THE DESIGN AND CONSTRUCTION OF AN AXENIC SYSTEM, AND THE SURVIVAL, GROWTH, AND BASELINE MICROBIAL LOAD OF THE REARING ENVIRONMENT, FOOD SOURCE AND INTERNAL MICROBIOTA OF A CICHLID FISH

By

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“A self-denial, no less austere than the saint's, is demanded of the scholar. He must worship truth, and forgo all things for that, and choose defeat and pain, so that his treasure in thought is thereby augmented.”

~ Ralph Waldo Emerson

Abstract

A novel axenic apparatus was designed and constructed for use as a research platform in germ-free fish larvae culture and the development of antibiotic alternatives. The system contains many innovations to the systems most used in germ-free aquaculture research today. Using a cichlid (*Synspilum*) species and a cichlid hybrid (*Synspilum* x *Amphilophus*), the system was tested under holoxenic conditions to ensure that fish can survive in such an apparatus. The system has six chambers, of which three were stocked with *Synspilum* and three with the hybrid (n=15 each). Two control tanks were set up and one was stocked with the *Synspilum* and one with the hybrid (n=45 each). The experiment was run for a duration of 16 days. Survival and growth (length, mm and weight, g) measured and compared between the *Synspilum* and the hybrid, as well as individuals reared in the apparatus and those in control tanks. Using microbiological culture techniques, a baseline bacterial load was determined for the rearing environment, the feed source, the source water, and the fish gut, within the apparatus and a set of control tanks. These were compared for *Synspilum* and the hybrid, as well as for individuals in the apparatus versus those in control tanks.

Using R statistical software to perform a statistical analysis, no significant differences were found in survival between groups (two-sample t (5.311) = -1.528, p= 0.184). Significance did occur in a linear model comparing the final length and survival ($F_{1,6}$= 5.529, p= 0.0569, α= 0.1, adjusted R^2=0.392). However, no differences were found in fish length or weight (two-sample t (5.162)= 1.443, p= 0.207; two-sample t (5.919)= -0.348, p= 0.740).It was determined that the baseline bacterial load of the chambers and tanks were not significantly different (two-sample t (1.007)=-1.018, p=0.493). Also, no differences were found in the baseline bacterial load of the fish gut between those in chambers and in tanks (two-sample t (1.007)=-1.018, p=0.493). The maximum bacterial load observed in the rearing environment was $2.26x10^6$ CFU/ml, and $1.800x10^6$ CFU/ml for the three-fish PBS-sample suspension (Phosphate buffered saline). These are considered the baselines. These findings reveal that *Synspilum* and *Synspilum* x *Amphilophus* perform similarly to each other and within the apparatus and control tanks under holoxenic conditions. This is evidence that the apparatus is ready for further testing under axenic conditions and that these fishes are good model species to use within the system.

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Definitions

Gnotobiotic animal (gnotobiote)- “an animal derived by aseptic cesarean section (or sterile hatching of eggs) which are reared and continuously maintained with germ-free techniques under isolator conditions and in which the composition of any associated fauna and flora, if present, is fully defined by accepted current methodology” (Gnotobiotes, 1971).

Axenic animal or conditions- “A gnotobiote which is free from all demonstrable associated forms of life including bacteria, viruses, fungi, protozoa, and other saprophytic or parasitic forms” (Gnotobiotes, 1971).

Axenic system- a system or apparatus used for the rearing of axenic and gnotobiotic animals

Holoxenic animal or conditions (conventional)- opposite of an “axenic” animal; an animal exposed to or reared in conditions open to the environment, with *normal*, undefined, and unmanipulated microbiota.

Microbiome- “The complete genetic content of all the microorganisms that typically inhabit a particular environment, especially a site on or in the body, such as the skin or the gastrointestinal tract.” *(*American Heritage Dictionary of the English Language, 2011)

Microbiota- “an ecological community of commensal, symbiotic, and pathogenic microorganisms” (Lederberg and McCray, 2001).

Pathogenicity- “the quality of producing or the ability to produce pathologic changes or disease” (Miller-Keane Encyclopedia and Dictionary of Medicine, 2003).

Virulence- “the degree of pathogenicity of a microorganism as indicated by case fatality rates and/or its ability to invade the tissues of the host; the competence of any infectious agent to produce pathologic effects” (Miller-Keane Encyclopedia and Dictionary of Medicine, 2003).

Chapter 1

Introduction

In light of current human population growth trends, demand for food has grown rapidly and is expected to continue to grow as the human population continues to expand. The current human population is around 7.25 billion people and is projected to *peak* at 9.22 billion people in 2075 (UN). Fish and other aquatic organisms are a major food resource for humans, and exploitation of wild stocks and capture fisheries have been relied upon throughout history. In fact, the global per capita rate of fish consumption is 20 kg per year (FAO 2016), and the growing demand for these resources surpasses the ability of wild stocks in capture fisheries to be sustainably exploited. Therefore, new food production methods are needed to be able to meet subsequent demands for resources and aquaculture, or the intentional rearing of aquatic organisms in tanks or ponds, will become increasingly important. The industry currently provides more than 50% of fish for direct human consumption, and is projected to provide around 62% by 2030 (Cruz et al., 2012; FAO, 2014). Considering this, aquaculture contributions are very significant to the overall food supply. Over 220 aquatic species are produced through aquaculture and these include both fish and crustaceans (FAO, 1999; Naylor et al., 2000). Cruz et al. (2012) highlighted four primary reasons for growth in aquaculture production from the 2012 State of World Fisheries and Aquaculture: fisheries are being exploited at unsustainable rates, the tastes and preferences of consumers are changing, demand is increasing for nutritious, high protein aquatic resources, and aquaculture contributes very little to climate change compared to other agricultural production systems.

It is desirable to see increased production of economically important species as demand increases so as to reduce pressure on natural populations. There are three ways to do this. First, it must be shown that there is an economic incentive to invest in fish farms so that the number of farms may increase in order to meet the growing demand for food. Second, there are barriers to production which need to be evaluated. Third, each of these barriers should be addressed so that production at each farm will increase.

There are many barriers to aquaculture which impose limitations to the growth of the industry. These include nutrition, waste management, and growth, but these are all linked to disease in some way. This work seeks to address the barrier of disease, specifically, so that production of economically important aquaculture species increases and continues to meet the demand. Herein, the problems associated with disease, current treatments and novel treatments are addressed, and a potential solution for finding alternative treatments for disease

is described and tested. In chapter 2, a review of the current literature is presented. Chapter 3 contains a list of research objectives. Chapter 4 contains a complete description of the axenic apparatus that has been designed, as well as the protocols and theoretical (currently untested but proposed) procedures for cleaning, maintaining, and operating the system. Chapter 5 describes an experiment performed to evaluate survival and growth between individuals reared in the axenic apparatus under holoxenic conditions. Then a baseline bacterial load is determined for the fish gut, rearing environment, water source, and live feed source, under holoxenic conditions. Chapter 6 provides a discussion of the overall implications of the system and the future of germ-free research in aquaculture.

Chapter 2

Background and Justification

Introduction

The body of literature relating to disease in aquaculture is extensive. The current treatments and their associated problems are well represented and novel treatments have been proposed. However, many of these proposed treatments are still being developed and tested to ensure their efficacy. In this section, a discussion on the current literature related to disease is presented, which includes the impacts of disease on the aquaculture industry, as well as the current treatments, new treatments, prevention, and potential solutions to disease, which includes the use of axenic systems.

Disease in Aquaculture

Disease in aquaculture operations has many potential consequences for both society and producers which can include decreased yields, leading to financial losses for producers and local economies, as well as losses to global food supplies. This leads to increases in dependence on already stressed wild fish populations.

The prevalence of disease in aquaculture is largely a result of standard farming practices. Optimum fish production requires intensive farming techniques, or “Rearing at high densities, compression of rearing cycles, use of brood stock with limited host genetic diversity, and accepting endemic disease in cultured populations,” (Kennedy et al., 2016). These practices increase the susceptibility of fish through increased stress and increased contact with other individuals. Additionally, these practices are leading to the increased virulence and pathogenicity of disease causing agents (Kennedy et al., 2016; Sahu et al., 2008; Lewin, C.S., 1992; Cruz et al., 2012). Ultimately, the push to increase production in aquaculture facilities is leading to decreased production in many cases due to the consequences of each of these factors as they relate to disease.

The aquaculture industry in many places is shrinking due to decreased production, as a consequence of disease. China, for instance, reported losses of $750 million in 1993, and India reported losses of $210 million between 1995 and 1996, all associated with disease (Bondad et al., 2005; Cruz et al., 2012). Globally, disease-associated losses are estimated to be worth several billion dollars (U.S. Currency) annually (Defoirdt et al., 2011; FAO, 2014). Therefore,

finding new solutions for disease prevention and treatment, to decrease mortality and increase yields, is necessary if aquaculture is to remain a viable enterprise.

Fish reared in aquaculture systems are threatened by exposure to many pathogens including viral, fungal, and especially bacterial. Mortality and failure to thrive leading to growth issues are often the result of infected fish stocks if not treated properly. Some common pathogens include *Aeromonas* spp., *Citrobactor* spp., *Edwardsiella* spp., *Photobacterium* spp., and *Vibrio* spp. (Pirarat et al., 2006; Kesarcodi-Watson et al., 2007; Defoirdt, 2011; Suantika et al., 2013). Each of these pose their own challenges and are often treated using broad spectrum therapies, but these can lead to an entirely new set of risks.

Current Treatments for Disease and the Impacts to Aquaculture Systems

Chemotherapeutic drugs are often administered in aquaculture systems to prevent or treat disease and of these, antibiotics are the primary treatment. 18,000 tons of antibiotics are produced annually within the U.S. and 12,600 tons of those are used for non-therapeutic purposes as growth promoters in agriculture, and some characterize this as *antibiotic overuse* (SCAN, 2003; Sahu et al., 2008). This overuse, or otherwise misuse, continues to contribute to the rise and persistence of antibiotic resistant pathogens (Benbrook, 2002; Kesarcodi-Watson et al., 2008; Defoirdt et al., 2011). Despite this, the only preventative measure used conventionally and considered effective against *Edwardsiella tarda*, for example, is chemotherapy (Pirarat et al., 2006).

Antibiotic resistance in bacterial pathogens occurs through the acquisition of plasmids via horizontal gene transfer (Sahu et al., 2008; Cruz et al., 2012). Bacteria are very promiscuous in nature and can transfer plasmid to adjacent bacteria via a sex pilus. It is within this bacterial DNA or plasmid that genes reside, which when expressed, convey resistant traits within the origin and recipient bacteria. This means that a bacterium can acquire resistance without ever having been exposed to an antibiotic, and one individual can transfer plasmid to multiple individuals which is indicative of an exponential growth trend in bacteria conveying some resistance.

Antibiotic use can be costly to purchase in the potential impacts to aquaculture. For instance, unregulated antibiotic use and the subsequent rise of resistance in pathogens has affected shrimp production in the Philippines by causing major crashes in those systems. There, between 1995 and 1997, shrimp production dropped by 55%, from 90,000 tons to 41,000 tons annually (Kesarcodi-Watson et al., 2008). Kesarcodi-Watson et al. (2008) report that according

to FAO (2007), the industry was previously worth $760 million and is now only worth $240 million and it has not recovered to any degree. Likewise, production dropped by 41% in Thailand between 1994 and 1997 (FAO, 2007; Kesarcodi-Watson et al., 2008). In both cases, antibiotic resistant pathogens were the cause. In the long-term, antibiotic use is proving counterproductive, for this reason.

An additional negative impact of antibiotic use is the non-species specific or broad spectrum action. Not only do antibiotics kill or otherwise inhibit the growth of pathogenic microbes, they do the same for non-pathogenic microbes and often these types of microbes have beneficial impacts on the host organism such that their destruction is detrimental to the health of the host. Understanding this relationship is imperative to a holistic view of solving the problem of disease in aquaculture.

These factors have led the European Union to ban antibiotic growth promoters in livestock and continue to phase out antibiotic use in non-human animals altogether (Cogliani et al., 2011). Therefore, if antibiotics are having such detrimental impacts on human and animal health, are being rendered ineffective due to the rise of resistant pathogens, and are being banned throughout large parts of the world, new treatments will need to be developed to address disease in agriculture in general, but especially in aquaculture.

Human and Wildlife Implications of Antibiotic Use

In terms of antibiotic resistant pathogens, there is even some concern that bacteria from aquaculture systems may transfer resistant genes to human pathogens. For example, a cholera epidemic between 1991-1994 in Ecuador, was a result of resistance in terrestrial *Vibrio cholera* which was incurred via transfer from a shrimp-pathogenic *Vibrio* sp. (Kesarcodi-Watson et al., 2008; Cruz et al., 2012). More recently, however, the bacterium *Clostridium difficile* has become problematic in humans due to overgrowth which is a direct result of antibiotic overuse; antibiotic overuse changes intestinal flora communities (Stevens et al., 2011). According to the CDC (2015), 500,000 people were affected by *Clostridium difficile* infection and 29,000 deaths occurred, in 2014. Researchers have noted that in healthy humans, with healthy microbiomes, this bacterium poses few problems as *Clostridium difficile* overgrowth is kept in check via competition from pre-existing microbiota within the intestines. However, the incidence of this type of infection is expected to increase into the future and for this reason, minimizing antibiotic use in humans and non-human animals alike is extremely important (CDC, 2015). It is unclear whether antibiotic use in aquaculture has a direct influence on *Clostridium difficile* infection in

humans but pharmaceutical antibiotics in general are ubiquitous within the environment and both humans and wildlife are exposed inadvertently. Antibiotics are an emerging contaminant in aquatic environments and are routinely found persisting in wastewater treatment facilities (Petrovic et al., 2003). They have even been detected in drinking water from municipal water supplies around the country due to insufficient means of removal during treatment. It is unclear whether this low dose exposure has any effect on *C. difficile* overgrowth within human systems but it is even more unclear as to the extent of impacts to wildlife, fish, macroinvertebrate, and plant communities and the prevalence of disease therein.

Importance and Origins of Microbiomes in Humans and in Fish

In humans, bacterial cells outnumber eukaryotic cells 10:1 and in healthy humans, 100 trillion bacterial cells exist internally and externally. This bacterial load can weigh up to 5 lbs. (ASM, 2008). However, less than 1% of all bacteria on the planet are harmful to humans (NIH, 2015). Microbiomes are extremely important to any given host, including fish, for their ability to regulate gene expression, but also for the links between microbiota and behavioral, neurological, immune system, and digestive function; they have positive effects on each (Heselmans et al., 2004; Cruz et al., 2012; Faith et al., 2014). Intestinal microbiota in humans are connected to brain health and function through multiple pathways including spinal pathways, the vagus nerve, tryptophan metabolism and neurotransmitter release, cortisol levels and other pathways (Cryan and Dinan, 2015). Cruz et al. (2012) explain that the antimicrobial effect of microorganisms occurs by altering microbial communities within the intestines, producing antibiotic compounds, competing with pathogens for space and nutrients, and by neutralizing pathogen toxins. The antimicrobial substances produced, as is the case with *Bacillus* spp., are lysozyme, protease, hydrogen peroxide, polymyxin, colistin, and circulin, all of which exhibit this antibacterial effect (Verschuere, 2000; Rosario et al., 2005). For these reasons, the internal microbiome is very important to host health.

A host's normal microbiota originates from several places. Initial neonatal microbial exposure occurs through the vertical transmission of the microbiota of the parent gastrointestinal tract to offspring, but subsequent microbial inoculation occurs via exposure to extrinsic environmental microorganisms and the early diet (Mackie et al., 1999). However, in fish the primary source of the internal microbiota is thought to be the consumption of food resources at first feeding, within their immediate environment (Dehler et al., 2017). Internal microbial communities can then be altered via changes in habitat and subsequently diet, and this can be the primary source of microbial exposure over the lifetime of the fish thus acting as a stabilizer

of the internal microbiome. Therefore, the consumption of food is a major driver in the transmission of microorganisms from the environment to the fish gut.

Novel Treatments for Disease in Aquaculture: Probiotics

There are several areas of research related to the development of new and improved disease treatments in agriculture, including aquaculture: prebiotics, probiotics, phage therapy, and vaccines. Probiotics will be the primary treatment addressed here and are defined as “live microbial food supplements that benefit the host (human or animal) by improving the microbial balance of the body” (Fuller, 1989). Probiotics have the potential to decrease vulnerability and incidence of diseases, increase growth and nutrition, and yield other desirable phenotypes in all organisms, including fish (Verschuere et al., 2000).

The literature does contain some probiotic research in aquaculture. Pirarat et al. (2006) tested the effects of *Lactobacillus rhamnosus*, a human probiotic, against *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*) and showed that mortality was lowest in individuals that received high amounts of probiotics. Cumulative mortality was reduced in proportion to the concentration of probiont cells ingested per day. Additionally, immunohistochemistry analysis confirmed that probiotic bacteria have a beneficial effect against immunosuppression by *E. tarda* and phagocytosis is also increased. Dawood et al. (2016) found that Red Sea Bream (*Pagrus major*) fed probiotic enriched diets had increased feed utilization via improved digestion, enhanced immune response, increased protease enzyme activity, and increased growth. Liu et al. (2017) found increased resistance to disease in hybrid tilapia fed probiotic enriched diets containing seven *Lactobacillus* spp. There are many more studies which have revealed the befits of probiotics.

However, in testing probiotics, researchers often fail to account for the individual and synergistic effects of pre-existing internal and external microbiota which could potentially be major confounding influences in phenotypic responses of the host organism. If other microbes within a given system are not accounted for, there may be inter- and intra- specific interactions between microbes, and these could yield misinterpretations of results. Therefore, to better understand the relationship between fish microbiomes and disease, growth, waste management, diet, and nutrition, it is imperative that systems be developed in which microbial communities can be closely observed and manipulated. Axenic (germ-free) systems are a promising new arena for testing many aspects of host-microbe interactions as well as the microbial effects on environmental conditions that subsequently impact fish health.

Axenic Systems

The use of axenic systems is one technology that can further our understanding of host-pathogen, host-probiont, and host-pathogen-probiont interactions. This can eventually lead to alternative disease treatments in aquaculture. They are systems used for the culture of a single organism in an environment void of all other organisms, in this case microbes. The literature contains studies related to tilapia, sea bass, turbot, zebrafish, and other fish species cultured in axenic systems.

Suantika et al. (2013) tested the effects of probiotic bacteria against *Aeromonas hydrophila,* the pathogen responsible for Aeromonads Syndrome in Common Carp (*Cyprinus carpio* L.) in axenic culture. *Bacillus coagulans* and *Bacillus firmus* were found to have a probiotic action in individuals challenged with the pathogen and axenic culture was found to increase growth. Pham et al. (2008) created a protocol for generating and colonizing gnotobiotic Zebrafish for the purposes of Zebrafish research as a model organism for other vertebrate species including humans.

Issues in Current Axenic Systems Research

There are many challenges to be encountered in axenic research and each of these must be addressed to continue progress in this promising field. Many studies have failed to achieve complete axenicity. Pham et al. (2008) achieved 80-90% axenicity within their system while Suantika et al. (2013) only achieved 66% axenicity. Rekecki et al. (2009) achieved 100% axenicity but had very little contact or intervention in their system. The failure to achieve complete axenicity may be a result of the system design in combination with the frequency of interventions. These systems all require the input of sterilized air and are primarily based on the system designed by Lesel and Dubourget (1979), including Pham et al. (2008), which utilized a gnotobiotic isolator (Illustrations 1 and 2). However, air may be more difficult to sterilize than non-turbid water (Gutsol et al., 2008). Also, while filtration is often relied upon for air and water sterilization, the occasional passage of some bacteria and viruses does occur (Rutala et al., 2008). For this reason, air and water filtration should never be solely relied upon when attempting complete sterilization and elimination of microbes. The systems above do not account for these possibilities. However, if redundancies in disinfection and sterilization techniques and procedures are utilized, they may be able to achieve higher percent axenicity for longer durations with less risk of contamination during active experiments. Progress in axenic

systems design must include the use of redundant disinfection and sterilization techniques by design.

Another challenge that is encountered in axenic research is in identifying a viable and appropriate food source to be administered to individuals within the system, and this is perhaps the greatest challenge of all. Pham et al. (2008) used autoclaved pelletized fish feed but the high heat and pressure of autoclaving may degrade or denature nutrients and proteins within the feed, thereby introducing the potential for nutrient deficiencies and possible deformities. Both may confound results related to growth and survival within axenic systems. Cooking has been shown to significantly reduce key amino acid concentrations, fat, carbohydrate fractions, ash, minerals, and B- vitamins (Alajaji and El-Adawy, 2006). Live feed is a viable solution to this problem although the generation of germ-free live feed is a challenge unto itself. Tinh et al. (2006) were able to generate germ-free rotifers by altering the rotifer environment to sub-optimum conditions in order to promote the production of amictic eggs which were disinfected and hatched in sterile conditions. However, this process is time consuming and would be a challenge to accomplish on a large scale such that large quantities of germ-free rotifers could be acquired for use as a live feed source. Hache et al. (2016) attempted to generate axenic *Artemia* nauplii using high salt concentrations but these high salt concentrations can potentially alter the salinity of the fish environment to the detriment of the fish. Rinsing *Artemia* treated in this manner is an option to reduce the risk of altering the salinity but the process of rinsing *Artemia* while maintaining germ-free status is difficult and increases the risk of contamination.

Limitations to experiment duration have also been a challenge. Pham et al. (2008) found that maintaining germ-free fish for more than eight days post hatching (DPH) may be problematic due to negative phenotypic responses, including deformities that are a result of increases in fish metabolite concentrations within the system. Rawls et al. (2004) generated germ-free Zebrafish but encountered several morphological variations and encountered 100% mortality at 20 days post-fertilization (DPF). To remedy this issue, it is necessary to appropriately address the build-up of fish metabolites within the system. Pham et al. (2008), Lesel and Dubourget (1979), and others did not attempt to remove fish metabolites from their respective systems, although Pham et al. did add sterile water to their tanks periodically and make recommendations addressing the accumulation of metabolites. Any future work in axenic systems design must include some method for removing metabolites from the system to prevent the issues experienced by Rawls et al. (2004). This will potentially increase the potential

duration of experiments and reduce the risk of metabolite induced variation in individuals reared in axenic systems versus more conventional methods.

Finally, the financial and space limitations of research facilities may limit the entry into the field of germ-free research in fish. For instance, Pham et al. (2008) use a gnotobiotic isolator (Illustration 2) but this equipment is costly and requires more lab space than is often available in existing aquaculture labs. It is imperative to identify ways to cut costs and remove the financial barriers to entry into this area of research.

Implications of Current Germ-free Research

In generating germ-free fish, the origins of fish microbiomes must be understood and addressed as potential routes of contamination. Germ-free research and the design of a germ-free system and protocols is ultimately a venture in risk-management, where contamination of the system is an impending risk and the management of this risk comes in the form of removing as many routes of contamination as possible. Preventing contamination of the axenic environment requires the reduction of intervention while experiments are in progress. Water samples must be taken as few times as possible. Aseptic technique, as is standard in microbiological work, is necessary for any interventions that do occur. To prevent contamination via feeding, it must be done as few times as possible while at the same time maintaining proper fish nutrition. All inputs to these systems must be quarantined and undergo microbial analysis prior to introduction to the system. Germ-free fish must be generated using the spawning method with which the vertical transmission of microflora from parent to progeny is minimized or eliminated. If each of these risks are averted, the success rate will theoretically increase.

Overall, there have been few *major* advances in axenic systems design. The majority of germ-free fish studies still use the same or similar system developed by Lesel (1979, Illustration 1) and few changes have been made since. If this area of research is to progress, it is important to develop a novel apparatus which can be used as a standardized research platform. Any such system and subsequent protocols must be able to achieve high percent axenicity, limit the number of interventions and routes of contamination, utilize nutritionally complete food sources that are appropriate to the species of interest, allow for longer term studies of larval development under axenic and gnotobiotic conditions, and be cost and space efficient to allow more facilities to contribute to research in this field. Furthermore, germ-free research related to economically important fish species in aquaculture specifically, are grossly under-represented within the literature and more work should be done in this area.

Chapter 3

Research Objectives

Research Objectives

Progression in the field of axenic research requires a novel axenic apparatus is necessary to account for the issues experienced by other researchers and remedy the shortcomings of current system designs. In order to accomplish this task, the work performed here had four primary objectives or phases:

Phase 1. The design of an axenic system for use as a research platform in germ-free larvae culture that will be used to identify and evaluate alternative treatments for disease in aquaculture systems.

Phase 2. Construct the designed system.

Phase 3. Perform preliminary testing of the system under holoxenic conditions and modify the system to optimize performance and functionality.

Phase 4. Experimentation to begin acquiring growth and survival data as well as a baseline bacterial load within the system and the microflora of the fish model.

Overall, the goal of the research performed here is to develop a new, innovative apparatus that can be sterilized in the future, and used to perform germ-free studies. The apparatus presented here limits the number of interventions and routes of contamination, utilizes food sources which are appropriate to the species of interest and nutritionally complete, may allow for longer term studies of larval development under axenic and gnotobiotic conditions, and is cost and space efficient to allow more facilities to contribute to research in this field. It is imperative that fish survive and grow similarly within the system and tanks open to the environment under holoxenic conditions, before moving on to germ-free trials. A baseline bacterial load must be acquired before attempting to evaluate the efficacy of sterilization procedures in germ-free trials. The experiment described here accomplishes these objectives.

Chapter 4

Phase 1 and 2: Axenic Apparatus Design, Construction, and Procedures

Introduction

The following chapter is a description of the system components and procedures for operating and maintaining the apparatus. The section entitled "*Issues in Current Axenic System Research*" in chapter 3, contains many criticisms of current axenic system designs. The system described here seeks to remedy the majority of these issues to create a more effective platform. These innovations are described here. This system, and subsequent protocols, contain sterilization redundancy by design, to ensure that it can be maintained under axenic conditions as effectively as possible. It addresses the issue of fish metabolite accumulation and potential methods for germ-free live food source inputs. It also is designed to be less costly both financially and space-wise so that barriers to entry within this field are limited. Finally, perhaps the greatest innovation of this system is a shift in focus from aeration through the input of air sterilized via filtration, to oxygenation of sterile water using pure compressed oxygen and the transfer of this sterile oxygenated water to the incubation chambers. This final prototype is primarily a result of all changes made to the original prototype following many trials testing the functionality of the components and fish performance. The system, as described, was used in the experiment presented in chapter 5. This section also describes the theoretical (currently untested but proposed) protocols that have been created based on the literature pertaining to microbiology, germ-free research, and sterilization techniques. No germ-free embryos or live feed have been generated for use in the system thus far. The system has not been sterilized for use in germ-free experiments either. The protocols for these tasks are presented here to explain how the system works and what protocols are intended for use with the apparatus.

This system was loosely based on the system described by Lesel (1979, Illustration 1). That system used a large vessel of unspecified volume as an incubation environment. Cork stoppers were used as a closure in which three holes had been drilled. A catheter was inserted into one and glass tubing through the others, one being used for an air stone (circulation) and an air output through the other. The air output tubing flowed into a test tube with a liquid medium to prevent exposure to the external environment. Inflowing air was sterilized using an inline air filter. Each unit was placed in a water bath to maintain proper temperatures for incubation. While this system was effective, there are many changes that could be made to improve its design and function. I have made many changes to this original design and again, have

implemented as many redundancies as possible. In this chapter, a description of the components of the system, how to assemble the system, and the theoretical cleaning protocols, are presented. (Illustration 3)

Water Reservoir

The water reservoirs are constructed of 20 L rectangular carboys (Nalgene, Rochester, NY). Three holes were drilled into each of two carboys which have been fitted with three stainless steel female valved quick-release fittings (CPC St. Paul, MN) with 3/16 inch hose barbs. A stainless steel washer and a silicone rubber washer were threaded onto each quick-release fitting before inserting into the drilled holes. Inside the carboy, a rubber and stainless steel washer was threaded and a stainless steel nut was used to fasten the fittings tightly to the carboy wall. A 4 mm O.D. Tygon flexible tubing (Saint-Gobain Malvern, PA) of approximately 4 cm length was placed on the hose barb of fitting 2, inside the chamber. To the other end, a 0.5 micron sintered stainless steel air stone (PPD Inc., Milford, CT) with a hose barb, was attached. Fitting 1 is for the connection of the system water supply line and fitting 2 is for the connection of the incoming oxygen supply line. This oxygen supply line is equipped with an inline stainless steel one-way check valve (CPC, St. Paul, MN) with 3/16 inch hose barbs to prevent water from entering the line from the carboy. Fitting 3 is for the connection of a stainless steel inline pressure release valve (Pneumadyne Inc., Plymouth, MN), equipped with a 0.2 micron inline membranous air filter (Whatman, GE Healthcare, Wauwatosa, WI) to prevent contamination from the external environment. The pressure release valve is opened to release pressure after oxygenation of the reservoir and to allow the inflow of sterile air during flushing to prevent a vacuum from building within the reservoir, which would reduce the flow rate. The reservoir is placed above the chambers so that the movement of water through the water supply line is assisted by the force of gravity. The pressure release valve is accessible from the ground.

Ultraviolet Germicidal Lamp

Before entering the incubation chambers, water passes from the water reservoir and through an inline stainless steel ultraviolet lamp (Philips Lighting, USA). This is the next line of defense against contamination of the system. UV irradiation (252 nm) is an effective disinfectant under specific conditions. Through induction of thymine dimers, it destroys nucleic acids, thus inactivating microorganisms with the exception of bacterial spores (Rutala et al., 2008). For this reason, UV irradiation alone is not sufficient for maintaining germ-free conditions but can dramatically decrease the microbial load of air and water at close ranges and over long

exposure times. Therefore, when used in conjunction with other treatments, UV irradiation can assist in the overall elimination of microbes from the system.

Manifolds and Incubation Chambers

The water supply line connects the UV lamp to the inflow manifold which distributes the incoming water among each incubation chamber. The manifolds are constructed of anodized aluminum, which inhibits corrosion. To maintain independence between incubation chambers, the manifolds are equipped with six one-way stainless steel check valves (Brasscraft Mfg., Novi, MI), to prevent the backflow of water. Each check valve has a 4 mm hose barb to which the water supply line for an individual chamber is connected. The inflow manifold has seven valves, while the outflow manifold has six. One of the inflow check valves is used to flush the manifold and supply line before flushing the system, to prevent heated water from the UV lamp from entering the chambers. Each of the six remaining valves feeds a single chamber.

Six 1000 ml borosilicate glass media bottles (Pyrex, Corning, NY) serve as incubation chambers in which fish are stocked. Each has a stainless steel 3-port insert, a rubber seal, and a plastic closure (ChemGlass, Vineland, NJ). The three ports on the lid have specific functions within the system. One port is capped by an injectable 4 mm self-sealing rubber septum (Sigma-Aldrich, St. Louis, MO) in which a 12 inch arterial-ventral (AV) fistula with a 16 gauge needle (Becton-Dickinson, Franklin Lakes, NJ), is inserted. The opposite end of the AV fistula is equipped with a Luer lock with a threaded removable cap. A hose clamp is used to control water flow and the fistula itself is used for the syringe injection of fish embryos and live feed as well as the collection of water samples for microbial analyses. The other two ports are used for water exchange within the system, with one port serving as the inflow from the inflow manifold and the other serving as the outflow leading to the outflow manifold. A removable plastic screen cap (Thoren, Hazleton, PA) is placed on the outflow tube inside each chamber to allow debris to pass but prevent fish from being flushed from the chambers. For smaller fish, a small piece of mesh fabric may be placed inside the cap before attaching to the outflow tube. Finally, to each chamber a 2 cm magnetic stir rod is added.

Each of the six chambers are placed within a 20 L plastic water bath (Rubbermaid, Newell Brands, Hoboken, NJ) containing 5 L of chlorinated water. An iodine solution is added to this water to inhibit microbial growth. An 8 inch stainless steel stick heater (Odyssea-aquarium Co., ltd, Guangdong, China) is placed in the water bath to maintain heat within the chambers and is plugged into a thermostat with a temperature probe. An air stone is used to maintain

agitation and the diffusion of heat throughout the water bath. The entire water bath is placed on a stainless steel multi-position stir plate (Velp Scientifica, Usmate, Italy).

Pre-assembly Cleaning

No single method of disinfection or sterilization is completely effective at eliminating living microbes (Rutala et al., 2008). Therefore, cleaning must contain redundancies to ensure that all microbes have been eliminated. Disinfection must only be an initial strategy to reduce the number of microbes within the system, whereas sterilization should be the primary tool in bringing the system to an initial germ-free state. All components of the system must be washed with soap and clean water, then rinsed, and soaked in a bleach solution for 30-90 minutes at 25 C to disinfect (Rutala et al., 1997). Next, the components must be thoroughly rinsed so that no residue remains, as any residue could potentially harm the fish. Each part should be dried and autoclaved on a dry cycle. New Tygon hoses should be used for each new experiment, as they are difficult to clean. These should first be cut to the appropriate lengths and autoclaved inside an autoclavable plastic bag, with the rest of the system components. Following the autoclave cycle, the autoclavable bag should be sealed and all items should then be transferred to a laminar flow hood.

Assembly of the System and Post-Assembly Cleaning

The system must be assembled using aseptic techniques (Cote, 2001) under a laminar flow hood, wearing a lab coat, sterile gloves, and a facemask. All glass and metal components should be rinsed with ethanol and flamed using a Bunsen burner immediately before making connections. First, the chambers must be set up, then the manifolds, and finally all connections must be made. Once the system has been completely assembled, the water supply line is connected to the ozone generator (DEL Ozone, San Luis Obispo, CA). Ozone is an extremely effective decontamination agent and has been shown to reduce the number of colony-forming units by 3 $\log_{10}$, including medically important pathogens such as MRSA (Rutala et al., 2008; Sharma and Hudson, 2008).

Before proceeding, an ozone destruct device (Ozone Solutions, Inc., Hull, IA) is properly connected and the valve opened so that ozone only flows through the catalyst. The catalyst used is granulated Carulite, which is composed of manganese and copper oxides. This facilitates the breakdown of ozone and limits the risks to human health (Rutala et al., 2008). The oxygen cylinder is connected to the generator and the valve is opened slowly to begin pushing oxygen gas through the ozone generator. The pressure must not exceed 5 PSI as doing so may

rupture the membranes (per manufacturer recommendation for this specific unit). The target range should be 2.5-4 PSI and the flow rate should not exceed 5 standard cubic feet per hour (SCFH) to prevent damage to the ozone membranes. The ozone generator produces 0.25 grams of gaseous ozone per hour. Each chamber should be ozonated individually for 10 hours to completely inundate the chamber. Ozonation of the system will take a minimum of 2.5 days.

While the system is undergoing ozonation, one carboy is filled with 20 L of embryo water as described by Oyarbide et al. (2015), which is a sterile solution of several salts. This solution is autoclaved and in the other reservoir (Carboy A), a mixture of 20 L Kanamycin, Amphotericin, and Ampicillin solution is prepared (Oyarbide et al., 2015). Following ozonation of the system, the ozone generator is disconnected and the water supply line is connected to Carboy B containing the antibiotic solution. This is used to fill the chambers. Carboy B is disconnected and the system is allowed to sit for 24 hours. Immediately, Carboy A containing sterile embryo water, is connected. The antibiotic solution is then flushed from the system using the embryo water. Carboys A and B are then filled with water using the procedures described in “Filling and Flushing.” Carboy A is used to flush the system once more. The water level within each chamber is reduced to 300 ml to allow easy access to the surface by larvae post- hatching so that proper swim bladder inflation can occur. The system is now filled and ready for use. Carboy B is used for system flushes for the first day of experiments. After the final rinse, a microbial analysis should be performed to confirm sterile conditions for each chamber (See Pham et al., 2008; Oyarbide et al., 2015).

Filling the System, Flushing, and Water Quality

To maintain control of water quality within the system, the system is flushed multiple times per day. The number of flushes is dependent upon the species, size of fish, mortality within the chambers and the duration of the experiment, with flushings typically increasing as an experiment progresses. Additionally, system flushes must be adaptive, as the internal environment changes over time. Flushing is the primary method for oxygenation of the chamber environment, removal of fish metabolites and debris, as well as acquiring water samples for water quality analyses.

Flushing and Refilling the Reservoir

In order to flush and fill the system, the sterile water reservoir is prepared by filling the clean reservoir with 20 L of 3ppt sterile salt water. 3 L of autoclaved 20 ppt saltwater (121C, 15 PSI, 20 minutes) is added to 17 L of 0 ppt water which has been filtered through a vacuum

driven 0.22 micron Millipore membrane filter. All valve openings are covered with foil, the lid loose and then the entire reservoir is autoclaved. The reservoir lid is sealed immediately following the cycle, before removing from the autoclave. To connect the reservoir to the system, each hose must be connected via the three quick-release valves, first removing the foil, and spraying both the male and female fittings with an ethanol or iodine solution. Immediately connect the first hose to the reservoir and move on to the next hose. The system hose is connected to valve 1 on the front of the reservoir and the oxygen line is connected to valve 2. Then, the pressure release hose is connected to valve 3 located on top of the reservoir, first ensuring that the release valve is closed. The system is now ready to be flushed.

Before flushing the chambers, the inflow line to the system must be flushed to remove heated water from the inline ultraviolet sterilizer using the manifold flush valve. 700 ml must be withdrawn, discarding the first 200 ml, and using the remaining 500 for water quality analysis. Next, the magnetic stir rod speed is increased for one minute to agitate the internal environment and remove debris from the floor of the chambers. This must be done carefully, observing the stir rod to ensure no fish collisions or injuries are occurring during this time. Each chamber is flushed individually by first engaging the pump, opening the pressure release valve on the water reservoir, and then turning on the pressurized oxygen cylinder valve. The oxygen pressure must be adjusted to 10 psi using the pressure regulator on the cylinder. Next, the chamber one outflow valve on the outflow manifold and then the chamber one inflow valve on the inflow manifold, is opened. Fresh, oxygenated water then begins to flow through that chamber, simultaneously mixing with the water within the chamber as water is flushed out through the outflow line on the pump into a 500 ml beaker. In total 700 ml of water is removed from each chamber at each flushing, with the first 200 ml being discarded. After discarding the 200 ml, 500 ml is withdrawn and saved for water quality analysis. This procedure must be done for each chamber.

Water Quality Analyses

Water quality analysis must be performed immediately following the flushing of each chamber, as the parameters begin to change rapidly upon removal from the system or tank. The chamber water quality cannot be measured directly as this would require opening the chambers, exposing them to the external environment, thus increasing the risk of contamination. Another option to acquire water for analysis is to withdraw a sufficient volume using a large syringe connected to the access-injection line but this introduces an additional route of contamination. To reduce this risk through the elimination of unnecessary routes of contamination, water quality

analysis is performed using the water flushed from the system (see Flushing and Refilling the Reservoir). To standardize measurements, a 3 cm magnetic stir rod is added and the beaker containing the 500 ml of chamber, reservoir, or control tank water is placed on a magnetic stir plate which is adjusted to 125 rpm. A probe connected to a handheld multiparameter YSI meter is secured with a three-prong clamp, which is attached to a retort stand using a boss head. The probes are then lowered into the water sample and allowed to stabilize for 3 minutes before logging the reading. It must be noted that what is being measured here, is the mixture of the incoming water from the reservoir and the pre-existing water within the chambers. Therefore, a correction must be made to acquire a more accurate estimate of the internal concentrations of the water quality parameters of interest. Due to the incorporation of a magnetic stir rod in each chamber, it is assumed that the water within the chamber is well mixed. This correction can be made using equation 1,

$$C= \{[C_f * (V_f + V_c)] - (C_s * V_f)\}/ V_c \qquad \text{Eq. 1}$$

where C= the actual concentration of the parameter of interest prior to flushing, C_f= the concentration in the water flushed from the chambers, C_s= the concentration in the source water from the reservoir, V_f= the volume of water flushed from the chambers (in this case, 0.7 L), and V_c= the volume of water contained within the chambers (in this case, 1.16 L when full).

Feeding

Feeding must be done only after a chamber has been flushed to prevent food loss during flushing. A fresh live feed stock must be prepared each day and the food of choice is primarily dependent upon the species and/or size of the fish in each experiment. Fish are fed on an as needed basis to prevent excess food from settling to the bottom of the chambers. If allowed to do so, decomposition may occur which could degrade the water quality within a chamber. The number of feedings and the amount of food/meal in each trial must be increased slowly over the duration of the experiment to account for increases in the dietary requirements of growing larvae.

The amount of live feed per fish is based on the nutrient requirements for the species, as well as the density of the prepared live feed stock. For the feed stock, the density is calculated via microscopy, counting the number of individuals per 100 microliters three times, taking the mean and extrapolating the number of individuals per ml. Before each counting, the organisms must be euthanized using acetic Lugol's solution (5%), which also serves as a stain, making the organisms more visible and therefore easier to count. The mean number of organisms per ml

will determine how many ml is to be withdrawn from the stock to achieve the appropriate number of organisms for each chamber. This amount is then withdrawn from the stock and sieved using the appropriate sieve mesh size for the feed organism. It is then rinsed using 3 ppt saltwater and then flushed from the sieve. The live feed solution is then diluted with the same water to an appropriate volume that would accommodate the feeding of 5 ml per chamber, whatever is to be fed to control tanks, and an extra 5 ml of feed solution. A 5 ml sterile syringe is used to draw up 5 ml of the resulting solution, agitating first to ensure that the live feed is equally distributed throughout. The syringe is then connected to the access-injection line via the Luer lock. The outflow valve for the chamber being flushed, on the outflow manifold, is opened and the access-injection line hose clamp is disengaged. Then the feed is injected into the chamber. The hose clamp is then engaged and the 5 ml syringe is removed and a 10 ml sterile syringe containing 10 ml of sterile 3ppt saltwater, is connected and injected to flush the access-injection line. This is to ensure that no feed is left in the line. Before disconnecting the 10 ml syringe the hose clamp and outflow valve for that chamber must be re-engaged to prevent entry of air from the external environment. The syringe is removed, and the access-injection line is dipped into ethanol and the cap is replaced. The end is clean again and the feeding procedures are repeated for each subsequent chamber. Here again, aseptic techniques and new sterile syringes must be used for each chamber to prevent contamination.

Embryo Injection and Water Level Adjustment

Germ-free embryos can be generated using the protocol created by Pham et al. (2008) or Oyarbide et al. (2015). These embryos are then injected into the chambers. However, the appropriate needle gauge must be determined before attempting injection as this will vary by species. Preliminary testing of the syringe injection process revealed no differences in the hatching rate or survival of Zebrafish between injected (16 gauge needle) individuals and non-injected individuals. This procedure is intended to reduce the risk of contamination by allowing embryos to be added to the chambers without opening the lid and exposing the internal environment to external microbes. Following the protocols for filling the chambers, the water level is then raised to the full volume of the chambers immediately before first feeding, after all hatching has occurred.

Chapter 5

Growth, Survival, and a Microbial Baseline for Both the Rearing Environment, Internal Microbiota, and Feed of a Cichlid and a Cichlid Hybrid

Introduction

Numerous trials have been run to evaluate the functionality of the components and fish performance within the system. This trial had several objectives. First, it was necessary to test the system components following changes made in the previous trials. These changes are too numerous to explain here, but are significant to the functionality of the system. Next, the changes to protocols were tested, especially the flushing protocols. Then, a fish model was evaluated for use within this system, and the growth and survival of this species was measured. Finally, the baseline microbial load was acquired for the system, the food source, and the source water before and after UV irradiation, under holoxenic conditions. Baselines were also determined for the internal microbiota of the model. For this trial, embryos were not used, though this is the ideal stage at which fish should be introduced to the chambers.

Methods

Stocking the Chambers

To accomplish these objectives, an experiment was set up and run for a duration of 16 days. The fish model selected for this trial was *Synspilum* and *Synspilum* x *Amphilophus* hybrid larvae, which were both stocked within the chambers. The larvae were acquired from conventionally reared broodstock and at six days post-hatching (DPH), they were stocked into the chambers at a density of 15 individuals per chamber. Three chambers received *Synspilum* and three received the *Synspilum* x *Amphilophus* hybrids. This was done by adding five individuals at a time to each of three containers until 15 fish per container was reached, for both Synspilum and the hybrid. They were then added to the chambers. Two control tanks were set up outside of the system, one stocked with *Synspilum* (n=45) and one with the hybrid (n=45), using the same procedures. Both groups were maintained within separate water baths at a target temperature of 28.5 C.

Water Quality

Flushing and water quality analyses were performed using the procedures described in chapter 4. For this experiment, the flushing frequency was adaptive to the conditions within the internal environment of the chambers. In previous trials, the protocols were tested to find an

optimum flushing frequency for each species reared in the system in order to maintain stable water quality conditions. However, conditions were found to vary so widely that it may be necessary to perform flushes based on the water quality measurements. In Trial 1, flushing was performed once per day and the system crashed on day 6 (6 DPF) of the experiment. Dissolved oxygen concentration was determined to be the limiting factor. In Trial 2, flushing was performed twice per day and the system crashed on day 14 of the experiment (14 DPF) and here again, dissolved oxygen was the limiting factor. In Trial 3, the system was flushed up to three times per day for 7 days. Until day 3 of that experiment, the system was flushed once per day but the dissolved oxygen concentrations in the chambers began to reach critical levels so an additional flushing was performed until day 4. While the dissolved oxygen concentrations increased, they were still too low and an additional daily flushing was performed from day 4 until the conclusion of that experiment. Flushing the system three times per day allowed for recovery of the dissolved oxygen levels, which seemed to stabilize.

Although an adaptive flushing strategy has been shown to be effective, as the number of flushes increases the risk of contamination in germ-free trials also increases. An increase in flushing means that more inputs from the external environment flow through the system and more reservoir changes are needed. Each reservoir change comes with some inherent risk of contamination during the autoclaving, transport, and connection procedures required. Also, if flushing increases, the temperature will fluctuate more and could potentially result in excessive thermal stress for the fish. For these reasons, the water reservoirs were super-saturated with pure oxygen from a compressed oxygen cylinder, by pressurizing the carboys as described in chapter 4. In doing so, oxygen was forced into solution within the reservoirs. Additionally, oxygen was diffused through the stainless steel air stones within the reservoirs during all flushes. These steps were taken to limit the number of daily flushes needed while maintaining control over the water quality of the chamber environment. Additionally, more water was removed from the system with each flushing. For this trial, 700 ml was removed from each chamber per flush, and of that, 500 ml was used for measuring water quality as described in chapter 4.

Feeding

Feeding was performed as described in chapter 4, using *Artemia* nauplii as a live food source. A more adaptive approach was taken for this trial compared to previous trials with this system, and fish were fed only as needed two to four times per day. As such, they were fed when no *Artemia* were left either in suspension, on the bottom, or in the fish gut. This was done

to avoid the potential effects of the microbial decomposition of food residues and subsequent degradation of water quality. A general increase in the size of each meal occurred each day to keep up with the growing nutritional demands of the fish.

Microbial Analyses

To acquire a baseline environmental microbial load for this system under holoxenic conditions, water samples were collected at 0, 4, 8, 12, and 16 days. Using aseptic techniques, 5 ml of water was collected from each chamber, the *Artemia* feed stock, the water reservoir before and after ultraviolet irradiation, as well as the control tanks. Before water samples could be withdrawn from the pre-axenic system, the access-injection line was flushed by withdrawing 10 ml of water using a 10 ml sterile syringe connected to the Luer lock. This water was discarded. Next, using the same access-injection line, the water sample was withdrawn using a separate 5 ml sterile syringe. This was done to ensure that the water sample contained no water from the access-injection line. Water samples were collected from the *Artemia* stock by placing a 50 micron mesh sieve into the stock tank, inserting a sterile syringe into the sieve, and then withdrawing the sample. This was done to avoid collecting *Artemia* with the sample. Water from the water reservoir before UV irradiation was collected by dipping the syringe into the water and drawing up the appropriate volume. The same process was used for the collection of samples from the control tanks. For each sample, new sterile syringes were used to prevent cross-contamination, and all samples were transferred to 10 ml culture tubes under a laminar flow hood equipped with a high efficiency particulate air filter (HEPA), and then transferred to refrigeration at 4 C. All samples were stored for six days following each sampling event, prior to microbial analysis.

After the six-day storage period, serial dilutions were prepared using each sample. These ranged from 10^{-0} to 10^{-5} for the chambers, water reservoir and inflow line, as well as the control tanks. The *Artemia* stock water samples were diluted to 10^{-10}. 9 ml phosphate buffered saline (PBS*,* 4x stock solution) dilution blanks were prepared in 20 ml test tubes. For the *Artemia* dilutions, dilution blanks were prepared in the same way but 20 ppt NaCl solution was made using purified molecular-grade water (EMD Millipore, Darmstadt, Germany). All dilution blanks were placed in an autoclave for 20 minutes at 121 C under 15 pounds of pressure and following cooling, were stored until needed. For each sample, 1 ml was added to the first dilution blank. Each dilution thereafter was carried out by transferring 1 ml of the previous dilution to the subsequent dilution blank and vortexed before making the next dilution. 0.1 ml of each dilution was plated on two 60 x 125 mm petri plates containing 25 ml brain heart infusion (BHI) agar

(Becton-Dickinson, Franklin Lakes, NJ). BHI is a non-selective rich media for culturing fastidious and non-fastidious aerobic and anaerobic microorganisms (Difco, 2009).

Multiple incubation temperatures and oxygen conditions were used to foster the growth of as many colonies as possible. To accomplish this, one plate from each dilution was placed in an incubator within an anaerobic chamber and the rest of the plates were incubated under aerobic conditions. The plates were incubated at 28.5 C for 72 hours, after which they were incubated at 20-25 C for 72 hours. The bacterial growth was observed and colonies counted at 48, 72, and 144 hours. Only plated dilutions in which the number of colonies were between 30 and 300 were considered to be countable. In cases where more than one plated dilution of the original sample was considered countable, all plates were considered by using the mean colony count for that sample (ASTM D5465-93, 1998; ASM, 2008). The colony counts were used to calculate the number of colony forming units (CFU) per ml of sample.

To compare the gut microbiota, specifically the internal microbial load of individuals in the chambers with those in the control tanks, three fish from each chamber and control tank were sacrificed. Under a laminar flow hood, fish were euthanized using tricaine methanosulfate (ms-222; 250 mg/l) and then placed in an ethanol solution (70% v/v) to disinfect the exterior surface of the fish. The fish were then pinned on an ethanol soaked sponge bed and dissected under a dissection microscope to remove the digestive tract. Once removed, the digestive tracts from each chamber were placed in a sterile culture tube with 2 ml of sterile PBS buffer. The sample was then vortexed. The tissues were immediately macerated using a 3 ml sterile syringe with a sterile 23 gauge needle by drawing and evacuating the entire sample into the syringe four times and then drawn back up into the syringe a fifth time but before pushing the sample out, the needle was removed and a 50 micron mesh fabric filter was inserted between the needle and syringe and the needle was replaced. The sample was then pushed through the filter back into the culture sterile tube. A new syringe, needle and filter was used for each sample and discarded. Serial dilutions from 10^{-0} to 10^{-10} were prepared and plated using the same techniques as in the environmental microbial analysis. The plates were incubated at 28.5 C and were counted at 24 and 72 hours. At 72 hours, the plates were removed from the 28.5 C incubator and then incubated at 20-25 C for another 72 hours. Final colony counts were performed at this time and the CFU/ml were calculated for the combined three-fish sample. This process was performed for both aerobic and anaerobic plates as described above for the water samples.

The baseline bacterial loads for the rearing environment, gastrointestinal tract, and food source water sample were determined by using the maximum mean bacterial count within each analysis. These were then graphically compared post hoc and will be used as baselines for future work.

Growth and Survival

Growth was observed by weighing (g) and measuring the length (mm) of a subsample of individuals from each group: *Synspilum* and *Synspilum* x *Amphilophus* hybrids. This was done before and at the conclusion of the experiment, a subsample of individuals within each chamber and tank was weighed and measured. Survival was observed by counting the number of fish still alive in each chamber or tank at the end of the experiment. It was calculated using the initial number stocked.

Statistical Analyses

All statistical analyses were performed using R (version 3.3.1) statistical software and Microsoft Excel (2016). The raw survival data was arcsine transformed to approximate a normal distribution. Welch's T-tests were used to determine whether significant differences exist in final survival, final weight, and final length between the *Synspilum* and the *Synspilum* x *Amphilophus* hybrid groups. Using the same test, final survival, final weight, and final length was compared between the individuals reared within the chambers and those reared in the control tanks. A regression analysis was performed to determine whether any relationship existed between the mortality of individuals reared in the chambers and the final weights and lengths to determine whether the fish density within the chambers had any effect on growth.

Next, the rearing environment microbial data was analyzed. A Welch's two-sample T-test was performed to determine whether significant differences exist in the mean CFU/ml between daily water samples prior to UV irradiation and after UV irradiation of the source water. The number of CFU/ml in the daily chamber water samples versus those of the control tanks were also compared (one-way ANOVA) to determine differences in microbial load between the closed chambers and the open tanks. A maximum CFU/ml value was then determined for all samples regardless of the source which serves as a baseline microbial load.

The internal microbiota of the fish was then analyzed. First, Welch's T-tests were performed to compare the internal microbial load between "species" groups and then between

individuals reared within the closed apparatus versus those in the open tanks to determine whether environmental exposure has any effect on the gut flora of the fish.

Results

Water Quality Analyses

The water quality parameters of interest, ammonium [NH4] (mg/L), ammonia concentration [NH3] (mg/L), pH, and temperature were relatively stable for both the control tanks and the chambers (Table 1). The dissolved oxygen concentration [DO] (mg/L), fluctuated widely in the chambers compared to the control tanks (Figure 1). [DO] within the chambers was between -8.676 mg/L and 33.128 mg/L post-corrections, while [DO] in the control tanks was between 8.54 mg/L and11.5 mg/L. Oxygenation of the chambers began at 60 hours, following a high mortality event. In the chambers, peak [DO] was reached on day 8 (12-hr period=16).

Growth and Survival

The *Synspilum* and *Synspilum* x *Amphilophus* hybrids reared in the chambers had a mean proportion (±standard deviation) survival of 0.600±0.521 and 0.778±0.278, respectively (Figure 2). These differences were not statistically significant (t (3.158)= 0.702, p= 0.531). The overall mean proportion (±standard deviation) survival of the individuals reared in the chambers and tanks was 0.689±0.386 and 0.978±0.031, respectively. The mean survival in the chambers includes one chamber which experienced a complete mortality event. Here, this was considered an outlier based on an inter-quartile analysis and was not included in the statistical analysis. Without the outlier, the mean proportion survival in the chambers was 0.827±0.209 (Figure 3). These were not found to be significantly different (t (5.311) = -1.528, p= 0.184). The linear model evaluating the relationship between the final weight and mean proportion survival was not significant ($F_{1,6}$= 0.681, p= 0.441, adjusted R^2= -0.048). Conversely, the linear model evaluating the relationship between the final length and survival was significant ($F_{1,6}$= 5.529, p= 0.0569, α= 0.1, adjusted R^2=0.392) (Figure 4).

The mean weights of the *Synspilum* and *Synspilum* x *Amphilophus* hybrids reared within the chambers were 0.041±0.022 grams (g) and 0.028±0.012 g, respectively, and these were not significantly different (t (2.738)= -0.025, p= 0.982) (Figure 5). The mean weights of individuals reared within the chambers and those reared in tanks were 0.033±0.016 g and 0.016±0.001 g, respectively (Figure 6). These differences were not statistically significant (t (5.162)= 1.443, p= 0.207).

The *Synspilum* and *Synspilum* x *Amphilophus* hybrids reared within chambers had mean lengths of 11.221±0.355 millimeters (mm) and 12.831±1.684 mm, respectively (Figure 7). These differences were not statistically significant (t (2.269)= 1.383, p= 0.287). The mean lengths of individuals reared in chambers and tanks were 12.187±1.492 mm and 10.980±1.534 mm, respectively, and were not statistically different (t (5.919)= -0.348, p= 0.740) (Figure 8).

Microbial Analyses

No anaerobic plates from any dilution of any of the rearing environment water samples, except a few collected on day 4 of the experiment, had countable colonies within the 30-300 colony target range. Specifically, none exceeded the lower limit. Only one plated *Artemia* water sample resulted in a countable plate ($8.000x10^4$ CFU/ml), but all others exceeded the 300 colony maximum of the target range or else had zero to ten colonies.

Over all samples, a maximum aerobic bacterial load of $2.26x10^6$ CFU/ml was observed for the rearing environment. The mean aerobic bacterial load for the source water before and after UV irradiation was $7.662x10^4$± $4.123x10^4$ CFU/ml and $1.542x10^6$±$1.067x10^6$ CFU/ml, respectively (Table 2). These differences were not statistically significant (t (2.004)= 2.378, p= 0.140). The mean aerobic bacterial load in the chamber water samples versus those of the control tanks was $1.019x10^5$±$1.007x10^5$ CFU/ml and $3.067x10^4$±$3.043x10^4$ CFU/ml, respectively, and were not significantly different ($F_{7, 30}$= 1.058, p= 0.413). (Table 3)

The aerobic mean bacterial load of the *Synspilum* gastrointestinal tract was $1.325x10^5$±$1.485x10^4$ CFU/ml PBS-sample suspension and $2.020x10^5$±$1.412x10^5$ CFU/ml for the *Synspilum* x *Amphilophus* hybrid and these were not significantly different (t (2.066)= 0.846, p= 0.484). Likewise, the anaerobic bacterial load of the *Synspilum* versus the hybrid were not significantly different (t (1.001)= 0.980, p= 0.506). The *Synspilum* had a mean of $8.920x10^2$± $1.129x10^3$ CFU/ml PBS-sample suspension while the hybrid had a mean of $4.203x10^4$±$5.935x10^4$ CFU/ml. The total aerobic and anaerobic mean bacterial load of the gut of the *Synspilum* x *Amphilophus* hybrid reared in chambers was $2.300x10^5$±$1.421x10^5$ CFU/ml PBS-sample suspension and $1.335x10^5$±$1.344x10^4$ CFU/ml for the *Synspilum*. These differences were not statistically significant (t (2.053)= 1.169, p= 0.360). (Table 4, Figure 9)

The mean aerobic bacterial load of the gastrointestinal tract of individuals reared in in the closed chambers was $1.742x10^5$±$1.071x10^5$ CFU/ml and $9.800x10^5$±$1.117x10^6$ CFU/ml of PBS-sample suspension for the individuals in the open tanks (Figure 9). These were not significantly different (t (1.007)=-1.018, p=0.493). The differences in the anaerobic microbial

load of these fish were not significant either (t(1.058)= -1.021, p= 0.486). The individuals reared in chambers had a mean of $2.146x10^4 \pm 4.170x10^4$ CFU/ml and the fish reared in tanks had a mean of $1.484x10^5 \pm 1.734x10^5$. The combined aerobic and anaerobic mean intestinal microbiota for the fish reared in chambers was $1.914x10^5 \pm 1.137x10^5$ CFU/ml and $1.131x10^6 \pm 9.468x10^5$ CFU/ml for fish reared in the tanks. No significant differences were found in the overall bacterial load of the individuals reared in the closed chambers compared with those reared in the tanks (t (1.012)= -1.399, p=0.393). Overall, the maximum combined anaerobic and aerobic bacterial load of the fish gut that was observed was $1.800x10^6$ CFU/ml of the three-fish PBS-sample suspension. (Table 4)

Discussion

Overall, the apparatus performed as expected in this trial. The components functioned properly and many of the problems experienced in other trials were remedied through modifications made, whether to the components or processes, before the start of this trial. In terms of the water quality analysis, all parameters in this trial were stable and similar between chambers and the control tanks, except dissolved oxygen. It was known from previous trials that the dissolved oxygen concentration of the rearing environment within the system is a limiting factor. This seemed to be the case in this trial as well. The high mortality that occurred between the evening of day 2 and the morning of day 3, seems to have been an acute response to a drastic drop in [DO] immediately prior to the event (Figure 1). An additional mortality event occurred and is speculated to have occurred following the next drop in [DO]. Prior to the drastic decrease in [DO], an attempt was made to recover the [DO] by using the oxygenation procedures and adaptive flushing, as an emergency protocol. This was done to prevent further mortalities. No complete chamber mortalities occurred thereafter and [DO] was maintained using oxygenation for the remainder of the trial. However, the use of pure oxygen to oxygenate the source water proved unpredictable. Major fluctuations occurred in [DO] while using this method. Future research should seek to remedy this issue due to the potential that erratic fluctuation in [DO] may act as a chronic stressor for individuals within the system. Despite this, the adaptive approach to flushing and oxygenation allowed for an increase in the duration of the experiment, when compared to previous trials. Low dissolved oxygen concentrations were recoverable via oxygenation of the source water and this method is promising for future work with this system.

Growth of individuals in the chambers was very similar to that of individuals within the control tanks. No significant differences were found in length or weight between groups. The

same was true between the *Synspilum* and the hybrid. Both findings are promising in that, if the system is to be capable of testing the effects of axenicity on fish performance, the effects of a gnotobiotic challenge, or any other topic of interest, individuals within the system must grow similarly to those reared using conventional methods. This rules out covariates which affect fish performance in the system and ensures that any differences are a result of the manipulations performed using the system. Likewise, survival must also be similar between groups and in this case, no significant differences occurred in survival between rearing environments or the *Synspilum* versus the hybrid. Though no significant differences occurred in weight or length between groups or fish "species," a linear model evaluating a relationship between fish final length and survival was significant. The opposite was found between fish weight and survival. However, this may warrant more research to determine the effect size of fish length or weight on survival and to explore what size limitations may exist for individuals of this species reared within this system.

The microbial analysis revealed issues with the use of UV irradiation within this trial. The microbial load of the source water entering the chambers from the reservoir post-irradiation, was not statistically different from the microbial load prior to irradiation. This indicates that either, the efficacy of UV is overestimated or that the specific UV lamp used here is ineffective. Regardless, this further supports the notion of redundancy in the antimicrobial and germicidal components and processes of the system and protocols. More work should be performed with this and other lamps to determine whether a change in model or lamp size would remedy the issue.

The microbial analysis of the rearing environments revealed that in the absence of sterilization techniques, the microbial load within the chambers is statistically the same as the external environment. This is likely because the fish acquired their internal and external microbiomes from the same parent and they were fed the same diet from the same source. It is speculated that if these two factors are eliminated a difference in microbial load would occur. However, this has not been tested with this system, but should be in future work. However, the lack of differences between the chamber group eliminates covariates and supports the idea that any differences in microbial load within or between the chambers and control tanks is a result of the manipulations performed by the experimenter.

The origin of microbes in this trial is not known but some attempt was made to track the origin of microbes within the system. Again, there are three routes of microbe exposure including contact of eggs with the parent fishes intestinal and external microbiota, contact with

the pre-existing microbes within the rearing environment, and the food source. Here, there is insufficient evidence to determine the primary source in these conditions. However, anecdotally the *Artemia* plates were the most prolific of all plates in this trial. They had so much growth that only one plate was countable. The others experienced rapid colony growth and the differentiation between colonies was impossible. Once it was understood that this level of growth was occurring, attempts were made to acquire countable plates in several ways. First, dilutions were adjusted such that the initial dilution using the PBS blanks was increased by using less of the original dilution. When this did not work, the dilution blank solution was altered. Instead of using PBS, a sterile 20 ppt saltwater solution of neutral PH was used. This was done to imitate the rearing environment of the *Artemia* and it was speculated that the lack of growth in plates cultured with higher dilutions was a result of a decrease in salinity with each step and therefore, suboptimal growth conditions for microbes that are acclimated to life in 20 ppt saltwater. Both of these were mostly ineffective with the exception of a single plate. This plate had less than ten colonies and considering the dilution factor and volume plated, provided an estimate of 8.000×10^4 CFU/ml for the *Artemia* water sample on day 16. This estimate was less than the estimated baseline microbial load for the system and appears to be unreliable considering the massive growth observed visually on the *Artemia* plates compared with the plated rearing environment samples. More work should be done to determine the primary sources of microbial exposure within the system. This can help confirm areas of focus in terms of eliminating microbes and routes of contamination in germ-free trials. A true baseline for the food source must also be acquired as attempts here could only provide anecdotal evidence. Visually, the primary source of microbes within the system is from the food source, in this case *Artemia*.

Like the microbial load of the rearing environments, the gut microbial baseline for individuals within the chambers and control tanks were statistically similar but a low sample size may have contributed to this. This further suggests that without active manipulation of the microbial load through sterilization procedures in the system, no differences occur. This is important for future work in ensuring that all differences are a result of manipulations performed by the experimenter. However, it was observed that the cultured gut flora of fish reared both in the system and control tanks was more diverse in colony color and morphology, based on visual inspection. This would suggest that the microbiota of the fish gut was either acquired outside of the system or that the fish gut provided the optimum growth conditions for microbes living within the rearing environment but not growing. Regardless, more work should be done to determine the relationship between the microbial load of the fish gut and the microbial load of the rearing

environment. This should also be done for the food source. Ultimately this will provide an even better understanding of how fish are influenced by both their environment and their food source, and this will allow for a better understanding of how to prevent contamination of the system under germ-free conditions. Overall, the baseline bacterial loads of the rearing environment, the gastrointestinal tract, and the food source, as determined by the maximum mean (±SD) bacterial load within each of these groups, revealed similarities between each group but once again, the environment and food source baselines do not include anaerobic bacterial counts (Figure 10).

These findings are not without limitations. It must be acknowledged that the microbial analysis methods used here are insufficient for acquiring an accurate estimate of the microbial load. Using the culture method only provides results for culturable bacteria and less than 2% of all environmental bacteria and 50% of human oral flora can be cultured in a laboratory setting (Wade 2002). It is expected that the culturable gut flora of fish would be somewhere between these figures.

Another limitation is that the conditions presented in this trial may not have promoted the optimum growth of the most species was promoted. For example, although a general-purpose media was used, it still may not have contained sufficient nutrients for some microbes to grow visible colonies. Also, the temperature ranges used may not have encompassed or overlapped the optimum ranges of some microbes. The incubation time may have also been insufficient. Therefore, only microbes that had growth ranges for each parameter within the conditions used here were cultured. Molecular analysis of the samples would eliminate these issues altogether. This method would also account for any anaerobic microbes present in the sample which would provide a more complete picture. In this trial, only a few anaerobic plates were successful at producing growth. However, none of these were countable except for those for the fish gut samples. Any growth that was achieved under anaerobic conditions was most likely that of facultative anaerobes rather than obligate anaerobes. This is because obligate anaerobes would have likely been killed through exposure to oxygen during sampling and plating. If culture is used at all in the future, anaerobic samples must be collected and cultured via culture tubes rather than plates. Plates could be used for this if the anaerobic samples were acquired under anaerobic conditions, then plated, and immediately placed within a Gas Pak system to minimize oxygen exposure. Regardless the microbial baselines for the rearing environments only includes culturable aerobic bacteria and the gut microbial baseline includes the culturable facultative

anaerobes and aerobic bacteria. More work should be done to improve the methodology of microbial analyses for similar future trials.

Culture is also an extremely labor intensive and time consuming endeavor. Overall, >700 mixed environmental cultures, and >140 gut flora cultures were produced and counted. All media was mixed, autoclaved and poured by hand. PBS was prepared in house and dilutions blanks were made. Dilutions for every sample had to be prepared. These tasks require significant time and labor, making it difficult to perform the microbial analyses simultaneously with the daily operation and maintenance of the system. Molecular analyses of the samples would eliminate this issue.

Wade (2002) explains that a more accurate and less labor intensive approach to the identification and quantification of microbial species within a sample, can be learned from molecular phylogeny. Here, a comparison of 16S rRNA subunits from samples can be made with databases containing the sequences of that subunit for up to 12,000 species. He suggests that the number of unculturable bacteria within a sample can be determined via the molecular analysis of 16S rDNA, the culture of the sample and subsequent 16S rRNA analysis of the culture, subtracting the 16S rRNA results from the 16S rDNA results. The samples from this trial can be analyzed in this way to acquire more accuracy in baseline microbial load estimation (Boon et al., 2002; Ovreas et al., 1997). Identification of the microbial species within this system was beyond the scope of the trial but these molecular methods can certainly accomplish that task as well.

Overall, a greater understanding and more accurate baseline microbial load of the source water, the rearing environment, the food source, and the fish gut is necessary before proceeding with germ-free trials. This baseline provides for a comparison of the microbial load of each of these sources before and after manipulation via sterilization procedures. This ultimately will provide a measure of success and a means for further troubleshooting during attempts to achieve complete axenicity of the system.

Chapter 6

Implications of this work and potential future work

The findings from experiments performed here will serve as baselines for the biological parameters of the species of interest, and the developed protocols may be used to replicate the system in aquaculture departments worldwide. Overall, a greater understanding and more accurate baseline microbial load of the source water, the rearing environment, the food source, and the fish gut is necessary before proceeding with germ-free trials. This baseline provides for a comparison of the microbial load of each of these sources before and after manipulation via sterilization procedures. This ultimately will provide a measure of success and a means for further troubleshooting during attempts to achieve complete axenicity of the system. These experiments offer only preliminary findings related to fish performance within this system under non-germ-free conditions. Before experimentation with probiotics or pathogen challenges, this experiment must be replicated under germ-free conditions. This is to ensure that the model selected for use here can survive under germ-free conditions as well, with no changes in growth or survival.

Axenic systems in aquaculture can be used to study many phenomena and the work performed here will be foundational to future work performed with this system, contributing to the beginning of a new scientific discipline in aquaculture. Overall, this system may lead to the development of new disease treatments in aquaculture and ultimately a reduction in the prevalence of persistent pharmaceutical antibiotics within the environment. Therefore, humans, wildlife and fish may experience lower exposure and avoid the health implications that are a consequence of over-exposure to antibiotics. Furthermore, this may contribute to a reduction in antibiotic resistant pathogens.

Future work may include studies related to the testing of microbial regulation of gene expression in controlled systems such as these. Microbes have the potential to induce or regulate gene expression (Marques et al., 2006; Pham et al., 2008). Heselmans et al. (2004) reported that some bacteria regulate up to 400 genes in Zebrafish. If this is true, then it may be true that testing single microbial species, followed by the testing of entire microbial assemblages and their effect on gene expression, may lead to the development of targeted probiotic supplementation to control specific genes. This could be of help in achieving desirable phenotypes for economically important fish species, which could result in increased yields through positive influences on biological parameters such as growth, nutrient acquisition and

allocation, metabolic efficiency, or reproduction. Nutrient concentrations could also possibly be increased in fish tissues to the benefit of consumers. Using this system, specific microbiomes may be engineered to induce desired phenotypes in aquaculture species or in wild strains, which can increase disease resistance, growth, and behavior/neurological function or other qualities, of which similar work has been done using gnotobiotic mice but not fish (Faith et al., 2014). The potential of these manipulations has yet to be fully recognized, especially with aquaculture species such as tilapia and Yellow Perch (*Perca flavescens*), which are economically important. At this time, there is no published work combining these concepts with Yellow Perch and Nile Tilapia (*Tilapia niloticus*) aquaculture, and the species used here may serve as a translational model to the health of these species.

Additionally, the microbiomes from healthy wild specimens could be characterized, identifying the specific ratios and demography of bacterial species compared to the overall microbial assemblage. Once accomplished, the same could be done with domesticated specimens reared in a laboratory environment. In humans, significant microbiome differences have been observed between wild (hunter- gatherer) groups and domestic (metropolitan) groups. Additionally, the incidence of disease has been linked to these differences in microbiomes (Schnorr et al., 2014). The microbiomes of a group of hunter-gathers, the Hadza tribe of Tanzania, was found to be significantly more diverse and abundant in internal and external microbiota, and positive and direct correlations have been found in the incidence of degenerative diseases in this culture compared to that of sample populations in Italian metropolitan groups. Based on comparisons of microbiomes in domestic aquaculture species versus their wild counterparts, and how these are linked to disease resistance, the development of *optimal* microbial assemblages for overall fish health may be achieved.

Furthermore, the system may be used to compare existing fish diets including live feed sources, improve them, or develop new formulated diets, including probiotic diets. Germ-free rotifers and *Artemia* can be tested as a viable option for delivery of probiotics to host fish larvae (Tinh et al., 2006). Currently, probiotics for rotifers and *Artemia* exist, which presumably contribute to the internal microbiota of anything predating on these organisms (Dehler et al., 2017), but few studies have been done to test the efficacy of such methods of delivery in controlled environments such as axenic systems. This could potentially identify areas for improvement in rotifer and *Artemia* culture as it relates to use as a gnotobiotic live feed probiont administration mechanism.

References

Alajaji, S.A. and T.A. El-Adawy, 2006. Nutritional composition of chickpea (*Cicer arietinum* L.) as affected by microwave cooking and other traditional cooking methods. Journal of Food Composition and Analysis 18(8):806-812.

American Heritage Dictionary of the English Language, Fifth Edition. 2011. Retrieved March 19 2017 from http://www.thefreedictionary.com/microbiome.

ASM. 2008. Scientists study bacterial communities inside us to better understand health and disease [Press Release]. American Society of Microbiology. Accessed from: http://www.eurekalert.org/pub_releases/2008-06/asfm-ssb052908.php

ASTM D5465-93.1998. Standard Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods, ASTM D5465-93 International, West Conshohocken, PA, 1998, www.ASTM.org

Benbrook, C.D. 2002. Antibiotic drug use in U.S. aquaculture. IATP Report. http://www.iatp.org/files/421_2_37397.pdf (Accessed 16.04.21).

Bondad, M.G., Subasinghe, R.P., and J.R. Arthur. 2005. Disease and health management in Asian aquaculture. Veterinary Parasitology 132: 249-272.

Boon, N., De Windt, W., Verstraete, W., and E.M. Top. 2002. Evaluation of nested PCR-DGGE (denaturing gradients gel electrophoresis) with group specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiology and Ecology. 101-112.

CDC. 2015. Nearly half a million Americans suffered from *Clostridium difficile* infections in a single year [Press Release]. Centers for Disease Control and Prevention. Accessed from: http://www.cdc.gov/media/releases/2015/p0225-clostridium-difficile.html.

Cogliani, C., Goossens, H., and C. Greko. 2011. Restricting antimicrobial use in food animals: lessons from Europe. Microbe 6:274-279.

Coté, R. J. 2001. Aseptic Technique for Cell Culture. Current Protocols in Cell Biology. 00:1.3:1.3.1–1.3.10.

Cruz, P.M., Ibanez, A.L., Hermosillo, O.A.M., and H.C.R. Saad. 2012. Use of probiotics in aquaculture. Microbiology 2012:1-13.

Cryan, J.F. and T.G Dinan. 2015. More than a Gut Feeling: the Microbiota Regulates Neurodevelopment and Behavior. Neuropsychopharmacology Reviews. 40:241-242.

Dawood, M.A.O., Koshio, S. Ishikawa, M., El-Sabagh, M., Esteban, M.A., and A.I. Zaineldin. 2016. Probiotics as an environment-friendly approach to enhance red sea bream, Pagus major growth, immune response and oxidative status. Fish and Shellfish Immunology 57:170-178.

Defoirdt, T., Sorgeloos, P., and P. Bossier. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology 14:251-258.

Dehler, C.E., Secombes, C.J., and S.A.M. Marten. 2017. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (*Salmo salar* L.). Aquaculture 467:149-157.

Difco. 2009. Difco and BBL Manual: Manual of microbiological culture media. Becton-Dickinson 1-700.

Faith, J.J, Ahern, P.P., Ridaura, V.K., Cheng, J., and J.I. Gordon. 2014. Identifying gut microbe-host phenotype relationships using combinatorial communities in gnotobiotic mice. Science Translational Medicine 6: 220ra11.

FAO. 1999. Food and Agriculture of the United Nations. The State of World Fisheries and Aquaculture.

FAO. 2007. Food and Agriculture of the United Nations. The State of World Fisheries and Aquaculture.

FAO. 2009. Food and Agriculture of the United Nations. The State of World Fisheries and Aquaculture.

FAO. 2012. Food and Agriculture of the United Nations. The State of World Fisheries and Aquaculture.

FAO. 2014. Food and Agriculture of the United Nations. The State of World Fisheries and Aquaculture.

FAO. 2016. Food and Agriculture of the United Nations. The State of World Fisheries and Aquaculture.

Fuller, R. 1989. Probiotics in man and animals. Journal of Applied Bacteriology 66:365-378.

Gnotobiotes: Standards and Guidelines for the Breeding, Care, and Management of Laboratory Animals. 1971. National Academy of Sciences, Washington, D.C., 1970. ISBN 0-309-01858-7.

Gutsol, A. 2008. Plasma for Air and Water Sterilization. In: Güçeri S., Fridman A., Gibson K., Haas C. (eds) Plasma Assisted Decontamination of Biological and Chemical Agents. NATO Science for Peace and Security Series Series A: Chemistry and Biology. Springer, Dordrecht

Hache, R., Lanteigne, C., and Y. Hebert. 2016. Salt as a decontamination agent to control bacterial load in *Artemia salina* cultures. Aquaculture 452: 24-27.

Heselmans, M., Reid, G., Akkermans, L.M.A., Savelkoul, H., Timmerman, H., F.M. Rombouts. 2004. Gut flora in health and disease: potential role of probiotics. Current Issues in Intestinal Microbiology. 6:1-8.

Kennedy, D.A., Kurath, G., Brito, I.L., Purcell, M.K., Read, A.F., Winton, J.R., and A.R. Wargo. 2016. Potential drivers of virulence evolution in aquaculture. Aquaculture Magazine 42(1):16-19.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., and L. Gibson. 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture 274:1-14.

Lederberg, J. and A.T. McCray. 2001. 'Ome Sweet 'Omics—a genealogical treasury of words. Scientist. 15: 8.

Lesel, R. and Dubourget. 1979. Obtention of axenic ovoviviparous fish- technical improvement. Annales de Zoologie Ecologie Animale 11:389.

Lewin, C.S. 1992. Mechanisms of resistance development in aquatic microorganisms. Chemotherapy in Aquaculture: from Theory to Reality 288-301.

Liu, W., Wang, W., Ran, C., He, S., Yang, Y., and Z. Zhou. 2017. Effects of dietary scFOS and lactobacilli on survival, growth, and disease resistance of hybrid tilapia. Aquaculture 470:50-55.

Mackie, R.I., Sghir, A., and H.R. Gaskins. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. American Journal of Clinical Nutrition 69(5):1035s-1045s.

Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P., and P. Bossier. 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. Journal of Applied Microbiology 100:903-918.

Miller-Keane Encyclopedia and Dictionary of Medicine, Nursing, and Allied Health, Seventh Edition. 2003. Retrieved March 19 2017 from http://medical dictionary.thefreedictionary.com/pathogenicity.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., and M. Troell. 2000. Effect of aquaculture on world fish supplies. Nature 405:1017-1024.

NIH. 2015. Bacterial infections. The National Institutes of Health and Prevention, Medline Plus. U.S. National Library of Medicine. Accessed from: http://nlm.nih.gov/medlineplus/bacterialinfections.html.

Ovreas, L., Forney, L., Daae, F.L., and V. Torsvik. 1997. Distribution of bacterioplankton in mermictic Lake Saelevannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. Applied Environmental Microbiology 63:3367-3373.

Oyarbide, U., Rainieri, S., Iturria, I. and M.A. Pardo. 2015. Use of gnotobiotic Zebrafish to study *Vibrio anguillarum* pathogenicity. Zebrafish 12: 71-80.

Petrovic, M., Gonzales, S., and D. Barcelo. 2003. Analysis and removal of emerging contaminants in waste water and drinking water. Trends in Analytical Chemistry 22(10):685-696.

Pham, L.N., Kanther, M., Semova, I., Rawls, J.F. 2008. Methods for generating and colonizing gnotobiotic zebrafish. Nature Protocols 3(12):1862-1875.

Pirarat, N., Kobayashi, T., Katagiri, T., Maita, M., Endo, M. 2006. Protective effects and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against experimental Edwardsialla tarda infection in tilapia (*Oreochromis niloticus*).

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rawls, J. Samuel, B.S., and J.I. Gordon. 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proceedings of the National Academy of Sciences of the United States of America 101: 4596-4601.

Rekecki, A., Dierckens, K., Lareau, S., Boon, N., Bossier, P., Van den Broeck, W. 2009. Effect of germ-free rearing environment on gut development of larval sea bass (*Dicentrarchus labrax* L.). Aquaculture 293:8-15.

Rutala, W.A, and D.J. Weber. 1997. Uses of inorganic hypochlorite (bleach) in health-care facilities. Clinical Microbiology Reviews 10(4):597-610.

Rutala, W.A., Weber, D.J., and Healthcare Infection Control Practices Advisory Committee. 2008. Guideline for disinfection and sterilization in healthcare facilities. CDC 1-158.

Sahu, M.K., Swarnakumar, N.S., Sivakumar, K., Thangaradjou, T., Kannan, L. 2008. Probiotics in aquaculture: importance and future perspectives. Indian Journal of Microbiology 48:299-308.

SCAN. 2003. Opinion of the scientific community on animal nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance. Eurpoean Commission Health and Protection Directorate- General.

Schnorr, S.L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., Turroni, S., Biagi, E., Peano, C., Severgnini, M., Fiori, J., Gotti, R., De Bellis, G., Luiselli, D., Brigidi, P., Mabulla, A., Marlowe, F., Henry, A.G., Crittenden, A.N. 2014. Gut microbiome of the Hadza hunter-gatherers. Nature Communications 5:3654

Sharma, M., and J.B. Hudson. 2008.Ozone gas is an effective and practical antibacterial agent. American Journal of Infection Control Online 36(8):559-563.

Stevens, V., Dumyati, G., Fine, L.S., Fisher, S.G., van Wijngaarden. 2011. Cumulative antibiotic exposures over time and the risk of *Clostridium difficile* infection, Clinical Infectious Diseases 53:42-48.

Suantika, G., Aditiawati, P., Astuti, D.I., Sjarmidi, A., Lim, N., Khotimah, Z.F. 2013. Evaluation of probiotic bacteria against Aeromonads syndrome in Common Carp (*Cyprinus carpio* L.) in simple axenic larviculture. Fish and Shellfish Larviculture Symposium 433-436.

Tinh, N.T.N., Phuoc, N.N., Dierckens, K., Sorgeloos, P., Bossier, P. 2006. Gnotobiotically grown rotifer *Brachionus plicatilis* sensu strictu as a tool for evaluation of microbial functions and nutritional value of different food types. Aquaculture 253:421-432.

UN (United Nations). 2004. World Populations to 2300. United Nations Department of Economic and Social Affairs/Population Division, Report ST/ESA/SER.A/236.

Verschuere, L., Roumbaut, G., Sorgeloos, G., Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Review 64:655-671.

Wade, W. 2002. Unculturable bacteria- the uncharacterized organisms that cause oral infections. Journal of the Royal Society of Medicine 95:81-83.

Appendix A: Illustrations, Tables, and Figures

Illustrations

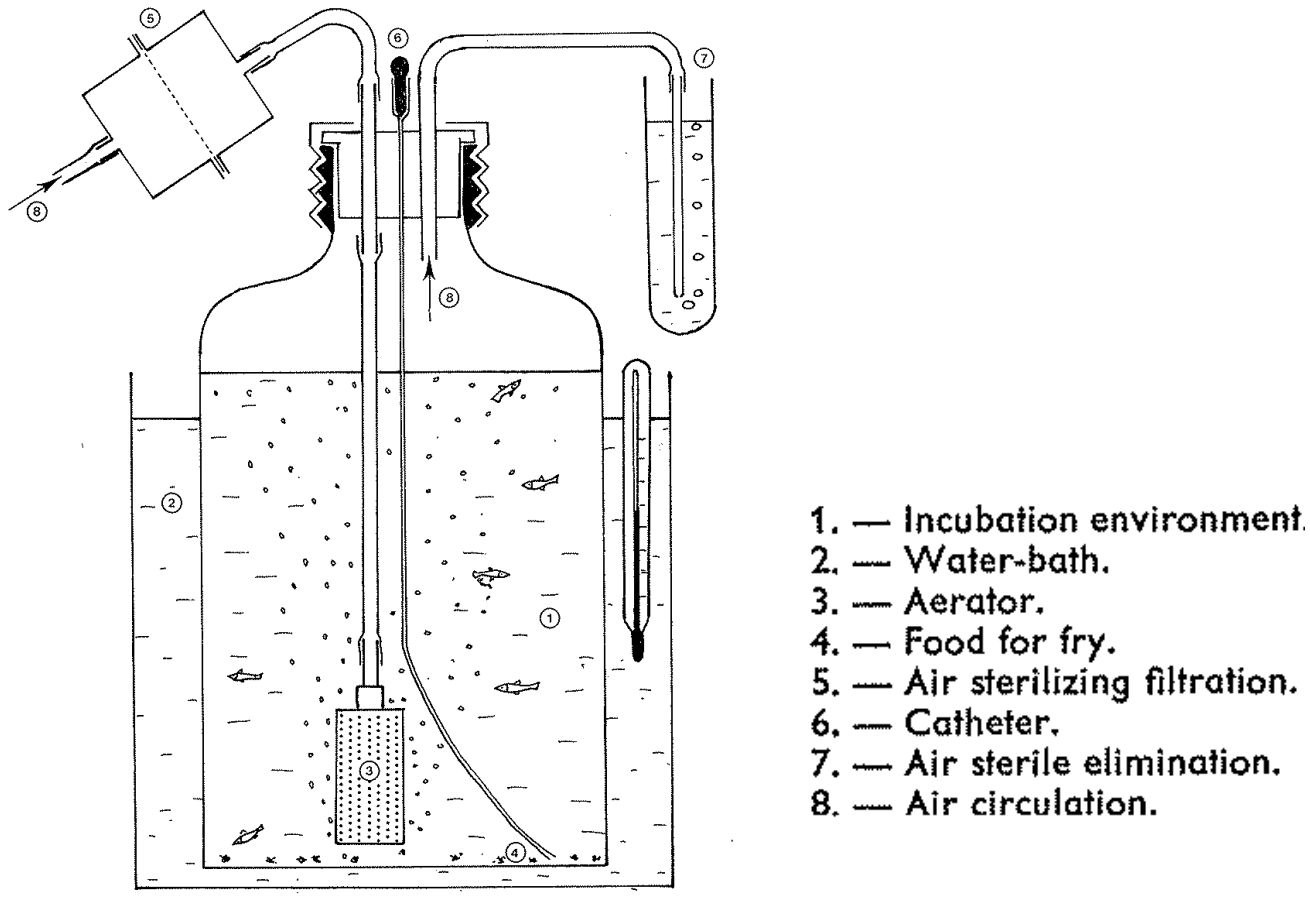
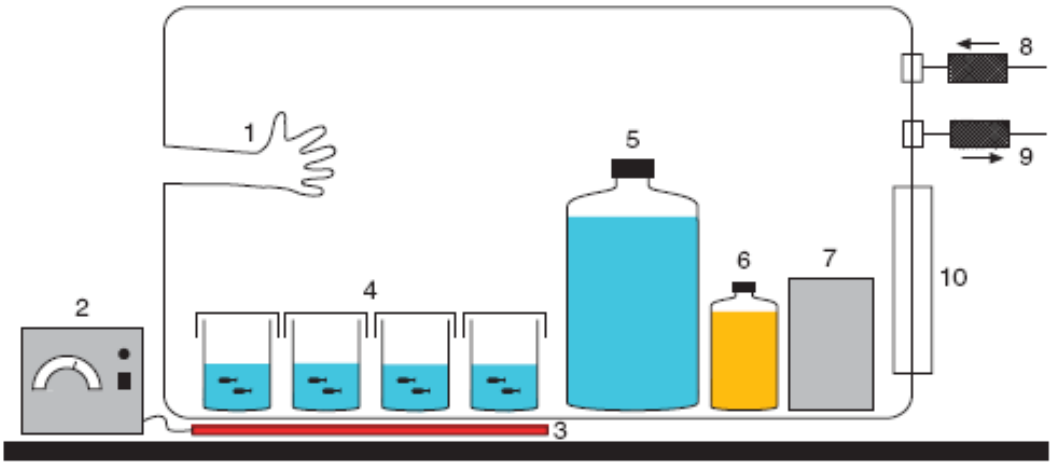


Illustration 1: An axenic system designed by Lesel and Dubourget (1979) uses filtration to sterilize air pumped into the chamber for aeration (5) and an air-lock (7) to prevent contaminating microbes from entering the air outflow.



1. Attached gloves
2. Heated water pump
3. Circulating warm water pad
4. Foil-covered beakers containing zebrafish in GZM
5. Bottles of sterile GZM
6. Bottles of sterile zebrafish food
7. Supplies
8. HEPA-filtered forced air supply
9. HEPA-filtered air exhaust
10. Sealed port for transfer of materials to/from isolator

Illustration 2: An axenic system designed by Pham and Rawls (2008) uses a gnotobiotic isolator. Air is pumped in through a high efficiency particulate air filter (HEPA, 8). Zebrafish larvae are fed autoclaved dry feed rather than live feed.

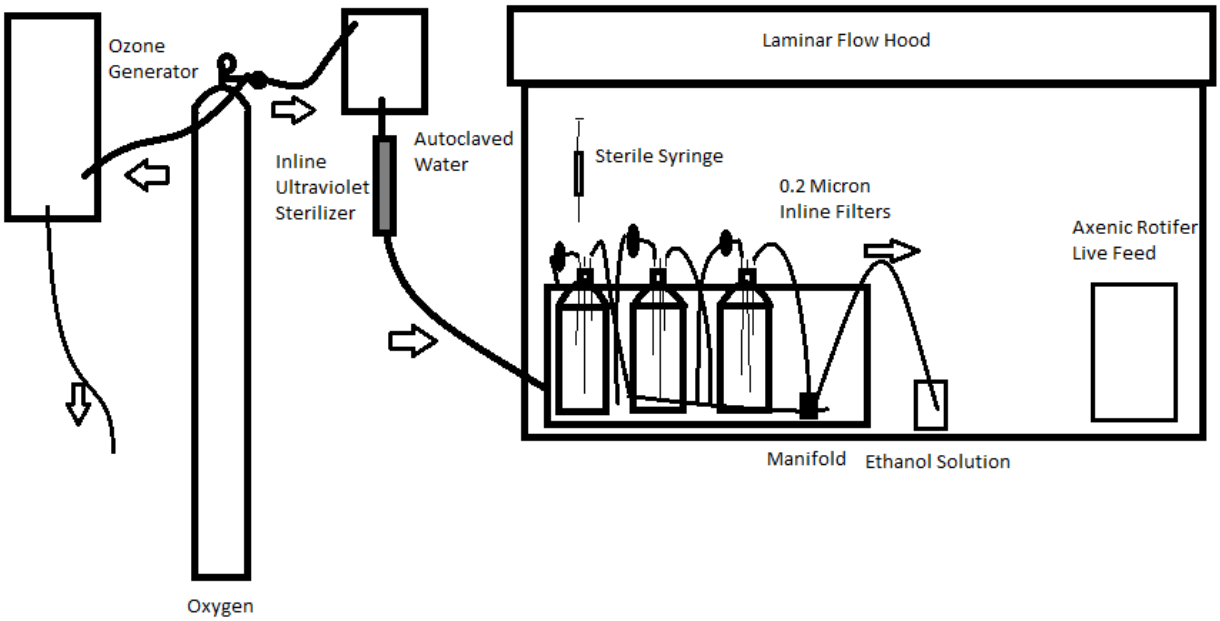


Illustration 3: A schematic illustrating the overall setup of the axenic apparatus that was designed and described in chapter 4. All system components are contained within a laminar flow hood except for the oxygen cylinder, water reservoir, and ozone generator. Pure oxygen is used to oxygenate the water reservoir. The water from the reservoir flows through an inline UV germicidal lamp before entering the inflow manifold. The water is diverted into the six chambers, flowing through inline 0.2 micron filters. This water mixes with the existing water in the chambers and flows through the outflow manifold individually. An axenic live food source can be kept under the same hood for ease in feeding. The system is accessed via sterile syringe.

Tables

Table 1: In Trial 5, the water quality parameters within the chambers and control tanks were relatively stable throughout the duration of the experiment, with the exception of the dissolved oxygen concentrations [DO] (mg/L) within the chambers.

		Temp. (°C)	pH	[DO] mg/L	[NH3] mg/L	[NH4] mg/L
Chamber 1	Mean	28.23	7.50	7.92	0.06	2.11
	sd	1.44	0.32	4.07	0.09	0.24
	Range	26.44- 31.24	7.00- 8.30	-0.93- 14.03	-0.04-0.30	1.56- 2.42
Chamber 2	Mean	29.43	7.40	9.43	0.04	2.26
	sd	1.54	0.37	4.93	0.12	0.25
	Range	26.72- 32.53	6.92- 8.23	0.67- 20.43	-0.28- 0.37	1.70- 2.71
Chamber 3	Mean	29.44	7.36	9.90	0.02	2.15
	sd	1.49	0.37	5.90	0.07	0.23
	Range	27.24- 32.53	6.93- 8.19	0.33- 23.83	-0.15-0.14	1.57- 2.43
Chamber 4	Mean	29.54	7.34	10.88	0.02	2.15
	sd	1.41	0.38	5.34	0.07	0.21
	Range	27.97- 32.33	6.90- 8.11	0.30- 21.54	-0.10- 0.19	1.57- 2.39
Chamber 5	Mean	29.44	7.34	9.65	0.02	2.09
	sd	1.59	0.36	5.12	0.07	0.27
	Range	27.40- 32.53	6.87- 8.06	0.09- 19.28	-0.13- 0.16	1.23- 2.43
Chamber 6	Mean	29.45	7.47	16.01	0.03	2.03
	sd	1.20	0.32	7.50	0.09	0.21
	Range	27.10- 31.88	7.05- 8.34	2.32- 27.47	-0.28- 0.16	1.46- 2.32
Polyround 7	Mean	25.85	7.96	10.25	0.19	2.17
	sd	0.89	0.81	2.44	0.09	0.21
	Range	24.55- 27.40	5.06- 8.80	5.19- 8.36	0.08- 0.44	1.87- 2.65
Polyround 8	Mean	26.02	8.15	9.44	0.34	2.35
	sd	0.91	0.71	1.15	0.17	0.40
	Range	24.55- 28.00	5.57- 8.88	5.33- 10.59	0.16- 0.71	1.84- 3.22

Table 2: The mean (± sd)daily bacterial load (CFU ml^{-1}) of the source water from the water reservoir before and after UV irradiation. The UV germicidal lamp appears to be ineffective, according to the data.

	24-Hour Period						
	0	**4**	**8**	**12**	**16**	**Mean Daily**	**sd**
Pre- UV	2.13E+04	8.31E+04	6.37E+04	1.36E+05	7.90E+04	7.66E+04	4.12E+04
Post- UV	No Growth	No Growth	2.05E+06	2.26E+06	3.16E+05	1.54E+06	1.07E+06

Table 3: The mean (± sd) daily bacterial load (CFU ml^{-1}) of the water within the rearing environment, beginning at time zero and ending on day 16. This data is for aerobic bacteria as no anaerobic plates were near the countable range (30-300 colonies).

Origin	0	4	8	12	16	Mean Daily	sd
Chamber 1	1.38E+05	6.60E+03	2.11E+05	6.66E+04	1.05E+05	1.05E+05	7.66E+04
Chamber 2	8.90E+04	7.40E+03	4.63E+04	1.40E+05	2.96E+04	6.25E+04	5.27E+04
Chamber 3	2.04E+05	2.72E+04	1.06E+05	1.52E+05	4.32E+04	1.06E+05	7.38E+04
Chamber 4	5.50E+04	5.00E+04	3.59E+05	1.01E+04	1.15E+05	1.18E+05	1.40E+05
Chamber 5	1.72E+05	1.22E+04	4.13E+05	8.90E+03	1.57E+05	1.53E+05	1.65E+05
Chamber 6	1.47E+05	na	3.95E+04	3.66E+04	9.00E+03	5.80E+04	6.09E+04
Polyround 7	9.00E+02	7.97E+04	1.04E+04	2.75E+04	na	2.96E+04	3.52E+04
Polyround 8	7.20E+03	5.30E+03	6.50E+04	6.40E+04	1.60E+04	3.15E+04	3.04E+04

Table 4: The combined three-fish gut bacterial load (CFU ml^{-1}) for each chamber and polyround control tank, on the final day of the experiment (Day 16). Chamber 6 (***) had complete mortality and therefore, no individuals remained to acquire samples from.

	Day 16		
Origin	**Total Aerobes**	**Total Anaerobes**	**Total Bacterial Load**
Chamber 1	3.54E+05	6.90E+01	3.54E+05
Chamber 2	1.77E+05	8.40E+04	2.61E+05
Chamber 3	7.50E+04	1.69E+03	7.67E+04
Chamber 4	1.22E+05	na	1.22E+05
Chamber 5	1.43E+05	9.40E+01	1.43E+05
Chamber 6* **	na	na	na
Polyround 7	1.90E+05	2.71E+05	4.61E+05
Polyround 8	1.77E+06	2.58E+04	1.80E+06

Figures

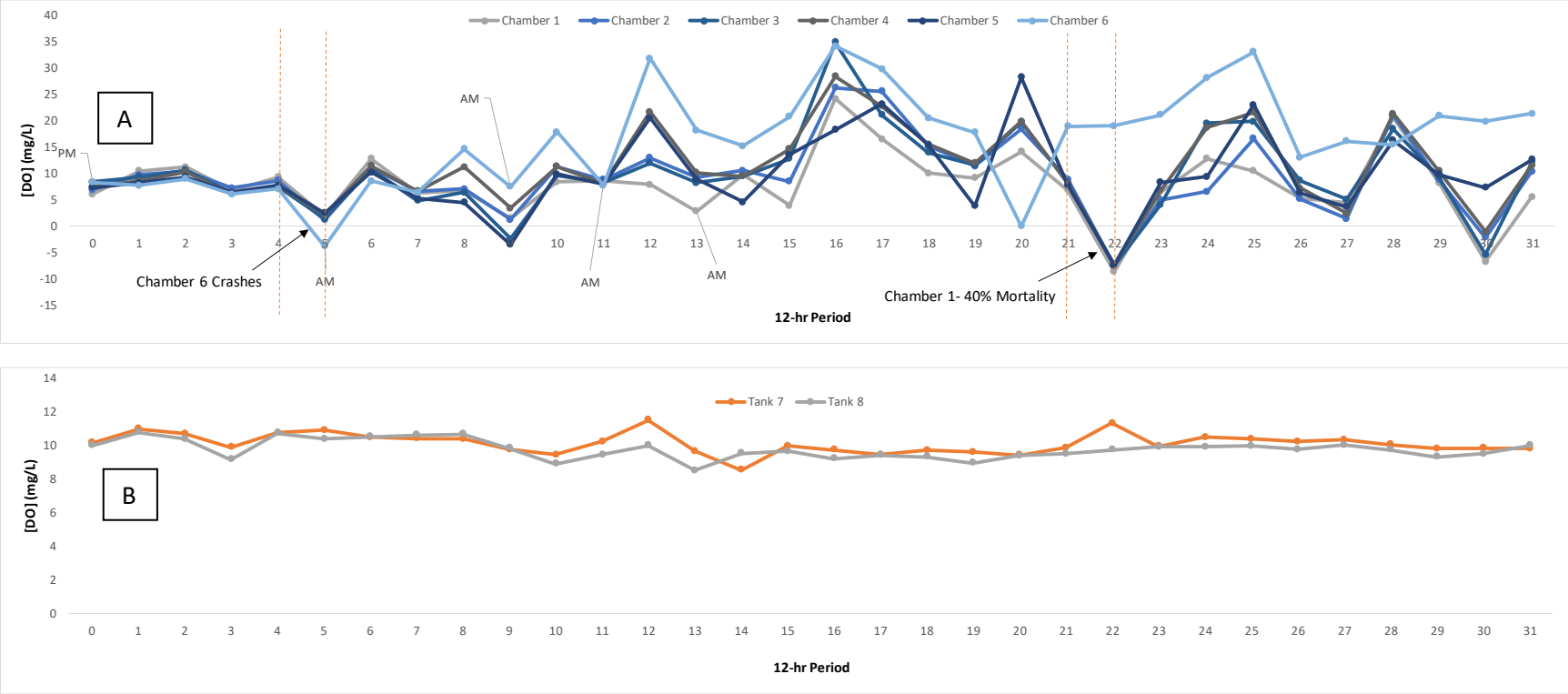


Figure 1: Part A depicts the fluctuations of dissolved oxygen concentrations (mg L^{-1}) within the chambers under holoxenic conditions over the duration of Trial 5. Part B depicts the dissolved oxygen concentrations of the control tanks over the same period. The chamber concentrations were much more unstable with peaks in the PM and valleys in the AM. Morning dissolved oxygen concentrations were much lower as no system flushes were performed overnight. After measuring the water quality in the mornings, the reservoir was oxygenated using a compressed oxygen cylinder. The system was flushed 1-4 times between 7AM and 7PM but not after. The control tanks were aerated using an airstone connects to an air pump.

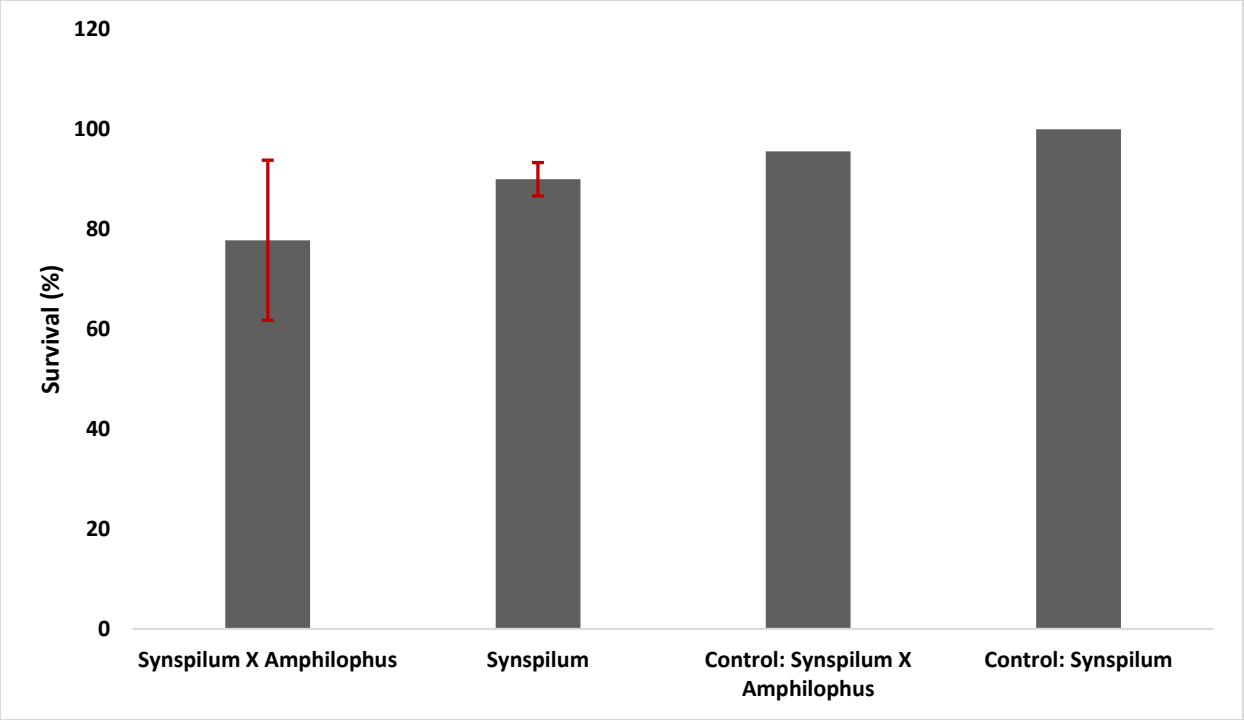


Figure 2: Mean proportion survival (±SE) was not significantly different between the *Synspilum* and the hybrid within the chambers (t (3.158)= 0.702,

p= 0.531).

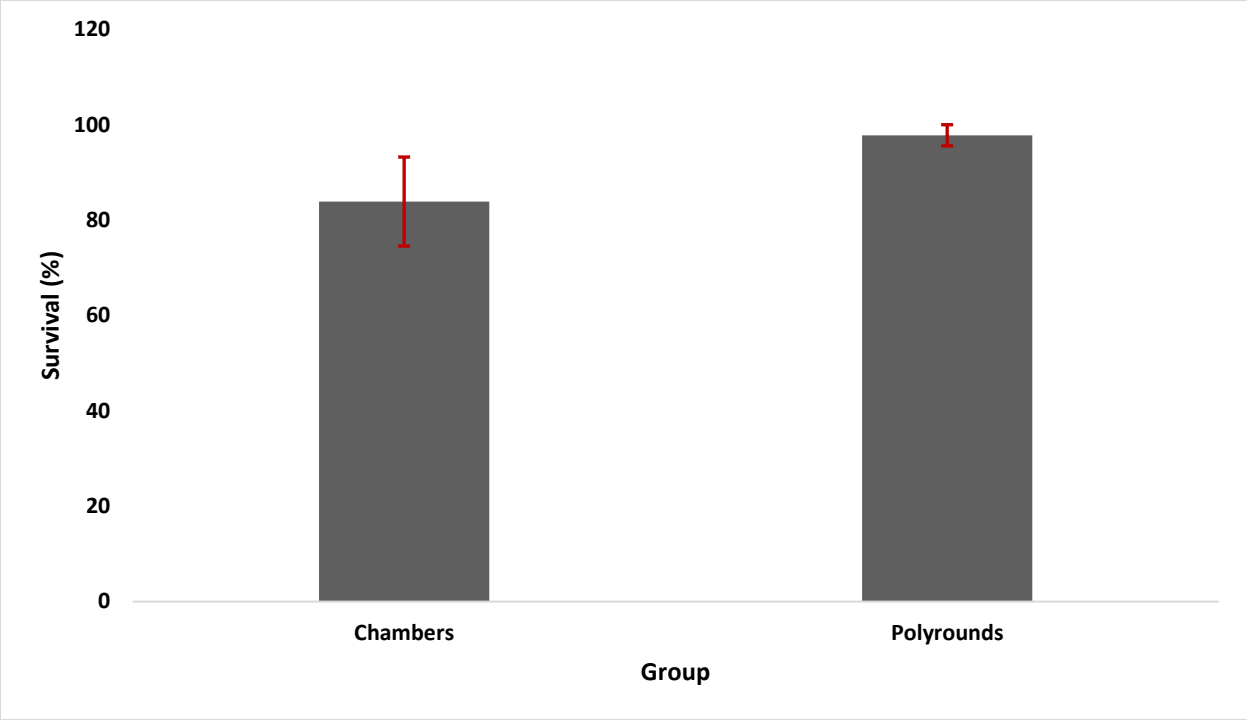


Figure 3: Mean proportion survival (±SE) was not significantly different between the chambers and the polyrounds (t (5.311) = -1.528, p= 0.184).

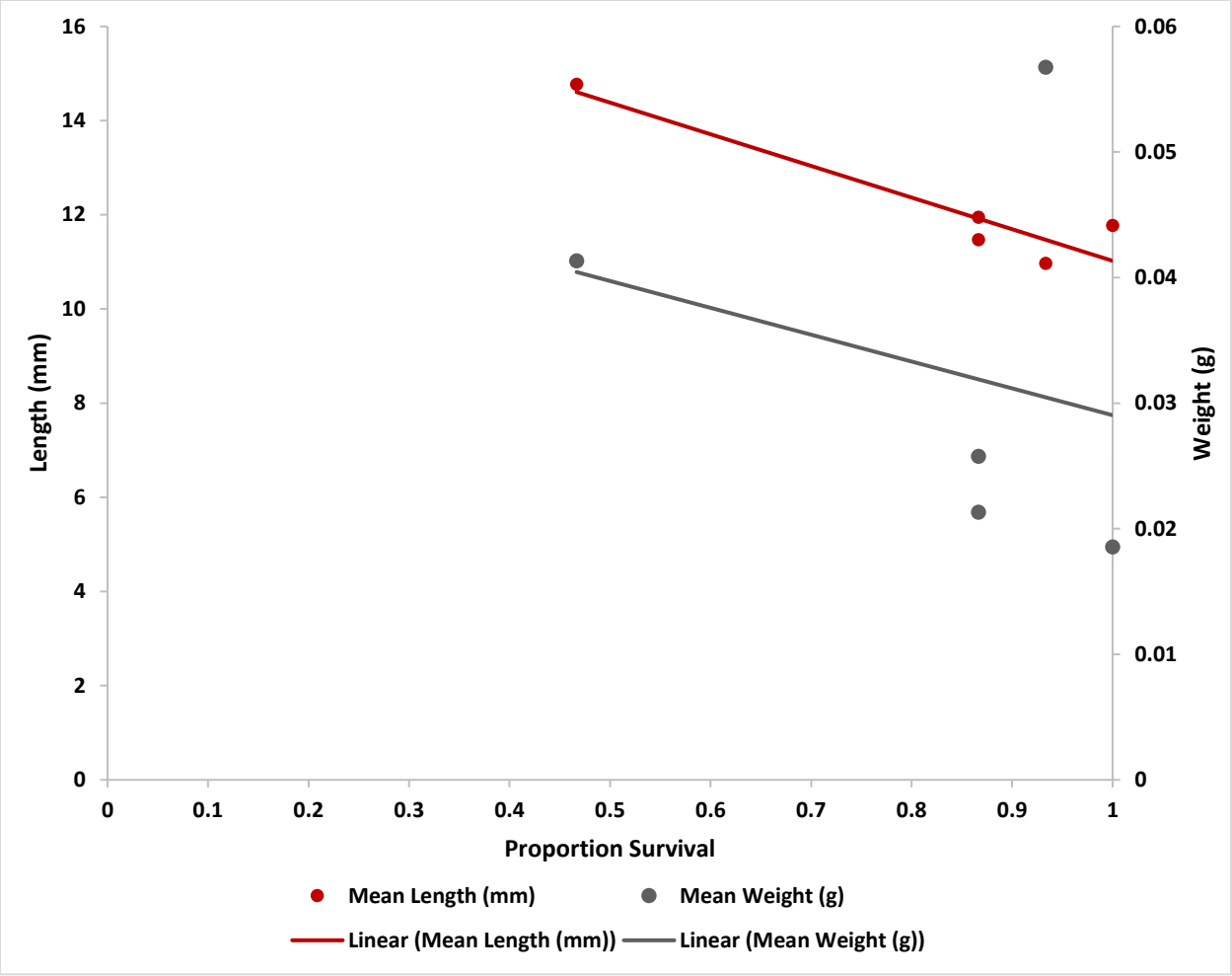


Figure 4: A regression analysis comparing chamber survival with the length and weight of a subsample of individuals within chambers (n=4) indicated a significant relationship between survival and length ($F_{1,6}$= 5.529, p= 0.0569, α= 0.1, adjusted R^2=0.392) but not between survival and weight ($F_{1,6}$= 0.681, p= 0.441, adjusted R^2= -0.048). As survival increases, the length of fish decreases.

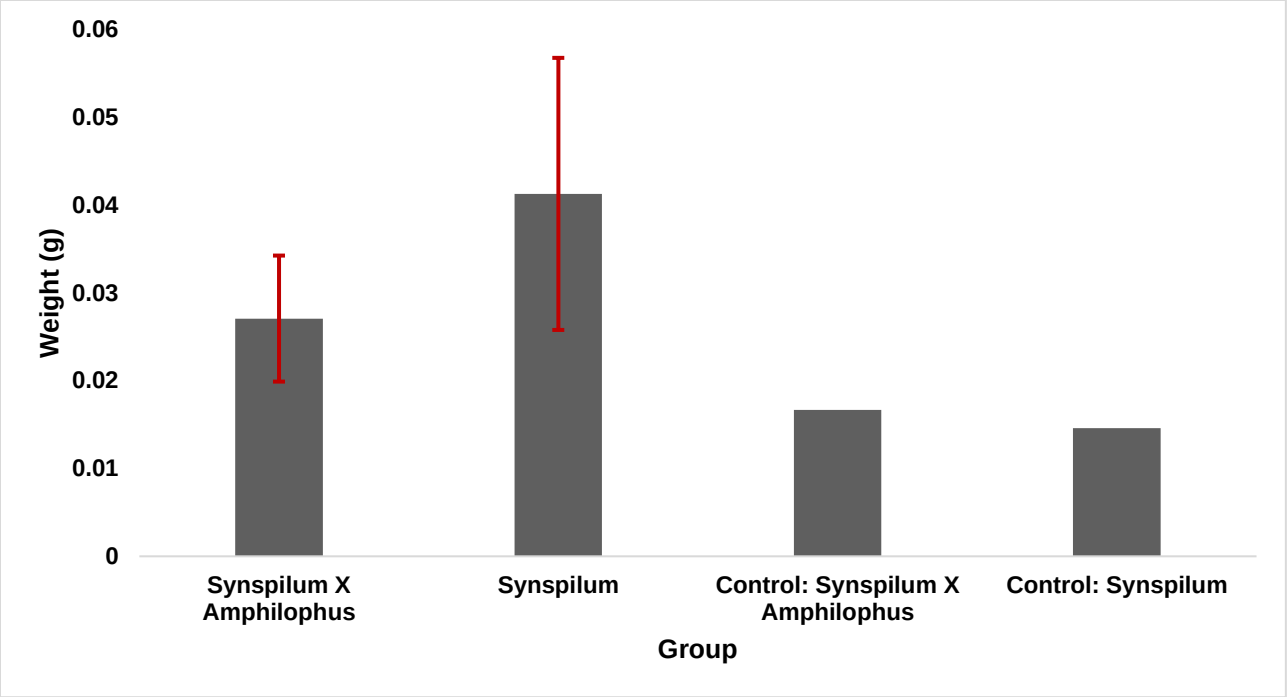


Figure 5: The mean weights (±SE) between the *Synspilum* and the hybrid were not significantly different (t (2.738)= -0.025, p= 0.982).

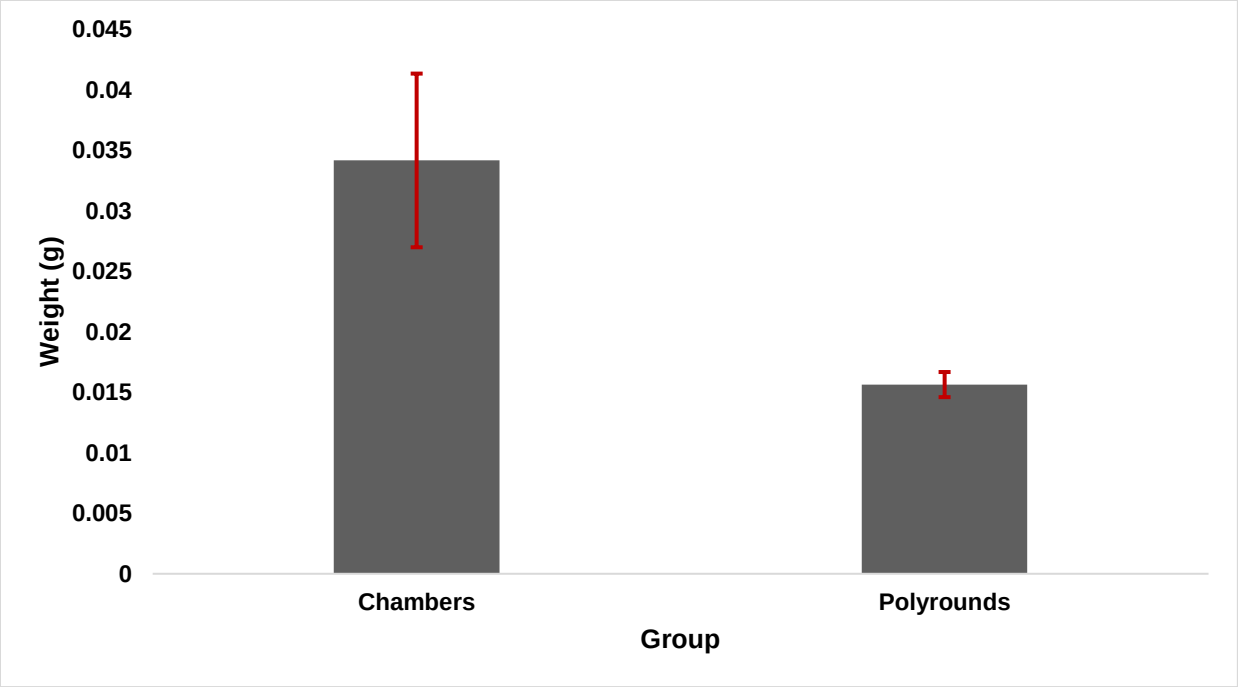


Figure 6: The mean weights (±SE) between the chambers and polyrounds were not significantly different (t (5.162)= 1.443, p= 0.207).

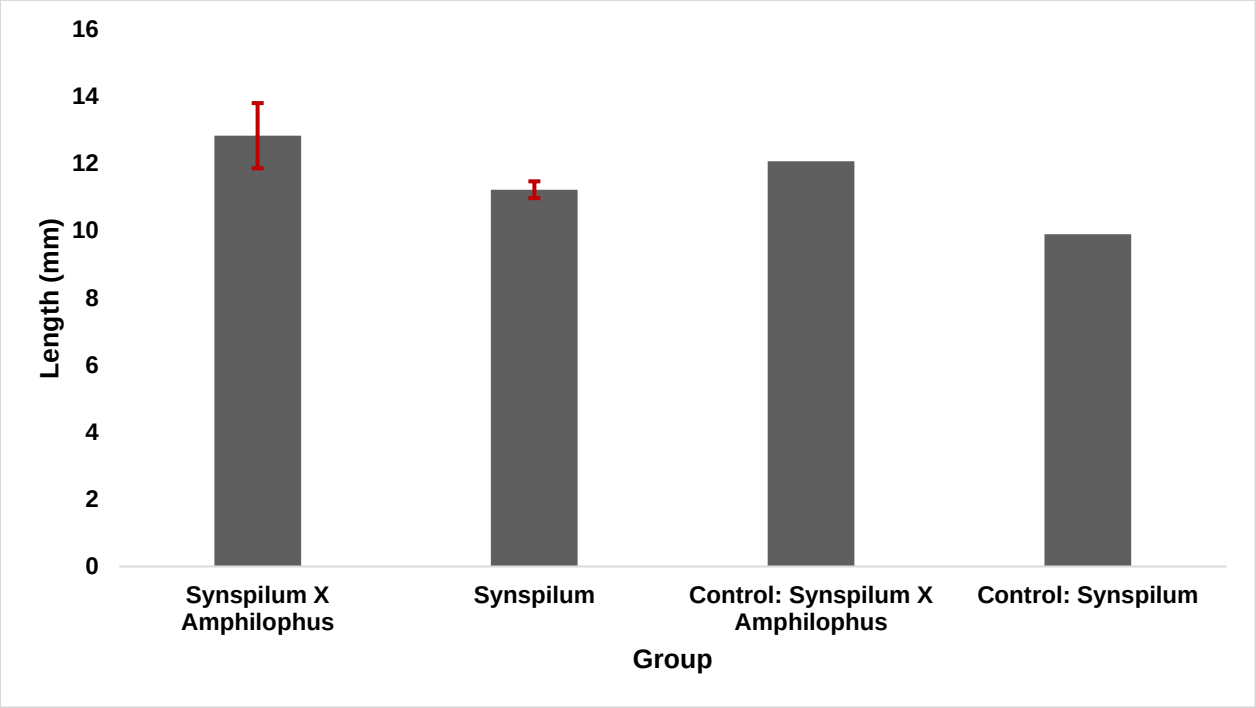


Figure 7: The mean length (±SE) between the *Synspilum* and the hybrid were not significantly different (t (2.269)= 1.383, p= 0.287).

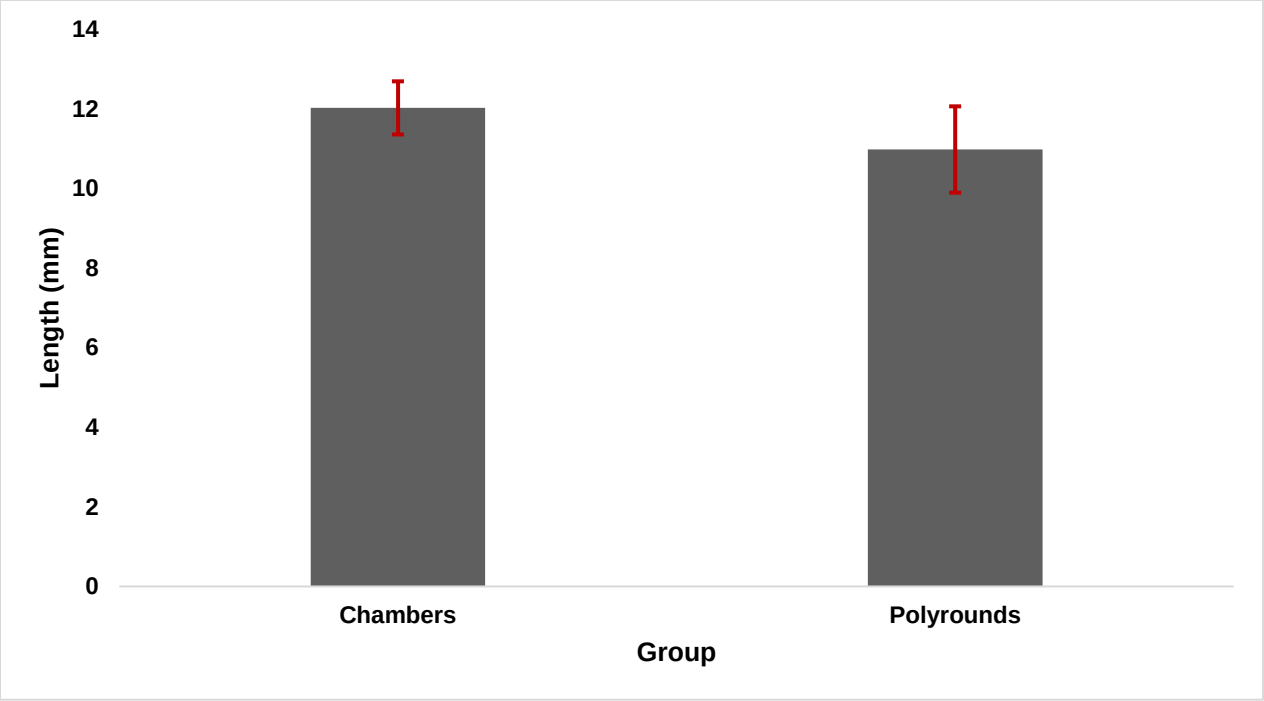


Figure 8: The mean length (±SE) between the chambers and polyrounds were not significantly different (t (5.919)= -0.348, p= 0.740).

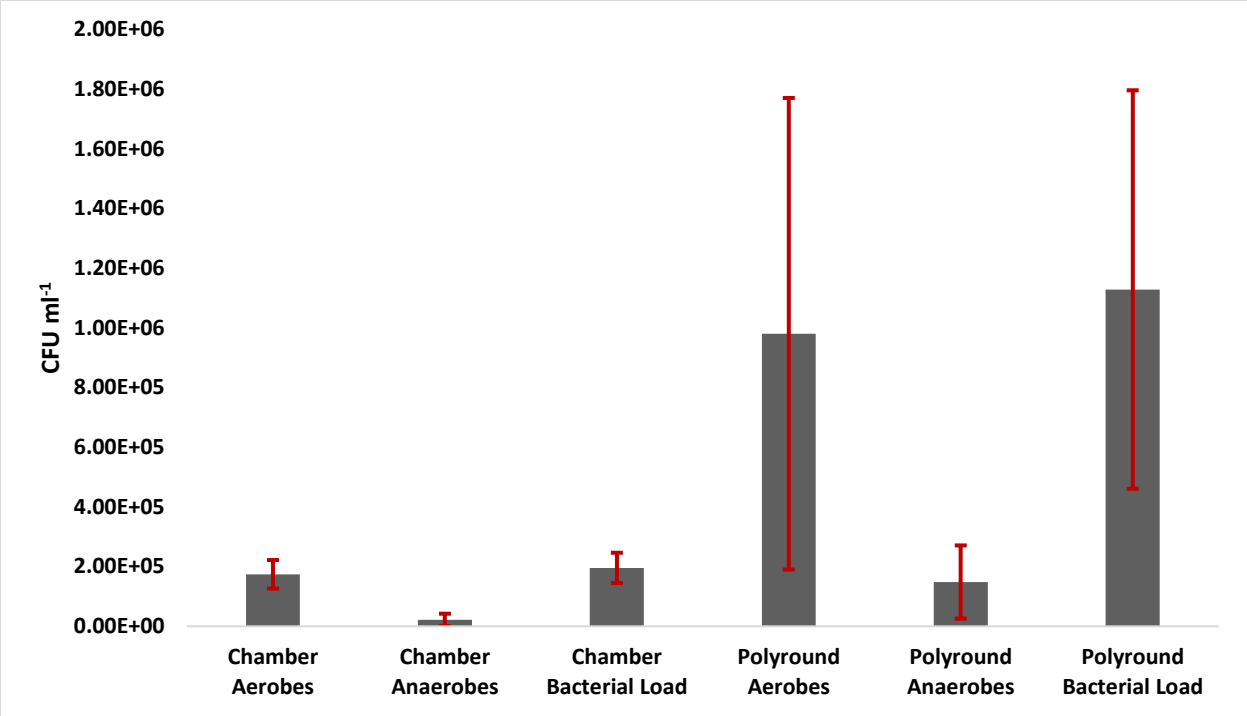


Figure 9: The mean (±SE) bacterial load of the three-fish gut sample suspension (PBS) on the final day of the experiment (day 16) in chambers and polyrounds (control tanks) by number of aerobes (CFU ml^{-1}) and number of anaerobes. No significant differences were found in aerobic bacteria (t (1.007)=-1.018, p=0.493), anaerobic bacteria (t (1.058)= -1.021, p= 0.486), and bacterial load (aerobic and anaerobic combined; t (1.012)= -399, p=0.393).

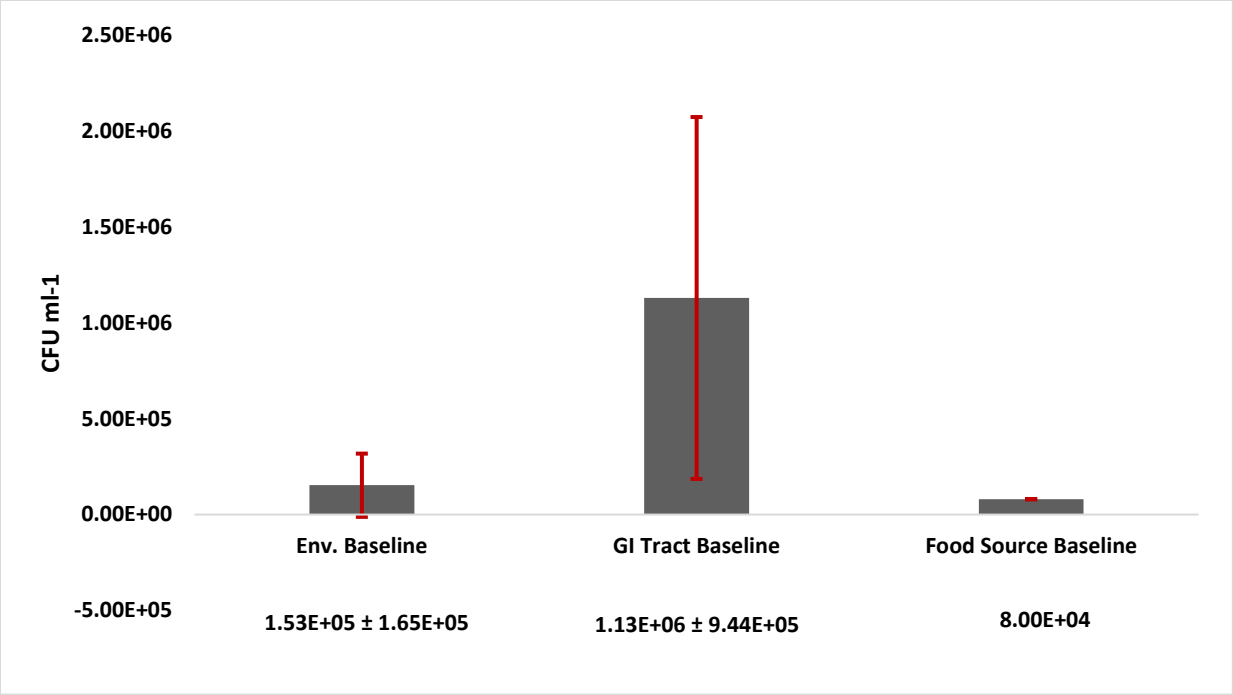


Figure 10: The baseline bacterial loads of the rearing environment, the gastrointestinal tract, and the food source determined by the maximum mean (±SD) bacterial load within each. The environment and food source baseline does not include anaerobic bacterial counts. The food source baseline was determined based on the colony count of a single plate as only one plate contained colonies near but not within the target range (30-300 colonies) required for plates considered countable.