Fish Out of Water: Understanding the Impacts of Regional Species Pool Variation on Local Community Assembly in a Host-Microbiome Model System

by

Kayla C. Evens

A dissertation accepted and approved in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in Biology

Dissertation Committee:

William Cresko, Chair

Brendan Bohannan, Advisor

Karen Guillemin, Core Member

Lauren Hallett, Core Member

Nelson Ting, Institutional Representative

University of Oregon

Winter 2024

DISSERTATION ABSTRACT

Kayla C. Evens

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Title: Fish Out of Water: Understanding the Impacts of Regional Species Pool Variation on Local Community Assembly in a Host-Microbiome Model System

The host-microbiome is essential to many aspects of host health and function, and acts as a useful model system in which to investigate broader questions of community assembly. Since the composition of host-microbiomes is, in part, determined by the input of microbes from the surrounding environment, it is integral to understand how variation in the environmental microbiome may influence host-microbiome assembly. For my dissertation research, I used a species pool conceptual framework to investigate drivers of variation in the microbiomes of aquaculture research facilities and the fish they house.

Zebrafish (*Danio rerio*) are used extensively as model organisms in scientific research, especially in studies investigating host-microbiome dynamics. For in-vitro experimentation utilizing model organisms, we rely on the reproducibility of results to make broadly applicable conclusions about the host-microbiome. However, evidence suggests that inter-facility variation may influence zebrafish gut microbiome composition via acquisition of microbes from the environment, potentially leading to phenotypic differences among fish housed at different aquaculture facilities.

To investigate the relationship between aquaculture facility water and fish gut microbiomes, I first characterized spatial and temporal variation across multiple aquaculture facilities on the University of Oregon campus. Facility water microbiota not only varied over time, but patterns of spatial variation in each facility were consistent despite differences in host

species. I then used this information to guide an expanded, intensive sampling of water and fish across four zebrafish facilities in Oregon and Norway. I observed significant variation in microbiome composition both within and between facility water systems and zebrafish gut samples. Further, there was evidence that variation in the water microbiome was a source of variation in zebrafish gut microbiomes. Finally, as differences in facility management and technical specifications can make directly linking microbiome variation in the water to variation in fish difficult, I attempted to isolate the effects by experimentally manipulating the water microbiome in a laboratory study using germ-free larval fish. My results indicate that microbial inputs from live feed overwhelmed any potential influence of water microbiome variation in early-life microbiome assembly.

Overall, this dissertation provides a comprehensive look at the drivers of environmental microbiome variation and how these may mediate aspects of host-associated microbiota. My results have implications for fish microbiome research and suggest that research conducted with zebrafish sourced from a single facility may be heavily influenced by facility-specific effects.

CURRICULUM VITAE

NAME OF AUTHOR: Kayla C. Evens

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, OR, USA
New College of Florida, Sarasota, FL, USA

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2024, University of Oregon
Bachelor of Arts, Environmental Science, 2017, New College of Florida

AREAS OF SPECIAL INTEREST:

Host-microbiome dynamics
Community ecology
Aquaculture systems

PROFESSIONAL EXPERIENCE:

Teaching Assistant (Ecology, Biology, Ocean Science, Animal Behavior), University of Oregon, 2018-2020
GIS Research Assistant, U.S. Geological Survey, 2017-2018
GIS Research Assistant, New College of Florida, 2016-2017
Research Assistant, U.S. Antarctic Program, 2015-2016

GRANTS, AWARDS, AND HONORS:

NSF Non-Academic Research Internships for Graduate Students, University of Oregon, 2023
Department of Biology Best Poster Award, University of Oregon, 2022
NSF Graduate Research Fellowship Program, National Science Foundation, University of Oregon, 2020
Gulf Coast Community Foundation scholarship, New College of Florida, 2018
Sarasota-Manatee CEO Forum Student scholarship, New College of Florida, 2017-2018
Deborah Marsha Herbstman Endowed In-State scholarship, New College of Florida, 2016-2017
New College of Florida Presidential scholarship, New College of Florida, 2016-2017
U.S. Department of Defense Antarctica Service Medal, New College of Florida, 2016

NSF Research Experiences for Undergraduates award, Rutgers University, New College of Florida, 2015
Bright Futures Florida Academic scholarship, New College of Florida, 2013-2017

PUBLICATIONS:

Mackin, H., Shek, K., Thorton, T., **Evens, K.C.**, … & Roy, B.A. (2021) The "black box" of plant demography: How do seed type and climate affect grass seed germination and fungal communities? New Phytologist 6:2319-2332.

ACKNOWLEDGMENTS

Funding for this work was provided in part by the National Science Foundation Graduate Research Fellowship Program (grant no. DGE 2236419), the National Institutes of Health (grant no. P01GM125576), and the Research Council of Norway (project no. 322546).

The data in Chapter 2 and Chapter 3 were generated with the permission of Dr. Emre Yaksi, Dr. Fredrik Jutfelt, Dr. William Cresko, Zoltán Varga, and Tim Mason. I am indebted to you all for granting me access to your aquaculture facilities and the free reign to collect samples. Extra special thanks to David Lains, Mark Currey, Eline Rypdal, and Andreas Moe Nygård for their hands-on assistance with sampling.

I would like to acknowledge and give my most heartfelt appreciation to my advisor, Dr. Brendan Bohannan, who made this work possible. You have provided nothing but unwavering support and encouragement for my academic and personal growth over the past five years. Thank you for all your patience, compassion, and pieces of chocolate.

I would also like to thank all the members of the Bohannan Lab I've had the pleasure of working with- especially Caitlin Smith, Andrew Morris, Killian Campbell, Diana Christie, Karen Adair, Hannah Tavalire, Bob Week, Beth Miller, and Becca Maher- for their guidance and comedic relief. Because of you all, I fear every group I work with in the future will pale in comparison to the generous and collaborative spirit the Bohannan Lab has embodied. Special thanks to Karen Adair for assisting me through literally every part of my research and keeping the lab afloat.

I extend my appreciation to all those in my academic sphere who have been a part of this journey. Thank you to the IPAMA group for providing great insight into the world of fish and especially to Dr. Ingrid Bakke for bringing us all together. Thank you to Alex Fiedler and

Madeleine Gundersen for providing a warm welcome to Norway and for the mutual support as we raced to the finish line of our degrees. And thank you to the members of IEE, who have made it a pleasure to come in to work every day.

Finally, completing this PhD would have seemed impossible without the support of a few special people. First, thank you to my dearest friends, Maxine Wren and Ash Frieswyk. You two have provided more laughs, shoulders to cry on, and board game distractions than anyone could possibly hope for in a lifetime. To my husband, Andrew, I almost don't have the words. Thank you for being my biggest advocate and best editor. I cannot possibly thank you enough. And lastly, the most significant acknowledgment is for my family. Your support has been lifelong, and for that, I am always grateful.

For TJ and Gary,

who would have been overjoyed to welcome me into the ranks of certified, card-carrying nerds.

I love you and miss you dearly.

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CHAPTER 1: LITERATURE REVIEW AND INTRODUCTION

A central goal of community ecology is to understand the drivers of community assembly to accurately predict the impact of environmental change on biodiversity. However, the processes that drive community assembly remain major areas for investigation in community ecology (Nemergut et al., 2013). This is particularly true regarding host microbiomes- communities of microorganisms living in and on animals and plants- since these communities have only recently been comprehensively sampled yet play a vital role in host function (Christian et al., 2015). Ecological theory is useful for understanding the complexities of the microbiome as we can conceptualize the host as an ecosystem or habitat patch in (and on) which a community of microorganisms inhabit (Costello et al., 2012; Miller et al., 2018; Morar & Bohannan, 2019).

The application of community assembly theory to host-microbiome research is essential. Without knowledge of how microbiomes establish or persist, we cannot manipulate the microbiome to pursue questions of process and function relevant to areas like host health. The research covered in this dissertation expands our current knowledge of drivers of microbiome community assembly in an important model host (the zebrafish, *Danio rerio*) commonly used to address questions in developmental biology, physiology, toxicology, and many other areas of life science research.

1.1 Processes in community assembly theory

Community assembly theory has been adapted into a conceptual framework used to understand the formation of communities, and the drivers of their variation in space and time. This framework asserts that the species composition of a community can be conceptualized as

the passage (or impediment) of species from a "regional species pool" through a series of hierarchical filters before successfully forming a local community (Figure 1) (Keddy, 1992; Pearson et al., 2018). These "assembly filters" or "processes" are a metaphor for the often concurrent abiotic and biotic factors in an environment that select for or against specific species (Kraft et al., 2015).

The study of community assembly has historically maintained two viewpoints: the niche view (in which local processes are paramount) and the colonization view (that emphasizes regional processes). The niche view has been traditionally pre-eminent in theoretical and empirical studies of community assembly and highlights strong habitat filters that restrict recruitment of species to those that can maintain viable populations at the local scale (Chesson, 2000; Leibold, 1995). However, the colonization view stresses that assembly is a result of a random series of colonization and demographic stochasticity events and has more recently challenged the traditional approach of solely looking at the role of local processes (Adler et al., 2007; Hubbell, 2001).

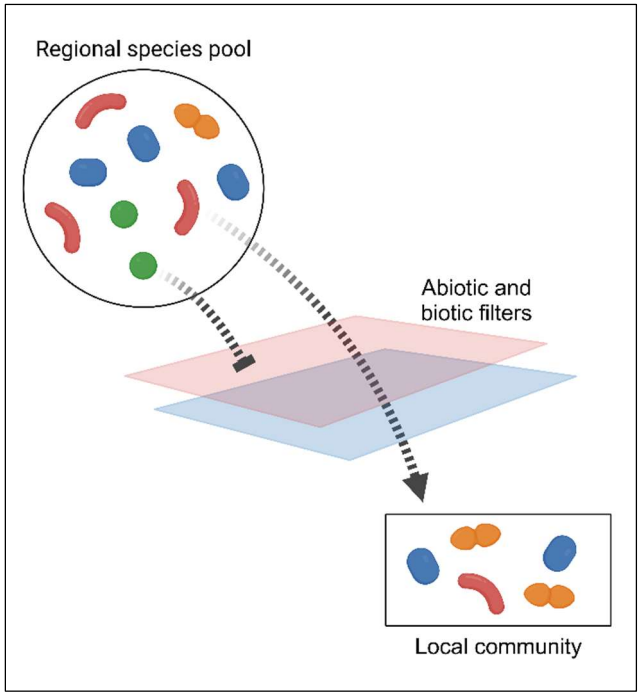

Figure 1: A conceptual model of the community assembly framework. Species represented in a local community are a filtered selection of species dispersed from the regional species pool. *Created with Biorender.com.*

As our understanding of community assembly has expanded, it has become evident that communities more likely exist on a continuum between the extremes of the niche and

colonization viewpoints. One important area of investigation is the influence of the regional species pool, hereby referred to as the 'species pool'. The species pool is defined as the set of species that could potentially colonize and persist in a local community and is a persistent and central concept in community assembly theory. The structure of a local community is thus directly influenced by factors such as the diversity, abundance, and richness of its respective species pool (Cornell & Harrison, 2014).

In some cases, the influence of the species pool can overwhelm the effects of local assembly factors. For example, in a study of the global distribution of birds, mammals, and amphibians, regional richness was almost always positively correlated with local richness, independent of environmental conditions (Belmaker & Jetz, 2012). Compared to local community dynamics, there have been relatively few studies that explicitly investigate regional dynamics, how a species pool itself is formed and maintained, or how variation in the species pool influences the development of local communities despite their importance to community assembly.

1.2 Origins and challenges of species pool theory

Regional processes were included in ecological analyses of local community assembly as early as 1909 (Taylor et al., 1990). Ecologists have since begun to integrate species dispersal (a regional process) with the traditional view of competition and environmental filtering (local processes) when determining drivers of local community composition, primarily through empirical studies of plants (Clements, 1916; Gleason, 1927; Spalding, 1909). This integration has allowed for the influence of large-scale processes to be included in our understanding of local community assembly. For example, the "species pool hypothesis" posits that species

richness is relative to the commonness of a habitat because greater habitat size and frequency should allow for more speciation opportunities (Harper, 1977).

There are several integral theories that have informed the species pool concept. Island biogeography theory challenged the emphasis on local processes of community assembly. Put simply, island biogeography theory proposes that island species diversity depends on island size and distance to a mainland species pool (MacArthur & Wilson, 1977). Neutral theory, a descendant of island biogeography theory, postulates that local communities are a stochastic sample from a species pool where dispersal probability is related to the distance and abundance of a species in the species pool. Though directly utilizing the species pool concept, neutral theory emphasizes community assembly as the absence of niche factors, not explicitly an in-depth understanding of how regional processes drive interactions at the local scale (Hubbell, 2001).

Lastly, metacommunity theory, which postulates that the biotic world can be envisioned as a set of local communities linked through dispersal, was developed in tandem with the species pool concept. Metacommunity theory envelops several different concepts, including neutral theory, and similarly to the species pool hypothesis, emphasizes the interaction between local and regional scale processes (Leibold et al., 2004). However, metacommunity theory assumes that local and regional processes occur on the same timescale, which has been difficult to observe in the field (Cornell & Harrison, 2014). Combined with the empirical explorations in community ecology at the time, these theoretical origins have seeded the continuum approach to community assembly we utilize today.

Despite the potential importance of the species pool in determining local community diversity, the drivers of species pool diversity and composition are not well-understood. There is

a longstanding assumption that the species pool is relatively static, and in the rare instance that change does occur, it is predominately structured by historic-evolutionary processes such as speciation (Ricklefs, 2004; Terborgh & Faaborg, 1980). This assumption is based, in part, on the notion that the species pool exists at much larger scales of space and time than local communities. However, though the species pool and the local community are differentiated by scale, with the regional species pool encompassing the local community, definitions of "local" and "regional" vary dramatically in the literature.

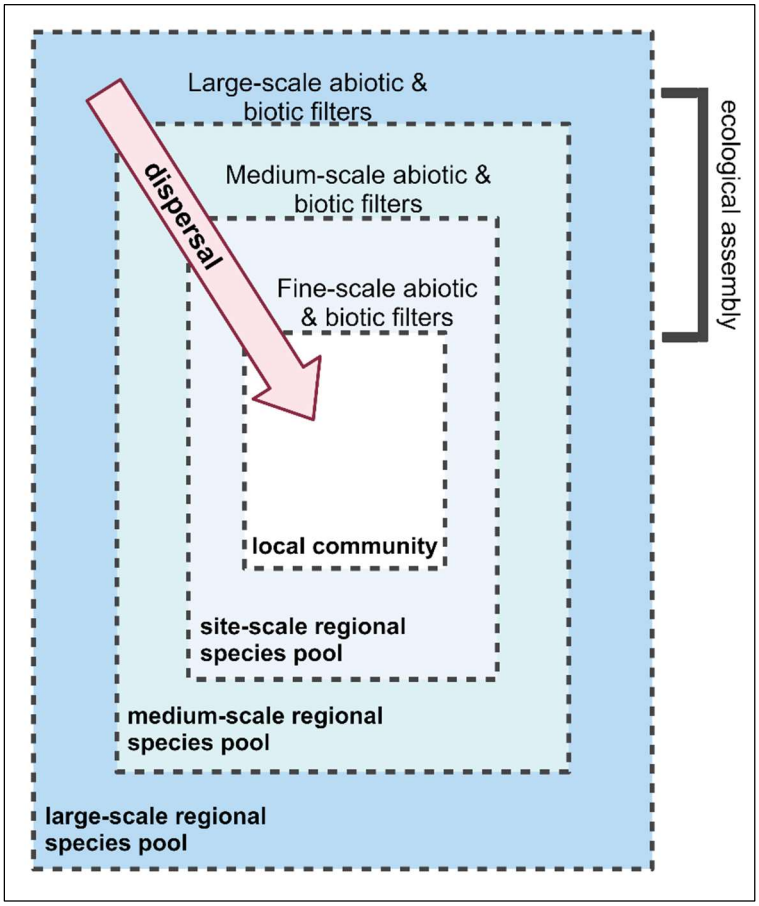

Figure 2: Alternative conceptual model of the community assembly framework that incorporates multi-scale regional species pools and processes. *Created with Biorender.com.*

Under certain defined scales of a regional species pool, the species pool is under the same influence of assembly processes commonly attributed to local communities- such as selection and drift. In other words, the relative influence of local and regional processes in community assembly is context-dependent on the size and scope of the defined species pool (Russell et al., 2006; Zobel, 1997). As an example, Figure 2 presents an attempt to conceptualize this multiscale contextualization of the regional species pool by integrating it into an alternative conceptual representation of the classic community assembly framework. Other ecologists have

made similar arguments, inferring that discrete spatial scales of species pools and local communities are arbitrary (Schemske et al., 2009; Vellend, 2010). It is increasingly recognized that the composition of the species pool is dynamic and can be influenced by interactions that occur within and between local communities. However, relatively little attention has been focused on such feedback (Mittelbach & Schemske, 2015). What is crucially lacking are studies focused on the drivers of species pool attributes at multiple scales and the subsequent impact of species pool variation on local community composition.

1.3 A community assembly framework for the host-microbiome

Nearly every macroorganism maintains assemblages of associated symbiotic, commensal, and pathogenic microorganisms, jointly referred to as the "host-microbiome" (Hammer et al., 2019; Parfrey et al., 2018; Robinson et al., 2018). Host-microbiomes have recently been proposed as a potentially ideal system to examine the drivers of community assembly (Miller et al., 2018). They can be thought of as local communities, whose composition and dynamics are at least partially determined by dispersal of microbes from the environment (the regional species pool) and partially determined by host selection (Figure 3).

Host-microbiomes are well-suited for studies of the interplay between regional and local processes in community assembly because host microbiomes are distinctly bounded (by their

individual hosts) and clearly nested within a larger species pool (the surrounding environmental microbiota) (Christian et al., 2015). This eliminates some of the issues associated with determining where a local community ends and a species pool begins, as discussed earlier. Within certain study systems, microbes in a host's environment can even be experimentally manipulated to observe direct and indirect effects on the microbiome. Many animal models have been extensively developed for use in host-microbiome studies, such as *Drosophila*, zebrafish, and mice, providing an established and accessible suite of tools and protocols for research (Kostic et al., 2013).

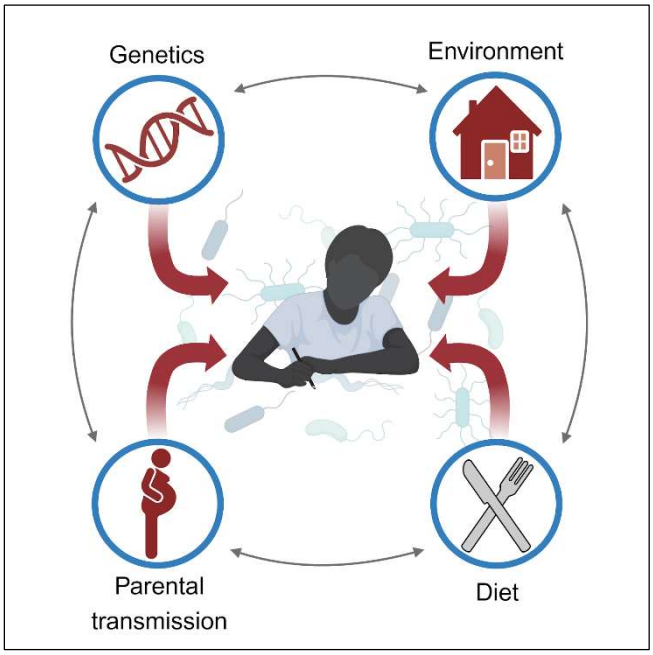

Figure 3: Host-microbiome assembly is a dynamic process that can be influenced by many factors. Several known contributors to the host-microbiome include host genetics, parental transmission during prenatal development and birth, environmental exposure, diet, and the interactions between these. *Created with BioRender.com.*

For decades, researchers have expanded our understanding of the host-microbiome and elucidated its tremendous role in aspects of host health and function. The microbiome has been linked to an extensive suite of processes such as metabolism (Cox et al., 2022), neurological function (Cryan et al., 2020), nutrition (Hacquard et al., 2015), and immunology (Ottman et al., 2012; Zheng et al., 2020). Despite its direct ties to host health and function, the microbiome can exhibit significant variability within and across individuals. Microbiome variation has been correlated with several major indicators, such as host phylogeny, geographical location, diet, and even cultural and ethnic identity, though this remains far from an exhaustive list (Costello et al.,

2009; Gupta et al., 2017; Moraitou et al., 2022; Rojas et al., 2021; Singh et al., 2017). Understanding how this variation in the microbiome occurs remains a major area of inquiry in host-microbiome research.

A great deal of research has focused on exploring host-microbiome interactions as potential drivers of microbiome variation, such as host genetics selecting for specific microbial members within the host (Bonder et al., 2016) or microbiota directly altering host phenotypic traits (Rolig et al., 2017). However, there is growing evidence that microbial dispersal from the environment may play an even greater role in microbiome assembly, sometimes even overwhelming the effects of host genetics (Belmaker & Jetz, 2012; Rothschild et al., 2018; Tavalire et al., 2021), consistent with many studies of non-host systems that have observed substantial influence of the species pool on local community composition (Belmaker, 2009; Lessard et al., 2012; White & Hurlbert, 2010) . Yet, elucidating the role of the environmental 'species pool' of microbiota in host-microbiome assembly remains an integral but understudied area.

1.4 Life history and use as model organisms: zebrafish and threespine stickleback

Zebrafish (*Danio rerio*) and threespine stickleback *(Gasterosteus aculeatus*) have long served as tractable model organisms for research in disciplines such as evolution and development, vertebrate biology, toxicology, and biomedicine (Figure 4) (Cresko et al., 2007; Dooley & Zon, 2000; Eisen, 2020; Ostlund-Nilsson et al., 2006; Tanguay, 2018). However, these teleost fish have only recently been established as a model to study host-microbiota interactions (Roeselers et al., 2011).

Zebrafish and stickleback have distinct advantages as model organisms in host-microbiome research over the more commonly used murine models, which currently account for approximately 95% of all laboratory animals (Hickman et al., 2017). Zebrafish and stickleback are smaller, can be reared in larger numbers, reproduce more frequently, and develop more quickly than mice and rats. Therefore, we can perform frequent, well-powered, and highly controlled experiments for a more robust sampling of the microbiome (Lescak & Milligan-Myhre, 2017).

Figure 4: Adult zebrafish and threespine stickleback specimens. Adult zebrafish average 2-3 cm in length. Threespine stickleback measure 3-8 cm in length at adulthood but average around 5 cm. *Zebrafish image: Mirko Rosenau, Adobe Stock. Stickleback image: adapted from Jacobs, R. P., O'Donnell, E. B., and Connecticut DEEP. (2009). A Pictorial Guide to Freshwater Fishes of Connecticut. Hartford, CT.*

The zebrafish, a hardy, freshwater fish native to south Asia, has been gaining popularity as an animal model in research communities over the last half-century. Though well-known in the pet trade, Dr. George Streisinger was the first to use zebrafish in a laboratory setting in the late 1960s while at the University of Oregon. Many key aspects of zebrafish physiology make them an ideal animal model. The zebrafish genome has been extensively sequenced, and approximately 70% of zebrafish genes have at least one orthologous human gene (Howe et al., 2013). This makes them attractive candidates for the study of vertebrate genetics and models for human genetic disease. Additionally, they can reach sexual maturity as early as 2 months post-fertilization and spawn year-round, producing upwards of 400 eggs per clutch (Hickman et al., 2017). Zebrafish embryos are translucent and develop over the course of 24-48 hours, which

aided in their rise in popularity for studying vertebrate embryonic development in the decades following their introduction as an animal model (Tanguay, 2018).

Research by Dr. Nikolaas Tinbergen first introduced stickleback as a model in the late 1930s (Tinbergen, 1939). Threespine stickleback are small, bony fish named for the line of protective spines along their dorsal side. They can be found in both marine and freshwater environments in the Northern Hemisphere. Over evolutionary time, usually following periods of glacial retreat, populations of marine stickleback have colonized freshwater environments, resulting in phenotypically divergent forms that coexist in nature (McKinnon & Rundle, 2002). Because of this, threespine stickleback have gained popularity as animal models in the fields of evolution, genetics, and developmental biology. In nature, stickleback follow a paternal care system. Males will build nests and care for eggs until hatching. In laboratory settings, researchers utilize *in vitro* fertilization to cross populations that would otherwise be reproductively limited (Heng et al., 2016).

1.5 State of research in zebrafish and threespine stickleback-microbiome assembly

Over the past decade, zebrafish and threespine stickleback have been increasingly used in studies of host-microbiomes (Eisen et al., 2020; Reid et al., 2021). Zebrafish and stickleback embryos can be derived germ-free (i.e. axenic) and reared free from cellular microorganisms through early development (Melancon et al., 2017; Milligan-Myhre et al., 2016). This is particularly advantageous in host-microbiome studies as we can utilize germ-free fish to monitor microbiome assembly over time using various consortia of introduced microbes. Several recent studies have helped identify significant factors that influence microbiome assembly in zebrafish and stickleback including, but not limited to, developmental age, host-selection, and dispersal

(Rawls et al., 2006; Roeselers et al., 2011; Smith et al., 2015; Stephens et al., 2015; Steury et al., 2019).

In a study of gut microbial composition across time, researchers surveyed the intestinal bacteria of sibling zebrafish reared under identical environmental conditions at key developmental time points. They found that microbial composition in gut samples from approximately 135 fish significantly clustered by development stage. Interindividual variation remained high throughout the study period, but as fish aged, their microbiota became more differentiated from the microbiota in their environment, suggesting a stronger influence of host selection (Stephens et al., 2015). Another study utilized the Stephens et al. (2015) dataset to discern the contribution of neutral processes (chance colonization and dispersal from the environment) to gut microbiome assembly. Further supporting their initial findings, neutral models indicated that the relative importance of neutral processes to community assembly decreased over host development (Burns et al., 2015). In other words, developmental age significantly impacted microbiome composition.

A 2006 study further identified that, even though it increases over time, host-selection remains a strong governing force on microbiome assembly at a young age. Researchers inoculated germ-free zebrafish with gut microbiota collected from conventionalized mice and sampled resulting gut microbiota in the zebrafish three and seven days later. Though the lineages in the resulting gut microbiota resembled the original mouse-gut, the relative abundances of the lineages changed to match those reflective of a normal zebrafish gut (Rawls et al., 2006). Similarly, when researchers compared gut microbiota across domesticated adult zebrafish housed in different aquaculture facilities and to wild-caught zebrafish, they found evidence for shared

microbiota across all individuals. They concluded that strong host-selection factors in the gut maintain a consortium of shared microbiota in the zebrafish gut regardless of environment (Roeselers et al., 2011).

Similar findings were reported in several threespine stickleback studies. One paper sampled six genomically and phenotypically diverse stickleback populations from coastal and freshwater environments in northwestern Oregon. Using population genetic and gut microbial data, they found that gut microbial diversity was better explained by population genetic divergence than environment and geography. This indicates that host genetics imposes a strong selection effect on the microbiota inhabiting the host, though microbial diversity was also partitioned by environment and geography (Steury et al., 2019). Another study examined similar data from ten stickleback populations (two marine, eight freshwater) in and around Vancouver Island, British Columbia. Researchers also collected samples of water and common prey and quantified microbial DNA from these sources. Resembling results found in the previous study, stickleback gut microbial composition and diversity were strongly associated with population genetic divergence. Food microbiota, water body geomorphology, and habitat type were also associated with population-level variation to a lesser extent (Smith et al., 2015).

The studies discussed previously are united by their focus on host-level interactions with the microbiome. Yet, recent work has highlighted the importance of measuring dispersal between the host and its external environment in studies of host-microbiome assembly- a mode of assembly commonly overlooked. Within host-microbiome research, this process of dispersal is sometimes referred to as ‘horizontal transmission’. We are in the relatively early stages of looking at the host-microbiome at a broad ecological scale, but research in humans (Sharma et

al., 2019; Song et al., 2013; Tavalire et al., 2021), other vertebrates (Bik et al., 2016; Bornbusch et al., 2022; McCafferty et al., 2013), and even invertebrates (Eckert et al., 2021; Unzuenta-Martinez et al., 2022) have provided strong evidence that dispersal from the environment is a major contributor to microbiome composition.

Current research in the role of environmental dispersal in zebrafish microbiome assembly, though limited, has suggested that fish follow similar patterns to those found in non-fish systems. One study raised zebrafish with fully functional immune systems and immunodeficient zebrafish (a nearly isogenic mutant *myd88*- line) in different housing conditions designed to either limit or allow for dispersal between hosts via the environment. Wild-type zebrafish and *my88*- zebrafish were either reared in solitude (one fish per flask), in single genotype groups, or in mixed-genotype groups (ten fish per flask). When cohoused, interhost dispersal overwhelmed host factors, eliminating any difference seen in the intestinal microbiota of mixed-genotype groups (Burns et al., 2017). In another study, researchers passaged a zebrafish bacterial isolate, *Aeromonas veronii*, through populations of larval zebrafish, each time using *Aeromonas* isolated from the gut of the previous population to inoculate the next. Over time, they observed increased adaptation of *Aeromonas* to dispersal into and colonization of the host (Robinson et al., 2018). Without the consideration of the processes occurring outside of the host, we would be missing critical biological insights into microbiome assembly.

1.6 Research aquaculture facilities: broadening the scale of host-microbiome assembly

I have established that zebrafish and threespine stickleback are ideal models for understanding the processes of host-microbiome assembly and broader themes regarding general community assembly. However, one criticism of studies that have attempted to quantify the

influence of environmental microbiota on microbiome assembly in animal models is that these derived laboratory experiments can largely differ from "real world" microbial communities and scales of interactions between host and environment (Greyson-Gaito et al., 2020; Leray et al., 2021). On the other hand, human and wild animal studies can make it difficult to identify drivers of assembly due to the overwhelming complexity inherent in studies of organisms in the wild (Glowacki et al., 2021).

Examining host-microbiome assembly in research aquaculture facilities- where fish used for experimental research are bred and housed- provides a unique opportunity to work in a system that balances these extremes on the spectrum of experimental design. Research aquaculture facilities- hereby referred to as "facilities"- are heavily monitored and maintained due to strict animal care and use guidelines and to ensure healthy stocks of fish for experimentation (National Research Council, 2011). These standards reduce some of the variation and complexity across sites (i.e. facilities) that would be overwhelming in more "natural" systems. However, local differences in the details of facility management (e.g. frequency of tank cleaning, specific food used, etc.) result in interesting and potentially important variation across facilities. In addition, although research facilities are not "wild" (i.e. unmanaged) they represent the actual environment in which research animals are raised (often over many generations), making information about inter- and intra-facility microbiome variation directly applicable to animal husbandry.

Aquaculture facilities also provide an ideal system in which to study community assembly across varying spatial scales as tanks in each facility are organized in a way that allows for sampling at nested scales- from an individual tank to a single closed recirculating water

system to entire facilities. Since these facilities predominantly utilize closed loop water systems, the species pool is theoretically shared among all connected tanks within a given facility.

Finally, the importance of research in aquaculture facilities extends beyond their relevance as a powerful host-microbiome model system. Zebrafish and stickleback are used as laboratory animal models, and understanding the dynamics of how their microbiome forms and functions is essential to maintaining a reproducible model for experimental manipulation. Furthermore, aquaculture facilities used to rear fish for research are very similar to those used to rear fish for commercial purposes. Aquaculture accounts for nearly 50% of the world's fish supply and is one of the fastest growing food sectors (FAO, 2019). As such, results from research can help promote fish health and welfare by informing the microbial management of aquaculture facilities in general.

In summary, aquaculture facilities can be used as an experimental system to investigate general questions about community assembly such as the role of the species pool in driving local community variation, the frequency and nature of feedback between local communities and the species pool, and the importance of variation in species pools across space and time in driving local community variation.

1.7 Host-microbiome research in aquaculture

Studies comparing aquaculture microbiota have supported the connection between the environment and host microbiome, as many have highlighted that facility water varies in its microbial composition and those compositional differences can correlate with variation in fish gut microbiota and vice versa. In 2019, researchers recorded the change in both water and

zebrafish gut microbiota during a water system switch at Wayne State University in Detroit, Michigan. As the water microbial community changed during the transition, so did the zebrafish gut microbiome (Breen et al., 2019). Vestrum et al. (2018) highlighted compositional differences in the microbiome of Atlantic cod (*Gadus morhua)* larvae reared in water sourced from facilities utilizing different water filtration systems. Both water and fish microbial communities differed significantly across facility systems and clustered in PCoA analyses according to facility source.

In the previous papers, researchers directly measured both microbiota in the host and in the facility they were housed in. However, there are many more studies that have not directly measured the microbiota in the environment, and rather, described the influence of facility-level "factors" on the host microbiome. These facility "factors", which can refer to things such as the selected diet, the temperature of the enclosure, or even the number of people within the facility, in many ways can influence the environmental microbiome as much as the host microbiome. As such, we can assume that, to some degree, these facility "factors" may be influencing the host microbiome indirectly via changes to the environmental microbiome.

Some examples of this phenomena are represented in the Roeselers et al. (2011) paper, which was discussed earlier. Zebrafish from lab facilities across the U.S. had unique gut microbiota that correlated with the seeding history of their respective facility. Seeding refers to when microbiota from a facility (or commercial source) is used to prime a new aquaculture facility for use. Some forms of this may include adding fish and/or biofilter media from an established facility to the tanks and/or biofilter attached to the water system of another facility. In another study of the gut microbiota of reared Atlantic cod larvae demonstrated large variation among a gradient of facility management types as well as substantial inter-individual variation

(Fjellheim, 2011). Lastly, in mice, a recent study revealed that the microbiome of wild-type and disease model mice was heavily influenced by which facility they were sourced from, independent of cage, sex, or sequencing-related influence. Researchers replicated microbiome experiments in endothelin receptor type B knock-out mice (Ednr +/-) and a common in-bred mouse strain (wild-type C57BL/6J). Facility-specific differences were major drivers of not only gut and fecal microbiome composition at all taxonomic levels but also genotype-based differences in the microbial composition and diversity of the wild-type versus knock-out mouse models (Parker et al., 2018).

1.8 Aquaculture facility design

In modern zebrafish aquaculture, facilities are land-based and can vary substantially in the technical aspects of their design. However, all but one of the facilities studied in this dissertation use recirculating aquaculture systems (RAS), which were developed for use in intensive fish farming in areas with low water availability. These systems allow up to 99% of the wastewater in the system to be recycled by recirculating through a complex network of water treatments (Badiola et al., 2012; Figure 5). RAS water treatment systems include at least a mechanical and a biological filter. Mechanical filters, such as sand filters and settling tanks, remove solid waste and other particulates from the water column. Biological filters serve to remove dissolved waste from the water system and utilize beneficial bacteria that convert ammonia and nitrite into less harmful forms. Modern 'biofilters' are often some form of floating plastic media that serve as a surface for formation of beneficial biofilms. Additionally, RAS systems often include some form of a disinfection stage and ways to manage water oxygenation, quality control, and circulation. Two common water disinfection techniques are passage through

a UV or ozone filter. More intensive systems often include automated water quality technologies, such as pH and salinity sensors, for continued monitoring of the abiotic and biotic environment within the system (Xiao et al., 2018).

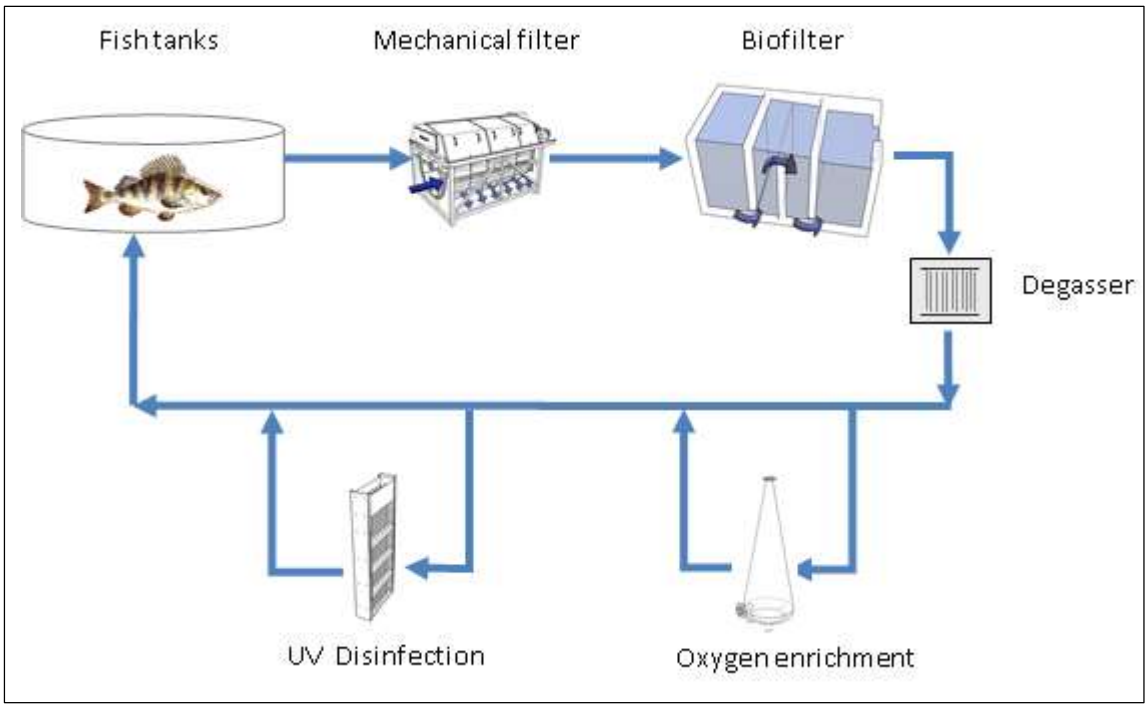

Figure 5: The basic water treatment loop for a recirculating aquaculture system includes mechanical filtering, biological filtering, and aeration. Additional steps, including UV-disinfection and water oxygenation, can be used. *Diagram from Bregnballe, J. 2022. A guide to recirculation aquaculture– An introduction to the new environmentally friendly and highly productive closed fish farming systems. Rome. FAO and Eurofish International Organisation. https://doi.org/10.4060/cc2390en.*

The other facility represented in this dissertation uses a flow-through microbial maturation system (MMS). A flow-through aquaculture system relies on water exchange to maintain a suitable rearing environment for fish. Clean water, either from freshwater or saltwater sources, is diverted and pumped into rearing tanks. These systems rely on the flow of water

created from input to drainage to remove solid and dissolved waste (Fornshell & Hinshaw, 2008).

Though flow-through systems are one of the most widely used aquaculture systems across the world, due, in part, to their lower operation and labor costs compared to RAS systems, fish health and mortality rates are notably worse (Eurofish, 2021). For example, in studies of cod, turbot, and Atlantic salmon, fish survival and overall health in RAS systems show remarkable improvement over flow-through systems, attributed partly to the maintenance of mature microbial communities in the water system (Attramadal et al., 2014; Kolarevic et al., 2014; Salvesen et al., 1999).

As such, MMS are used as an intermediary between flow-through and RAS systems. MMS uses a flow-through system paired with a microbial pre-treatment, and thus, maintains the relative operational simplicity and low cost while utilizing the beneficial microbial management component of RAS systems. Input water, prior to being distributed to tanks, is microbially "matured" by passage through a biofilter, often seeded with ammonium and nitrite oxidizers, and wastewater is removed from the system.

1.9 Aquaculture study sites

The facilities used in my research included five aquaculture facilities spanning two geographical regions. I sampled from two zebrafish and one stickleback facility on the University of Oregon campus in Eugene, Oregon, USA, and two zebrafish facilities on the Norges teknisk-naturvitenskapelige universitet, or Norwegian University of Science and Technology (NTNU), Gløshaugen and Øya campuses in Trondheim, Norway.

The facilities represented in this dissertation are particularly useful for answering questions regarding the interaction between the fish and water microbiome for several reasons. First, these facilities are clustered in geographic space, thus providing an excellent system to test questions regarding species pool variation across multiple scales. For example, I can investigate the influence of variation in the water microbiome within a facility, between facilities in close proximity to one another, and between facilities on separate continents to develop a more robust understanding of the interaction of fish and water microbiomes across space. Second, the facilities represent a variety of modern approaches to rearing fish for research use. The technical specifications of each facility, such as the life support systems used, are similar, but the size of the facility, number of fish, range of genotypes, and approach to care vary. Due to these aspects, the results of this dissertation research can be considered broadly applicable to other aquaculture research facilities despite potential variation in geographical distance or management. Finally, including a facility that houses a different species of fish (stickleback, rather than zebrafish) will allow me to explore the possible role of host species in the assembly of host-microbiomes.

All the facilities on the University of Oregon campus use RAS systems and include the Aquatic Animal Care Services zebrafish facility (referred to as Ore1 throughout this dissertation), the Zebrafish International Resource Center (Ore2), and the Cresko Stickleback Lab (OreSB).

The Ore1 facility was established in 2012 and received its first fish from commercial suppliers. The facility covers 1,000 square meters with an average capacity of 50,000 fish. There are around 2,900 different genotypic strains represented in the facility. All fish are kept in 3.5-liter acrylic tanks. The facility life support system utilizes a RAS system containing automated

mechanical and biological filters in addition to ultra-violet disinfection. The facility has two separate, closed-loop RAS systems with a UV-treatment step supplying the main housing. There is an additional quarantine area with a separate flow-through system (T. Mason, personal communication, September 20, 2023).

The Ore2 facility was built in 1999 and the first fish were introduced in 2001. Ore2 is a 900 sq. m. facility with a maximum capacity of 150,000 fish and maintains live populations of 45 genotypic lines on average. This facility serves as a worldwide resource for zebrafish, maintaining approximately 44,000 different genotypic lines in cryogenic storage. Ore2 maintains the same layout of life support systems as the Ore1 facility and fish are kept in a mix of both 3.5-liter acrylic and 75-liter glass tanks (D. Lains, personal communication, January 20, 2022). This facility is approximately 300 meters from both Ore1 and OreSB.

The OreSB facility represents the only stickleback facility studied in this dissertation. It was started in 2004 as an experimental facility for the lab of Dr. William Cresko and is located approximately 150 meters from Ore1 and 300 meters from Ore2. OreSB is approximately 185 square meters in total and is divided into two separate rooms that mimic aspects of the sticklebacks' natural environment throughout the year. Stickleback spawn in the spring and summer, so the first of these rooms, the "Summer Room", houses stickleback in their larval (hatching to ~3 months) and reproductive adult stages (12+ months). Larval fish are housed in 3.5-liter acrylic tanks while larger adult fish are housed in 75-liter glass tanks. This room is kept at 20 ℃ and follows a day-night cycle of 14 light/ 19 dark hours to mimic the longer days in the spring and summer. The second room, the "Winter Room", is on a 10 light/ 14 dark hour light cycle and is also kept at 20 ℃. This room primarily houses juvenile fish between the larval and

reproductive adult stages (3-12 months) in 75-liter tanks. Each room has its own closed-loop RAS system (M. Currey, personal communication, February 12, 2024).

The Norwegian zebrafish facilities are located on the Norwegian University of Science and Technology Trondheim campuses in Trondheim, Norway. One facility (referred to as Nor2A and Nor2B), is maintained by the Jutfelt research group and is located on the Gløshaugen campus. Originally built as a general animal laboratory in the 1990s, Dr. Fredrik Jutfelt repurposed the lab to house fish from 2015 through 2020. Nor2 is made up of five different rooms (around 10-30 square meters each) and maintains both populations of zebrafish and guppies (*Poecilia reticulata*). Because the facility consists of multiple small rooms with separate life support systems within each, I reference Nor2 as separate facilities (Nor2A and Nor2B) throughout this dissertation although they are technically maintained under the same administrative body. I conducted my research in two zebrafish-only rooms within the facility, each approximately 10 square-meters. The first room (A) contains approximately 50, 3.5-liter acrylic tanks utilizing a standalone RAS system with UV-treatment. The second room (B) houses approximately 14, 75-liter glass tanks with a flow-through filtration system using a maturation tank without UV-treatment. An aspect of this facility that makes them particularly unique from the others is that they maintain no common laboratory zebrafish genotypic strains. The ones housed in the facility today are direct descendants (~6 to 10 generations) of wild zebrafish caught in India in 2016 (F. Jutfelt, personal communication, January 13, 2024).

Lastly, the second Norwegian facility, the Yaksi Lab (Nor1), is located on the Øya campus, approximately 800 meters from Nor2A and Nor2B, and maintains approximately 10,000 zebrafish in a 70 square-meter facility. Built in 2014 for the lab of Dr. Emre Yaksi to study brain

function in health and diseases, the Nor1 facility houses over 100 unique genotypic strains. Fish in this facility are housed in 3.5-liter acrylic tanks attached to a RAS system with UV-treatment. There is an additional quarantine area that uses a flow-through system with approximately 50 tanks (E. Yaksi, personal communication, October 10, 2023).

1.10 Dissertation overview and objectives

In this dissertation, I investigate the dynamic interaction between the environmental microbiome and fish microbiome in several zebrafish and three-spine stickleback research aquaculture facilities in the United States and Norway.

In Chapter 2, I examine broad scale spatial and temporal variation in the water microbiome of three aquaculture facilities in Oregon, United States, and measure patterns of variation across facilities to identify the greatest magnitude of variation in the regional pool of microbiota. Using the results of this chapter, I designed an expanded, intensive sampling of water and fish microbiomes across four zebrafish facilities in Oregon and Norway (described in Chapter 3).

In Chapter 3, I characterize variation in the water microbiome at a finer spatial scale and measure the consequences on variation in the zebrafish gut microbiome across four aquaculture facilities in Oregon and Norway. Results from this survey were used to design an experimental exploration of the impact of water microbiome variation on fish gut microbiome variation (described in Chapter 4). Finally, in Chapter 4, I attempted to directly measure the influence of the water microbiome on the zebrafish gut microbiome by experimentally manipulating the environmental microbiome in a controlled laboratory environment. By removing the effects of facility-specific differences, such as husbandry and management, I assess the relative

contributions of the environmental microbiome on the fish gut microbiome during initial assembly and after exposure to a novel environmental microbiome.

CHAPTER 2: WATER MICROBIOMES IN ZEBRAFISH AND STICKLEBACK AQUACULTURE FACILITIES SHARE GENERAL PATTERNS OF SPATIAL AND TEMPORAL VARIATION

2.1 Introduction

Community assembly theory assumes the existence of a species pool from which species that can disperse through a series of biotic and abiotic filters may colonize the local community. However, communities under similar conditions in different regions may demonstrate idiosyncratic species assemblages, suggesting a broader scale influence on local diversity (Cornell & Harrison, 2008). We can utilize inherent variation in a defined species pool— an aquaculture facility— to understand how regional richness combines with environmental filters to determine local diversity— a host-microbiome. In this chapter, I focus on identifying patterns of variation in the regional species pool- the water microbiome. In particular, I am interested in 1) identifying whether the facility water microbiome demonstrates temporal stability and 2) determining whether there is spatial variation in the facility water microbiome and if these patterns are consistent across different facilities.

Aquaculture facilities are quickly adopting the use of recirculating aquaculture systems (RAS) to filter and reuse water. The microbial composition within a RAS has a direct impact on fish health and development (Attramadal et al., 2014). However, microbiota within aquaculture facilities are rarely monitored, save for key culturable pathogens. Preliminary data suggests that different aquaculture facilities have unique species pool compositions, with differential impacts on host function. For example, tissue development (specifically pancreatic β-cell proliferation) and growth proceed at different rates in larval zebrafish inoculated with water from two separate University of Oregon aquaculture facilities- both of which are included in this dissertation (Ore1

and Ore2)- despite their proximity and general technical similarity (Adair, 2019; Massaquoi & Black, 2019). Understanding the factors shaping the species pool available for host colonization could inform facility management and husbandry protocols that minimize pathogen spread through facilities and promote reproducibility in research by accounting for known variation within and across facilities (Franklin & Ericsson, 2017).

This study compares all of the aquaculture facilities on the University of Oregon campus, which includes two zebrafish and one stickleback facility. Although this dissertation primarily focuses on microbiome variation in zebrafish and zebrafish facilities, the inclusion of a stickleback facility is useful for several reasons.

Like zebrafish, stickleback are used as animal models in research, and investigating how the regional pool of microbiota in their facilities varies across time and space can help promote reproducibility in stickleback as an animal model. It also extends our understanding of whether the patterns I observe in this study are generalizable to aquatic host-microbiome systems and RAS aquaculture or specific to zebrafish and zebrafish aquaculture only.

In this study, I quantified water microbiome variation across three aquaculture facilities over the course of four years. I looked at how the water microbiomes in each facility varied over time as well as in space by using a nested sampling design that captured total variation in water between tanks at the Housing, Row, and Shelf levels to identify where the greatest amount of variation was occurring. I found that the microbiome composition of each facility varied across time and compositional shifts in all facilities were largely driven by Proteobacteria. Within each facility, PERMANOVAs reveal that the greatest magnitude of variation among tanks occurs at an intermediary scale at the Row or Shelf level, depending on the size and layout of the facility.

These results are used to help inform more intensive sampling of both fish and tank water in later chapters.

2.2 Methods

2.2.1 Study sites. In this project, I investigated three aquaculture facilities used for academic research on the University of Oregon (UO) campus in Eugene, Oregon. Two of these facilities housed zebrafish and one housed threespine stickleback for experimental research (Figure 1).

The first facility- the Aquatic Animal Care Services Zebrafish Facility (Ore1)- is located in Huestis Hall on the UO campus and houses approximately 50,000 zebrafish in a 1,000 square-meter facility. This facility utilizes a recirculating aquaculture system (RAS) containing mechanical and biological filters in addition to ultra-violet disinfection to clean water used in fish husbandry. Two separate RAS systems filter water in the 'East' and 'West' housing areas.

Facility Name	Abbr.	Years sampled
Aquatic Animal Care Services: Huestis Facility	Ore1	2020, 2022
Zebrafish International Resource Center	Ore2	2019, 2020, 2022
Cresko Stickleback Lab	OreSB	2019, 2020, 2022

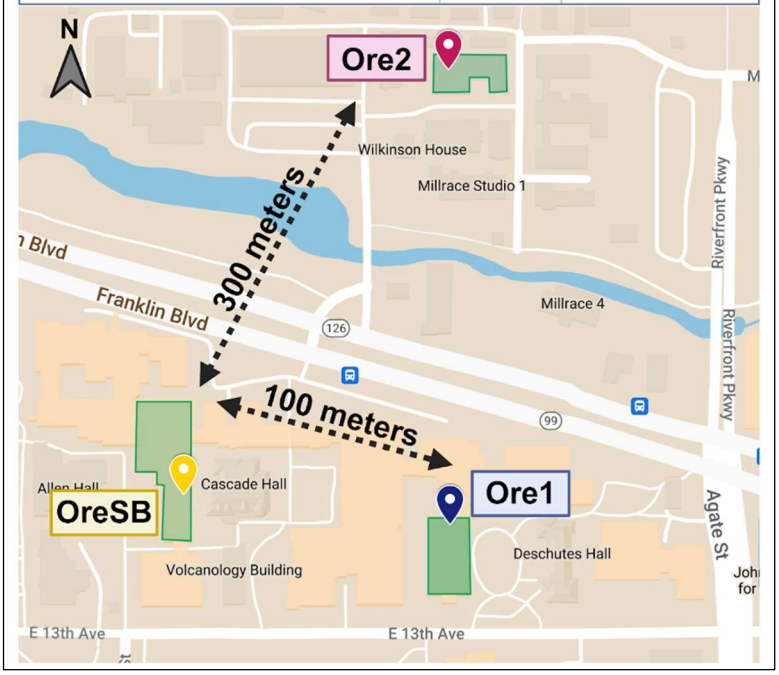

Figure 6: Sample site locations on the University of Oregon campus in Eugene, Oregon. The table includes information on the name of the facility, the abbreviations used in this study, and the years sampled for this study. *Map image adapted from Google Earth, 2022. Figure created with Biorender.com.*

The Zebrafish International Resource Center (Ore2) is the second zebrafish facility. This facility is located on the Riverside campus at UO, approximately 300 meters from both the Ore1 and OreSB facilities. It houses an average of 30,000 zebrafish (maximum capacity is around 150,000) in a 900 square-meter facility, and like the Ore1 facility, maintains two separate RAS systems that supply two housing areas.

The only stickleback facility, the Cresko Lab (OreSB) is located in the Pacific Hall building at UO. It is approximately 185 square-meters and houses 2,500 fish on average (maximum capacity 3,500 fish). The RAS water filtration system mimics the other two facilities, though the separate housing areas are unique in their operational capacity, as discussed in Chapter 1.

2.2.2 Sample collection and processing. Tank water was collected in 2019 and 2020 using a nested sampling design (Figure 2), though the actual number of tanks sampled was dependent on facility size and housing setup (*n* = Ore1: 2020 (*29*), 2022 (*27*); Ore2: 2019 (*47*), 2020 (*18*), 2022 (*17*); OreSB: 2019 (*51*), 2020 (*29*), 2022(*28*)). I sampled tanks from both Housing areas in each facility. Within each Housing, I randomly selected three 'Rows', or large sets of shelves. For each Row, I randomly selected three shelves, and then sampled three random tanks on each selected shelf. In 2022, I used a smaller scale version of the whole-facility sampling design. I selected one to two sets of shelves

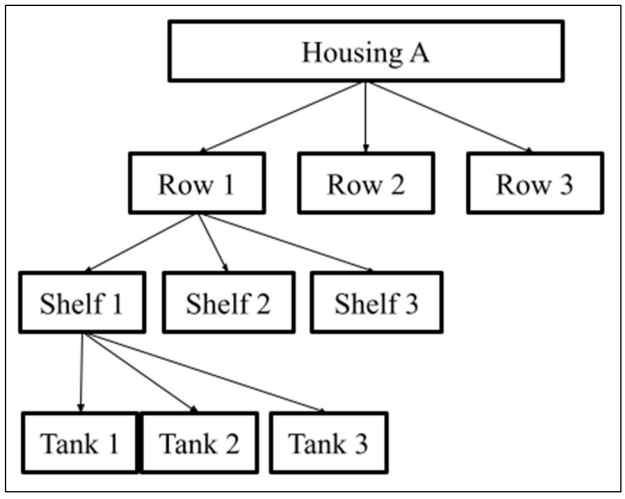

Figure 7: Facility nested sampling design.

immediately adjacent to one another connected within the same Housing. I then sampled following the same nested scheme. 150-mL of tank water was passed through a Sterivex filter cartridge (MilliporeSigma, Massachusetts, USA) using a 60-mL syringe. Sterivex filters were capped and kept on ice until processing. If DNA extraction could not be performed immediately, dry cartridges were stored at -80°C.

2.2.3 Microbial DNA extraction and processing. DNA extractions for samples collected in 2019 were performed using the DNeasy Blood and Tissue kit (QIAGEN, Carlsbad, California, USA) following the adapted in-cartridge '$SX_{CAPSULE}$' extraction method from Spens et al., (2017). Extractions for samples collected in 2020 and 2022 were performed using the DNeasy PowerWater Sterivex kit (QIAGEN, Carlsbad, California, USA). DNA was extracted following the standard protocol provided in the kit handbook (2009, pg. 9).

2.2.4 16S rRNA gene amplification, library preparation, and sequencing. DNA extraction products were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR amplification of the V4 region of the 16S rRNA gene was performed using a dual-indexed 515F-806R primer combination based on protocols described in Caporaso et al. (2011). Each 515F (AATGATACGGCGACCACCGAGATCTACACxxxxxxxxTATGGTAATTGTGTGCCAGCMGCCGCGGTAA) and 806R (CAAGCAGAAGACGGCATACGAGATxxxxxxxxAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT) primer pair were modified with unique barcodes (represented by italicized portion of primer above). PCR mixtures contained 12.5 µL NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 10.5 µL DNA template, 1 µL Bovine Serum

Albumin (Thermo Fisher Scientific), and 1 µL mixed primers (12.5 mM concentration). PCR cycling steps were denaturation at 98°C for 30 seconds, 30 amplification cycles at 98°C for 10 seconds, 61°C for 20 seconds, and 72°C for 20 seconds, followed by a final extension at 72°C for 2 minutes. Samples were held at 4°C until removal from thermocycler.

PCR products were visually checked for successful gene amplification via agarose gel electrophoresis. Amplicon libraries were cleaned twice with Mag-Bind RxnPure Plus isolation beads according to an adapted kit protocol using a 0.8x reaction volume (Omega Bio-Tek, Norcross, Georgia, USA). Concentrations of amplicon libraries were quantified with the Quant-iT 1x dsDNA HS Assay kit (Thermo Fisher Scientific) using a SpectraMax M5E Microplate Reader (Molecular Devices, San Jose, CA, USA) and multiplexed at equimolar concentration. Amplicons were then sequenced by the University of Oregon Genomics & Cell Characterization Core Facility on the Illumina MiSeq (Illumina, San Diego, CA, USA) with 150-bp paired-end reads.

2.2.5 Bioinformatics and statistics. All bioinformatics processing and statistical analyses were performed in 'R' (R Core Team, 2018). Sequences were demultiplexed and then denoised to construct an amplicon sequence variant (ASV) table using DADA2 1.16 (Callahan et al., 2016). Taxonomy was assigned to sequences using the RDP Classifier and Silva NR99 v.138.1 16S rRNA gene reference database (Wang et al., 2007; Quast et al., 2012). Sample contaminants were evaluated using the frequency method with 'DECONTAM' (Davis et al., 2018). Samples with less than 1,000 reads were considered unreliable and trimmed.

To analyze water microbiome composition, ASV sequences were first transformed to relative abundances and plotted using the 'fantaxtic' package, which selects the most abundant

taxa averaged across all samples at any selected taxonomic levels (Teunisse, 2022). I calculated and compared beta-diversity of facility water microbiomes using Bray-Curtis distance by testing a difference in centroid and dispersion using permutational analysis of variance (PERMANOVA) with the 'adonis2' function from the 'vegan' package in R (McArdle & Anderson, 2001; Okansen et al., 2019). Pairwise comparisons between sampling time points were made using the pairwise PERMANOVA function, 'pairwise.adonis2' from the 'pairwiseAdonis' package (Martinez Arbizu, 2020).

To identify taxa most associated with differences between facility microbiome composition, I used SIMPER analyses with a Bray-Curtis distance matrix (Clarke, 1993). I additionally constructed principal coordinate analyses (PCoA) using Bray-Curtis distances to visualize clustering in microbial composition.

2.3 Results

Samples with less than 1,000 reads were considered unreliable and trimmed. After amplicon sequencing data was normalized to 2,556 reads per sample, I identified a total of 1,104 unique genera in water microbiome samples, representing 53 phyla across all aquaculture facilities and time points.

2.3.1 Water microbiomes display both temporal and spatial intra-facility variation.

Zebrafish facilities

To look at patterns of within-facility variation, I used nested PERMANOVAs to evaluate spatial and temporal differences in microbial composition. When testing for temporal variation in

both zebrafish facilities, Ore1 and Ore2, PERMANOVA analyses showed significant differences in the microbiota composition among sampling time points (Ore1: p = 0.001; Ore2: p = 0.001).

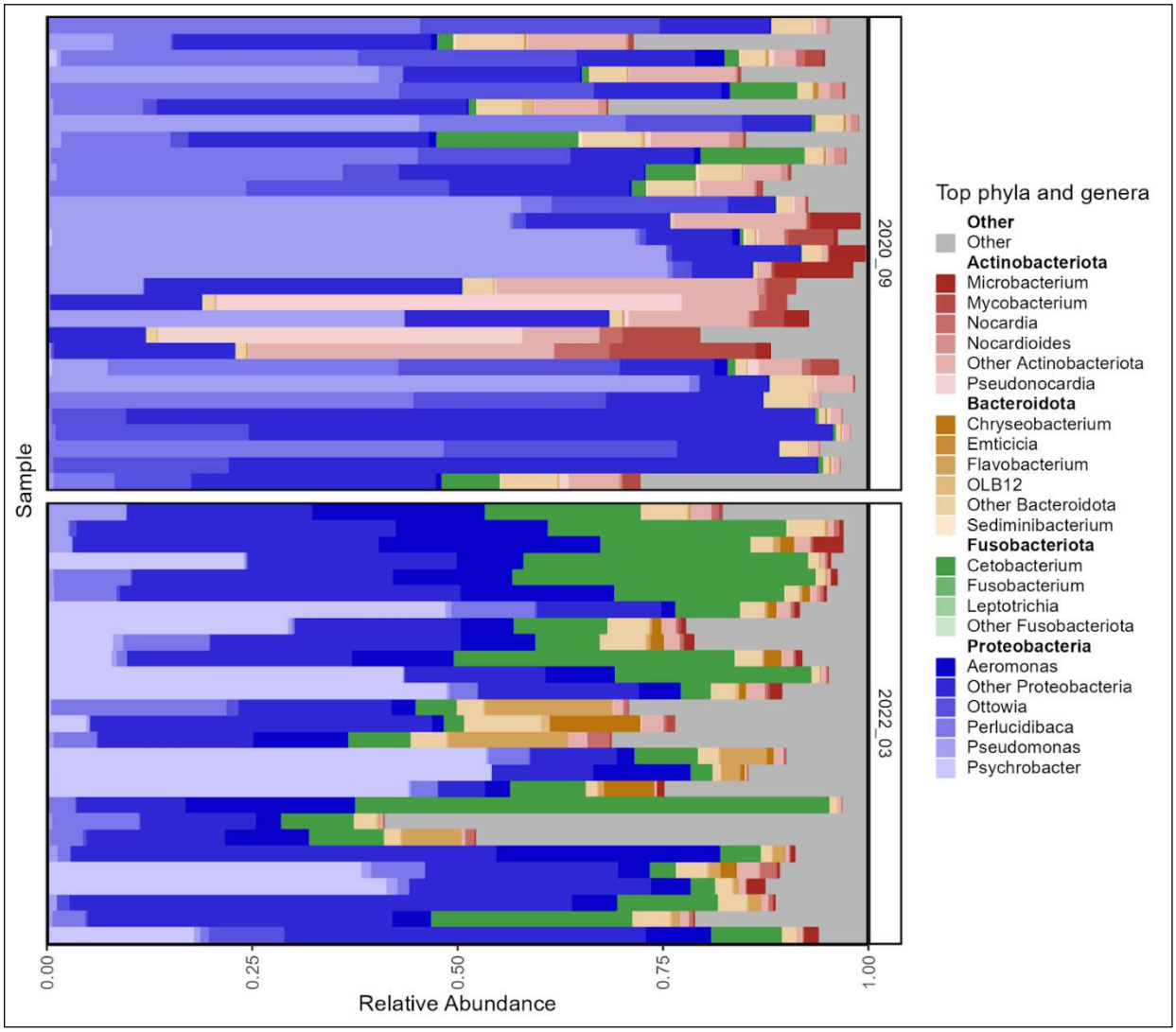

Figure 8: Phylum and genus-level taxonomic composition of Ore1 water microbiome samples collected in 2020 and 2022. The mean relative abundance is grouped by sampling time point and is displayed for only the top four most abundant phyla and top five genera within each phylum as averaged across samples.

Large shifts in composition can be observed in graphs of relative abundance over the sampling time points, especially within the phyla Proteobacteria and Fusobacteriota. In Ore1, Proteobacteria was the most prevalent phylum in both 2020 and 2022 (Figure 3). However, each sampling time point was characterized by large compositional shifts in dominant taxa. In 2020,

Proteobacteria represented an average of 71% of all sequences and the most prevalent taxa were *Pseudomonas* and *Perlucidibaca*. In 2022, Proteobacteria represented 60% of sequences and the most prevalent taxa was *Psychrobacter*.

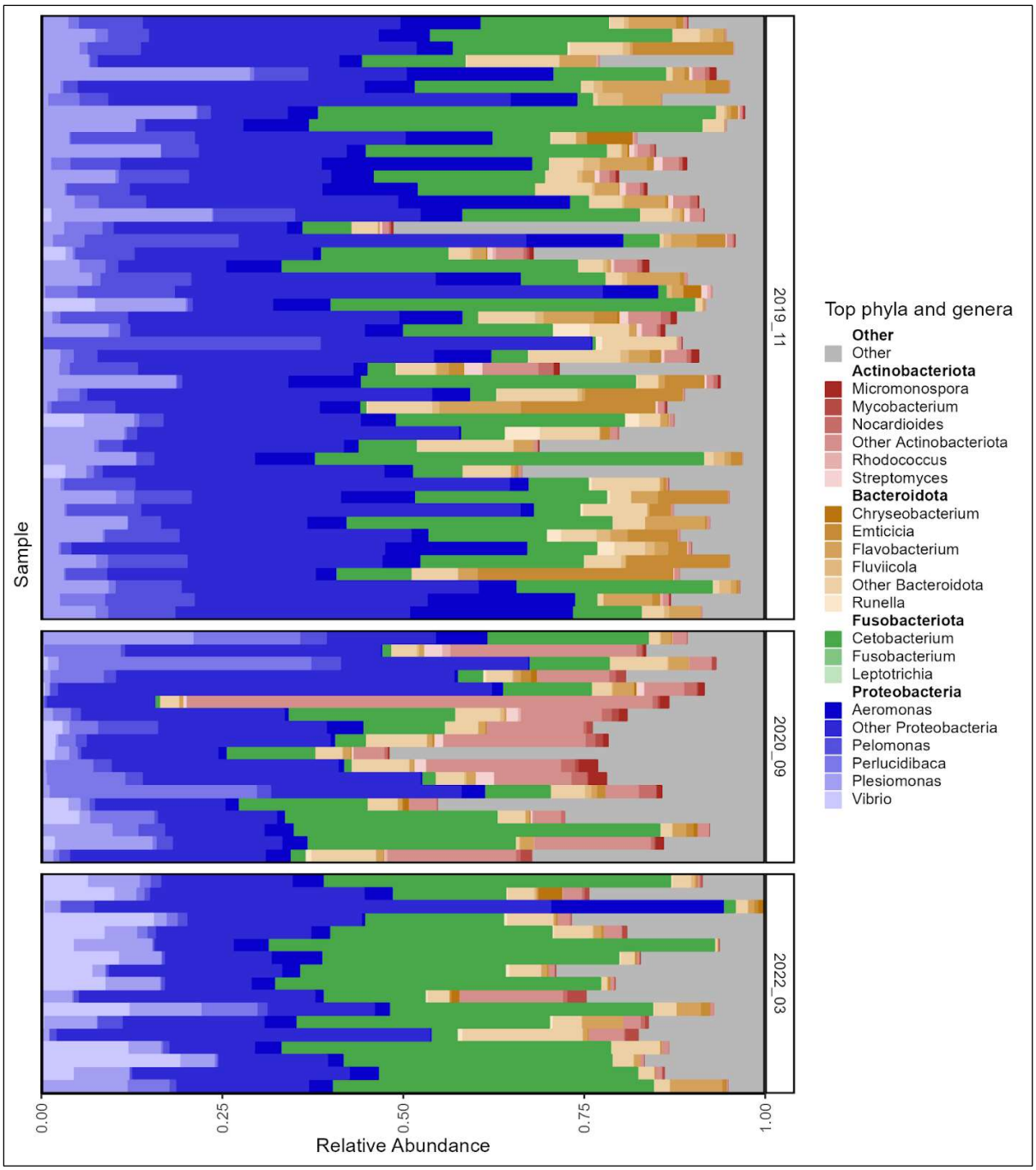

Figure 9: Phylum and genus-level taxonomic composition of Ore2 water microbiome samples collected in 2019, 2020, and 2022. The mean relative abundance is grouped by sampling time point and is displayed for only the top four most abundant phyla and top five genera within each phylum as averaged across samples.

Ore2 was characterized by similar patterns (Figure 4). Proteobacteria was the dominant phyla across all sampling time points (2019: 55%; 2020: 43%; 2022: 43%). However, the most prevalent taxa within Proteobacteria changed over the sampling time points from *Aeromonas* in 2019, *Perlucidibaca* in 2020, and *Vibrio* in 2022. Additionally, the relative abundance of *Cetobacterium* (phylum Fusobacteria) changed substantially over the years. SIMPER analyses of both facilities indicated that shifts in the most dominant Proteobacteria taxa as well as *Cetobacterium* (phylum Fusobacteria) were the largest contributors to variation in composition between sampling time points, though each only accounted for around 7-10% of observed differences.

Ore1

	Df	SumOfSqs	R2	F	Pr(>F)
Sampling_Round	1	3.8402197	0.1975913	19.873199	0.001
HousingID	1	0.7276789	0.0374413	3.765750	0.001
Sampling_Round:HousingID:RowID	7	5.0651579	0.2606181	3.744610	0.001
Sampling_Round:HousingID:RowID:ShelfID	6	2.0726692	0.1066453	1.787683	0.002
Residual	40	7.7294447	0.3977040	NA	NA
Total	55	19.4351704	1.0000000	NA	NA

Ore2

	Df	SumOfSqs	R2	F	Pr(>F)
Sampling_Round	2	3.0811076	0.1344874	7.120602	0.001
HousingID	1	0.7201879	0.0314355	3.328784	0.001
Sampling_Round:HousingID:RowID	11	4.2192711	0.1841672	1.772901	0.001
Sampling_Round:HousingID:RowID:ShelfID	18	4.2882089	0.1871763	1.101142	0.128
Residual	49	10.6012295	0.4627336	NA	NA
Total	81	22.9100050	1.0000000	NA	NA

Table 1: Complete PERMANOVA models of Ore1 and Ore2 facilities using Bray-Curtis distances.

To look at spatial variation within facilities, I used a nested PERMANOVA, constrained by sampling time point, to test the influence of various scales of sampling (e.g. groups of tank water samples belonging to the same Housing, Row, or Shelf) on overall microbial composition. The analyses indicated significant variation across all sampling scales within the facilities-

Housing, Row and Shelf (Ore1, Housing: p = 0.002, Row & Shelf: p = 0.001; Ore2, Housing: p = 0.003, Row: p = 0.001, Shelf: p = 0.032; complete model Table 1). However, the greatest variation was often seen between tanks at the intermediary Shelf or Row level.

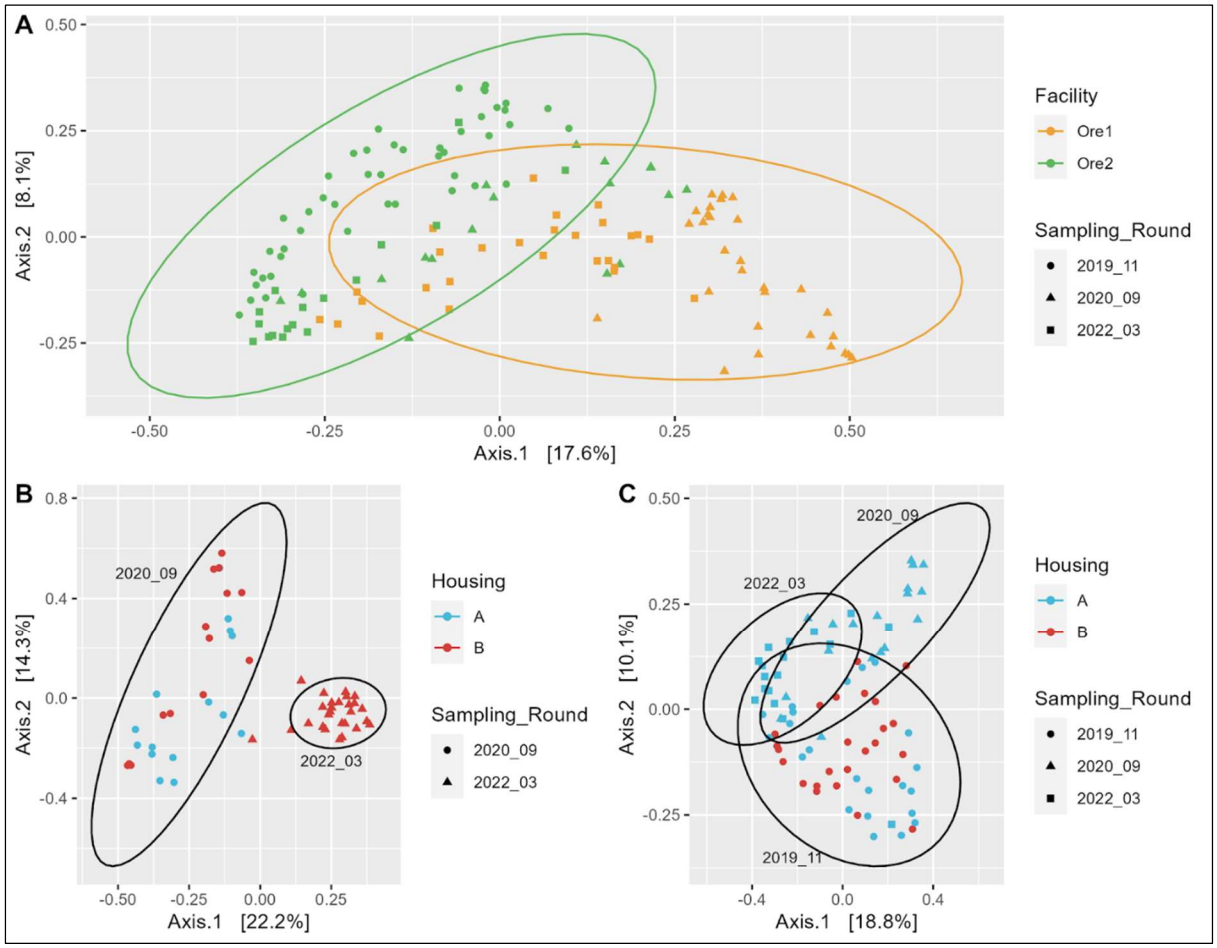

Figure 10: Beta diversity of zebrafish facility water samples using Bray-Curtis dissimilarity. A principal coordinate analysis (PCoA) (**A**) grouped by aquaculture facilities ('Facility') and sampling time point ('Sampling_Round'). Colors of normal confidence ellipses (95%) group aquaculture facilities. PCoAs of (**B**) Ore1 and (**C**) Ore2 colored by 'Housing' and sampling time point. Normal confidence ellipses (95%) are labeled and group sampling time point.

In Ore1, Row accounted for 40% of variation in the model while Shelf accounted for 10%. While in Ore2, Row and Shelf both accounted for about 21% of variation. In PCoA visualizations for Ore1 and Ore2, communities clearly cluster by facility and sampling time point (Figure 5). However, within these clusters, the influence of finer spatial scales within each facility- such as by Row, or Shelf- are difficult to visualize.

Stickleback facility

In the OreSB facility, sampling time point also had a significant influence on water microbial communities and explained 36% of variation in the Bray-Curstis dissimilarity (p = 0.001, Table 2). Like the zebrafish facilities, though the most prevalent phylum was the same across all time points- Proteobacteria-, the most abundant genera within that phylum changed year to year (Figure 6).

OreSB

	Df	SumOfSqs	R2	F	Pr(>F)
Sampling_Round	2	12.7136798	0.3656800	44.816513	0.001
HousingID	1	2.2493740	0.0646981	15.858367	0.001
Sampling_Round:HousingID	2	2.5046709	0.0720411	8.829121	0.001
Sampling_Round:HousingID:RowID	4	0.8965411	0.0257870	1.580182	0.013
Sampling_Round:HousingID:RowID:ShelfID	23	5.7648537	0.1658129	1.767084	0.001
Residual	75	10.6381100	0.3059810	NA	NA
Total	107	34.7672296	1.0000000	NA	NA

Table 2: Complete PERMANOVA model of the OreSB facility using Bray-Curtis distances.

In 2019, *Rheinheimera* and *Roseateles* were the most abundant taxa while *Pseudomonas* dominated in 2020 and 2022. Notably, *Rheinheimera* remained in abundance of >1% across all sampling time points. Another major contributor to changes seen across time was Planctomycetota. This was the second most abundant phyla in 2019 and represented about 21% of sequences. However, this phylum was greatly reduced in both the 2020 and 2022 time points, making up approximately 0.4% of total sequences in 2020 and 1.2% in 2022. According to a SIMPER analysis, changes in *Pseudomonas* prevalence alone explained an average of 12-15% of the differences seen between communities at all time points.

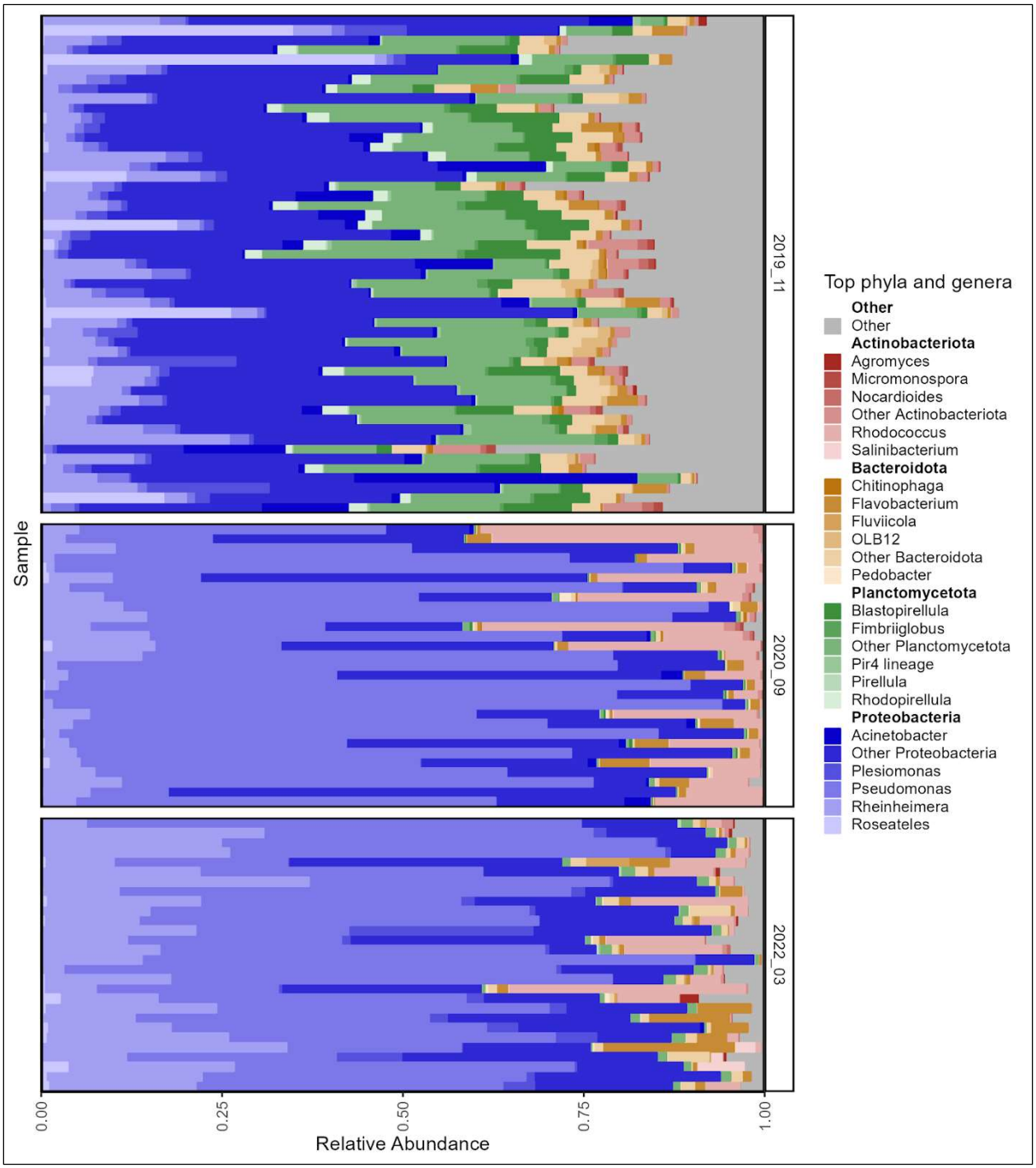

Figure 11: Phylum and genus-level taxonomic composition of OreSB water microbiome samples collected in 2019, 2020, and 2022. The mean relative abundance is grouped by sampling time point and is displayed for only the top four most abundant phyla and top five genera within each phylum as averaged across samples.

Results from a nested PERMANOVA showed significant differences within the facility at all spatial scales ($p = 0.001$; Table 2). Much like the Ore1 and Ore2 facilities, however, Row

and Shelf explained the majority of the variation in Bray-Curtis dissimilarity at 16% and 21%, respectively. In a PCoA plot of distance values, clusters of water samples can be seen by both sampling time point and Housing (Figure 7).

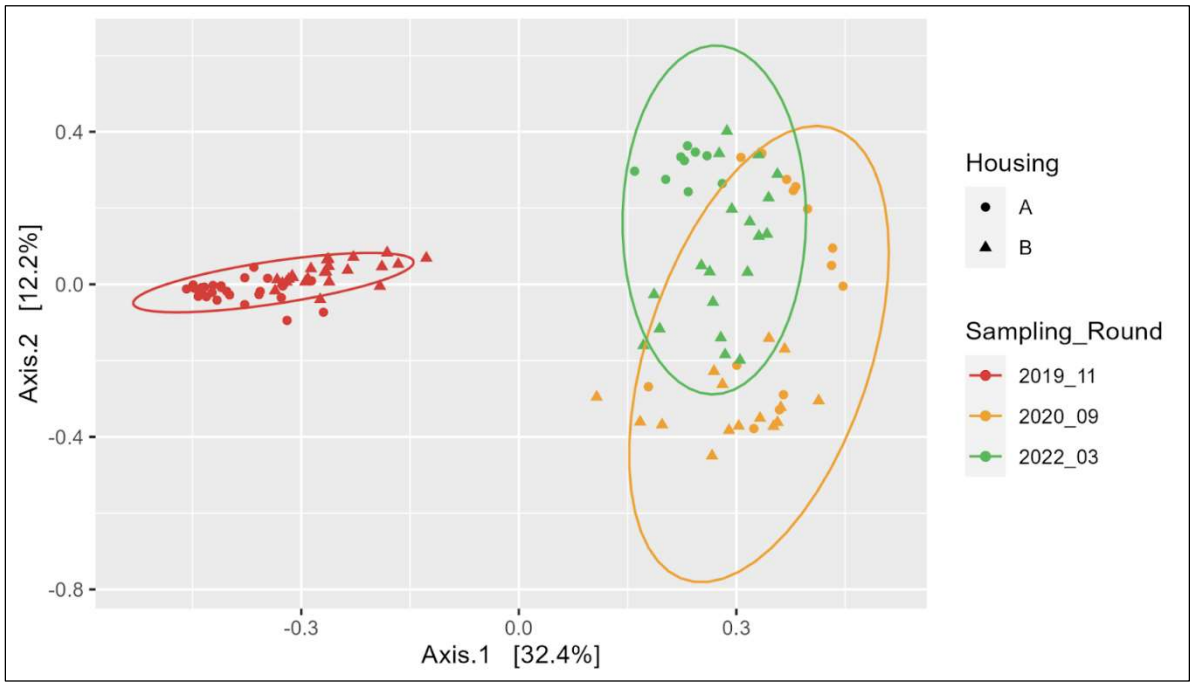

Figure 12: Beta diversity of OreSB facility water samples using Bray-Curtis dissimilarity. A principal coordinate analysis (PCoA) grouped by Housing and sampling time point ('Sampling_Round'). Colors of normal confidence ellipses (95%) group sampling time points.

2.3.2 Aquaculture facilities maintain unique water microbiomes but share patterns of variation across facilities.

To quantify the similarity of tank water microbial communities between facilities, I conducted several PERMANOVA analyses using Bray-Curtis distance. The PERMANOVA showed significant effects of species, facility, and sampling time point as well as the interaction between these factors ($p = 0.001$) (Table 3). Species accounted for the largest proportion of variance in the model (16.1%) and facility the second largest (10.9%). The significant interaction effects between sampling time point, species, and facility were unsurprising given the broad variation in microbial composition within facilities across time and unequal sampling effort across facilities.

Bray-Curtis					
	Df	SumOfSqs	R2	F	Pr(>F)
Species	1	16.014854	0.1619776	65.788302	0.001
Sampling_Round	2	10.796531	0.1091984	22.175832	0.001
Facility	1	4.681244	0.0473471	19.230338	0.001
Species:Sampling_Round	2	7.855647	0.0794537	16.135323	0.001
Sampling_Round:Facility	1	1.586148	0.0160426	6.515825	0.001
Residual	238	57.936369	0.5859806	NA	NA
Total	245	98.870792	1.0000000	NA	NA

Table 3: Complete PERMANOVA model of all aquaculture facilities and time points using Bray-Curtis distances.

I further used a nested PERMANOVA constrained by facility to test the overall pattern in within-facility spatial variation. Water microbial communities across all facilities significantly varied by Housing, Row, and Shelf ($p = 0.001$), and Row and Shelf accounted for most of the variation within the model, much like the individual models.

2.4 Discussion

I sampled water microbiota from tanks in three aquaculture facilities that are in close geographic proximity over the course of four years. Two of these facilities house zebrafish and one houses stickleback, though all use a RAS water treatment life support system. Despite the relative proximity and general similarity in facility technical layout and design, I found both a significant species and facility-specific effect on the composition of the water microbiomes. Within each facility, there were significant temporal and spatial influences on microbiota that were common to all facilities sampled. Though there was a significant effect on water microbiome composition at every spatial scale I tested, the magnitude of variation within every facility was largest at either the Row or Shelf level.

Within individual aquaculture facilities, I observed both substantial spatial and temporal variation in the water microbiome. For all facilities, both zebrafish and stickleback, taxa within the Proteobacteria phyla were responsible for the largest compositional shifts year to year. Taxa belonging to the Proteobacteria phylum are consistently found to be some of the most abundant bacteria in both zebrafish and stickleback (Roeslers et al., 2011; Sharpton et al., 2021; Small et al., 2023; Steury et al., 2019). Thus, it is not unexpected that these were also the most dominant taxa found across the water microbiomes in the study facilities, regardless of species housed.

Both Ore2 and OreSB had noticeable shifts in composition between sampling time points 2019_11 (November 2019) and 2020_09 (September 2020). After, in Ore2, the water microbiome composition at the 2022_03 (March 2022) sampling time point was more similar to the 2019 sampling time point than 2020, while in OreSB, the 2022 was largely similar to the community in 2020. Notably, the time span between the 2019 and 2020 sampling efforts included the onset of the 2019 SARS-CoV-2 pandemic, which was first confirmed in the U.S. in January 2020 (Centers for Disease Control and Prevention, 2023). The pandemic was responsible for major changes in the operation of the facilities during this time as physical access was limited following state and local guidelines for pandemic response. In both the Ore2 and OreSB facilities, staffing numbers in the facility were greatly reduced and a large portion of the fish in each of the facilities at the time were culled; in Ore2 alone, fish numbers were reduced from 48,000 to 30,000 (D. Lains, personal communication, January 20, 2022; M. Currey, personal communication, February 12, 2024). Ore1 also underwent similar changes to facility operation during 2020, but since I do not have prior data, I cannot say for certain that this affected the Ore1 water microbiome in a similar capacity to Ore2 and OreSB (T. Mason, personal communication, September 20, 2023).

In both the Ore2 and OreSB facilities, this reduction in staffing also meant an overall reduction in the amount of time that remaining personnel were physically in the facilities. Between the 2020_09 and 2022_03 time points both facilities resumed operations similar to those prior to 2020. However, some practices adopted during the pandemic in the facilities, like increased use of personal protective equipment, have largely remained in use.

One potential limitation to the interpretation of the temporal variation seen within the facilities is the change in microbial extraction methodology. Bacterial DNA from water microbiome samples collected in 2019 were extracted following an adapted method from Spen et al. (2017) using a Qiagen DNeasy Blood & Tissue kit, while those collected in 2020 and 2022 were extracted following the methodology included in the DNeasy PowerWater Sterivex kit (Qiagen, 2009). However, both methodologies follow the same general principles (cell lysis and extraction *within* the Sterivex filter cartridge), and the extraction kit used was produced by the same manufacturer. As such, it is very unlikely that all variation observed from 2019 to 2020 in the water microbiome samples is solely due to differences in extraction methodology.

To measure spatial variation within the facilities, I grouped water samples across spatial scales at the 'Housing', 'Row', and 'Shelf' level. Though every scale within the facility significantly influenced water microbiome composition in my PERMANOVA models, I observed the greatest magnitude of variation in the water microbiome at the intermediary scale- e.g. individual shelves ('Shelf') or groups of shelves ('Row'). These results suggest that tank-to-tank variation is not the largest contributor to overall water microbiome variation within the facilities, but rather, certain groupings of tanks.

This is somewhat surprising, given that other studies have noted tank-to-tank variation in aquaculture facilities and experimental systems (albeit intermediary scales of variation are not

usually included in analyses), but may be a result of common approaches to the organization of fish within research aquaculture facilities (Bakke et al., 2013; Breen et al., 2019; Schmidt et al., 2015; Stagaman et al., 2017). At least in the facilities investigated in this dissertation, fish tanks were often grouped within the facility by some identifying information about the fish.

In Ore2, sets of tanks containing specific genotypes were commonly grouped in one area of the facility. Further, within these groups of genotypes, tanks containing fish bred from the same stocks- in other words, genetically related siblings, half-siblings, and so on- were also commonly in the same general proximity relative to others.

In Ore1, tanks within the facility are primarily arranged by research group. Many research groups employ specific staff to care for fish and may keep only one or two genotypes specific to their research needs. For example, my research group- the Bohannan Lab-, at the time of the publication of this dissertation, maintains around six tanks of zebrafish in the Ore1 facility. These six tanks are spread across two shelves, immediately adjacent to one another, and contain only ABxTu and AB wild-type fish from the same stock pools. Immediately across from these tanks, on a separate 'Row', another research group maintains several Casper and other CRISPR-Cas9-knockout lines cared for by a different staff member. Given the evidence for genotype-specific variation in the fish microbiome, variation in the water microbiome, at this scale within the facility, may be partially due to the microbial influence of fish (Burns et al., 2017; Lu et al., 2021; Okazaki et al. 2018; Thormar et al., 2024).

For OreSB, the facility layout is slightly different than the zebrafish facilities and suggests that the observed water microbiome variation may be due to more than just the spatial groupings of fish. Though the Housings retain very different developmental stages of fish, within each Housing, fish within tanks across Rows and Shelves are not necessarily grouped by

genotype or lineage, yet this intermediary scale still explains the largest proportion of variance. After discussion with personnel in each of the facilities, one early- and frequently repeated-suggestion for why this variation may be occurring was the proximity of the fish tanks to overhead light sources, since exposure to ultraviolet radiation can promote bacterial and algal growth in water (Hörtnagl et al., 2010; Lindell et al., 1995).

However, when included in my models, proximity to light was not statistically significant. I additionally looked at the relative abundance of Cyanobacteria, a phylum containing some of the most common photosynthetic bacteria in aquatic environments, across tanks but saw no clear pattern that would align with this hypothesis. While this is obviously not an exhaustive approach to examine all potential photoautotrophic bacteria in the water microbiome, there is little evidence that this is a viable source of variation. Some other common suggestions for potential sources of variation from discussion with facility personnel included differences in bacterial biofilm composition and accumulation in the plumbing throughout the facility as well as potential exposure to air or falling debris from higher shelves. These things were not measured in this study, however, and remain as speculation for future investigation.

Beyond identifying and quantifying variation in the water microbiome throughout these aquaculture facilities, one of the main objectives of this study was to determine whether the observed patterns in water microbiome variation were generalizable across facility systems. I found evidence for consistent patterns of variation compared across all our study facilities. Facilities not only varied year to year, but at least in our sites, Proteobacteria was the main driver of these changes.

In terms of spatial variation, analyses also indicated that the greatest magnitude of variation was occurring at the ‘Row’ or ‘Shelf’ level. Though in our sampling scheme, these are

considered different scales this is not always the case. For example, Housings within OreSB did not have Rows in the same capacity of Ore1 or Ore2 (as there were far fewer tanks in OreSB), and thus the classification of spatial scales is somewhat subjective. However, regardless of the technical difference in layout, it was clear that intermediary sampling scale explained the largest proportion of variance in all the facilities and more metadata and greater sampling power would be needed to draw any definite conclusions about why this is occurring.

Knowing how and to what capacity the microbiome of facility water varies over time and in space is not only an important ecological question, but also an integral aspect to aquaculture facility management. Facility managers monitor changes in the water microbiome to combat pathogen spread and maintain appropriate environments for fish (Barton et al., 2016; Lawrence & Mason, 2012). To make generalizable recommendations about microbial management, however, we first need to establish how facility water systems may differ and which aspects remain the same. Additionally, this is only one piece of the puzzle, as it remains unclear whether the variation in the water microbiome observed in this study has any consequence on fish microbiomes. As such, future investigations should attempt to connect variation within the water and fish microbiome in aquaculture facilities.

2.5 Bridge

The study discussed in this chapter functioned as a proof-of-concept that guides the rest of my dissertation. To use the aquaculture facilities as a model system to investigate the influence of regional pool variation in local community assembly, I needed to determine three things: 1) whether there was microbial variation in the aquaculture facility water systems, 2) whether it could be quantified, and 3) where the magnitude of variation within the facilities was greatest to inform future sampling. In other words, since zebrafish are a valuable resource and I

utilize destructive sampling (notably, however, care should be taken to minimize destructive sampling of organisms regardless of research utility), I wanted to determine the scale at which I could sample both fish and water to accurately capture the range of variation within each facility while minimizing the extent of destructive sampling. I use my results from Chapter 2 to inform my sampling approach in the following chapter.

CHAPTER 3: AQUACULTURE FACILITY-SPECIFIC WATER MICROBIOTA SHAPE THE ZEBRAFISH GUT MICROBIOME

This chapter has been formatted and prepared for submission to the Animal Microbiome journal. As such, the order of sections deviates from other chapters and some material in the Introduction and Methods may be repeated elsewhere in this dissertation.

3.1 Introduction

Over the past several decades, research has elucidated the influence of the microbiome on the development and fitness of their vertebrate hosts, through mediation of nutrition, metabolism, physiology, and immunology, among others (Teame et al., 2019; Cryan et al., 2020). However, substantial variation in the microbiome can be observed between and even within hosts (Adair & Douglas, 2017; Kostic et al., 2013). To fully comprehend why variability may occur and how that impacts animal host health and function, it is important to first determine how the microbiome is acquired and maintained.

The structure and composition of the host-microbiome is partially determined by factors outside of the host individual, which includes, but is not limited to, food, other host individuals, and the environmental microbiome (Adair & Douglas, 2017). While factors such as diet have been well-studied, the role of the environmental microbiome in driving microbiome variation remains unclear. Previous studies have indicated that variation in the environment surrounding vertebrate hosts correlates with variation in the microbiome, but few have characterized the potential for environmental acquisition of microbes by measuring both the host microbiome and

the environmental microbiome simultaneously (Kers et al., 2019; Moraitou et al., 2022; Shigeno et al., 2022).

Zebrafish (*Danio rerio*) are ideal for investigating these questions of host and environment as they are already used extensively as a laboratory animal model in host-microbiome research due, in part, to their rapid development, high reproductive rate, and ability to be derived germ-free (reared without microbes; Eisen, 2020; Stagaman et al., 2020). Broad conclusions made from experimental manipulation of zebrafish, however, are only useful if the results can be widely replicated and reproduced. In other studies of laboratory animals, including mice (Parker et al., 2018; Rausch et al., 2016) and non-human primates (Flynn, 2022; Shigeno et al., 2022), genotype-matched individuals displayed marked variation in their microbiota correlating with housing condition and vendor source. This variation, if unaccounted for, could potentially confound cross-study comparisons of microbiome-sensitive research.

Similar studies in zebrafish have noted both intra-facility variation (Breen et al. 2019) and inter-facility variation (Roeslers et al., 2011; Sharpton et al., 2021) in gut microbiota. To the best of my knowledge, however, no studies have attempted to directly characterize the contribution of the environmental microbiome to zebrafish gut microbiome variability across multiple modern research aquaculture facilities. Therefore, in this study, I used 16S rRNA amplicon sequencing to quantify variation in the environmental microbiome (tank water) both within and across representative aquaculture facilities and identify the consequences on the zebrafish gut microbiome.

In modern aquaculture facilities, zebrafish of different ages and genetic backgrounds are housed in large aquaculture systems that can range widely in their technical specifications and

management. I was particularly interested in investigating the relationship between the host and environmental microbiome in facilities that supply zebrafish for basic research, as there should be some general similarity between their operation and management due to the sensitive nature of rearing laboratory animals. By comparing similar aquaculture facilities, I can better isolate the interaction between host and environment as well as provide helpful insight into the reproducibility of results derived from zebrafish sourced from different facilities.

The facilities investigated in this study are in two geographic locations: Trondheim, Norway and Eugene, Oregon. Within each region, facilities are located within 300 meters of each other on the University of Oregon campus and within 700 meters on the Norwegian University of Science and Technology campus (Figure 1). These facilities are representative of zebrafish research facilities because they

Facility Name	Region	Abbr.	System type	Samples analyzed (water/fish)
Aquatic Animal Care Services: Huestis Facility	Oregon	*Ore1*	RAS	27 / 55
Zebrafish International Resource Center	Oregon	*Ore2*	RAS	17 / 5
Yaksi Lab	Norway	*Nor1*	RAS	3 / 15
Jutfelt Lab RAS	Norway	*Nor2A*	RAS	7 / 36
Jutfelt Lab MMS	Norway	*Nor2B*	MMS	6 / 23

Figure 13: Aquaculture facilities (region, name, abbreviation, and water system type) and samples collected from tank water and zebrafish. The two photos of zebrafish tanks (Ore1- left; Nor2B- right) provide an example of the variety and organization of tanks common in modern aquaculture facilities. *Image of Ore1 facility courtesy of Kelley Christensen, 2024, University of Oregon. Image of Nor2B facility, Kayla Evens, 2022. Created with Biorender.com.*

predominantly utilize recirculating aquaculture systems (RAS) to filter their water. One facility Nor2B- uses a similar system called a microbial maturation style system (MMS). RAS systems, as the name suggests, filter outflow water and recirculate it back through the closed-loop system. Interest in these types of facility water systems has grown over the last several decades as they minimize waste and energy usage. Studies have indicated they can be useful for maintaining high loads of beneficial bacteria in the water system, or at the very least, reduce the abundance of pathogenic bacteria, because the long retention times characteristic of RAS systems allows for the "maturation" of the water microbial community (i.e. the replacement of fast-growing pathogens with slow-growing but competitively superior non-pathogens; Attramadal et al., 2014; Attramadal et al., 2016; Salvesen et al., 1999).

MMS systems use a similar theory of creating a mature microbial community by lengthening retention time of water prior to it being pumped out to tanks, but outflow "dirty" water is removed from the system instead of being recirculated. During the establishment of both types of aquaculture systems, the water is seeded with beneficial bacteria, such as nitrifying bacteria, as part of the biofilter. In addition to inputs and manipulation of the microbial community through husbandry, management, and other factors, the progression of this initial microbial seeding can greatly diversify even the most similar of aquaculture facilities.

Despite the general shared goal of maintaining safe, clean rearing conditions for zebrafish, the microbial communities in aquaculture facility water systems are very likely to vary in composition. As such, if the microbiome of zebrafish is mostly assembled via acquisition from the environment, this facility-level variation could potentially drive significant variation among zebrafish housed in different facilities. However, it is thought that the zebrafish intestine is at

least somewhat selective since a “core” intestinal microbiome has been suggested to exist across zebrafish facilities (Roeselers et al., 2011; Sharpton et al., 2021). Selection by the zebrafish gut would reduce the effect of facility on the composition of the gut microbiome. Here, I address three main questions, (1) How does the environmental microbiome in aquaculture facilities vary?, (2) Can we identify patterns of covariation between the environmental microbiome and the zebrafish gut microbiome?, and (3) At what scale(s) does such variation occur? By using paired samples of fish and water from five locations, I show significant variation in both the water and fish gut microbiomes across facilities despite the relative technical similarities of their water systems. Furthermore, I show that overall, the similarity between fish and water microbiomes is far greater within facilities. Finally, I use source tracking methods to demonstrate a consistent influence of the environmental microbiome on the zebrafish gut microbiome.

3.2 Results

3.2.1 Tank water. The goal of this study was to investigate the consequences of variation in the tank water microbiome on the zebrafish gut microbiome across aquaculture facilities. However, to answer this question I first needed to determine whether there was variation in the tank water microbiome within and between facilities. To address this, I examined metrics of both community composition and diversity within and across tank water samples. A total of 1,625 unique amplicon sequence variants (ASVs) were identified across 50 tank water samples (n = Ore1 (27); Ore2 (17); Nor1 (7); Nor2A (6); Nor2B (3)) after the amplicon sequence data were normalized to 1,369 reads per sample.

Variation in the tank water microbiota is driven by shifts in dominant bacterial taxa.

I first looked at α-diversity metrics to compare the communities within each facility. Across the five facilities, α-diversity of water microbiota did not differ significantly using the Shannon-Wiener index (Kruskal-Wallis: $p = 0.13$), but in pairwise comparisons, the Nor2A and Nor2B facilities varied significantly from the Nor1 facility (pairwise Wilcoxon test: Nor2A vs. Nor1, $p = 0.008$; Nor2B vs. Nor1, $p = 0.017$). However, the Inverse Simpson index, which gives greater weight to numerically dominant taxa, varied significantly across facilities (Kruskal-Wallis: $p = 0.029$) and pairwise comparisons using this index resulted in significant differences between the Nor1 facility and both the Ore1 and Ore2 facilities (pairwise Wilcoxon test: Ore1 vs. Nor1, $p = 0.004$; Ore2 vs. Nor1, $p = 0.013$) (Figure 2).

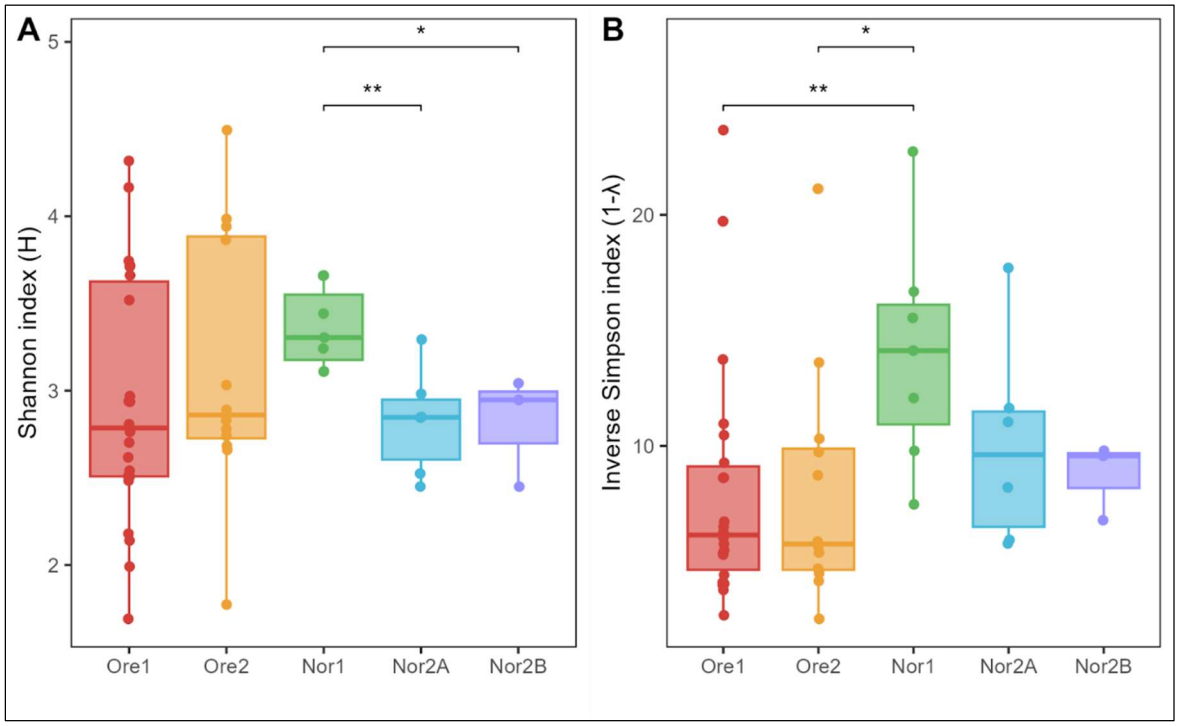

Figure 14: Alpha diversity metrics of tank water microbiota with (A) Shannon diversity and (B) Inverse Simpson diversity. Tukey-style box and whisker plots display the median (center horizontal line) and interquartile range (upper and lower bounds of the box), while whiskers extend +/- 1.5 times the interquartile range. Significance calculated with pairwise Wilcoxon rank-sum test with Benjamini-Hochberg p-value correction (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = non-significant).

The α-diversity metrics indicated that variation seen in the tank water was most influenced by shifts in numerically dominant taxa, as indicated by the significant variation between facilities detected using the Inverse Simpson index. To further elucidate variation between facilities, the composition of tank water microbiota was compared between the five facilities. Though no one phylum was shared across every tank water sample, seven phyla were detected across the facilities at a mean relative abundance threshold of 1%. Proteobacteria was by far the dominant phylum in every facility, making up anywhere from 52% (Ore1) to 92% (Nor2A) of the water microbial community. There were additionally 19 abundant (>1%) genera represented across all facilities. Of these abundant taxa, none were unique to water samples and all were shared with samples of fish gut communities. The Ore1 and Ore2 facility water microbiomes were both dominated by *Cetobacterium*, representing an average of 20.2 and 42% of overall sequences, respectively. Ore1 was further characterized by high relative abundances of *Psychrobacter* (19%) and *Aeromonas* (12%). *Pseudomonas* was most prevalent in the Nor1 facility (35% on average) while *Rheinheimera* was most prevalent in the Nor2A facility (33%). Compared to the other facilities, Nor2B had no one dominant taxon, and instead was characterized by a number of Proteobacteria including *Acidovorax* (11%), *Nevskia* (10%), and *Limnobacter* (9%) among others (Figure 3).

A SIMPER Analysis, a method that assesses which taxa are primarily responsible for observed differences between groups, indicated that the abundances of members of the Fusobacteria and Proteobacteria were primary contributors to the differences seen among all five facility water communities (Clarke, 1993). *Cetobacterium* was responsible for 8 to 16% of the observed differences between the Oregon and the Norwegian facilities. A number of

Proteobacteria, including both *Pseudomonas* and *Rheinheimera*, were responsible for 7 to 8% of the observed differences among the three Norwegian facilities.

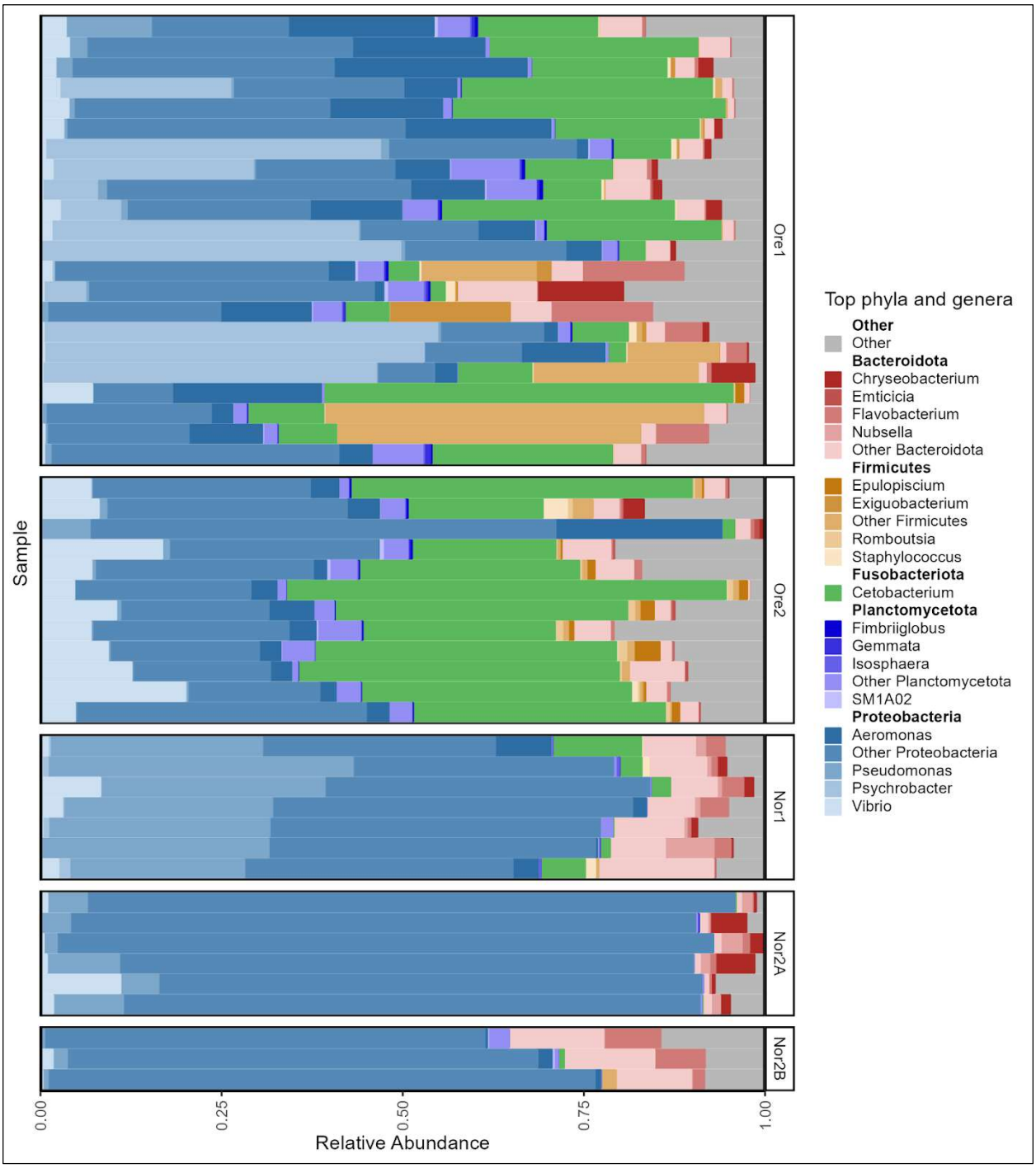

Figure 15: Phylum and genus-level taxonomic composition of tank water microbiome samples across aquaculture facilities. The mean relative abundance is grouped by aquaculture facility and is displayed for only the top five most abundant phyla and top four genera within each phylum as averaged across samples.

Aquaculture facilities are a major contributor to variation in tank water microbial communities.

Bray-Curtis

pairs	Df	SumsOfSqs	F.Model	R2	p.value	p.adjusted	sig
Ore1 vs Ore2	1	1.8801363	9.061010	0.2206719	0.001	0.01	*
Ore1 vs Nor2A	1	2.5048624	10.524188	0.2881430	0.001	0.01	*
Ore1 vs Nor2B	1	1.6105481	6.939968	0.2317961	0.005	0.05	.
Ore1 vs Nor1	1	2.4533282	10.775905	0.2852587	0.001	0.01	*
Ore2 vs Nor2A	2	2.5150135	6.751745	0.4737486	0.001	0.01	*
Ore2 vs Nor2B	1	1.6893445	11.302569	0.4650771	0.002	0.02	.
Ore2 vs Nor1	1	2.3208669	14.334089	0.4574599	0.001	0.01	*
Nor2A vs Nor2B	1	0.9079807	4.261691	0.3784237	0.011	0.11	
Nor2A vs Nor1	1	1.5323010	7.325676	0.3997493	0.001	0.01	*
Nor2B vs Nor1	1	1.3919969	7.679089	0.4897663	0.010	0.10	

Unweighted UniFrac

pairs	Df	SumsOfSqs	F.Model	R2	p.value	p.adjusted	sig
Ore1 vs Ore2	1	1.1775325	5.886370	0.1553691	0.001	0.01	*
Ore1 vs Nor2A	1	1.6593681	9.030955	0.2577993	0.001	0.01	*
Ore1 vs Nor2B	1	0.8515854	4.331148	0.1584693	0.005	0.05	.
Ore1 vs Nor1	1	1.1684623	6.093103	0.1841200	0.001	0.01	*
Ore2 vs Nor2A	2	1.4231007	3.633566	0.3263614	0.001	0.01	*
Ore2 vs Nor2B	1	0.7616227	3.691215	0.2211472	0.002	0.02	.
Ore2 vs Nor1	1	0.9970251	5.077958	0.2300012	0.001	0.01	*
Nor2A vs Nor2B	1	0.8264406	5.466732	0.4385056	0.011	0.11	
Nor2A vs Nor1	1	0.8317548	5.338801	0.3267560	0.001	0.01	*
Nor2B vs Nor1	1	0.7611186	4.174308	0.3428785	0.010	0.10	

Table 4: Pairwise PERMANOVA on Bray-Curtis and unweighted UniFrac distance of aquaculture facility water microbiome. p-value adjusted with Benhamini-Hochberg correction.

The composition of the water microbiomes varied significantly across all facilities. A permutational analysis of variance (PERMANOVA) indicated a strong effect of both geographical location (Norway versus Oregon; $p < 0.001$) and individual facility ($p < 0.001$) on both Bray-Curtis and unweighted UniFrac dissimilarity. Location explained 20.4% of variation in the Bray-Curtis and 12.7% in the unweighted UniFrac dissimilarities. Meanwhile, individual facilities explained 26% of the variation in the Bray-Curtis and 21.5% in the unweighted UniFrac dissimilarities. However, a pairwise PERMANOVA demonstrated that though the taxonomic

dissimilarity of the water microbial community differed strongly by facility, there was weaker variation among the Norwegian facilities, particularly between Nor2A and Nor2B (Bray-Curtis and unweighted UniFrac: p-adj = 0.11) and Nor2B and Nor1 (Bray-Curtis and unweighted UniFrac: p-adj = 0.10) (Table 1).

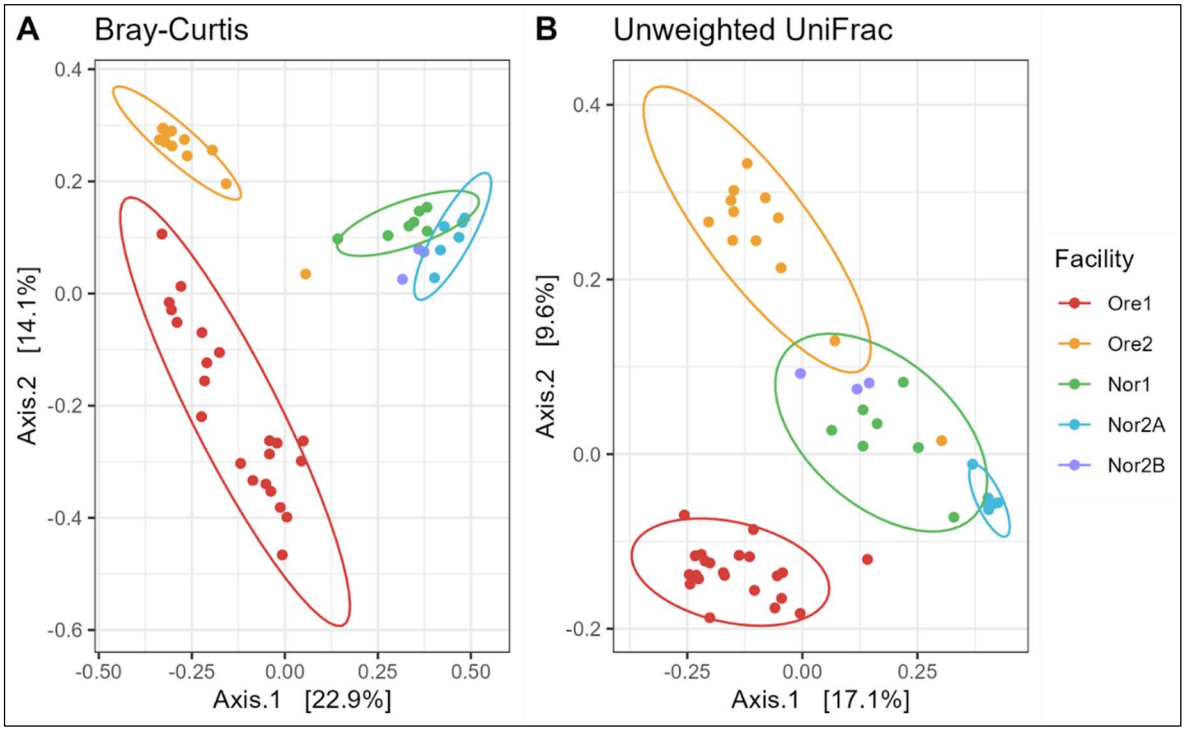

Figure 16: Beta diversity of tank water samples across aquaculture facilities. A principal coordinate analysis (PCoA) of (A) Bray-Curtis and (B) unweighted UniFrac dissimilarity. Colors represent facilities with normal confidence ellipses (95%).

A PCoA plot based on the Bray-Curtis and unweighted UniFrac dissimilarity indices further corroborated the results of the PERMANOVA. The water microbiomes clustered strongly according to aquaculture facility, with some overlap seen primarily in the Norwegian facilities (Figure 4).

3.2.2 Zebrafish. To address whether the zebrafish gut microbiome also varied across tanks and facilities, I used similar analyses of community composition and diversity. A

total of 1,281 unique ASVs were identified across 134 fish samples (*n* = Ore1 (55); Ore2 (5); Nor1 (15); Nor2A (36); Nor2B (23)) after data were normalized to 1,171 reads per sample.

Differences in the zebrafish gut microbiota are driven by shifts in dominant bacterial taxa.

Zebrafish gut microbiomes varied significantly in α-diversity metrics, both across all facilities (Kruskal-Wallis: Shannon: $p < 0.001$; Inverse Simpson: $p = 0.003$) and between pairs of facilities (pairwise Wilcoxon test: Shannon: Ore1 vs. Nor2A: $p < 0.001$; Ore2 vs. Nor2A, $p < 0.001$; Nor2A vs. Nor2B, $p < 0.001$; Nor2A vs. Nor1, $p < 0.001$, Inverse Simpson: Ore1 vs. Nor2A: $p = 0.008$; Ore2 vs. Nor2A, $p < 0.001$; Nor2A vs. Nor2B, $p = 0.002$; Nor2A vs. Nor1, $p < 0.001$). Taxonomic richness was consistently lowest in the Nor2A facility (Figure 5).

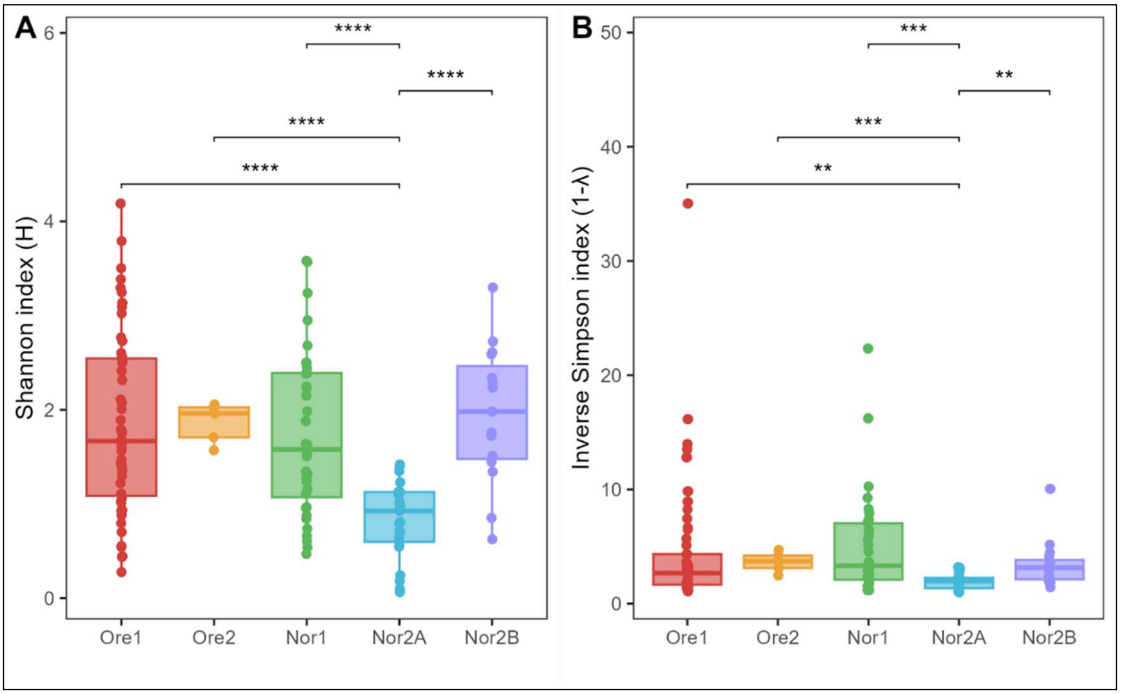

Figure 17: Alpha diversity metrics of zebrafish gut microbiota with (A) Shannon diversity and (B) Inverse Simpson diversity. Tukey-style box and whisker plots display the median (center horizontal line) and interquartile range (upper and lower bounds of the box), while whiskers extend +/- 1.5 times the interquartile range. Significance calculated with pairwise Wilcoxon rank-sum test with Benjamini-Hochberg p-value correction (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, **ns** = non-significant).

Zebrafish gut microbiomes included 15 taxa at a mean relative abundance threshold of 1% averaged across all facilities. Three of these abundant taxa were shared across all facilities- *Cetobacterium* (phylum Fusobacteriota), *Aeromonas* (phylum Proteobacteria), and *Plesiomonas* (phylum Proteobacteria). Additionally, of the identified taxa three were unique to the fish gut microbiome, all members of the order Lactobacillales (phylum Firmicutes)- *Lactococcus*, *Lactilactobacillus*, and *Pediococcus*.

In the Ore1 and Ore2 facilities, *Cetobacterium* represented 56% and 47% of all sequences, respectively. The Nor2B and Nor1 facilities were primarily dominated by *Aeromonas* (60 and 24%, respectively), while in the Nor2A community, *Vibrio* made up an average of 47% across all sequences. *Vibrio* was also identified as an abundant taxon in zebrafish gut microbiomes in all facilities except for Nor1 (Figure 6).

A SIMPER analysis of zebrafish gut microbiomes produced results similar to those found in water, with members of the Fusobacteria and Proteobacteria phyla contributing substantially to the variation observed between the Oregon and Norwegian facilities. *Cetobacterium* accounted for approximately 20% of the observed differences between the Oregon and Norwegian facilities. Members of the Proteobacteria, *Vibrio* and *Aeromonas*, accounted for 13-22% of the differences observed among the Norwegian facilities.

Aquaculture facility is a major driver of variation in the zebrafish gut microbiome.

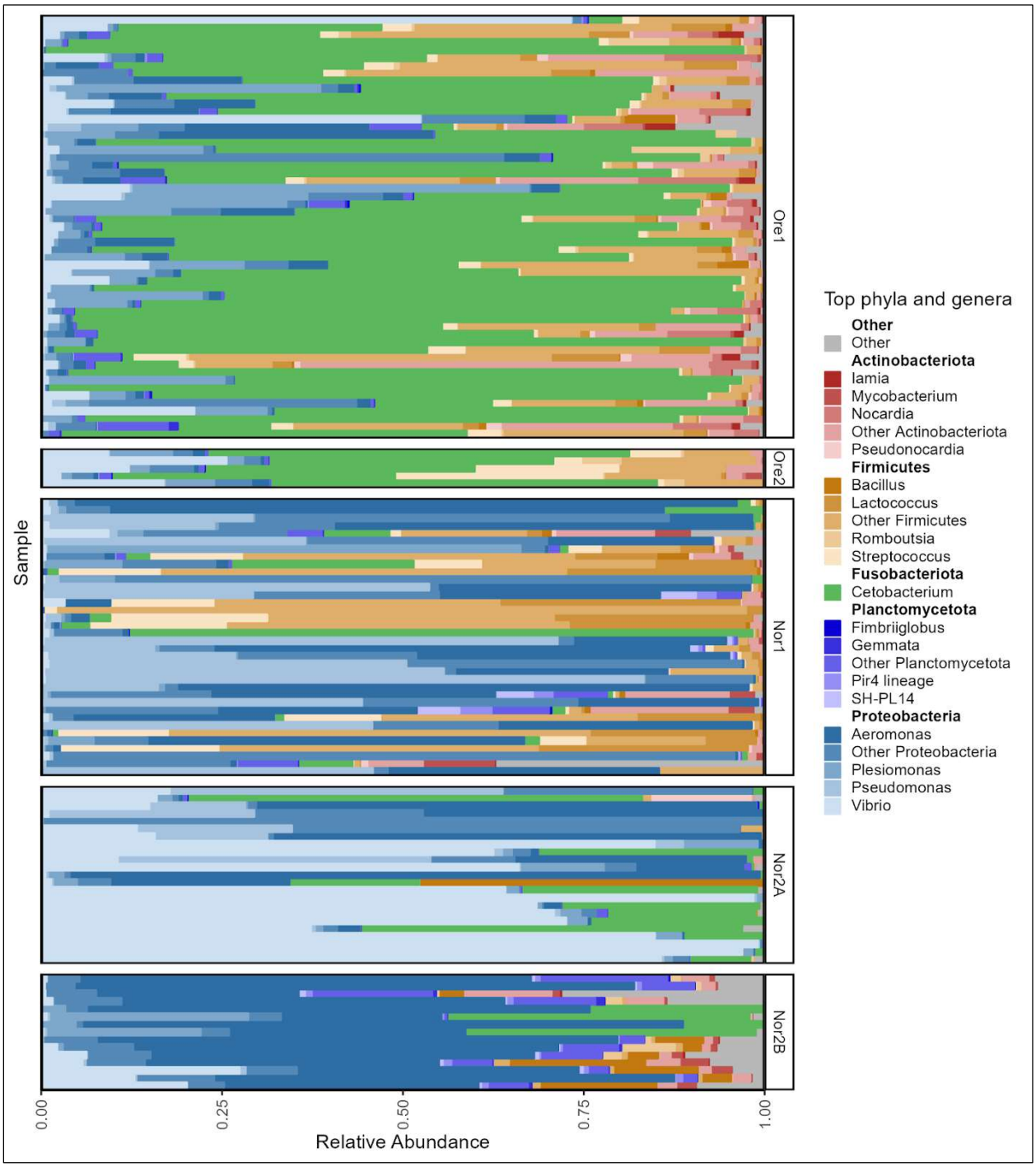

Figure 18: Phylum and genus-level taxonomic composition of zebrafish gut microbiome samples across aquaculture facilities. The mean relative abundance is grouped by aquaculture facility and is displayed for only the top five most abundant phyla and top four genera within each phylum as averaged across samples.

Compositional differences in zebrafish microbiomes were visualized using a PCoA based on Bray-Curtis and unweighted UniFrac dissimilarity (Figure 7). Despite apparent overlap in the plot, the beta-diversity of zebrafish gut microbiomes varied significantly by both location and facility (PERMANOVA; Bray-Curtis: $p < 0.001$, unweighted UniFrac: $p < 0.001$). Location explained 21.9% of variation in the Bray-Curtis and 12.4% in the unweighted UniFrac dissimilarities, and facility identity explained 14.4% of the variation in the Bray-Curtis and 14.4% in the unweighted UniFrac dissimilarities. Pairwise PERMANOVAs revealed significant variation between each pair of facilities (p-adj. = 0.01), with the sole exception of the Ore1 and Ore2 microbiomes using Bray-Curtis dissimilarity (p-adj. = 0.07). UniFrac dissimilarity values, however, indicated significant variation between these two facilities (p-adj. = 0.01).

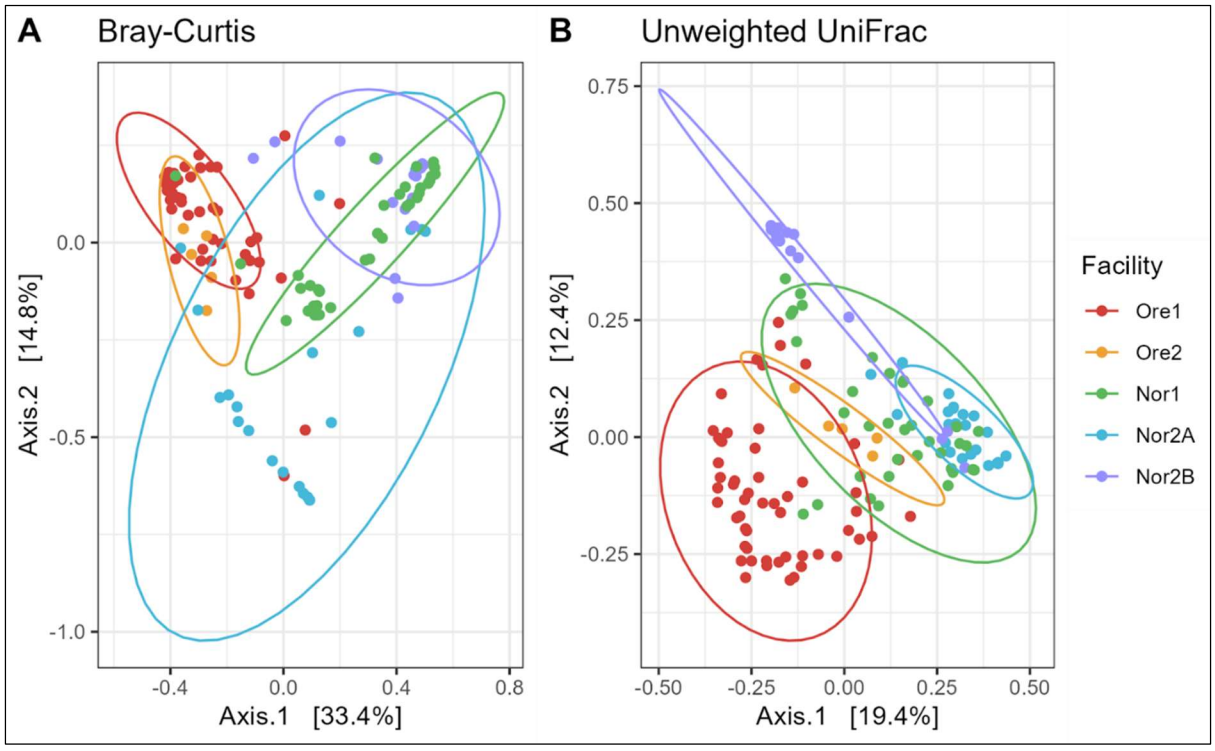

Figure 19: Beta diversity of zebrafish gut samples across aquaculture facilities. A principal coordinate analysis (PCoA) of (A) Bray-Curtis and (B) unweighted UniFrac dissimilarity. Colors represent facilities with normal confidence ellipses (95%).

3.2.3 Covariation between the zebrafish gut and water microbiome.

Once I confirmed significant variation in both the fish and water microbiome across facilities, I asked if there was significant covariance between fish and water microbiomes. I accomplished this by examining overlap in microbiome composition between matched fish and water samples across and within each facility. I then used source tracking methods to identify the relative proportion of water microbiota that could be found in zebrafish guts and vice versa.

Fish and water microbiomes share greater similarity within than between facilities.

Overall, there were 179 ASVs shared between fish and water microbiomes, nearly half of which (81 ASVs or 45%) were members of the Proteobacteria phylum. The other dominant taxa belonged to the Actinobacteriota (27 ASVs, 15%), Firmicutes (17 ASVs, 9.4%), Bacteriodota (14 ASVs, 7.8%), and Plantomyceota (12 ASVs, 6.7%) phyla. Many of these shared taxa were abundant in either fish or water (average relative abundance >1%), but just 12 were abundant in both. Of these, members of the Cetobacterium, Pseudomonas, and Aeromonas genera were shared at >5% average relative abundance in both fish and water.

I conducted pairwise comparisons of Bray-Curtis dissimilarity for fish and water collected from their own tank, tank water from elsewhere in the same facility, and water from tanks in other facilities (Figure 8). Overall, fish gut microbiomes were significantly more similar to both tank water from the same tank and within the same facility than tank water from other facilities (Kruskal-Wallis test: $p < 0.001$; pairwise Wilcoxon test: same tank vs. same facility, p-adj. = 0.013; same tank vs. other facility, p-adj. < 0.001; same facility vs. other facility, p-adj. <

0.001). Consistently, variation between fish and water microbiomes within a facility was significantly less than variation between fish and other facility water microbiomes.

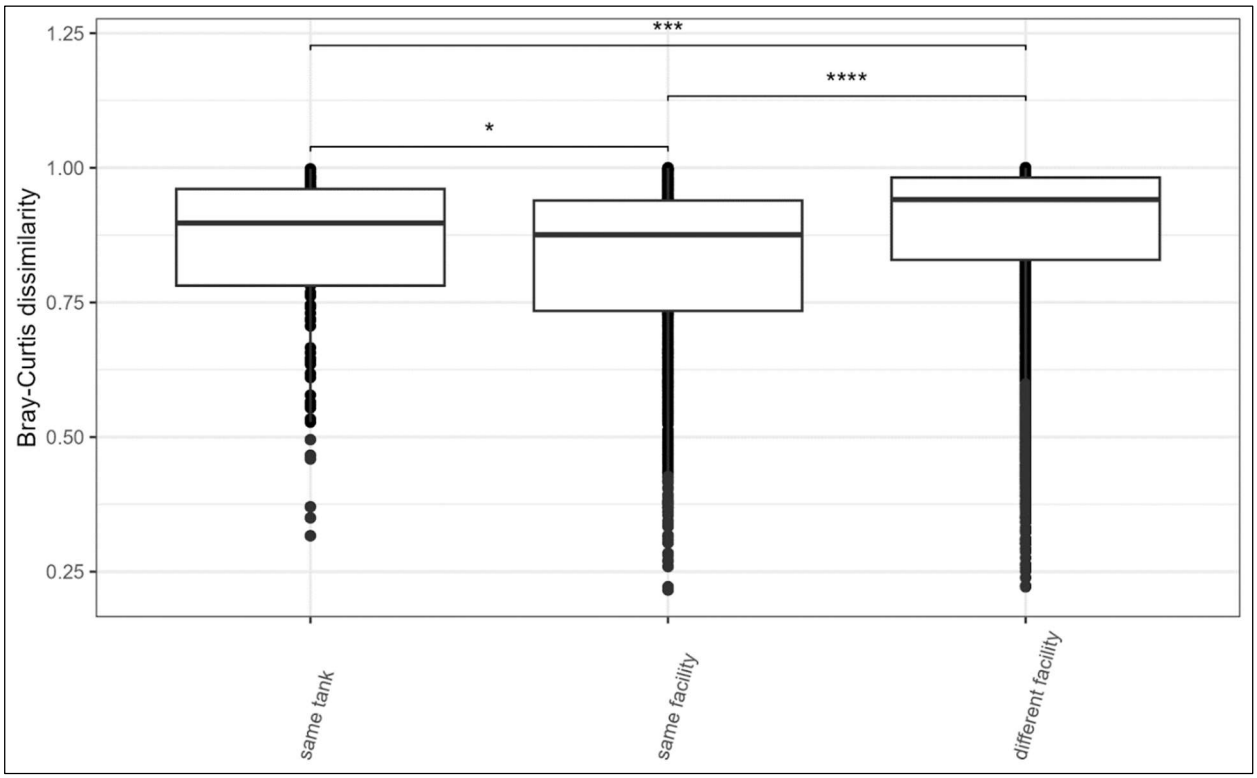

Figure 20: Tukey-style box and whisker plot featuring pairwise beta-diversity values using Bray-Curtis distance for fish gut microbiome samples paired with either 1. water from the same tank ('same tank'), 2. water from tanks within the same facility but not in the same tank ('same facility'), and 3. water from tanks in other facilities ('different facility'). Significance calculated with Kruskal-Wallis and pairwise Wilcoxon rank-sum test with Benjamini-Hochberg p-value correction (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, **ns** = non-significant).

The relative influence of the water microbiome on the zebrafish gut microbiome varies by facility.

To further assess potential acquisition of fish microbiota from the tank water environment, I performed source tracking analysis, calculated using probabilistic models in the Fast Expectation-maximization microbial Source Tracking (FEAST) R package, to identify the relative source proportion of water microbiota found in the fish gut microbiome. The FEAST package requires the assumption of directionality, so fish were first classified as 'sinks' and

water samples, ‘sources’. As directionality between species pools is difficult to determine, and this process can often be dynamic, I also performed source tracking analyses where fish were classified as ‘sources’ and water samples were classified as ‘sinks’ (Miller et al., 2018).

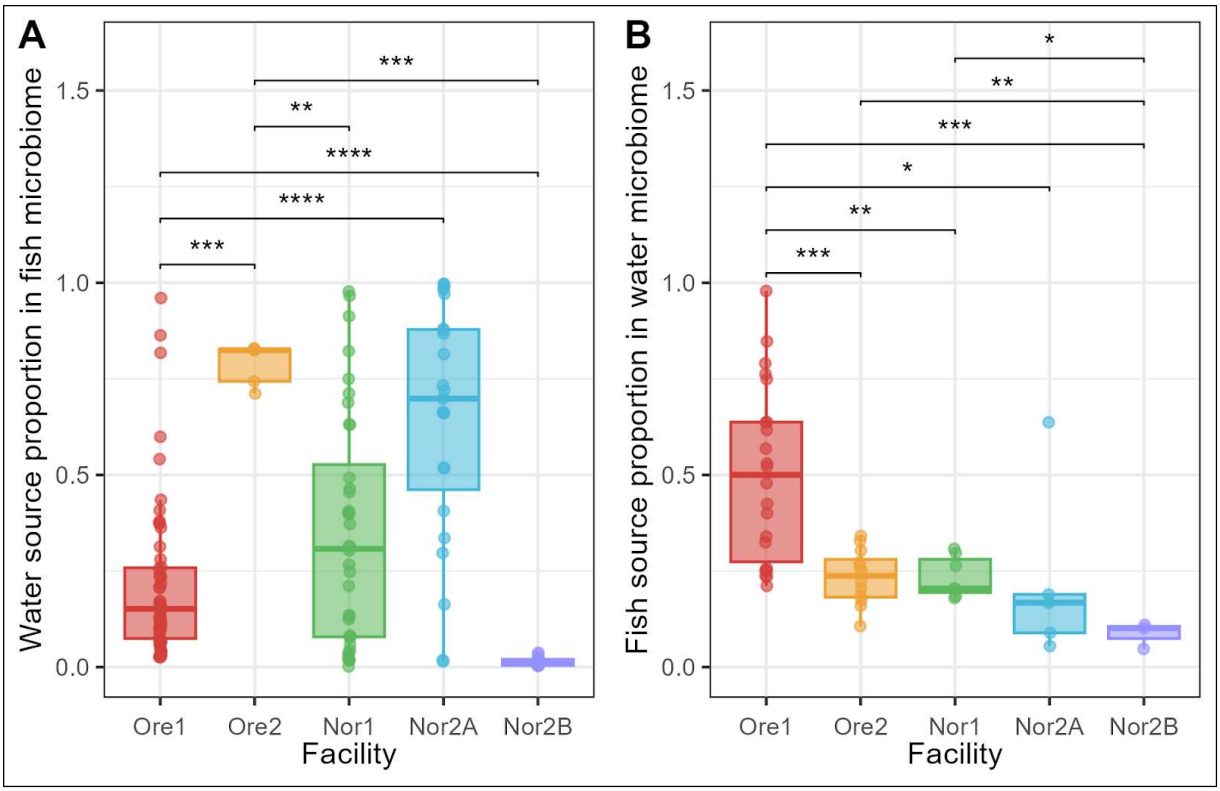

Figure 21: Source proportions for (A) water microbiota found in the zebrafish gut microbiome and (B) fish microbiota found in water microbiomes. Proportions were calculated using probabilistic models within the R package ‘FEAST’. Significance calculated with pairwise Wilcoxon rank-sum test with Benjamini-Hochberg p-value correction (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, **ns** = non-significant).

The proportion of tank water microbiota found in zebrafish gut samples varied significantly across facilities (Kruskal-Wallis: $p < 0.001$). Gut microbiota of fish in Nor2A and Ore2 facilities had significantly greater proportions of tank water microbiota than Ore1, Nor1, and Nor2B (Figure 9A). In the converse analysis with fish as the source, the proportion of fish microbiota found in water samples also varied significantly across facilities ($p < 0.001$). The Ore1 and Nor2B facilities were the only facilities where fish microbiota were found in larger proportions in water than water microbiota in fish (Figure 9B), though overall, Nor2B fish

consistently contained the lowest proportion of shared microbiota out of all facilities tested. This demonstrates that the facility water microbiome clearly exerts an influence on the fish gut microbiome, but the strength of this process is dynamic and dependent upon facility.

3.3 Discussion

In this study, the objective was to characterize microbial variation across aquaculture facilities and identify the potential for microbial sharing between the fish and water microbiomes. I assessed paired tank water and zebrafish gut microbiomes from five different aquaculture facilities that produce zebrafish for research use across the globe. The results indicate that facility factors have a significant effect on both water and fish gut microbial communities, and though water and fish microbiomes remain compositionally distinct from one another, microbial taxa are widely shared between the two. Furthermore, zebrafish microbiomes, on average, share greater similarity to water within their own facility than other facilities, and there is a significant effect of tank identity on within-facility variation. Finally, source tracking analyses demonstrate that the water microbiome influences the zebrafish gut microbiome. However, the strength of this interaction varies across facilities and the reciprocal analyses of fish microbiota indicate that there may be substantial feedback (i.e. mutual exchange of microbiota between fish and water), especially in some facilities.

3.3.1 Variation in facility water. Beta-diversity analyses of the facility water microbial communities showed differences in microbiome composition between regions (i.e. Oregon versus Norway) as well as variation among facilities within a region. Some clues to the driving force in the division of these facilities can be gathered through inspection of their community composition. Of the abundant taxa, two phyla represented greater than 75% of the

reads within most samples. These were the Proteobacteria and Fusobacteriota. While Proteobacteria were represented by multiple genera, only one genus (*Cetobacterium*) was represented from the Fusobacteriota. Both Oregon facilities were dominated by *Cetobacterium*. In fact, differences in composition between Ore1 and Ore2 were largely driven by variation in the relative abundance of *Cetobacterium* alone. Multiple studies have identified this as one of the 'core' members of the zebrafish gut microbiome (Roeselers et al., 2011; Sharpton et al., 2021), though they are commonly found in a wide range of other freshwater fish species (Ofek et al., 2021; Ramirez et al., 2018; Yajima et al., 2023). Stephens et al. (2015) has previously shown that *Cetobacterium* appears to be enriched in the intestines of adult zebrafish. Additionally, there is some evidence that its presence is negatively associated with parasite burden, can facilitate glucose homeostasis, and some *Cetobacterium* strains have shown promising results as a candidate probiotic for liver health (Gaulke et al., 2019; Wang et al., 2020; Xie et al., 2022). Other highly prevalent taxa in both facilities, including *Psychrobacter* and *Vibrio*, are also widely found in fish guts (albeit many *Vibrio* spp. are notable pathogens; Manchanayake et al. 2023; Quero et. al 2022; Zhao et al., 2022). Given that results of source tracking analyses indicated that in the Ore1 and Ore2 facilities, source proportions of fish microbiota accounted for upwards of 50% and 25% of the water microbiome, it's clear that microbial inputs from fish may be one of the aspects driving differences in the water microbiomes of the Oregon facilities.

In contrast, water samples from the Norwegian facilities were dominated by Proteobacteria. While Proteobacteria is a phylum that contains a large range of bacterial taxa, the most abundant members found within our water samples were primarily isolated from open water and sediment, though their common appearance in samples of fish microbiota may indicate a "niche preference for fish" (Sylvain et al., 2022). For example, in the Nor2B facilities, the most

abundant taxa included *Acidovorax* and *Nevskia*- both of which have predominantly isolated from wastewater and open water environments (Babenzien & Cypionka, 2015; Wang et al., 2024), though one study has also noted enrichment of *Nevskia* in larval zebrafish exposed to microplastics (Zhao et al., 2021). Nor1, similarly, had many abundant taxa (i.e. *Delftia*) commonly found in water and soil (Bhat et al., 2022; Goldschmidt-Clermont et al., 2008), though also seen sporadically in pathogen-challenged zebrafish guts (Stressmann et al., 2020; Vargas et al., 2021). Finally, in Nor2A, the most abundant genera were *Rheinhemera*. This taxon is often found in aquatic systems and soil (Presta et al., 2017; Sheu et al., 2018), but is often enriched in zebrafish exposed to antibiotics, often through the germ-free derivation process (Phelps et al., 2019; Weitekamp et al., 2019).

One hypothesis for the difference between the Oregon and Norwegian facilities is their relative age and density of housed fish. The Ore1 and Ore2 facilities have been in operation since 2012 and 2001, respectively, while the Nor1 facility was established in 2014 and the Nor2A and Nor2B in 2015. Given that these facilities use RAS systems, water housing fish in Oregon has been recirculating through the same closed loop system for years longer than those in Norway. Additionally, there are just far more fish in both the Oregon facilities than the facilities in Norway (i.e. approx. 50,000 in Ore1 and 30,000 in Ore2 vs. 10,000 in Nor1 and 100-200 in Nor2A and Nor2B). This would allow the microbial communities within the biofilter, the water column, and the various biofilms throughout the facility plumbing to mature and possibly become more reflective of the fish housed within them. Similar effects can be seen in the microbiota of other built environments, such as office buildings and homes, where the presence, number, and activity of hosts can lead to the environmental microbiome becoming more reflective of the host-microbiome over time (Young et. al 2023, Meadow et. al 2014).

Additionally, there are far more disease-model genotypes housed in the Norwegian facilities compared to the Oregon facilities- most likely due to the nature of the research of the lab groups associated with each-, which may explain the prevalence of microbiota associated with pathogen and antibiotic-challenged fish.

Another possible explanation for why I observed an influence of facility location (Oregon vs. Norway) is the general maintenance and care routines of the physical tank environment. The Oregon facilities maintain the strictest protocols, changing tanks once every two weeks or earlier if visible algal or biofilm growth is observed (T. Mason, Ore1 facility manager, personal communication, September 20, 2023; D. Lains, Ore2 facility manager, personal communication, January 20, 2022). The Norwegian facilities Nor2A and Nor2B similarly follow a two-week cleaning schedule (F. Jutfelt, Nor2A and Nor2B facility manager, personal communication, January 13, 2024), while the Nor1 facility changes tanks once every two to three months (E. Yaksi, Nor1 facility manager, personal communication, October 10, 2023). All facilities regularly use personal protective equipment to limit potential cross-contamination within the facilities.

Although similar protocols were maintained in each facility, anecdotally, nearly all the tanks sampled in the Norwegian facilities had at least some visible biofilm and algal growth. This was not observed in the Oregon facilities. Thus, the high proportion of fish-associated microbiota found in the water microbiome of the Oregon facilities may be explained by the disturbance of the tank environment through regular tank cleaning and replacement. Regular disruption of the environmental microbiome in the Oregon facilities may reduce competitive water-associated taxa and allow open niche space for fish-associated microbiota that are

dispersed into the environment via the zebrafish host to proliferate. In contrast, by allowing competitive taxa found in biofilm and algal communities to grow in the tank water of the Norwegian facilities, dispersal of microbiota from fish into the environment could have less of an influence on the tank water microbiome, resulting in a more stable water microbiome. If the tank water microbiome converged towards a similar stable state, this could offer another explanation as to why we observed no significant differences among the Norwegian facility tank water microbiota.

3.3.2 Variation in zebrafish. The zebrafish gut samples shared many similarities to patterns seen in the tank water samples. Although variation in alpha-diversity between facilities was far greater, the composition of the zebrafish microbiome showed significant influence of both location and facility origin. In Roeselers et al. (2011), the authors compared the microbiomes of wild and domesticated zebrafish. In their paper, Proteobacteria represented over 99% of all reads in wild-caught zebrafish raised in laboratory aquaculture facilities but made up considerably smaller proportions of the microbiome of zebrafish genotypes that had been maintained for many generations in such facilities. Notably, this pattern was seen in both the Nor2A facility and the Nor2B facility, where Proteobacteria comprised the greatest proportion of reads- upwards of 95% and 75%, respectively- while making up a far lesser proportion of reads in the other facilities.

Unlike the other facilities, which maintain many domesticated zebrafish lines, the Nor2B zebrafish are approximately sixth generation descendents of wild zebrafish collected in India in 2015. Those in Nor2A are a cross between these wild descendents and a domesticated Casper line. This proximity to “wildness” could explain some of the compositional patterns seen in these facilities. However, both the Fusobacteriota that dominates the Oregon facilities and the

Proteobacteria abundant in the Norwegian facilities are considered part of the shared 'core' microbiome (Roeselers et al., 2011; Sharpton et al., 2021).

Effects of genotype on the composition and structure of the host microbiome have been well documented in zebrafish research (Lu et al., 2021). As such, I attempted to include genotype as a factor in our mixed models. In the facilities, there were many different lines and sublines of zebrafish that could be considered either wild-type or a genetic modification of these lines and were often confounded with facility (i.e. wild-type strain 'ABCxTu' was only found in Ore1 while the genetically modified strain 'HuC/GCaMP' was only found in Nor1). Therefore, I created a binary classification where all lines that could be considered wild type were coded as 'WT' and all lines that were genetically modified for a specific experimental purpose as 'GM'. Though genotype coded in this manner was consistently not a significant factor in my models, I can assume that the number and variety of zebrafish lines housed in a facility could explain at least some of the differences observed between facilities given the complexity introduced through decades of cross-breeding and genetic alteration (Sprague et al., 2001; Trevarrow & Robison, 2004).

3.3.3 Zebrafish gut and water covariation. Lastly, in trying to link the variation seen in the microbial communities of both fish and water, I observed that many of the patterns in diversity and composition unique to individual facilities were reflected in both sample types, suggesting that there is active sharing of microbiota between the two. It is difficult to establish whether this sharing was reciprocal or primarily in one direction, so I took two approaches to infer the directionality of transfer. First, I considered the identity of the shared bacterial taxa, and whether they were considered primarily aquatic or fish-associated. A caveat to this approach is that information is extremely limited on the associations of specific bacterial taxa and

associations and function can be dependent on specific locations or lines as well as being limited by our sequencing technique (Blazewicz et al., 2017; Lebov et al., 2020).

To complement this approach, I also used a source tracking method to estimate whether the shared taxa likely originated from the water or the fish guts. The source tracking analysis I used, conducted with the R package 'FEAST', requires the assumption of directionality in the transmission of microbiota. Since there is no prior conclusive evidence for the direction of transmission between fish and water in these systems, I performed the source tracking analyses once with fish designated as the 'source' and once as the 'sink'. The goal of this reciprocal analysis was to determine if there was any clear pattern of directionality; for example, if there were large proportions of water microbiota found in fish but little to no fish microbiota found in water.

In the Oregon facilities, there was significant overlap between ASVs shared between fish and water samples. Compared to the Norwegian water samples, the tank water community in Ore1 and Ore2 had a large proportion of taxa from the phylum Fusobacteriota and Firmicutes. As discussed previously, the only genus identified in Fusobacteriota- *Cetobacterium*- has been widely found associated with freshwater fish. Though not definitive, this was the case with many of the abundant taxa from the Firmicutes as well. For example, two of the most abundant Firmicutes taxa in Oregon water samples included *Epulopiscium* and *Exiguobacterium*, both of which there is evidence of association with host gut and skin, respectively (Angert, 2021; Krotman et al., 2020). In these two facilities, the abundance of fish-associated microbiota found in the water microbiome suggests there may be substantial microbial input from the fish, seeding the water microbiome.

In the Norwegian facilities, there were fewer abundant taxa in the water microbiome that were also found abundantly in the fish gut microbiome. Conversely, when looking at the dominant taxa in the water microbiome, these were also not necessarily found at high abundances in the fish microbiome. Although the composition of both the water and fish microbiomes across all the facilities appear similar to one another in regard to the relative proportion of Proteobacteria each share, highly abundant water microbiota were not necessarily seen in proportionally greater abundance in the zebrafish microbiome. Instead, the variation in fish across facilities was largely driven by compositional shifts in abundant microbial taxa already generally considered to be part of a normal zebrafish gut microbiome. However, zebrafish and water did share several rare ASVs that were unique to each facility, suggesting that in addition to shifts in numerically dominant taxa, key rare taxa are also contributing to the differences seen between facilities (Schmidt et al. 2015).

In general, results from source tracking analyses highlighted the highly variable nature of microbial sharing between fish and water seen across aquaculture facilities. Water microbiota appeared to be far more prevalent in fish reared in RAS systems (Ore1, Ore2, Nor1, and Nor2A) than MMS (Nor2B). Considering we only investigated one MMS facility in this study, however, these results may be anomalous to this facility alone. Beyond that, there were no clear patterns across facilities as to what proportion of water microbiota could be found in the zebrafish microbiome. Reciprocally, facilities with smaller source proportions of water microbiota in the fish microbiome did not necessarily have large source proportions of fish microbiota in the water microbiome. The Ore1 water microbiome, by far, showed the largest influence of fish microbiota. Other than Ore1, however, the fish input of microbiota appeared relatively stable across all facilities. This may indicate that fish have a relatively stable input of microbiota to the

environment, but the influence of the environmental microbiome on the fish is greatly facility specific.

These source tracking results are generally consistent with my observations of the relationship between the zebrafish and water microbiomes across facilities. Variations in water microbiomes do not appear to exert predictable compositional shifts in the zebrafish microbiome, but there is evidence that microbes are readily shared between the two. This observation is consistent with observations of other aquatic organisms, such as common bottle-nose dolphins, California sea lions, Atlantic cod, and Mediterranean gilthead sea bream; while host and environmental microbiomes share some similarities, environmental abundance and diversity does not necessarily correlate with changes in the host, and the microbiomes of each remain largely distinct (Bakke et al., 2013; Bik et al., 2016; Quero et al., 2022).

A major potential limitation to this study is that the microbial communities captured by my sampling efforts represent the microbiomes of both fish and water at a singular time point. Based on my observations in Chapter 2, we have evidence that the composition of the water microbiome varies significantly over time. Given that variation in the water microbiome is correlated with variation in the fish gut microbiome, this temporal variation may mean that the relative influence of the water microbiome changes over time and repeated sampling efforts at more time points would provide more accurate insight into the interactions between fish and their environment.

While I cannot define the mechanism and directionality of transmission between the zebrafish gut microbiome and the environmental microbiome of the surrounding tank water, my results indicate that zebrafish and tank water microbiomes shared large proportions of microbiota, and both were heavily influenced by aquaculture facility and geographic location.

Zebrafish are used as animal models in foundational research, and variation in their microbiome can have significant effects on the reproducibility of phenotypes. As stated at the start of this paper, the host-microbiome mediates many aspects of host health and function, and studies in mice and primates have already noted disparate experimental outcomes directly linked to microbial variation associated with facility origin (Mandal et al., 2020; Parker et al., 2018; Shigeno et al., 2022). The microbiome variation I observed across facilities and regions could reduce the reproducibility of zebrafish research focused on microbiome-mediated processes. My results suggest that one potential driver of this variation could be differences in hygiene practices across the facilities I sampled; increasing consistency in such practices across facilities could increase reproducibility. Future work should focus on identifying other facility-specific factors which could be altered to reduce microbiome variation, and potentially increase experimental reproducibility, across zebrafish facilities.

3.4 Conclusion

My results demonstrate that even in relatively controlled aquaculture facilities, facility-specific differences can be seen in both water and zebrafish gut microbial communities. I found evidence that variation in the zebrafish microbiome was being driven by environmental acquisition of microbiota from the tank water, though the magnitude of this influence varied across facilities. Microbial sharing between zebrafish and their environment is most likely just one part of a much larger landscape of facility-specific factors shaping the zebrafish gut microbiome, and future studies should consider isolating potential factors to characterize mechanisms and magnitude of transmission. This study expands our current understanding of the factors influencing the microbiome of a popular animal model, and I highlight that more in depth

sampling and monitoring needs to be done to identify the factors shaping zebrafish gut microbiome assembly in aquaculture facilities. Further, I caution that care should be used when using zebrafish models sourced from single facilities for research given that microbiome variation could greatly influence experimental outcomes.

3.5 Methods

3.5.1 Study sites. The study sites in this Chapter included five zebrafish aquaculture facilities used for academic research in the United States and Norway. Both facilities in the U.S. are hosted on the main University of Oregon campus in Eugene, Oregon. The first, the Aquatic Animal Care Services Zebrafish Facility (Ore1), is a 1,000 square-meter facility with an average capacity of 50,000 fish (maximum capacity~88,000). The facility life support system utilizes a recirculating aquaculture system (RAS) containing mechanical and biological filters in addition to ultra-violet disinfection. The facility has two separate RAS systems with a UV-treatment step supplying the main housing and a quarantine area with a separate flow-through system.

The second U.S. facility, the Zebrafish International Resource Center (Ore2), is a 900 square-meter facility with an average capacity of 30,000 fish (maximum capacity~150,000). Ore2 maintains the same layout of life support systems as the Ore1 facility.

The Norwegian zebrafish facilities are located on the Norwegian University of Science and Technology Trondheim campuses in Trondheim, Norway. The first facility, the Yaksi Lab (Nor1), is located on the Øya campus and maintains approximately 10,000 zebrafish in a 70 square-meter facility. It utilizes a singular RAS system with UV-treatment.

The other Norwegian facility, the Jutfelt Lab, is located on the Gløshaugen campus. The Jutfelt Lab fish facility maintains both populations of zebrafish and guppies (*Poecilia reticulata*). Because the facility consists of multiple small rooms with separate life support systems within each, we reference Jutfelt as separate facilities (Nor2A and Nor2B) in our analyses though they are technically maintained under the same administrative body. We conducted our research in two zebrafish-only rooms within the facility, each approximately 10 square-meters. The first room (Nor2A) contains approximately 50, 3.5L tanks utilizing a standalone RAS system with UV-treatment. The second room (Nor2B) houses approximately 14, 75L tanks with a flow-through filtration system using a maturation tank without UV-treatment. The entirety of the facility houses around 2,000 fish.

3.5.2 Sample collection and processing.

Tank water

Tank water was collected from a representative number of tanks based on the criteria that they were on a single RAS system and contained adult zebrafish. I used a nested sampling design, though the actual number of tanks sampled was dependent on facility size and housing setup (n = Ore1 (*27*); Ore2 (*17*); Nor1 (*7*); Nor2A (*6*); Nor2B (*3*)). For the nested sampling design, I selected one to two sets of shelves immediately adjacent to one another connected to the same RAS system. For each set of shelves, I then selected three random shelves. On each shelf, I sampled from three randomly selected tanks. If there were less than three shelves or tanks (for example, in Nor2B, which had two shelves containing two tanks each) I sampled from all available. 150-mL of tank water was passed through a Sterivex filter cartridge (MilliporeSigma, Massachusetts, USA) using a 60-mL syringe. Sterivex filters were capped and kept on ice until

processing. If DNA extraction could not be performed immediately, dry cartridges were stored at -80°C.

Zebrafish

Zebrafish were selected from matching tank water samples (*n* = Ore1 (*55*); Ore2 (*5*); Nor1 (36); Nor2A (*23*); Nor2B (*15*)) and euthanized via rapid chilling. I sampled four fish per tank. In the event there were not enough fish, or I was otherwise limited in my sampling, I sampled as many as were available. The whole intestine was aseptically dissected the same day and placed into a 2-mL RHINO screw cap tube containing zirconium oxide beads and 400 µL enzymatic lysis buffer (20mM Tris-Cl, pH-8.0; 2mM sodium EDTA; 1.2% Triton X-100). If DNA extraction could not be performed immediately, tubes were stored at -80°C.

3.5.3 Microbial DNA extraction.

Tank water

DNA extractions were performed using the DNeasy PowerWater Sterivex kit (QIAGEN, Carlsbad, California, USA). DNA was extracted following the standard protocol provided in the kit handbook (2009, pg. 9).

Zebrafish

DNA extractions for all zebrafish gut samples were performed following an adapted protocol based on the DNeasy Blood & Tissue Kit Quick-Start protocol as described in Stephens et al. (2015).

3.5.4 16S rRNA gene amplification, library preparation, and sequencing. DNA extraction products were quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher

Scientific, Waltham, MA, USA). PCR amplification of the V4 region of the 16S rRNA gene was performed using a dual-indexed 515F-806R primer combination based on protocols described in Caporaso et al. (2011). Each 515F (AATGATACGGCGACCACCGAGATCTACAC*xxxxxxxx*TATGGTAATTGTGTGCCAGCMGCCGCGGTAA) and 806R (CAAGCAGAAGACGGCATACGAGAT*xxxxxxxx*AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT) primer pair were modified with unique barcodes (represented by italicized portion of primer above). PCR mixtures contained 12.5 µL NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 10.5 µL DNA template, 1 µL Bovine Serum Albumin (Thermo Fisher Scientific), and 1 µL mixed primers (12.5 mM concentration). PCR cycling steps were denaturation at 98°C for 30 seconds, 30 amplification cycles at 98°C for 10 seconds, 61°C for 20 seconds, and 72°C for 20 seconds, followed by a final extension at 72°C for 2 minutes. Samples were held at 4°C until removal from thermocycler.

PCR products were visually checked for successful gene amplification via agarose gel electrophoresis. Amplicon libraries were cleaned twice with Mag-Bind RxnPure Plus isolation beads according to an adapted kit protocol using a 0.8x reaction volume (Omega Bio-Tek, Norcross, Georgia, USA). Concentrations of amplicon libraries were quantified with the Quant-iT 1x dsDNA HS Assay kit (Thermo Fisher Scientific) using a SpectraMax M5E Microplate Reader (Molecular Devices, San Jose, CA, USA) and multiplexed at equimolar concentration. Amplicons were then sequenced by the University of Oregon Genomics & Cell Characterization Core Facility on the Illumina MiSeq (Illumina, San Diego, CA, USA) with 150-bp paired-end reads.

3.5.5 Bioinformatics. All bioinformatics processing was performed in 'R' (R Core Team, 2018). Sequences were demultiplexed and then denoised to construct an amplicon sequence variant (ASV) table using DADA2 1.16 (Callahan et al., 2016). Taxonomy was assigned to sequences using the RDP Classifier and Silva NR99 v.138.1 16S rRNA gene reference database (Quast et al., 2012; Wang et al., 2007). Sample contaminants were evaluated using the frequency method with 'DECONTAM' (Davis et al., 2018).

3.5.6 Statistics. I performed all statistical analyses in 'R' (R Core Team, 2018). I calculated metrics of alpha diversity (Shannon and Inverse Simpson) and beta diversity (Bray-Curtis and unweighted UniFrac) using the 'vegan' package and tables of ASVs (Bray & Curtis, 1957; Oksanen, 2022). All plots were created with 'ggplot2' (Wickham, 2016). To test for differences in alpha diversity between the water and zebrafish gut microbiomes by facility, I used the non-parametric tests Kruskal-Wallis (Hollander & Wolfe, 1973) and pairwise Wilcoxon rank-sum with Benjamini-Hochberg p-value adjustment (Benjamini & Hochberg, 1995; Wilcoxon, 1945). To test the effects of Location and Facility on both fish gut and water microbiomes, I used Permutational Multivariate Analysis of Variance (PERMANOVA) on Bray-Curtis and unweighted UniFrac distance values. Pairwise PERMANOVA using the 'pairwise.Adonis' package was conducted to test for the pairwise effects of facility (Martinez Arbizu, 2020). Additionally, I used a principal coordinate analysis to visualize clustering of microbiome composition.

Relative abundance plots were created by first agglomerating ASV data by taxonomic classification level. Relative abundance values were then calculated by dividing sums of ASVs by total counts. Nested bar plot graphs were created using the 'fantaxtic' package in R (Teunisse, 2022).

To determine which ASVs were contributing to differences in fish and water microbiota between facilities, I used a 'Similarity Percentage' (SIMPER) analysis with Bray-Curtis dissimilarity (Clarke, 1993). This analysis partitions Bray-Curtis dissimilarity and calculates the average contribution of taxa to the difference between every sample pair.

For pairwise beta-diversity plots, I classified paired Bray-Curtis dissimilarity values of fish and water across all facilities as either 1) from the same tank, 2) not from the same tank but from the same facility, and 3) not from the same tank and not from the same facility. Values from all facilities were then plotted by classification status. I tested differences between classification status with nonparametric Kruskal-Wallis (Hollander & Wolfe, 1973) and pairwise Wilcoxon rank-sum with Benjamini-Hochberg p-value adjustment tests (Benjamini & Hochberg, 1995; Wilcoxon, 1945).

Finally, to test if water microbiota were present in the zebrafish gut and vice versa, I used the package 'Fast expectation-maximization microbial source tracking', or 'FEAST' (Shenhav et al., 2019). 'FEAST' uses maximum-likelihood probabilistic models and machine-learning classification to model microbial source tracking (Shenhav et al., 2019). Using matched fish and water samples from one facility at a time, I assigned all the water samples from within a singular facility as potential 'sources' for every fish sample- assigned as 'sinks'. Source proportion values were then summed across every input source for each sink and plotted as a singular value. For example, for every fish, the relative proportion from each potential water source was summed. This was then repeated with the source and sink assignments flipped. To test for differences in source proportion values between facilities, I used a non-parametric Wilcoxon rank-sum test with a Benjamini-Hochberg p-value adjustment (Benjamini & Hochberg, 1995; Wilcoxon, 1945).

3.6 Bridge

In Chapter 3, I investigated the dynamic interaction between zebrafish and tank water microbiomes within various research aquaculture facilities located in both the United States and Norway. Despite functional parallels, such as water treatment specifications, I detected a significant impact of each facility's identity on both zebrafish and water microbial communities. I further attempted to ascribe causation of zebrafish gut microbiome variation to facility water microbiome variation by examining compositional overlap and using source tracking analyses. However, unique aspects of each facility- such as differences in fish feed and husbandry protocols- confound my results and make it difficult to directly link regional pool and local community variation in this system, though it serves as a useful observation of "real-world" dynamics. In Chapter 4, I will attempt to isolate the effects of variation in the water microbiome on zebrafish gut microbiome assembly by experimentally manipulating the regional species pool of water microbiota in a controlled laboratory environment.

CHAPTER 4: INFLUENCE OF DIET OVERWHELMS EFFECTS OF REARING WATER DURING EARLY LIFE MICROBIOME ASSEMBLY IN LARVAL ZEBRAFISH

4.1 Introduction

Empirical studies have suggested that the richness and absolute abundance of species pools can be used to predict average richness of local communities (Belmaker & Jetz, 2012; Gerhold et al., 2008). However, the extent to which species pools influence local communities is not fully understood. Given the dynamic interplay between regional and local processes, the species pool may be predictive of biodiversity in light of habitat degradation, global change, and anthropogenic pressures (Harrison & Cornell, 2008). However, one of the main obstacles to understanding how species pools and local communities are connected is that boundaries of species pools are often difficult to delineate, and studies run the risk of overestimating regional diversity (Zobel, 2016). As was discussed earlier in this dissertation, a zebrafish model in an aquaculture system is especially useful for testing these ecological assembly questions because the local community (zebrafish gut microbiome) and species pools (tank water) can be distinctly bounded.

In Chapter 3, I examined the dynamic relationship between the zebrafish and tank water microbiome in several research aquaculture facilities across the United States and Norway. Though there were, functionally, many similarities shared among these facilities- such as water treatment specifications-, I observed a significant influence of facility identity on both zebrafish and water microbial communities. One possible reason for why I saw this effect was variation in the numerous facility-specific factors that could influence the microbial environment in a facility.

Two such factors are diet and genotype (Burns et al., 2017; Okazaki et al., 2019; Sieler Jr. et al., 2023). While each facility fed their fish an overall similar diet- most fish received rotifers, brine shrimp, and dry fish flakes at similar developmental stages across their lifetimes-, every facility sourced their feed from different locations or manufacturers. Additionally, while some facilities maintained primarily wild-type lines of zebrafish, others reared (almost) exclusively transgenic lines. Variation in these two factors alone could potentially lead to drastic differences in the microbial inputs into the microbiome of the aquaculture facilities, and these examples are far from the only observable differences.

The differences in facility factors observed among the aquaculture facilities we investigated in Chapters 2 and 3, though reflective of a "real-world" environment, potentially confound our ability to directly examine the influence of variation in the environmental microbiome on zebrafish microbiome assembly. To address this issue, I experimentally manipulated environmental microbiome variation in a controlled system for 12 days to test the isolated effect on host-microbiome assembly. In my experiment, germ-free larval zebrafish were initially reared in flasks inoculated with water from two aquaculture facilities previously investigated in this dissertation (Ore1 and Ore2). After six days of exposure to the aquaculture facility water, a subset of zebrafish were moved to new flasks inoculated with either the same facility water or different facility water. In the first part of the experiment, the goal was to isolate the influence of variation in the facility water microbiome and test whether it would cause divergence in gut microbiome assembly across related individuals. Though my previous chapters focused on adult fish, I used larval fish for this experiment as they can be reared axenically, and this would allow for control over their initial microbial exposure. The second part of the experiment was to investigate whether the magnitude of variation in the water microbiome we

observed across the aquaculture facilities in this dissertation was enough to influence the gut microbiome once established. I generated 16S amplicon sequences from samples of zebrafish gut, fish food, and water taken across the course of the experiment.

In my comparative studies in earlier chapters, I observed significant covariation between fish gut microbiomes and facility water microbiomes. However, the complexity of the aquaculture facility environment prevented the establishment of causality between fish and water microbiome variation. As such, the main goal of this study was to address the questions 1) What is the relative influence of species pool variation in early life microbiome assembly?, and 2) How does the range of variation in the environmental microbiome alter the host-microbiome after establishment? In my experiment, I observed that water microbiomes significantly varied by inoculum source, but zebrafish gut microbiomes did not. Evidence suggests microbial inputs from food were sufficient to overwhelm any effect of microbial input from water in the zebrafish gut. Other considerations for why a relationship was not observed could be that the water variation generated was not enough to observe an effect in zebrafish gut microbiomes, that selection by fish associated with genotype overwhelmed the effect of water variation, and/or that it requires a lifetime of exposure to variation in aquaculture facility water to generate an effect on the gut microbiome. However, these theories require further experimentation.

4.2 Methods

4.2.1 Experimental design and zebrafish husbandry. All zebrafish experiments were performed using standard protocols approved by the University of Oregon Institutional Care and Use Committee.

I set up approximately 40 wild-type AB fish from a single tank housed in the Huestis facility at the University of Oregon in crossing cages. The crossing cages were outfitted with dividers separating two males and two females per cage. Cages were left overnight. The following day, fish were transferred to new crossing cages with fresh water. I removed the dividers and allowed the fish to spawn for one hour. Afterwards, I removed adult fish from the crossing cages and collected fertilized embryos into a petri dish containing antibiotic embryo media (ABEM). After collection, I selected approximately 300 of the best embryos and transferred them to another sterile petri dish with fresh ABEM. After one hour had passed, I selected the best 200 embryos and repeated the process of moving them to a new petri dish. After another hour, I selected the final 100 best embryos and moved them to a sterile petri dish containing filter-sterilized ABEM. These embryos were made "germ-free" (absent of microbes) following established protocols (Melancon et al., 2017).

Part One

Germ-free embryos were evenly split among 9, 500-mL Erlenmeyer flasks for a total of 8 embryos per flask. Each flask was filled with 390 mL filter-sterilized E4 media (salinity adjusted reverse-osmosis water; Aquatic Animal Care Services, 2016). Then, I inoculated each flask with 10 mL of water collected from either 1) an AB tank in the Ore1 facility, 2) an AB tank in the Ore2 facility, or 3) molecular biology grade water. I filtered 50 mL of remaining tank water used for inoculation of flasks using a Sterivex filter cartridge. Molecular grade water and filter sterilized E4 media were plated on prepared Difco Nutrient Broth (Becton Dickinson, Franklin Lakes, NJ, USA) agar plates and incubated overnight at 34 ℃ to check for microbial growth.

Zebrafish were grown out in their respective flasks for six days. Every day, starting at 1 d.p.f. (the day after inoculation), I exchanged 300 mL of media in each flask for fresh E4 media

and measured ammonia levels. Though standard protocol dictates that media changes do not need to be performed until after larval fish have hatched and started feeding- as long as ammonia levels stay low- I did so to reduce the influence of variation in husbandry throughout the course of the experiment. Prior to media changes at 5 and 6 d.p.f., I filtered 50 mL of media from each flask using Sterivex cartridges. Fish were fed rotifers sourced from the Ore1 facility every day after media changes starting at 5 d.p.f. (Figure 1).

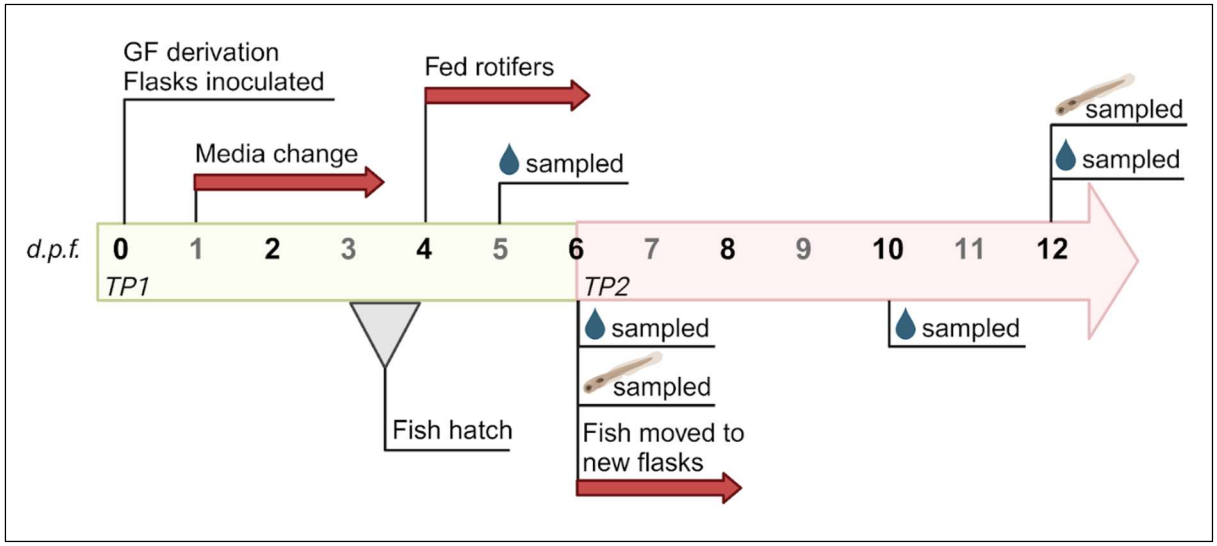

Figure 22: Experimental timeline of important data collection and husbandry events. Black lines indicate single-day events while red arrows indicate repeated events. *Figure created with Biorender.com.*

Part Two

At 6 d.p.f., prior to media change, I removed three larval fish from each flask and euthanized them via lethal overdose of tricaine methanesulfonate (MS-222). The intestines were dissected for 16S rRNA amplicon sequencing as described in Chapter 3.

After dissection, I added 390 mL of fresh E4 media to 9 new Erlenmeyer flasks. Three replicate flasks were then inoculated with 10 mL of either 1) an AB tank in the Ore1 facility, 2) an AB tank in the Ore2 facility, or 3) molecular biology grade water. Tank water used for inoculation was filtered for later 16S sequencing, and water and E4 media was plated to check

for microbial growth. The remaining fish were moved to new flasks following a factorial design as seen in Figure 2. Prior to moving, fish were netted and rinsed with sterile E4 media.

Fish were reared for an additional six days in these new flasks using the same husbandry protocols as described above. Water was sampled prior to media changes at 10 and 12 d.p.f. At 12 d.p.f., the remaining zebrafish were euthanized and dissected as described previously.

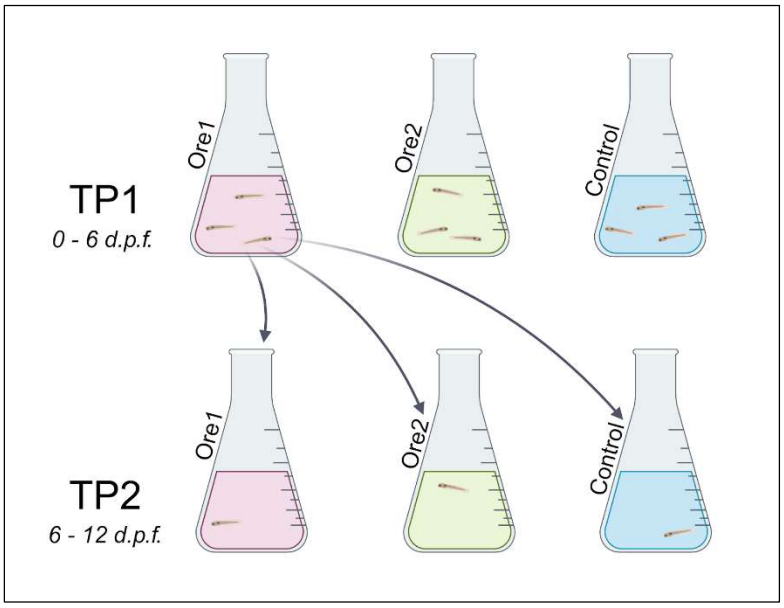

Figure 23: Part Two flask switch design. During the inoculant switch at 6 d.p.f., three fish from each flask were moved into flasks containing media with either a novel inoculant or an inoculant matching that at initial exposure (0 d.p.f.). For example, of the three remaining fish in flask Ore1(A), one was moved into a flask containing E4 media inoculated with tank water from the Ore1 facility, one was moved into a flask containing E4 media inoculated with tank water from the Ore2 facility, and the third was moved into a flask containing E4 media inoculated with molecular-grade water. *Figure created with Biorender.com.*

4.2.2 Microbial DNA extraction. All DNA extractions for fish and water were performed as described in Chapters 2 and 3. Rotifer DNA extractions were performed following the same protocols as used for fish.

4.2.3 16S rRNA gene amplification, library preparation, sequencing, and bioinformatics.

All post-extraction processing, sequencing, and bioinformatics followed the same protocols as described in Chapter 2 and 3.

4.2.4 Statistics. All statistical analyses were performed in 'R' (R Core Team, 2018). To calculate metrics of alpha diversity (Shannon and Inverse Simpson) and beta diversity (Bray-Curtis), I used the 'vegan' package and our table of ASVs (Bray & Curtis, 1957; Oksanen, 2022). All plots were created with 'ggplot2' (Wickham, 2016). Differences in alpha diversity by sample type, time point, and treatment groups were tested using a Kruskal-Wallis test using the 'kruskal.test' function (Hollander & Wolfe, 1973).

To test for multivariate differences in microbial community composition by treatment group, sample type, and time point, I used a PERMANOVA from the 'adonis2' function in the 'vegan' package using the Bray-Curtis distance. Pairwise comparisons between sample groups were tested using the 'wilcox.test' (Wilcoxon, 1945) with the Benjamini-Hochberg p-value adjustment (Benjamini & Hochberg, 1995). Further, I used Bray-Curtis distance values to create a principle coordinate analysis (PCoA) to visualize clustering in microbial composition.

Finally, to identify potential sources of microbiota found in zebrafish guts, I used maximum-likelihood probabilistic models to estimate the proportion of the microbial community in each 'sink' that could be attributed to each 'source' in the package 'FEAST' (Shenhav et al., 2019).

4.3 Results

To reveal variation in zebrafish microbiota due to variation in facility water treatment, I characterized the microbial composition of zebrafish guts, flask water, tank water used for initial inoculation, and zebrafish feed.

4.3.1 What is the relative influence of species pool variation in early life microbiome assembly?

I isolated results from TP1, prior to the switch in facility water inoculum, to investigate the relative influences of regional variation on host microbiome development. To characterize regional variation, I looked at the similarity between the initial aquaculture facility inocula and the resulting flask water microbiomes. The initial facility water inocula (Inoc1) explained 52% of the variation between flask water microbiome samples ($p < 0.001$), and there were no significant differences among replicate flasks ($p = 0.168$). Flask water microbiome composition was completely dominated by Proteobacteria, namely the genera *Novosphingobium*, which accounted for 29-59% of all sequences across flasks, and *Vibrio*, which accounted for 18 and 21% of sequences in flask water inoculated with Ore1 water and Ore2 water, respectively.

Comparatively, there was no significant influence of facility water inoculum on the fish gut microbiome in TP1 ($p = 0.511$), nor was there any evidence of a flask effect ($p = 0.238$). *Vibrio* was the most common taxa, making up > 50% of reads across all fish samples.

The dissimilarity and general taxonomic microbiome composition between the Ore1 and Ore2 facility water used as inoculants was similar to that seen in previous chapters (average Bray-Curtis distance ~0.88) though significance could not be tested as there were too few samples. Proteobacteria dominated samples from both facilities; *Perlucidibaca* (30% average) and *Limnobacter* (10% average) were most abundant in Ore1, and *Vibrio* (14% average) and *Cetobacterium* (30% average; phylum Fusobacteriota) were the most abundant taxa in Ore2. Similarly, the Proteobacteria *Vibrio* (77% average) and *Pseudomonas* (22% average) were the two most abundant taxa identified in rotifer samples.

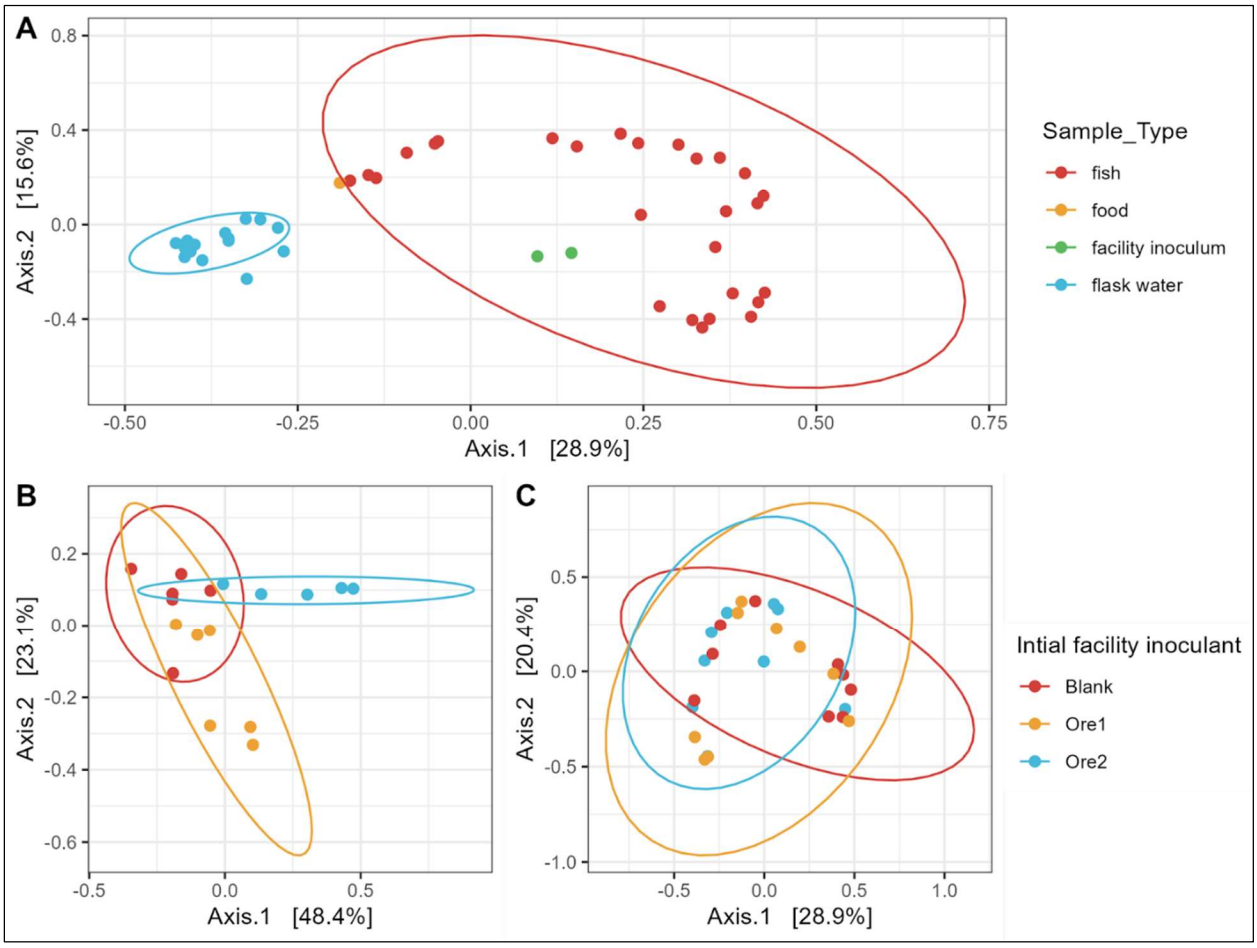

Figure 24: Beta diversity of zebrafish gut, fish food, facility water inoculants, and flask water microbiome samples using Bray-Curtis dissimilarity for TP1 (0-6 d.p.f.). A principal coordinate analysis (PCoA) of (A) all sample types, (B) flask water, and (**C**) fish gut microbial samples. Colors in (**A**) represent sample type while colors in (**B**) and (**C**) represent the facility water source used to inoculate flask water at 0 d.p.f. Normal confidence ellipse (95%) groupings match each plot legend.

PERMANOVA results are supported by a PCoA; overall microbiome samples cluster by sample type. Within each sample type, there is slight clustering in water microbiome samples by initial facility inoculum, but fish gut microbiome samples completely overlap (Figure 3).

To evaluate the potential sources of zebrafish microbiota, I used FEAST to perform source tracking on all potential source samples (i.e. food and initial facility inoculant; Figure 4). Rotifers (food) had the greatest relative source proportion of microbiota in zebrafish across all experimental treatments. Very few, if any, zebrafish microbiota could be attributed to initial facility inoculants. This suggests that, in my experiment, the assembly of the fish gut

microbiomes was greatly influenced by the immigration of microbiota from diet sources rather than from the surrounding tank water. This could also include indirect acquisition via the environment if microbiota from rotifers are first passed to flask water and then dispersed into fish guts.

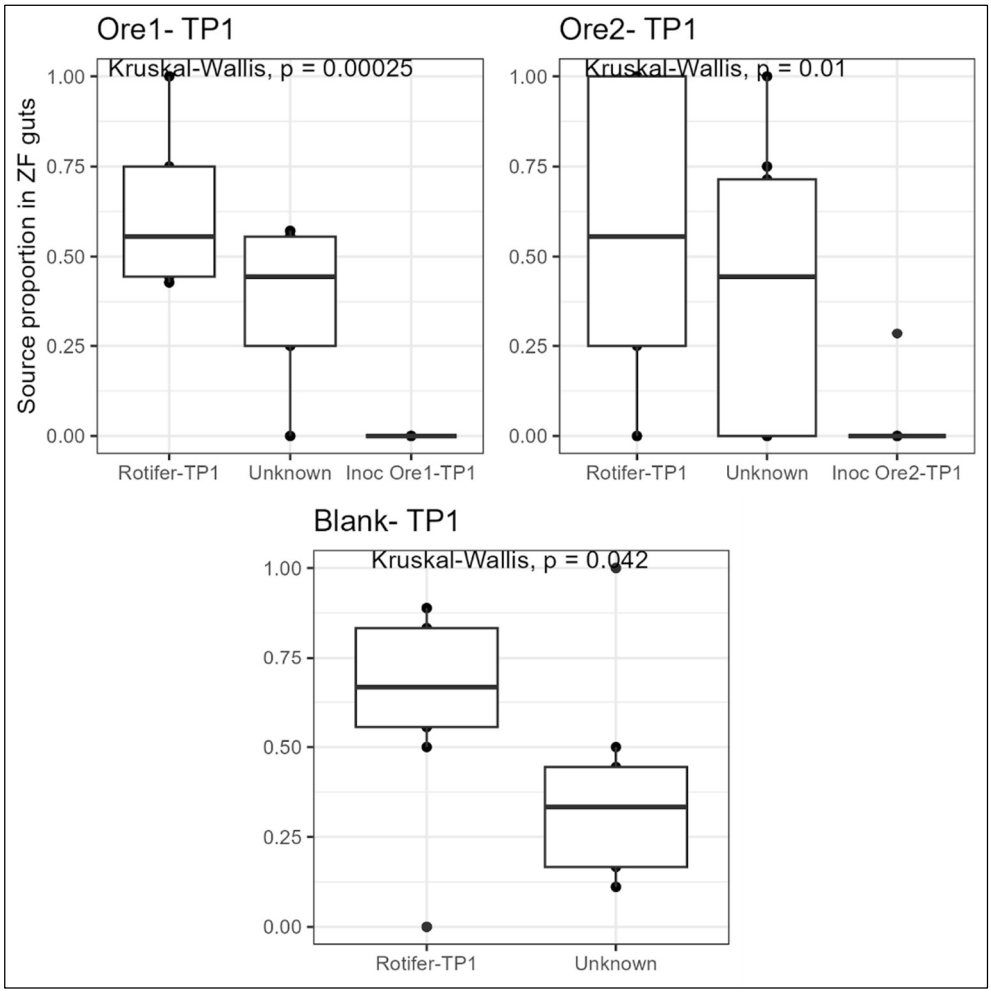

Figure 25: Source proportions of food ('Rotifer'), facility inoculant ('Inoc'), and a default 'Unknown' source microbiota in zebrafish gut microbiomes for each treatment in TP1. Proportions were calculated using probabilistic models within the R package 'FEAST'. Significance calculated with a Kruskal-Wallis test.

4.3.2 How does the range of species pool variation alter host microbiome development when a microbiome is already established?

To answer the second question- how species pool variation may influence an established host-microbiome- I first looked at microbial communities in TP2. Unlike TP1, after switching facility water inoculant, flask water microbiomes no longer exhibited significant variation among facility water inoculant, even when controlling for initial inoculant in TP1 (Inoc 1: $p = 0.973$; Inoc2:Inoc1, $p = 0.824$). Additionally, all water microbiomes regardless of facility water inoculum in TP1 or TP2 were now primarily dominated by *Pseudomonas*, *Vibrio*, and *Flavobacterium*. These were not only in greater abundance than in TP1, (i.e. *Pseudomonas* accounted for an average of >30% of all reads in TP2 vs. 14% in TP1), they were uniformly present in almost equal abundance across all flasks.

In fish, results were very similar to those found in TP1. There appeared to be no significant effect of facility water treatment on gut microbiome samples (Inoc1: $p = 0.518$; Inoc2:Inoc1, $p = 0.237$), nor was there any significant difference between replicate flasks ($p = 0.214$). Overall community composition was so similar to TP1 that a differential abundance analysis identified no significant taxa. *Vibrio* was the most dominant taxa in all flasks, making up an average of at least 50% across all fish samples. Fish that were inoculated with Ore1 in TP1 had the highest average relative abundance, at 81% of all reads.

Like fish, facility water inoculant looked very similar to samples in TP1. Rotifer samples in TP2 were dominated by *Microbacterium* though *Vibrio* still accounted for about 25% of reads. In a PCoA, although microbiome samples were still clustered by sample type, there was complete overlap in both fish and water individually (Figure 5).

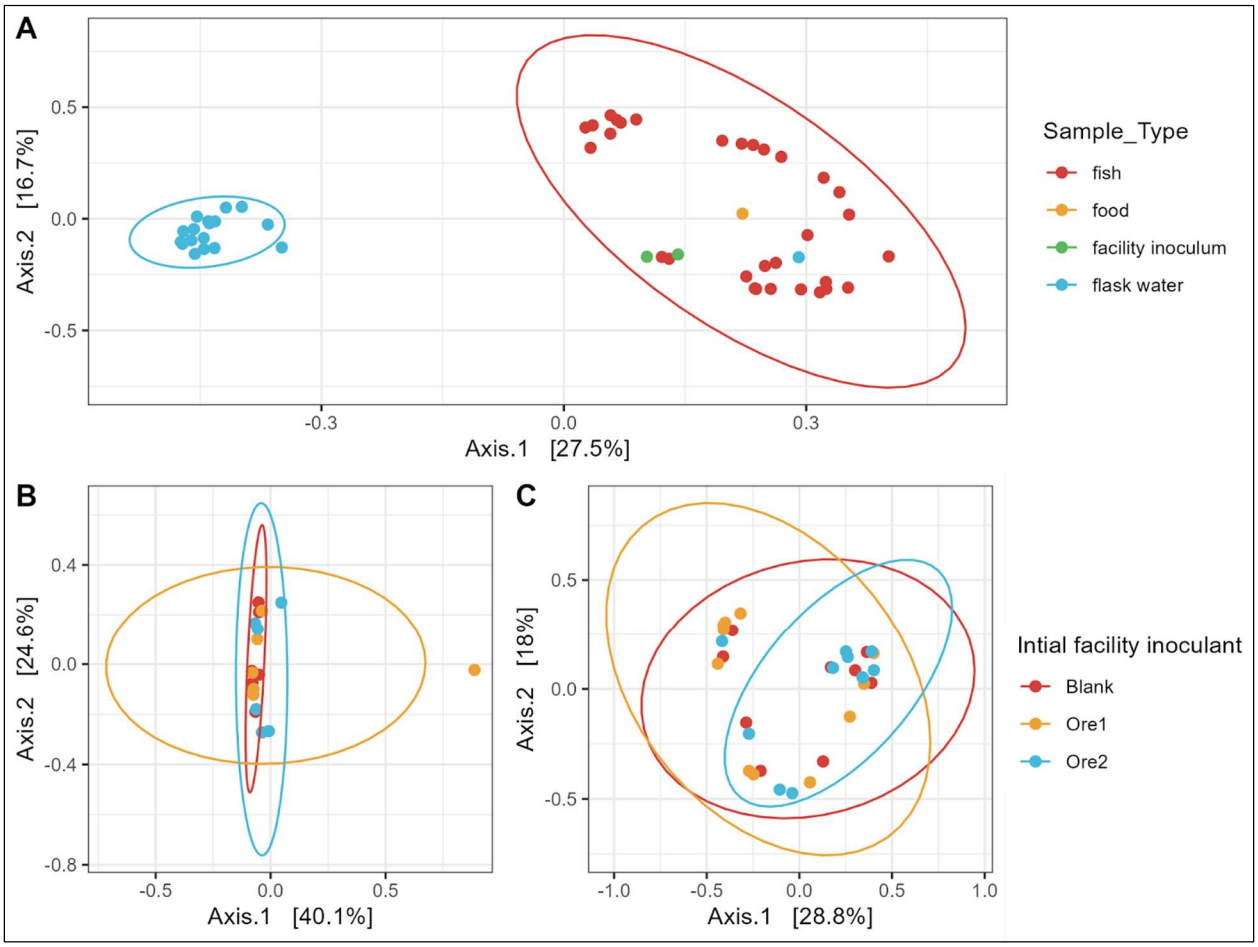

Figure 26: Beta diversity of zebrafish gut, fish food, facility water inoculants, and flask water microbiome samples using Bray-Curtis dissimilarity for TP2 (6-12 d.p.f.). A principal coordinate analysis (PCoA) of (**A**) all sample types, (**B**) flask water, and (**C**) fish gut microbial samples. Colors in (**A**) represent sample type while colors in (**B**) and (**C**) represent the facility water source used to inoculate flask water at 0 d.p.f. Normal confidence ellipse (95%) groupings match each plot legend.

To identify potential sources of zebrafish microbiota, I repeated FEAST source tracking analyses. Since zebrafish microbiota sampled in TP2 were most likely affected by variables across the entirety of the experimental timeline, all food and relevant facility inoculant samples from both TP1 and TP2 were included in the analyses (Figure 6). Results again show significant differences among measured sources and indicate that the largest source proportion of microbiota found in the zebrafish gut microbiome could be attributed to rotifers in TP1 (Kruskal-Wallis: Ore1-TP2, $p < 0.001$; Ore2-TP2, $p = 0.004$; Blank-TP2, $p = 0.004$). Almost no microbiota could

be attributed to facility water inoculant, nor any of the measured variables in TP2. This suggests that, in this experiment, the influence of initial microbiome assembly in the zebrafish gut outweighs later exposure to variation in the water microbiome.

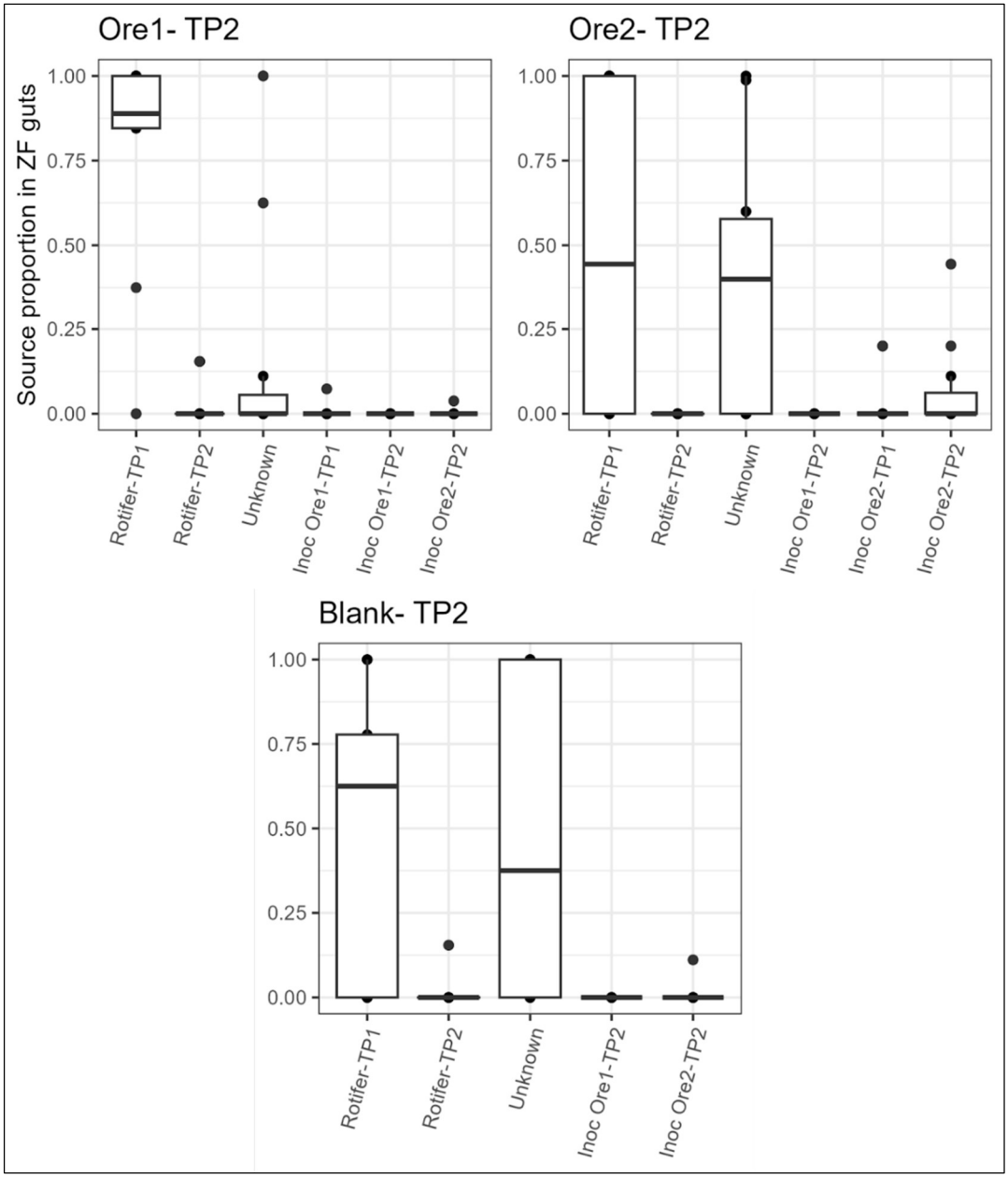

Figure 27: Source proportions of food ('Rotifer'), facility inocula ('Inoc'), and a default 'Unknown' source microbiota in zebrafish gut microbiomes for each treatment in TP2. Food and inocula from both TP1 and TP2 were included as sources to identify potential legacy effects of source contributions over time. Plot titles refer to the initial facility inocula used in TP1. Proportions were calculated using probabilistic models within the R package 'FEAST'. Significance calculated with a Kruskal-Wallis test.

4.4 Discussion

In previous Chapters, I observed significant covariation between tank water and zebrafish gut microbiomes across multiple aquaculture facilities. However, the complex nature of these facilities, including differences in water treatment and husbandry, prevented me from determining whether there was a causal link between variation in water and fish. In this study, I aimed to determine whether variation in the water microbiome could directly influence zebrafish gut microbiome assembly by standardizing husbandry, diet, and zebrafish genotype while manipulating the regional pool of microbiota. I did this by inoculating the rearing water of GF larval zebrafish with water from different aquaculture facilities to establish unique microbial regional pools and measured the gut microbiome of a subset of the larval fish after six days. After this initial measurement, remaining fish were moved to different flasks that were inoculated with either water from the same facility or a different facility to determine the influence of water microbiome variation after a microbiome had been assembled.

Overall, even though the facility water inoculants were enough to induce variation in the flask water microbiome, I found no evidence that this variation in the flask water was enough to influence variation in zebrafish microbiome assembly. Instead, source tracking analysis indicated that diet was the largest source proportion of microbiota in the zebrafish gut microbiome.

In humans and other vertebrates, researchers have long highlighted the strong influence diet can have on host-microbiome assembly (Hacquard et al., 2015; Singh et al., 2017). It has also been widely studied in aquaculture, since diet is, arguably, one of the most accessible methods of microbial management and has direct, measurable effects on fish health (Bruno et al., 2023; Infante-Villamil et al., 2020; Leeper et al., 2023; Parata et al., 2020; Sieler Jr. et al., 2023).

However, there are several different ways in which we may consider how diet influences the fish gut microbiome. The first, and often primary, way we consider the influence of diet is through the direct influence on gut physiology. Gut microbiota metabolize a portion of consumed food, thus influencing the overall structure and composition of the gut microbiome based on the amount and type of available nutrients (Zhang, 2022). Another way to consider the influence of diet, especially live food, is as a source of microbiota itself. Rotifers and brine shrimp- common live diets used for zebrafish and stickleback- maintain their own unique microbiomes, and their microbiota can be dispersed to the fish either directly via consumption or indirectly via the environment (Eckert et al., 2021; Lee et al., 2023). Live food is not always eaten immediately by fish, and as such, some portion of their microbiota can be dispersed to the surrounding environment, integrated into the tank water microbiome, and potentially passed on to the fish via their interaction with the tank water.

Only one of the studies cited earlier- Bruno et al. (2023)- considered diet as a potential source of microbiota through direct measurements and comparisons of the compositions of diet, water, and fish gut microbiomes. Though the influence of diet in aquaculture systems most likely always acts by direct influence of host gut microbiota *and* indirect influence via dispersal of microbiota, the study in this chapter is one of the few that directly tests the latter in fish, especially in zebrafish.

Conclusions about the relative influence of the rearing water microbiome compared to the influence of diet on the microbiome of larval fish diverge (Vadstein et al., 2018). In some studies, such as in Bakke et al. (2013) (another study that considered diet as a microbial source), the authors fed cod larvae three different diets and found no statistically significant differences in their gut microbiomes. Moreover, despite different diets, cod gut microbiomes became more

similar over time and more closely resembled the tank water rather than the live feed. However, in a recent study by Sieler Jr. et al. (2023), zebrafish fed different common laboratory diets displayed significant variation in their gut microbiomes at all developmental stages and responded differently when challenged with a common pathogen. Similar results to Sieler Jr. et al. (2023) have been seen in Delacroix et al. (2014) in larval sea bass and Michl et al. (2017) in larval rainbow trout.

Whether this means diet will always overwhelm the effects of water microbiome variation, however, is yet to be determined. In my study, I wanted to experimentally test the water microbiome variation I observed in the aquaculture facilities investigated earlier in this dissertation. Though the fish I sampled in Chapter 2 were adults, I used larval zebrafish in my study because they can be reared germ-free, allowing for direct manipulation of their initial microbial exposure (Melancon et al., 2017). It has been well-documented that zebrafish larvae experience rapid and age-specific changes in their microbiome (Stephens et al., 2015). As I did not sample into adulthood, my results are solely applicable to the larval stage and may not be reflective of the relative influence of environment and diet at later developmental stages.

Additionally, though I attempted to impart variation in the flask water microbiome that mimicked the variation observed in facility water, this effect was largely diminished due to the technical setup of my experiment. Unlike the RAS systems in the aquaculture facilities, where water in tanks is recirculated and thus, microbial inputs via water are continually renewed, the flask water in this experiment was largely stagnant. Moreso, around 70% of the flask water was removed every day after initial inoculation and replaced with ‘sterile’ media. Though I was still able to observe an effect on the flask water microbiome, the average dissimilarity among flasks

dropped dramatically by Day 6 (average Bray-Curtis distance = 0.4) compared to the initial inocula (average Bray-Curtis distance = 0.88).

In the second part of this experiment, fish were moved from their flasks in TP1 into new flasks inoculated with either water from the same facility source as in TP1 or a new facility source, following a fully factorial design (see Figure 2). While in TP1 I was interested in isolating the effects of water microbiome variation on zebrafish microbiome assembly, the goal of TP2 was to determine whether variation in the water microbiome could influence an already established zebrafish microbiome. It is important to note that, in this case, the term 'established' means that the zebrafish gut has already been colonized by some subset of microbiota and is not meant to reference the stability of the microbiome, as it fluctuates quite a bit throughout development (Stephens et al., 2015). In TP2, I did not observe a significant effect of aquaculture facility inoculant on either flask water microbiomes or zebrafish gut microbiomes, and results from source tracking analyses indicated that diet was still a major source of microbiota for the zebrafish microbiome.

In results from previous chapters, I observed that, in some facilities (e.g. Ore1), the influence of microbial inputs from fish on the water microbiome far outweighed the reciprocal influence of water on the fish microbiome. Compared to TP1, fish in TP2 were larger, more active, and were fed more food on a more frequent schedule. If the variation imparted on the flask water microbiome via facility water inoculant was not enough to observe any significant influence on zebrafish microbiomes in TP1, then it makes sense that, with fish now more actively interacting with their environment and larger inputs of food, the variation in flask water in TP2 was unlikely to have an impact on the zebrafish microbiome.

Future studies could address the limitations in this experiment by conducting trials on a scaled-down RAS system. Or, at the very least, by mimicking the recirculation action of tank water by continually replenishing microbial inputs to the tank water microbiome to ensure an accurate representation of the magnitude of variation in tank water microbiota seen in aquaculture facilities. Additionally, it would be helpful to extend this study through the adult developmental stage in order to determine what proportion of variation in the zebrafish gut microbiome is driven by early-life exposure. These studies would allow us to build a more holistic understanding of how the environmental microbiome interacts with the zebrafish microbiome under a variety of conditions in aquaculture and potentially how these can be manipulated to better zebrafish health and welfare.

CHAPTER 5: CONCLUSION

Community assembly theory suggests that variation in the regional species pool can act as a source of variation in a local community. In my dissertation, I leveraged species pool theory to empirically test how regional species pool variation drives local community assembly in a host-microbiome model system. The microbiome plays a critical role in aspects of host health and development, yet the drivers of microbiome assembly and sources of variation remain major questions in host-microbiome research. This is especially true in research aquaculture facilities, where fish used in basic experimental research are bred and housed. Variation in the microbiome of zebrafish and stickleback, two animal models commonly used in biomedical and evolutionary genetics research, can lead to disparate phenotypes, potentially influencing research outcomes. Identifying drivers of variation in fish microbiome assembly not only helps to promote reproducibility in an important animal model, but also provides a broader understanding of how regional and local community dynamics may play a role in host-microbiome assembly in general.

Using a set of aquaculture facilities on the University of Oregon campus, I characterized temporal and spatial variation in the regional pools- the water microbiome. My research suggests that facilities maintain unique water microbiomes that vary over time. Despite the differences in their water microbiomes, the patterns of spatial variation within facilities were shared, and the greatest magnitude of variation within each water system occurred at the intermediary levels of Shelf or Row. I used my findings in Chapter 2 to guide my experimental approach in Chapter 3.

In my third chapter, I extended my study to connect regional pool variation in the facility water microbiome to local community variation in the zebrafish gut microbiome. I expanded the number of facilities I investigated to include an additional geographic region and sampled tank

water microbiomes more intensively over a smaller spatial scale to minimize destructive sampling of zebrafish while ensuring the capture of as much variation in the water microbiome as possible. I found a significant influence of both regional location and facility identity on fish gut and tank water microbiomes. I also examined covariation between fish and water within facilities to determine the directionality in microbial sharing between the two. Results suggest that variation in the water microbiome does impact the fish gut microbiome, but the relative magnitude of this influence is facility-dependent. The inherent differences in each facility, from the way they are managed to the density of fish housed- are representative of the differences we see between almost all modern zebrafish research facilities but can restrict our ability to completely isolate the effects of the water microbiome on the zebrafish gut microbiome.

As such, in my final chapter I attempted to address this issue by experimentally manipulating the water microbiome to measure the direct influence on the zebrafish gut microbiome. I found that the microbial inputs from live feed were the primary drivers of zebrafish gut microbiome assembly, and that, by standardizing feed across all water treatments, the consistent microbial inputs from live food most likely negated all influence of flask water on microbiome variation.

My results highlight that interactions between the fish gut microbiome and their tank water microbiome are highly dynamic, and due to the microbial sharing between the two, variation in facility water microbiomes can act as a driver of variation in the fish microbiome. However, the specific conditions of the rearing environment and select host physiological features, like developmental stage, could play major roles in determining the magnitude at which regional pool water microbiota impacts gut microbiome composition. Future studies should continue to identify the sources of variation in aquaculture facility water systems to better

understand how the fish gut and water microbiome may interact under different conditions. This research ultimately provides insight into the housing effects on zebrafish in aquaculture systems and advocates for a deeper understanding of what drives microbiome variation in zebrafish to promote reproducibility in our animal models.

REFERENCES CITED

A

Adair, K. (2019). Unpublished raw data.

Adair, K. L., & Douglas, A. E. (2017). Making a microbiome: The many determinants of host-associated microbial community composition. *Current Opinion in Microbiology*, *35*, 23–29. https://doi.org/10.1016/j.mib.2016.11.002

Adler, P. B., HilleRisLambers, J., & Levine, J. M. (2007). A niche for neutrality. *Ecology Letters*, *10*(2), 95–104. https://doi.org/10.1111/j.1461-0248.2006.00996.x

Angert, E. R. (2022). Epulopiscium spp. *Trends in Microbiology*, *30*(1), 97–98. https://doi.org/10.1016/j.tim.2021.11.004

Attramadal, K. J. K., Minniti, G., Øie, G., Kjørsvik, E., Østensen, M.-A., Bakke, I., & Vadstein, O. (2016). Microbial maturation of intake water at different carrying capacities affects microbial control in rearing tanks for marine fish larvae. *Aquaculture*, *457*, 68–72. https://doi.org/10.1016/j.aquaculture.2016.02.015

Attramadal, K. J. K., Truong, T. M. H., Bakke, I., Skjermo, J., Olsen, Y., & Vadstein, O. (2014). RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae. *Aquaculture*, *432*, 483–490. https://doi.org/10.1016/j.aquaculture.2014.05.052

B

Babenzien, H.-D., & Cypionka, H. (2015). Nevskia. In *Bergey's Manual of Systematics of Archaea and Bacteria* (pp. 1–6). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781118960608.gbm01233

Badiola, M., Mendiola, D., & Bostock, J. (2012). Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. *Aquacultural Engineering*, *51*, 26–35. https://doi.org/10.1016/j.aquaeng.2012.07.004

Bakke, I., Skjermo, J., Vo, T. A., & Vadstein, O. (2013). Live feed is not a major determinant of the microbiota associated with cod larvae (Gadus morhua). *Environmental Microbiology Reports*, *5*(4), 537–548. https://doi.org/10.1111/1758-2229.12042

Barton, C. L., Johnson, E. W., & Tanguay, R. L. (2016). Facility Design and Health Management Program at the Sinnhuber Aquatic Research Laboratory. *Zebrafish*, *13*(S1), S-39. https://doi.org/10.1089/zeb.2015.1232

Belmaker, J. (2009). Species richness of resident and transient coral-dwelling fish responds differentially to regional diversity. *Global Ecology and Biogeography*, *18*(4), 426–436. https://doi.org/10.1111/j.1466-8238.2009.00456.x

Belmaker, J., & Jetz, W. (2012). Regional Pools and Environmental Controls of Vertebrate Richness. *The American Naturalist*, *179*(4), 512–523. https://doi.org/10.1086/664610

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bhat, S. V., Maughan, H., Cameron, A. D. S., & Yost, C. K. (2022). Phylogenomic analysis of the genus Delftia reveals distinct major lineages with ecological specializations. *Microbial Genomics*, *8*(9). https://doi.org/10.1099/mgen.0.000864

Bik, E. M., Costello, E. K., Switzer, A. D., Callahan, B. J., Holmes, S. P., Wells, R. S., Carlin, K. P., Jensen, E. D., Venn-Watson, S., & Relman, D. A. (2016). Marine mammals harbor unique microbiotas shaped by and yet distinct from the sea. *Nature Communications*, *7*(1), Article 1. https://doi.org/10.1038/ncomms10516

Blazewicz, S. J., Barnard, R. L., Daly, R. A., & Firestone, M. K. (2013). Evaluating rRNA as an indicator of microbial activity in environmental communities: Limitations and uses. *The ISME Journal*, *7*(11), 2061–2068. https://doi.org/10.1038/ismej.2013.102

Bonder, M. J., Kurilshikov, A., Tigchelaar, E. F., Mujagic, Z., Imhann, F., Vila, A. V., Deelen, P., Vatanen, T., Schirmer, M., Smeekens, S. P., Zhernakova, D. V., Jankipersadsing, S. A., Jaeger, M., Oosting, M., Cenit, M. C., Masclee, A. A. M., Swertz, M. A., Li, Y., Kumar, V., … Zhernakova, A. (2016). The effect of host genetics on the gut microbiome. *Nature Genetics*, *48*(11), Article 11. https://doi.org/10.1038/ng.3663

Bornbusch, S. L., Greene, L. K., Rahobilalaina, S., Calkins, S., Rothman, R. S., Clarke, T. A., LaFleur, M., & Drea, C. M. (2022). Gut microbiota of ring-tailed lemurs (Lemur catta) vary across natural and captive populations and correlate with environmental microbiota. *Animal Microbiome*, *4*(1), 29. https://doi.org/10.1186/s42523-022-00176-x

Bray, J. R., & Curtis, J. T. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349. https://doi.org/10.2307/1942268

Breen, P., Winters, A. D., Nag, D., Ahmad, M. M., Theis, K. R., & Withey, J. H. (2019). Internal Versus External Pressures: Effect of Housing Systems on the Zebrafish Microbiome. *Zebrafish*, *16*(4), 388–400. https://doi.org/10.1089/zeb.2018.1711

Bruno, A., Sandionigi, A., Panio, A., Rimoldi, S., Orizio, F., Agostinetto, G., Hasan, I., Gasco, L., Terova, G., & Labra, M. (2023). Aquaculture ecosystem microbiome at the water-fish interface: The case-study of rainbow trout fed with Tenebrio molitor novel diets. *BMC Microbiology*, *23*(1), 248. https://doi.org/10.1186/s12866-023-02990-y

Burns, A. R., Miller, E., Agarwal, M., Rolig, A. S., Milligan-Myhre, K., Seredick, S., Guillemin, K., & Bohannan, B. J. M. (2017). Interhost dispersal alters microbiome assembly and can

overwhelm host innate immunity in an experimental zebrafish model. *Proceedings of the National Academy of Sciences*, *114*(42), 11181–11186. https://doi.org/10.1073/pnas.1702511114

Burns, A. R., Stephens, W. Z., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. (2015). Contribution of neutral processes to the assembly of gut microbial communities in the zebrafish over host development. *The ISME Journal*, *10*(3), Article 3. https://doi.org/10.1038/ismej.2015.142

C

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), Article 7. https://doi.org/10.1038/nmeth.3869

Centers for Disease Control and Prevention. (2023, March 15). *CDC Museum Covid-19 Timeline*. CDC Museum COVID-19 Timeline. https://www.cdc.gov/museum/timeline/covid19.html

Chesson, P. (2000). Mechanisms of Maintenance of Species Diversity. *Annual Review of Ecology and Systematics*, *31*(1), 343–366. https://doi.org/10.1146/annurev.ecolsys.31.1.343

Christian, N., Whitaker, B., & Clay, K. (2015). Microbiomes: Unifying animal and plant systems through the lens of community ecology theory. *Frontiers in Microbiology*, *6*. https://www.frontiersin.org/articles/10.3389/fmicb.2015.00869

Clarke, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology*, *18*(1), 117–143. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

Clements, F. E. (1916). *Plant Succession: An Analysis of the Development of Vegetation*. Carnegie Institution of Washington.

Cornell, H. V., & Harrison, S. P. (2014). What Are Species Pools and When Are They Important? *Annual Review of Ecology, Evolution, and Systematics*, *45*(1), 45–67. https://doi.org/10.1146/annurev-ecolsys-120213-091759

Costello, E. K., Lauber, C. L., Hamady, M., Fierer, N., Gordon, J. I., & Knight, R. (2009). Bacterial Community Variation in Human Body Habitats Across Space and Time. *Science*, *326*(5960), 1694–1697. https://doi.org/10.1126/science.1177486

Costello, E. K., Stagaman, K., Dethlefsen, L., Bohannan, B. J. M., & Relman, D. A. (2012). The Application of Ecological Theory Toward an Understanding of the Human Microbiome. *Science*, *336*(6086), 1255–1262. https://doi.org/10.1126/science.1224203

Cox, T. O., Lundgren, P., Nath, K., & Thaiss, C. A. (2022). Metabolic control by the microbiome. *Genome Medicine*, *14*(1), 80. https://doi.org/10.1186/s13073-022-01092-0

Cresko, W. A., McGuigan, K. L., Phillips, P. C., & Postlethwait, J. H. (2007). Studies of threespine stickleback developmental evolution: Progress and promise. *Genetica*, *129*(1), 105–126. https://doi.org/10.1007/s10709-006-0036-z

Cryan, J. F., O'Riordan, K. J., Sandhu, K., Peterson, V., & Dinan, T. G. (2020). The gut microbiome in neurological disorders. *The Lancet Neurology*, *19*(2), 179–194. https://doi.org/10.1016/S1474-4422(19)30356-4

D

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. *Microbiome*, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

Delcroix, J., Gatesoupe, F.-J., Desbruyères, E., Huelvan, C., Le Delliou, H., Le Gall, M.-M., Quazuguel, P., Mazurais, D., & Zambonino-Infante, J. l. (2015). The effects of dietary marine protein hydrolysates on the development of sea bass larvae, Dicentrarchus labrax, and associated microbiota. *Aquaculture Nutrition*, *21*(1), 98–104. https://doi.org/10.1111/anu.12139

Dooley, K., & Zon, L. I. (2000). Zebrafish: A model system for the study of human disease. *Current Opinion in Genetics & Development*, *10*(3), 252–256. https://doi.org/10.1016/S0959-437X(00)00074-5

E

Eckert, E. M., Anicic, N., & Fontaneto, D. (2021). Freshwater zooplankton microbiome composition is highly flexible and strongly influenced by the environment. *Molecular Ecology*, *30*(6), 1545–1558. https://doi.org/10.1111/mec.15815

Eisen, J. S. (2020). Chapter 1—History of Zebrafish Research. In S. C. Cartner, J. S. Eisen, S. C. Farmer, K. J. Guillemin, M. L. Kent, & G. E. Sanders (Eds.), *The Zebrafish in Biomedical Research* (pp. 3–14). Academic Press. https://doi.org/10.1016/B978-0-12-812431-4.00001-4

F

FAO (2019). *The State of the World Fisheries and Aquaculture 2019: Meeting the Sustainable Development Goals.* FAO, Rome.

Fjellheim, A. J., Playfoot, K. J., Skjermo, J., & Vadstein, O. (2012). Inter-individual variation in the dominant intestinal microbiota of reared Atlantic cod (Gadus morhua L.) larvae. *Aquaculture Research*, *43*(10), 1499–1508. https://doi.org/10.1111/j.1365-2109.2011.02952.x

Flynn, J. K., Ortiz, A. M., Herbert, R., & Brenchley, J. M. (2022). Host Genetics and Environment Shape the Composition of the Gastrointestinal Microbiome in Nonhuman Primates. *Microbiology Spectrum*, *11*(1), e02139-22. https://doi.org/10.1128/spectrum.02139-22

Fornshell, G., & Hinshaw, J. M. (2008). Better Management Practices for Flow-Through Aquaculture Systems. In C. S. Tucker & J. A. Hargreaves (Eds.), *Environmental Best Management Practices for Aquaculture* (1st ed., pp. 331–388). Wiley. https://doi.org/10.1002/9780813818672.ch9

Franklin, C. L., & Ericsson, A. C. (2017). Microbiota and reproducibility of rodent models. Lab Animal, 46(4), 114-122.

G

Gaulke, C. A., Martins, M. L., Watral, V. G., Humphreys, I. R., Spagnoli, S. T., Kent, M. L., & Sharpton, T. J. (2019). A longitudinal assessment of host-microbe-parasite interactions resolves the zebrafish gut microbiome's link to *Pseudocapillaria tomentosa* infection and pathology. *Microbiome*, *7*(1), 10. https://doi.org/10.1186/s40168-019-0622-9

Gerhold, P., Pärtel, M., Liira, J., Zobel, K., & Prinzing, A. (2008). Phylogenetic structure of local communities predicts the size of the regional species pool. *Journal of Ecology*, *96*(4), 709–712. https://doi.org/10.1111/j.1365-2745.2008.01386.x

Gleason, H. A. (1927). Further Views on the Succession-Concept. *Ecology*, *8*(3), 299–326. https://doi.org/10.2307/1929332

Glowacki, R. W. P., Engelhart, M. J., & Ahern, P. P. (2021). Controlled Complexity: Optimized Systems to Study the Role of the Gut Microbiome in Host Physiology. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.735562

Goldschmidt-Clermont, E., Wahli, T., Frey, J., & Burr, S. E. (2008). Identification of bacteria from the normal flora of perch, Perca fluviatilis L., and evaluation of their inhibitory potential towards Aeromonas species. *Journal of Fish Diseases*, *31*(5), 353–359. https://doi.org/10.1111/j.1365-2761.2008.00912.x

Greyson-Gaito, C. J., Bartley, T. J., Cottenie, K., Jarvis, W. M. C., Newman, A. E. M., & Stothart, M. R. (2020). Into the wild: Microbiome transplant studies need broader ecological reality. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1921), 20192834. https://doi.org/10.1098/rspb.2019.2834

Gupta, V. K., Paul, S., & Dutta, C. (2017). Geography, Ethnicity or Subsistence-Specific Variations in Human Microbiome Composition and Diversity. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.01162

H

Hacquard, S., Garrido-Oter, R., González, A., Spaepen, S., Ackermann, G., Lebeis, S., McHardy, A. C., Dangl, J. L., Knight, R., Ley, R., & Schulze-Lefert, P. (2015). Microbiota and Host Nutrition across Plant and Animal Kingdoms. *Cell Host & Microbe*, *17*(5), 603–616. https://doi.org/10.1016/j.chom.2015.04.009

Hagen, D. W. (1967). Isolating Mechanisms in Threespine Sticklebacks (Gasterosteus). *Journal of the Fisheries Research Board of Canada*, *24*(8), 1637–1692. https://doi.org/10.1139/f67-138

Hammer, T. J., Sanders, J. G., & Fierer, N. (2019). Not all animals need a microbiome. *FEMS Microbiology Letters*, *366*(10), fnz117. https://doi.org/10.1093/femsle/fnz117

Harper, J. L. (1977). Population biology of plants. *Population Biology of Plants.* https://www.cabdirect.org/cabdirect/abstract/19781671245

Harrison, S. P., & Cornell, H. V. (2008). Toward a better understanding of the regional causes of local community richness. *Ecology Letters*, *11*, 969-979.

Heng, K., Thompson, A., Chu, D., & Kingsley, D. M. (2016). Three cheers for the three-spined stickleback. *Lab Animal*, *45*(11), Article 11. https://doi.org/10.1038/laban.1142

Hickman, D. L., Johnson, J., Vemulapalli, T. H., Crisler, J. R., & Shepherd, R. (2017). Commonly Used Animal Models. *Principles of Animal Research for Graduate and Undergraduate Students*, 117–175. https://doi.org/10.1016/B978-0-12-802151-4.00007-4

Hollander, M. & Wolfe, D.A. (1973). Nonparametric Statistical Methods. John Wiley and Sons, New York.

Hörtnagl, P., Pérez, M. T., & Sommaruga, R. (2011). Contrasting effects of ultraviolet radiation on the growth efficiency of freshwater bacteria. *Aquatic Ecology*, *45*(1), 125–136. https://doi.org/10.1007/s10452-010-9341-9

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G.-J., White, S., … Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), Article 7446. https://doi.org/10.1038/nature12111

Hubbell, S. P. (2001). The Unified Neutral Theory of Biodiversity and Biogeography. In *Monographs in Population Biology, 32*. Princeton University Press. https://doi.org/10.1515/9781400837526

I

Infante-Villamil, S., Huerlimann, R., & Jerry, D. R. (2021). Microbiome diversity and dysbiosis in aquaculture. *Reviews in Aquaculture*, *13*(2), 1077–1096. https://doi.org/10.1111/raq.12513

K

Keddy, P. A. (1992). Assembly and response rules: Two goals for predictive community ecology. *Journal of Vegetation Science*, *3*(2), 157–164. https://doi.org/10.2307/3235676

Kers, J. G., Velkers, F. C., Fischer, E. A. J., Hermes, G. D. A., Lamot, D. M., Stegeman, J. A., & Smidt, H. (2019). Take care of the environment: Housing conditions affect the interplay of nutritional interventions and intestinal microbiota in broiler chickens. *Animal Microbiome*, *1*(1), 10. https://doi.org/10.1186/s42523-019-0009-z

Klinkhardt, M. (2021, October 5). Are recirculating aquaculture systems the future of mariculture? Advantages and risks of land-based fish production. *Eurofish*, *5*, 17–21.

Kolarevic, J., Baeverfjord, G., Takle, H., Ytteborg, E., Reiten, B. K. M., Nergård, S., & Terjesen, B. F. (2014). Performance and welfare of Atlantic salmon smolt reared in recirculating or flow through aquaculture systems. *Aquaculture*, *432*, 15–25. https://doi.org/10.1016/j.aquaculture.2014.03.033

Kostic, A. D., Howitt, M. R., & Garrett, W. S. (2013). Exploring host–microbiota interactions in animal models and humans. *Genes & Development*, *27*(7), 701–718. https://doi.org/10.1101/gad.212522.112

Kraft, N.J.B., & Ackerly, D.D. (2014). Assembly of Plant Communities. In *Ecology and the Environment, The Plant Sciences 8* (pp. 67-88). Springer Science & Business Media, New York, New York.

Krotman, Y., Yergaliyev, T. M., Alexander Shani, R., Avrahami, Y., & Szitenberg, A. (2020). Dissecting the factors shaping fish skin microbiomes in a heterogeneous inland water system. *Microbiome*, *8*(1), 9. https://doi.org/10.1186/s40168-020-0784-5

L

Lawrence, C., & Mason, T. (2012). Zebrafish Housing Systems: A Review of Basic Operating Principles and Considerations for Design and Functionality. *ILAR Journal*, *53*(2), 179–191. https://doi.org/10.1093/ilar.53.2.179

Lebov, J. F., Schlomann, B. H., Robinson, C. D., & Bohannan, B. J. M. (2020). Phenotypic Parallelism during Experimental Adaptation of a Free-Living Bacterium to the Zebrafish Gut. *mBio*, *11*(4), 10.1128/mbio.01519-20. https://doi.org/10.1128/mbio.01519-20

Lee, E., Lee, K.-W., Park, Y., Choi, A., Kwon, K. K., & Kang, H.-M. (2024). Comparative Microbiome Analysis of Artemia spp. And Potential Role of Microbiota in Cyst Hatching. *Marine Biotechnology*, *26*(1), 50–59. https://doi.org/10.1007/s10126-023-10276-7

Leeper, A., Sauphar, C., Berlizot, B., Ladurée, G., Koppe, W., Knobloch, S., Skírnisdóttir, S., Björnsdóttir, R., Øverland, M., & Benhaïm, D. (2023). Enhancement of Soybean Meal Alters Gut Microbiome and Influences Behavior of Farmed Atlantic Salmon (Salmo salar). *Animals*, *13*(16), Article 16. https://doi.org/10.3390/ani13162591

Leibold, M. (1995). The niche concept revisited: mechanistic models and community context. *Ecology*, *76*(5), 1371-1382.

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., Holt, R. D., Shurin, J. B., Law, R., Tilman, D., Loreau, M., & Gonzalez, A. (2004). The metacommunity concept: A framework for multi-scale community ecology. *Ecology Letters*, *7*(7), 601–613. https://doi.org/10.1111/j.1461-0248.2004.00608.x

Leray, M., Wilkins, L. G. E., Apprill, A., Bik, H. M., Clever, F., Connolly, S. R., León, M. E. D., Duffy, J. E., Ezzat, L., Gignoux-Wolfsohn, S., Herre, E. A., Kaye, J. Z., Kline, D. I., Kueneman, J. G., McCormick, M. K., McMillan, W. O., O'Dea, A., Pereira, T. J., Petersen, J. M., … Eisen, J. A. (2021). Natural experiments and long-term monitoring are critical to understand and predict marine host–microbe ecology and evolution. *PLOS Biology*, *19*(8), e3001322. https://doi.org/10.1371/journal.pbio.3001322

Lescak, E. A., & Milligan-Myhre, K. C. (2017). Teleosts as Model Organisms To Understand Host-Microbe Interactions. *Journal of Bacteriology*, *199*(15), e00868-16. https://doi.org/10.1128/JB.00868-16

Lessard, J.-P., Borregaard, M. K., Fordyce, J. A., Rahbek, C., Weiser, M. D., Dunn, R. R., & Sanders, N. J. (2011). Strong influence of regional species pools on continent-wide structuring of local communities. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1727), 266–274. https://doi.org/10.1098/rspb.2011.0552

Lindell, M. J., Granéli, W., & Tranvik, L. J. (1995). Enhanced bacterial growth in response to photochemical transformation of dissolved organic matter. *Limnology and Oceanography*, *40*(1), 195–199. https://doi.org/10.4319/lo.1995.40.1.0195

Lu, H., Li, P., Huang, X., Wang, C. H., Li, M., & Xu, Z. Z. (2021). Zebrafish model for human gut microbiome-related studies: Advantages and limitations. *Medicine in Microecology*, *8*, 100042. https://doi.org/10.1016/j.medmic.2021.100042

M

MacArthur, R. & Wilson, E. (1977). The Theory of Island Biogeography. Princeton University Press, Princeton, NJ.

Manchanayake, T., Salleh, A., Amal, M. N. A., Yasin, I. S. M., & Zamri-Saad, M. (2023). Pathology and pathogenesis of Vibrio infection in fish: A review. *Aquaculture Reports*, *28*, 101459. https://doi.org/10.1016/j.aqrep.2022.101459

Mandal, R. K., Denny, J. E., Waide, M. L., Li, Q., Bhutiani, N., Anderson, C. D., Baby, B. V., Jala, V. R., Egilmez, N. K., & Schmidt, N. W. (2020). Temporospatial shifts within commercial laboratory mouse gut microbiota impact experimental reproducibility. *BMC Biology*, *18*(1), 83. https://doi.org/10.1186/s12915-020-00810-7

Martinez Arbizu, P. (2020). pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4.

Massaquoi, M. & Black, R. (2019). Unpublished raw data.

McArdle, B. H., & Anderson, M. J. (2001). Fitting Multivariate Models to Community Data: A Comment on Distance-Based Redundancy Analysis. *Ecology, 82* (1), 290-297.

McCafferty, J., Mühlbauer, M., Gharaibeh, R. Z., Arthur, J. C., Perez-Chanona, E., Sha, W., Jobin, C., & Fodor, A. A. (2013). Stochastic changes over time and not founder effects drive cage effects in microbial community assembly in a mouse model. *The ISME Journal*, *7*(11), Article 11. https://doi.org/10.1038/ismej.2013.106

McKinnon, J. S., & Rundle, H. D. (2002). Speciation in nature: The threespine stickleback model systems. *Trends in Ecology & Evolution*, *17*(10), 480–488. https://doi.org/10.1016/S0169-5347(02)02579-X

McPhail, J. D. (1969). Predation and the Evolution of a Stickleback (Gasterosteus). *Journal of the Fisheries Research Board of Canada*, *26*(12), 3183–3208. https://doi.org/10.1139/f69-301

Meadow, J. F., Altrichter, A. E., Bateman, A. C., Stenson, J., Brown, G. Z., Green, J. L., & Bohannan, B. J. M. (2015). Humans differ in their personal microbial cloud. *PeerJ*, *3*, e1258. https://doi.org/10.7717/peerj.1258

Melancon, E., Gomez De La Torre Canny, S., Sichel, S., Kelly, M., Wiles, T. J., Rawls, J. F., Eisen, J. S., & Guillemin, K. (2017). Chapter 3—Best practices for germ-free derivation and gnotobiotic zebrafish husbandry. In H. W. Detrich, M. Westerfield, & L. I. Zon (Eds.), *Methods in Cell Biology* (Vol. 138, pp. 61–100). Academic Press. https://doi.org/10.1016/bs.mcb.2016.11.005

Michl, S. C., Ratten, J.-M., Beyer, M., Hasler, M., LaRoche, J., & Schulz, C. (2017). The malleable gut microbiome of juvenile rainbow trout (Oncorhynchus mykiss): Diet-dependent shifts of bacterial community structures. *PLOS ONE*, *12*(5), e0177735. https://doi.org/10.1371/journal.pone.0177735

Miller, E. T., Svanbäck, R., & Bohannan, B. J. M. (2018). Microbiomes as Metacommunities: Understanding Host-Associated Microbes through Metacommunity Ecology. *Trends in Ecology & Evolution*, *33*(12), 926–935. https://doi.org/10.1016/j.tree.2018.09.002

Milligan-Myhre, K., Small, C. M., Mittge, E. K., Agarwal, M., Currey, M., Cresko, W. A., & Guillemin, K. (2016). Innate immune responses to gut microbiota differ between oceanic and freshwater threespine stickleback populations. *Disease Models & Mechanisms*, *9*(2), 187–198. https://doi.org/10.1242/dmm.021881

Mittelbach, G. G., & Schemske, D. W. (2015). Ecological and evolutionary perspectives on community assembly. *Trends in Ecology & Evolution*, *30*(5), 241–247. https://doi.org/10.1016/j.tree.2015.02.008

Moraitou, M., Forsythe, A., Fellows Yates, J. A., Brealey, J. C., Warinner, C., & Guschanski, K. (2022). Ecology, Not Host Phylogeny, Shapes the Oral Microbiome in Closely Related Species. *Molecular Biology and Evolution*, *39*(12), msac263. https://doi.org/10.1093/molbev/msac263

Morar, N., & Bohannan, B. J. M. (2019). The Conceptual Ecology of the Human Microbiome. *The Quarterly Review of Biology*, *94*(2), 149–175. https://doi.org/10.1086/703582

N

National Research Council (2011). *Guide for the Care and Use of Laboratory Animals.* Washington: National Academy Press.

Nemergut, D. R., Schmidt, S. K., Fukami, T., O'Neill, S. P., Bilinski, T. M., Stanish, L. F., … & Ferrenberg, S. (2013). Patterns and processes of microbial community assembly. *Microbiology and Molecular Biology Reviews, 77*(3), 342–356.

O

Ofek, T., Lalzar, M., Laviad-Shitrit, S., Izhaki, I., & Halpern, M. (2021). Comparative Study of Intestinal Microbiota Composition of Six Edible Fish Species. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/articles/10.3389/fmicb.2021.760266

Okazaki, F., Zang, L., Nakayama, H., Chen, Z., Gao, Z.-J., Chiba, H., Hui, S.-P., Aoki, T., Nishimura, N., & Shimada, Y. (2019). Microbiome Alteration in Type 2 Diabetes Mellitus Model of Zebrafish. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-018-37242-x

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., . . . & Wagner, H. (2019). vegan: Community ecology package. R package version 2.6-4.

Ostlund-Nilsson, S., Mayer, I., & Huntingford, F. A. (Eds.). (2006). *Biology of the Three-Spined Stickleback*. CRC Press. https://doi.org/10.1201/9781420004830

Ottman, N., Smidt, H., de Vos, W. M., & Belzer, C. (2012). The function of our microbiota: who is out there and what do they do? *Front. Cell Infect Microbiol.*, *2*(104).

P

Parata, L., Mazumder, D., Sammut, J., & Egan, S. (2020). Diet type influences the gut microbiome and nutrient assimilation of Genetically Improved Farmed Tilapia (Oreochromis niloticus). *PLOS ONE*, *15*(8), e0237775. https://doi.org/10.1371/journal.pone.0237775

Parfrey, L. W., Moreau, C. S., & Russell, J. A. (2018). Introduction: The host-associated microbiome: Pattern, process and function. *Molecular Ecology*, *27*(8), 1749–1765. https://doi.org/10.1111/mec.14706

Parker, K. D., Albeke, S. E., Gigley, J. P., Goldstein, A. M., & Ward, N. L. (2018). Microbiome Composition in Both Wild-Type and Disease Model Mice Is Heavily Influenced by Mouse Facility. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/articles/10.3389/fmicb.2018.01598

Pearson, D. E., Ortega, Y. K., Eren, Ö., & Hierro, J. L. (2018). Community Assembly Theory as a Framework for Biological Invasions. *Trends in Ecology & Evolution*, *33*(5), 313–325. https://doi.org/10.1016/j.tree.2018.03.002

Phelps, D., Brinkman, N. E., Keely, S. P., Anneken, E. M., Catron, T. R., Betancourt, D., Wood, C. E., Espenschied, S. T., Rawls, J. F., & Tal, T. (2017). Microbial colonization is required for normal neurobehavioral development in zebrafish. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-10517-5

Presta, L., Bosi, E., Fondi, M., Maida, I., Perrin, E., Miceli, E., Maggini, V., Bogani, P., Firenzuoli, F., Di Pilato, V., Rossolini, G. M., Mengoni, A., & Fani, R. (2017). Phenotypic and genomic characterization of the antimicrobial producer *Rheinheimera* sp. EpRS3 isolated from the medicinal plant *Echinacea purpurea*: Insights into its biotechnological relevance. *Research in Microbiology*, *168*(3), 293–305. https://doi.org/10.1016/j.resmic.2016.11.001

Q

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

Quero, G. M., Piredda, R., Basili, M., Maricchiolo, G., Mirto, S., Manini, E., Seyfarth, A. M., Candela, M., & Luna, G. M. (2023). Host-associated and Environmental Microbiomes in an Open-Sea Mediterranean Gilthead Sea Bream Fish Farm. *Microbial Ecology*, *86*(2), 1319–1330. https://doi.org/10.1007/s00248-022-02120-7

R

R Core Team. (2021). R: A language and environment for statistical computing. Vienna, Austria. https://www.R-project.org/

Ramírez, C., Coronado, J., Silva, A., & Romero, J. (2018). Cetobacterium Is a Major Component of the Microbiome of Giant Amazonian Fish (*Arapaima gigas*) in Ecuador. *Animals*, *8*(11), Article 11. https://doi.org/10.3390/ani8110189

Rausch, P., Basic, M., Batra, A., Bischoff, S. C., Blaut, M., Clavel, T., Gläsner, J., Gopalakrishnan, S., Grassl, G. A., Günther, C., Haller, D., Hirose, M., Ibrahim, S., Loh, G., Mattner, J., Nagel, S., Pabst, O., Schmidt, F., Siegmund, B., … Baines, J. F. (2016). Analysis of factors contributing to variation in the C57BL/6J fecal microbiota across German animal facilities. *International Journal of Medical Microbiology: IJMM*, *306*(5), 343–355. https://doi.org/10.1016/j.ijmm.2016.03.004

Rawls, J. F., Mahowald, M. A., Ley, R. E., & Gordon, J. I. (2006). Reciprocal Gut Microbiota Transplants from Zebrafish and Mice to Germ-free Recipients Reveal Host Habitat Selection. *Cell*, *127*(2), 423–433. https://doi.org/10.1016/j.cell.2006.08.043

Reid, K., Bell, M. A., & Veeramah, K. R. (2021). Threespine Stickleback: A Model System For Evolutionary Genomics. *Annual Review of Genomics and Human Genetics*, *22*(1), 357–383. https://doi.org/10.1146/annurev-genom-111720-081402

Ricklefs, R. E. (2003). A comprehensive framework for global patterns in biodiversity. *Ecology Letters*, *7*(1), 1–15.

Robinson, C. D., Klein, H. S., Murphy, K. D., Parthasarathy, R., Guillemin, K., & Bohannan, B. J. M. (2018). Experimental bacterial adaptation to the zebrafish gut reveals a primary role for immigration. *PLOS Biology*, *16*(12), e2006893. https://doi.org/10.1371/journal.pbio.2006893

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., & Rawls, J. F. (2011). Evidence for a core gut microbiota in the zebrafish. *The ISME Journal*, *5*(10), Article 10. https://doi.org/10.1038/ismej.2011.38

Rojas, C. A., Ramírez-Barahona, S., Holekamp, K. E., & Theis, K. R. (2021). Host phylogeny and host ecology structure the mammalian gut microbiota at different taxonomic scales. *Animal Microbiome*, *3*(1), 33. https://doi.org/10.1186/s42523-021-00094-4

Rolig, A. S., Parthasarathy, R., Burns, A. R., Bohannan, B. J. M., & Guillemin, K. (2015). Individual Members of the Microbiota Disproportionately Modulate Host Innate Immune Responses. *Cell Host & Microbe*, *18*(5), 613–620. https://doi.org/10.1016/j.chom.2015.10.009

Rothschild, D., Weissbrod, O., Barkan, E., Kurilshikov, A., Korem, T., Zeevi, D., Costea, P. I., Godneva, A., Kalka, I. N., Bar, N., Shilo, S., Lador, D., Vila, A. V., Zmora, N., Pevsner-Fischer, M., Israeli, D., Kosower, N., Malka, G., Wolf, B. C., … Segal, E. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature*, *555*(7695), Article 7695. https://doi.org/10.1038/nature25973

Russell, R., Wood, S. A., Allison, G., & Menge, B. A. (2006). Scale, Environment, and Trophic Status: The Context Dependency of Community Saturation in Rocky Intertidal Communities. *The American Naturalist*, *167*(6), E158–E170. https://doi.org/10.1086/504603

S

Salvesen, I., Skjermo, J., & Vadstein, O. (1999). Growth of turbot (Scophthalmus maximus L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. *Aquaculture*, *175*(3), 337–350. https://doi.org/10.1016/S0044-8486(99)00110-6

Schemske, D. W., Mittelbach, G. G., Cornell, H. V., Sobel, J. M., & Roy, K. (2009). Is There a Latitudinal Gradient in the Importance of Biotic Interactions? *Annual Review of Ecology,*

Evolution, and Systematics, *40*(1), 245–269. https://doi.org/10.1146/annurev.ecolsys.39.110707.173430

Schmidt, V. T., Smith, K. F., Melvin, D. W., & Amaral-Zettler, L. A. (2015). Community assembly of a euryhaline fish microbiome during salinity acclimation. *Molecular Ecology*, *24*(10), 2537–2550. https://doi.org/10.1111/mec.13177

Sharma, A., Richardson, M., Cralle, L., Stamper, C. E., Maestre, J. P., Stearns-Yoder, K. A., Postolache, T. T., Bates, K. L., Kinney, K. A., Brenner, L. A., Lowry, C. A., Gilbert, J. A., & Hoisington, A. J. (2019). Longitudinal homogenization of the microbiome between both occupants and the built environment in a cohort of United States Air Force Cadets. *Microbiome*, *7*(1), 70. https://doi.org/10.1186/s40168-019-0686-6

Sharpton, T. J., Stagaman, K., Sieler, M. J., Arnold, H. K., & Davis, E. W. (2021). Phylogenetic Integration Reveals the Zebrafish Core Microbiome and Its Sensitivity to Environmental Exposures. *Toxics*, *9*(1), Article 1. https://doi.org/10.3390/toxics9010010

Shenhav, L., Thompson, M., Joseph, T. A., Briscoe, L., Furman, O., Bogumil, D., Mizrahi, I., Pe'er, I., & Halperin, E. (2019). FEAST: Fast expectation-maximization for microbial source tracking. *Nature Methods*, *16*(7), 627–632. https://doi.org/10.1038/s41592-019-0431-x

Sheu, S.-Y., Chen, W.-T., Young, C.-C., & Chen, W.-M. (2018). Rheinheimera coerulea sp. Nov., isolated from a freshwater creek, and amended description of genus Rheinheimera Brettar et al. 2002. *International Journal of Systematic and Evolutionary Microbiology*, *68*(7), 2340–2347. https://doi.org/10.1099/ijsem.0.002838

Shigeno, Y., Liu, H., Sano, C., Inoue, R., Niimi, K., & Nagaoka, K. (2022). Individual variations and effects of birth facilities on the fecal microbiome of laboratory-bred marmosets (Callithrix jacchus) assessed by a longitudinal study. *PLOS ONE*, *17*(8), e0273702. https://doi.org/10.1371/journal.pone.0273702

Sieler, M. J., Al-Samarrie, C. E., Kasschau, K. D., Varga, Z. M., Kent, M. L., & Sharpton, T. J. (2023). Disentangling the link between zebrafish diet, gut microbiome succession, and Mycobacterium chelonae infection. *Animal Microbiome*, *5*, 38. https://doi.org/10.1186/s42523-023-00254-8

Singh, R. K., Chang, H.-W., Yan, D., Lee, K. M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T. H., Bhutani, T., & Liao, W. (2017). Influence of diet on the gut microbiome and implications for human health. *Journal of Translational Medicine*, *15*(1), 73. https://doi.org/10.1186/s12967-017-1175-y

Small, C. M., Beck, E. A., Currey, M. C., Tavalire, H. F., Bassham, S., & Cresko, W. A. (2023). Host genomic variation shapes gut microbiome diversity in threespine stickleback fish. *mBio*, *14*(5), e00219-23. https://doi.org/10.1128/mbio.00219-23

Smith, C. C., Snowberg, L. K., Gregory Caporaso, J., Knight, R., & Bolnick, D. I. (2015). Dietary input of microbes and host genetic variation shape among-population differences in stickleback gut microbiota. *The ISME Journal*, *9*(11), Article 11. https://doi.org/10.1038/ismej.2015.64

Song, S. J., Lauber, C., Costello, E. K., Lozupone, C. A., Humphrey, G., Berg-Lyons, D., Caporaso, J. G., Knights, D., Clemente, J. C., Nakielny, S., Gordon, J. I., Fierer, N., & Knight, R. (2013). Cohabiting family members share microbiota with one another and with their dogs. *eLife*, *2*, e00458. https://doi.org/10.7554/eLife.00458

Spalding, Volney M., F. E. (1909). Distribution and Movements of Desert Plants. *Science*, *31*(805), 863–866. https://doi.org/10.1126/science.31.805.863

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T., Sigsgaard, E. E., & Hellström, M. (2017). Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: Advantage of enclosed filter. *Methods in Ecology and Evolution*, *8*(5), 635–645. https://doi.org/10.1111/2041-210X.12683

Sprague, J., Doerry, E., Douglas, S., & Westerfield, M. (2001). The Zebrafish Information Network (ZFIN): A resource for genetic, genomic and developmental research. *Nucleic Acids Research*, *29*(1), 87–90. https://doi.org/10.1093/nar/29.1.87

Stagaman, K., Burns, A. R., Guillemin, K., & Bohannan, B. J. (2017). The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. *The ISME Journal*, *11*(7), Article 7. https://doi.org/10.1038/ismej.2017.28

Stagaman, K., Sharpton, T. J., & Guillemin, K. (2020). Zebrafish microbiome studies make waves. *Lab Animal*, *49*(7), 201–207. https://doi.org/10.1038/s41684-020-0573-6

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. M. (2015). The composition of the zebrafish intestinal microbial community varies across development. *The ISME Journal*, *10*(3), Article 3. https://doi.org/10.1038/ismej.2015.140

Steury, R. A., Currey, M. C., Cresko, W. A., & Bohannan, B. J. M. (2019). Population Genetic Divergence and Environment Influence the Gut Microbiome in Oregon Threespine Stickleback. *Genes*, *10*(7), Article 7. https://doi.org/10.3390/genes10070484

Stressmann, F. A., Bernal-Bayard, J., Perez-Pascual, D., Audrain, B., Rendueles, O., Briolat, V., Bruchmann, S., Volant, S., Ghozlane, A., Häussler, S., Duchaud, E., Levraud, J.-P., & Ghigo, J.-M. (2021). Mining zebrafish microbiota reveals key community-level resistance against fish pathogen infection. *The ISME Journal*, *15*(3), Article 3. https://doi.org/10.1038/s41396-020-00807-8

Sylvain, F.-É., Leroux, N., Normandeau, E., Custodio, J., Mercier, P.-L., Bouslama, S., Holland, A., Barroso, D., Val, A. L., & Derome, N. (2022). *Fish-microbe systems in the hostile but highly biodiverse Amazonian blackwaters* (p. 2022.10.22.513327). bioRxiv. https://doi.org/10.1101/2022.10.22.513327

T

Tanguay, R. L. (2018). The Rise of Zebrafish as a Model for Toxicology. *Toxicological Sciences*, *163*(1), 3–4. https://doi.org/10.1093/toxsci/kfx295

Tavalire, H. F., Christie, D. M., Leve, L. D., Ting, N., Cresko, W. A., & Bohannan, B. J. M. (2021). Shared Environment and Genetics Shape the Gut Microbiome after Infant Adoption. *mBio*, *12*(2), e00548-21. https://doi.org/10.1128/mBio.00548-21

Taylor, A. D. (1990). Metapopulations, Dispersal, and Predator-Prey Dynamics: An Overview. *Ecology*, *71*(2), 429–433. https://doi.org/10.2307/1940297

Teame, T., Zhang, Z., Ran, C., Zhang, H., Yang, Y., Ding, Q., Xie, M., Gao, C., Ye, Y., Duan, M., & Zhou, Z. (2019). The use of zebrafish (*Danio rerio*) as biomedical models. *Animal Frontiers*, *9*(3), 68–77. https://doi.org/10.1093/af/vfz020

Terborgh, J. W., & Faaborg, J. (1980). Saturation of Bird Communities in the West Indies. *The American Naturalist*, *116*(2), 178–195. https://doi.org/10.1086/283621

Teunisse, G. M. (2022). Fantaxtic - Nested Bar Plots for Phyloseq Data. R package version 2.0.1. https://github.com/gmteunisse/Fantaxtic

Thormar, E. A., Rasmussen, J. A., Mathiessen, H., Marana, M. H., Clausen, C. G., Hansen, M., Kodama, M., von Gersdorff Jørgensen, L., & Limborg, M. T. (2024). A zebrafish model to elucidate the impact of host genes on the microbiota. *Environmental DNA*, *6*(1), e513. https://doi.org/10.1002/edn3.513

Tinbergen, N. (1939). On the Analysis of Social Organization Among Vertebrates, with Special Reference to Birds. *The American Midland Naturalist, 21*(1), 210-234.

Trevarrow, B., & Robison, B. (2004). Genetic Backgrounds, Standard Lines, and Husbandry of Zebrafish. In *Methods in Cell Biology* (Vol. 77, pp. 599–616). Academic Press. https://doi.org/10.1016/S0091-679X(04)77032-6

U

Unzueta-Martínez, A., Scanes, E., Parker, L. M., Ross, P. M., O'Connor, W., & Bowen, J. L. (2022). Microbiomes of the Sydney Rock Oyster are acquired through both vertical and horizontal transmission. *Animal Microbiome*, *4*(1), 32. https://doi.org/10.1186/s42523-022-00186-9

V

Vadstein, O., Attramadal, K. J. K., Bakke, I., Forberg, T., Olsen, Y., Verdegem, M., Giatsis, C., Skjermo, J., Aasen, I. M., Gatesoupe, F.-J., Dierckens, K., Sorgeloos, P., & Bossier, P. (2018). Managing the Microbial Community of Marine Fish Larvae: A Holistic Perspective for

Larviculture. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2018.01820

Vargas, O., Gutiérrez, M. S., Caruffo, M., Valderrama, B., Medina, D. A., García, K., Reyes-Jara, A., Toro, M., Feijóo, C. G., & Navarrete, P. (2021). Probiotic Yeasts and Vibrio anguillarum Infection Modify the Microbiome of Zebrafish Larvae. *Frontiers in Microbiology*, *12*. https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2021.647977

Vellend, M. (2010). Conceptual synthesis in community ecology. *The Quarterly Review of Biology*, *85*(2), 183-206.

Vestrum, R. I., Attramadal, K. J. K., Winge, P., Li, K., Olsen, Y., Bones, A. M., Vadstein, O., & Bakke, I. (2018). Rearing Water Treatment Induces Microbial Selection Influencing the Microbiota and Pathogen Associated Transcripts of Cod (Gadus morhua) Larvae. *Frontiers in Microbiology*, *9*. https://www.frontiersin.org/articles/10.3389/fmicb.2018.00851

W

Wang, A., Zhang, Z., Ding, Q., Yang, Y., Bindelle, J., Ran, C., & Zhou, Z. (2020). Intestinal Cetobacterium and acetate modify glucose homeostasis via parasympathetic activation in zebrafish. *Gut Microbes*, *13*(1), 1–15. https://doi.org/10.1080/19490976.2021.1900996

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology*, *73*(16), 5261-5267. https://doi.org/10.1128/aem.00062-07

Wang, Y., Fei, S., Gao, X., Wu, H., Hong, Z., & Hu, K. (2024). Mechanical abrasion stimulation: Altered epidermal mucus composition and microbial community in grass carp (*Ctenopharyngodon idella*). *Aquaculture Reports*, *35*, 101936. https://doi.org/10.1016/j.aqrep.2024.101936

Weitekamp, C. A., Phelps, D., Swank, A., McCord, J., Sobus, J. R., Catron, T., Keely, S., Brinkman, N., Zurlinden, T., Wheaton, E., Strynar, M., McQueen, C., Wood, C. E., & Tal, T. (2019). Triclosan-Selected Host-Associated Microbiota Perform Xenobiotic Biotransformations in Larval Zebrafish. *Toxicological Sciences*, *172*(1), 109–122. https://doi.org/10.1093/toxsci/kfz166

White, E. P., & Hurlbert, A. H. (2010). The Combined Influence of the Local Environment and Regional Enrichment on Bird Species Richness. *The American Naturalist*, *175*(2), E35–E43. https://doi.org/10.1086/649578

Wickham, H. (2016). *ggplot2: Elegant graphics for Data Analysis.* Springer, Cham.

Wilcoxon, F. (1945). Individual Comparisons by Ranking Methods. *Biometrics Bulletin*, *1*(6), 80–83. https://doi.org/10.2307/3001968

X

Xiao, R., Wei, Y., An, D., Li, D., Ta, X., Wu, Y., & Ren, Q. (2019). A review on the research status and development trend of equipment in water treatment processes of recirculating aquaculture systems. *Reviews in Aquaculture*, *11*(3), 863–895. https://doi.org/10.1111/raq.12270

Xie, M., Hao, Q., Xia, R., Olsen, R. E., Ringø, E., Yang, Y., Zhang, Z., Ran, C., & Zhou, Z. (2022). Nuclease-Treated Stabilized Fermentation Product of Cetobacterium somerae Improves Growth, Non-specific Immunity, and Liver Health of Zebrafish (Danio rerio). *Frontiers in Nutrition*, *9*. https://www.frontiersin.org/articles/10.3389/fnut.2022.918327

Y

Yajima, D., Fujita, H., Hayashi, I., Shima, G., Suzuki, K., & Toju, H. (2023). Core species and interactions prominent in fish-associated microbiome dynamics. *Microbiome*, *11*(1), 53. https://doi.org/10.1186/s40168-023-01498-x

Young, G. R., Sherry, A., & Smith, D. L. (2023). Built environment microbiomes transition from outdoor to human-associated communities after construction and commissioning. *Scientific Reports*, *13*(1), Article 1. https://doi.org/10.1038/s41598-023-42427-0

Z

Zhang, P. (2022). Influence of Foods and Nutrition on the Gut Microbiome and Implications for Intestinal Health. *International Journal of Molecular Sciences*, *23*(17), 9588. https://doi.org/10.3390/ijms23179588

Zhao, F., Guo, M., Zhang, M., Duan, M., Zheng, J., Liu, Y., & Qiu, L. (2022). Sub-lethal concentration of metamifop exposure impair gut health of zebrafish (*Danio rerio*). *Chemosphere*, *303*, 135081. https://doi.org/10.1016/j.chemosphere.2022.135081

Zhao, Y., Qin, Z., Huang, Z., Bao, Z., Luo, T., & Jin, Y. (2021). Effects of polyethylene microplastics on the microbiome and metabolism in larval zebrafish. *Environmental Pollution*, *282*, 117039. https://doi.org/10.1016/j.envpol.2021.117039

Zheng, D., Liwinski, T., & Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Research*, *30*(6), Article 6. https://doi.org/10.1038/s41422-020-0332-7

Zobel, M. (1997). The relative of species pools in determining plant species richness: An alternative explanation of species coexistence? *Trends in Ecology & Evolution*, *12*(7), 266–269. https://doi.org/10.1016/S0169-5347(97)01096-3