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Development of Standard Operational Procedures for Bacterial Management in Marine Fish Hatcheries

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UNIVERSITY OF MIAMI

DEVELOPMENT OF STANDARD OPERATIONAL PROCEDURES FOR BACTERIAL MANAGEMENT IN MARINE FISH HATCHERIES

By

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Development of Standard Operational Procedures for Bacterial Management in Marine Fish Hatcheries

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Live feeds are utilized in marine fish hatcheries to feed and promote the health of finfish larvae due to their nutritional advantages. The presence of detrimental bacteria in rotifer culture can cause disease outbreaks in larval rearing. Nevertheless, the use of UV application to disinfect seawater is not very effective to eliminate and inactivate all pathogenic agents presented in the raw surface water. To investigate new methods of disinfection, two experiments were conducted at the University of Miami Experimental Hatchery (UMEH) to quantify and test for antibiotic susceptibility for Vibrio spp. and the total coliforms by using plate counting method and treating with two water-soluble antibiotics, Tobramycin and Minocycline, at concentrations of 30 μg/mL, 100 μg/mL and 200 μg/mL. In the first experiment, water samples from Virginia Key Bear Cut, Florida were collected from five locations beginning from surface water, settling tank, sand filter, after a 120 watts UV instrument and after a 80 watts UV instrument respectively. No fecal coliform colonies were observed by plate counting method after UV disinfections, implying a complete inactivation by UV irradiation, but *V. vulnificus*, *V. parahaemolyticus* and

V. alginolyticus were observed in all samples. In the second experiment, the isolation of several pure strains of bacteria was performed to test the susceptibility to these antibiotics on Mueller Hinton Agar (MHA). After measuring the zone of inhibition, all bacteria strains isolated after the 80 watts UV instrument were highly sensitive to the antibiotics, but when antibiotics were applied to bacteria in UV treated water, resistance to all dosages of antibiotics was observed indicating the presence of multi-antibiotic resistance (MAR) bacteria in the water. The transmission of multi-antibiotic resistance bacteria through the food chain may cause serious problems to consumers and different treatments and technologies are needed, such as ozone and ultrasound, to completely disinfect incoming and discharge water to generate pathogen free water.

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Chapter 1

Introduction

Aquaculture utilizes large amounts of water and requires that water should be of good quality. The biological and physiochemical characteristics that play an important role in the water quality provide suitable conditions for the growth of fish and other biota. These water bodies are often contaminated by bacteria from human and animal wastes. Harmful bacteria may find their way into surface water bodies through rainwater run-off, or other persistent ways including leaking septic tanks, untreated run-off from the agricultural fields and unwanted discharge from pipes.

Wastes from animals make up the largest source of bacteria found in water surfaces and they include; Staphylococcus, Streptococcus, Clostridia, and Lactobacillus. A gram of human feces is estimated to carry close to twelve billion bacteria including harmful bacteria. Moreover, feces may have pathogenic parasites, viruses, and protozoa. The quantity of pathogenic microbes in surface water is difficult to identify and isolate, and usually vary according to their type or characteristic (Perkins et al., 2009).

It is recommended that surface water should be tested regularly for *E. coli* and other microbes that can cause harm to human health. However, some of the microbes found in water surfaces metabolize organic waste materials while others metabolize dissolved wastes including ammonia, nitrates, nitrites and other organic compounds (Kaeberlein et al., 2002).

1.1 Importance of water quality in aquaculture

Water quality is an important aspect necessary for the growth of marine organisms. Water quality refers to the quality that provides for successful propagation of organisms that thrive in water. Water is a matrix which holds minerals (inorganic substances), dissolved gases, and organic matter. Water also supports the life of microorganisms, animals, and plants and acts as a medium of exchange of chemicals among these living organisms (MADEP, 2007).

Good water quality is signified by presence of a sufficient amount of oxygen and limited amounts of metabolites. Microorganisms including bacteria and algae are responsible for producing these metabolites. Food found in water provides the primary nutrient source for the microbes. Levels of microorganisms can increase in aquaculture systems exponentially. Exponential increase takes place during the second half of the culture period due to availability of nutrients (Sugita et al., 2005). During the third quarter, approximately 30% of feed consumed is available in water and during the last quarter, 50% of the feed is loaded. The population of microorganisms increases exponentially until growth factors become limiting. This leads to a sudden decrease in population also termed as collapse, or die-off. The rapid increase and decrease in population of microorganisms can cause major changes in parameters of water quality which in turn affect growth.

1.2 Types of bacteria in seawater

Bacteria found in surface water can be classified into two major groups: heterotrophic bacteria which include bacteria utilizing organic compounds including

peptides, amino acids, lipids or carbohydrates for their metabolic functions and chemoautotrophic bacteria such as those that derive their source of carbon from carbon dioxide and energy through oxidation of inorganic compounds such as nitrogen, iron and sulphur (Sharrer et al., 2005). The autotrophic bacteria, such as nitrifying bacteria, remove ammonia effectively to ensure that water quality is maintained at levels that minimize exposure to the aquatic organisms. The nitrifying bacteria oxidize ammonia to nitrate and nitrites using two bacteria called Nitrobacter and Nitrosomonas. Nitrifying bacteria have a long generation time because 80% of their energy is used in fixation of carbon dioxide while the rest is utilized for growth.

Heterotrophic bacteria, on the other hand, play major roles in consumption of oxygen, releasing metabolic products after cellular lyses, causing disease in aquatic organisms such as fish, and rivalry for space, oxygen and nutrients with autotrophic bacteria. Heterotrophic bacteria include denitrifiers such as *Pseudomonas, Paracoccus, Rhodobacter and Ralstonia*. Surface water is capable of supporting growth of many heterotrophic microorganisms such as opportunistic and pathogenic bacteria (Hill et al., 2003).

1.3 *E. coli* as an indicator

One way of assessing the quality of water is through carrying out regular examinations for fecal indicator microbes. Coliforms are used as indicators of microbial quality of water due to the assumption that coliforms occur in high numbers in human and animal feces and that the bacteria will likely be present in water if the feces have entered even after numerous dilutions. The fecal indicator bacteria should

be present in high quantities in feces of humans and animals, should be easily detectable by simple techniques, and should be capable of growing in natural water. *E. coli* is the indicator of choice for carrying out microbiological examination (Bissett et al., 2006).

Escherichia coli belong to the family Enterobacteriaceae and possess the enzymes β-galactosidase and β-glucuronidase. *E. coli* grows at temperatures of approximately 45°C on complex media. It is involved in fermentation of lactose and mannitol to produce acid, gas, and indole from tryptophan. There is an abundance of *E. coli* in human and animal feces, reaching concentrations of 10^9 per gram. *E. coli* can be traced in natural waters and soil subjected to fecal contamination, sewage and treated effluents. The presence of *E. coli* in water and food is not necessarily a direct indicator of the presence of pathogenic microbes in specimen but it indicates the heightened risk of the availability of other microbes found in feces such as bacteria and viruses including *Salmonella pp.* and Hepatitis A virus, many of which are considered pathogenic (Garzio-Hadzick et al., 2010).

E. coli is more specific compared to fecal coliforms because the test carried out for fecal coliforms is capable for identifying even the thermo-tolerant non-fecal coliform bacteria. *E. coli* test gives confirmation of presumptive fecal coliforms through carrying out test for absence enzymes selective for *E. coli.*

1.4 Pathogenic agents in water

Aquaculture is a fast-rising, food-generation industry worldwide but the area is greatly affected by diseases that result in major economic losses. The major

pathogens affecting the aquaculture industry include bacteria, viruses, fungi, and other parasites (Cole et al., 2009).

Bacteria are capable of surviving in the aquatic environment independent of the hosts and as such are a major hindrance to the growth of aquaculture. Bacteria have been shown to be pathogenic to aquatic organisms and some of the well-known genera include: gram-negative bacteria such as *Pseudomonas, Vibrio, Aeromonas, Edwardsiella, Photobacterium, Flavobacterium, Yersinia, and Francisella* and gram-positive bacteria including *Streptococcus, Lactococcus, and Renibacterium* (Reid et., 2009).

Fish cultured in fresh water and marine fish include catfish, eels, trout, tilapia, salmon, sturgeon, carp, perch, and bass are affected by bacterial diseases.

1.4.1 *Vibrio spp.*

Vibrio species are the most prevalent agents that cause bacterial infections in aquaculture organisms. The most deleterious and prominent pathogens of crustaceans, marine fish and mollusks are *Vibrio vulnificus, Vibrio parahaemolyticus,* and *Vibrio anguillarum*. They are capable of persisting in nutrient free water for over a year and can multiply a thousand times in sea water as a result of waste discharge rich in carbohydrates. *Vibrio harveyi* have a great impact on crustaceans and majorly affect the shrimps. Other species of Vibrio include *V. pectenicida* and *V. alginolyticus* which infect the bivalve larvae (Reid et al., 2009).

Vibrio bacteria affect fingerlings, grouper fry, broodstock, juveniles and adults. Vibrio is a serious pathogen causing severe hemorrhages and necrosis. It

causes hemorrhaging of the gastrointestinal tract, gills and other internal organs. The affected fish may lose balance and show abnormal behavior when swimming.

1.4.2 *Pseudomonas spp.*

Pseudomonas spp. are plentiful in the aquatic environment. They infect fish, shellfish and other microorganisms when exposed to environmental stressors including extreme changes in water temperature, poor water quality, poor nutrition and overcrowding.

Pseudomonas spp. cause pseudomonad hemorrhagic septicemia and majorly affect all the stages of grouper. This pathogen also causes severe hemorrhages of the internal organs of the infected organisms. Other effects include skin ulcerations, corneal opacity and hepatic vein thrombosis and embolism (Rao et al., 2005)

1.4.3 *Flexibacter spp.*

Flexibacter spp. are gram-negative bacteria. They are long, rod shaped cells with parallel sides and have rounded ends. These bacteria do not have flagella and therefore move by gliding. Flexibacter spp. are known to be opportunistic pathogens and they include *Flexibacter columnare* and *Flexibacter maritimus*. Flexibacter spp are causative agents of bacterial gill disease and fin rot (Muroga, 1995).

These bacteria normally attack fingerlings and groupers. They cause anorexia, lethargy and dark colorations among the affected fish. The fish remain close to the surface and their operculum may be flaring. The gills may also produce large amounts of mucus and become yellowish in color which signifies gill rotting.

1.4.4 *Aeromonas spp.*

Aeromonas salmonicida are gram-negative bacterial pathogens that greatly affect the salmonids and other species of fish. This pathogen was first isolated from brown trout in Germany. *A. salmonicida* are causative agents of atypical several diseases among wild and cultivated fish species in both marine and freshwater environments. More than thirty species of fish have been reported to be affected by *A. salmonicida*. There are four well-described sub species of *A. salmonicida* which include: *S.. salmonicida, S. smithia, S. masoucida and S. achromogenes* (Bissett et al., 2006).

A. salmonicida is a causative agent of furunculosis, a disease causing hemorrhages, septicemia, inflammation of lower intestines, enlargement of spleen and even death among populations of fish including goldfish, salmonids, turbot, cod, eel and halibut in aquatic environments. *A. salmonicida* affects fish populations due to poor water quality. Other causative factors include high temperatures, overcrowding and trauma.

1.4.5 *Streptococcus spp.*

Streptococcus are gram-positive bacteria that infect a wide variety of fish including tilapia, salmon, golden shiner, mullet, sea trout, pinfish, sturgeon, striped bass and eel. Streptococcal diseases affecting fish are not common, but when they occur, they result in major losses. The first incidence of streptococcal disease was reported to have affected rainbow trout in 1957 in Japan. The closely related bacteria

responsible for causing similar diseases include *Lactococcus, Vagococcus* and *Enterococcus* (Muroga, 1995).

Streptococcal infections result in high mortality rates among fish within three to seven days. They may also cause abnormal swimming behavior characterized by spinning or spiraling. Other clinical signs include hemorrhages, corneal opacity, lethargy, ulcerations, darkening and loss of buoyancy.

Streptococcal infections mainly occur due to poor water quality and can thus be prevented by ensuring good quality in water and provision of proper nutrition.

A summary of the bacterial pathogens in aquaculture is shown in table 1.

Table 1: Major pathogens in aquaculture

Pathogen	**Host**	**Disease**
Vibrio spp. e.g. *V. vulnificus, V. parahaemoltticus*	Marine fish, invertebrates	Vibriosis: hemorrhages, skin ulcers, septicemia,
Pseudomonas spp.	Fresh water and marine fish	Hemorrhages, skin ulcers, corneal opacity and thrombosis
Flexibacter e.g. *F. columnare*, *F. maritimus*	Marine fish	Tenacibaculosis: anorexia, lethargy, dark colorations

Aeromonas spp. e.g. *A. hydrophila* and *A. salmonicida,*	Fresh water and marine fish esp. salmons	Furunculosis, and atypical furunculosis
Edwardsiella tarda	Fresh water, marine fish, invertebrates	Edwardsiellosis: hemorrhages, organomegally, exophthalmia
Francisella spp. e.g. *F. noatunensis* and *F. piscicida,*	Fresh water, sea fish e.g. salmon, tilapia, mollusks	Granulomatous inflammation, septicemia
Streptococcus iniae	Fresh water and marine fish	Streptococcosis: hemorrhages, septicemia, skin lesions
Mycobacterium species. i.e. *M. marinum*	Marine fish	Damage of tissue by mycolactone generation

1.5 UV irradiation and its inactivation of bacteria

The most important section of light spectrum in bacterial management is the wavelengths between 100-400 nm in which Ultra Violet light lies (Bassiri, 2012). UV spectrum is subdivided into UV-A or long-wave UV which is black light with a wavelength of between 320 to 400 nm with truncated energy levels with low penetrability. The second one is UV-B that falls between 280 to 320 nm wavelength and under moderate penetration power that makes them potent mutagens. The third one is UV-C which has wavelength of between 100 to 280 nm, thus making them very penetrative with strong mutagenic impact on biological structures (Bassiri, 2012). When UV light strikes a bacterium, rays are absorbed by DNA molecules thus

leading to alterations which may be damaging to cell survival. The inactivation of bacteria occurs when the bacteria cells absorb the UV short wavelength rays which goes on to intermingle with water molecules in the cell to generate free (-OH) radicals (Bassiri, 2012). Given that the (-OH) free radicals do not have some electrons, they attack the other molecules like cell proteins and DNA by stealing the electrons. The stolen electrons results in the covalent fusion of the two thymine bases and thus creating a thymine dimer. The thymine dimer goes on to cause alterations in the profile of the DNA such that when there is another DNA replication, there is an irregular integration of the base at the thymine dimer location (Bassiri, 2012). This then results in a mutation location such that when a protein is involved it becomes a lethal outcome for the bacteria.

Several experimental studies have shown the impact of UV in the killing of waterborne bacteria. When it comes to UV destruction of waste water and aquaculture system bacteria, a low pressure mercury-lamp is used to create UV short-wavelength Angstroms that are fatal to micro- organisms (Spottel & Gary, 1981).

1.6 Antibiotics that affect bacteria

Antibiotics inhibit bacteria in two major ways. Some antibiotics exhibit bactericidal activity, which makes them capable of killing the bacteria. Others are bacteriostatic and act by inhibiting bacterial growth. The activity of bacteriostatic antibiotics is reversible. Antibiotics can be categorized as broad-spectrum antibiotics or narrow-spectrum antibiotics. The antibiotics that have broad-spectrum activity are effective against a large number of bacteria whereas antibiotics that possess narrow spectrum activity act against a limited number of bacterial species. The activity

spectrum of an antibiotic is characterized by its action against bacteria. Penicillin, for example, is bactericidal acting by hindering the synthesis of the cell wall. It has a narrow spectrum of activity because it acts against gram-positive bacteria only. On the other hand, tetracyclines act by inhibiting protein synthesis. Tetracyclines are broad spectrum antibiotics and they hinder development of gram-positive and gram - negative bacteria (Tamaki et al., 2005). In this study, two antibiotics, Minocycline and Tobramycin, were used to test their effects on coliforms and vibrio spp. Minocycline is a broad-spectrum antibiotic that represents the next generation of tetracycline group, and has bacteriostatic effect on bacteria cell wall. The antibiotic mixes well with water due to solubility, which allow the bioactive components to inhibit protein synthesis of all bacteria either gram-negative or gram-positive. Tobramycin is a bactericidal antibiotic derived from *Streptomyces tenebrarius* and belongs to aminoglycoside family. The antibiotic is water-soluble and it kills organisms by blocking bacterial protein synthesis.

Table 2: Activity spectrum of commonly used antibiotics

Drug	**Primary effect**	**Drug spectrum**
Chloramphenicol	Bacteriostatic	Broad (gram +/-; Rickettsia, Chlamydia)
Erythromycin	bacteriostatic	Narrow (gram +, mycoplasma)
Penicillin	bactericidal	Narrow (gram +)
Streptomycin	bactericidal	Broad (gram +/-; mycobacteria
Sulfonamides	Bacteriostatic	Broad (gram +/-)
Tetracyclines	Bacteriostatic	Broad (gram +/- rickettsia, Chlamydia)

Some other antibiotics interfere with the synthesis of nucleic acids or metabolites. These include the naladixic acid and sulfonamides.

1.7 Antibiotic resistance and sensitivity

The continued utilization of antimicrobial agents in aquaculture and other forms of farming and controlling human health has led to the formation of resistant strains to these agents. As a result, the resistant bacterial strains can have a negative impact on the management of diseases of fish and other marine organisms. Resistance to antibiotics has been reported in shrimps, eels, shellfish, finfish and aquaculture environments worldwide.

Antibiotic resistance can occur due to a variety of reasons. For instance, the bacteria can alter its cell membrane structure thus preventing drug entry into the cell. There also could be production of enzymes that destroy the drugs. Alternatively, the bacterial cell can counteract the activity of the drug. Emergence of antibiotic resistance has therefore led to need for intensified surveillance concerning use of antibiotics and antibiotics resistance (Kha and Hung, 2007).

Nevertheless, the three main genetic methods through which aquaculture based bacteria acquire resistance is transformation, transduction and conjugation. Under transformation, the recipient cell gains the resistant genes from either free-moving DNA molecules that are in the water in form of dead-cells or from laboratory (Adekunle, 2012). The difference is that in lab plasmid DNA is made possible unlike in the natural environment where only chromosomal DNA is transferred (Adekunle, 2012). The process begins with the extraction of DNA using chemical approach from

the donor bacteria and then integrated into a culture comprising recipient bacteria. Afterwards, the competent cells generate certain proteins which bind on donor DNA fragments such that the recipient bacterium gets at least one inheritable characteristics from the donor bacterium and thus become transformed. However, for a given bacteria to acquire the transformed characteristic, they must be growth receptive and in the late logarithm stage of their growth.

Under transduction, a bacteria acts as a transporter of DNA from the donor bacteria to another recipient bacteria (Adekunle, 2012). This is where the phage comprising nucleic-acid is attached to the phage of the other bacteria using appendages, and when it inserts its DNA into the host-cell there is replication of the phage DNA even as the bacteria DNA is being ruined. Later on, the phage DNA belonging to host-cells takes controls of synthesis of a new phage-protein such that a new phage DNA particles mixes with the phage proteins to create other complete phages that are released when the host-cell crumbles. However, there are fragments from the host bacteria DNA which may be integrated into the head of the new phage rather than the DNA. As a result, the fragments in the new phage affects another bacterium when injected into their DNA fragments, but this time round the transducing phages will not destroy the new host since they do not possess the whole viral DNA. Hence, the fragments experience recombination with new host chromosome and as a result become permanent segment making the subsequent bacterial host to attain at least one of the genes (Adekunle, 2012).

Under conjugation, gene transfer is on a cell to cell interaction (Adekunle, 2012). In this case, the plasmids undergo horizontal transfer whereby the DNA is transferred

directly from one bacterium to the other. However, the conjugation does not entail fusion of two gametes to create a solitary cell, but instead a direct transfer of plasmid from donor bacteria to the recipient. The other way is where huge chunk or entire donor bacteria cell chromosome are relocated to the recipient cell (Labella & et al, 2013).

Objectives:

The aim of this study was to examine the presence of detrimental microorganisms in raw seawater at UMEH, particularly Vibrio spp. and total coliforms, and also test the susceptibility of these microbes to UV treatments and antibiotics. At the UMEH, these bacteria survived two UV monochromatic irradiation instruments (120 watts and 80 watts) and were present in live feeds culture. The survival pathogenic opportunistic bacteria replicate and thrive within rotifer culture environment due to the density and the high level of excess nutrients that give an advantage to these bacteria to dominate the culture. Two water-soluble antibiotics, Tobramycin and Minocycline, were used to eliminate any pathogenic bacteria that may transfer within rotifer in order to feed larvae and potentially cause massive mortalities.

This study aimed to answer the following questions: a) Are antibiotics a good practice to inhibit and control unwanted bacteria to obtain axenic live feeds culture?; b) Will there be any signs of antimicrobial resistance acquired as a result of using

excessive amounts of antibiotics?; and, 3) Does UV disinfect and inactivate Vibrio spp. in incoming raw seawater?

Chapter 2

Material and Methods

In the hatchery, seawater from Virginia Key Bear Cut is drawn through dual intakes by two 900-gpm electric pumps. The water flows into six chambers of a 200,000-L settling tank to remove settable solids and any particulate matter. After gravity separates large suspended particles, the water leaves the settling tank through two 500-gpm pumps into two large sand filters filled with broken glass media (AFM, Advanced Filtering Media) to filter particles down to 20 µm. Solid filtering completes the process of basic mechanical filtration and the water moves through the 120 watts UV filter to eliminate any microorganisms within water body. After water passes the UV irradiation, the water is pumped through pipelines inside the greenhouse where it goes through 4 cartridges for additional filtration to particle sizes of 10 µm, 5 µm, 3 µm and 1 µm, prior to being treated with another 80 watts UV instrument. The disinfected water is supplied to rotifer culture to prevent any pathogenic bacteria from infecting rotifers and transferring to early developmental stages of fish potentially causing severe mortalities.

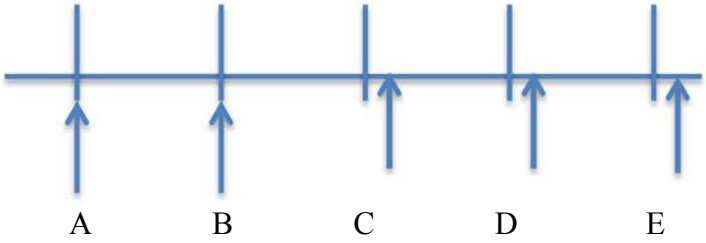
2.1 Seawater samples locations

Five 1-liter samples were taken from five different sites: intake water from Virginia Key Bear Cut, settling tank at National Oceanic Atmospheric Administration (NOAA), University of Miami Experimental Hatchery (UMEH) sand filter, after the 120 watts UV instrument, and after the 80 watts UV instrument. Four sets of samples were taken from each location between (Sep-2016 – Dec-2016), and samples were

transferred to the Aqua Lab to estimate bacterial concentration using membrane filtration method. Bottles were kept for 48 hours in the refrigerator at 4°C until they were used again for further analyses.

Figure 1: Satellite image of University of Miami Experimental Hatchery

Figure 2: Samples locations at UMEH



* Arrows point out the samples location before or after each site, location A represents surface water, location B representing settling tank, location C represents sand filter, location D represents the 120 watts UV instrument, and location E represents the 80 watts UV instrument.

2.2 Filter membrane technique

First, the bench was disinfected using isopropyl 70% and the bottom funnel was connected to the blue cap. WHATMANS 0.45 µm-sterile filter paper circle shape was used. Sterile tweezers were used to remove the white foam paper grid and leave the blue paper in packet. The white foam grid was placed on top of the blue cap. Sampled water was poured on the blue cap and the vacuum was turned on to drain the water as the foam grid works as a filter to collect bacteria load from sampled water. The filter paper containing bacteria was removed and placed on top of the media ECC for coliforms (obtained from Hardy Diagnostics) and covered. Plates were placed upside down in an incubator at 35°C. This protocol was repeated again for Vibrio media (obtained from Hardy Diagnostics).

2.3 Isolate pure bacterial strains

After 24 hours of culturing 1 mL samples of intake water, both media showed a variety of bacterial colonies that covered the entire foam grid. In both media, colonies were isolated based on the color to have a pure culture of every strain. A sterile wire loop was used to take one to two colonies of each strain and these colonies were re-plated again on the same media to have a pure culture.

After 24 hours, pure strains were obtained of the following types of bacteria: *V. parahaemolyticus* (turquoise color on vibrio media), *V. alginolyticus* (colorless on vibrio media), *V. cholera or V. vulnificus* (Purple color on vibrio media), *Klebsiella* or *Enterobacter (*turquoise color on ECC media)*, E. coli* (purple on ECC media) and other gram negative bacteria (white or colorless on ECC media). Information regarding colonies color was obtained from Hardy Diagnostics website.

2.4 Total bacteria count

To determine total bacteria count, sample volume of 1 mL was taken by using a pipette from the settling tank water. Tank water was chosen over intake water simply because the settling tank is the last station to separate fecal coliforms from other total coliforms. Harmful microbes, *E.coli* particularly, if they pass UV treatment could cause huge mortalities to either rotifer culture or larval rearing. Sample volume of 1 mL was used as described above with membrane filtration by using ECC media and incubated at 35°C.

To insure the detection of *E. coli*, another medium that is dry compact ECC (obtained from Hardy Diagnostics) was used to determine the presence of fecal coliform in

settling tank water. Two samples were used: 1 mL of settling tank water and 0.5 mL diluted with 0.5 mL 0.9% Nacl, and were incubated at 35°C for 24 hours.

For vibrio medium, sample volume of 1 mL was incubated for 24 hours.

2.5 Disk diffusion method

Muller-Hinton Agar (obtained from Hardy Diagnostics) was used for susceptibility tests because it gives acceptable growth of malicious pathogenic bacteria. After isolating pure strains of bacteria in the previous media, liquid agar, namely tryptic soy broth TSA (Obtained from Carolina Biological website) was used to obtain bacteria suspensions for each species of vibrio and coliforms. A sterile wire loop was used to choose 3-5 colonies of each bacteria strain to inoculate multiple liquid broths and obtain bacterial suspensions. The suspensions were incubated for 16 hours at 35°C to achieve or exceed the turbidity of 0.5 McFarland standard and adding sterile saline may reduce turbidity. Sterile cotton swabs were dipped into the suspension and any excess inoculum was removed by gently pressing the swab against the tube wall. Wet swabs were streaked on MHA and rubbing was repeated while rotating the plate by 60 degrees. Surface was uncovered for 3-5 minutes to allow absorption of any moisture. Antimicrobial disk assays (disk diameter is 6 mm) were dipped in antibiotics solution to test the effect of two water-soluble antibiotics tobramycin and minocycline at individual concentrations of 30 μg/mL and 100 μg/mL, and mix of both antibiotics at concentration of 200 μg/mL. Sterile tweezers were used to pick Kirby Bauer disks loaded with antibiotic agents. Disks were placed firmly on the agar surface and gently pressed on it to ensure complete contact. Disks

positions were not changed as antibiotic activity occurs right away, and distances between disks were 24 mm and no closer than 10 to 15 mm from the edge of petri dish. Plates were incubated at 35°C for 24 hours for observing zone of inhibition.

2.6 Using different dosages of antibiotics on rotifer culture

Total of six 1-L beakers were used to maintain rotifer cultures supplied with UV water and rotifer density was kept at 200 per mL. Rotifers were fed UM Enrichment that consists of 75% yeast, 15% protein plus, 10% RotiGrow as an algae paste and 35.175g/L Amquel plus. All ingredients were mixed well and kept in a bucket inside a refrigerator at 4^{o}C. Daily, 0.2 mL of algae paste was dispensed using a micropipette to feed rotifer culture in every beaker and Cloro-maX powder was added to maintain pH levels. Tobramycin and minocycline were applied at first day at the concentrations previously mentioned. Also, live feeds, especially rotifer, have high metabolic rates that need constant aeration to prevent the culture from being under stress. Therefore, an air stone was attached to airline connected to air pumps was applied to each beaker to remain dissolved oxygen (DO) above 4 mg/L.

Chapter 3

Results:

After 24 hours of culturing bacteria from settling tank water on ECC media, 180 colony forming unit (cfu) formed with the 1 mL sample. The sample showed other colorless gram-negative bacteria colonies and one single colony represented the coliforms *Klebsiella spp* or *Enterobacter spp*. Colorless colonies were plated on Vibrio media but no presence of Vibrio species appeared, suggesting other types of bacteria, possibly including *Pseudomonas*, *Aeromonas*, *Flexibacter*, and others. In addition, both dry media cultures showed 1 cfu and 2 cfu White/Beige colonies. After 48 hours, two purple colonies appeared representing *E. coli*.

In addition, sample volume of 1 mL was showed 21 colonies including *V. parahaemolytics* and *V. alginolyticus,* and *V. vulnificus*.

Figure 3A Coliforms colony forming units found in water samples from UMEH locations

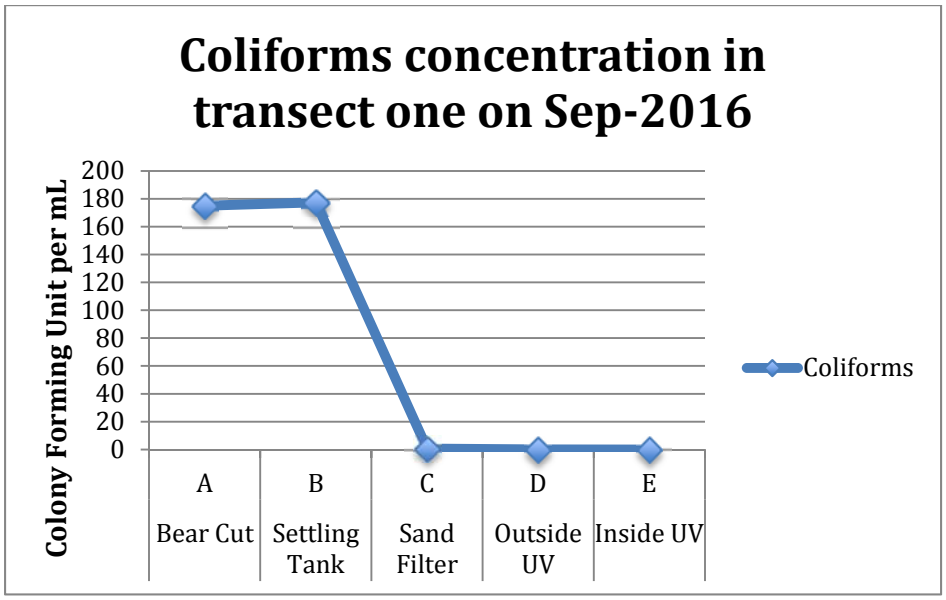


Figure 3B: Coliforms colony forming units found in water samples from UMEH locations

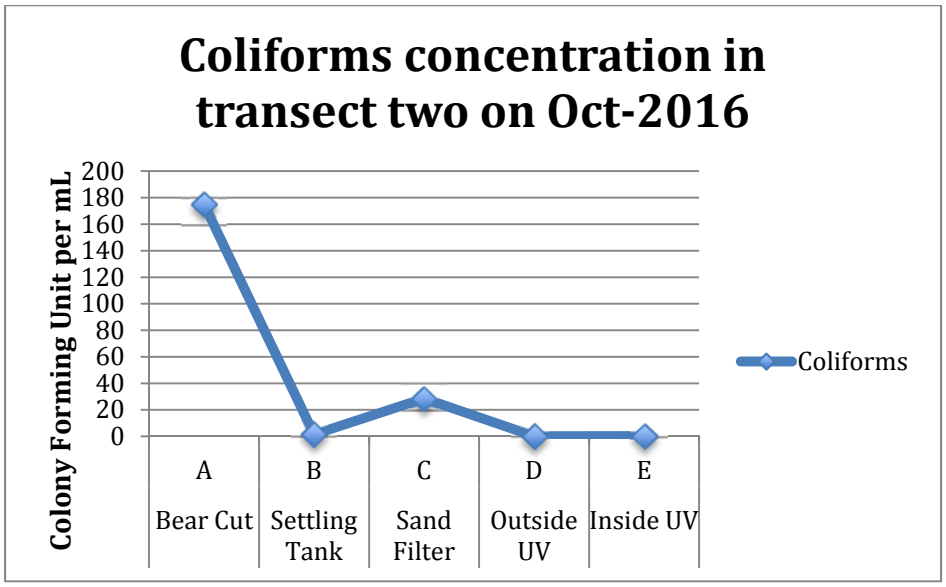


Figure 3C: Coliforms colony forming units found in water samples from UMEH locations

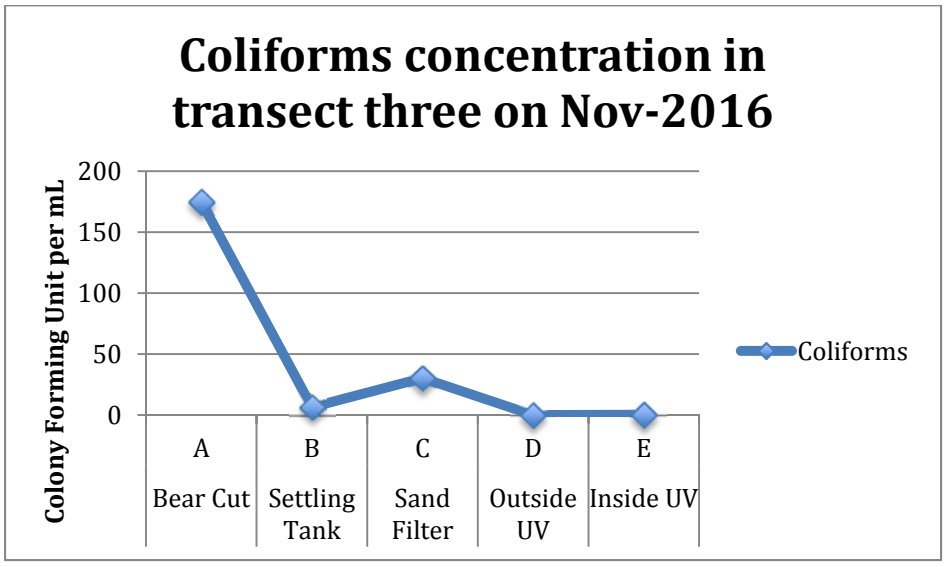


Figure 3D: Coliforms colony forming units found in water samples from UMEH locations

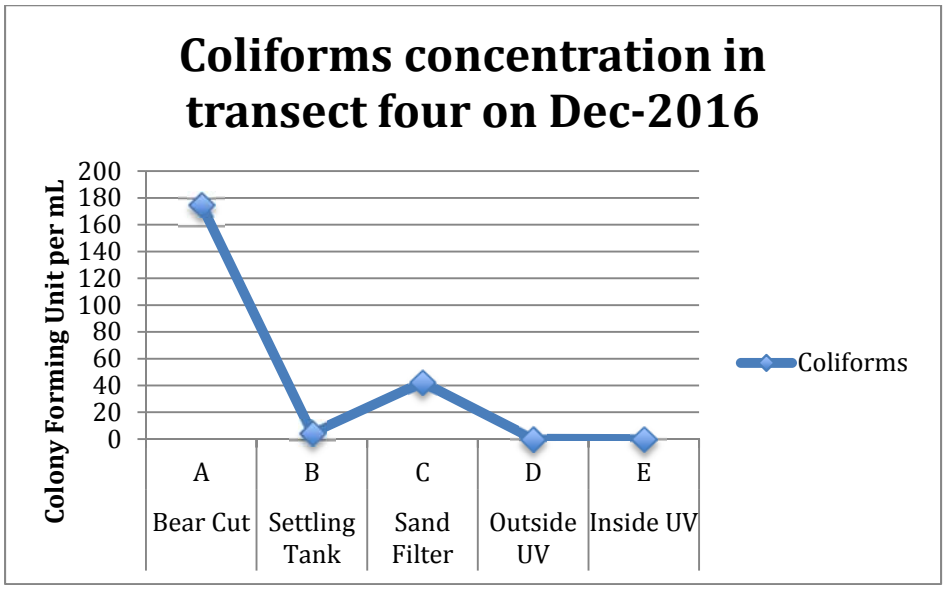


Figure 4A: Vibrio spp. colony forming units found in water samples from UMEH locations

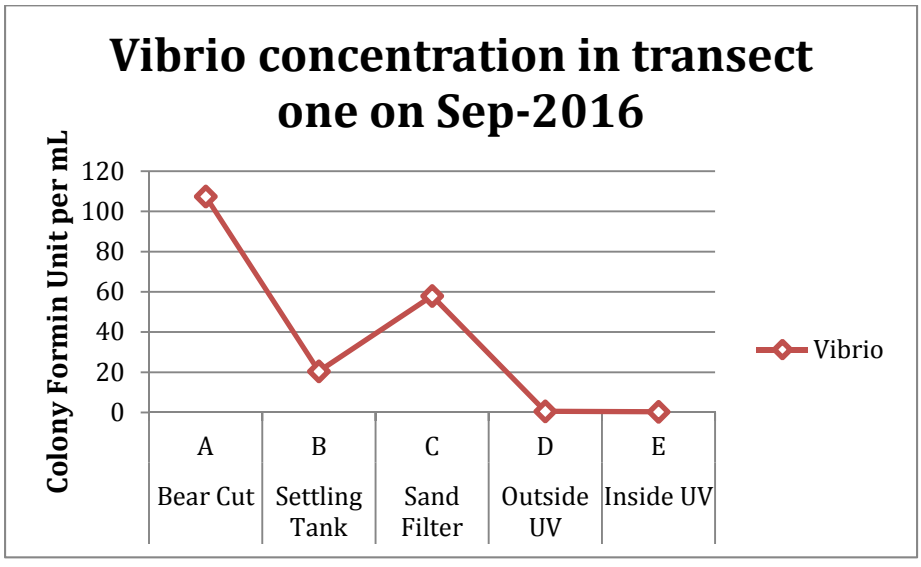


Figure 4B: Vibrio spp. colony forming units found in water samples from UMEH locations

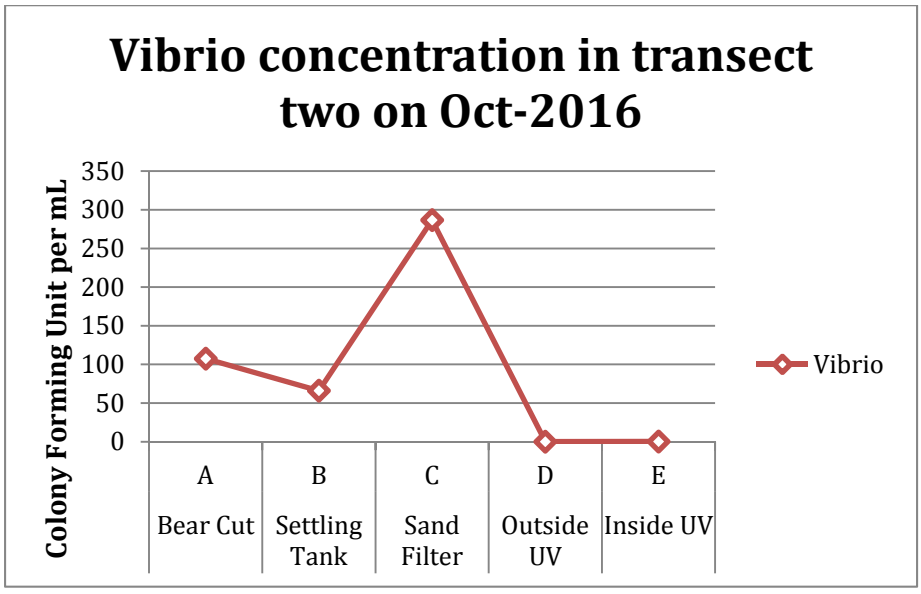


Figure 4C: Vibrio spp. colony forming units found in water samples from UMEH locations

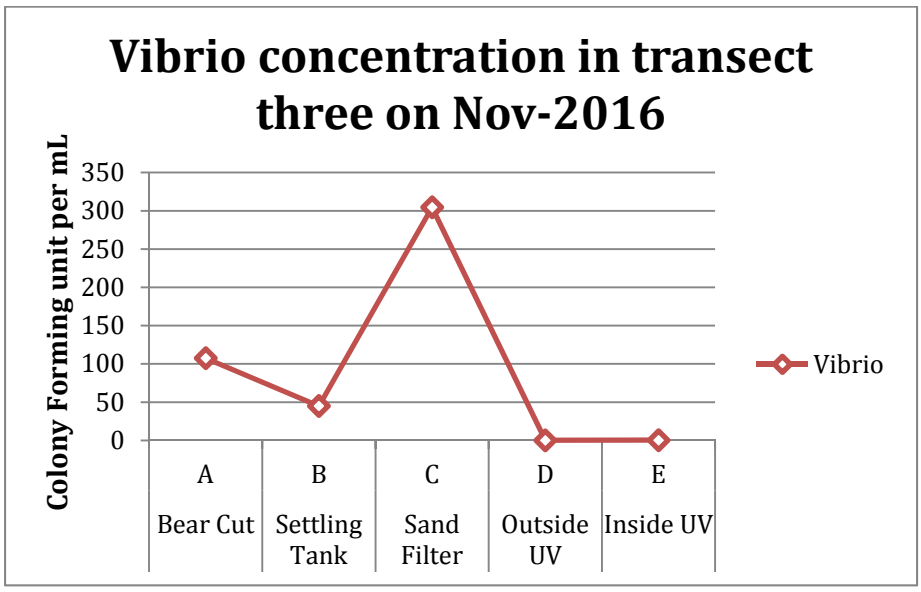
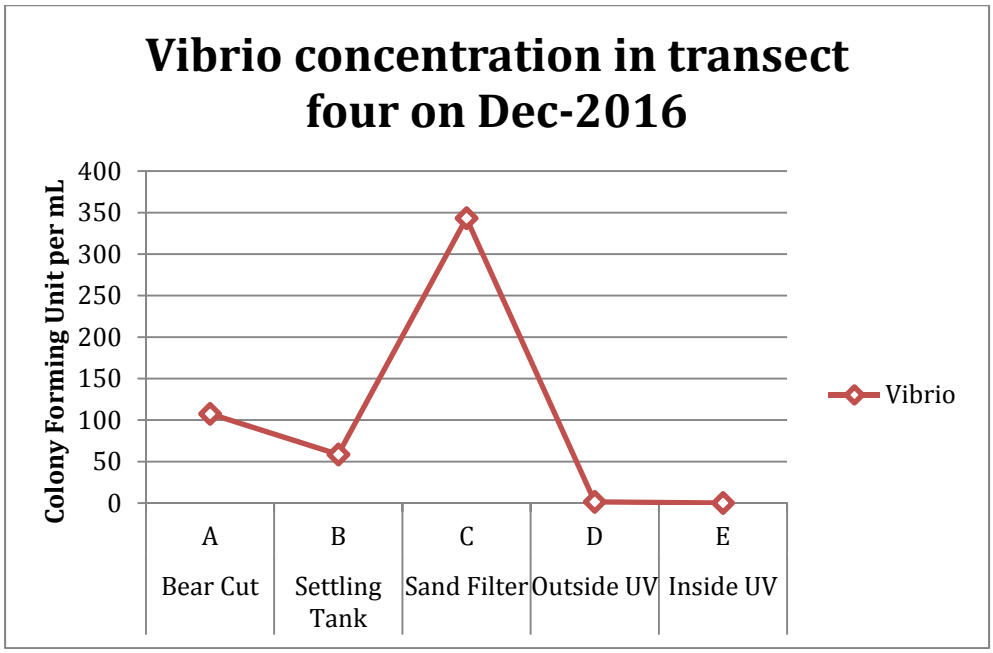


Figure 4D: Vibrio spp. colony forming units found in water samples from UMEH locations



As expected for Coliforms, the number of colonies reduced after water moved further through UV treatments. However, in the sand filter, bacterial concentration was elevated slightly.

There was an unexpected increase in number of Vibrio colonies in the sand filter, which was observed in all samples. This happened since Vibrio spp. appeared to settle in the sand filter where they could replicate and produce before water flow to UV treatments.

To test the influence of antibiotics on the bacteria, Table 5 below shows that all bacteria strains were sensitive to Tobramycin and Minocycline at different dosages. No signs of microbial resistance or intermediate resistance were observed within the

zone of inhibition on Mueller Hinton Agar. This result suggested that Tobramycin and Minocycline might be good chemical agents to eliminate detrimental bacteria present in seawater to obtain pathogen-free water to use in fish hatcheries.

Table 3:Antibiotic susceptibility patterns of bacterial isolates from raw seawater.

Microorganisms	**Zone of Inhibition (mm)**				
	Minocycline (µg/mL)*		**Tobramycin (**µg/mL)*		**Mix (**µg/mL)*
	30	**100**	**30**	**100**	**200**
V. Vulnificus	≤ 20		≤ 25		≤ 23
V. parahaemolyticus	≤ 19		≤ 30		≤ 27
Klebsiella	≤ 26		≤ 18		≤ 20
V. alginolyticus	≤ 20		≤ 22		≤ 20
E. coli	≤ 19		≤ 16		≤ 17

*** This concentration represents the concentration of antibiotics on a filter paper dissolved in sterile seawater and placed on MHA.**

In rotifer culture, samples from each beaker were taken and plated on ECC and Vibrio media to detect the presence of microbes. All plated media showed high concentrations at least 10^3 of *V. vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* indicating multi-resistant strains of these bacteria that were able to resuscitate in the rotifer culture.

UV disinfection for seawater reduced the numbers of Vibrio species and fecal coliforms colonies but did not eliminate them. This is an indication of viable but non-culturable (VBNC) state that bacteria may use to escape from harsh environments. For example after UV treatment, bacteria may regenerate in rotifer culture where a massive nutrient load is available.

Chapter 4

Discussion

UV treatments reduced Vibrio species, but did not inactivate them all. *V. parahaemolyticus*, *V. vulnificus,* and *V. alginolyticus* were able to regenerate in rotifer cultures. In contrast, (Mounaouer & Abdennaceur, 2012) showed the kill rate of UV-irradiation on *P. aeruginosa* bacterial strains. The experiment observed that UV results in the inactivation of the bacteria strain because the attack occurs at the locations where the DNA molecules with double thymine bases are neighboring each other. Furthermore, an increase in the bacteria inactivation rate is observed even at extreme temperatures of 10°C and highs of 50°C since the water molecules in the cell generate free (-OH) radicals which go on to steal electrons from cell proteins. In this expeirment at UMEH, flow through system, UV light achieved complete inactivation of fecal coliforms but did not eliminate total coliforms which were still present in rotifer cultures. This is may be related to the turbidity of incoming raw seawater and high levels of organic particles. Also, two UV disinfections were used with low flow rate to allow more exposure time to insure inactivation of microbes. Due to this low flow rate, particle content may accumulate on quartz sleeve glass and reduce UV light intensity. This may lead to reduced efficacy of UV treatments and a build up of heat inside the glass which would reduce the life of UV lamp significantly.

On the other hand, Sharrer et al, 2005, reported that the entire coliform bacteria population in a recirculating aquaculture system (RAS) is vulnerable to UV inactivation process. The experiment showed that a complete inactivation of coliform

bacteria is attained even at lower UV dosage of 77 mW s/cm^2. the average inactivation rate of UV in waterborne bacteria is 99.9% and at a UV dosage of 1.0 * 10^5 µW sec/cm^2 and lasting less than one minute (Hiasae et al, 2002; Sharrer et al, 2005; Spottel & Gary, 1981; Bassiri, 2012). Accordint to Spottel & Gary (1981) experiments showed that even at a kill-rate of 99.99% in a cell-density of 10^4 / mL, at least 1.0 / mL is left intact. Thus, this concentration is still sufficient to spread diseases during intensive culture given high growth potential of waterborne bacteria. Their experiments also showed that both cutaneous and systemic infections are transmitted to captive animals like dolphins and whales since yeast called *Candida albicans* flourishes at cell counts as low as 100 / mL (Spottel & Gary, 1981). In addition, (Mounaouer & Abdennaceur, 2012) reported that in a system, the mass of pathogens may never go to zero because UV radiation cannot stop the spread of pathogens in recirculating systems. According to our results and those of other studies, relying on UV disinfiction is not enough to inactivate all pathogenic bacteria from occurring in rotifer cultures.

UV radiation is quite effective in treatment of raw influent-water since it is able to reduce pathogen numbers and decrease the likelihood organisms entering from external sources (Wennberg & et al, 2013). However, according to Zhang & et al, (2015), the presence of viable but non-culturable condition (VBNC) in bacteria often misjudges the health risks posed by raw waste water when treatment is done using UV. Also, disinfectants like chlorine can turn into VBNC chemical inducers in secondary treated wastewater. Prokaryotic organisms under the VBNC state often fail to create colonies under appropriate environmental stresses, such that the cells stay

alive and thus making them capable of reemerging their metabolic actions under suitable stimulation. This is because there are many factors in the natural environment that can induce Prokaryotic organisms into the VBNC state, like antagonistic nutrition levels, extreme temperatures, or adverse osmotic pressures. Based on our results, the reduction of Vibrio colonies were observe after UV treatments on the plate count method but were regenerated massively in rotifer cultures. Plate count method was not a proper technique for determining the state of VBNC, implying that stress free environments with appropriate nutrients induce microorganisms to restore their metabolic activities.

Another reason UV is insufficient to completely disinfect is because bacteria have developed biological mechanisms that seek to fix the thymine dimers through a DNA process that utilizes protease enzymes (Kreuzer, 2013). The bacteria repair the DNA by trying to fix the impaired base. In the experiment, after UV treatments Vibrio population was revived in rotifer cultures upon the availability of high levels of nutrients. This may explain why UV disinfection is not reliable to eliminate all Vibrio species, as microbes still can fix their damaged DNA to survive and regenerate.

The number of Vibrio colonies rose drastically in the sand filter as shown in the figure 4 (A,B,C and D) respectfully. When water with high turbidity comes into contact with material like broken glasses, which makes up the sand filter, this induces Vibrio to settle in the mechnical filteration apparatus. Therefore, in the hatchery, the sand filter is made from activated filter media which is bio-resistant crushed green glass. This media is developed to prevent any bio-film to form in the filter bed.

However, Kogure et al (1998) illustrated the attachment of *V. alginolyticus* to a glass surface, which is dependent on time, bacterial concentration, organic concentration, Na^+ concentration, type of flagella, and an inhibitor of Na^+ motive flagellar rotation and concluded that *V. alginolyticus* depend on swimming speed to attach to glass surface. In this experiment, backwashing procedure along with high particle content may have incentivized Vibrio to distribute and attach to the sand filter. Vibrio may remain on the sand filter media, replicate, and then flow to UV treatments. Vibrio species tend to attach to host using adsorption technique and are able to form biofilm (slime layer) on pipelines and then reproduce upon the avialability of nutrients. Another filter of proper mesh size (e.g. less than 1 μm) after the sand filter is crucial to stop large particles from passing to UV treatments.

This study found that in rotifer cultures, antibiotic treatments were not effective to kill detrimental bacteria because Vibrio species may acquire resistance genes. However, isolated pure strains from the settling tank of all types of *Vibrio* and the coliforms showed sensitivity to the antibiotics. This suggests that other strains of *Vibrio* were present in seawater which were multi-resistant to antibiotics. Moreover, one of the major threats facing intensive aquaculture farming is infectious diseases caused by bacteria which have high resistance against antibiotics. One of these bacteria is *Vibrio parahaemolyticus* which is a halophile gram-negative pathogen that causes lethargy, abdominal distension, and high mortalities in cultured fish. The other one is *Vibrio vulnificus* which causes external hemorrhages from the gill, fin and head to the ventral body. Recent studies such as that of Elmahdi & et al (2016) shows that these two species' antibiotic resistance is multiple and is in most places caused by

misuse of antibiotics when controlling infections during breeding. The multi-resistance is a result of fish farms often applying antibiotics in fodder with the intention of stimulating growth, and this makes the drug molecules bind with ions in the water, hence significantly decreasing the biological action of the drugs (Elmahdi et al., 2016). In particular, the resistance profiles are in drugs like ampicillin with a resistance rate of 90%, carbenicillin with a resistance of 45.2%, oxytetracycline at 90%, penicillin at 90%, ceftazidime at 45.2% and tetracycline at 90% and makes these two Vibrio spp. bacteria to account for 95% of aquacluture fish related deaths (Costa & et al, 2014). These Vibrio species acquire antimicrobial resistance through mobile genetic features and horizontal gene-transfer (Labella & et al, 2013). This is attributed to the existence of R-factors in a given aquaculture system population such that gene control of plasmids and chromosomes creates a resistance which is transferred vertically either through heredity or in certain cases horizontally (Reboucas & et al, 2011). The resistance is also caused by creation of inducible chromosomal beta-lactamases, which makes the bacteria susceptible to drugs like beta-lactams (Igbinosa, 2016). Cultured fish species like cobia, Atlantic halibut and salmon which are bred in aquaculture settings are prone to these antibiotic resistant bacterial because they are exposed to horizontal gene transfer due to survival instinct, and as a result of several environmental stresses like temperature, variations in pH, or high antibiotic concentration. This involves transfer of genes by either by conjugation within bacteria, natural gene transformation or gene transduction (Guo et al., 2015).

Obtaining rotifers from one supplier without knowing how they culture them or if they were using antibiotics is a factor of increased risk of these microbes acquiring

resistance genes. This must be taken into consideration when acquiring rotifers from external sources and should be incorporated in any hatchery standard operating procedures a part of a strict biosecurity program.

Chapter 5

Recommendations

Combining several advanced technologies for treating inflow raw seawater to minimize particle content and eliminate pathogenic agents is essential to ensure supply of good water quality. Relying only on UV disinfection is insufficient to inactivate and eliminate pathogenic bacteria. This is because the effectiveness of UV radiation is restricted to only *in vitro* settings such that only free-floating pathogens are destroyed. In particular, organisms attached to the external sides of their hosts are not destroyed by UV and instead need chemotherapeutic methods to destroy them (Summerfelt, 2003). What this implies is that UV sterilization is only effective for treating incoming water supplies and the finishing effluent from culture-systems as these two systems are single-pass. In addition, UV irradiation, even in large dosages is not able to penetrate particulate matter around the bacteria and thus is unable to reach entrenched bacteria (Sharrer & et al, 2005). Therefore, additional disinfection methods should be applied to ensure high water quality.

5.1 Ozone (O_3)

Ozone has been reviewed by several research studies to be a stronger oxidizing agent that can be applied in aquaculture for disinfecting and improvement of water quality (Summerfelt, 2003). Ozone deactivates sedentary fish pathogens by oxidizing the organic wastes and nitrite. Ozone is produced through electrical-corona discharge in a feed-gas filled with oxygen (Summerfelt, 2003). Ozone is able to eliminate at least 99.9% of bacterial cells by producing total residual oxidants (TROs) in one minute or

less (Hisae, Yoshimizu, & Ezura, 2002). Properly designed and managed, ozone is a very effective mechanism to remove bacteria from hatchery systems and its use should be considered.

5. 2 Advanced Oxidation Process (AOP)

Another disinfecting process is Advanced-Oxidation-Process, which involves using UVc/O_3/H_2O_2 or UVc/H_2O_2. Through an adsorption process, the UVc/ozone/hydrogen-peroxide or the UVc/hydrogen-peroxide are able to generate hydroxyl-radicals (HO•) which, when targeted using heterogeneous photocatalysts such as TiO_2, easily generates the radicals H_2O_2 and O_3 which are toxic to the bacteria cells and membrane functions (Chávez, Pizarro, & Araya, 2016). AOP is very useful in sterilization of intake water since it kills pathogenic intruders, while at the same time temporarily reducing the natural competition between dissimilar bacterial species. This is because AOP generates HO• which is a very strong oxidant of internal cell-structures that goes on to inactivate the pathogens. UV is very important in AOP, since radiation with wavelength 254 nm produces photolytic dissociation reactions of hydrogen peroxide and this creates hydroxyl radicals that are used in several other oxidation pathways. UV-C is also important in removing ozone byproducts through adsorption process. This involves eliminating ammonia, chemical oxygen demand, reduction in bromine concentration and the decomposition of H_2O_2, through adsorption (Chávez, Pizarro, & Araya, 2016).

5.3 Photodynamic Antimicrobial Chemotherapy (PACT)

Another possible treatment is Photodynamic Antimicrobial Chemotherapy using singlet oxygen, referred to as $^1O_2$, as another potent and sustainable wastewater treatment technique. The technique has a 100% elimination of antibiotic resistant pathogens like *V. parahaemolyticus* (Malara & et al, 2016). The process involves irradiation using a combination of UV-B and light porphyrin photosensitizers, and takes around 45 minutes (Malara & et al, 2016). The photosensitizer is the compound responsible for releasing singlet oxygen, which is a very reactive and toxic oxygen species that kills the bacteria cells.

5.4 Electrolytic water treatment

Disinfection of seawater to be used in hatcheries is commonly performed using electrolytic treatment since it has a 99.8% reduction rate and a minimal time of only one minute (Hisae et al., 2002). Electrolysis is particularly effective in eliminating antibiotic resistant *V. anguillarum* since it results in the release of hypochlorite ion which behaves as a strong oxidizing agent that attacks the bacteria cell walls, thus destroying their membranes (Jorquera et al., 2002). In hatcheries containing salmon, disinfection is applied to eliminate pathogens from egg surfaces. This technique ensures that the vertical diffusion of bacterial pathogens from brood stock to eggs is stopped. Secondly, the hypochlorite created from continuous drifting of electrolyzed hatchery wastewater reduces the number of other bacteria by at least 99%.

5.5 Ultrasound technology

Ultrasound waves are longitudinal sound waves with frequencies greater than 20 kHz (Doosti et al., 2012). The energy spread by these waves results in a vibration of molecules whereby a cavitation process causes the growth and breakdown of micro-bubbles in small time intervals. The technique comprises the use of membrane purification, turbidity, and a whole suspension solid reduction system. It also incorporates algae removal process using ultrasonic irradiation followed by a bacterial disinfection process using UV. Ultrasound irradiation inactivates the bacteria using acoustic cavitation whereby there is an attack on the membranes by generated hydroxyl radicals. Afterwards, high pressure and temperature created by a micro-streaming force induces bubbles that then collapse the bacteria, leading to cell death (Doosti, Kargar, & Sayadi, 2012).

5.6 Phage therapy

New strategies are needed in controlling aquaculture to avoid extensive misuse of antibiotics. One of these strategies is phage therapy, which works as bio-control agent and is applied to remove antibiotic resistant pathogens. The advantage is that it is based on host specificity and thus is safe for human applications (Elmahdi,et al., 2016). In laboratory experiments, lytic phages are isolated and categorized to determine if the phage is suitable for therapeutic use. One such experiment was performed by Elmahdi & et al (2016) whereby a phage from *V. vulnificus* was used in a mouse models infected with this multiple antibiotic-resistance strain. The phage-treated mice survived the fatal oral and intra-peritoneal infection since the SSP002 Phage considerably protected the mice for up to two months. Phage therapy is an

excellent approach that can be applied to fish larvae production to prevent infection from pathogenic bacteria.

5.7 Detecting the presence of VBNC

There are three effective ways of identifying Prokaryotic organisms in VBNC state that is 16S rRNA, ribotyping PCR and PCR-based methods (Chatterjee and Haldar, 2012). Other methods include reverse-transcriptase quantitative-polymerase-chain reaction or RT-qPCR, and the propidium-monoazide quantitative polymerase-chain-reaction (PMA-Q PCR). All these methods are used to determine the induction and recovery of bacteria into the VBNC state. (Zhang et al, 2015).

5.8 Establishing axenic rotifer culture

Rotifers, which are the most commonly used live feeding approaches, are vulnerable to bacteria especially those sheltering in the rotifer culture (Suga et al, 2011). In order to make rotifer cultures axenic, antibiotics such as ampicillin, kanamycin, nalidixic acid, or streptomycin are used at a recommended amount of 30-100 µg/mL. These antibiotics encourage the reproduction of rotifers without any toxic outcome. The product is used to treat the eggs but when combined with around 0.25% (w/v) of sodium hypochlorite for three minutes, they render the rotifer-culture bacteria-free (Suga et al, 2011).

Chapter 6

Conclusion

UV treatments reduced the number of Vibrio and coliform colonies but were insufficient to fully inactivate the microbes. High turbidity of seawater may decrease the efficacy of UV disinfections and without using proper mesh size after the sand filter, to prevent large organic particles to flow through UV treatments, pathogenic agents can be introduced in rotifer cultures. In this study, antibiotic agents were not highly active against *V. vulnificus*, *V. parahaemolyticus,* or *V. alginolyticus* presenting in rotifer culture*,* implying that these Vibrio spp. were able to develop a resistance mechanism to resist antibiotics. The excessive use of antibiotics induces pathogenic agents to evolve their structures and remain unaffected in different environments. Relying on agar plate count is not adequate to determine the presence of microbes in the VBNC state, but using more specific instruments such as PCR may facilitate the detection of DNA fragments from pathogenic microbes. In fish hatcheries, supplying good water quality, and controlling infections along with using proactive health management strategies (e.g. prophylaxis with freshwater or approved chemicals such as hydrogen peroxide) other than antibiotics are essential to prevent multi-resistant bacteria from causing disease outbreaks.

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