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Variations of Vibrio Populations in Pacific White Shrimp, *Litopenaeus vannamei*, Aquaculture Raceways

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VARIATIONS OF *VIBRIO* POPULATIONS IN PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*, AQUACULTURE RACEWAYS

by

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ABSTRACT

VARIATIONS OF *VIBRIO* POPULATIONS IN PACIFIC WHITE SHRIMP, *LITOPENAEUS VANNAMEI*, AQUACULTURE RACEWAYS

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Reducing incidence of vibrioses in intensive aquaculture systems is essential to maintaining yields. This study performed real-time qPCR (quantitative polymerase chain reaction) using primers targeting 16s rDNA sequences for total bacteria, the *Vibrio* genus, and *Vibrio parahaemolyticus* in aquaculture tanks over the growing season. Total DNA concentrations in each sample were measured with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Inc. USA). These data were compared to two levels of bio-floc solids removal in addition to physical and chemical tank parameters such as temperature, ammonia, nitrite, nitrate and phosphate. The total bacteria gene copy abundances were higher ($p = 0.0081$) in the low solids tanks but the quantity of *Vibrio* genus and *Vibrio parahaemolyticus* DNA copy numbers did not significantly differ between treatments. Regressions performed between the bacterial populations, shrimp specific growth rate (SGR) and nutrient concentrations were found to support the concept that bio-floc in the high solids tanks processes nutrients and suppresses *Vibrio* populations. Optimizing the quantity of bio-floc is essential for controlling nutrient concentrations in order to select for beneficial bacteria that can improve shrimp health and growth. High nutrient concentrations can stress livestock and promote the incidence of disease causing bacteria and should therefore continue to be monitored and controlled.

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ARDRA	Amplified ribosomal DNA restriction analysis
BAL	Belize Aquaculture, Ltd.
BLAST	Basic local alignment search tool
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
FISH	Fluorescence in situ hybridization
GCRL	Gulf Coast Research Laboratory
LAMP	loop-mediated isothermal method
qPCR	quantitative polymerase chain reaction
RAPD	random amplified polymorphic DNA
REP-PCR	repetitive extragenic palindromic elements – polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SGR	specific growth rate
TAN	total ammonia nitrogen
TCBS	thiosulphate- citrate-bile salt sucrose
TDH	thermostabile direct hemolysin
TLH	thermolabile hemolysin
TRH	thermostabile direct-related hemolysin

CHAPTER I

LITERATURE REVIEW

Bio-floc Systems

Bio-floc particles are composed of phytoplankton, organic material, bacteria and grazers of bacteria such as protozoa and rotifers (Hargreaves 2006, Figure 1). Burford et al. (2003) found that particulate material composed mostly of dead phytoplankton also contained 40% of the total bacterial population in densely-stocked shrimp ponds in Belize. Bacterial abundance in bio-floc may be explained in part by their association with phytoplankton and zooplankton (Asplund et al. 2011; Rehnstam-Holm et al. 2010). Dying algae release large amounts of dissolved organic carbon (DOC) and other materials which are available to bacteria although they may also derive nutrition from algal extracellular products (Cole 1982). Organic material derived from zooplankton feces or exoskeletons containing chitin can be utilized by heterotrophic bacteria such as *Vibrio* that are normally associated with these organisms (Colwell 1984). Riemann and Azam (2002) found that all *Vibrio* strains tested were able to hydrolyze chitin and absorb the break down products. All the strains were also facultative anaerobes which likely gave *Vibrio* species an advantage in low-oxygen conditions that could occur in floc particles (Riemann and Azam 2002).

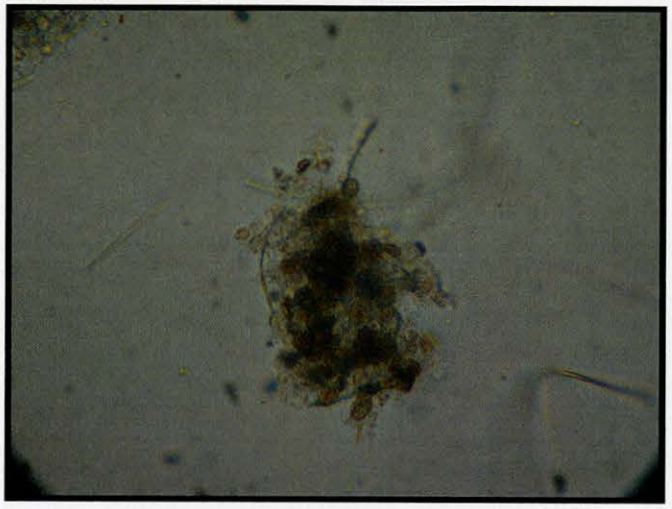

Figure 1. Bio-floc particle as seen under 100X magnification. Photo provided by Andrew Ray.

Bio-floc particles play an important role in processing toxic, nitrogenous waste products such as ammonia and nitrite by making them available to colonies of bacteria residing within. The structure of the porous, aggregate material causes nutrient-laden water entering the particle to slow down and change direction, creating increased opportunities for nutrient uptake by the attached microbial community (Bossier and Verstraete 1996; Deschryver et al. 2008). As nutrient loads in the system increase, the proportion of nitrogen in the floc also increases, placing a higher burden on the community. However, in most aquatic habitats, sessile bacteria are more metabolically active than free living bacteria due to less energy expenditure on activities such as motility and chemotaxis and are able to assimilate additional nitrogen (Hargreaves 2006). As an example, in a short biofilm study, Thompson et al. (2002) measured shrimp growth and water parameters in tanks both with and without biofilm. The researchers found that the bacteria in the biofilm more effectively processed ammonium and phosphorus and provided a food source to contribute significantly to shrimp growth (Thompson et al.

2002). The reduction in nutrient levels can also reduce stress on livestock and the likelihood of disease (Thompson et al. 2002).

As heterotrophic bacteria grow and consume carbohydrates, they assimilate nitrogen in order to form peptides and proteins such as enzymes and peptidoglycan (Avnimelech 1999; Tremblay and Benner 2006). Production of these bacterial proteins can be stimulated in order to increase the feed protein conversion ratio by livestock by adding a labile carbon source to increase the carbon nitrogen ratio (C:N ratio) to at least 10 (Avnimelech 1999). Heterotrophic bacterial nitrogen uptake and assimilation is a more efficient and rapid process than nitrification due to the rapid growth of heterotrophic bacteria relative to nitrifiers and because glycolysis is comparatively inexpensive metabolically (Hargreaves 2006). Indeed, the proliferation of heterotrophs can outcompete nitrifiers and reduce their efficiency by 70% even at low C:N ratios so care must be taken to add sufficient carbon to stimulate total ammonia nitrogen (TAN) uptake (Zhu and Chen 2001). Another caveat is that strong aeration must be utilized to compensate for an increased oxygen demand as carbohydrate breakdown is an aerobic process (Hargreaves 2006). Failure to keep oxygen levels above 2 mg/L can further inhibit nitrification and negatively affect livestock health (Hargreaves 2006). However, low oxygen would still allow the growth of facultative, heterotrophic bacteria like *Vibrio* spp., which can use nitrate as an electron acceptor during respiration (Madigan & Martinko 2006).

Hari et al. (2004) examined *Penaeus monodon* in ponds fed high or low protein feed either with or without carbon addition. Tapioca flour was used as a carbon additive to successfully reduce TAN and increase total heterotrophic bacterial abundance as well

as shrimp production. Additionally, shrimp fed 25% protein feed had a significantly higher SGR (7.9 ±0.1) than shrimp fed the higher protein feed (40%, 7.7 ±0.1). Shrimp fed the low protein feed with carbon addition generated 400% more profit because their large size brought a higher market price (Hari et al. 2004).

Aquaculturalists taking advantage of carbon addition can potentially reduce costs and lessen environmental losses by using microbial proteins as a significant part of their stocks' nutritional requirements, replacing a portion of more expensive, partially fish-meal based feed (Avnimelech 2007). Additionally, some bacteria store excess carbon as poly-β-hydroxybutyrate which has been shown to act as a source of fatty acid nutrition and have a protective effect on *Artemia* from *Vibrio campbellii* (Defoirdt et al. 2007; Halet et al. 2007). Bio-floc particle size can be optimized for effectiveness and ease of consumption by increasing the mixing rate in order to decrease the overall circumference (Biggs and Lant 2000). Burford et al. (2004) demonstrated that the addition of 1-3% of 15N isotopically labeled ammonium was ultimately incorporated as shrimp biomass, presumably consumed as a flocculated particle (Burford et al. 2004). De Schryver (2008) calculated the savings of lower protein feed while considering the usage of acetate as a carbon source to be 10% (De Schryver et al. 2008). Reducing protein in feed is an important part of reducing pressure on fisheries for fish oil and fish meal products for aquaculture (De Schryver et al. 2008).

Boyd and Clay (2002) evaluated a bio-floc system used by Belize Aquaculture, Ltd. (BAL). Large inland lined ponds were filled with filtered brackish water and treated with fertilizer and mixed grains to stimulate microbial activity. A zeolite byproduct, sodium silicate, was distributed into the ponds for the purpose of providing sites for

microbes to attach. After stocking with disease-resistant shrimp strains, a low-protein (18%) feed was gradually replaced by a higher protein (30%) feed over the growing season. The low-protein feed was used to further stimulate bacterial growth and reduce the early buildup of nitrogenous wastes. Heavy aeration in the form of paddle wheel aerators and pumps were used to prevent low oxygen levels. Sludge buildup was removed with settling basins. The average shrimp yield in these ponds was 11,231 kg/ha per crop. The pond with the highest production rate (27,200 kg/ha) used AquaMats (Calverton, MD, USA) that float vertically in the water column and serve as large substrates for microbial biofilm growth, which provided an additional food source for the shrimp and reduced territoriality. In this study, the AquaMats brought the survival rate from 65-78% to 91%. The BAL ponds demonstrate that high yields and survival rates are possible in intensive systems (Boyd and Clay 2002).

The idea behind AquaMats is similar to the periphyton-based systems being utilized in some developing countries. In these systems, available substrate such as bamboo is placed in the ponds to serve as attached sites for periphyton growth which helps to control nutrient concentrations and serves as food for pond inhabitants. Azim et al. (2004) analyzed ponds with increasing amounts of substrate and found increasing shrimp yields of 114%, 168% and 209% over controls (R^2=0.93). Additionally, ammonia levels in control ponds were significantly higher than those with substrate, demonstrating the nutrient processing ability of the periphyton (Azim et al. 2004). Periphyton-based systems are an efficient use of scarce resources in these countries.

Vibrio Population Dynamics in Aquaculture

Vibrios have been shown to be extremely adaptable members of the bacterial community and they are able to utilize a variety of resources. *Vibrio fischeri* has even been shown to consume 3':5'-cyclic nucleotides (e.g., cAMP) from its host to provide its energy, carbon, nitrogen and phosphorous needs (Callahan et al. 1995). Ammerman and Azam (1985) discovered that in addition to alkaline phosphatase, many bacterioplankton including *Vibrio* spp. possess a 5'-nucleotidase that hydrolyzes nucleotides and facilitates the uptake and eventual recycling of phosphate. They postulated that this enzyme could supply phytoplankton with half of its phosphate demand (Ammerman and Azam 1985). Kim et al. (2000) demonstrated that *V. alginolyticus* colonized on a submerged biofilter removed up to 2000 ppm of ammonia gas above the solution using glucose as a carbon source.

It has been known for decades that increasing temperature can positively influence the number of *Vibrio* spp. present. In 1970, Baross and Liston found the abundance of *V. parahaemolyticus* and *V. alginolyticus* in oyster meat correlated well with water temperature (Baross and Liston 1970). In Laguna de Balandra, Baja California Sur, Mexico, the mangrove sediments in this arid region are nitrogen and phosphate limited. Without excess nutrients, temperature was shown to be the primary determining factor of *Vibrio* spp. abundance (Gonzalez-Acosta et al. 2006).

In mesocosm studies, *V. cholerae, V. vulnificus, V. parahaemolyticus and V. alginolyticus* all showed a >10 fold increase in abundance in response to organic material from *Nodularia spumigena*, a coastal, summer-blooming cyanobacteria (Eiler et al. 2007). While monitoring ribosomal ribonucleic acid (rRNA) levels in bacteria in

seawater, Eilers (2000b) found that *Vibrio* spp., even from a starved state, were better able to take advantage of a rapid increase in nutrient levels than many other types of bacteria due to the retention of extra ribosomes (Kramer and Singleton 1992). The researcher attributed this to their lifestyle of attachment to metazoans (Eilers et al. 2000b).

Vibriosis in Shrimp Aquaculture

In order to cause infection, *Vibrio* spp. must first overcome the immune system of the shrimp. Shrimp possess hemocytes that are involved in the phagocytosis of microbes or small particulate matter. These cells can also stimulate the release reactive oxygen species or respiratory bursts, the production of melanin through phenoloxidase, and lysozyme which can break down bacterial cell walls (Burgents et al. 2005; Cheng et al. 2005). The hemocytes also contain antimicrobial compounds called penaedins, the location of which had not been known until 2000 (Destoumieux et al., 2000). Burgents et al. (2005) challenged *Litopenaeus vannamei* with green fluorescent protein-producing, kanamycin resistant *V. campbellii* and determined that the hemolymph and the lymphoid organs were the sites with the greatest ability to stop the growth and reproduction of bacteria. Increasing temperature, or non-neutral pH (low or high) conditions have been shown to reduce immune system functions such as total hemocyte count, phenoloxidase activity, respiratory burst, and superoxide dismutase which may play significant role in disease problems at higher temperatures (Cheng et al., 2005; Li & Chen, 2008).

Vibrio harveyi can be highly pathogenic to shrimp, causing quorum sensing mediated luminous vibriosis (Austin and Zhang 2006). Alavandi et al. (2006) found that *Vibrio harveyi* isolates need not be luminescent to cause disease. The researchers' trials

with several sucrose positive, hemolytic isolates with varying degrees of virulence all caused significant mortality in *Penaeus monodon* larvae (Alavandi et al. 2006). In trials where *P. monodon* larvae were challenged with pathogenic *V. harveyi*, tanks with the highest salinity (35 ppt) and the highest temperature (32˚C) had the highest mortality rates of 73% and 85% respectively (Alavandi et al. 2006). In India, this organism has also been associated with loose shell syndrome and white gut disease (Jayasree et al. 2006).

Normally, increased virulence is associated with increasing temperatures. However, *V. penaeicida*, which causes "Syndrome 93" in New Caledonia was shown to release cytotoxic exotoxins in response to decreases in temperature. This corresponds to reports surrounding outbreaks of the disease (Goarant et al. 2000). Affected shrimp show signs of discoloration, disorientation, difficulty swimming and lethargy (Goarant et al. 1999).

Vibrio parahaemolyticus has been most commonly associated with red disease or red leg disease, which alters the chromatophores on the pleopods causing a reddish coloration (Alapide-Tendencia and Dureza 1997; Jayasree et al. 2006). Mortality in shrimp ponds infected with *V. parahaemolyticus* can be up to 100% (Sudheesh and Xu 2001). This pathogen has also been reported in shrimp as a secondary bacterial infection when infected with white spot syndrome virus (WSSV) (Jayasree et al. 2006). Recently, *V. parahaemolyticus* has been identified as the causative agent of Early Mortality Syndrome, a high-mortality illness usually affecting young shrimp. The initial symptoms include inactivity and a softening and spotting of the carapace (Lightner 2012). This

disease is phage mediated whereby the lysogenic *V. parahaemolyticus* produces a toxin that allows it to penetrate the gut lining and cause septicemia (Tran 2013).

Vibrio Control Methods in Aquaculture

Misuse of antibiotics in aquaculture has contributed to the development of antibiotic resistant bacterial strains that are a threat to environmental and human health and their use has been highly restricted in many countries (Jayasree et al. 2006). If the system water is not properly treated, resistant organisms are often released into the environment. Bacterial genes coding for antibiotic resistance can be transferred, perhaps to a pathogen, once it gets out into the environment (Heuer et al. 2009). In some cases, antibiotics have been rendered almost ineffective for treatment of disease in aquaculture systems. These systems may serve as reservoirs for virulent and resistant organisms (Defoirdt et al. 2007). This review will focus on other methods of control.

One alternative to antibiotics has been the use of immunostimulants. Chang challenged *L. vannamei* that had been fed Zingerone, an antioxidant constituent of ginger, at varying dosages. The treatment groups gained more weight with a dosage effect at the highest dose and demonstrated increased immune system activity such as phenyloxidase levels, respiratory bursts, lysozyme levels and phagocytic activity (Chang et al. 2012). Other successful disease challenge studies with shrimp have utilized extracts of seaweeds such as *Sargassum hemiphyllum* (Sargassum) (Huynh et al. 2011), *Gracilaria fisheri (Ogonori)* (Kanjana et al. 2011) and *Ricinus communis* (Castor Oil Plant) (Immanuel 2004). Future studies may elucidate whether increased immune system vigilance can be maintained throughout the growing season without harming the health of the shrimp.

Work with salmonids demonstrated that *V. alginolyticus* can function as a probiotic against a limited number of pathogens (Austin et al. 1995). This lab-based study also found that *V. alginolyticus* had colonized the gut of the salmonids which could be a benefit in-situ under real exposure conditions. *V. alginolyticus* was not as effective, however, in a challenge on *L. vannamei* with *V. parahaemolyticus*. Mortality in this study was slightly less than the control group (Balcázar et al. 2007). However, Moriarty (1999) supported the use of probiotics but has questioned the use of species of *Pseudomonas* or other *Vibrio* spp. that are closely related to pathogenic strains and may be able to incorporate and act as carriers for virulence plasmids.

Balcázar et al. (2007) fed groups of shrimp *Bacillus subtilis, Roseobacter gallaeciensis*, *Pseudomonas aestumarina* or *V. alginolyticus* for 28 days then challenged them with *V. parahaemolyticus*. All of the shrimp gained weight compared to the control group possibly due to digestive stimulation or the secretion of extracellular products by the bacteria. The shrimp that received the *Roseobacter* had the highest weight gain and survival rate (96%±1.98). The *V. parahaemolyticus* strain was not highly virulent, however, as the control group had a survival rate of 89.75%±1.96 (Balcázar et al. 2007). Dead *Lactobacillus* spp. were also shown to boost shrimp immune function and survival rate if fed daily or every three days but not if fed every six days (Flores-Miranda et al. 2011). Other *Lactobacillus* species have been used in *V. harveyi* trials on farmed shrimp with some success (Kongnum and Hongpattarakere 2012). Rotifer cultures, which can be fed to larval shrimp, are sometimes a source of pathogens. Mixed cultures of *B. subtilus* and *B. cereus* increased rotifer populations and reduced *Vibrio* spp. in the cultures through the excretion of antibiotic compounds called bacteriocins (Murillo and Villamil

2011). Adding probiotics to the larval feed cultures would prevent the growth of pathogens in the cultures and reduce the growing shrimp larvae's susceptibility to disease. The use of probiotics is very promising in the fight against disease and has additional health benefits for livestock (Defoirdt et al. 2007).

Bacteriophage therapy is an attractive method to control bacterial pathogens in that it is species specific, targeting only the problem organism and multiplying in-situ as needed (Nakai and Park 2002). However, development of resistant strains, just as with antibiotic treatment, will likely be a problem (Nakai and Park 2002). Chrisolite et al. (2008) has found that *V. harveyi* populations persist at low levels in the shrimp larval maturation tanks despite high numbers of bacteriophage present.

Defoirdt et al. utilized brominated furinones to disrupt quorum sensing and significantly raise survival rates in *Artemia* challenged with *V. harveyi, V. campbellii* and *V. parahaemolyticus* (Defoirdt et al. 2006). The virulence of the bacteria was reduced by interfering with bacterial toxin production which is regulated through quorum sensing. It is unclear whether or not compounds like this can be used in aquaculture due to toxicity concerns.

One unusual method of Vibrio control is the green water technique using water taken from Tilapia ponds which has a high abundance of *Chlorella* algae but its protective mechanisms are unknown. Huervana et al. (2006) was able to reduce *Vibrio harveyi* counts to below detection with this technique but was unable to determine the cause. The researchers speculated that fish mucus may have played a role since water from the brood stock tanks had a quicker effect than water from the juvenile Tilapia tanks (Huervana et al. 2006).

Vibrio Detection Methods

Biochemical-based identification methods are an easy way to identify organisms for which no primers are readily available, when an additional form of identification is desired or when molecular methods give ambiguous results. Commonly, used identification systems are API 20E (BioMerieux, Inc. Marcy I'Etoile, France) and Biolog GenIII (Biolog, Inc., Hayward, CA, USA) which use company-built databases derived from biochemical identification keys (Alsina and Blanch 1994). Chromagenic media, if available for the target organism, can be useful as a direct plating method or after enrichment but not as a definitive identification method. Researchers in one study greatly preferred CHROMagar Vibrio media (PBI International, Milan, Italy) to thiosulphate-citrate-bile salt sucrose (TCBS) (Oxoid, Hampshire, UK) due to ease of isolation and greater specificity (Di Pinto et al. 2011). Specialty agars such as *V. harveyi* agar have also been developed to assist in isolating the desired organism (Harris et al. 1996).

The random amplified polymorphic DNA (RAPD) PCR "fingerprinting" technique is a quick and easy way to generate a bacterial species-specific signature because random, nonspecific primers are used (Hernández and Olmos 2004). Though RAPD-PCR's resolution is considered moderate, it is able to distinguish pathogenic and nonpathogenic *V. harveyi* isolates (Alavandi et al. 2006). Unfortunately, reproducibility is poor with this assay (Cano-Gomez et al. 2009).

Repetitive Extragenic Palindromic Elements (REP-PCR) with its high resolution was the method used to determine that thirty-nine out of fifty closely related, misidentified strains were *V. campbellii* and not *V. harveyi* (Gomez-Gil et al. 2004). This

method involves targeting conserved, repeated sequences in the genome. The resulting gel pattern can distinguish organisms at the strain level (Cano-Gomez et al. 2009).

Amplified ribosomal DNA restriction analysis (ARDRA) is another identification method performed by first amplifying rRNA with a universal primer set, then running a digestion on the products with five enzymes. The resulting banding pattern does not always distinguish between species. Sometimes another enzyme can be used but that does not always solve the problem (Kita-Tsukamoto et al. 2006).

Bej (1999) developed a multiplex PCR based detection method for *Vibrio parahaemolyticus* that targeted the virulence factor genes for thermolabile hemolysin (TLH), thermostabile direct hemolysin (TDH) and thermostabile direct hemolysin-related (TRH). He also showed that the possession of the *tdh* gene correlated with the Kanagawa phenomenon or the ability to lyse red blood cells (Bej et al. 1999; Miyamoto et al. 1969). Labreuche et al. (2012) demonstrated that his multiplex assay for amplification of *V. nigripulcritudo* and *V. penaeicida* would also function to type *V. nigripulcritudo* by displaying a band for a virulence plasmid (Labreuche et al. 2012).

Fluorescence in situ hybridization (FISH) is useful in enumeration as well as identification. Samples are dried on Teflon-coated slides and fixed. Samples are then hybridized to the appropriate oligonucleotide probe and counterstained. Counts can be done manually while viewing with a fluorescent microscope equipped with the proper filters or with the appropriate software (Eilers et al. 2000a).

The loop-mediated isothermal method (LAMP) utilizes four primers that target six regions of DNA in close proximity. The primers are designed to anneal between 60˚C and 65˚C and the products form a continuous stem and loop structure that continues

building on itself with each cycle. No thermocycler is necessary for the reaction and so much product is produced that it can be perceived by turbidity or with the aid of a dye. The detection limit is six copies of the targeted DNA sequence and is highly specific since four primers are used (Notomi et al. 2000). Cao et al. (2010) designed a LAMP method protocol to detect *V. harveyi*. The reaction ran at 65°C for one hour and could be read visually. Chang et al. (2011) has incorporated the LAMP protocol into a microfluidic, automated unit that is capable of detecting four pathogens at once (*Streptococcus agalactiae*, koi herpes virus, Iridovirus and *Aeromonas hydrophila*) with a detection limit of twenty DNA sequence copies (Chang et al. 2011).

Flow cytometry can also be used in some aquaculture settings to detect pathogens. Although expensive and not as specific as other methods without additional sample processing, assays can be completed in just a couple of minutes unless additional sample processing time is needed (Endo et al. 2000). Endo (2000) was able to distinguish two bacterial species by treating samples with a fluorescent antibody technique prior to processing (Endo et al., 2000). Microbial identification can also be made by utilizing fluorescent oligonucleotides targeting RNA or DNA (Davey and Kell 1996). With flow cytometry, it would be possible to increase the sample size and build a database of information about what changes in the community lead to outbreaks over time.

A group at Chulalongkorn University in Bangkok, Thailand developed an immunosensor for *V. harveyi* consisting of immobilized monoclonal antibodies bound to a gold electrode on a quartz crystal microbalance. The sensor can be regenerated with a hydrochloric acid solution and rinsed with distilled water for reuse. Testing for the target organism was specific and successful with a minimum detection level of 10^3 colony

forming units per mL (Buchatip et al. 2010). However, it has not been tested in-situ or against closely related organisms. Advancements in this area are greatly needed in aquaculture and food safety and represent the future of microbial detection.

Real-time qPCR (used in the study herein) is similar to standard PCR except that SYBR green or an internal, labeled probe is used on the target gene between the forward and reverse primer. This probe, of which there are several formats, allows the hardware to read the fluorescent signal which would theoretically increase exponentially with each reaction cycle and extrapolate the number of gene targets present in the sample. A comparison is made to a standard curve generated by an identical reaction mixture run against serial dilutions of known DNA concentrations. Real-time qPCR generates immediate results without the need for electrophoresis (Fierer et al. 2005).

Importance of *Vibrio* Control in Prevention of Seafood-Related Illness

Vibrio vulnificus is a cause of septicemia in the United States, particularly in those with hepatic cirrhosis or immune system disorders (Barton and Ratard 2006). Symptoms can also include shock, intestinal bleeding and bullous skin lesions (Morris and Nair 2011). This pathogen has been isolated in shrimp, crabs, fish, oysters, eels, etc. (Oliver 2005; do Nascimento et al. 2001). Gopal et al. (2005) was able to identify fifteen species of Vibrio out of aquacultured shrimp hemolymph samples in India, including *V. vulnificus*; many more were not identified.

Vibrio parahaemolyticus is responsible for a significant portion of seafood-borne illness worldwide. This pathogen usually causes gastroenteritis but can cause wound infections and uncommonly septicemia (Daniels et al. 2000). Consuming raw oysters during summer months increases the risk of disease from this pathogen (Daniels et al.

2000; DePaola et al. 2003; Kelly and Stroh 1988). In the years 2004-2006, over 11,000 clinical cases of the serotype O3:K6 pandemic clonal *V. parahaemolyticus* strain occurred in Chile. It was thought that the cause was a significant increase in water temperature (Fuenzalida et al. 2006).

Other closely-related organisms are present in seafood and can present a danger to consumers, especially if the seafood is raw, undercooked or contaminated. In Peru, *V. furnisii* was found in samples from fourteen people during an epidemiological study on cholera. Of the fourteen, only six were symptomatic with diarrhea (Dalsgaard et al. 1997). Strains taken from sixteen patients in Brazil were tested and found to possess cytolysins and hemolysin (Magalhães et al. 1993).In 2002, researchers in Mexico grew eighty-two strains of *Aeromonas* spp. from frozen Tilapia they had purchased. Many of the strains were virulent, possessing genes for hemolysins, proteases and other enzymes and antibiotic resistance (Castro-Escarpulli et al. 2003). In 1978, eleven people in Louisiana became infected with the El Tor variant of *V. cholerae,* after eating locally-caught, cooked crabs (Blake et al. 1980). After Hurricane Rita in 2005, a couple in Louisiana contracted *V. cholerae* after consuming cooked shrimp that had subsequently come into contact with the ice the raw shrimp had been stored on (CDC 2006).

CHAPTER II

INTRODUCTION

Aquacultured seafood is an increasingly important contributor to the world's food supply (FAO, 2010). Disease caused by bacteria in the genus *Vibrio*, or Vibriosis, causes billions of dollars in losses to the seafood farming industry each year (Lundin 1996). Control of this group of pathogens, some of which, like *Vibrio parahaemolyticus*, cause human disease, is not only essential for successful aquaculture but also for economic stability, food safety and consumer confidence in aquacultured products (FAO 2010).

Vibrios are ubiquitous in marine environments, commonly found in association with chitinous animals such as copepods, crabs and shrimp (Colwell 1984; Vanderzant et al. 1971). Some *Vibrio* spp. such as *Vibrio alginolyticus* are considered normal flora of Pacific White Shrimp, *Litopenaeus vannamei*, and are constantly present in the aquaculture tanks and ponds (Colwell 1984; Johnson et al. 2008). However, these bacteria are opportunistic pathogens that can overwhelm an animal weakened by stresses such as poor water quality, over-crowding, low oxygen (Moriarty 1997; Selvin and Lipton 2003; Sung et al. 1999) or poor nutrition (Lavilla-Pitogo et al. 1998). Environmental factors such as high ammonia (Liu and Chen 2004) and nitrite concentrations (Tseng and Chen 2004) as well as increasing temperature (Cheng et al. 2005) have also been shown to reduce shrimp immune system effectiveness, making them susceptible to disease.

Other water quality parameters in aquaculture waters may also select for *Vibrio* over other types of bacteria. High organic matter concentrations, low oxygen levels, high temperatures and high salinity have all been shown to increase the number of *Vibrio* spp.

present in the system (Baross and Liston 1970; Gopal et al. 2005; Noriega-Orozco et al. 2007; Pfeffer et al. 2003; Williams and Larock 1985). Chitin, a polymer of N-acetylglucosamine, is abundant in shrimp aquaculture waters as it is a main component of their exoskeletons (Souza et al. 2011). Chitin-rich shrimp molts can encourage *Vibrio* biofilms and support their growth, even under suboptimal conditions (Nahar et al. 2011). Additionally, it has been found that a higher abundance of *Vibrio* spp. can increase the risk of a disease outbreak (Cheng et al. 2005; Lavilla-Pitogo et al. 1998; Moriarty 1997; Sung et al. 1999). *V. parahaemolyticus* is considered a major pathogen of penaeid shrimp (Lightner 1975; Jayasree et al. 2006; Alavandi et al. 2006; Austin 2010). This study quantified deoxyribonucleic acid (DNA) copy numbers of total bacteria, total *Vibrio,* and *V. parahaemolyticus* in each raceway using real-time qPCR. The resulting DNA concentrations and the total quantity of DNA were compared to each tank's temperature and nutrient concentrations (ammonia, nitrite, nitrate, and phosphate) to investigate the conditions that may select for Vibrio spp., several species of which are potentially pathogenic. Bacterial data were also compared to the shrimp SGR in the raceways to determine their effects.

In intensive aquaculture systems , high nutrient levels result in the generation of bio-floc, macroscopic aggregates consisting of detritus, bacteria, protozoa, algae, fungi and inorganic particles (Burford et al. 2004; Johnson et al. 2008). Microorganisms in the bio-floc take up materials such as nitrogenous compounds which are then recycled as the bio-floc is consumed by shrimp (Burford et al. 2004; Johnson et al. 2008; Ray et al. 2010) thereby lowering feed costs (Crab et al. 2007). However, bio-floc contributes to the total biomass in the system and can increase oxygen demand and consumption of

alkalinity (Ray et al. 2010). In this study, eight aquaculture raceways were randomly divided into four high and four low rates of bio-floc removal using settling chambers as in Ray et al. 2011. Quantified DNA copy numbers of targeted bacterial groups were analyzed to determine the effect of high and low solids removal on bacterial populations. The amount of DNA present in the sample and the SGR were also compared across the treatments.

The objectives of this study were as follows:

1. To determine the effects of the high and low solids treatments on total DNA µg/µL, eubacteria copy number, *Vibrio* genus copy number, *V. parahaemolyticus* (Vp) copy number.
2. To examine correlations between tank parameters (temperature and nutrient concentrations) and variations in the bacterial community and their possible effect on shrimp growth rate.

Although *Vibrio* are constantly present in shrimp aquaculture systems, their dominance in the bacterial community may be indicative of poor water quality. Many of the same environmental conditions that can increase Vibrio populations can also reduce shrimp disease resistance. Information gathered about the objectives of this study could help improve control of bacterial populations by confirming which parameters positively influence growth rate while reducing risk of disease in shrimp, aquaculture workers and consumers. Furthermore, optimizing the amount of nutrient export via solids removal is a valuable tool in maintaining the best water quality possible. It is hoped that an improved understanding of the factors that shape the bacterial community will result in a more preventative rather than reactive strategy in controlling Vibrio populations.

CHAPTER III

MATERIALS AND METHODS

Sample Processing

Eight aquaculture raceways at the Gulf Coast Research Laboratory (GCRL) were sampled each week throughout a thirteen-week growing season from May 18 to August 9, 2010 (Figure. 2). Four randomly-selected tanks had a higher rate of bio-floc removal by varying the flow rate through large settling chambers, resulting in four high solids and four low solids raceways (Ray et al. 2010). 25-mL water and floc samples were scooped using sterile, graduated, 50-mL conical vials, taking care to avoid any floating mats. Samples were vortexed for 30 seconds and then allowed to settle for 5 minutes. Five mL of settled bio-floc was taken from the bottom of the vial with a serological pipet and used to fill 3 labeled 1.5 mL centrifuge tubes. The tubes were centrifuged for 10 minutes at 14,100 x g. The supernatant was poured off and the remaining sample material was aseptically combined into a new tube by pipetting. The 3 samples combined filled one 1.5 mL centrifuge tube which was stored at -20° C until DNA extractions could be performed. Concentrating the samples in this way helped ensure that the DNA concentrations would yield results above the detection limit for the quantitation method.

Figure 2. Shrimp Raceways at the Gulf Coast Research Lab in Ocean Springs, Mississippi.

Bulk DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen Corp., USA). After thawing, samples were lysed overnight at 56 °C, vortexing occasionally with 180 µL buffer ATL and 40 µL proteinase K. After lysing, 4 µL of RNase A (100 mg/ml solution, Qiagen Corp., USA) was added with pulse vortexing and incubated for two minutes as directed. 2 µL of the resulting DNA solution was placed on analyzer of the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Inc. USA) in order to measure total DNA concentrations.

Nutrients

All nutrient samples were processed by GCRL aquaculture staff. Below surface water samples were taken near pump intakes. Ammonia-nitrogen (NH_3-N) concentrations were measured using Hach method 8155. This test can detect NH_3-N in a range of 0.01-0.50 mg/L (Hach Company, Loveland, Colorado, USA). Although the term, NH_3-N, denotes the equilibrium between ammonia and ammonium, this variable is

also referred to as ammonia throughout the study, as that is the toxic compound of interest.

Nitrite concentrations were detected colormetrically as in Strickland and Parsons (1972). The range of the test is 0.46 – 115.01 µg/L. Acceptable errors are 0.23 – 1.38 µg/L depending on the amount of NO_2 present in the sample (Hach Company, Loveland, Colorado, USA).

Total nitrite and nitrate concentrations were measured with a chemiluminescence nitrogen analyzer according to Braman and Hendrix (1989). Nitrate concentrations were determined by subtracting nitrite concentrations from the total test results. The detection limit for nitrite-nitrogen with this test is 2-3 ng.

Real-time qPCR

Molecular methods are increasingly chosen over culture-based methods for their ease of use and enhanced sensitivity and accuracy. Culture-based methods can be time-consuming requiring media preparation, multiple dilutions and plating of samples and long incubation periods, sometimes weeks for isolates that are difficult to grow (Matulewich et al. 1975). Molecular methods are more sensitive, particularly for species that exist in low numbers like *V. parahaemolyticus* that normally require an enrichment step by incubating overnight in a stimulating broth to increase their numbers (Thompson et al. 2004). Culture-based methods are only capable of enumerating bacteria that are not dormant and are able to grow on the media selected for the study. The resulting discrepancies can be substantial. For example, according to most studies conducted with culture-based enumeration, *Vibrio* represent approximately 10% of culturable bacteria. In contrast, most studies using molecular methods show that *Vibrio* are less than 1% of

all bacteria (Thompson et al. 2004). The difference of these results is likely a reflection of the ease with which *Vibrio* spp. grow on commonly-used bacterial media and that so many other bacteria are not culturable. For this study, *Vibrio* spp. may have been easily quantified with traditional methods but error associated with the data for total bacteria would have been unacceptably large. When identifying bacteria, accuracy is improved with molecular methods, especially with isolates that are difficult to identify phenotypically like *V. harveyi* (Cano-Gomez et al. 2009). Real-time qPCR is relatively quick and easy to perform compared to older molecular methods, requiring only DNA extraction, sample preparation and PCR. The results are read during the PCR amplification, eliminating additional steps that would each introduce variability. A few disadvantages of using molecular methods are expense, difficulty in designing and validating primers and that not all DNA present in the sample is from physiologically active or alive organisms. In this case, the advantages for real-time qPCR outweighed the disadvantages.

Analysis of rDNA been used for bacterial community analysis for years using methods such as hybridization, fluorescence and PCR (Zhang and Fang 2006). Ribosomal DNA (rDNA), sometimes referred to as functional genes or rRNA genes, are the genes that code for ribosomal RNA and are part of the genome, not the ribosome itself. The slowly-evolving nature of these genes make them ideally suited for bacterial identification and enumeration (Weisburg et al. 1991). However, the number of copies of rDNA per cell must be known in order to determine the number of cells present in the sample (Bach et al. 2002). Nonetheless, rDNA quantification is commonly used to

determine the overall genetic abundance of a target bacterial group (Zhang and Fang 2006).

Validated primer sets (Table 1) were chosen to amplify 16S rDNA specific to Eubacteria (Fierer et al. 2005) and the *Vibrio* Genus (Thompson et al. 2004). A primer and probe set was used to target *V. parahaemolyticus* (Nordstrom et al. 2007). For specificity, the pre-optimized annealing temperatures were not altered. Amplification products for the standard curve were located using BLAST (Basic local alignment search tool: http://www.ncbi.nlm.nih.gov/BLAST/). The resulting oligonucleotide sequence was ordered as an "Ultramer" from IDT (Integrated DNA Technologies, Inc., USA).

Table 1

Primers and Products.

Target	Name (s)	Sequence	Annealing Temp °C	Reference
Eubacteria	Eub338 F Eub518 R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	53	Fierer 2005
5- **[ACTCCTACGGGAGGCAGCAG]** TGGGGAATATTGGACA ATGGGCGAAAGCCTGATCCAG CCATGCCGCGTGTGTGAAGAAGGTCTTCGG ATTGTA AAGCACTTTAAGTTGGGAGGA AGG GCAGTAAGTTAATACCTTGCTGTTTTG ACGTTACCAACAGAATAAGCACCGGCTAACTTCGTG **{CCAGCAGCCGCGGTAAT}** -3				
Vibrio Genus	567 F 680 R	GGCGTAAAGCGCATGCAGGT GAAATTCTACCCCCCTCTACAG	64	Thompson 2004
5- **[GGCGTAAAGCGCATGCAGGT]** GGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTG GCAGACTAGAGTA **{CTGTAGAGGGGGGTAGAATTTC}** -3				
V. parahaemolyticus	tlh F tlh R	ACTCAACACAAGAAGAGATCGAC AAGATGAGCGGTTGATGTCCAA	59	Nordstrom 2007
V. parahaemolyticus	tlh probe	/56-FAM/CCGCAAATACCTACGGG TCAAAGAGGG/3BHQ_1/	59	Nordstrom 2007
5- [ACTCAACACAAGAAGAGATCGACAA] ATTCGTGCGAAAGTGCTTGAGATGAACGAGTTCATCAAGGCACAAGCGATGTACTACAAA GCGCAAGGTTATAACATCACGTTGTTTGATACTCACGCCTTATTCGAGACGCTAACTTCT GCGCCAGAAGAGC'**ACGGTTTCGTGAACGCGAGCG**'ATCCTTGT **{TTGGACATCAACCGCTCATC}** -3				

Note: Each primer set is shown followed by the synthesized product with the [forward], {reverse}, and 'probe' binding sites marked.

Real-time qPCR was performed on the extracted DNA using the LightCycler 480 system (Roche Diagnostics Corp., USA). The reactions were run in triplicate on 96 well plates using each primer set and SYBR Green 1 Master Mix or the 480 Probes Master Mix for *V. parahaemolyticus* (Roche Diagnostics Corp., USA). Standard curves for the real-time absolute quantitation analysis were constructed using serial dilutions of known quantities of each primer product.

Analysis

Eubacteria, *Vibrio* genus and *V. parahaemolyticus* DNA copy numbers for all the samples were calculated in triplicate with the Roche LightCycler software using the absolute quantitation, Second Derivative Maximum method (Roche Diagnostics Corp., USA). The means of these triplicate values were used for statistical analysis. The Total DNA, Eubacteria DNA copy number, *Vibrio* Genus copy number and *V. parahaemolyticus* copy number (Vp) for the triplicate samples were calculated, averaged and used for further analysis with JMP 10 (SAS Institute Inc., North Carolina, USA). The specific growth rate (SGR) of shrimp for each of the raceways was also calculated using weight data obtained from Ray et al. 2011 with the following formula:

$$SGR = \frac{(\ln w_2 - \ln w_1)}{(t_2 - t_1)} \times 100$$

Where ln= natural log, w_1= initial weight at time t_1 and w_2= final weight at time t_2 (Immanuel 2004).

Most of the variable distributions were lognormal; therefore, Log 10 transformations were performed. All of the bacterial count distributions and Total DNA were brought into normality with this method. Ammonia was greatly improved but was still not normal. Nitrite and SGR were only slightly abnormal and were worsened by the

transformation. The result, however, was close to normality with a slight skew and acceptable kurtosis. Temperature, phosphate concentrations and TSS were normal before and after transformation (data not shown).

Multiple analysis techniques were employed to analyze the dataset. Differences in bacterial count and SGR data between treatment groups were analyzed using repeated measures ANOVAs. Least squares linear regressions were used to detect possible relationships between the quantified bacterial and nutrient concentrations. Additional regressions were performed by treatment group. A stepwise forward multiple regression was employed to elucidate variable interactions and reduce the number of terms.

CHAPTER IV

RESULTS

Table 2

Descriptive Statistics for Study Data.

Variable	Mean	SD	Range
EB copies/mL	$1.86x10^{13}$	$3.49x10^{13}$	$1.51x10^{5} - 2.1x10^{14}$
VG copies/mL	$8.81x10^{6}$	$1.15x10^{7}$	$1.19x10^{5} - 9.23x10^{7}$
Vp copies/mL	$2.92x10^{3}$	$4.97x10^{3}$	$6.67x10^{2} - 2.42x10^{4}$
DNA ng/μL	7.18	4.51	0.73 – 23.61
SGR	3.48%	2.78	-1.46 – 10.60%

Note: Total DNA and total bacteria, *Vibrio* genus and *V. parahaemolyticus* DNA copy numbers are stated per mL of concentrated sample. The specific growth rate is given as a percentage. All data is untransformed.

High and Low Solids Treatments

Total bacteria DNA copy numbers trended upwards initially, then fell rapidly and stayed relatively stable for the rest of the study period (Figure 3). Eubacteria DNA copy numbers were significantly higher in the low solids raceways (p=0.0081). *Vibrio* genus DNA copy numbers were highest at the beginning of the season and trended downwards while *V. parahaemolyticus* was undetectable early in the study and increased, becoming a higher proportion of the *Vibrio* population (Figures 4 and 5). Although percentage of DNA copy numbers of *V. parahaemolyticus* as part of the *Vibrio* genus was higher in the low solids tanks, 0.02% in the high solids tanks and 0.04% in the low solids tanks, there were no significant differences in the *Vibrio* populations between the treatment groups.

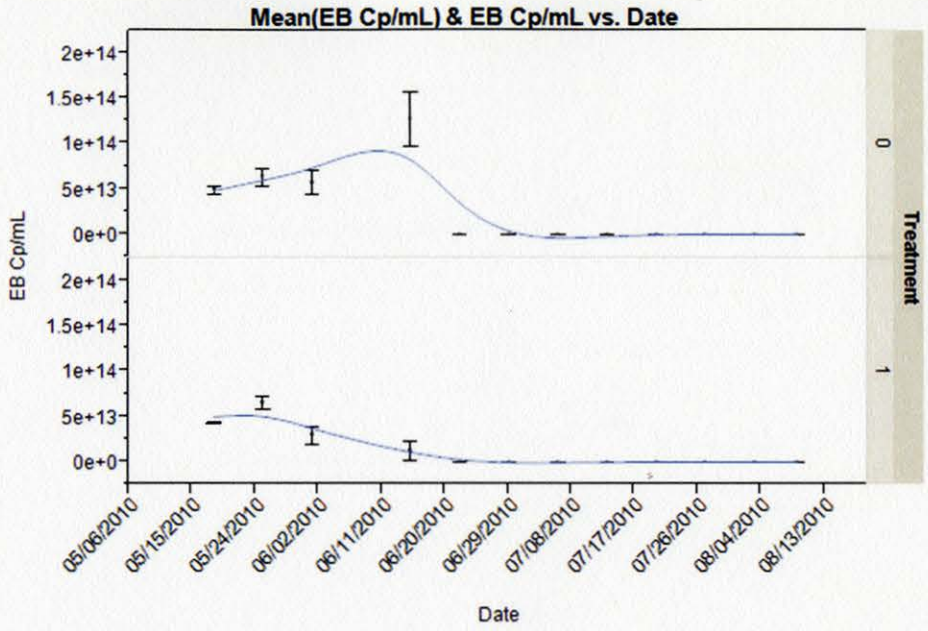

Figure 3. Total Bacteria Between Treatment Groups. Eubacteria DNA copy number between the low (0) and high (1) solids treatment groups. Each error bar is constructed using 1 standard error from the mean.

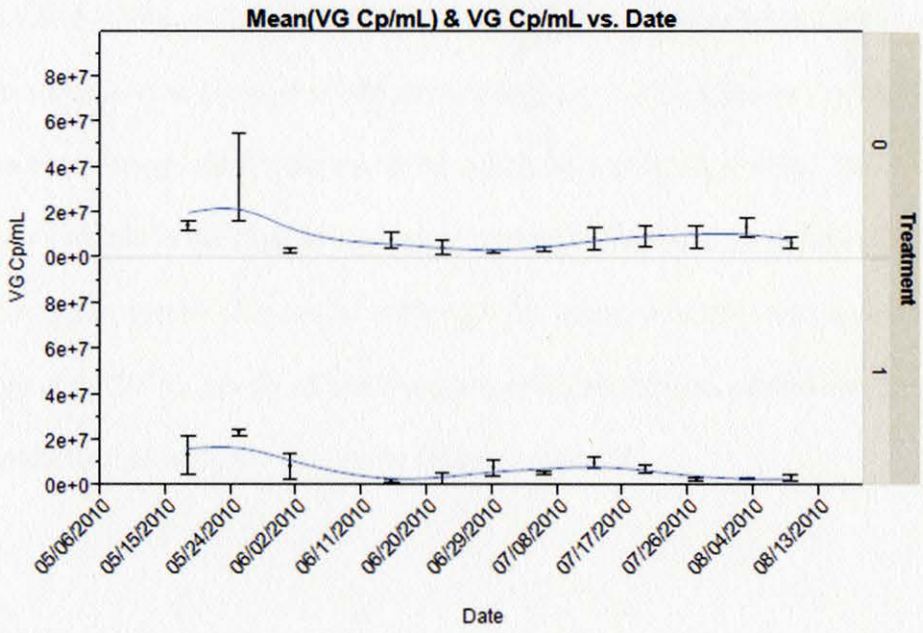

Figure 4. Vibrio Genus Between Treatment Groups. *Vibrio* genus DNA copy number between the low (0) and high (1) solids treatment groups. Each error bar is constructed using 1 standard error from the mean.

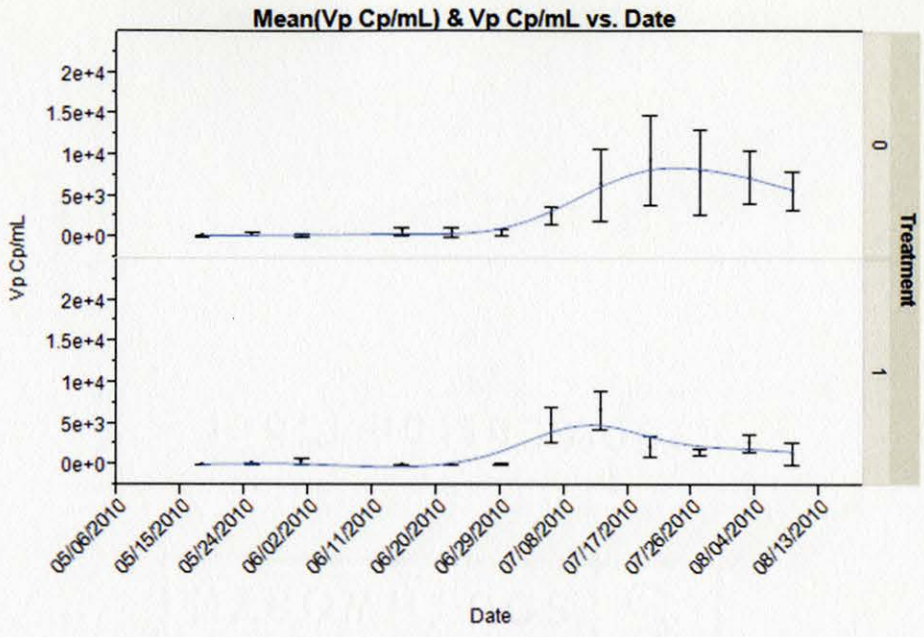

Figure 5. V. parahaemolyticus Between Treatment Groups. *V. parahaemolyticus* DNA copy number between the low (0) and high (1) solids treatment groups. Each error bar is constructed using 1 standard error from the mean.

Total DNA concentrations were significantly higher in the high solids raceways (p=0.0062, Figure 6). In addition, the data shows increased variability in DNA concentrations in the high solids treatment group. In both groups, however, DNA concentration gradually increased throughout the growing season. The SGR was also more variable in the high solids group, although there was no significant difference in growth rate overall (Figure 7). Although the water chemistry data is part of another study (Ray et al. 2011), graphs of statistically significant factors, nitrite, nitrate, ammonia and phosphate, are included for clarity (Figures 8 and 9).

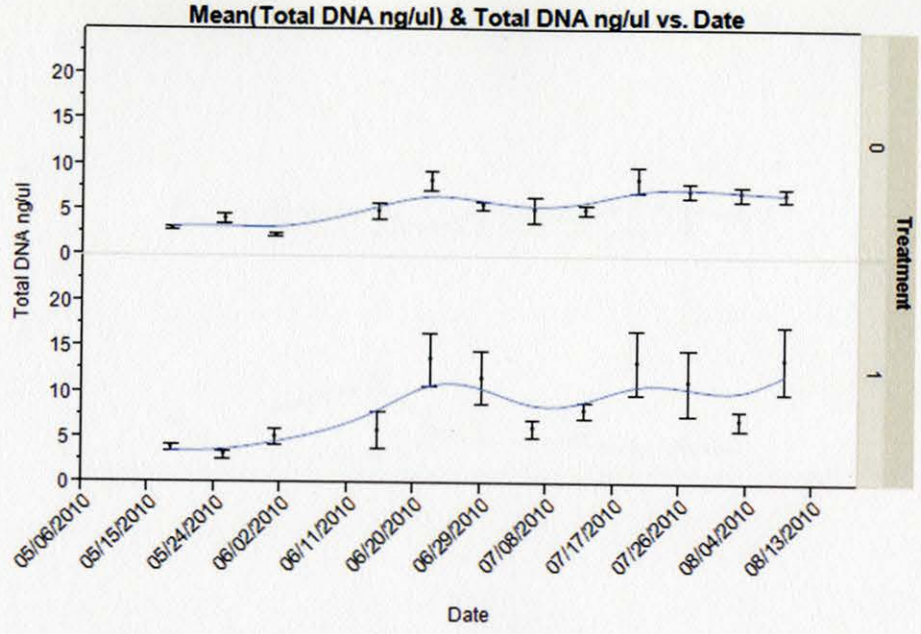

Figure 6. DNA Concentrations Between Treatment Groups. DNA concentrations between the low (0) and high (1) solids treatment groups. Each error bar is constructed using 1 standard error from the mean.

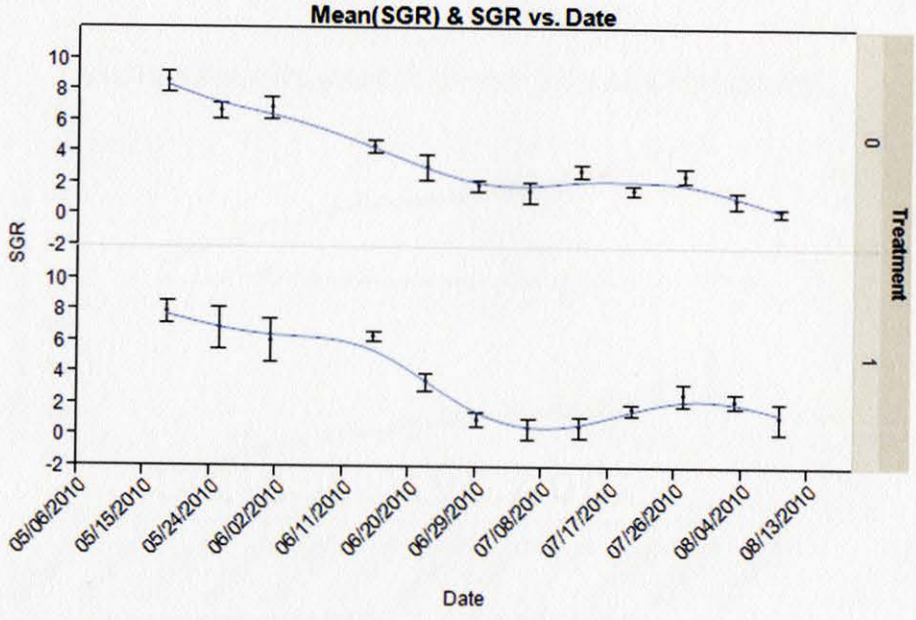

Figure 7. Specific Growth Rate Between Treatment Groups. Specific growth rate as a percentage between the low (0) and high (1) solids treatment groups. Each error bar is constructed using 1 standard error from the mean.

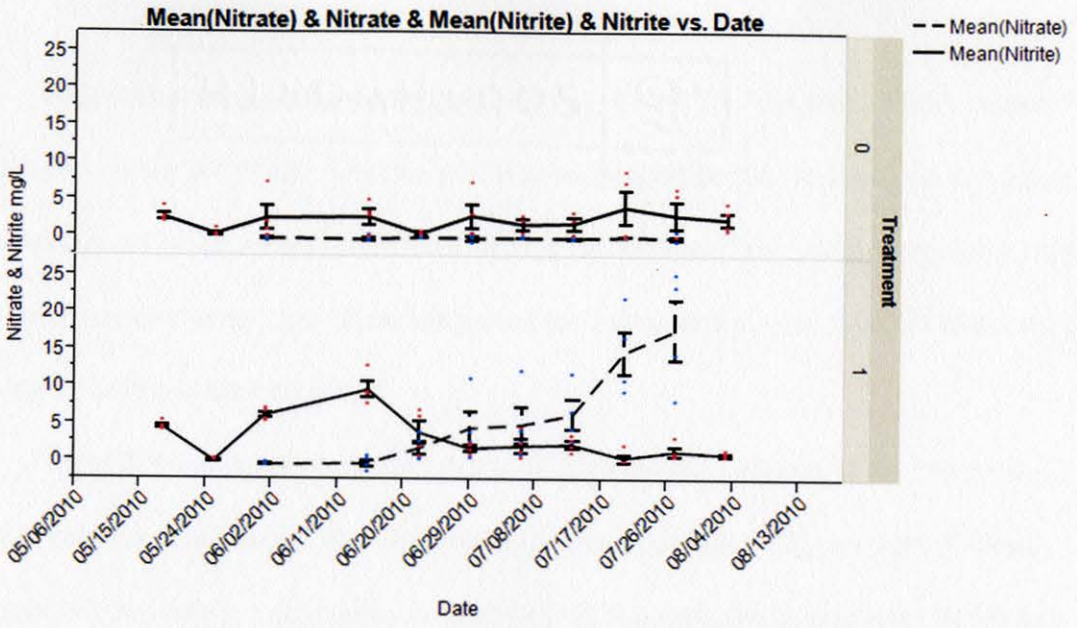

Figure 8. Nitrite and Nitrate Concentrations Between Treatment Groups. Nitrite and nitrate concentrations were higher in the high (1) solids treatment group (p=0.007 and p=0.000, respectively). Data from Ray et al. 2011. Each error bar is constructed using 1 standard error from the mean.

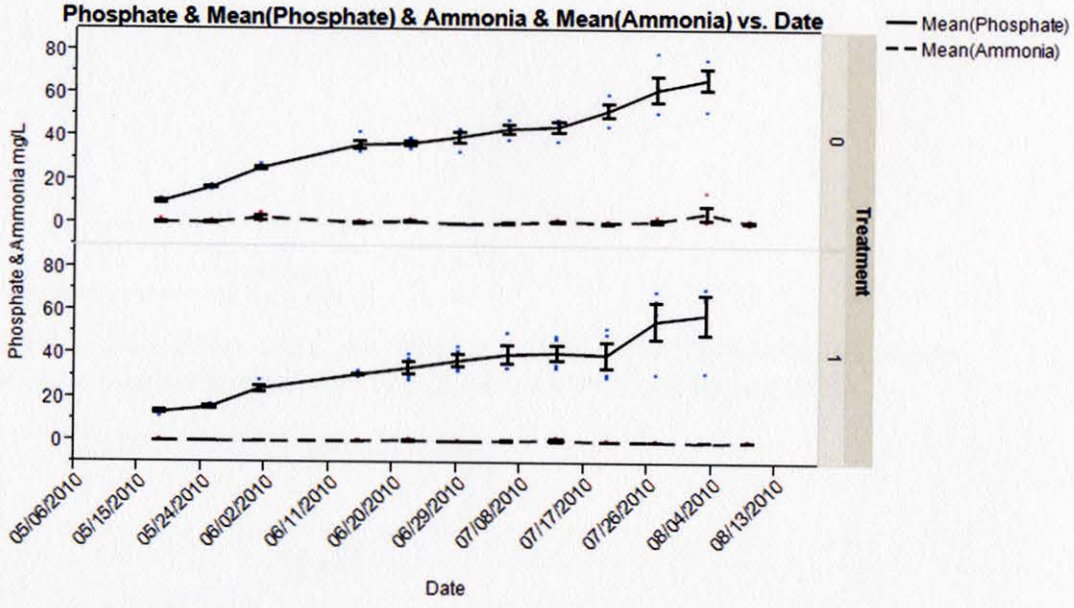

Figure 9. Phosphate and Ammonia Between Treatment Groups. Phosphate and ammonia concentrations were higher in the low (0) solids treatment group (p=0.003 and p=0.021, respectively). Data from Ray et al. 2011. Each error bar is constructed using 1 standard error from the mean.

Regression Analysis

Regression analysis was performed on total DNA, total bacteria, *Vibrio* genus, *V. parahaemolyticus* and SGR. The analysis was performed on the total data set in addition to the treatment group subsets to determine how the high and low solids treatments affect the significance or effect size of each regression. The treatments and overall effect sizes are similar unless otherwise stated.

Total DNA was not correlated to nitrite or ammonia concentrations. However, a slightly positive relationship was observed with nitrate (R^2=0.37, Figure 10). Further evaluation revealed the relationship to exist only in the high solids treatment (R^2=0.44). DNA was positively correlated to temperature only in the low solids treatment (R^2=0.20) but was related to phosphate in all groups (R^2=0.26, Figure 11).

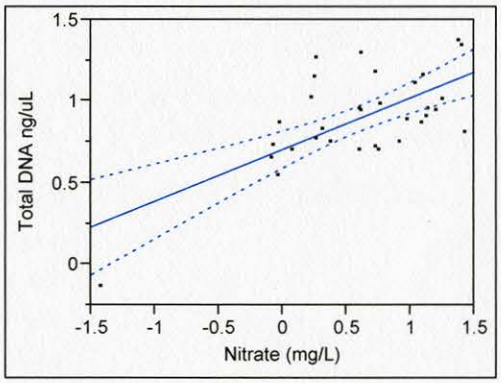

Figure 10. Total DNA (ng/µL) by Nitrate (mg/L) in the High Solids Treatment (R^2=0.44). Graphical results are displayed with 95% confidence intervals.

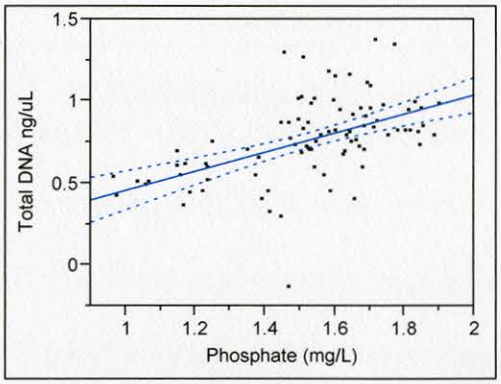

Figure 11. Total DNA (ng/μL) by Phosphate (mg/L) in both Treatments (R^2=0.26). Graphical results are displayed with 95% confidence intervals.

Analysis of total bacteria DNA copy numbers did not yield a relationship with nitrite or ammonia. Nitrate had a negative relationship with total bacteria (R^2=0.23) which was shown to be only in the high solids treatment (R^2=0.51, Figure 12). Bacteria appeared to be negatively affected by temperature (R^2=0.10) in all groups and phosphate, particularly in the high solids treatment (R^2=0.63, Figure 13).

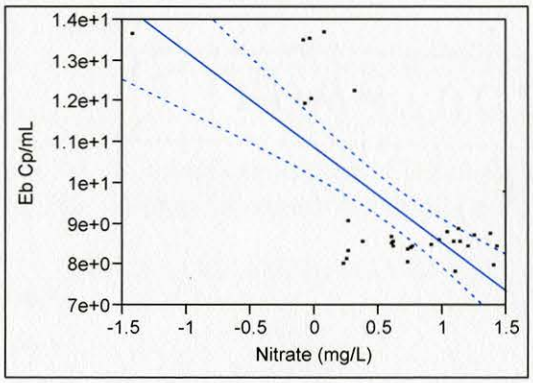

Figure 12. Total Bacteria (Copies/mL) by Nitrate (mg/L) in the High Solids Treatment (R^2=0.51). Graphical results are displayed with 95% confidence intervals.

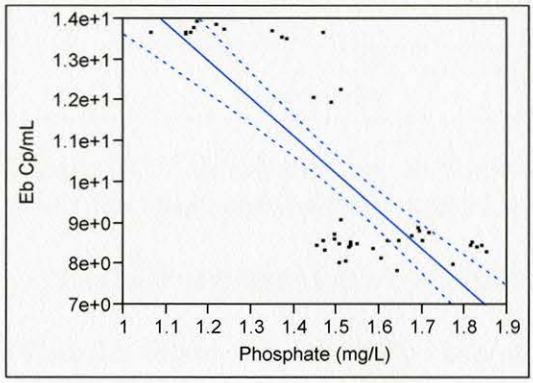

Figure 13. Total Bacteria (Copies/mL) by Phosphate (mg/L) in the High Solids Treatment (R^2=0.63). Graphical results are displayed with 95% confidence intervals.

The *Vibrio* genus DNA copy numbers were only correlated with phosphate (R^2=0.07). This negative effect only appeared in the high solids treatment (R^2=0.10). *Vibrio parahaemolyticus* DNA copy numbers were not related to nitrite, but appeared to

be positively influenced by nitrate (R^2=0.07) only in the high solids treatment (R^2=0.26) and ammonia in the low solids treatment (R^2=0.07). Positive correlations were also found with temperature (R^2=0.28, Figure 14) and phosphate (R^2=0.29, Figure 15).

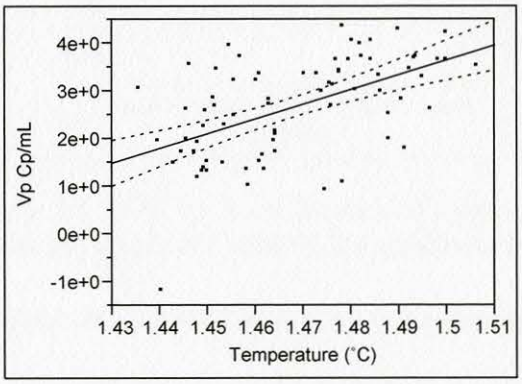

Figure 14. V. parahaemolyticus (Copies/mL) by Temperature (°C) in Both Treatments (R^2=0.28). Graphical results are displayed with 95% confidence intervals.

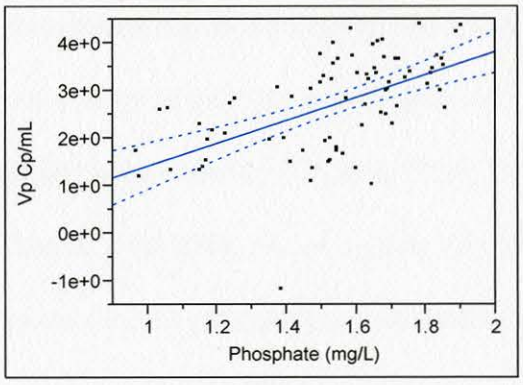

Figure 15. V. parahaemolyticus (Copies/mL) by Phosphate (mg/L) (R^2=0.29). Graphical results are displayed with 95% confidence intervals.

The SGR displayed a positive correlation only with total bacteria DNA copy numbers (R^2=0.43, Figure 16). This effect was slightly stronger in the low solids (R2=0.51) than the high solids (R2=0.37). SGR was negatively associated with total DNA (R2=0.10) and V. parahaemolyticus DNA copy numbers (R2=0.10). No significant relationship could be determined between SGR and the *Vibrio* genus DNA copy numbers.

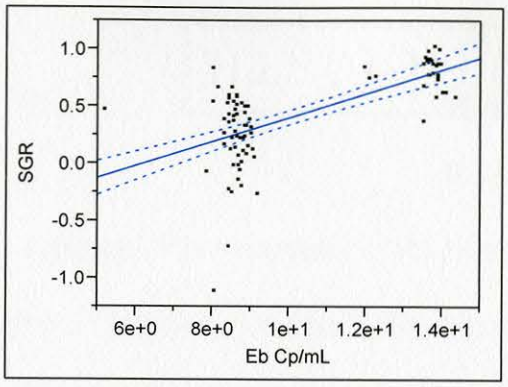

Figure 16. SGR by Total Bacteria (Copies/mL) in both Treatments (R^2=0.43). Graphical results are displayed with 95% confidence intervals.

Stepwise forward multiple regression analysis was performed to create models of the factors predicting each segment of the bacterial community. The model for total bacteria DNA copy numbers showed that measured parameters explained 65% of the variance in this group (adj. R^2=0.65, F(8,47)=13.54, p<0.0001). Significant predictors in ascending order were nitrate*phosphate (β=0.3063, p=0.0148), temperature (β=0.3134, p=0.0024), DNA*nitrate (β=-0.3228, p=0.0167), DNA*phosphate (β=0.3317, p=0.0092), nitrate (β=-0.3436, p=0.0059) and phosphate (β=-0.5686, p=<0.0001). The model for the *Vibrio* genus did not yield a significant result. However, 45% of the variance in *V. parahaemolyticus* DNA copy numbers could be explained with this method (adj. R^2=0.45, F (6,33)=6.30, p<0.0002). Factors affecting the result were nitrite (β=0.2943, p=0.0324), DNA (β=-0.3476, p=0.0482), nitrate*phosphate (β=-0.4165, p=0.0045), nitrate (β=0.4421, p=0.0090) and phosphate (β=0.5730, p=0.0020).

CHAPTER V

DISCUSSION

Relationships

Figure 17 is a summary of the relationships in the data found by linear regression analysis. Visualizing the data in this way helps illuminate the pathways whereby SGR can ultimately be affected and the complexity of the interactions between the variables. Increasing DNA and *V. parahaemolyticus* DNA copy number concentrations both had a small negative effect on SGR. Pathogenic *Vibrio* spp. have been shown to inhibit shrimp growth rate (Cano-Gomez et al. 2009; Lavilla-Pitogo et al. 1998). Increasing total bacterial DNA copy numbers, however, was the only positive effect on SGR. This supports the idea that bacterial growth serves as a food source for shrimp (Burford et al. 2004; Hargreaves 2006; Kuhn et al. 2009). In addition, we know that carbon addition increases the bacterial population and contributes to shrimp growth (Hari et al. 2004; Schneider et al. 2005). Therefore, increasing nitrate and phosphate would reduce SGR by increasing DNA and *V. parahaemolyticus DNA copy numbers* and reducing the copy numbers of total bacteria. Increasing temperature also had a negative effect on SGR through its negative effect on total bacteria DNA copy numbers and positive effect on *V. parahaemolyticus* DNA copy numbers. Temperature has long been associated with an increase in *Vibrio* spp. (Baross and Liston 1970a; Noriega-Orozco et al. 2007; Thompson et al. 2004; Johnson et al. 2010). It is possible that these relationships could be indicators of stress. An increase in temperature would also reduce the amount of available oxygen which could stress shrimp and limit their growth. This study confirms the well-known idea that high nutrient concentrations reduce the health of shrimp but also

demonstrates that a possible mechanism for shrimp growth inhibition is through the manipulation of the bacterial community.

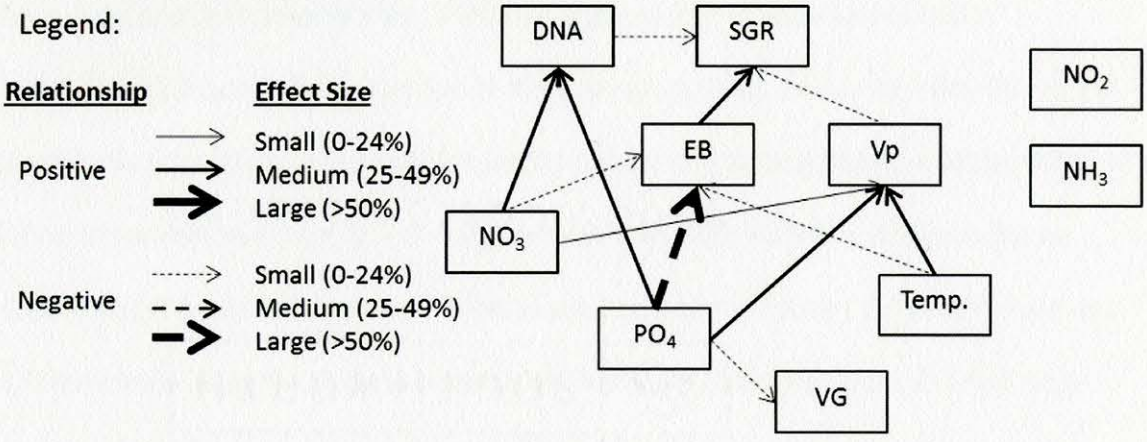

Figure 17. Summary of Significant Relationships. Regression effect sizes as illustrated by the font size of arrows. Positive effects are shown with a solid arrow and negative with a dotted arrow.

Overall Trends

Total bacteria DNA copy numbers displayed a precipitous drop from week five to seven, calling results into question. However, a closer inspection of other parameters during this time period shows that the raceways were in a period of flux. In week five, nitrite concentrations were beginning to fall and nitrate was beginning to accumulate, signaling the end of the cycling period. These changes were accompanied by spike in TSS and then a sharp reduction, dropping 27% from week six to week seven (Ray et al. 2011). Temperatures were rising and oxygen was falling, demanding constant liquid oxygen additions after week five (Ray et al. 2011). Total DNA concentrations also spiked on week 6 and were reduced by week 9. Nitrate and temperature were both shown to have a negative effect on total bacteria DNA copy numbers. The drop in bacteria was more pronounced in the low solids raceways, perhaps because there appeared to be more bacteria present. It is also possible that a large shift in the makeup of the bacterial

community to a group that has less rDNA gene target copy numbers per cell could create the appearance of a larger decrease in abundance than what actually occurred.

DNA copy numbers representing *Vibrio* as a genus and *V. parahaemolyticus*, contrastingly, began a sharp increase in week seven, perhaps due to the reduction in the number of competitors, increased temperature, increased shrimp biomass or the ability of *Vibrio* to survive and grow in low oxygen conditions. The rDNA abundance should directly relate to the cell counts of V. parahaemolyticus and closely relate to the amount of Vibrio in the samples as there is much less variation in the numbers of rDNA copy numbers per cell in these smaller groups.

High and Low Solids Treatments

One would expect the total DNA to be higher in the high solids tanks, due to the increased biomass present in the high solids raceways. However, the higher numbers of rDNA templates for total bacteria in the low solids tanks were surprising. Explaining this phenomenon through data analysis is not entirely possible in this study. Although higher nitrates in the high solids raceways would have depressed bacterial growth, perhaps through competitive exclusion, higher phosphates in the low solids tanks would also have reduced the numbers of bacteria. The action of the settling chambers may have influenced the bacterial population by removing algal and fungal competitors. Although bacteria can lyse algae and feed on dead algae or their extracellular products, they may also be inhibited by algae through the depletion of nutrients. Some algal species produce antibiotics to inhibit competition (Cole 1982). Larger amounts of putative algal and fungal species were observed in the high solids tanks as the growing season progressed but were not measured. Some fungal species, (*Rhodotorula sp., Saccharomyces sp.,*

Candida sp., Penicillium sp., mycelia sterilia,), isolated from tilapia green water grow out tanks were shown to inhibit *V. harveyi* (Lio-Po et al. 2005). It is possible that heretofore unknown inhibitory products could have been released by algal and fungal species when in high densities that selectively inhibited bacterial populations in the high solids raceways.

The accumulation of ammonia in the low solids tanks suggests the inhibition of nitrification, possibly due to carbon addition (Zhu and Chen 2001). The high rate of solids removal would have reduced the phytoplankton population and lessened their ability to absorb ammonia, placing a higher burden for waste processing on the bacterial community. Moreover, the higher quantity of bio-floc aggregates in the high solids raceways presented a vast network of surface area for bacterial communities to colonize, increasing their efficiency through attachment and better access to nutrients, similar to the human body's use of capillary networks in addition to arteries and veins. The lower ammonia in the high solids tanks illustrates the increased capacity for absorption by the total microbial community in the high solids raceways. Higher nitrate in the high solids raceways demonstrates the completion of nitrification. However, the buildup of nitrite in the high solids tanks also suggests nitrifier inhibition, possibly by low oxygen caused by increased oxygen consumption in the bio-floc. Regardless of the reason, settling chambers could be adjusted to decrease solids retention in order to lower ammonia concentrations.

Regression Models

The multiple regression models that were created in this study did not explain all of the variance in the bacterial community's total DNA or DNA copy numbers (65% for

total bacteria and 45% for *V. parahaemolyticus*). More data is needed to fully explain the conditions that select for *Vibrio* or increase total bacteria. For example, full-time oxygen monitoring would reveal times of oxygen depletion that could change the makeup of the microbial community. Several terms, including interaction terms, were included in each model, illustrating the complex relationships that exist. The importance of variables like phosphate and nitrate is demonstrated by their presence as both individual and interaction terms.

Optimizing the Process

Perhaps the best way to reduce stress and the incidence of disease in livestock would be a multi-faceted approach. We already use carbon supplementation and should continue to do so. It would be desirable to lower the protein content in our feed at the beginning of the grow-out and transition to the higher protein feed to keep nitrogenous waste at a minimum. Our water flow has been increased but perhaps aeration could be improved so that flow and aeration are optimized to function similarly to a BAL system. This would keep sludge suspended for collection and prevent the formation of anoxic zones and possibly microzones. The use of a good probiotic mixture would compete with pathogens and prevent them from gaining a foothold. A balanced approach to solids removal would maintain enough bio-floc particulate matter to process the waste being generated by the livestock and would also serve as supplemental feed. Lastly, integrated aquaculture using one of the outdoor ponds to grow seaweeds would yield an extra valuable product while processing nitrogenous waste products in the used raceway water (Neori et al. 2004; Schneider et al. 2005; Crab et al. 2007). This could be done in tandem with the shrimp aquaculture although they would not have to be permanently connected

systems to reduce the risk of spreading disease. However, this could be used as a reservoir of reduced nutrient, high oxygen content water that could be utilized in the event of an outbreak or to improve water quality. The multifaceted approach of carbon addition, flow and aeration, lower protein feed, probiotic, reduced solids removal and integrated aquaculture are useful because they focus on prevention of the ecological conditions that lead to disease outbreaks. Once the outbreak occurs, treatment options such as antibiotics carry negative consequences in the form of the creation of antibiotic resistant bacteria. The BAL systems and others like them were able to increase heterotrophic bacteria while managing nutrients to a certain extent and keeping *Vibrio* populations to a minimum. This seems to be the key to success.

Future Work

A environmentally sustainable future in which aquaculture can meet the world's growing food needs hinges on making significant advancements in four key areas:

1. Rapid detection of pathogen species
2. Increased control over microbial community members
3. Increased use of modular, integrated aquaculture units operated in series to recycle proteins
4. Quality control/food handling techniques to eliminate human pathogens in food products

Mitigating an outbreak caused by a suspected pathogen requires a quick diagnosis. For example, having bacteriophage for *V. harveyi* on hand is of no benefit if the detection method takes three days. Inexpensive, quick assays that can be done with minimal equipment would be ideal. Of the methods reviewed here, a machine using a

LAMP protocol and SYBR green programed to enumerate target pathogens would be my chosen method for development. This machine would function as a real-time PCR machine without needing a thermocycler or expensive reagents. A machine like this could have results in less than six hours including DNA extraction. Another aspect of detection in need of development is specificity. For certain pathogens, for example, *V. harveyi*, primers are still needed that do not also amplify other species.

Ultimately, carbon addition, aeration, high water flow, probiotics and nutrient control are all attempts at controlling the members of microbial community. By keeping oxygen levels up, for example, the aquaculturalist hopes to keep his livestock healthy but also to not select for facultative anaerobes such as *Vibrio* in the pond or raceway. Probiotic bacteria are administered in order to competitively exclude undesirable organisms with these preferred ones. In each case, standard ecological principles are used to guide the composition of the community members in order to have an effect on the health and growth rate on the macroinhabitants. With advanced monitoring capabilities like parameter monitoring probes and flow cytometry, exciting opportunities exist to gather real-time data relating environmental changes to differences in microbial populations. Robust data flows, unlike once-per-week snap shots could greatly improve our understanding of the complex relationships between the various community members and how they might better be controlled.

Aquaculture has evolved from a business model that involved destroying wetlands and dumping high-nutrient wastewater into one that uses open-water systems or land-based recirculating systems. The next step is to incorporate integrated, modular systems that re-use the waste products produced as an input in the next module. The initial

investment of protein feed is reused. Integrated systems are already in use, usually featuring seaweeds in combination with fish, shrimp or bivalves (Neori et al. 2004; Pang et al. 2006;Wang 2003) However, one hypothetical system includes five modules. Examples of what can be included in the modules include fish, shrimp, macro and microalgae, worms, bivalves and bacteria (for nutrient processing) (Schneider et al. 2005). Ecological concerns demand that we find a way to get more aquacultured product out of the protein sources such as menhaden that we remove from our environment. These types of recirculating systems will process most of the nutrients resulting in less stress and less likelihood of disease.

With fisheries in decline, advances in aquaculture are essential in making sure that we are able to provide high-quality, disease-free products (USCOP 2004). Technology is an important part of these advances but will never replace following sound ecological principles in aquaculture management.

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