PROBIOTICS AND THEIR USE IN COMMERCIALLY IMPORTANT AQUACULTURE SPECIES IN THE NORTHEASTERN UNITED STATES

by

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A THESIS

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Dedication

Husband. You know what you did.

Acknowledgements

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Probiotics and their use in Commercially Important Aquaculture Species in the Northeastern United States

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Thesis Abstract

Probiotics have shown to be beneficial to aquatic farming in aspects such as growth and development. This study, using three commercial aquaculture species, intends to ascertain if certain strains of *Bacillus* and *Shewanella* bacteria could possibly be used in aquaculture to improve finfish growth and survival, and be classified as true probiotics. Trials run at the DSU ARDF dose Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and hybrid striped bass (*Morone chrysops x M. saxatilis*) with three probiotics *Bacillus spp.* (Iso 5 and Iso 11) and *Shewanella spp.* (Iso 12). Mortality was observed in three finfish trials over a 56-day trial or until one tank in the trial attained 10% survival. Ammonia, nitrite and nitrate were monitored throughout the trial. Feed conversion ratios, specific growth rates, weight gains and mortality were assessed at the end of the study.

During the Nile tilapia experiment control tanks lost a total of 42 of 75 fish. ISO 12 (60/75), ISO 5 (56/75), and ISO 11 (58/75), treated tanks had statistically higher survival rates then the control tanks. Significant differences were also seen in survival during the hybrid striped bass experiment between the control tanks (16/30), ISO 12 (24/30) and ISO 11 (30/30). No significant differences were seen in survival for the rainbow trout experiment, but no treatment lost more than 10 finfish during the entirety of the experiment.

Significant differences were observed for nitrite concentrations among tanks with probiotic treatments in the hybrid striped bass as well as with the rainbow trout experiment. ISO 5 treated tanks had a significantly lower concentration (0.107 ± 0.027 mg/l) of nitrite when compared to control tanks (0.231 ± 0.027 mg/l) during the rainbow trout experiment. Tanks treated with ISO 12 were significantly higher in nitrite concentration (0.038 ± 0.002 mg/l) than tanks treated with ISO 5 (0.030 ± 0.002 mg/l), and significantly higher than those treated with ISO 11 (0.029 ± 0.002 mg/l) during the hybrid striped bass experiment. The difference in ISO 5 and ISO 12 nitrite concentrations could be attributed to the ability of *Bacillus spp.* bacteria to metabolize ammonia into nitrate and nitrite, jumpstarting the nitrogen cycle. ISO 12 treated tanks very high nitrite concentration in comparison, might also be due to *Shewanella spp.* ability to denitrify nitrate into nitrite. If the experiment had lasted longer, perhaps an eventual decrease in nitrite would have been seen as the nitrite was converted into ammonium, a benign gaseous output.

All three tested bacteria have the potential to be probiotics and used in aquaculture. Higher survival of finfish in the probiotic treatment tanks and the control tanks suggest that all three probiotics have the ability to increase survival in warm water (24 ºC) conditions, though no significant results were observed for cooler water (19 ºC). ISO 5, *Bacillus cereus,* and ISO 12, *Shewanella spp.,* were both shown to have an effect on the concentrations of nitrite, but ISO 5 decreased the nitrite concentration while ISO 12 increased the concentrations when compared to ISO 5 and ISO 11, not the control. Further testing is required for ISO 12 to see if it could possibly lower the nitrite over a longer time period. Further testing is required for all three

probiotic strains, and different systems as well as temperatures and salinities should be used to better understand the functions of these bacteria.

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CHAPTER 1: INTRODUCTION

According to the 2013 USDA (United States Department of Agriculture) Census of Aquaculture, finfish aquaculture was valued at approximately $1.4 billion. In the Northeast (NE) region, finfish aquaculture was valued at $146 million, representing 11% of the finfish revenue nationally (USDA 2014). This is comprised of three primary production sectors *food fish*, or species intended for direct human consumption, *sports fish*, or species intended for release into the wild, and *ornamental fish,* species intended for display. Out of the $146 million of finfish value in the NE, $9.3 million (32%) of the value was food fish, and $1.4 million (1%) was ornamental fish, and the value for sport fish was $356,000 (<1 %). Crustaceans, bait fish and assorted other aquaculture categories made up the remaining sales. The same census showed that 42% of domestic foodfish farms were in the NE (Table 1-1).

Fishermen's journals like Bassmaster and American Angler rate rainbow trout (*Oncorhynchus mykiss*) and hybrid striped bass (*Morone chrysops x Morone saxatilis*) to be among the most sought-after freshwater sports fish in America. According to the U.S. Department of Fish and Wildlife, between 2016 and 2018 there was a million person rise (500,000 persons per year) in the number of sport fishermen, whereas in the previous ten years the same census showed an average increase of only 100,000 persons per year (US DOI *et al.* 2018). The increase reported in the two most recent fishing license censuses is a measure of how many new anglers have come to the sport in the last few years. With the increased popularity of the sport, sport fishing waters need to be stocked with more finfish to keep up with the increased

fishing pressure. Rainbow trout and hybrid striped bass are bred in aquaculture facilities and then released into the wild before the season starts allowing more fishing licenses to be sold. Between 2017 and 2018 fishing license sales were $1.4 billion in the U.S. (US DOI *et al.* 2018).

Table 1-1. Value of finfish aquaculture (thousands of dollars) in the Northeast (NE) region vs. the whole U.S. of reporting farms in the 2013 USDA Census of Aquaculture (USDA 2014).

State	**# Farms**	**Total Fish Value**	**# Food Fish Farms**	**Food Fish Value**
Vermont	6	$132	6	$132
New Hampshire	7	$759	4	(D)
West Virginia	19	$1,604	19	$1,499
Rhode Island	21	$5,734	(D)	(D)
Maryland	18	$6,158	2	(D)
Pennsylvania	56	$6,927	44	$5,714
New York	44	$7,491	21	$1,586
New Jersey	59	$13,835	4	(D)
Massachusetts	145	$18,065	10	(D)
Connecticut	28	$28,676	3	$378
Maine	35	$57,326	11	(D)
Delaware	3	(D)	2	(D)
Totals Northeast	441	$146,707	126	$9,309
Totals United states	3093	$1,371,707	1,296	$732,147

(D)- "Withheld to avoid disclosing data for individual farms" (USDA 2013). To avoid giving away data for individual farms, the data was not reported.

Rainbow trout and hybrid striped bass may be prized in the sports fishing world, but they are also among the most consumed finfish in the U.S. ranking in the top ten list of best tasting freshwater finfish caught in the U.S. (NOAA 2011). NOAA (National Oceanic and Atmospheric Association) has compiled a list of the top ten most popular finfish eaten in the U.S. and while these two have not made the top ten for that list, another popular finfish has, Nile tilapia. Nile tilapia is the number 5 on the top ten of seafood consumed in the U.S. as it is one of the most

versatile finfish to cook. Tilapia was being eaten at approximately 0.59 kg per person in 2011 (NOAA 2011).

Nile tilapia, rainbow trout and hybrid striped bass are in high demand as they account for 33% (Table 1-2) of total income for finfish in the NE region (USDA 2013). Sometimes in an effort to produce more finfish from the same available space, farmers turn to higher stocking densities. Higher stocking densities in tanks can often be harmless as some aquaculture systems have been built to recycle water, cleaning it as it is returned to the production area. This can be fine if water recycling rates remain high and water is properly cleaned but can have disastrous effects if finfish get over stressed or sick.

Table 1-2. Farm gate value in the NE of three individual species of interests. the U.S. total value of the three commercially important finfish species (thousands of dollars) (NMFS 2015).

Species	**Value by Year**	
	2008-2010	**2011-2013**
Nile tilapia	*$140,359	*$150,299
Rainbow trout	$149,081	$200,831
Hybrid striped bass	$85,890	$107,056
US total of 3 fish	$375,330	$458,186

*Nile tilapia was not documented for these years by state, numbers are U.S. based

While higher stocking densities can allow for greater yields and increased profit for farmers, it can also cause degradation of water quality, thereby increasing the possibility of a disease outbreak (Laura-Florez 2014, Perez-Sanchez *et al.* 2013 and Wang and Li 2008). The routine uses of antibiotics to combat highly virulent bacteria is leading to increasing number of antibiotic resistant bacteria (Laura-Flores 2014) but has been the only option for many years.

The use of antibiotics can weaken the immune system of the finfish and make it harder for them to defend against pathogenic bacteria creating a need to use more antibiotics (Romero *et al.* 2012). Increased use of antibiotics over the long term to increase animal health has caused the emergence of antibiotic resistant bacteria in finfish (Verschuere *et al.* 2000). Without being able to use antibiotics farmers inability to fight virulent bacteria is leading scientists to find alternative ways to boost aquaculture health (Mizock 2015, Ghosh *et al.* 2008, Naylora *et al.* 2009 and Rahman *et al.* 2009). A workable solution to combat microbial warfare without using antibiotics is probiotics.

Probiotics are bacteria that are shown to have a beneficial impact on their host and may originate from the finfish themselves, another species or their environment. Larval finfish in the wild acquire their internal bacteria from their environment (the mixture of bacteria coming from the water, stirred up soil as well as other environmental factors) and the food that they consume (Sales 2003). Selection of possible probiotic bacteria over other background bacteria requires research to see if they meet certain requirements. To be classified as a probiotic, an organism can enter a host through feeding, or absorption through the gills and dermis (Hai 2015), and that treating with probiotic bacteria must be harmless and make a positive and measurable difference in the overall health, growth or development of the host (Perez-Sanchez 2013, Fuller 1989). These differences can include improved water quality, growth, or survival depending on the species of bacteria and its ability to work with the host (Table 1-3). There are several species of bacteria that are known to work as probiotics in tandem with finfish; many are in the family *Bacillaceae*, and less common are marine species from the *Shewanellaceae* family (Table 1-3).

Table 1-3. Comparison of observed functions of *Bacillaceae* and *Shewanellaceae*, two families of probiotic bacteria used in aquaculture. This table indicates the genus of the probiotic tested, the host species and the observed benefit(s).

Bacteria	Host	Water Quality	Growth	Survival	Body Weight
Bacillus spp.					
	Nile tilapia[10]			X	
	Gilthead sea bream[11]		X	X	
	Rainbow trout[4]		X	X	X
	Molly[3]	X	X	X	X
	Platy[3]	X	X	X	X
	Carp[2,12]	X	X	X	
	Rohu[13]	X	X	X	
	Sea bass[8]	X	X		X
Shewanella spp.					
	Abolone[9]		X	X	X
	Prawn[4]		X	X	
	Gilthead sea bream[1]	X	X		
	Senegalese sole[5,7]		X		

Makridis *et al.* (2015)[1], Kumar *et al.* (2006)[2], Ghosh *et al.* (2008)[3], Rahiman *et al.* (2010)[4], Merrifield *et al.* (2010)[5], Tapia-Paniagus *et al.* (2011)[6], De la Banda *et al.* (2012)[7], Franke *et al.* (2013)[8], Jiang *et al.* (2013)[9], Del'Duca *et al.* (2013)[10], Cerezuela *et al.* (2013)[11], He *et al.*(2011)[12], Bairagi *et al.*(2004)[13]

Probiotics can outcompete other less active bacteria and assist the host with the digestion of nutrients (Balcazar *et al.* 2006, Franke *et al.* 2013). Mizock *et al.* (2015), describes probiotic function as assistance with extraction of nutrients and vitamins from food, while helping the body to absorb them better. While this description was created to describe the effects of probiotics on human flora, finfish gut bacteria function in quite the same way. Bergh *et al.* (1994) showed finfishes were capable of limited self-regulation of gut bacteria numbers and types, showing that a beneficial bacterium may be a better option for the finfishes' overall survival tactic and that certain types of bacteria may be more beneficial then others. This observable movement of certain bacteria in some finfish show that some bacteria may be more beneficial in aiding the basic digestive functions (i.e. absorption of nutrients, breakdown of large

molecules, etc.) and that finfish are selecting for these better bacteria to help them survive stressful situations. While farmers do their best to maintain clean water and give plenty of food, the optimum growth and survival may not be possible for our finfish without the aid of many different types of bacteria that may not be available to them in current set ups. This may mean that specific nutrients from their food may not be available to them without specific bacteria, and the bacteria might not be available if not supplied by the farmer. If bacterial additions are made to the aquatic environment of farmed finfish, they may be able to help their host attain previously unattainable nutritional resources, improving growth and development.

As basic digestive functions are not readily observable, the benefits they impart (i.e. improved growth and survival in harsh or stressful conditions) need to be measured to determine if the bacteria are truly probiotics. Trials run by Bergh *et al.* (1994), Balcazar *et al.* (2006), Franke *et al.* (2013) and Mizock *et al.* (2015) are looking at growth, development of finfish as well as survival and water quality changes to determine if the dosed bacteria are truly beneficial to their hosts. For my thesis, I tested three potential probiotics that were isolated from the mummichog (*Fundulus heteroclitus*) as part of an earlier research project (McIntosh *et al.* 2016). Mummichog are versatile finfish that are often used as a model system to screen potential environmental dangers and toxins and are often found in a range of salinities and water temperatures (Stegeman *et al.* 2018).

Probiotics are often quite specific in their application, typically being limited to use in freshwater or saltwater environments. Mummichog's ability to survive in varied environments may also mean that their internal microflora can as well, making them ideal candidates as

possible probiotics for use in other hosts a similar diversity of environmental requirements. 230 possible bacteria strains were isolated from gills, mucus layers and stomach lining of the mummichogs. Only three bacteria species were testable for probiotic use, as all others were deemed *Vibrio. spp*., and potentially harmful to their hosts. The three strains of probiotics being investigated in this project are two *Bacillus spp*., and one *Shewanella spp*. For my thesis research, I specifically tested the three bacterial strains to see if they have an effect on growth and survival.

CHAPTER 2: LITERATURE REVIEW

High stocking densities and poor water quality can lead to high losses of finfish, as well as income for farmers. High finfish mortality can occur due to difficulties in biofilter maintenance needed for full filtration, proper regulation of high stocking densities and regulation of water quality parameters such as ammonia, nitrite and nitrate (Balcazar *et al.* 2006, Lara-Flores 2011, Perez-Sanchez *et al.* 2013). The health and size of finfish in the market place can be increased using growth hormones and other types of growth supplements (Naylora 2009, Mizock 2015 and Kesarcodi-Watson *et al.* 2008) but another option for improving both water quality and growth is starting to present itself: probiotics. Probiotics have been seen to have a significant effect on finfish weight and overall growth; Yanbo and Zirong (2006) noted that using a lyophilized *Bacillus* strain, their probiotics increased finfish body length by 5% and SGR (specific growth rate) significantly over 60 days. Ghosh *et al.* 2008 showed that their *B. subtillius* strain probiotic improved survival of guppy (*Poecillia reticulata* and *P. sphenops*) and swordtail (*Xiphophorus helleri* and *X. maculatus*) by 7% over a 90-day period. They also noted a 2% increase in growth in their probiotic treated groups compared to their controls (Ghosh *et al.* 2008). Rainbow trout (*Oncorhynchus mykiss)* fry treated with a *Bacillus spp*. probiotic showed an increase in survival (3.1%), weight (71%), SGR (18%) and overall length (7%), and an 18% decrease in FCR (feed conversion ratio) versus the control groups over 63 days (Bagheri *et al.* 2011).

Studies using freshwater prawn (*Macrobrachium rosenbergii*) and Pacific white shrimp (*Litopenaeus vannamei*), both showed increases in survival when using *Bacillus* strains compared to controls (27.9% and 3.5%, respectively) (Rahiman *et al.* 2012, Olmos *et al.* 2011). Common carp (*Cyprinus carpio*) and cobia (*Rachycentron canadium*) studies showed increases in SGR and a lowering of FCR when using a *Bacillus* probiotic (He *et al.* 2011, Geng *et al.* 2011). The cobia study also showed a 29% leukocyte activity increase over the control (Geng *et al.* 2011). *Bacillus* strains of probiotics have also shown to increase the leukocytes numbers in rainbow trout (Bagheri *et al.* 2011) which help the finfish to counteract foreign bacteria and fight disease.

Several studies have shown that strains of *Shewanella* probiotic can increase immune-stability (or the ability of the target host to help resist harmful foreign bacteria) in some species by causing an increase in leukocytes and cross-reactive antibodies which could help them defend against virulent bacteria (Avella *et al.* 2010, Divya *et al.* 2012, Ghosh *et al.* 2008, Kumar *et al.* 2006 and Franke *et al.* 2013). *Shewanella spp*. probiotics have also shown the ability to increase the fry's metabolic rates, functionally lessening the amount of big lipid inclusions, allowing for better absorption of lipids and vitamins already in formulated diets. *Shewanella spp*. have been shown to help the fry's gut develop longer microvilli; increasing intestinal surface area and absorption and use of nutrients (Cahu *et al.* 2003). De la Banda *et al.* (2012), using Senegalese sole (*Solea senegalensis*), showed that feeding of *Shewanella* bacteria over two months increased SGR by 9.5% over the controls, with a corresponding 25% increase in survival. *Shewanella spp*. probiotics have also been tested in Pacific abalone (*Haliotis discus*), resulting in a 50%

improvement in survival for the probiotic treated group over the control groups over a period of four weeks (Jiang *et al.* 2013).

CHAPTER 3: MATERIALS AND METHODS

In this experiment, I assessed the possible probiotic effects of two *Bacillus spp*. (ISO 5, ISO 11) and one *Shewanella spp*. (ISO 12) on growth and survival of three finfish species (Table 3-1): Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*) and hybrid striped bass (*Morone chrysops x M. saxatilis*). The economic importance and availability of these finfish made them ideal candidates for testing (Table 1-2). This experiment was conducted at the Delaware State University Aquaculture Research and Demonstration Facility (DSU ARDF). Bacteria species were grown at the Institute of Marine Education and Technology by Dr. Hal Schreier for specific use in this study. Nile tilapia and rainbow trout were obtained from Cheyney University with the help of Dr. Steven Hughes. Rainbow trout were delivered as eggs and hatched in an indoor recirculating system specifically set up for finfish eggs. Hybrid striped bass came from Keo Fish Farms Inc. with the help of Mike Freeze. They were delivered to the ARDF already several days post hatch.

Table 3-1. Bacteria in use for the trials detailed in this thesis. Each of the probiotics were taken from the Mummichog (*Fundulus heteroclitus*) in a previous effort (McIntosh *et al.* 2016) and then evaluated in this experiment to see if they were potentially harmful or helpful.

Family	**Species**	**Designation**
Bacillaceae		
	Bacillus cereus	ISO 11
	Bacillus cereus	ISO 5
Shewanellaceae		
	Shewanella spp.	ISO 12

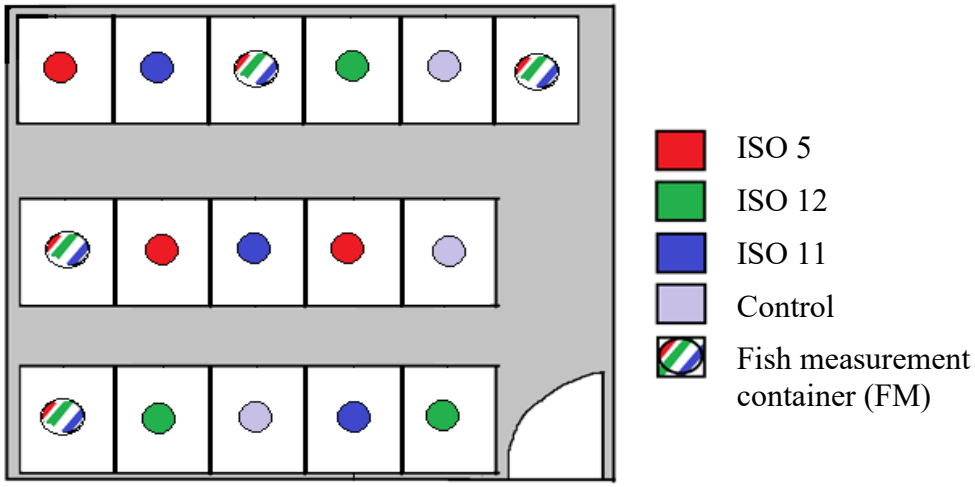
My study was conducted in an isolated probiotic room, which was partitioned to have three rows with five or six individual stations per row (Figure 3-1). Individual stations were created using rigid foam insulation to minimize the likely hood of cross contamination from aerosolized probiotic bacteria after they were applied to the respective experimental containers. An in-wall timer was used to maintain the lighting regime at 12:12 (light:dark) for the rainbow trout and hybrid striped bass. Since the Nile tilapia were very young (1-2 days old) and it was a concern that too much light would stress them out as well as make it more difficult for small eyes to see food, this trial was run under red light condition with no photoperiod as was recommended Bason 2018. Temperatures in the experimental containers were controlled by adjusting the ambient conditions in the study room (Table 3-3).

For each species trial, 16 2-L polycarbonate containers (RFSCW2135, Cambro, Huntington Beach, CA) were used, with individual experimental containers assigned to one of four treatment groups (three probiotics plus a control) and arranged in a completely randomized design (Figure 3-1). Lids (RFSCWPP190, Cambro, Huntington Beach, CA) were placed on all containers to hold air stones, prevent finfish loss, and minimize cross contamination due to aerosolization of probiotic bacteria. Aeration was supplied to each experimental container by a regenerative blower feeding a common air distribution system, with one ceramic 1.23-cm x 4-cm air stone (AS1, Pentair Aquatic Eco-Systems, Apopka, FL) in each experimental container.

Each 2-L experimental container was filled with 1-L of water (conditioned seawater or fresh water depending on finfish species) and maintained at each finfish's required temperature (Table 3-3). For hybrid striped bass, full strength seawater (31 ± 2 ppt) was collected from the

Indian River Inlet, DE and stored in holding tanks at the DSU ARDF. Raw seawater was disinfected by chlorinating with 10 mg/l Cl, aerating for 24 h, and then removing residual chlorine with sodium thiosulfate. Salinity was adjusted to 22 ppt (Table 3-2) with well water. For the freshwater species, well-water was obtained from the on-site well at the DSU ARDF. For all trials, additional make-up water was stored seven days in advance in separate holding containers to ensure this was at the desired temperature for each finfish species (Table 3-2).

Figure 3-1. Schematic of the isolated probiotic experiment room at the DSU ARDF. The white squares depict the individual stations created from the insulation sheets, and the colored circles show a representative placement of the 2-L experimental containers used in the growth and survival trials.



Finfish for all experiments were held in a 64-L tank to acclimate to lab conditions. After seven days finfish were netted from the acclimation tank and placed into each experimental container at a density of 10 - 25 individuals/L depending on species (Table 3-3). Once stocked, individual experimental containers were assigned to one of four treatment groups (Table 3-1) and arranged in a completely randomized design (Figure 3-1). In an effort to control stress and

preventing unmeasured stressors from effecting the study data, one experimental container from each treatment was designated as the finfish measurement container (FM). The FM containers from each treatment group were the only containers removed from the study area while the trials were underway; this was done only to assess growth in order to adjust feed rations.

Table 3-2. Selected finfish and their respective culture conditions. Optimal ranges, feed rates and finfish densities were selected from the literature, and the study strived to stay within those reported scales.

Species	**Temp. (ºC)**	**Salinity (ppt)**	**Feed Type**	**Feed Rate**	**Densities**
Nile tilapia[1]	24	N/A	Zeigler Freshwater Finfish Starter	30% of body weight	25 fish/L
Rainbow trout[3]	19	N/A	Zeigler Freshwater Finfish Starter	30% of body weight	10 fish/L
Hybrid striped bass [2]	24	0.01-32	Freshwater Finfish Starter	30% of body weight	10 fish/L

El-Sayed,Abdel-Fattah 2002[1], Hedayati and Bagheri 2009[2], North *et al.* 2006[3]

Each experimental container was marked and numbered for clarity during the trials. Once the individual experimental containers were stocked, placed into their respective stations in the probiotic room, and randomly assigned to a treatment group, 0.001 ml of prepared probiotic was added to each container to create an effective concentration of 10^6 CFU (colony forming units)/ml. To prepare the probiotics for use, dosing vials are removed from a -80 ºC freezer five minutes prior to use and allowed to thaw to room temperature.

Each morning, surviving animals in all experimental containers were counted, and any dead were immediately removed and weighed. Following the daily counting, a 75% water exchange was performed to remove detritus from the bottom of the experimental containers.

Containers were then refilled with clean make-up water to maintain the 1-L working volume. Feed amounts were adjusted daily after accounting for mortality in containers. Following the morning survival count and water exchange, the first of three daily feedings (09:00, 12:00, 15:00) were conducted. Every other day, following the daily water exchange, an additional 0.001 µl aliquot of prepared probiotics was added to the appropriate tanks to maintain the desired treatment concentration (10^6 CFU/ml).

To monitor concentrations of ammonia, nitrate and nitrite in the experimental containers, one sample was collected from each treatment group daily. Samples taken were spaced out so that water in an individual tank was only sampled every four days. Collected samples consisted of water that was removed from the tanks during the water exchanges. Each four-day water quality sampling period was termed a "time cycle." Water quality samples were refrigerated at the DSU ARDF until they were ready to be analyzed later the same day for ammonia, nitrite and nitrate (Table 3-4). Temperature was measured for the room and the water holding tanks using an electric thermometer, and salinity was measured in the holding tanks using a refractometer before daily water changes.

Growth was assessed weekly to adjust feed rations so as to ensure that finfish in the experimental containers have adequate feed. To minimize handling stress associated with weight sampling, only the FM container from each treatment group was weighed. In addition, on scheduled sampling days, the 09:00 (pre-weigh) and 12:00 (post-weigh) rations were withheld from all experimental containers. The FM containers were removed from the study room and finfish were weighed individually using an APX-100 digital scale (Denver Instruments,

Bohemia, NY). Finfish were collected using a dip net, blotted dry with a paper towel, and placed into a tared petri dish one by one. After weighing, finfish were returned to their respective FM tanks and returned to the study room. FM tanks were specifically used only for weekly weight sampling to adjust feed, and not included in any of the subsequent statistical analysis.

Each trial was slated to run for eight weeks or until any one experimental container had 90% mortality. At the end of each trial, all surviving finfish from each experimental container were euthanized with a lethal dose of MS-222 (Tricaine methane sulfonate, Sigma-Aldrich, St. Louis, MO), in their home containers. After finfish have stopped reacting to stimuli, they were poured through a net to collect. Finfish were individually counted and weighed as described above. Collected data was archived and verified in MS Excel (Microsoft Office 2016 MS0 16.0.9126.2282, Redmond, WA). Statistical analysis was performed with SPSS (Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

Table 3-4. Water quality parameters measured as part of the larval growth and survival experiment. Also included are the testing frequency and analysis methods employed.

Parameter	**Frequency**	**Unit of Measure**	**Method**
Temperature	Daily	ºC	Thermometer
Salinity	Daily	PPT	Refractometer
pH	Daily	N/A	Insta-test analytical wide range pH[2]
Ammonia	Daily	mg/L	Salicylate method (385)[1]
Nitrite	Daily	mg/L	USEPA diazotization method (371)[1]
Nitrate	Daily	mg/L	Cadmium reduction method (355)[1]

HACH DR-3900 Spectrophotometer, HACH CO. Loveland, CO[1]
Insta-test, LaMotte, Chestertown MD[2]

Weight gain (WG) was calculated by subtracting the initial weight from the final weight for each tank; this was also used to calculate specific growth rate (SGR) as well as feed conversion ratio (FCR) using equations found in appendix (Appendix A). Treatment differences in SGR, FCR and WG were tested with a one-way ANOVA in SPSS. To ensure the validity of my conclusions I verified the data assumptions: normality was tested using Shapiro- Wilks test, outliers were looked for using box plots (Appendix B, C and D), and homogeneity of variance was tested using Laverne's test of homogeneity of variances.

Kaplan-Meier survival analysis (Kaplan and Meier 1958) was conducted in SPSS to compare four different treatments (control, ISO 5, ISO 11 and ISO 12) for their effect on finfish survival. A log-rank test was run to determine if there were any differences in finfish survival distributions among treatments. Log-rank pairwise comparisons were then run to determine which treatments had significantly different survival distributions. A Bonferroni correction was made in SPSS with statistical significance accepted at the $\alpha = 0.05$ level.

All six violations (exclusivity of events, measured time, avoidance of 'left censored' data, even censoring, secular trends, and normality of censorship) were verified. Normality of censorship, i.e. pattern and percentage, were verified using a scatterplot to ensure that there was a varied amount of censorship over time. During set up of Kaplan-Meier all events were labeled with either 0 or 1 denoting mortality on date of occurrence, this allowed for verification of exclusivity of events and measured time as both are determined in set up. Avoidance of 'left centered' data was verified as all treatments in trial started on the same day. Due to an unintended drop of temperature, mortality during T3 in the Nile tilapia experiment was censored

as ‘non-trial related death.’ This meant that there was a censorship of two trial tanks from the ISO 12 group. This censorship did not cause the trial to fail normality.

A repeated-measures ANOVA in SPSS was used determine if there were any significant differences among treatments with respect to mean concentrations of ammonia, nitrite and nitrate for treatments. Ammonia, nitrite and nitrate were run in time cycles (T1, T2, T3 etc.) as described above. The cycles were used then as the time stamps to run the repeated measures resulting in a ‘time cycle’ that represents the complete collection of all 16 tanks water samples in a four-week cycle. Analysis was only run on 12 of the 16 containers as the four FM containers were not used to assess anything other than feed changes over the course of the study. The assumptions for a repeated measures ANOVA were assessed in SPSS. Outliers were observed using boxplots and normal distribution was assessed using Shapiro-Wilk test for each of the time periods. The assumption for sphericity was assessed using Maulchy’s test and Greenhouse-Geisser corrections were applied to data that failed the assumption. For all tests an $\alpha = 0.05$ determined significance. All data is presented in in a mean ± standard deviation. A Bonferroni confidence interval adjustment was made for estimated marginal means in the SPSS software.

CHAPTER 4: RESULTS

The three bacterial strains used in this study were identified to their genus and strain name as *Shewanella spp.* (ISO 12), *Bacillus cereus.* (ISO 11), and *Bacillus cereus* (ISO 5). They concurrently will be referred to in this thesis as their given name (Table 3-1). Each bacterium was trialed with each of the three finfish species: Nile tilapia, rainbow trout and hybrid striped bass. Survival, FCR, SGR, WG were calculated at once each trial was concluded. Changes in ammonia, nitrite and nitrate concentrations were monitored for change over the course of the study.

Tilapia

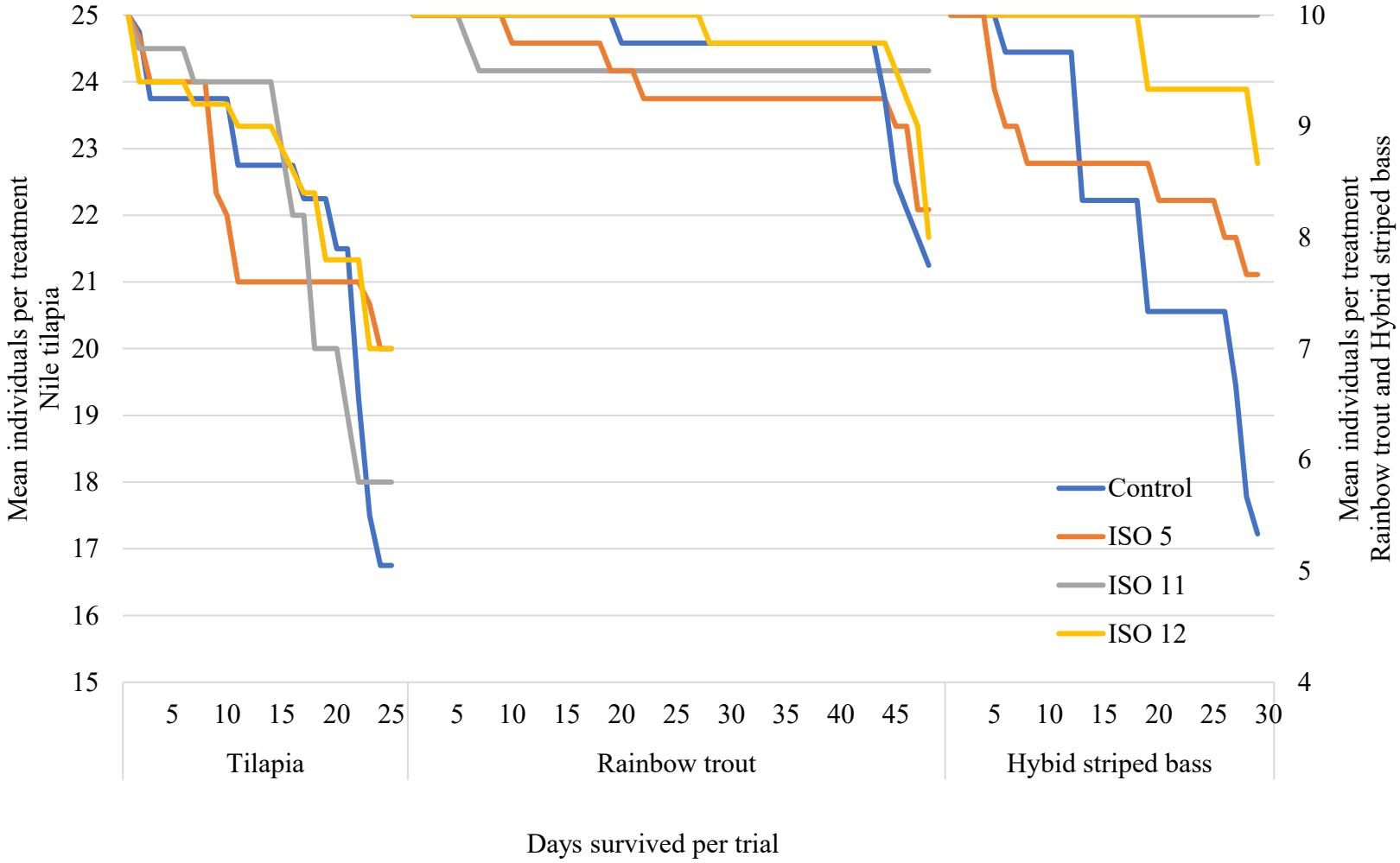
Mortality

The Nile tilapia experiment lasted 28 days, losing 84 (n = 300) finfish over the course of the experiment. In the first three days of the experiment all four treatments had light (1-2 finfish per treatment) finfish mortalities. The three tanks treated with ISO12 lost fish every three days for the first 16 days, while the control, ISO 11 and ISO 5 treated tanks lost one finfish every four days. During the final seven days of the experiment, ISO 12 and ISO 5 treated tanks had mortalities on two days, the control tanks had losses on four of the seven days and ISO 11 treated tanks had mortality on five of the seven days (Fig. 4-1). During this experiment a sharp decrease in temperature (-4ºC) caused heavy mortality in some of the finfish tanks. These losses occurred on the 19th day of the study and mortality continued for three days after. Tanks being treated with ISO 12 lost a total of 19/75 fish over the course of two days, and another 6 over the next three days. Tanks treated with ISO 11 lost 8/75, and the control and ISO 11 treated tanks lost

none. Heavy losses seen in the ISO 12 treated tanks eventually caused the end of the experiment as the 24% loss of fish occurred mainly in two tanks, one of which already had a dwindling number of survivors.

After a censoring of the finfish lost to temperature change, survival distributions increased for control vs. ISO 11 (χ 2 (3) = 6.347, p = 0.012), ISO 12 (χ 2 (3) = 8.571, p = 0.003), and ISO 5, (χ 2 (3) = 5.005, p = 0.025). Tanks treated with ISO 5 had 2.75% longer survival times over the course of the study than the control tanks. In ISO 11 treated tanks survival time was 6.6% longer than the control and ISO 12 treated tanks, with censored finfish numbers removed, lived 5.4% longer then the control finfish. Nile tilapia experiment had a mean survival of 22 ±0.0327 days for all four treatments. Tanks with the control treatment (n = 75) had a mean survival period of 22 (95 % CI, 20 to 23) days (Fig. 4-1) matching the overall mean for the experiment. Tanks treated with probiotics had mean survival periods of 22 (95% CI, 21 to 24) days for ISO 5, 23 (95% CI, 22 to 24) days for ISO 12 and 23 (95% CI, 22 to 24) for ISO 11. Overall lower bounds for the survival interval was 22 days with the upper bound at 23 days. Tanks treated with ISO 11 saw 77% survival with 58/75 finfish surviving the entire 28-day experiment, ISO 12 treated tanks lost only 15 fish with 60/75 surviving (80% survival) and ISO 5 treatment tanks had 56/75 survive (74% survival) versus the control which only had 42/75 finfish survive (56% survival).

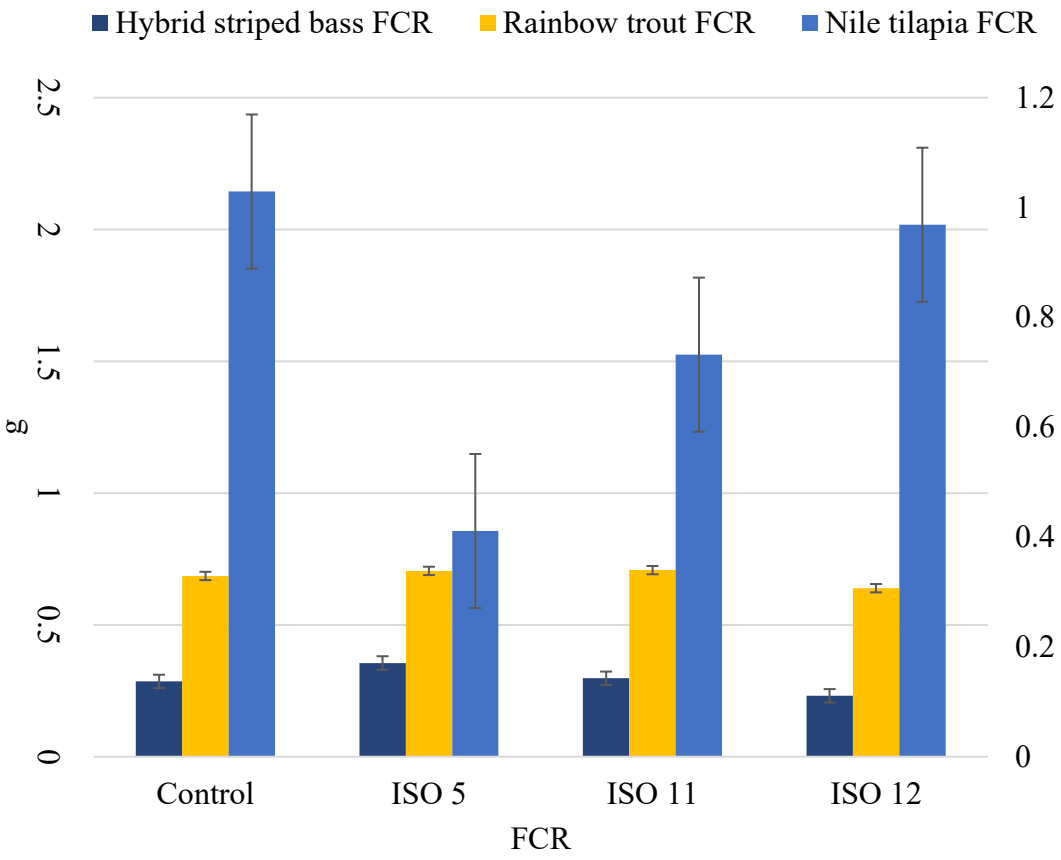
Figure 4-1. Survival graph generated using Kaplan-Meier means for Nile tilapia, rainbow trout and hybrid striped bass over the course of each trial. Each trial was run until 10% survival was seen in one out of 12 tanks, mortality was counted daily each morning to assure that survival graphs would be accurate to the day.



FCR, SGR and WG

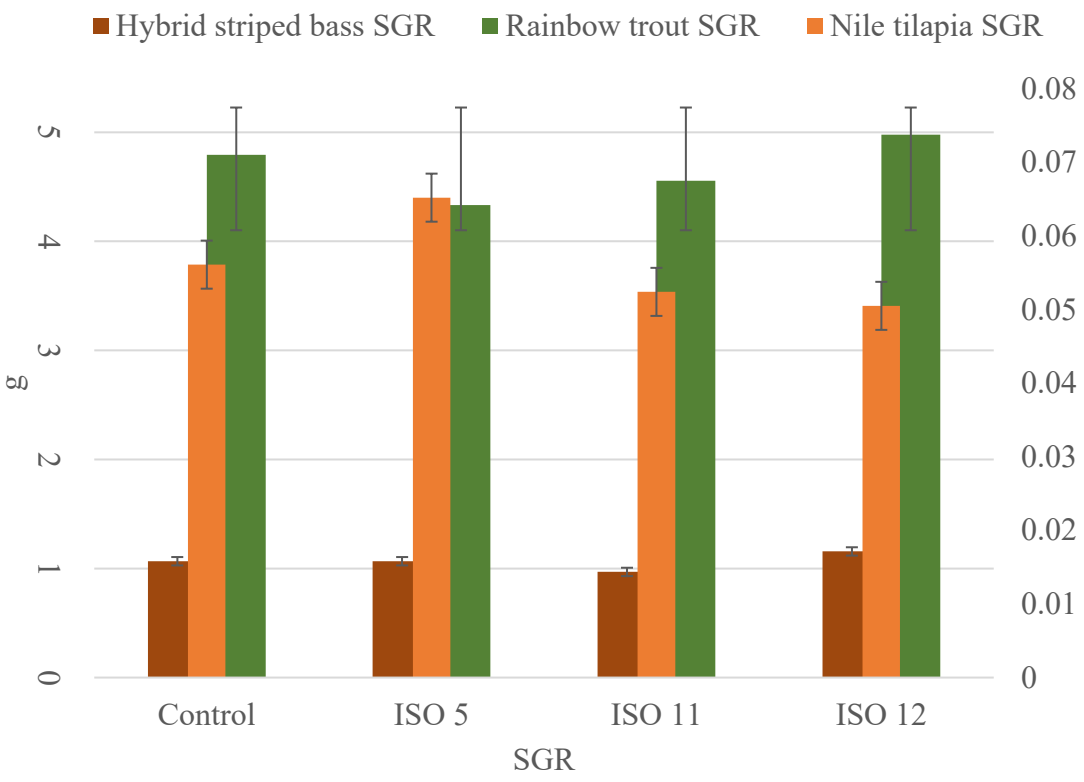
Nile tilapia FCRs had an overall mean of 1.636 ± 2.529 (n = 12). Finfish treated with ISO 5 (0.86 ± 0.19) and ISO 11 (1.53 ± 1.08) had FCR values below the mean, but not significantly so. ISO 12 treated tanks (2.02 ± 5.52) as well as the control (2.14 ± 1.42) tanks were above the mean but again, not significantly (Fig. 4-2). No significant differences were seen among the treatments for tilapia FCRs ($F_{3,8} = 0.12$, $p = 0.95$).

Figure 4-2. Feed conversion ratios by treatment. Data was assessed in SPSS using a one-way ANOVA, with error bars set at 95% confidence interval.



Nile tilapia SGR means increased from ISO 12 (0.05 ± 0.008 g), to ISO 11 (0.05 ± 0.01 g), to control (0.06 ± 0.02 g), to ISO 5 (0.07 ± 0.004 g) with the overall species mean 0.056 ± 0.012 (Fig 4-3). ISO 12 and ISO 11 were lower (0.001 g and 0.002 g, respectively) than the overall species mean, and ISO 5 was 0.0091g higher. The minimum SGR for this experiment was .04 g and the maximum was .08 g. Even with the spread of treatment SGRs above and below the overall experimental mean for the Nile tilapia experiment, SGRs for the Nile tilapia experiment were not significantly different ($F_{3,8} = 0.867$, $p = 0.497$).

Figure 4-3. SGRs calculated using the first and last weigh day of the experiment, by treatment. Data was assessed in SPSS using a one-way ANOVA, with error bars set at 95% confidence interval.



Mean WG for Nile tilapia experiment was 0.712 ± 0.596 g and treatment mean increased from ISO 12 (0.13 ± 0.34 g), to control (0.75 ± 0.66 g), to ISO 11 (0.80 ± 0.66 g), to ISO 5 (1.17 ± 0.36 g). ISO 12 was the lowest with a 0.58 g decrease from the overall study mean, and ISO 5 was the highest with 0.46 g increase in WG. The mean minimum WG for all treatments was -0.21 g and the mean maximum was 1.58 g. No significant difference among the treatments ($F(3,8) = 2.009$, $p = 0.191$) was observed for WG measurements.

Ammonia

Nile tilapia ammonia concentrations were significantly different over time, $F(5, 40) = 7.790$, $p < 0.0005$, η2 0.493 (Fig. 4-3) and increased as the experiment continued for 29 days. Overall mean ammonia concentrations were 4.7793 ± 0.401 mg/l for all time periods (T) over the 29 day experiment. T1, T2 and T3 (3.338 ± 0.058 mg/l) and T5 (6.925 ± 0.643 mg/l) had significant differences among the time periods ($F(3,6) = 20.030$, $p = 002$, $\eta^2 = 0.909$). The difference between T3 and T5 (+ 3.588 ± 0.601 mg/l, $p = 0.005$) was significant and the spike in ammonia occurred in the time period just after the temperature decrease (on the 19th day of study) led to mortality in experiment containers.

Mean concentrations of ammonia among treatments were similar (Fig 4-3). Overall mean of ammonia concentration was 4.7793 ± 0.3808 mg/l among the four treatment groups. Concentrations increased from ISO 11 (4.236 ± 0.401 mg/l), to the control (4.619 ± 0.401 mg/l), to ISO 12 (4.978 ± 0.401 mg/l) and ISO 5 (5.284 ± 0.401 mg/l) with a range of 3.854 mg/l to 5.7048 mg/l. No significant differences were observed among the means of concentration of ammonia in treatment tanks ($F(3,8) = 1.271$, $p = 0.348$, $\eta^2 = 0.323$). There was no significant

difference in the means of concentration of ammonia over time between the treatments ($F(15,40) = 0.803$, $p = 0.668$, $\eta 2 = 0.231$).

Nitrite

Overall mean for Nile tilapia nitrate concentration over the course of the experiment was 0.109 ± 0.018 mg/l and the ranged from 0.066 to 0.152 mg/l. Nitrite concentrations were significantly different over time ($F(1.55, 12.391) = 14.928$, $p = 0.001$, $\eta 2 = 651$) with significant differences were observed (Fig. 4-3) among T1 (0.231 ± 0.025 mg/l) and T2 (0.073 ± 0.010 mg/l), T3 (0.029 ± 0.003 mg/l), T4 (0.047 ± 0.013 mg/l) and T5 (0.057 ± 0.016 mg/l) as well as between T4 and T6 (0.219 ± 0.045 mg/l). Mean concentrations of nitrite significantly decreased from T1 to T2 (-0.158 ± 0.022 mg/l, $p = 0.001$) and from T1 again to T3 (-0.202 ± 0.027 mg/l, $p = 0.001$). Nitrite concentrations continued to significantly drop from T1 to T4 (-0.185 ±0.036 mg/l, $p = 0.013$) and from T1 to T5 (-0.175 ±0.018 mg/l, $p = 0.018$). A significant increase in mean concentrations of nitrite was observed between T4 and T6 (+0.173 ± 0.035 mg/l, $p = 0.018$) as well as T5 and T6 (+0.163 ±0.037 mg/l, $p = 0.035$). The concentrations of nitrite lowered until at the lowest (T4) they increased again to match the concentrations at the beginning of the study.

Concentrations for nitrite among treatments increased from the control (0.086 ± 0.017 mg/l) to ISO 5 (0.086 ± 0.017 mg/l), to ISO 12 (0.0131 ± 0.017 mg/l to ISO 11 (0.0134 ± 0.017 mg/l) with an overall experimental mean of 0.109 ± 0.017 mg/l. The concentrations in treatment tanks ranged from 0.0707 to 0.1479 mg/l and mean concentration differences were highest between the control, ISO 5 and the mean. Differences were not statistically significant among

the Nile tilapia treatment groups (F (3,8) = 2.555, p = 0.128, η^2 = 0.489). While there were significant differences in the mean concentrations of nitrite among the time periods, there was no significance among the means for the treatments or for the treatments over time.

Nitrate

The concentration of nitrate in the Nile tilapia experiment were significantly different among the time points (F 1.957, 15.656) =4.837, p = 0.024, η^2 = 0.377) and increased over time for the entirety of the study. Differences between T1 (9.858 ± 1.522 mg/l) and T3 (3.208 ± 0.385 mg/l), F (4,3) = 10.695 p = 0.046 were observed to be statistically different. A significant decrease in mean concentration of nitrate between T1 and T3 (-6.650 ± 1.591 mg/l, p = 0.046) was observed during (Fig 4-4) but did continue to rise throughout the remainder of the study with final concentrations of 7.458 ± 1.179 in T6. There was no significant difference of the nitrate concentration among the treatment tanks for the experiment.

Rainbow trout

Mortality

The rainbow trout experiment lasted 49 days with an overall survival rate of 83.8%. A total of five fish were lost in the first 28 days of the experiment (n = 120), with the control losing three before the 22nd day (n = 30). Tanks treated with ISO 5 (n = 30) and ISO 11 (n= 30) lost one fish for each set of three tanks. Two tanks treated with ISO 11 lost a fish each with in the first six days and one tank treated with ISO 5 lost a fish on the 20th day. No more finfish mortality occurred until the 44th day of study when tanks started to once again loose finfish, one of the tanks treated with ISO 5 lost nine fish in the last five days. Control tanks lost five more fish

during the last five days of the study and tanks treated with ISO 12 lost four fish. Tanks treated with ISO 11 did not lose any more fish after the sixth day of the study and lost only two fish for the full 49 days.

Days survived per finfish is averaged 47 days over the course of the study for all treatments. Rainbow trout tanks with the control treatment had a mean survival period of 46 (95 % CI, 44 to 48) days, at the lower bound end of the survival interval. Tanks treated with probiotics had mean survival periods of 48 (95% CI, 46 to 49) days for ISO 5, 48 (95% CI, 47 to 49) days for ISO 12 and 47 (95% CI, 44 to 48) for ISO 1, all three at the upper bound of the survival interval. Estimated means for all four treatments were within the overall bounds of the survival interval and as such did not observe any significance (χ 2 (3) = 4.877, p = 0.181). As there were no significant differences noted in the log-rank comparisons no further statistical tests were run.

FCR, SGR and WG

Rainbow trout FCRs increased from the control (0.67± 0.02), to ISO 12 (0.64 ± 0.08), to ISO 5 (0.71 ± 0.04), to ISO 11 (0.069 ± 0.04) with the overall mean of the rainbow trout experiment FCRs at 0.686 ± .057. None of the treatments however, were statistically significantly different (F (3,8) =0.894, p = 0.485) (Fig 4-2).

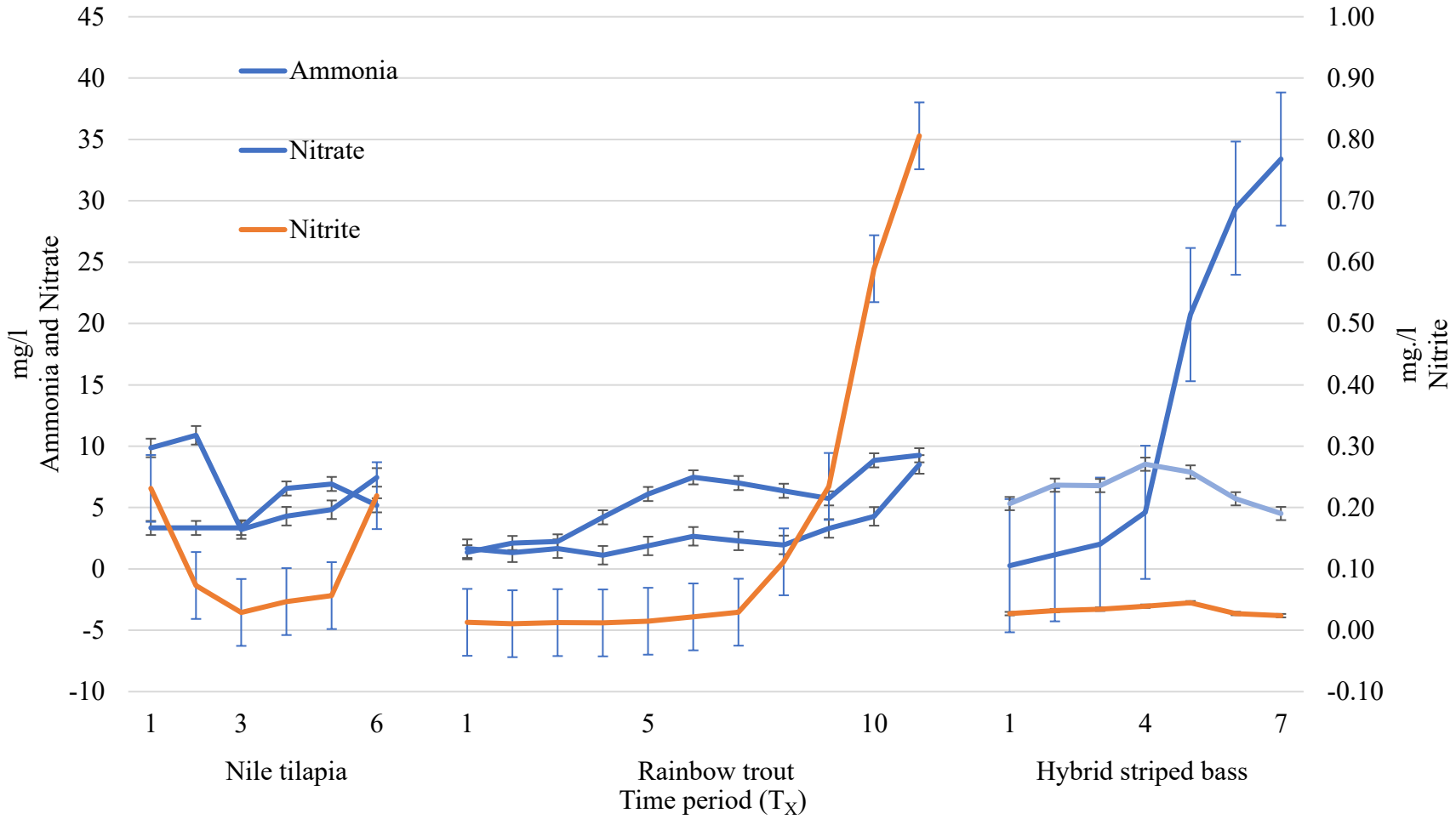
Increasing means for the rainbow trout SGRs went from ISO 5 (4.33± 0.55 g), to ISO 11 (4.55 ± 0.25 g), to control (4.80 ± 0.20 g), to ISO 12 (4.98 ± 0.54 g). Minimum SGRs for the rainbow trout experiment were 3.70 g (Fig 4-3), with maximums at 5.59. With the four

treatment groups falling with in the minimum and maximum ranges, SGR calculations for rainbow trout did not have significantly different means ($F_{3,8} = 1.385$, $p = 0.316$). Rainbow trout WG means increased from ISO 5 (1.37 ± 0.085), to ISO 11 (1.37 ± 0.20), to control (1.40 ± 0.04), to ISO 12 (1.54 ± 0.24) with a mean of 1.421 ± 0.158. Differences from treatment means to overall study mean were not statistically significant ($F_{(3,8)} = 0.773$, $p = 0.541$).

Ammonia

During the rainbow trout experiment, the means of ammonia concentrations were also significantly different over time, $F_{(2.122, 25.461)} = 20.444$, $p < 0.0005$, η^2 0.630 (Fig. 4-3) as expected for growing finfish. Significant differences were seen between all time periods as the concentrations increased from T1 (1.358 ± 0.165 mg/l) to T11 (9.263 ± 0.465 mg/l) with time periods overall means at 13.082 ± 1.4382 mg/l. Significant increases between mean concentrations of ammonia were observed (Fig 4-5) between T1and T11 (+7.905 ± 0.492 mg/l, $p < 0.0005$) as well as with in most other time periods (Appendix H) as all measures were well outside the lower and upper boundaries (9.7656 mg/l and 16.3984 mg/l). Ammonia concentrations per treatment increased from ISO 5 (5.261 ± 0.244 mg/l) to the control (5.423 ± 0.244 mg/l), to ISO 11 (5.544 ± 0.244 mg/l) to ISO 12 (5.852 ± 0.244 mg/l) but there was no significance between the treatment means, ($F_{(3,8)} = 1.331$, $p = 0.331$, $\eta^2 = 0.333$) or between means of treatments over time ($F_{(5.871,15.656)} = 0.405$, $p = 0.861$, $\eta^2 = 0.132$). Just like the Nile tilapia experiments, over time the concentrations of ammonia are increasing, but there is no significant difference among the concentrations in the treatment tanks (Fig 4-3), or among the treatments over time.

Figure 4-5. Nile tilapia, rainbow trout and hybrid striped bass water quality graphs over time, showing mean ammonia, nitrite and nitrate.



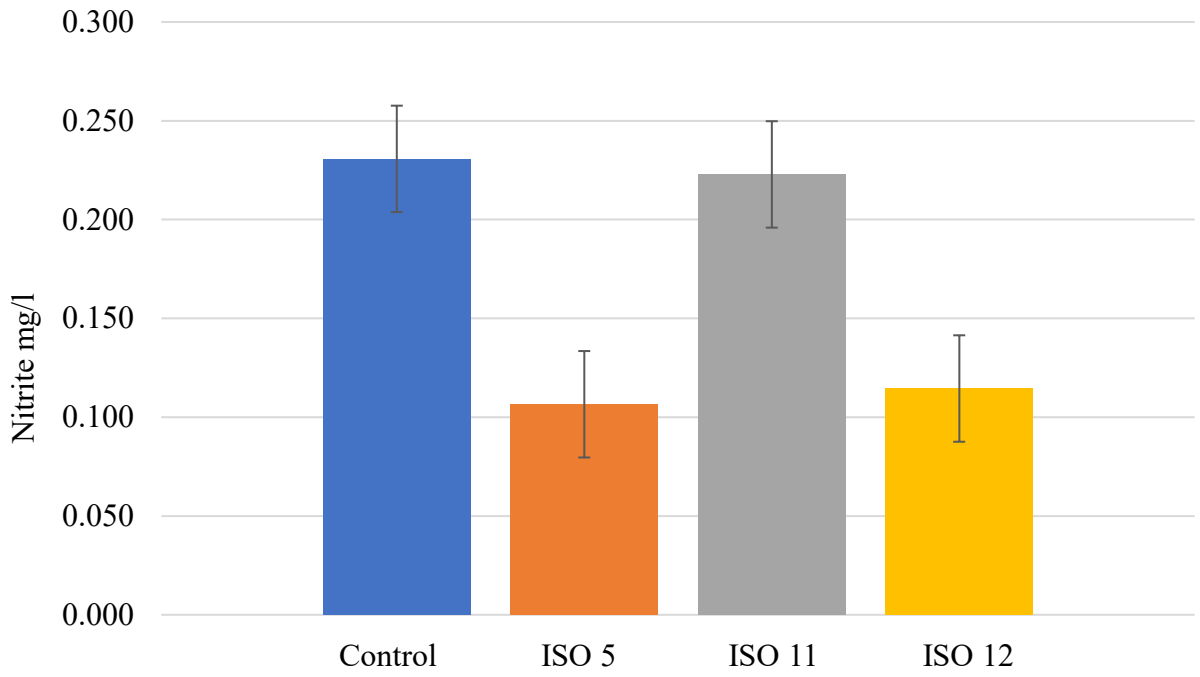
Nitrite

During the rainbow trout experiment, the means of nitrite concentrations were significantly different over time ($F(2.140, 25.682) = 34.858$, $p < 0.0005$, $\eta 2 = 0.744$) (Fig. 4-3) and overall mean was 0.1687 ± 0.0256 mg/l. Significant increases in nitrite means were observed from T1 to T11(+0.793 ± 0.106 mg/l, $p < 0.0005$). Significant increases were also observed in all other time periods between T10 and T11 (Figure 4-3). T1 to T8 were very similar in nitrite concentrations with mean differences averaging ± 0.0293 mg/l, and after T8 the rainbow trout experiment saw a rise in nitrite concentrations across all tanks. Tanks rose on average 0.007 mg/l between T7 and T8 and continued to rise in T9 through T11. T11 final concentration was 0.806 ± 0.105mg/l and it was significantly different from all other time periods except T10 due to its range being 0.377 to 0.802, and not significantly different.

There were significant differences between the means of concentration of nitrite in treatment tanks ($F(3,8) = 6.240$, $p = 0.008$, $\eta 2 = 0.609$) during the rainbow trout experiment (Fig 4-6). A statistically significant decrease in nitrite concentration we seen between the control (0.231 ± 0.027) and ISO 5 (0.107 ± 0.027) with ISO 5 nitrite concentration mean just below the average lower boundary (0.1099 mg/l) and the upper bound for ISO 5 (0.165 mg/l) being out of the lower bound interval for the control (0.172 mg/l). ISO 5 was the lowest mean concentration however, there was no significant differences between ISO 5 and either ISO 11 (0.223 ± 0.027) or ISO 12 (0.115 ± 0.027). A difference is observed between the control and ISO 12, but the upper bound interval for ISO 12 (0.173) is within the lower bound interval for control treatment means (0.172) so there is no significance. The difference between the control and ISO 5 was the

only significant difference among the treatments for concentration in nitrite. Significant increases were seen in concentration of nitrite over time, and significant differences were observed between the control and ISO 5 treatment, but no other significant differences were seen between treatments or between treatments over time.

Figure 4-6. Means of nitrite data for rainbow trout in control, ISO 5, ISO 11 and ISO 12 treatments. Mean units are milligrams per liter, error bars are standard error. Means and standard error were generated in SPSS using a repeated measure ANOVA and chart was built in Excel.



Nitrate

Mean nitrate concentration in the rainbow trout experiment was at 2.7826 ± 0.2796 mg/l and concentrations were significantly different over all time periods, $F(1.918, 23.016) = 23.845$, $p < 0.0005$, $\eta 2 = 0.665$ (Fig 4-5). Mean nitrate concentrations showed significant differences in

all time periods and T1 and T11, steadily increasing from T1 (1.663 ± 0.187 mg/l) to T11 (7.781 ± 0.987, p = 0.002 mg/l). T1 was significantly lower than the mean, and T11 was significantly higher. Mean concentration between the treatments was 2.7826 ± 0.2499 mg/l and treatment concentrations increased starting with ISO 12 (2.393 ± 0.238 mg/l) to ISO 5 (2.602 ± 0.238 mg/l), to control (2.955 ± 0.238 mg/l) and ISO 11 (3.193 ± 0.238 mg/l). There was no statistical significance between the mean concentrations of nitrate in probiotic treatments, $F(3,8) = 3.034$, $p = 0.093$, $\eta 2 = 0.532$ and very little difference between the treatment concentrations and the overall mean. Probiotic treatments did not have a statistically different means from the control tanks, meaning they also had no effect on the concentration of nitrate in the experiment over time, $F(5.754, 23.016) = 2.044$, $p = 0.103$, $\eta 2 = 0.338$.

Hybrid striped bass

Mortality

The hybrid striped bass experiment started seeing increased mortality on the 19th day, from there the study lasted another 10 days lasting 29 days total. During the first 19 days 14 finfish out of 120 died, eight coming from the control tanks followed by four from the tanks treated with ISO 12 and two from tanks treated with ISO 12. Another major loss occurred in control tanks on the 27th and 28th days of the study with ½ a tank dying. Nine fish in total were last for all the treatments in the last four days of the study. This mortality along with the deaths in the control tanks on day 28 ultimately led to the end of the study on day 29. ISO 11 treated tanks did not lose any fish for the entirety of the study.

Hybrid striped bass tanks with the control treatment had a mean survival period of 26 (95 % CI, 24 to 28) which was the lower bound of the survival interval for the experiment. The hybrid striped bass experiment lasted 29 days with tanks treated with probiotics having mean survival periods of 25 (95% CI, 22 to 28) days for ISO 12, 28 (95% CI, 27 to 29) days for ISO 5, and 28 (95% CI, 27 to 29) for ISO 11. ISO 5 and ISO 11 treated tanks had the highest survival and were closest to the upper bound interval (29 days) having some tanks that had 100% survival. Tanks treated with ISO 12 had estimated survival times 3.2% lower than the control tanks, while ISO 5 and ISO 11 treated tanks both had 9.5% longer survival times than the control. The survival distribution for ISO 5 (χ 2 (3) = 11.968, p = 0.001) and ISO 11 (χ 2 (3) = 8.357, p = 0.004) were significantly higher when compared to the control tanks. ISO 12 treated tanks had 73% overall survival but compared to control tanks at 53% there was no statistical difference between the two treatment groups (χ 2 (3) = 1.619, p = 0.203).

FCR, SGR and WG

Hybrid striped bass FCRs ranged from ISO 12 (0.23± 0.11), to control (0.29 ± 0.18), to ISO 11 (0.30 ± 0.06), to ISO 5 (0.36 ± 0.22). The overall mean FCR for hybrid striped bass was 0.292 ± 0.139. Tanks treated with ISO 11 had an estimated marginal means that were closest to the overall average value and ISO 5 treated tanks were 0.065 higher than the overall mean. While the FCR for the ISO 5 treatment was well above the overall mean, none of the three species experiments had any significance between the treated tanks' FCRs and the control tank FCRs and there was no pattern to the lowest FCR to the highest.

Hybrid striped bass SGRs increased from ISO 11 (0.97 ± 0.14 g), to control (1.07 ± 06 g), to ISO 5 (1.07 ± 0.79 g), to ISO 12 (1.16 ± 0.39 g). All four treatments were within the minimum and maximum statistics (0.488,1.301) with ISO 11 having the lowest SGR and ISO 12 having the highest. SGRs, while having a wide range of values, for hybrid striped bass did not have significantly different means, $F(3,8) = 0.061$, $p = 0.979$ for this experiment.

Hybrid striped bass WG means increased from ISO 11 (0.62 ± 0.11), to control (0.74 ± 0.49), to ISO 5 (0.78 ± 0.66), to ISO 12 (0.81 ± 0.27). Overall study mean for hybrid striped bass was 0.7382 ± 0.3784 g. ISO 11 was the lowest WG and was 0.1148 g lower than the overall study mean with ISO 12 being 0.0703 higher. The large spread between min and max (0.338 g and 1.538 g) was observed for hybrid striped bass WG. WG for hybrid striped bass did not have significantly different means ($F\ 3,8 = 0.105$, $p = 0.955$).

Ammonia

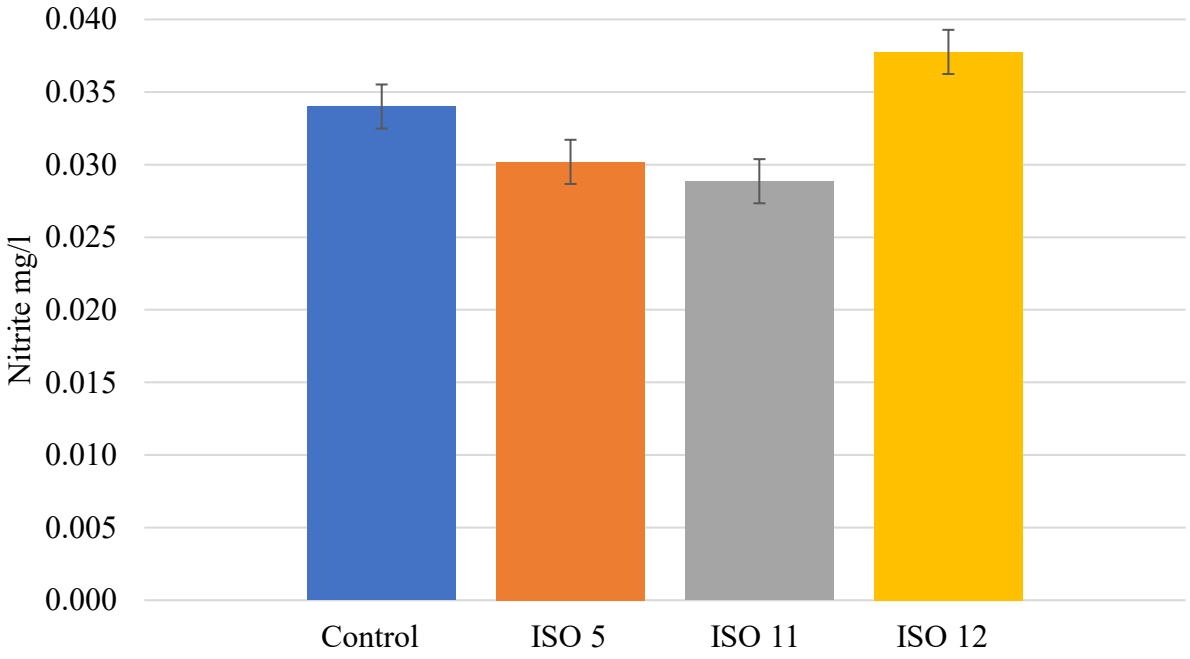
Hybrid striped bass ammonia concentrations increased over the course of the experiment as well and significance was observed among the individual time points, $F(1.575, 12.602) = 51.429$, $p < 0.0005$, $\eta 2 = 0.865$ (Fig. 4-4). Differences in the means of the concentration of ammonia from T1 to T5 were significant ($F(6,3) = 53.547$, $p = 0.004$, $\eta 2 = 0.991$) as the ammonia concentrations went from under 1 mg/l up to 20 mg/l. The increase here is seen just like in the Nile tilapia and rainbow trout studies, after T4 seems to be the time at which the ammonia concentrations start to rise and are less affected by the daily water exchanges (Fig 4-5). Ammonia concentrations in the treatment tanks increased in all four treatment tanks over the duration of the experiment, with an overall mean ammonia concentration of 13.082 ± 1.0777

mg/l. Total ammonia concentration means for each treatment increased from the control (10.941 ± 1.077 mg/l), to ISO 5 (11.545 ± 1.077 mg/l), to ISO 11 (13.266 ± 1.077 mg/l) and ISO 12 (16.576 ± 1.077 mg/l). Significant differences were not observed among the treatments (Fig 4-4) concentrations of ammonia. There were no significant differences between the means of concentration on ammonia in treatment tanks or the treatment tanks over time, $F(4.726,12.602) = 1.172$, $p = 0.374$, $\eta 2 = 0.305$.

Nitrite

Hybrid striped bass means of nitrite concentrations were significantly different over time, $F(6, 48) = 6.016$, $p < 0.0005$, $\eta 2 = 429$ (Fig. 4-3). T5 and T6. T5 had significantly higher means then T6 (+ 0.018 ± 0.004 mg/l, $p = 0.047$) which was the highest concentration level over the course of the entire experiment (Fig 4-5). After the T6 increase, there was a drop to T7 the final time period, where there was no significant difference and the experiment ended. Statistically different means among treatment concentrations of nitrite were observed in this experiment, $F(3,8) = 6.974$, $p = 0.013$, $\eta 2 = 0.723$. A significant increase in the concentration of nitrite was seen between ISO 12 and ISO11 (+0.009 ± 0.002 mg/l, $p = 0.019$) and between ISO 12 and ISO 5 (+0.008 ± 0.002 mg/l, $p = 0.047$) (Fig 4-7). Increases between the ISO 12 treatment groups and the ISO 5 and ISO 11 groups show that ISO 12 may have had a direct effect on the nitrite concentration in this experiment when compared to the other treatments. There were no other significant differences in the means among the treatments for the hybrid striped bass experiment, or between the control and any other treatment. There were no significant differences among the means of concentration on nitrite in treatment tanks over time, $F(18, 48) = 0.398$, $p = 0.982$, $\eta 2 = 0.130$.

Figure 4-7. Means of nitrite data for hybrid striped bass in control, ISO 5, ISO 11 and ISO 12 treatments. Mean units are milligrams per liter, error bars are standard error. Means and standard error were generated in SPSS using a repeated measure ANOVA and chart was built in Excel.



Nitrate

Hybrid striped bass nitrate means of nitrate concentrations were significantly different over some time periods, $F(2.624, 20.996) = 6.074$, $p = 0.005$, $\eta 2 = 0.432$ (Fig.4-3) with a range of 5.5199 to 7.511 mg/l. Significant differences in the means of concentration of nitrate were observed between T4 (8.533 ± 0.315 mg/l) and T7 (4.517 ± 0.404 mg/l, $p = 0.003$), T4 being the peak of the concentrations over seven time periods and T7 being the final time period ($F(6,3) = 101.064$, $p = 0.002$, $\eta 2 = 0.995$). The concentrations of nitrate among the four treatments; ISO 11 (5.857 ± 0.432 mg/l), ISO 5 (6.186 ± 0.432 mg/l), the control (6.362 ± 0.432 mg/l) and ISO

12 (7.657 ± 0.0432 mg/l) had no significant differences among the treatments observed, $F(3,8) = 3.342$, $p = 0.077$, $\eta 2 = 0.556$.

CHAPTER 5: DISCUSSION

Tilapia

The Nile tilapia experiment saw significant increases in survival between the control tanks and all treatment tanks. ISO 5 and ISO 11 are Bacillus cereus bacteria and tanks treated with both the ISO 5 and ISO 11 probiotics had 2.75% and 6.6% respectively longer survival times than the control tanks. Tanks treated with ISO 5 and 11 also saw higher survival with 74.7% and 77.3% respectively. The significantly high survival in the ISO 11 and ISO 5 treatments of Nile tilapia experiment when compared to control survival, shows what others have seen of other *Bacillus spp.* in Nile tilapia (Del'Duca *et al.* 2013). Nile tilapia tanks treated with ISO 12 also were significant with 5.4% longer survival times than the control. Survival also was 30% higher in ISO 12 treatment tanks when compared to control tanks in the Nile tilapia trial. While the finfish in the treated tanks had significantly higher survival they did not have significantly higher FCRs, SGRs or WGs.

Several studies treating Nile tilapia with a *Bacillus spp.* probiotic have shown that the finfish are affected by the probiotics and that the probiotics can have a significant effect on the FCRs, the growth as well as the weight gain (Yanbo and Zirong, 2006, and Del'Duca *et al.* 2013). Our experiment did not see any significant differences in the calculated FCR, SGR or the WG in the Nile tilapia finfish. This finding should not be a deterrent to using the probiotics as while there were no weight gains or decreases in feed conversions, the FCR SGR and WGs among the treatments did not drop. Even in the high stress environment the finfish had high survival and were able to maintain the same metabolic functions as the control tanks. While this

doesn't show that the probiotics helped increase metabolic function, the probiotics didn't slow down or retard the process either.

There was no significance among the treatments for water quality meaning there was no difference in the concentrations of ammonia, nitrite or nitrate between the control or the treatment tanks. Concentrations of ammonia in the Nile tilapia experiment were consistently high and by T3/4 in Nile tilapia ammonia concentrations were outpacing the water exchanges. Ammonia concentrations were expected to increase, and the 75% water exchange had hoped to control the increase in concentration as much as possible without removing too much of the probiotic treatments, a water exchange of 75% of 4 mg/l brings the water back down to 1 mg/l with approximately 0.0125 mg/l increase per hour over a 24 hour period. A study done in 2008 by Benali *et al.* showed that concentrations of 0.071 mg/l resulted in negative histopathological changes in gills, liver and kidney tissue in Nile tilapia adults, leading to eventual declines in health and increased mortality. Stress from high concentrations of ammonia could be a danger to finfish, the physiological changes cause a release of corticosteroids during periods of high stress. A study done in 2008 by Benali *et al.* showed that concentrations of 0.071 mg/l resulted in negative histopathological changes in gills, liver and kidney tissue in Nile tilapia adults, leading to eventual declines in health and increased mortality. Nile tilapia concentrations during our experiment were consistently high and high mortality should have been seen in all treatments.

The statistically high survival of the ISO 5, ISO 11 and ISO 12 treated tanks over the control treatment shows that the probiotics must be helping the finfish survive under the high stress caused by the high-ammonia, nitrite and nitrate concentrations. That the control tank had

shorter survival times and less finfish survived for the course of the study shows that without the probiotic treatment the finfish died sooner and higher mortality rates. For the Nile tilapia experiment all three probiotic treatments were successful at aiding the finfish in survival even during destructively high concentrations of ammonia, nitrite and nitrates.

Rainbow trout

The rainbow trout experiment saw no significant difference in survival times among the treatments. The experiment ran for 49 days and the rate of fish loss accelerated only in the last five days of the study. All four treatments in the rainbow trout experiment had similar means for length of finfish survival as well as fish growth and feed conversions. Among all the treatments FCR, SGR and WGs did have significant differences. This means that the control tank as well as all of the probiotic treatments survived a similarly long, had similar survival and similar growth with similar FCRs. This also means that the probiotics neither lengthened or decreased the finfish survival or manipulated the FCR, SGR or WGs of any tanks treated.

The rainbow trout experiment had very high survival over the course of the study for the *Bacillus cereus* treated tanks. All tanks treated with *Bacillus cereus* maintained 99% survival until the 44th day of the trial. Tanks treated with ISO 11 ended the trial with a 95% survival and tanks treated with ISO 5 77.5%. The difference between the two *Bacillus cereus* needs to be studied further, while there was no significance with survival, the control tanks had a higher survival (82.5%) than the ISO 5 treated tanks. The ISO 5 tanks also had significantly lower levels of nitrite when compared to the control. The ISO 5 treated tanks did not have significantly lower levels of ammonia or nitrates, but the significantly lower levels nitrite could be due to

Bacillus cereus ability to help increase water quality over time (Balcazar *et al.* 2006, Lara-Flores 2011, Perez-Sanchez *et al.* 2013).

The significantly lower nitrite may be a function of the *Bacillus cereus* probiotic to help increase water quality, but the ISO 5 treated tanks did not see less mortality or longer survival. The tanks treated with ISO 5 had lower survival, but there was no significance to link the change in water quality with the survival. The lack of significance in survival and significance in the drop of nitrites in tanks treated with ISO 5 means that ISO 5 should be further tested for its effect on water quality without affecting mortality, FCR, SGR or WG. The probiotic could have other functions that were not studied in this trial that may lend to it being used in aquaculture.

Hybrid Striped Bass

Tanks treated with ISO 5 and ISO 11 (both *Bacillus cereus)* had significantly higher survival (93% and 87%) when compared to the control tanks (54%) in the hybrid striped bass experiment. Tanks treated with ISO 12 did not have significantly different survival (73%) when compared to both the *Bacillus cereus* probiotics or the control. *Bacillus cereus* treated tanks in this experiment both had 9.5% longer survival times then the control while the ISO 12 was 3.2% shorter than the control. Only the ISO 5 and ISO 11, *Bacillus cereus*, treated tanks were significantly different from the control and showed that the probiotic addition of the *Bacillus cereus* had a positive impact on the survival time. Survival in the ISO 5 and ISO 11 tanks even increased when compared to the control despite the poor water quality in all containers.

Ammonia and nitrate concentrations among all treatments were not significantly different, but the nitrite concentrations in the ISO 12 treated tanks was significantly higher than the ISO 5 and ISO 11 treated tanks. The high nitrite concentrations and low survival time for ISO 12 treated thanks shows that the ISO 12 probiotic may influence the nitrite concentrations. The high nitrite concentrations may also have had an effect on the survival times, causing them to be lower than the ISO 5 and ISO 11 treated tanks, but they were not significantly lower than the ISO 5 and ISO 11 treated tanks or significantly higher than the control tanks. The difference in nitrite concentration is significant among the probiotic treatments, but it is not significant between ISO 5, ISO 11, ISO 12 and the control, so the effect that any of the three probiotics are having on water quality is not necessarily clear.

Probiotics

Bacillus cereus

Two different strains of *Bacillus cereus* were used in this experiment. ISO 5 and ISO 11 are both *Bacillus cereus* bacteria and as such were expected to perform well due to their family's history in probiotic research. The *Bacillus* probiotics used in this study were expected to have some effect on survival and water quality as they came from a warm estuarine environment, and the Nile tilapia and hybrid striped bass water parameters were very close to the estuarine environment that the mummichog bacteria were harvested from. *Bacillus spp.* are generally great candidates for probiotic use in aquaculture, being a spore, they are easily stored at room temperature, which means using them in feeds is feasible as they won't lose their activity due to small temperature fluctuations (Lalloo *et al.* 2009 & Buruinana *et al.* 2014). This makes food storage easier and cheaper for farmers. The spores also have another benefit, in that they are

able to survive the low pH of the gastero-intestines, meaning they are able to move past the barrier and colonize further down the gut where they can be beneficial over a longer period of time (Lalloo *et al.* 2009 & Buruinana *et al.* 2014). Previous studies have shown that *Bacillus* species are often useful for reducing stress and increasing survival when used in finfish aquaculture (Del'Duca *et al.* 2013 & Cerezuela *et al.* 2013). *Bacillus cereus* treated tanks in this experiment had significantly higher survival then the control in both the Nile tilapia and the hybrid striped bass experiments. Significant survival increases between the control and the *Bacillus cereus* treated tanks could come from the ability of some *Bacillus spp.* probiotics to help aquatic species better deal with stressful environments, like poor water quality and overcrowding (Olmos *et al.* 2011).

Our experiments showed that *Bacillus cereus* treated tanks in the rainbow trout experiments had statistically lower nitrite than the control tanks. This could be due to *Bacillus cereus* ability to help increase water quality over time (Balcazar *et al.* 2006, Lara-Flores 2011, Perez-Sanchez *et al.* 2013). A surprise came from the significantly low nitrite concentration in tanks treated with ISO 5 during the rainbow trout study, showing that this *Bacillus cereus* may function better in a colder (19 ºC) water and perhaps is a probiotic for the mummichog when it is changing to colder waters. This probiotic seems to be most useful and have the best significance for water quality in the cooler water as there was no significance observed in either the Nile tilapia or hybrid striped bass experiments. This experiment shows that both *Bacillus cereus* probiotics are useful for affecting survival in both the Nile tilapia and the hybrid striped bass study, and ISO 5 may be helpful in lowering nitrite in cooler waters as seen in the Rainbow trout study.

Shewanella spp.

Shewanella spp. is a marine bacterium that, while rarely pathogenic, has a bad reputation for causing infections and being highly tolerant to antibiotic treatment (Vignier *et al.*2013). Discussing *Shewanella spp.* in aquaculture means discussing *Shewanella putrefaciens*, a pathogenic bacterium in some finfish, but now used as a probiotic in gilthead seabream and senegalese sole, increasing survival and growth in both species (Austin and Austin 2012, Makridis *et al.* 2015 and De la Banda *et al.* 2012). When used in aquaculture *Shewanella spp.* probiotics are slowly gaining a reputation for improvements in growth, survival as well as reductions in stress when used long term (Merrifield *et al.* 2010, Jiang *et al.* 2013 and Rahiman *et al.* 2010). During this trial finfish were treated with a *Shewanella spp.* probiotic ISO 12. ISO 12 is still being identified and as such, only assumptions can be had about the *Shewanella* strain its self and not the specific species.

The warm water results from this experiment show that in warm water conditions (24 ºC) our *Shewanella spp.* probiotics can be used to increase survival in Nile tilapia. Our finding match those of other experiments in showing an increased survival when compared to the control for both the Nile tilapia (De la Banda *et al.* 2012, Ghosh *et al.* 2008 and Kumar *et al.* 2006). While other studies also saw an increase in SGR and FCR (De la Banda *et al.* 2012) our study did not see these results. This probiotic is a novel strain and as such may not have similar functions as the *Shewanella spp.* used in other experiments. While no significance was observed in FCR, SGR or WGs, significance was observed in the concentrations of nitrite in the hybrid striped bass experiments for tanks treated with the *Shewanella spp.* probiotcs.

Other strains of *Shewanella spp.* have been identified as denitrifying organisms (Cruz-Garcia *et al.* 2006) meaning with the ability to either denitrify or dissimilate nitrate to nitrite to ammonium, where it eventually becomes a gas that can escape the surface of the water leaving it harmless to the finfish. In the Cruz-Garcia *et al.* (2006) experiment *Shewanella oneidensis* was given nitrate and they observed the reduction to nitrite and eventually to ammonium. It is possible such high concentrations of nitrite were seen in the ISO 12 containers because of the reduction of the nitrate into nitrite. In our experiment the concentrations of nitrate and nitrite had very similar curves, with nitrate and nitrite peaking during the same time periods, and then a heavy drop of nitrate where nitrites are statistically higher than the other treatments. If *Shewanella spp*. ISO 12 is catabolizing the nitrates into nitrites, this may explain why ISO 12 treated tanks nitrite concentrations were statistically higher in the hybrid striped bass experiment.

ISO 12 has use in Nile tilapia to increase survival. In this study it was observed increasing the concentrations of nitrites, but this could be do to unmeasured factors as well as *Shewanella spp.* history of quickly converting ammonia and causing concentrations of nitrite to increase. Mores study is needed for this probiotic to see what affect if any it truly has on the water quality of select species of finfish. This bacterium is still recommended for further study and use as a probiotic for Nile tilapia as it increased survival.

CHAPTER 6: CONCLUSIONS

Aquaculture is faced with difficulties as we move forward into the future, feeding billions of people while still maintaining healthy rearing and optimal growing conditions. Where previously antibiotics and chemical treatments were relied upon to treat bacterial infection and reduce virulent bacteria in systems, probiotics may have the ability to work alongside them, if not replace them all together to address these challenges. Antibiotics and chemical treatments can have unintended side effects such as killing off multiple species of bacteria instead of targeting the harmful. The goal of this study was to evaluate the ability of probiotic bacteria ISO 5, ISO 11 and ISO 12 and see if they might influence survival in finfish trials. My hope is that the bacteria evaluated in this study may be able to help provide aquaculturists a better tool to use than traditional antibiotics and chemical treatments, and the unintended consequences these can have.

ISO 5, *Bacillus cereus*, treated tanks had consistently higher survival and longer survival times than the control tanks. It has significantly higher survival for both Nile tilapia and Hybrid striped bass. While there was no significant difference for survival in the rainbow trout experiment, ISO 5 did not have significant differences in survival between itself and the control, meaning that it did not do harm to the host and tanks treated with ISO 5 had the benefit of significantly lower nitrite concentrations during the rainbow trout studies. ISO 5 is a good candidate for probiotic status and should be studied further to determine its optimal temperature as it had different affects when going from warmer water to cooler.

ISO 11 treated tanks had significantly higher survival when compared to control tanks for both the Nile tilapia and the hybrid striped bass. ISO 11 survival did not go below 70% for any of the three experiments and while there was no statistical difference for the rainbow trout, ISO 11 was at 95% survival for the entirety of the study. The significantly higher survival for the Nile tilapia and hybrid striped bass as well as the lack of significance in the rainbow trout experiment prove that ISO 11 is viable for probiotic use. The treatment assisted the finfish and increased survival in warmer waters while not affecting the survival of the cool water rainbow trout and should be studied to see what other functions it might have in finfish. While ISO 11 treated tanks did not always have significantly lower concentrations of nitrite, it was consistently lower than the control and most of the other probiotics. Treating Nile tilapia, rainbow trout and hybrid striped bass with ISO 11 does not harm finfish.

Survival was expected to be significantly higher for both ISO 5 and ISO 11 treated tanks as they are members of the *Bacillaceae* family and a lot of research has already been done on this family of bacteria. Previous research shows that *Bacillaceae* are among the most known about graham-positive bacteria, and one of the better functioning probiotic groups (Kunst *et al.*1997). With this family legacy behind them ISO 11 and ISO 5 would be ideal for further study and experimentation as they could have other advantages that were not studied in these trials. Both probiotics need to be tested in different systems, water temperatures as well as salinities to further prove their usefulness to aquaculture. The *Shewanella spp.* probiotic should also be studied further.

ISO 12, *Shewanella spp.* has also proven to be useful for aiding in finfish survival in warm water conditions. High survival in the Nile tilapia experiments show that this bacterium could be lowering stressful conditions and is increasing survival for Nile tilapia. Further testing in multiple water temperatures and higher salinities would be useful before this probiotic to determine what other functions it might have as *Shewanella spp.* probiotics have been seen to greatly increase survival and change water quality. ISO 12 should be tested for more benefits as it maintained high survival rates, even while having the highest concentrations of nitrite during the hybrid striped bass experiment.

Through this experiment I can say that ISO 5, ISO 11 and ISO 12 have the makings of useful probiotics for the aquatic industry. These experiments were run in static systems with no bacteriological media filter, no running water and only one water exchange a day, and improved survival was seen for all three probiotic treatments in multiple species. These probiotics need to be trialed in aquaculture systems in large quantities of water to determine their true probiotic abilities over longer periods of time. While there was no significance in FCR, SGR or WG, these finfish were in small containers and trialed with very poor water quality conditions under high stress. If the study were to be run again to look for effects in growth and development of the finfish, I would recommend that the animals be placed in a rack system and that water quality parameters being maintained in optimal conditions to allow for growth and proper assessment of the parameters. This study has shown the ability of the probiotics to assist in survival, but no evidence for growth was seen. To truly grasp the scope of the aide these bacteria have in aquaculture more studies would need to be done.

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APPENDIX

APPENDIX

Appendix A. Specific growth rate (SGR) and Feed conversion ratio (FCR) was calculated using the equation below.

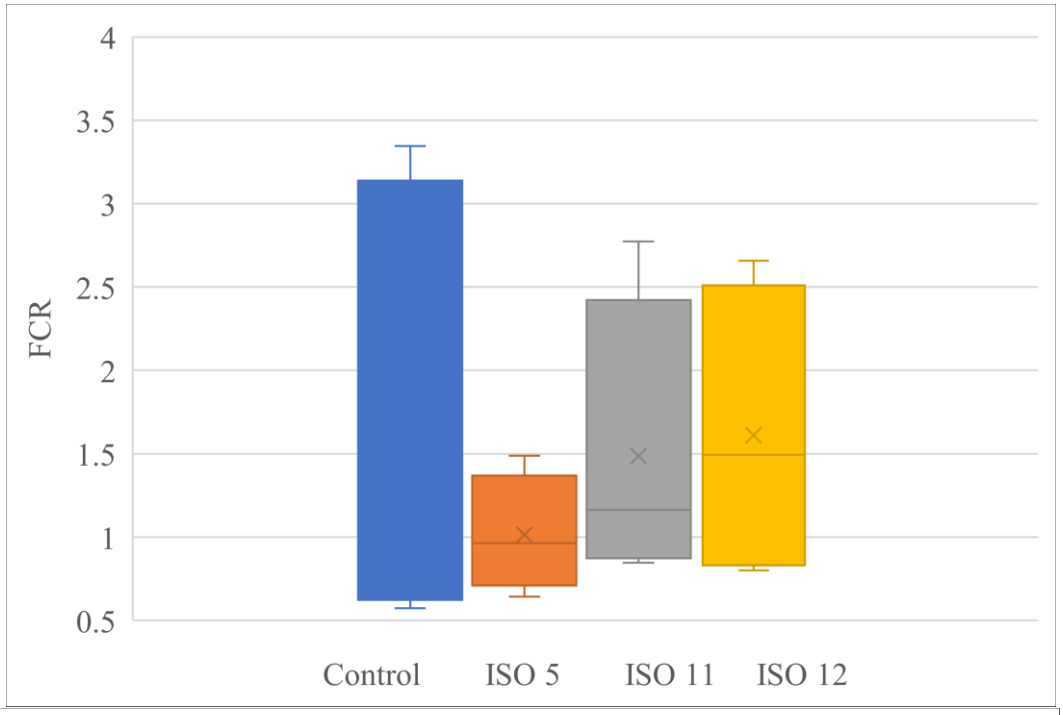
SGR = (LN(Wf)-LN(Wi)) *100/days

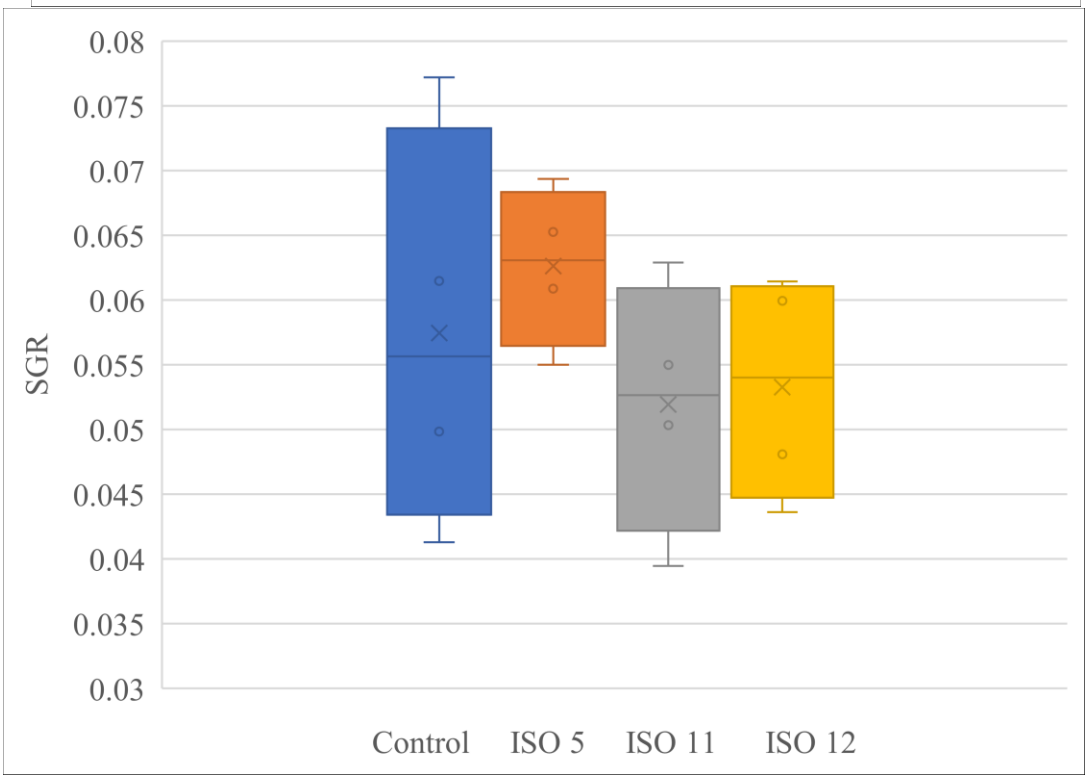
Where *Wf* is the final body weight of individuals, *Wi* is the initial body weight of individuals and days is the time, measured in days, between *Wf* and *Wi*.

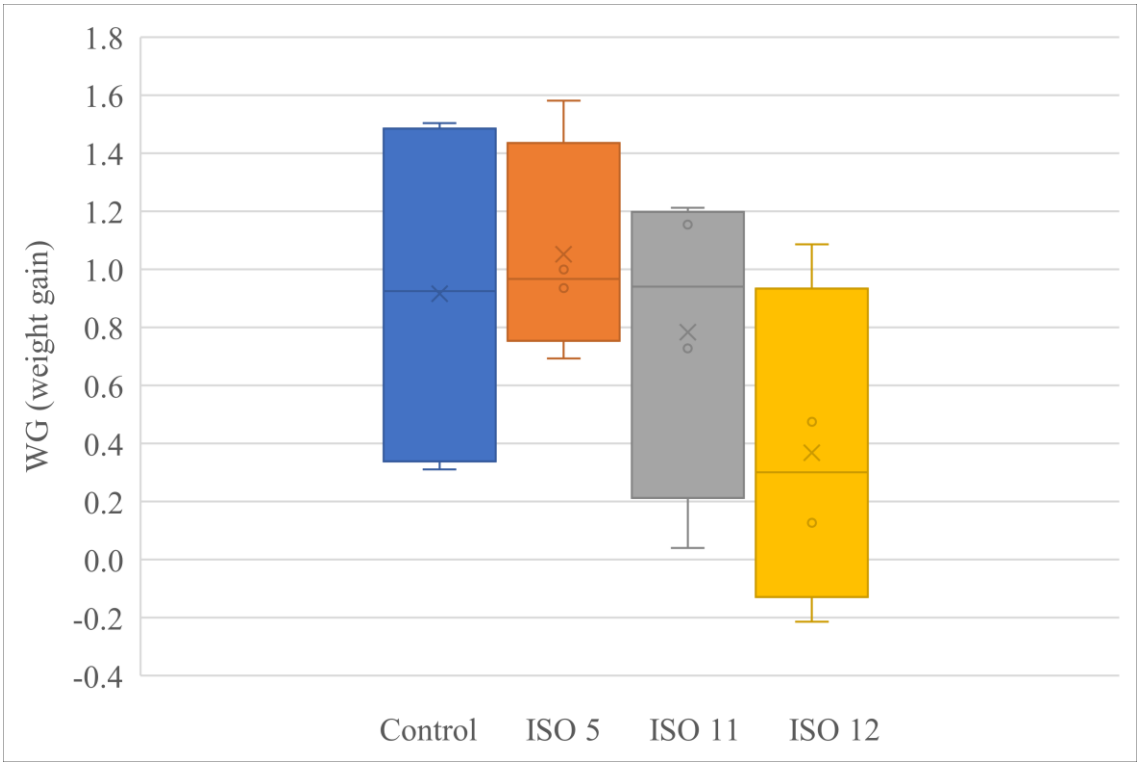
FCR = Fi/(Wf-Wi)

Where *Fi* is feed intake over course of study, *Wf* is final weight of individuals and *Wi* is initial weight of individuals.

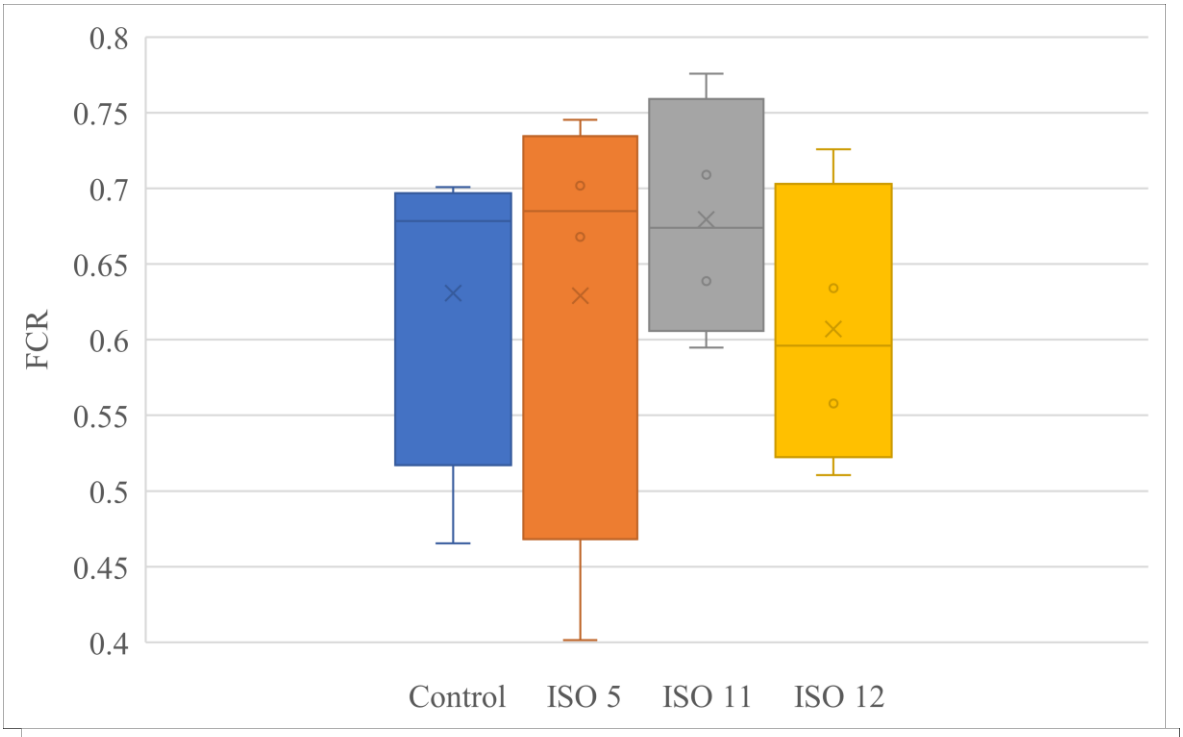
Appendix B. Box plot of Nile tilapia for feed conversion ratios, specific growth rates and weight gain, boxplot was created in SPSS and used to determine that there were no outliers before continuing with a one-way ANOVA in SPSS.

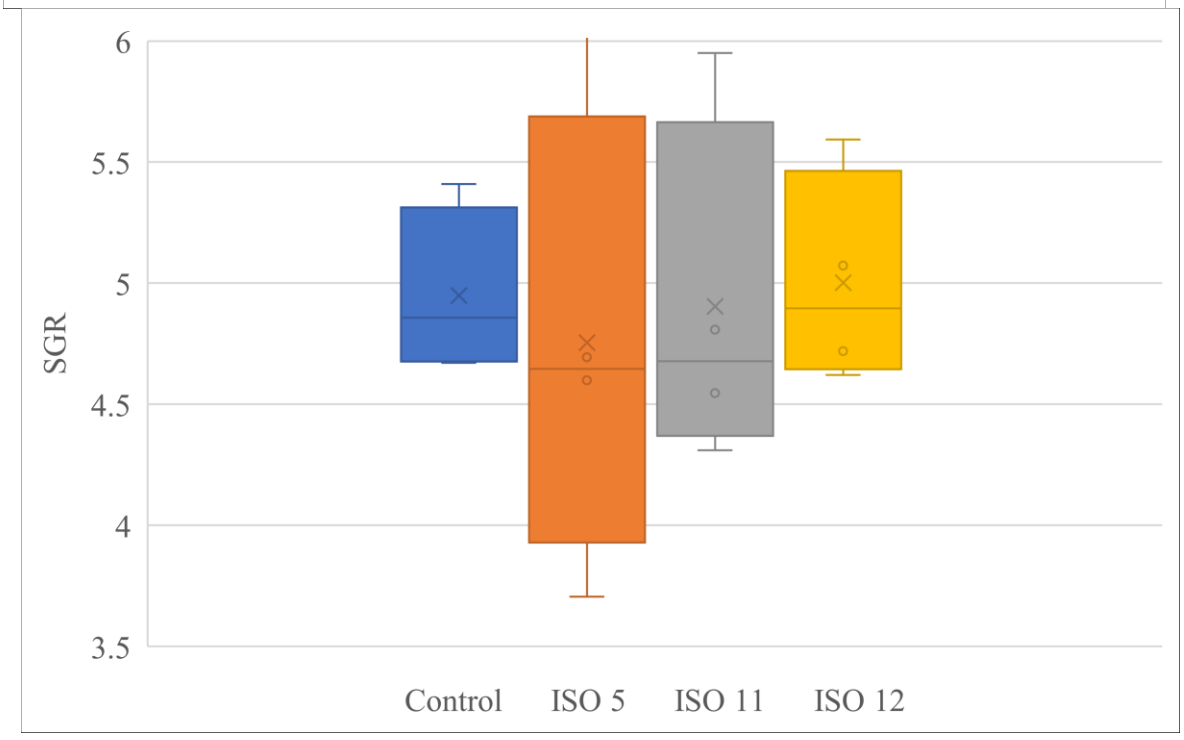


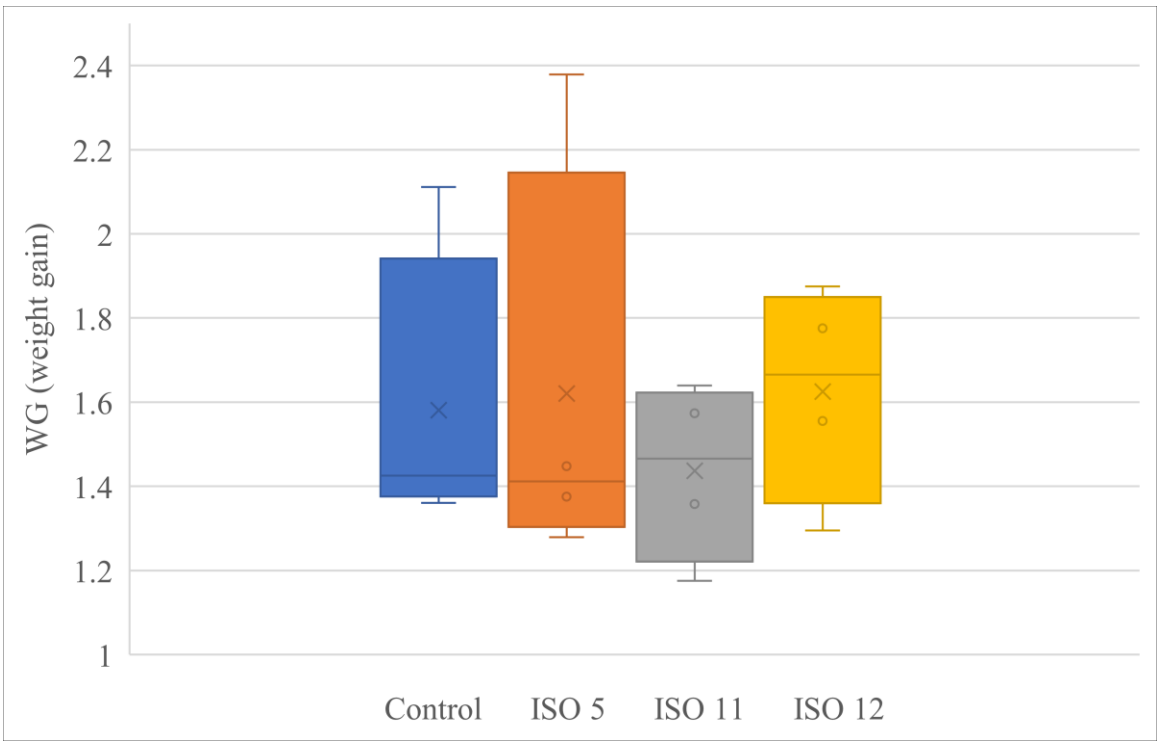



1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
WG (weight gain)
Control
ISO 5
ISO 11
ISO 12

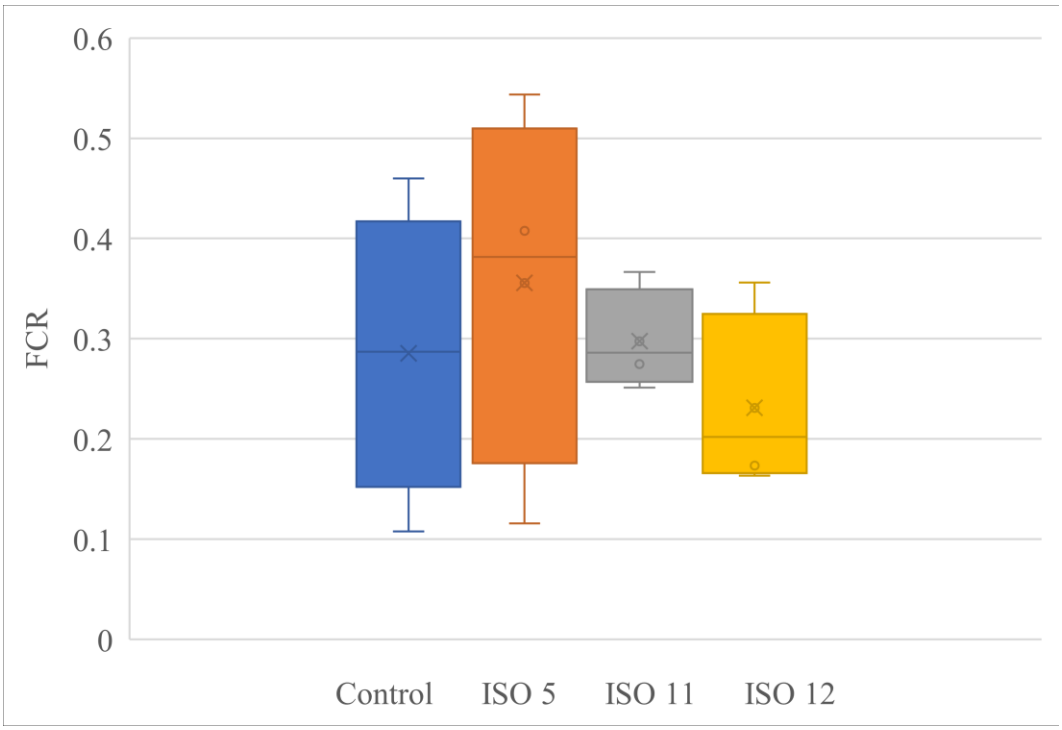
Appendix C. Box plots for feed conversion ratios, specific growth rates and weight gain of rainbow trout. Boxplots were created in SPSS and used to determine that there were no outliers before continuing with a one-way ANOVA in SPSS.

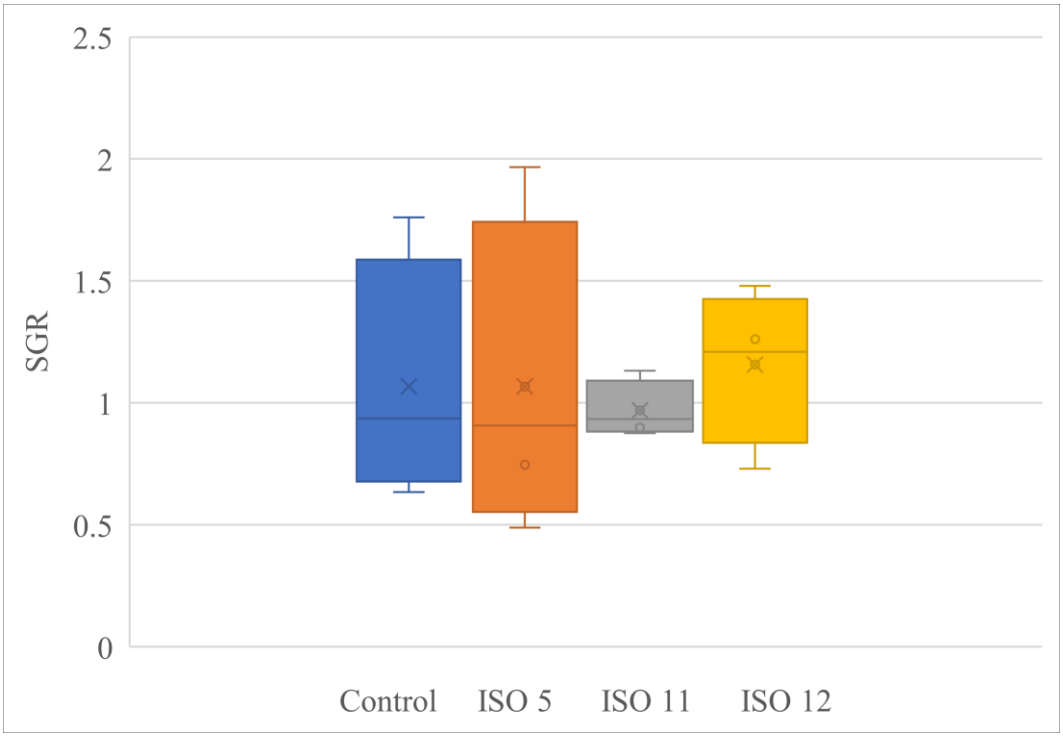


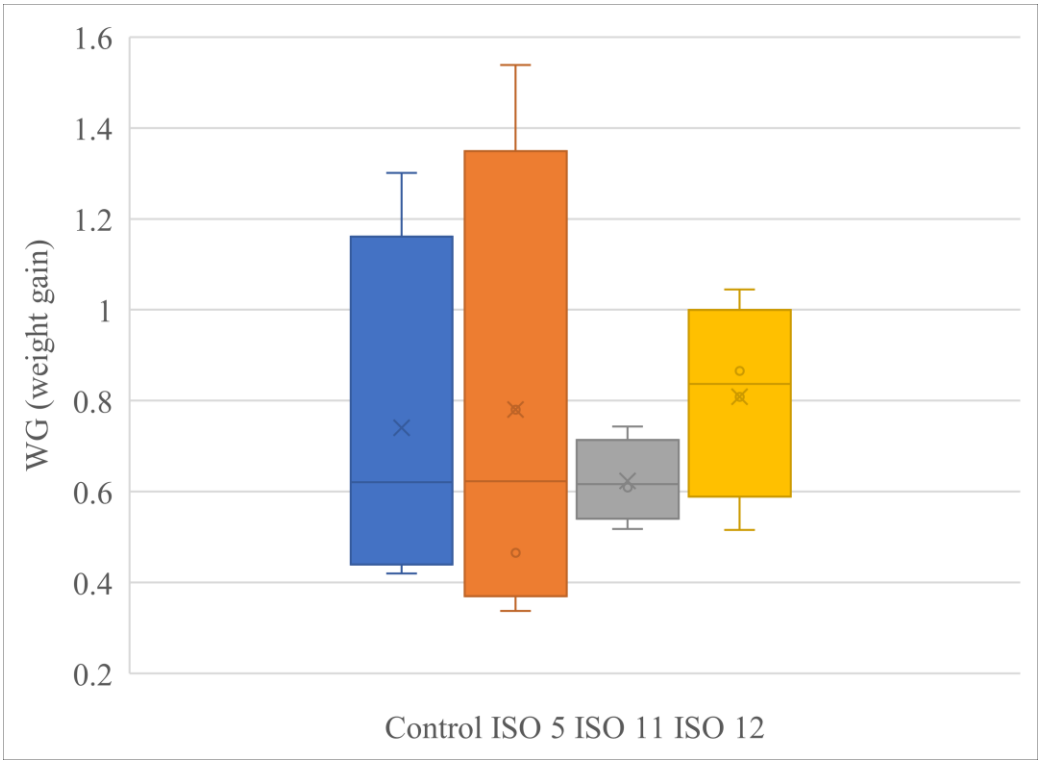



2.4
2.2
2
1.8
1.6
1.4
1.2
1
WG (weight gain)
Control
ISO 5
ISO 11
ISO 12

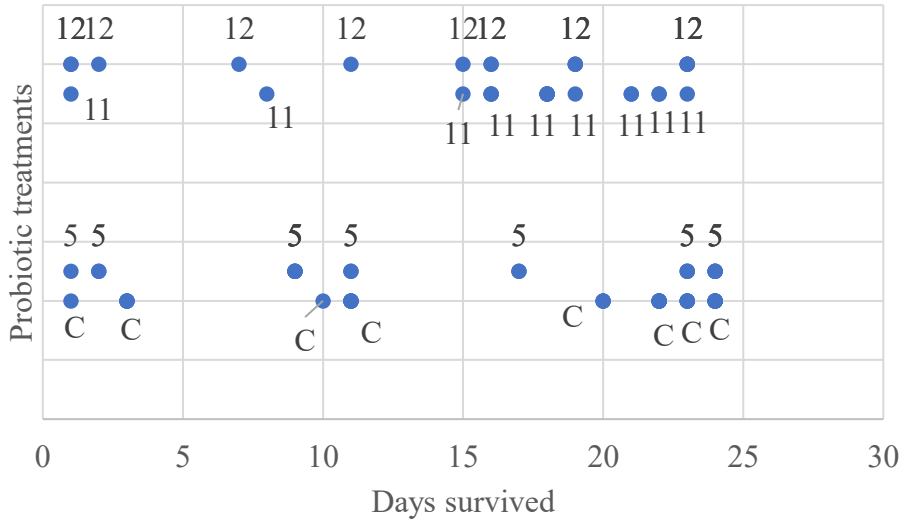
Appendix D. Box plot of hybrid striped bass for feed conversion ratio, specific growth rate, and weight gain, boxplot was created in SPSS and used to determine that there were no outliers before continuing with a one-way ANOVA in SPSS



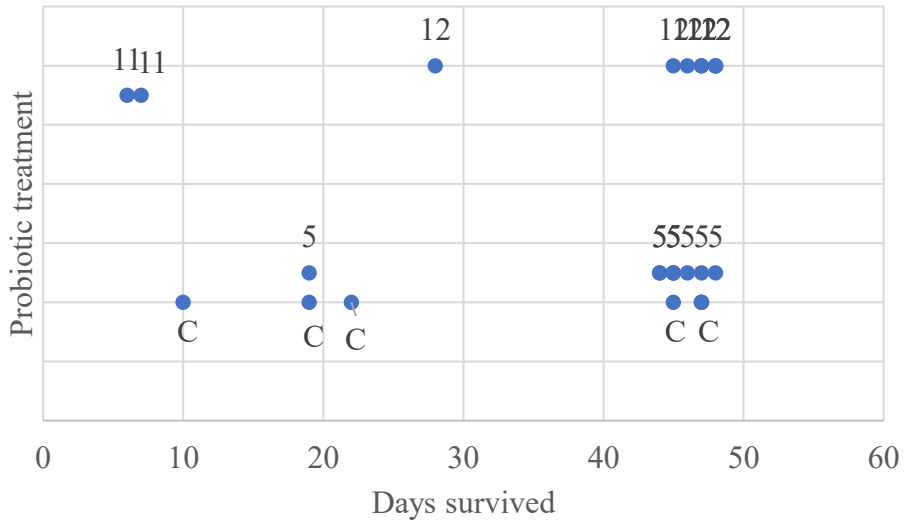




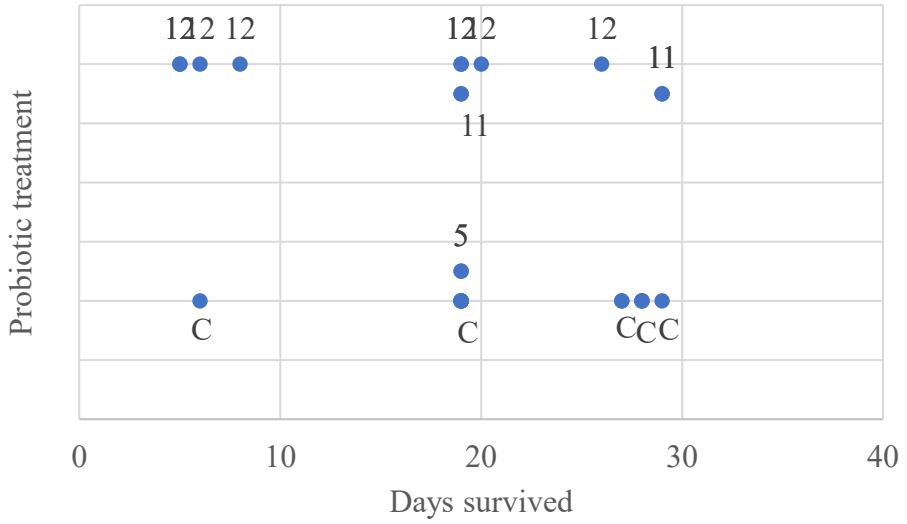
Appendix E. Nile tilapia survival graph for spread in patterns for censoring data produced in SPSS for a Kaplain-Meier statistical assessment. C is the control, each of the probiotics are labeled by their number.



Appendix F. rainbow trout survival graph for spread in patterns for censoring data produced in SPSS for a Kaplain-Meier statistical assessment. C is the control, each of the probiotics are labeled by their number.



Appendix G. hybrid striped bass survival graph for spread in patterns for censoring data produced in SPSS for a Kaplain-Meier statistical assessment. C is the control, each of the probiotics are labeled by their number.



Appendix H. Mean ammonia concentrations over time for rainbow trout trial, as assessed by a repeated measure and post hoc pairwise comparisons in ANOVA in SPSS.

(I) Time period		Mean Difference (I-J)	Std. Error	Sig. [b]	95% Confidence Interval for Difference [b] Lower Bound	Upper Bound
1	2	-0.755	0.194	0.119	-1.605	0.095
	3	-0.886	0.246	0.202	-1.964	0.191
	4	-2.849*	0.513	0.007	-5.091	-0.606
	5	-4.749*	0.268	0.000	-5.920	-3.577
	6	-6.105*	0.535	0.000	-8.444	-3.766
	7	-5.643	1.345	0.068	-11.524	0.239
	8	-5.011*	0.563	0.000	-7.473	-2.549
	9	-4.393*	0.797	0.007	-7.879	-0.906
	10	-7.492*	0.379	0.000	-9.148	-5.837
	11	-7.905*	0.492	0.000	-10.056	-5.754
2	1	0.755	0.194	0.119	-0.095	1.605
	3	-0.131	0.344	1.000	-1.635	1.373
	4	-2.094	0.512	0.083	-4.332	0.145
	5	-3.994*	0.269	0.000	-5.169	-2.819
	6	-5.350*	0.526	0.000	-7.649	-3.051
	7	-4.888	1.343	0.187	-10.761	0.986
	8	-4.256*	0.559	0.000	-6.702	-1.810
	9	-3.637*	0.821	0.045	-7.227	-0.048
	10	-6.737*	0.336	0.000	-8.207	-5.268
	11	-7.150*	0.517	0.000	-9.409	-4.891
3	1	0.886	0.246	0.202	-0.191	1.964
	2	0.131	0.344	1.000	-1.373	1.635
	4	-1.963	0.574	0.280	-4.473	0.548
	5	-3.863*	0.373	0.000	-5.492	-2.233
	6	-5.219*	0.623	0.000	-7.940	-2.497
	7	-4.756	1.433	0.336	-11.021	1.508
	8	-4.125*	0.603	0.001	-6.761	-1.489
	9	-3.506*	0.798	0.048	-6.994	-0.018
	10	-6.606*	0.462	0.000	-8.625	-4.587
	11	-7.019*	0.380	0.000	-8.679	-5.359
4	1	2.849*	0.513	0.007	0.606	5.091
	2	2.094	0.512	0.083	-0.145	4.332
	3	1.963	0.574	0.280	-0.548	4.473
	5	-1.900*	0.391	0.021	-3.609	-0.191
	6	-3.256*	0.645	0.016	-6.077	-0.435

	7	-2.794	1.779	1.000	-10.570	4.982
	8	-2.163	0.993	1.000	-6.505	2.180
	9	-1.544	0.783	1.000	-4.967	1.879
	10	-4.644*	0.625	0.000	-7.375	-1.913
	11	-5.056*	0.685	0.000	-8.050	-2.062
5	1	4.749*	0.268	0.000	3.577	5.920
	2	3.994*	0.269	0.000	2.819	5.169
	3	3.863*	0.373	0.000	2.233	5.492
	4	1.900*	0.391	0.021	0.191	3.609
	6	-1.356	0.502	1.000	-3.550	0.837
	7	-0.894	1.521	1.000	-7.543	5.755
	8	-0.262	0.737	1.000	-3.485	2.960
	9	0.356	0.755	1.000	-2.947	3.659
	10	-2.744*	0.512	0.009	-4.983	-0.505
	11	-3.156*	0.605	0.012	-5.800	-0.512
6	1	6.105*	0.535	0.000	3.766	8.444
	2	5.350*	0.526	0.000	3.051	7.649
	3	5.219*	0.623	0.000	2.497	7.940
	4	3.256*	0.645	0.016	0.435	6.077
	5	1.356	0.502	1.000	-0.837	3.550
	7	0.463	1.455	1.000	-5.897	6.822
	8	1.094	0.773	1.000	-2.288	4.475
	9	1.713	1.090	1.000	-3.053	6.478
	10	-1.388	0.738	1.000	-4.615	1.840
	11	-1.800	0.741	1.000	-5.038	1.438
7	1	5.643	1.345	0.068	-0.239	11.524
	2	4.888	1.343	0.187	-0.986	10.761
	3	4.756	1.433	0.336	-1.508	11.021
	4	2.794	1.779	1.000	-4.982	10.570
	5	0.894	1.521	1.000	-5.755	7.543
	6	-0.463	1.455	1.000	-6.822	5.897
	8	0.631	0.959	1.000	-3.559	4.822
	9	1.250	1.881	1.000	-6.975	9.475
	10	-1.850	1.252	1.000	-7.323	3.623
	11	-2.263	1.404	1.000	-8.400	3.875
8	1	5.011*	0.563	0.000	2.549	7.473
	2	4.256*	0.559	0.000	1.810	6.702
	3	4.125*	0.603	0.001	1.489	6.761
	4	2.163	0.993	1.000	-2.180	6.505
	5	0.262	0.737	1.000	-2.960	3.485
	6	-1.094	0.773	1.000	-4.475	2.288
	7	-0.631	0.959	1.000	-4.822	3.559
	9	0.619	1.146	1.000	-4.393	5.630

	10	-2.481	0.582	0.061	-5.026	0.063
	11	-2.894	0.674	0.058	-5.842	0.055
9	1	4.393*	0.797	0.007	0.906	7.879
	2	3.637*	0.821	0.045	0.048	7.227
	3	3.506*	0.798	0.048	0.018	6.994
	4	1.544	0.783	1.000	-1.879	4.967
	5	-0.356	0.755	1.000	-3.659	2.947
	6	-1.713	1.090	1.000	-6.478	3.053
	7	-1.250	1.881	1.000	-9.475	6.975
	8	-0.619	1.146	1.000	-5.630	4.393
	10	-3.100	0.898	0.262	-7.024	0.824
	11	-3.513	0.982	0.210	-7.807	0.782
10	1	7.492*	0.379	0.000	5.837	9.148
	2	6.737*	0.336	0.000	5.268	8.207
	3	6.606*	0.462	0.000	4.587	8.625
	4	4.644*	0.625	0.000	1.913	7.375
	5	2.744*	0.512	0.009	0.505	4.983
	6	1.388	0.738	1.000	-1.840	4.615
	7	1.850	1.252	1.000	-3.623	7.323
	8	2.481	0.582	0.061	-0.063	5.026
	9	3.100	0.898	0.262	-0.824	7.024
	11	-0.413	0.427	1.000	-2.280	1.455
11	1	7.905*	0.492	0.000	5.754	10.056
	2	7.150*	0.517	0.000	4.891	9.409
	3	7.019*	0.380	0.000	5.359	8.679
	4	5.056*	0.685	0.000	2.062	8.050
	5	3.156*	0.605	0.012	0.512	5.800
	6	1.800	0.741	1.000	-1.438	5.038
	7	2.263	1.404	1.000	-3.875	8.400
	8	2.894	0.674	0.058	-0.055	5.842
	9	3.513	0.982	0.210	-0.782	7.807
	10	0.413	0.427	1.000	-1.455	2.280

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Appendix I. Meant nitrite concentration for rainbow trout trial, as assessed by a repeated measure and post hoc pairwise comparisons in ANOVA in SPSS.

(I) Timeperiod		Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
1	2	0.002	0.001	1.000	-0.003	0.007
	3	0.000	0.002	1.000	-0.007	0.007
	4	0.001	0.002	1.000	-0.008	0.010
	5	-0.002	0.001	1.000	-0.008	0.004
	6	-0.009	0.002	0.199	-0.020	0.002
	7	-.017*	0.004	0.036	-0.032	-0.001
	8	-0.099	0.035	0.842	-0.251	0.054
	9	-.222*	0.042	0.012	-0.407	-0.036
	10	-.576*	0.097	0.004	-1.001	-0.151
	11	-.793*	0.106	0.000	-1.255	-0.331
2	1	-0.002	0.001	1.000	-0.007	0.003
	3	-0.002	0.001	1.000	-0.007	0.004
	4	-0.001	0.002	1.000	-0.010	0.007
	5	-0.004	0.001	0.586	-0.010	0.002
	6	-.011*	0.002	0.007	-0.020	-0.002
	7	-.019*	0.004	0.019	-0.035	-0.002
	8	-0.101	0.034	0.695	-0.251	0.050
	9	-.224*	0.043	0.011	-0.410	-0.038
	10	-.579*	0.098	0.004	-1.006	-0.151
	11	-.795*	0.105	0.000	-1.255	-0.335
3	1	0.000	0.002	1.000	-0.007	0.007
	2	0.002	0.001	1.000	-0.004	0.007
	4	0.001	0.002	1.000	-0.009	0.010
	5	-0.002	0.001	1.000	-0.008	0.004
	6	-.009*	0.002	0.024	-0.018	-0.001
	7	-.017*	0.003	0.008	-0.030	-0.003
	8	-0.099	0.034	0.735	-0.248	0.050
	9	-.222*	0.042	0.011	-0.406	-0.038
	10	-.577*	0.097	0.004	-1.003	-0.151
	11	-.793*	0.106	0.000	-1.255	-0.332
4	1	-0.001	0.002	1.000	-0.010	0.008

	2	0.001	0.002	1.000	-0.007	0.010
	3	-0.001	0.002	1.000	-0.010	0.009
	5	-0.003	0.002	1.000	-0.012	0.006
	6	-0.010	0.003	0.317	-0.023	0.003
	7	-0.017	0.004	0.117	-0.037	0.002
	8	-0.100	0.035	0.854	-0.254	0.055
	9	-.222*	0.043	0.014	-0.412	-0.033
	10	-.577*	0.098	0.004	-1.004	-0.150
	11	-.794*	0.106	0.000	-1.255	-0.332
5	1	0.002	0.001	1.000	-0.004	0.008
	2	0.004	0.001	0.586	-0.002	0.010
	3	0.002	0.001	1.000	-0.004	0.008
	4	0.003	0.002	1.000	-0.006	0.012
	6	-0.007	0.002	0.593	-0.017	0.003
	7	-.015*	0.003	0.041	-0.029	0.000
	8	-0.097	0.035	0.878	-0.248	0.054
	9	-.220*	0.042	0.012	-0.403	-0.036
	10	-.575*	0.097	0.004	-0.998	-0.151
	11	-.791*	0.106	0.000	-1.253	-0.329
6	1	0.009	0.002	0.199	-0.002	0.020
	2	.011*	0.002	0.007	0.002	0.020
	3	.009*	0.002	0.024	0.001	0.018
	4	0.010	0.003	0.317	-0.003	0.023
	5	0.007	0.002	0.593	-0.003	0.017
	7	-0.008	0.005	1.000	-0.027	0.012
	8	-0.090	0.034	1.000	-0.238	0.059
	9	-.213*	0.041	0.013	-0.393	-0.032
	10	-.568*	0.097	0.004	-0.993	-0.142
	11	-.784*	0.105	0.000	-1.242	-0.326
7	1	.017*	0.004	0.036	0.001	0.032
	2	.019*	0.004	0.019	0.002	0.035
	3	.017*	0.003	0.008	0.003	0.030
	4	0.017	0.004	0.117	-0.002	0.037
	5	.015*	0.003	0.041	0.000	0.029
	6	0.008	0.005	1.000	-0.012	0.027
	8	-0.082	0.034	1.000	-0.229	0.065
	9	-.205*	0.041	0.018	-0.386	-0.024
	10	-.560*	0.096	0.005	-0.981	-0.139
	11	-.776*	0.108	0.001	-1.248	-0.305
8	1	0.099	0.035	0.842	-0.054	0.251
	2	0.101	0.034	0.695	-0.050	0.251
	3	0.099	0.034	0.735	-0.050	0.248
	4	0.100	0.035	0.854	-0.055	0.254

	5	0.097	0.035	0.878	-0.054	0.248
	6	0.090	0.034	1.000	-0.059	0.238
	7	0.082	0.034	1.000	-0.065	0.229
	9	-0.123	0.042	0.713	-0.307	0.061
	10	-.478*	0.105	0.037	-0.937	-0.019
	11	-.694*	0.112	0.003	-1.186	-0.203
9	1	.222*	0.042	0.012	0.036	0.407
	2	.224*	0.043	0.011	0.038	0.410
	3	.222*	0.042	0.011	0.038	0.406
	4	.222*	0.043	0.014	0.033	0.412
	5	.220*	0.042	0.012	0.036	0.403
	6	.213*	0.041	0.013	0.032	0.393
	7	.205*	0.041	0.018	0.024	0.386
	8	0.123	0.042	0.713	-0.061	0.307
	10	-0.355	0.086	0.077	-0.731	0.021
	11	-.571*	0.116	0.019	-1.077	-0.066
10	1	.576*	0.097	0.004	0.151	1.001
	2	.579*	0.098	0.004	0.151	1.006
	3	.577*	0.097	0.004	0.151	1.003
	4	.577*	0.098	0.004	0.150	1.004
	5	.575*	0.097	0.004	0.151	0.998
	6	.568*	0.097	0.004	0.142	0.993
	7	.560*	0.096	0.005	0.139	0.981
	8	.478*	0.105	0.037	0.019	0.937
	9	0.355	0.086	0.077	-0.021	0.731
	11	-0.217	0.162	1.000	-0.923	0.490
11	1	.793*	0.106	0.000	0.331	1.255
	2	.795*	0.105	0.000	0.335	1.255
	3	.793*	0.106	0.000	0.332	1.255
	4	.794*	0.106	0.000	0.332	1.255
	5	.791*	0.106	0.000	0.329	1.253
	6	.784*	0.105	0.000	0.326	1.242
	7	.776*	0.108	0.001	0.305	1.248
	8	.694*	0.112	0.003	0.203	1.186
	9	.571*	0.116	0.019	0.066	1.077
	10	0.217	0.162	1.000	-0.490	0.923

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.