilms of healthy Lake Sturgeon egg isolates with river microbiome

(Chapter 3) as well as following successional studies on Lake Sturgeon eggs (Chapter 4) further supported this hypothesis. Moreover, these bacterial species may form coaggregates in pelagic form that will further promote their attachment to abiotic or biotic surfaces. To test this hypothesis a cell sorting method can be used *in vitro* in future studies.

Another noteworthy result was the remarkable boost in biofilm formation (initial attachment) of *Pseudomonas C22* (a healthy Lake Sturgeon egg isolate) in the presence of the fish pathogen *Flavobacterium columnare.* Moreover, according to the results of "invasion" tests, interaction of *F. columnare* in its pelagic form with already established *Pseudomonas C22* biofilms allowed the latter to form even larger biofilm biomass along with increased levels of metabolic activity. Importantly, mixing these species in the reverse order did not produce the same result. Although the underlying mechanism remains unknown, this result provides another line of evidence that history can play an important role in the development of bacterial communities of egg surfaces. Importantly, this effect can impact the fish eggs microbial community composition and, thus, its subsequent fate (Chapter 3 and Chapter 4). These findings on biofilm formation in the presence of *F. columnare,* previous *in vitro* antagonistic studies using soft-agar overlay [1, 2] and bacterial community analysis of Lake Sturgeon eggs during their development in the hatchery (Chapter 4) suggest that *Pseudomonas C22* can be a potential Lake Sturgeon egg mutualist, protecting eggs from pathogenic bacteria and fungi by producing antimicrobial compounds. This however, should be tested in the hatchery on live Lake Sturgeon eggs. Furthermore, the nature of *Pseudomonas C22*

antimicrobial activity should be studied in the future using methods such as gene expression profiling.

Metals are known to play an important part in bacterial biofilm formation [10-12], where Mg^{2+} and Ca^{2+} are known to provide structural support in some cases and Ni^{2+} is a stimulus for biofilm formation in several systems. As such, our goal here was to examine whether these metals also have positive effects on the biofilm formation of selected bacterial species. Our experiments, however, indicated that Nickel not only did not facilitate any biofilm growth, but also was the most effective (as compared with Mg^{2+} and Ca^{2+}) inhibitor of biofilm formation in bacterial species used in this study, especially *Brevundimonas*. This observation is of great interest since, as we discussed above, *Brevundimonas* appears to be an initial colonizer that promotes the attachment of different secondary colonizers that can be fish egg pathogens or mutualists. Although Nickel concentrations used in this study were much higher than those found in highly contaminated waters, it is important to account for potential presence and effect of this metal on egg-associated microbial communities when conducting studies in hatcheries in the future.

In experiments with Mg^{2+} and Ca^{2+}, we observed that calcium facilitates biofilm formation of *P. aeruginosa*, *Hydrogenophaga* and *Brevundimonas*, while inhibiting formation of double-species biofilms of *Brevundimonas* with either *Hydrogenophaga* F14 or *Acidovorax* F19. This result suggests that while Ca^{2+} can be beneficial for biofilm growth of certain bacterial species when they are present alone, it could have negative effects on the biofilm development in mixed cultures by potentially diminishing inter-cellular binding capability. This is an important observation as it suggests that high

levels of Ca^{2+} in water can have detrimental effect on egg survivability by inhibiting the attachment of some potential probiotics on the fish egg surface. Finally, magnesium appears to promote biofilm formation of *Hydrogenophaga* F14 alone without having statistically significant effect on any other bacterial species or their double-species mixtures.

Next, we studied how established biofilms of healthy Lake Sturgeon egg isolates interact with the river water microbiome (Chapter 3). Our goal in this experiment was to investigate secondary colonization of a preexisting biofilm due to exposure to the river water. Both the stability of the single- or double-species biofilms and the identification of specific river populations that invade or are recruited can be measured using amplicon-based sequencing (Illumina) of 16s rRNA gene. With this approach, we can establish unique fate maps for community assembly in which specific populations appear to select for secondary colonizers. Knowing the river water populations that are selected by specific established biofilms may assist in identification of mutualists among pre-existing and newly attached species and, thus, in the development of probiotics that can direct fish egg-associated microbial community assembly and reduce egg mortality. In order to distinguish between the dynamics of aggressive or collaborative interactions during secondary colonization, we compared the initial state of the founding population to its condition after 24 hours of exposure to the river water. Specifically, we were able to detect both highly resilient and weak biofilms, specific exclusions and recruitments of river populations by established biofilms of egg isolates, and apparent enhancements of biofilm development.

The results of this study suggested that even after 4 hours of exposure, the bacterial populations that are formed on the polystyrene and established biofilms are different from the pelagic community. When we investigated early attachments on Lake Sturgeon eggs we got similar results (Chapter 4). In fact, the community formed on Lake Sturgeon eggs was different from the pelagic community even as early as 15 minutes after exposure to water. These results suggest that different bacterial populations can attach to different surfaces based on specific topology and chemistry of these surfaces. As supported statistically, established biofilm communities selected for a unique collection of secondary colonizing populations from river water, indicating that historical contingency plays a role in the structuring of biofilms, the effect observed previously in our experiment with *Pseudomonas C22* and *F. columnare* (Chapter 2). Moreover, in both microtiter plates after exposure to river water and on Lake Sturgeon eggs before exposure to river water a potential fish pathogen, *Aeromonas,* was the most abundant genus. However, this was not the case with established biofilms and Lake Sturgeon egg communities after exposure to stream water. These results suggest *Aeromonas* is accepting of a hydrophobic surface (microtiter plates) but not as accepting of the same surface preconditioned with other biofilms, which is an indication that some members of the egg-associated bacterial community may be egg mutualist by inhibiting the attachment of pathogenic species such as *Aeromonas*.

Another interesting result was related to the recruitment of another potential fish pathogen, *Flavobacterium.* The abundance of *Flavobacterium* increased significantly in the established biofilm community of *Brevundimonas F16* after exposure to river water. Furthermore, each established biofilm recruited a specific set of OTUs associated with

this genus. Since not all members of the *Flavobacterium* genus are fish pathogens, this result suggests that while some established biofilms might recruit pathogenic *Flavobacterium* that will affect the wellbeing of the Lake Sturgeon eggs negatively, others may recruit *Flavobacterium* species that will not affect the health of eggs. While current study did not address the distinction between these populations, it is important to identify which isolates attract pathogenic *Flavobacterium* species. This can be tested in the future by isolating members of *Flavobacterium* genus from stream water used in the hatchery as well as healthy and unhealthy egg surfaces and exposing established biofilms of potential Lake Sturgeon egg probiotics *in vitro* to different bacterial species belonging to *Flavobacterium* genus including isolated species from eggs and stream water as well as known fish pathogens such as *Flavobacterium columnare, Flavobacterium spartansii* and *Flavobacterium psychrophilum.* In general, our observations suggest that using *in vitro* biofilm formation assays has the capacity to reveal unique features of multispecies bacterial biofilms and can be used as a rapid method to prescreen for probiotics that may direct egg-associated community assembly and reduce egg mortality in fish.

Finally, we studied assembly of bacterial communities on the Lake Sturgeon egg surfaces in the hatchery (Chapter 4). Previous work showed early intervention in the egg-associated bacterial community could change the fate of the eggs and reduce mortality significantly. Based on this observation, we chose two basic approaches to alter the pathways of community assembly. The first one was to add relatively high concentrations of healthy egg bacterial isolates shown to have strong biofilm forming capabilities *in vitro* (Chapters 2 and 3), to establish a robust population on the eggs

surface that, in turn, will change the subsequent egg-associated microbial community assembly. An alternative method was to alter binding patterns of bacterial populations by introducing a competitive inhibitor of binding, a specific saccharide that would block binding of certain bacterial populations. Our goal here was to identify which, if any, bacterial populations in stream water are inhibited from binding to eggs in the presence of a saccharide or potential probiotics.

Our results indicated that although the most abundant populations in stream water affect the initial (15 minutes) attachments on egg surfaces, as early as 120 minutes after exposure to stream water most of the egg bacterial community is composed of river populations that are significantly less abundant in stream water. This is an indication that Lake Sturgeon eggs as well as initial microbial colonizers of the egg surface actively select for specific populations from the river water. This dynamic selection continues during the eggs development and while one might speculate that processes such as maternally derived antimicrobial factors incorporated during vitellogenesis, expression of specific antimicrobial molecules by embryonic membranes, and the antagonistic and mutualistic interactions between initial microbial colonizers of the egg surface and river microbiome all play significant roles, the exact mechanisms that govern this process are not yet fully understood. Since it is expected that the trajectory of the community development play an important role in eggs wellbeing the reasons behind this selection need further investigation. Exposing the Lake Sturgeon eggs to known pathogenic, commensal and mutualistic bacterial species during their early development and comparing the resulting transcriptomic profiles will help to

identify the role of Lake Sturgeon eggs in selection of specific bacterial species from stream water in the future.

Interestingly, early intervention with monosaccharaides could change egg-associated bacterial communities; however, this change might not necessarily reduce the egg mortality. For example, we observed that presence of glucose or galactose in the stream water increased the abundance of *Aeromonas* and reduced the abundance of *Pseudomonas* on the egg surfaces remarkably. Based on our *in vitro* observations (Chapter 2) one might expect to find egg mutualists in *Pseudomonas* genus; as a result, this effect of glucose and/or galactose is expected to have negative consequences for the eggs wellbeing. In fact, using saccharides to prevent microbial infections by inhibiting adhesion on biotic surfaces such as fish egg requires further investigation since these molecules can inhibit the attachment of mutualistic microbial species while having no effect on the attachment of pathogens.

Investigating the bacterial communities associated with healthy and unhealthy egg surfaces showed a remarkable distinction between these communities. Healthy egg communities had considerably higher levels of *Rheinheimera* whereas unhealthy egg communities had higher levels of *Aeromnoas*, *Flavobacterium*, *and Massilia*. *Rheinheimera* has been shown as a producer of antimicrobial compounds [13, 14]. When we exposed the established biofilms of healthy Lake Sturgeon egg isolates to river microbiome in our *in vitro* experiments (Chapter 3), higher abundance of *Rheinheimera* appeared to have little to no effect on *Aeromonas* population, but it was in correlation with lower *Flavobacterium* abundance. Furthermore, our *in vivo* studies (Chapter 4) suggested presence of *Pseudomonas* is correlated with lower *Aeromonas*

and fungi contamination. These results suggest that some members of *Rheinheimera* and *Pseudomonas* are potential Lake Sturgeon egg mutualists that can protect eggs from pathogenic bacteria and fungi. Isolating members of these two genera from healthy Lake Sturgeon egg surfaces and examining how these isolates interact with river microbiome and eggs can lead to more effective methods for protecting eggs from pathogens and decrease high egg mortality.

Another approach for finding an effective method for reducing the egg mortality is to investigate which healthy Lake Sturgeon egg isolates recruit members of *Pseudomonas* and *Rheinheimera* from river water. For example our *in vitro* studies showed established biofilms of *Hydrogenophaga F14* recruit members of *Rheinheimera* genus from river water (Chapter 2). Furthermore, eggs treated with *Hydrogenophaga F14* during fertilization had the lowest egg mortality observed in our *in vivo* studies. These results indicate that treating eggs early on with bacterial species that can further recruit *Pseudomonas* and/or *Rheinheimera* from the river water can also change the eggs fate and reduce mortality. Moreover, previous studies on effect of probiotic species on the wellbeing of fish larvae showed probiotic species can enhance fish survival by decreasing the abundance of pathogenic bacteria. Further investigation showed some of these probiotic species neither colonize the fish nor existed in pelagic form in water, but formed biofilms on the surface of fish tanks [15]. These findings suggest that when conducting probiotic studies on Lake Sturgeon eggs in the hatchery, the microbial community of container surfaces where eggs are incubated or fertilized should also be analyzed.

Another noteworthy result that we observed throughout the course of our experiments on live eggs in the hatchery was the difference between the bacterial communities of eggs harvested from different females. Although this result was based on observations from just two females, it suggests that variations in egg features can select for different bacterial populations and, thus, affect microbial communities of egg surfaces. These variations, determined by female and embryo genetics, can be in the chemical composition of chorion, maternally derived antimicrobial factors, and specific antimicrobial molecules produced by embryonic membranes. As Lake Sturgeon is a polyploid species, the genetic diversity is expected to be high. Therefore, when studying egg-associated microbial communities higher number of females/mating pairs should be used.

Lastly, the egg mortality was generally high in both mating pairs used in our studies. One reason for this high egg mortality can be that eggs and/or sperm used in these studies were of low quality. Also, high abundance of *Aeromonas* on aseptically harvested eggs from both females could contribute to high egg mortality. Bacterial treatments used during fertilization could also be one reason for high mortality. For example, *Brevundimonas F16* treated eggs had the highest mortality. Furthermore, these eggs had the highest richness and diversity in their bacterial communities. This observation can be due to the presence of holdfasts in *Brevundimonas* cells. We also observed *Brevundimonas F16* treated eggs had higher fungi contamination. Fungi contamination is a key challenge in the hatcheries as it spreads quickly and causes developmental arrest due to anoxia in the eggs that are surrounded by affected eggs. Interestingly, the healthy eggs collected at day 5 post fertilization in our *in vivo* studies

stayed healthy during their development despite being surrounded by unhealthy, fungi contaminated eggs. We think the key to their health was their egg-associated bacterial community. However, further investigation of egg-associated fungal community and how it interacts with the bacterial community of the egg surfaces would be of interest in the future.

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