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ECOLOGÍA MICROBIANA DE BACTERIAS PATÓGENAS ASOCIADAS AL CAMARÓN DE CULTIVO Y SU PROCESO

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ABSTRACT

Most shrimp farmers tly lo control Vibrio population in !11eir ponds regardless their pat11ogenicity to hurnan or shrimp health. Some pa\1'1ogenic Vibrio t,p0lcies present in pond water and in aquaculture shrinip shell-skeleton could survive the harvest operation and freezing process. The exposure to low prncessin¡ temperatures will affecl the quanlity and diversity of V/brío species found at the end product, but the potential health risk of t11es(1 bacteria rnay still present. Therefore, the aims of this work were focused to study \he diversity of Vibrio specles found in a semi·intensive shrimp pond; correlt te the pres,1nce of those pathogenic Vibrio species detec\ed in pond water with sorne envimnmental parameters and determine those that are able to survlve harvest and freezing conditions and could be detected at (nd"product.

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RESUMEN

En el cultivo de camarón comúnmente se busca controlar la carga bacteriana de las especies del género Vibrio sin importar si estas son o no patógenos al camarón. Algunas especies del género Vibrio presentes en el agua de estanque, puedes adherirse al exoesqueleto o cáscara del camarón y sobrevivir a la cosecha e incluso su paso por la planta procesadora. Aún y cuando la exposición a bajas temperaturas durante el procesamiento del camarón puede afectar la cantidad y diversidad de las especies del género Vibrio encontradas en el producto final, el riesgo potencial de esta bacteria existe. Por lo anterior, los objetivos de este estudio se enfocaron a conocer la diversidad de las especies de Vibrio que pueden encontrarse en un cultivo de camarón semi-intensivo asociando la presencia de las especies patógenas a parámetros ambientales, así como determinar cuales de estos pueden encontrarse en el producto terminado (camarón congelado),

Para ello, se colectaron muestras de agua y tierra en tres estanques comerciales de una misma granja camaronera en el noroeste de México, durante un ciclo de engorda. Muestras de agua, hielo y superficies de contacto fueron colectadas durante las operaciones de cosecha, mientras que el camarón fue rastreado hasta la planta procesadora para colectar muestras de producto final de los estanques bajo estudio. Se realizaron determinaciones microbiológicas (cuenta total y NMP de especies de Vibrio spp) en las muestras colectadas. Se utilizaron patrones de restricción terminal (T-RFLP) en extractos de ADN de las muestras de agua, para conocer la comunidad bacteriana en las muestras de agua, utilizando las enzimas HhaI y HaeIII.

Se encontraron ligeras diferencias en el conteo de las especies del género Vibrio, más no para el conteo de bacterias viables a lo largo del ciclo de producción, Las fluctuaciones de temperatura y oxígeno disuelto mostraron un efecto sobre la diversidad bacteriana pero no sobre la población de Vibrio spp.

efecto sobre la diversidad bacteriana pero no sobre la población de Vibrfo spp. Considerando el número de fragmentos obtenidos por las en?..imas de restricción terminal, se observa una mayor diversidad bacteriam1 en las muestras de agua de los estanques si se comparan con el agua de entrada a los mis111üs. Se pudo observar qU(la comunidad bacteriana se i stabilizó a partir dr11 cuarto mues1.reo, predominado Vibrlo spp, alplla proleobacleria, y Cianol,acteria. Las cuentas de Vibrio spp. en camarón congelado variaron de 99 a 537 y 334 a 551 NMP de Vibrio spp / gr en camarón entero (parte comestible y cíiscara) y cáscara, respectivamente. La mayor recuperación de especit s del género Vibrlo se dio en las muestras de agua de los estanquc s. predominado los grupos de V. pan,/u1 1molyticus, V. ha1v1}yi y V. alolnolyticus. Por otro lado, *V.* mimicus, el cual no se encontró corno predominante en muestras de agua, fue la única especie patógena de Vi/Jrio identificada en el producto final.

ECOLOGÍA MICROBIANA DE BACTERIAS PATÓGENAS ASOCIADAS A CAMARÓN DE CULTIVO Y SU PROCESO.

Tradicionalmente la ecología microbiana se ha enfocado al estudio de las interacciones entre microorganismos y su medio ambiente (biótico y abiótico). En la década de los 60's y 70's, esta disciplina se enfoca al efecto de biorremediación que pueden tener estas interacciones sobre el medio ambiente (Atlas y Bartha,1998). Actualmente la ecología microbiana engloba mucho más que solo biorremediación, puesto que se enfocan a conocer la biodiversidad de los microorganismos (tipo), entender sus interacciones, además de medir su actividad y controlar sus efectos. Ejemplo de esto pueden ser los estudios enfocados a conocer el tipo y cantidad de bacterias presentes en diversos ambientes como sedimentos, agua, suelo, aire, heces o contenido intestinal de diversos animales (Ravenschlag *et al* 2001; Scala y Kerkof, 2000). Existen también estudios enfocados a conocer la ecología de una bacteria específica (Norton *et al*, 2001), generalmente patógena. El enfoque principal en estos casos es conocer la ruta que sigue esta bacteria a lo largo del ambiente de proceso en un alimento determinado, para conocer los mecanismos y/o posibles rutas de contaminación. En este caso, la diversidad de la comunidad bacteriana asociada al proceso en estudio pocas veces es considerada, a excepción de algunas bacterias utilizadas en la elaboración de alimentos fermentados (Giraffa y Neviani, 2001). Es claro que la ecología microbiana asociada a alimentos no ha sido propiamente estudiada, mucho menos la relación de bacterias patógenas y la microflora natural del alimento. Esto se debe a la dificultad que representa el recuperar la mayoría de las especies bacterianas en el alimento (Giraffa y Neviani, 2001). Muchas de las bacterias asociadas a los alimentos tienen necesidades específicas de crecimiento, lo que dificulta su estudio utilizando métodos tradicionales de cultivo. Además, en el caso de las bacterias

patógenas, normalmente no se espera encontrarlas en grandes cantidades en el alimento y muchas veces se encuentran dañadas o en una forma no cultivable a consecuencia del estrés sufrido durante el procesamiento o por lo complejo de la matriz de la mayoría de los alimentos.

En las últimas décadas, la comercialización de productos marinos así como la difusión de problemas asociados a la salud con el consumo de alimentos contaminados, ha dado como resultado un mayor interés por parte del consumidor y la industria en adoptar sistemas de aseguramiento de calidad que garanticen la inocuidad del alimento a lo largo de toda la cadena alimenticia (Beulens et al, 2005), mientras que en el ámbito de la investigación se ha despertado un mayor interés en las bacterias patógenas, las posibles rutas de contaminación a hacia los alimentos, así como la prevalencia de las mismas. El comercio internacional ha dado como resultado un incremento en el control de los parámetros de proceso y un mayor enfoque en las medidas preventivas que lleven aun mejor control de las bacterias patógenas y garantizar así la inocuidad de los alimentos (Schillhorn van Veen, 2005). Aunque la ecología de estas bacterias patógenas ha sido poco estudiada en el campo de la inocuidad alimentaria.

El desarrollo de nuevas técnicas microbiológicas, basadas en técnicas moleculares (PCR o polymerase chain reaction) ha acortados los tiempos de detección y/o facilitado la identificación de bacterias patógenas. Estas técnicas también han probado su utilidad y eficacia en muestras ambientales, donde la amplificación de los genes bacterianos facilita en gran medida su estudio (Kitts, 2001, Clement et al, 1998). Debido a que esta metodología permite la replicación *in vitro* de secuencias definidas de ADN y aumenta las probabilidades de detectar una secuencia específica en una mezcla heterogénea (Atlas y Bartha, 1998), puede mostrar las posibles interacciones entre las bacterias asociadas a un alimento.

Giraffa y Neviani (2001) estudiaron los inconvenientes para la recuperación de células bacterianas dañadas de una matriz alimentaria que impiden hacer una estimación real de las bacterias presentes. Debido a la

imposibilidad de estudiar la ecología bacteriana por métodos tradicionales proponen "métodos independientes de cultivo" basados en el análisis del ADN de la comunidad bacteriana, tales como el uso de huellas genéticas, PCR o hibridación *in situ* y técnicas de amplificación. Algunas de estas técnicas han sido probadas exitosamente en estudios de ecología microbiana. Pero el uso de patrones de restricción terminal (TRF) ha mostrado muy buenos resultados en matrices complejas como son las heces de rata (Kaplan et al, 2001) y han sido consideradas como una herramienta poderosa para el estudio de la ecología microbiana de ecosistemas cerrados (Giraffa y Neviani, 2001).

Los patrones de los fragmentos de restricción terminal (TRF) también conocidos como T-RFLP (terminal restricition fragment length polymorphisms) permiten un monitoreo rápido de las bacterias presentes basándose en una digestión restringida del ADN por una endonucleasa utilizando un "*primer*"o sonda terminal marcado fluorescentemente (Giraffa y Neviani, 2001, Kaplan et al, 2001). Este método ha sido utilizado en comunidades complejas y podría ser utilizado para la diferenciación de comunidades bacterianas y la identificación de organismos específicos en una comunidad (Dunbar et al, 2001). Además una vez identificados los principales grupos de una comunidad bacteriana o la bacteria de interés, es posible correlacionarla con los parámetros físico – químicos de su medioambiente (Kitts, 2001).

El estudio de la ecología bacteriana en alimentos, puede proveer información que lleve a comprender los patrones y la estructura bacteriana, la dinámica y su función en el alimento bajo condiciones ambientales cambiantes (Giraffa y Neviani, 2001). Además, sería de gran utilidad si estos cambios pudieran ser asociados a bacterias patógenas específicas a un alimento o a un determinado proceso (Becher et al, 1999). El aseguramiento de la inocuidad alimentaria podría ser mucho más eficiente si fuera posible establecer una correlación entre un grupo específico de bacterias, su cambio o el comportamiento de las bacterias patógenas (crecimiento o inhibición) y las condiciones biológicas en tiempo y espacio.

También es necesario el contar con estudios cuantitativos enfocados a la ecología microbiana de las matrices alimentarias para poder entender a las poblaciones bacterianas y sus efectos en la calidad y vida de anaquel del alimento (Padada, et al, 1999; Van der Vossen et. al, 1999). Esta necesidad ha sido mucho más clara, y por lo tanto mayor estudiada, en alimentos fermentados. En este tipo de productos la comunidad bacteriana puede influir grandemente no solamente en la calidad y características del alimento fermentado, sino también en la inocuidad y vida de anaquel. Es por ello que, en el área de alimentos, se ha puesto mayor atención al estudio de comunidades bacterianas fermentadoras, como son las bacterias lácticas (LAB). Además es en este campo donde se ha dado el desarrollo de "nuevas" bacterias lácticas utilizadas como iniciadoras en los procesos de fermentación para alimentos funcionales novedosos o preparaciones farmacéuticas; lo que ha generado mayor interés por parte del público y las agencias reguladoras con respecto a la inocuidad de estos productos (Giraffa y Neviani, 2001). La identificación a nivel de especies y cepas, así como estudios sobre su ecología para determinar la dinámica y composición de la comunidad, son necesarios para asegurar el nivel de inocuidad requerido por el consumidor o las agencias reguladoras.

El estudio de una comunidad de bacterias basado en su identificación y dinámica bajo consideraciones sanitarias, debería ser considerado en el área de alimentos. En los productos de la acuicultura estos estudios podrían brindar mayor información, puesto que se trata de un sistema semi-controlado. Donde el control de ciertos factores como temperatura, salinidad, concentración de oxígeno disuelto, calidad y cantidad del alimento suministrado así como los procesos de desinfección preventivos, determinan el medio ambiente donde las bacterias pueden o no desarrollarse. La posibilidad de controlar las condiciones ambientales ligadas a una bacteria o grupo de bacterias específicas ya ha sido utilizado por la acuicultura para el control de enfermedades (Verschuere et. al., 2000), pero únicamente ligado a calidad del agua y producción.

Ambientes Marinos

A pesar de que pudiera pensarse que la ecología de granjas acuícolas pudiera ser similar a la de los océanos, por contar como medio común el agua de mar, estos presentan condiciones completamente diferentes. En los océanos generalmente se pueden encontrar áreas con ambientes estables y relativamente homogéneos, debido a los efectos de las mareas, corrientes y termoclinas, las cuales favorecen un movimiento continuo y la mezcla del agua (Atlas y Bartha 1998). En términos generales, solo esperaríamos encontrar grandes cambios si comparamos la ecología de ambientes muy específicos como los encontrados en fosas hidrotermales, ambientes bentónicos (aguas someras) o pelágicos (aguas profundas), encontrando una mayor biomasa de microorganismos en las aguas superficiales que en aguas profundas. Naturalmente estuarios y áreas costeras presentan un mayor número de bacterias. En estos normalmente existe una mezcla de agua salada y dulce, presentándose condiciones completamente diferentes que en mar abierto. Aquí los gradientes de salinidad, incorporación constante de materia orgánica, cambios en la temperatura del agua y profundidad son mucho más variables.

En el caso de las granjas acuícolas se pueden utilizar diferentes sistemas de producción. Específicamente en el cultivo de camarón y sobre todo en el Noroeste de México, los cultivos se realizan en estanques con bordos de tierra contiguos a la zona costera. Generalmente, estos son construidos sobre extensiones planas, cercanas a esteros o salinas (Martínez, 1998) lo que garantiza un suministro continuo de agua. Se utilizan sistemas de producción semi-intensivos que invariablemente tienen un efecto sobre el ecosistema. En estos casos el uso de fertilizantes orgánicos, abonos, desechos, de organismos y el suministro de alimento artificial (*pelets*) provoca sistemas ricos en materia orgánica, produciendo fácilmente nutrificación, eutroficación y la creación de condiciones anóxicas. Además durante la etapa de engorda en el cultivo de

camarón, es común el uso de "cal común" (CaO) u otros desinfectantes químicos para controlar la calidad del agua o el uso de antibióticos (tetraciclina, cloranfenicol, estreptomicina, entre otros), para controlar las enfermedades en el cultivo. Estos compuestos pueden ocasionar cambios en la población natural de microorganismos además de crear bacterias resistentes en caso de utilizarse antibióticos (Vershuere, et. al., 2000).

La gran variedad de bacterias que soportan altas concentraciones de sal (20 a 40 partes por mil) que es posible encontrar de manera natural en el ambiente marino es difícil de estimar, debido a la falta de métodos para obtener cultivos viables. El conteo relativamente bajo de células viables en medios líquidos o sólidos (caldos y agares) con técnicas tradicionales, contra las altas cantidades de bacterias enumeradas por conteos directos, es una de las causas principales de esta controversia (Sherr, et. al, 2001). A pesar de ello, se estima que las bacterias aisladas por los métodos de cultivo tradicionales, representan la mayor parte de la población (Atlas y Bartha, 1998).

La mayoría de las bacterias asiladas de ambientes marinos son Gram negativas, móviles, aerobias o anaerobias facultativas y un gran porcentaje de ellas son proteolíticas. (Atlas y Bartha, 1998). Entre las especies más comúnmente reportadas se encuentran algunas bacterias patógenas como son *Vibrio spp.*, *Pseudomonas* spp., *Flavobacterium*, *Aeromonas*, *Clostridum botulinum* type E, entre otras (Huss et al, 2000; Dalsgaard, 1998). Aunque también son reportadas algunas bacterias Gram positivas y anaerobios estrictos, siendo estos últimos más comunes en el sedimento donde la acumulación de materia orgánica puede producir las condiciones necesarias para su crecimiento (Bouvier y Giorgio, 2002).

A pesar de que la mayoría de las granjas acuícolas cuentan con sistemas de bombeo y aireación del agua para ayudarse a mantener algunos de los parámetros físico – químicos homogéneos, se pueden dar cambios en estos parámetros en tiempos cortos. Debido a estas condiciones cambiantes y las prácticas comunes de manejo, es difícil mantener una comunidad bacteriana estable o similar al agua de mar utilizada para el cultivo a lo largo del tiempo de

engorda. Los ciclos discontinuos, las prácticas de desinfección o limpieza de los estanques y el incremento de la materia orgánica por una alimentación controlada, implican cambios en el ecosistema que impiden el establecimiento de comunidades por largos períodos de tiempo. Vershuere y colaboradores (2000), sugieren que para el establecimiento de una comunidad microbiana en un cultivo acuícola, intervienen factores determinísticos y estocásticos. Para los factores determinísticos como salinidad, temperatura, concentración de oxígeno y calidad y cantidad del alimento suministrado, generalmente se tiene un efecto de dosis – respuesta muy definido. Mientras que este efecto no existe para los factores estocásticos, ya que este se compone de un rango de probabilidades e incluso el azar. Por ejemplo, no es posible determinar la probabilidad de que una bacteria se encuentre en el momento justo en el lugar adecuado para su desarrollo (Moriarty, 2000).

En fecha reciente y en gran medida, debido a los problemas de enfermedades que han enfrentado las granjas camaroneras, se le ha dado mayor importancia a conocer el historial bacteriano de los estanques y la composición parcial de la población bacteriana, para controlar de esta manera la calidad del agua y la salud del cultivo. Con ello es posible establecer niveles "seguros" de bacterias para un determinado cultivo. El uso de probióticos para establecer un equilibrio biológico y controlar de esta manera la salud de los estanques, ha sido poco utilizado por la camaronicultura (Verschuere, et al, 2000) a pesar de los resultados positivos para el control de enfermedades en camarón de cultivo (Moriarty, 2000). .

Los análisis microbiológicos que rutinariamente se realizan en una granja camaronera, se centran en conteos de bacterias heterótrofas viables (BHV) y conteos de bacterias del género *Vibrio*, estas últimas por representar el grupo de bacterias de mayor prevalencia y potencial de patogenicidad sobre el cultivo. Para los estanques de engorda de camarón estos conteos bacterianos se realizan en muestras de agua y sobre todo en muestras de tejido, hemolinfa y hepatopáncreas (López-Torres, 2002).

Bacterias asociadas al camarón de cultivo

Las especies pertenecientes al género *Vibrio* han sido asociadas a ambientes marinos como son esteros y zonas costeras (Thompson et al, 2004). La importancia de estas bacterias ha ido en aumento por la asociación de algunas de estas bacterias con enfermedades trasmitidas por alimentos (Pérez-Rosas y Hazen, 1988; Venkateswaran, et. al. 1998), sin mencionar su importancia en el cultivo de camarón. Otras bacterias no naturales del medioambiente marino, como *Escherichia coli* o *Salmonella spp.*, que afectan la salud humana, han sido reportadas en las aguas costeras de zonas tropicales o cercanas a poblaciones, e incluso se ha reportado su supervivencia bajo condiciones de alta salinidad (Huss et al, 2000; Dalsgaard, 1998), y en algunos casos se tienen reportes de estas bacterias en productos acuícolas. La introducción de estos patógenos a la acuicultura se ha asociado con malas practicas de producción o durante el proceso en la planta procesadora, y hasta se ha reportado como vector de contaminación a las aves presentes en los ambientes costeros (Huss et al, 2000).

***Vibrio* spp.**

La familia *Vibrionaceae*, perteneciente al grupo de las *Gama-proteobacterias*, comprende a las especies del género *Vibrio*. Estas se caracterizan por ser bacilos Gram-negativos, generalmente móviles debido a un flagelo polar, mesófilos, y quimiorganotrofos y en su mayoría son oxidasa positivos (Thompson, 2004). La mayoría requieren de 1-2% NaCl para su crecimiento y son capaces de crecer en un medio selectivo alcalino y con sales biliares (agar tiosulfato-citrato- sales biliares-sacarosa o TCBS) (Massad y Oliver, 1987). Se han asociado a medios acuáticos, se les encuentran comúnmente en ambientes marinos (Pérez-Rosas y Hazen, 1998) y por

consiguiente están asociadas a productos de la pesca y acuicultura (Vandenberghe et al, 2003). Su asociación con temperatura y materia orgánica está bien documentada (Randa et al, 2004). En ambientes naturales, la ocurrencia de estas especies aumenta durante los meses cálidos cuando se registran las mayores temperaturas en el agua (Twedt, 1989). Recientemente se ha reportado que en ambientes semi-desérticos, con poca precipitación y altas temperaturas a lo largo del año, la materia orgánica tiene mayor influencia que la temperatura, encontrándose una correlación positiva con la presencia de *Vibrio parahaemolyticus* (Deepanjali et al, 2005).

La mayoría de las especies del género *Vibrio* encontrados en la naturaleza no representan un peligro para la salud del consumidor (Andrews, 2003), aunque se han reportado al menos 10 especies de importancia clínica , por asociarse a infecciones en la piel, septicemias y desordenes gastrointestinales de moderados a severos como en el caso de *V. cholerae* (Pérez- Rosas y Hazen, 1998; Venkateswaranet al, 1998). *V. cholerae*, *V. parahaemolyticus* y *V. vulnificus* son consideradas las especies de mayor riesgo para la salud humana por presentar serios efectos a la salud, mientras que *V. fluvialis, V. furnisii, V. harveyi, V. metschnikovii, V. hollisae, V. algynolyticus* y *V. mimicus* se han reportado en casos esporádicos o de menor severidad (Thompson et al, 2004, Elhadi, et al, 2004). Algunas de estas especies, también pueden ocasionar serias enfermedades y mortandades, con las consecuentes perdidas económicas, en la acuicultura incluyendo al cultivo de camarón (Goarant et al, 1999). Así tenemos que *V. angillarum, V. salmonicida, V. vulnificus* y *V. harveyi* son de alta relevancia para la acuicultura; siendo este ultimo de mayor importancia para la camaronicultura.

La diversidad genotípica y fenotípica de las bacterias que conforman el género *Vibrio* dificulta su rápida identificación y en los últimos con la difusión de métodos moleculares, este grupo ha crecido año con año. En 1994, Alsina y Blanch reportaron 30 especies pertenecientes al género *Vibrio*, mientras que actualmente se reconocen 63 especies pertenecientes a este género (Thompson et al, 2004). Al mismo tiempo que se incluyen más especies a este

grupo de bacterias, se ha reportado que algunas de ellas consideradas como no patogénicas, tienen la capacidad de producir tetradotoxina (TTX) y anhidrotetrodotoxina (Ottaviani et al, 2003).

De acuerdo a los registros de CDC (Centers for Disease Control and Prevention, 2005) de Estados Unidos de Norteamérica, durante el año 2003 el consumo de camarón, principalmente crudo, fue uno de los principales productos marinos asociados a Vibriosis, después del consumo de moluscos bivalvos y pescados crudos.

Por lo anterior, los objetivos de este estudio se encaminaron a identificar los principales grupos de bacterias asociados al camarón de cultivo, con especial énfasis en las especies del género *Vibrio*; analizando para ello los cambios poblacionales a nivel de granja (agua y suelo), operaciones de cosecha (hielo y superficies de contacto) y camarón congelado como producto final. Utilizando para la identificación de bacterias patógenas asociadas al medioambiente del camarón de cultivo, métodos dependientes e independientes de cultivo, para detectar las principales especies patógenas del género *Vibrio*. Mientras que los principales grupos bacterianos encontrados en el agua del estanque se determinaron analizando los fragmentos de secuencias amplificadas de ADN marcadas fluorescentemente, utilizando para ello enzimas de restricción terminal. Con ello se propone mostrar las posibles variaciones en la población bacteriana (dinámica) y determinar grupos de bacterias no detectables por los métodos tradicionales.

HIPÓTESIS Y OBJETIVOS

Hipótesis:

Existe una correlación entre la ecología microbiana del medio ambiente y la presencia de especies patógenas de Vibrio.

Objetivos

Objetivo general

a) Conocer la diversidad bacteriana asociada al camarón de cultivo, desde el estanque hasta su procesamiento como producto congelado.

Objetivos particulares

a) Analizar los cambios asociados a las principales especies patógenas de la familia *Vibrionaceae*, encontradas en estanques de producción de camarón de cultivo.
b) Conocer los patrones poblacionales de los principales grupos bacterianos asociadas al agua de cultivo.
c) Analizar los cambios ambientales de temperatura y oxígeno disuelto con relación a las poblaciones bacterianas encontradas en el agua de cultivo
d) Analizar las diferencias de especies patógenas .entre el agua del estanque antes de la cosecha y el producto final (camarón congelado).

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ARTÍCULO 1

Biochemical Diversity of *Vibrio* spp in Shrimp Ponds and its Association with Pathogenic Strains

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Biochemical Diversity of *Vibrio* spp in Shrimp Ponds and its Association with Pathogenic Strains

Abstract

Most shrimp farmers try to control *Vibrio* population in their ponds regardless their pathogenicity to human or shrimp health. This study focuses on the biochemical diversity of *Vibrio* species found in a typical semi-intensive shrimp pond and correlates environmental conditions to pathogenic *Vibrio* species detected in pond water. Bacterial diversity changed with time depending on water temperature. Such changes could be associated to dissolved oxygen, temperature and the pond itself. Growth conditions for pathogenic *Vibrio* species are particularly favorable during the harvest period for highly salt-tolerant species. Operational Taxonomic Units (OTUs) associated to *Vibrio parahaemolyticus* or *V. harveyi* increase their population from 3 to 460 MPN ml^{-1} at harvest time, while *V. cholerae* similar OTUs were only detected at initial stages of the production cycle at extremely low levels.

Keywords: *Vibrio*, shrimp, aquaculture, water

Introduction

Vibrio spp occurs naturally in aquatic environments (Thompson et al., 2004) and is one of the most commonly-occurring bacteria during shrimp farming (Vandenberghe, et al., 2003). The number of reported *Vibrio* species has increased rapidly in the last decade. Thompson, et al. (2004) have reported 63

environmental species comprising the genus *Vibrio*. Ten of them are of human concern (Twedt, 1989), since they have been associated with skin infections and severe gastrointestinal disorders (Andrews, 2004, Pérez-Rosas and Hazen 1998, Venkateswaran et al. 1998). Some of the pathogenic *Vibrio* species have also been reported as the causal agents of shrimp infections (Goarant et al., 1999). In contrast *Vibrio alginolyticus* have been reported also as a probiotic for shrimp aquaculture (Vandenberghe, 2003, Direkbusaram, et al., 1998).

Shrimp ponds are stressful environments compared to estuaries or other enclosed water bodies (Direkbusararam, et al., 1998). This is mainly due to high organic matter and dissolved oxygen fluctuations which affect the composition of natural bacterial communities. Under normal conditions, temperature increments will also bring about a greater diversity of *Vibrio* species but not necessarily a higher density of the organisms. At high temperatures and high salinity conditions some species such as *Vibrio parahaemolyticus* will predominate (Williams and LaRock, 1985).

In the last 15 years, shrimp farming has become an important economic activity in many countries of the world. Recently, an increasing number of shrimp farms have raised concern about water quality and shrimp health because of their economic impact on farm operations. Most farmers now recognize the relevance of *Vibrio* species in shrimp ponds and safety levels for viable heterotrophic bacteria (VHB) and *Vibrio* species are commonly used as environmental health indicators. Routine counts of those bacterial groups in water, and shrimp hepatopancreas and hemolymph are common (López-Torres, 2001). The routine determination of *Vibrio* species in farms is quite useful in terms of pond management decisions and in some cases the use of antibiotics is based on those results. On the other hand, preventive measures often include the use of disinfectant and antimicrobial solutions which alter the dynamics of bacterial communities and may induce antibiotic resistance (Verschuere, et al,. 2000). This is a worldwide problem and has been documented in several Mexican shrimp farms (Thompson, et. al., 2004).

Numerous studies of seasonal variation of pathogenic *Vibrio* species in natural environments can be found in the scientific literature (Williams, and LaRock, 1985; Venkateswaran, et al., 1989; Barbieri, et al., 1999; Pfeffer, et al., 2003; Hosseini, et al., 2004). Other studies refer to isolated strains from marine aquaculture systems (Gomez-Gil, et al., 1998; Vandenberghe, et al., 2003; George, et al., 2005), but there is a notable lack of information regarding changes of *Vibrio* species in aquaculture systems along the grow-out period. This information is critical from a food safety as well as from an economic impact perspective.

Therefore, the aim of this work was to study the variations of *Vibrio* species that can be found along the growout production cycle in shrimp pond water and soil, and its association with pathogenic *Vibrio* species.

Materials and Methods

Water and soil samples were collected along the growout production period, from seeding to harvest, in a shrimp (*Litopenaeus vannamei*) farm located in the coastal area of Hermosillo, Sonora, Mexico. Pond selection was made on the basis of pond size and age, seeding time, initial larvae density, water recharge, and history of antibiotic usage. A total of three ponds (A, B and C) were chosen for sampling. Five periodically sampling periods (S-1 to S-5) were considered from April to September starting two days after seeding the first pond under study. Three samples were collected from each pond at different locations: a) incoming water, collected right after the filter in the intake water supply; b) a composite water sample prepared from a mixture of bottom and surface water collected in the middle of the pond and water collected before the pond discharge outlet; and c) a soil sample, collected in the wet portion of the pond side. All samples were collected in sterile plastic containers and transported to the laboratory in insulated boxes to maintain low temperatures (below 5°C). Samples were analyzed the same day they were collected. Shrimp farm

management provided access to daily monitoring records of temperature and dissolved oxygen (two daily measures) and the historical feeding records to document antibiotic usage.

Samples were analyzed based on the Bacteriological Analytical Manual for the identification of *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus* and other *Vibrio* spp. (Elliot et al., 1992). Tenfold dilutions in alkaline peptone water (APW) were prepared in order to estimate - where possible - the MPN of pathogenic *Vibrio* species. Partial (6-8 hr) and complete (18-24 hrs) incubation at 35-37°C was carried out before transferring to TCBS (thiosulfate-citrate-bile salts-sucrose, Difco) agar plates. All yellow, green, and blue green colonies were selected for biochemical characterization (0%, 3%, 6%, 8% and 10% NaCl growth, motility, indole production, lysine and ornithine descarboxilase, sucrose, glucose and fructose fermentation, arginine dihydrolase, gelatinase and oxidase). The following CAIM (Collection of Aquatic Important Microorganisms) strains were included in the analysis: CAIM320 - *V. parahaemolyticus;* CAIM512 - *V. harveyi;* CAIM516 - *V. alginolyticus;* CAIM593 - *V. fluvialis;* CAIM 602 -*V. mimicus;* and CAIM610 - *V. vulnificus*. Also the *V. cholerae* non O1 strain, provided by the Mexican Health Department was used.

Statistical analysis included principal component analysis (PCA) and cluster analysis. Pond conditions and biochemical responses were combined for the PCA analysis using the Unscrambler® v9.2 (1996-2005 CAMO PROCESS AS). Dendrograms were obtained from the cluster analysis of biochemical responses using Bionumerics® - Version 3.0 (Applied Maths).

Results

According to farm records, no antibiotic use was detected at any pond under study, but lime (CaO) was used periodically (7 to 10 days, 50Kg / Ha) as a ponds disinfectants. Variations in pond's temperature and dissolved oxygen during the sampling period are summarized in Table 1.1. Those variables were

monitored twice a day by the farm, and daily fluctuations were calculated; average values were considered in the statistical analysis.

A total of 939 isolates from TCBS agar from 45 pond samples and control stains were characterized biochemically (data not shown). Loadings and scores from the PCA analysis considering pond conditions and biochemical responses are shown in Figure 1.1. Groups were obtained based on those variables with high correlations loadings.

Considering those results, cluster analysis was performed by pond and sampling time. Dendrograms obtained from cluster analysis of pond A at each sampling time (S-1 to S-5) are shown in Figure 1.2. Similar patterns along time for ponds B and C (not shown) were found.

OTUs (Operational Taxonomic Units) which present 100% similarity with control strains in cluster analysis were considered as presumptive pathogenic *Vibrio* species, and estimation of the MPN m^{-1} calculated (Table 1.2).

A full cluster analysis including all 939 OTUs and control strains was obtained. Sixty-six subgroups of at least four OTUs with 100% similarity were identified. Those subgroups were associated at 60% similarity with the control strains and eight groups were detected (Figure 1.3). Major groups and subgroups present along the sampling periods are shown in Table 1.3.

Discussion

Water temperature increased gradually until it reached 30°C but the daily average temperature fluctuation became significantly smaller. This resulted in a more stable temperature along the day (Table 1.1), a situation which favors bacterial growth. Dissolved oxygen drastically dropped from the averages registered in July to the end of the sampling period, but the daily fluctuation increased significantly favoring the development of facultative bacteria, capable of adjusting to very low oxygen levels in the environment. Both conditions in conjunction with lime used by the shrimp farm, resulted in very stressful

environment for pond bacteria. Furthermore, the bacterial population dynamically adapted to the evolving conditions and their relative abundance changed over time giving rise to a new bacterial community profile (Figure 1.1a and Figure 1.2).

Table 1.1. Pond conditions during the experiment.

Pond	*Sampling*	*Temp (°C)*	*DTF*	*DO*	*DDOF*
A	S1-April	19.8	3.60	5.9	0.70
	S2-May	22.9	2.62	7.4	1.40
	S3-June	26.5	2.53	6.0	1.96
	S4-July	29.3	1.90	3.9	2.02
	S5-Sept	31.0	2.03	3.3	2.02
B	S1-April	19.9	4.50	5.9	0.50
	S2-May	23.0	4.78	7.2	1.25
	S3-June	26.4	2.51	6.1	2.02
	S4-July	29.2	1.93	4.0	2.08
	S5-Sept	31.1	1.96	3.3	1.49
C	S1-April	19.9	3.70	5.8	0.30
	S2-May	23.1	3.23	7.4	1.42
	S3-June	26.4	2.25	6.0	2.02
	S4-July	29.3	1.95	4.0	1.89
	S5-Sept	31.0	1.89	3.0	1.61

DTF = Daily temperature fluctuation (°C).

DO = Dissolved oxygen (mg/l)

DDOF = Daily dissolved oxygen fluctuation (mg/l)

Water temperature was negatively correlated with dissolved oxygen and average temperature daily fluctuation (Figure 1.1d). A positive correlation

between *Vibrio* species and water temperature has been documented (Bariberi, et al., 1999; Pfeffer, et al., 2003); furthermore, temperature has shown a positive correlation with the presence of the cholerae toxin gene (*ctx*) (Huq, et al., 2005). Pfeffer et al. (2003) found positive correlations for *Vibrio* spp. and water temperature and negative ones for dissolved oxygen in estuarine waters. These authors found water temperature to be the most highly correlated variable to *Vibrio spp* abundance.

The results showed that, temperature, sampling time and dissolved oxygen were the principal ambient conditions affecting OTUs distribution (Figure 1.1b). The combined effect of these variables can explain the bacterial population change throughout the growout time. The largest bacterial diversity for *Vibrio* species was found at the middle of the production period. Once the new environmental conditions set in, diversity tends to decrease while the size of the bacterial subgroups increases considerably. The comparison of dendrograms for all sampling periods in pond A (Figure 1.2), clearly shows that at the earliest stages, suspected *Vibrio* populations were represented in a few large groups (Figure 1.2, S1, S2). By June the temperature rose about 6°C and population diversity increased substantially, but only with smaller groups (Figure 1.2 S-3). From this point on, daily temperature fluctuations were the lowest and bacterial population stabilized. Results from the S-4 sampling showed practically the same high bacterial diversity pattern than S-3, which then tends to diminish at the harvest period (S-5), where less but more abundant groups were present.

Ten species of *Vibrio* of concern to human health have been reported (Twedt, 1989) and seven of those were used as control strains in this study. Five of them (*V. cholerae* non O1, *V. mimicus, V. alginolyticus* and *V. parahaemolyticus* - *V. harveyi*), could be associated with isolated OTUs (Table 1.2). The major factors that affect their distribution were temperature and salt tolerance. The most abundant group was that comprised by OTUs clustered around *V. alginolyticus,* followed by those similar to *V. cholerae*. Two of the control strains, *V. vulnificus* and *V. fluvialis,* were not associated to any OTU in this study. Low salinities and

high temperatures have been associated to *V. vulnificus* found in shrimp shell (Yano, et al., 2004).

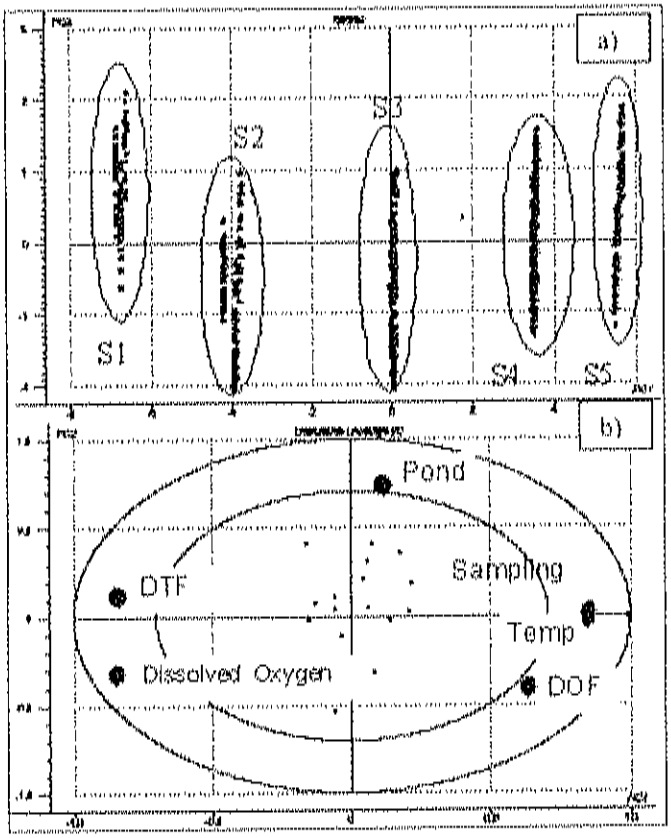

Figure 1.1. Principal component analysis (PCA) scores and loadings for PC1 and PC2: a) scores for samples grouped by average temperature and sampling time (S1 to S5), clearly shows time effect b) correlations loadings (DTF = Daily Temperature Fluctuation, DOF = Daily Dissolved Oxygen Fluctuation), shows OTUs variability highly influenced by temperature, dissolved oxygen and their fluctuations. A negative correlation for temperature and dissolved oxygen can be observed.

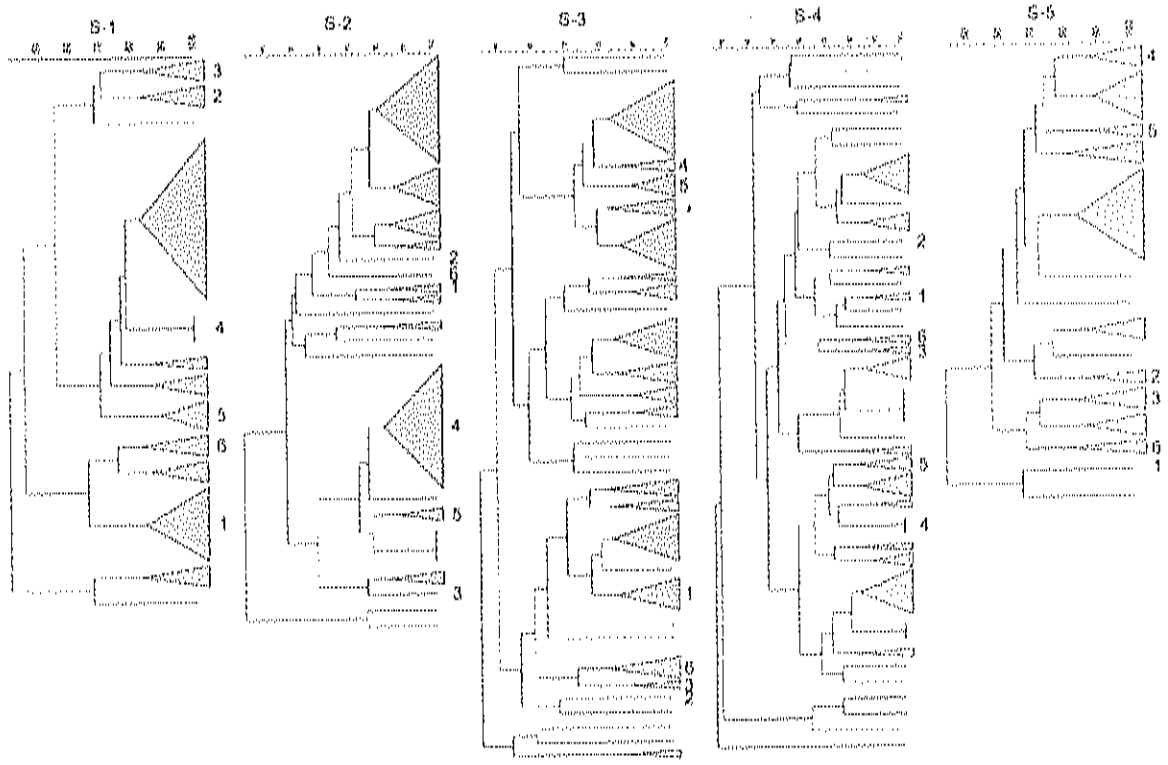

Figure 1.2. Cluster analysis for water and soil OTUs founded in pond A at different sampling time (S1- S5). OTUs are grouped based on 60% similarity, where 1: *V. fluvialis* group, 2: *V. vulnificus* group, 3: *V. mimicus* group, 4: *V. parahaemolyticus – V. harveyi* group, 5: *V. alginolyticus* group, 6: *V. cholerae* group.

Table 1.2. Pathogenic *Vibrio* species control strain with a 100% similarity with analyzed OTUs, sapling period where they were found, and their maximum and minimum estimated MPN ml^{-1}.

		V. cholerae	*CAIM 602*	*CAIM 593*	*CAIM 516*	*CAIM 320 or 512*	*CAIM 610*
Salt Tol		*3%*	6%	6%	10%	8%	6%
Pond A	S1	1	1	--	--	--	--
	S2	--	--	--	--	1	--
	S3	--	--	--	--	--	--
Sub Total		1	1	--	--	1	--
MPN ml^{-1}		43 – 3	3.6	---	---	43 – 3	--
Pond B	S1	1	1	--	--	--	--
	S3	--	--	--	--	2	--
	S4	--	--	--	1	--	--
	S5	--	--	--	--	1	--
Sub Total		1	1	--	1	3	--
MPN ml^{-1}		43 – 3	3.6	---	43 – 3	9.2 – 3.6	--
Pond C	S1	--	--	--	1	--	--
	S2	--	--	--	--	2	
	S4	--	--	--	--	1	--
	S5	--	--	--	--	1	--
Sub Total		--	--	--	1	4	-
MPN ml^{-1}		---	---	---	3.6	3 – 460	--
Total		2	2	--	2	8	--

Salt Tol = High salt tolerance reported (Alsina and Blanch, 1994)

-- = Not similar OTU founded; CAIM320 = *V. parahaemolyticus*; CAIM512 = *V. harveyi*; CAIM516 = *V. alginolyticus*; CAIM593 = *V. fluvialis*; CAIM 602 =*V. mimicus*; CAIM610 = *V. vulnificus*; *V. cholerae* = *V. cholerae* no 01, reference strain from the Mexican Health Department

Similar OTUs to *V. cholerae* and *V. mimicus* control strains, which are less salt tolerant (up to 3 and 6% respectively), were found only in the S-1, at the lowest recorded temperature (around 20°C). However, *V. mimicus* was identified in all periods using the Alsina and Blanch (1994) biochemical identification scheme. This difference could be explained by the biochemical variability in arginine dihydrolase and salt tolerance reported (Alsina and Blanch, 1994). Salinity and temperature have been reported as critical for *V. cholerae* in estuaries, with the best recovery rate between 16 and 24°C, and none at temperatures higher than 35°C (Williams and LaRock, 1985). On the other hand, *V. harveyi*, *V.alginolyticus* and *V. parahaemolyticus*, which are more salt tolerant bacteria (up to 8 and 10%) could be found at the end of the sampling period (S-4 and S-5), with prevalence of high temperatures. Minimal recovery water temperature for *V. parahaemolyticus* has been reported at 27°C in estuarine waters (Williams and LaRock, 1985). No difference between *V. parahaemolyticus* and *V. harveyi*, could be detected with the performed tests in this study, as they were always grouped in the same cluster. A total of thirty-one OTUs were clustered with this pathogenic bacteria group and eight of them did not show differences in biochemical responses with the control strain used (100% similarity). Even when the *V parahaemolyticus-V harveyi* group was not as abundant as *V. cholerae* or *V. alginolyticus* groups, that group encloses the major subgroup clustered at 100% similarity with one of the control strains. Those suspected pathogenic *Vibrio* species were well distributed throughout the growout period (S-2, S-4, S-5) and were found in water and soil samples. Most pathogenic *Vibrio* associated OTUs were present at low and stable concentration along the sampling period, except for *V. parahaemolyticus - V. harveyi* group, which increase drastically their estimated MPN ml^{-1} (from 3 to 460 MPN^{-1}) at the harvest stage (S-5). As mentioned before, when considered as a single group, they were not a major part of the total population, but it was the biggest pathogenic subgroup found and its importance stems from its ability to affect human health if present in the

end product. *Vibrio parahaemolyticus* has been associated in up to 70% of the seafood gastroenteritis in Japan (Deepanjali et al., 2005).

Even when *V. alginolyticus* was found to be the major group (31 subgroups from a total of a 66) (Figure 1.3) followed by those similar to *V. cholerae* (18 subgroups), only two fully similar OTUs were found. This could be explained by the high level of genetic variation reported among *V. alginolyticus* isolated from aquaculture shrimp, even when sampled in the same pond (Gorge et al., 2005). *V. alginolyticus* has also been reported as one of the main isolated *Vibrio* species in shrimp and shellfish (Hosseini et al, 2004; Parisi et al., 2004) and has been also associated at retail market in wild and aquacultured shrimp (Elhadi, et al., 2004). This human and shrimp pathogenic bacterium has been tested as a probiotic against other *Vibrio* species that can infect farmed shrimp (Sotomayor and Balcázar, 2003). Its variability and inhibitory effect against other *Vibrio* species could be the reason of the high subgroup abundance, compared with all identified groups.

Seven subgroups were detected in all the sampling periods; all of which belongs to those two more abundant groups (Table 1.3). All of them, except one, were highly salt tolerant. Those subgroups that were detected at the beginning but disappeared in further samplings, also showed high salt tolerances except for those that were not detected after S-3 or were detected in very small groups. These subgroups exhibited salt tolerance between 0 to 3%. Also, those subgroups that were not present at initial stages and were detected in later samplings, including harvest (S-5), were highly salt-tolerant.

Table 1.3.Principal subgroups along time, its group link and salt tolerance characteristics

Subgroup	No. OUT	Group	SALT	Sampling
Subgroups founded a long the sampling period				
B	71	6	3 – 10	S-1 to 5
D	46	6	3 – 10	S-1to 5
E	21	6	0 – 10	S-1to 5
K	59	1	3	S-1to 5
L	39	1	3 – 6	S-1to 5
N	14	1	3 – 6	S-1to 5
Subgroups that disappear along the sampling period				
A	35	6	0 – 10	S-1to 4
M	20	1	0 - 3	S-1- 4
U	3	1	0 - 3	S-1
44	5	4	3 – 6	S-1 to 4
55	10	1	0 – 3	S-1 to 3
W	4	7	0 – 10	S-1 to 3
Subgroups that appear along the sampling period				
F	32	6	3 – 10	S-2 to 5
G	29	6	3 – 10	S-3 to 5
H	13	5	3 – 8	S-2, 4, 5
48	6	3	0, 3, 8	S-2 to 3
54	6	1	3 – 6	S-2 to 4
61	12	1	3 - 6	S-4 to 5

1. *V. cholerae* group, 2. CAIM610 *V. vulnificus* group, 3. CAIM593 *V. fluvialis* group, 4. CAIM602 *V. mimicus* group, 5. CAIM320 *V. parahaemolyticus* and CAIM512 *V. harveyi* group, 6. CAIM516 *V. alginolyticus* group, 7 and 8. Unknown groups * = 100% similarity with its group

From the above discussion it becomes clear that pond conditions in shrimp farms may support the survival and growth of those high salt-tolerant pathogenic *Vibrio* species, such as *V. parahaemolyticus, V. harveyi* and *V. alginolyticus* and potentially *V. cholerae* in pond water at harvest time. Genotype studies have shown that pathogenic *Vibrio* species such as *V. cholerae, V. vulnificus* and *V. alginolyticus*, do not always express the virulence factors in their natural environment. A study reported by Baffone *et al.* (2000) found *V. alginolyticus* as the most common isolated pathogenic *Vibrio* in fresh seafood, but only 3 of 24 isolated strains shown cytotoxic effects. Rivera *et al.* (2001) isolated *V. cholerae* from environmental samples and concluded that in spite of the fact that those strains may not have their entire toxigenic factors; some environmental changes may trigger their virulent potential. Further studies should be conducted to determine virulence factor on those main groups found in shrimp water ponds. Therefore, if pathogenic *Vibrio* species are present in shrimp pond water, it is likely that they could also be present in the shrimp itself, with the implicit health risk for the end consumer, particularly in regions where raw shrimp is consumed as part of the traditional seafood dishes. They can also become a major vector for cross contamination when not properly handled.

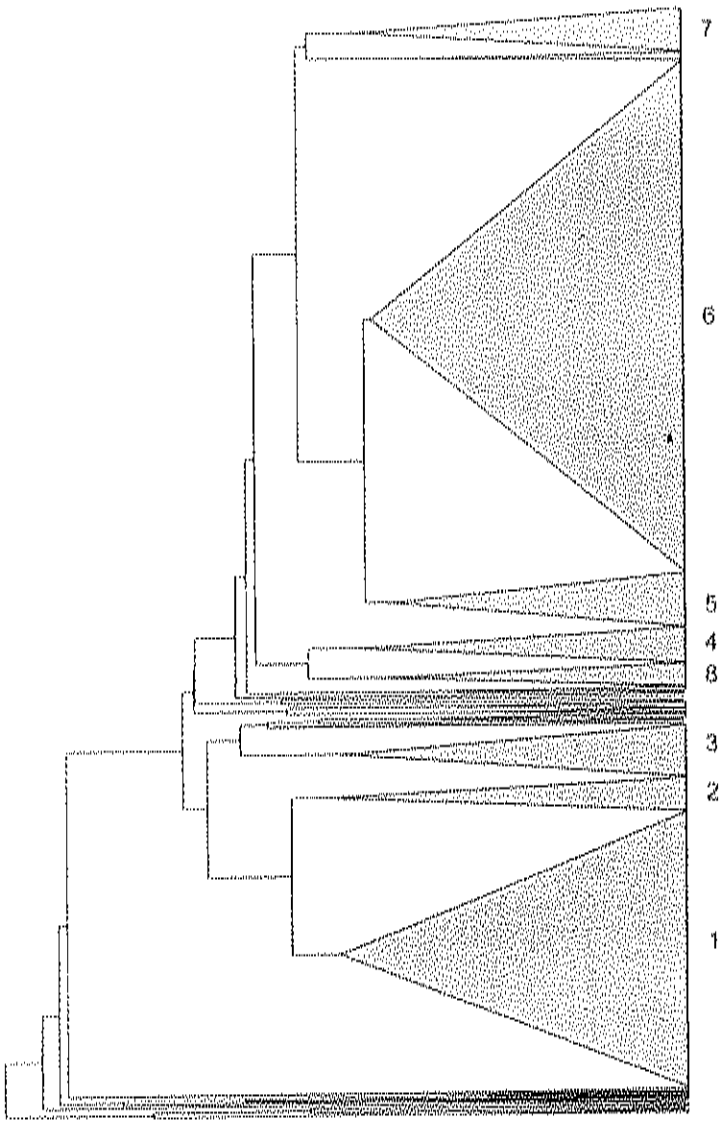

Figure 1.3. Dendrogram for all OTU's found at different sampling time at three shrimp ponds. Groups were formed around control strains using at least a similarity value of 60%, where 1: *V. cholerae* group, includes 18 subgroups (similarity 61.61%); 2: *V. vulnificus* group, includes 3 subgroups (similarity 60.11%); 3: *V. fluvialis* group, includes 3 subgroups (similarity 61.77%); 4: *V. mimicus* group, includes 3 subgroups (similarity 63.73%); 5: *V. parahaemolyticus* - *V. harveyii* group, includes 5 subgroups (similarity 67.06%); 6: *V. alginolyticus* group, includes 31 subgroups (similarity 65.38); 7: Unknown group, includes one subgroup (similarity 63.65%); 8: Unknown group.

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ARTÍCULO 2

Variation of Bacterial Communities in Shrimp pond Water Using Terminal Restriction Fragment Length Polymorphism (T-RFLP).

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Variation of Bacterial Communities in Shrimp pond Water Using Terminal Restriction Fragment Length Polymorphism (T-RFLP).

Abstract

Entrance and composite water samples, obtained from three commercial shrimp ponds in the Northwest of Mexico, were analyzed along one production cycle (from seeding to harvest). Total viable bacterial count (TBC) and MPN of *Vibrio* spp. were obtained in addition to T-RFLP patterns in order to study the bacterial community. *HhaI* and *HaeIII* enzymes were used for T-RFLP patterns. Differences in *Vibrio* population were found along the sampling period, but not for TBC, which show a stable tendency along the six sampling periods. Temperature and dissolved oxygen fluctuation shows to have an influence in bacterial diversity fluctuation, but not in Vibrio population. T-RFLP patterns showed that pond composite water (CW) samples present a bigger variability in bacterial composition based in the number of fragments sizes obtained. CW bacterial community stabilizes from S4 to harvest and a predominance of alpha-*proteobacteria*, *Cyanobacteria* and *Vibrio* spp, match with predominant fragments obtained from T-RFLP patterns.

Keywords: shrimp, T-RFLP, *Vibrio*

Introduction

It has been estimated that about 1% of the total bacterial present in marine environmental samples can be isolated by traditional culturable methods (Somerville et al, 1989). Thus, culture-dependent methods estimate only those viable bacteria, but the true marine bacterial population that can survive under seawater salinity concentrations is difficult to estimate (Sherr et. al, 2001). Different molecular methods have been developed to study the species richness in a bacterial community. The combination of PCR and fingerprinting methods have been widely used for the bacterial community studies (Urakawa et al., 2001). The use of restriction enzymes has been adopted for bacterial community characterization and terminal restriction fragment length polymorphism (T-RFLP) and they has been successfully used to determine the main bacterial groups in different environmental samples. Marine environments have not been the exception, and studies related to bacterial ecology in sediments (Urakawa et al, 2001) and waters (Moeseneder, et al., 1999) can be found in the scientific literature. There are also some studies that use restriction enzymes to differentiate between species among marine bacteria of great importance for aquaculture, such as *Vibrio* (Urakawa et al, 1998; Yoon et al, 2003).
Some natural marine environments such as estuaries or bay areas, are relative stable environments due to the influence of tides, thermoclynes and currents,

which promote mixing and water movement and a rather homogeneous composition of bacterial communities (Atlas and Bartha 1998). In contrast, important differences in bacterial populations due to salinity variations, constant organic matter incorporations, and temperature fluctuations are common when comparing benthonic and pelagic environments.

Shrimp farms usually depend on intake of estuarine water, and their typical managing operations create stressful environments for bacterial communities (Direkbusarakom et al., 1998).This can be explained for a higher organic carbon load coming from a variety of sources, including left-over artificial feed, fecal pellets, dead animals or shell skeletons from ecdysis. Organic matter is an excellent source of nutrients for bacterial growth, but at the same time it contributes to a significant reduction of the dissolved oxygen level in the pond which in turn influences marine bacterial population (Bissett et al., 2006). Water exchange is practiced to control dramatic variations in key parameters affecting shrimp growth and survival. The above conditions make it difficult to identify a stable marine bacterial community for a long period of time. Verschuere et al, (2000), have suggested that the establishment of a marine bacterial community in an aquaculture pond is dependant upon deterministic and stochastic factors. In the deterministic ones (temperature, salinity, dissolved oxygen, feed amount), there is a dose – response effect. In contrast, the effect of stochastic factors such chance and probabilities can not be modeled. For example, chances favor those bacteria that are in the right place at the right moment (Moriarty, 2000), and there is no way to measure it.

Shrimp production systems can easily allow eutrophic, nitrification and anoxic conditions, along the pond environment, with the significant effects on bacterial community. Furthermore, discontinuous production cycles and the use of water and soil disinfectants which are commonly applied to promote shrimp health contribute to natural bacterial depletion (Valle et al, 2002). All these factors together will promote bacterial community time variation and can affect the nearby environment once the pond is emptied during harvest. Therefore, the aim of this study was to understand the bacterial patterns associated to healthy shrimp pond water, under normal management conditions.

Materials and Methods

Pond water samples were collected along the production period, from seeding to harvest, in a Sonora, Mexico coastal area shrimp farm (28° N, 111° W). Pond for sampling were selected considering similar conditions regarding pond size and age, seeding time and initial larvae density, specie (*Litopenaeus vannamei*) water recharge, and historical antibiotic usage (negative use). Six sampling periods were established from April to October, starting two days after seeding the first pond under study. Two samples were collected at each pond: a) entrance water (EW), collected just after the filter in the intake water supply and b) composite water (CW) sample, a mixture of bottom and surface water collected in the middle of the pond and water collected before the discharge

outlet. All samples were collected in sterile plastic container and transported to the laboratory in insulated boxes at low temperatures (below 5 °) and analyzed the same day for total viable bacterial count and *Vibrio* population. The remaining sample were stored at -20°C until DNA extraction could be performed. Shrimp farm management provided access to daily monitoring records such as temperature and dissolved oxygen (two daily measures) and the historical feeding records to corroborate no antibiotic usage.

Bacterial Count: Ten - fold dilutions were used in spread plates of modified Long and Hammer agar (Koutsoumanis and Nychas, 1999) incubated at 25 °C / 5 days, for total viable bacteria count. Medium composition was as follows (grams per liter of distilled water): Proteose Peptone, 20; gelatin, 40; K_2HPO_4, 1; NaCl, 10; agar, 15; ammonium ferric citrate, 0.25. Dilutions in alkaline peptone water (APW) at 35-37°C were used for estimation of Vibrio population on TCBS (thiosulfate-citrate-bile salts-sucrose, DIFCO). The assumption that positive tubes were those showing yellow or green colonies after TCBS incubations was made for most probable number (MPN) calculation.

DNA extraction: DNA extraction was conducted based in Murmur Protocol (Johnson, 1991) and Barns et al. (1994) with some modifications. Thawed samples were vigorously mixed to allow homogenization. Triplicates of 100 ml were centrifuged (15 min @ 3000g), and the residue was washed with 10 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0), and centrifuged under the same conditions; re-suspended with 1 ml of buffer solution (500 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM sodium citrate) and the presence of lysozyme (15 mg,

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DNA extraction: DNA extraction was conducted based in Murmur Protocol (Johnson, 1991) and Barns et al. (1994) with some modifications. Thawed samples were vigorously mixed to allow homogenization. Triplicates of 100 ml were centrifuged (15 min @ 3000g), and the residue was washed with 10 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0), and centrifuged under the same conditions; re-suspended with 1 ml of buffer solution (500 mM Tris HCl pH 8.0, 100 mM NaCl, 1 mM sodium citrate) and the presence of lysozyme (15 mg,

Sigma) and mutanolyzyne (4 U). The mixture was incubated for 18 - 24 hours at 37°C. Proteinase K (2 mg, Invitrogen) was added and incubated at 50°C for 30 min. One volume of lysis solution (200 mM Tris HCl pH 8.9, 100 mM NaCl SDS 8% and PVPP 1.5%) was added and mixed by inversion. The mixture was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, vol:vol), mixed by inversion and centrifuged at 10,000 g for 5 minutes. One volume of cold isopropyl alcohol was added, mixed by inversion and kept at -20°C overnight. Cold mixture was centrifuged at 13,000 rpm for 20 min and supernatant discharged, before 50 µl RNAse – TE (2 mg/ml) was added. Extraction was visually corroborated by gel electrophoresis in 1% agarose gel.

PCR Amplification: PCR amplification was performed in a total reaction volume of 50 µl, as described by Kaplan *et al.* (2001), using 4 µl template DNA and Ba2F (GCYTAACACATGCAAGTCGA; GenSet, San Diego, California) as labeled primer and K2R (GTATTACCGCGGCTGCTGG) as unlabeled primer. Amplification was conducted in a Gen Amp PCR System 96000 (Perking Elmer), under the following conditions: 96°C (10 min), 1 cycle; 94°C (1 min), 46.5°C (1 min), 72°C (2 min), 30 cycles; 72°C (10 min) 1 cycle. The PCR product was visualized in gel electrophoresis in 1% agarose gel, stained with ethidum bromide in a UV transilluminator. The DNA was cleaned using MoBio UltraClean® PCR Cleanup (Mo Bio Laboratories, Inc. California) according the manufactures instructions. DNA concentration was determined using a microplate Fluorescence Reader FLX800 (Bio-Tek Instruments, Inc).

T-RFLP patterns: 100 ng of DNA template was digested in a total reaction volume of 40 µl, using *HhaI* (20 U) and *HaeIII* (8 U) (Bio Labs, New England) for four hours at 37°C, before enzyme inactivation. DNA fragments were recovered by ethanol precipitation, adding 2.5 volume of cold ethanol (95%), 2 µl of sodium acetate (3M, pH 4.6), 1 µl glycogen (20 mg/ml). This mixture was allowed to stand for at least 30 min at -20°C. Centrifugation for 15 min at 5300 rpm and 4°C to form the pellet was carried out and ethanol removing by decanting. Addition of 100 µl of cold ethanol (70%) and centrifuging for 5 min under the same conditions followed. Inverted microplate centrifugation for 1 min at 700 rpm and standing in a sterile chamber for 1 min was performed for removing all residual ethanol. Fragment length was determined using CEQ 8000 Genetic Analysis System (Beckman Coultier) using CEQ 600 pb standard. An algorithm based on peak area was used to sample alignment.

The Virtual Digest ISPaR found in the Microbial Community Analysis (MICA) website at the University of Idaho (http://www.sci.uidaho.edu/biosci/labs/forney/mica/) was used to predict the enzyme digestion considering the primers and enzymes used. This database was used to compare and analyze the fragments obtained from the samples in this study as described by Kent *et al* (2003) using the Phylogenetic Assignment Tool (PAT) (http://trflp.limnology.wisc.edu/index.jsp) from the North Template Lakes Microbial Observatory. Fragment size and pond conditions were analysed using Principal Component Analysis (PCA) with the Unscrambler® v9.2 (1996-2005 CAMO PROCESS AS). Cluster analysis for fragment size, bacterial counts

and pond conditions was conducted using Bionumerics® - Version 3.0 (Applied Maths).

Results

According to farm records, no antibiotic use was detected at any pond under study, but lime (CaO) was used periodically (7 to 10 days, 50Kg / Ha) as pond disinfectant. A total of 32 water samples were collected. Ponds were harvested at different times, and samples were collected from S1 to S5 for two ponds (B and C), pond A was sampled form S1 to S6 when harvest occurred. At the end of the sampling period, a tropical storm affected the study area lowering the water and ambient temperature by 5 °C for about three days, without a great effect in average temperature data. Temperature and dissolved oxygen variations along the study period, including the average daily fluctuation and the relation between those variables are shown in Figure 2.1.

Total viable bacterial counts (TBC), were more stable than those values found for *Vibrio* population. The behavior of TBC and *Vibrio* population at different sampling time is shown in Figure 2.2.

Correlations, loadings, and scores from PCA analysis results are shown in Figure 2.3. As it can be seen, temperature, dissolved oxygen and MPN of *Vibrio* populations were drawn as the most important variables under study. T-RFLP patterns and pond conditions cluster analysis is presented in Figure 2.4.

Fragment size frequency for entrance (EW) and composite water (CW) by sampling period for *HhaI* are shown in Figure 2.5. Similar results in terms of frequency were found for *HaeIII* enzyme (not shown). Some fragments were not present at all samples type; Table 2.1 shows those fragment sizes present in at least one EW sample and missing in all CW ample and vice versa (considering ±1 pb). The most frequent and higher peak fragments detected in Pond A and a list of those major groups for all samples including the bacterial group that present similar fragment with used enzymes according to PAT (Phylogenetic Assignment Tool, Kent et al, 2003) are shown in Figure 2.6 and Table 2.2 respectively.

Discussion

Water temperature increase along the sampling period, while temperature daily fluctuation (TDF) shows the opposite pattern (Figure 2.1a). At higher water temperatures, the differences along day were smaller; it gives a more stable environment in terms of temperature. The opposite pattern is found for dissolved oxygen and its daily fluctuation (DODF) (Figure 2.1a). Therefore, at higher and more stable temperatures, anoxic conditions are present with higher fluctuation rate. Similar patterns can be found in the bottom of estuaries, where anoxic conditions are common and alpha prteobacteria, cytophaga, flavobacteria and Gram positive bacterium are more common (Bouvier and Giorgio, 2002;

Boschker et al. 2005,). Figure 2.1b, shows the relation between temperature and dissolved oxygen. A significant drop of dissolved oxygen can be observed for temperatures higher than 25°C. It is possible to make a differentiation of pond conditions from the third sampling period. The S1, S2 and S3 sampling periods, show high stable dissolved oxygen content and the lowest and variable water temperatures, while sampling S4, S5, and S6 show lowest and variable dissolved oxygen content and high and stable water temperature.

No correlation between TBC or Vibrio population and temperature was found. Similar results for total culturable bacteria were reported by Stabili and Cavallo (2004) at Italian coastal waters. They found that no correlation between bacterial counts and temperature were present in area with continuous loading of nutrients, under those circumstances the temperature effect and other environmental factors were masked, although major bacterial density was found in warmer months.

The combined effect of temperature and dissolved oxygen could be minimized by the continuous high organic load (not measured) in shrimp ponds and the use of disinfectant (CaO) by the farm management. The latest could have a major negative impact on pond bacterial population. This effect is more appreciated in *Vibrio* populations than in the total viable bacterial count (TBC) (Figure 2.2). TBC were stable along the sampling period and similar but slight low values were found for entrance and composite water, with the exception of S5. Slight decrement for TBC can be observed in CW from S4 (29.27°C) to S6 (30.51°C), probably by pond management.

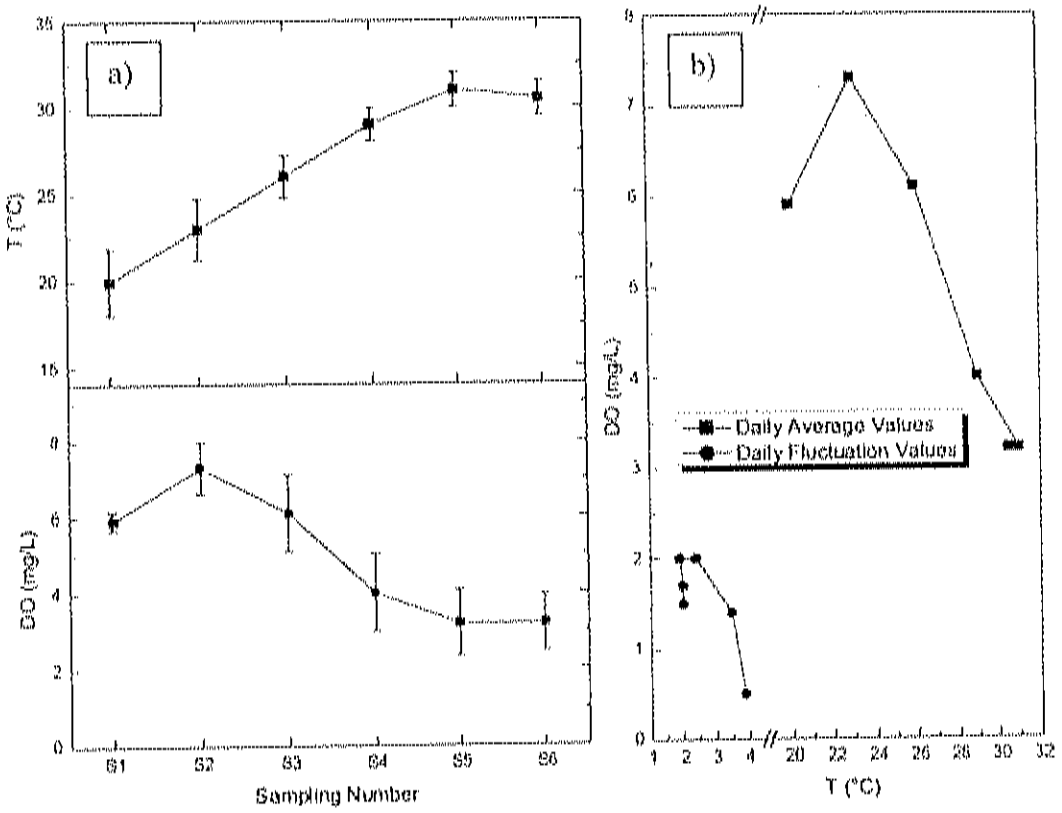

Figure 2.1a) Temperature (°C), dissolved oxygen (mg/l) and its daily fluctuation values (l) found at different sampling times. 1b) Relation between averages dissolved oxygen values and temperature (DO – dissolved oxygen, T – temperature °C).

As it was mentioned above, this study did not find correlation between *Vibrio* population and temperature contrary to most of the reported results for Vibrio in estuaries, seawater (Barberi, et al., 1999) and shellfish (Cook et al., 2002). In agreement to this study, Deepanjali et al. (2005), found no correlation between *Vibrio* abundance and temperature in tropical waters. They found that organic matter richness and rain will affect *Vibrio parahaemolyticus* counts more than temperature. They found that the highest incidence was at dry season and high organic load. The study farm is located in a dry and semi-desert area, which does not report a high rain level (>200 mm annual average from 1986 -2003, INEGI, 2004). Higher *Vibrio* populations can be observed for CW at initial stages; at S3 and S4 values were smaller that those found in EW probably affected by CaO used by the farm. An increment in both (EW and CW) was observed at S5 and a drastic decrement at S6, especially for EW. The last sampling period, was affected by a tropical storm a week before the sampling, its effect was not enough to affect the temperature and dissolved oxygen data, but it seem that *Vibrio* population in seawater could be affected by this natural event. This effect could not be corroborated because the pond was empty by harvest operation the same day the last sample was taken. In general, *Vibrio* population in CW showed a different pattern to TBC. A higher fluctuation and less stable population can be observed for Vibrio populations. Those fluctuations can be explained by pond management.

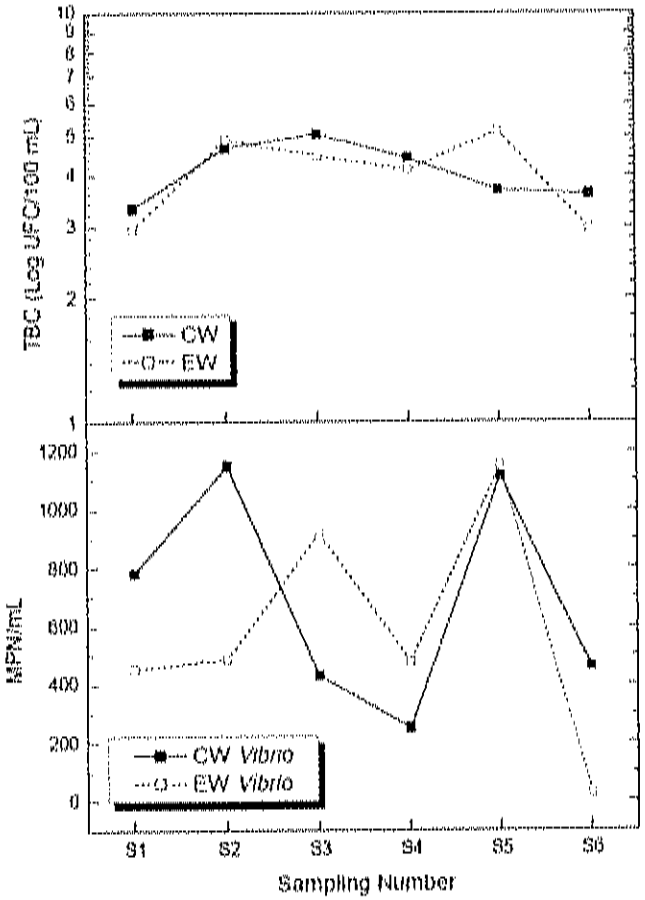

Figure 2.2 Time variation of total viable bacterial count (TBC Log UFC/ ml) and *Vibrio* populations (MPN/ml) for EW (entrance water) and CW (composite water).

MPN Vibrio population, temperature, dissolved oxygen and its daily fluctuations were the major variables affecting the system. Positive correlation for sampling period, temperature and dissolved oxygen daily fluctuation (ODF) was found and negative one for dissolved oxygen and temperature daily fluctuation (Figure 2.3).

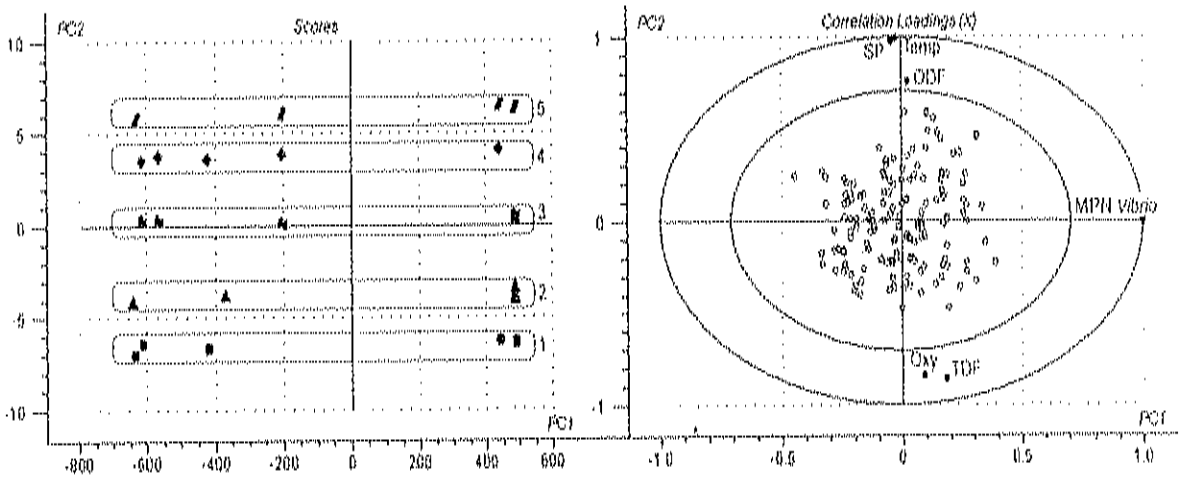

Figure 2.3. Correlation loadings and scores for fragment sizes obtained with *HhaI* and *HaeIII* enzymes, including other pond variables. Groups were made considering sampling period, temperature (T) and dissolved oxygen(DO), where 1: samples at S1, T 19.8–19.95 °C, DO 5.85–5.95 mg /l; 2: samples at S2, T 22.9–23.1 °C, DO 7.1–7.45 mg/l; 3:samples at S3, T 26.4–26.5 °C, DO 6.0–6.1 mg/l; 4: samples at S4, T 29.2–29-3 °C, DO 3.9-4.0 mg/l; 5: samples at S5-S6, 30.5–31.0 °C, DO 3.0-3.3 mg/l; SP: sampling period; Temp: temperature (°C); DOF: dissolved oxygen fluctuation (mg/l); Oxy: dissolved oxygen (mg/l); and TDF: temperature daily fluctuation (°C).

Those variables affect bacterial diversity, but not bacterial density, as it was explained earlier. Sample groups were made based on temperature and dissolved oxygen. Most of the scientific reports, agree that Vibrio is ubiquitous in the marine environment and one of the major bacterial group present in shrimp ponds (Vandenberghe et al., 2003), and its density also affects the sample distribution (Figure 2.3). But it was an independent variable from those considered in this study, and no correlation with any other variable was found.

Therefore, variables that affect changes in Vibrio population in the shrimp pond under commercial conditions can not be associated from this study.

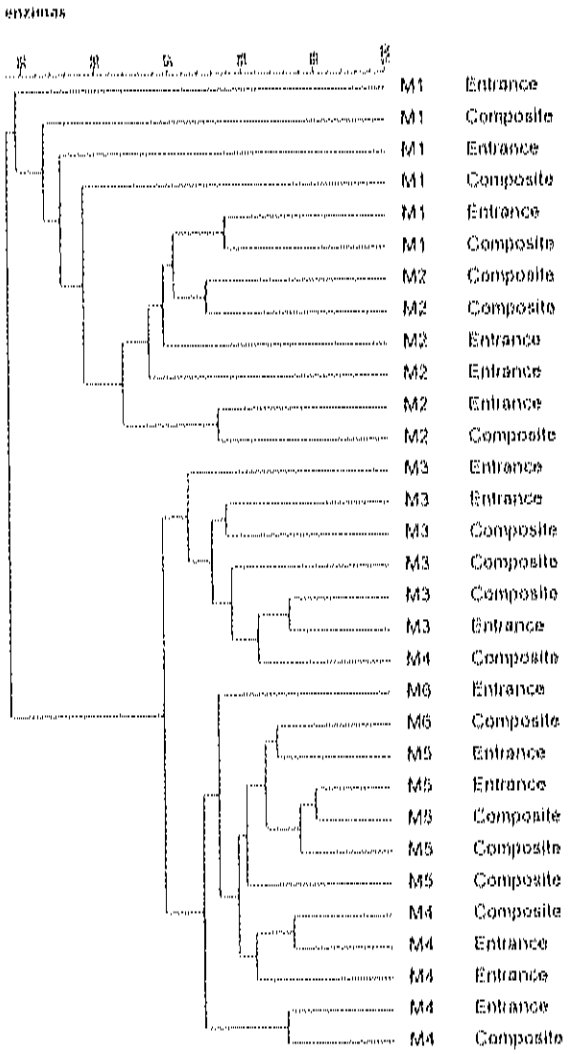

Figure 2.4. Dendrogram from cluster analysis including T-RFLP patterns and pond conditions (Rearson, UPGMA).

Groups from cluster analysis also are influenced by sampling period. PCA and cluster analysis showed a clear differentiation between sampling periods. It is possible to identify 3 groups the first one from S1 to S2 another one composed only for S3 and the third one from S4 to S6. (Figure 2.3 and 2.4). This corroborates that bacterial community changes along time could be determined by temperature and dissolved oxygen. Pond itself does not appear as a significant variable, even when small differences can be seen at individual fragment size levels. Therefore for explicative purposes we are going to refer in some cases to Pond A as an example of all ponds.

TRFLP- Patterns: CW samples present more diverse bacterial community, since they present a larger number of DNA fragments (with both enzymes); and it also was evident for the number of unique fragment lengths (Table 2.1). Three fragments sizes were present in most of the sampling period in EW (60, 435 and 488-489 bp), and seven in CW (56, 60, 62, 304, 433-435, 485-486 and 489-490 bp) for *HhaI* enzyme (Figure 2.5). Those fragments did not represent the major part of the bacterial population in most of the samples. Those fragments that were found in major intensity were varying along time (Figure 2.6), and were not necessarily the same for EW and CW. For example, 200 – 2001 bp fragment (possible Clostridia and/or gamma- proteobacteria) were present from S1 to S4 in entrance at low levels except for S4, an composite water in higher proportion. On the other hand 490-492 bp fragment were barely detected in CW, but were present as a major part of the population of entrance water in S1 and was clearly detected in most of the sampling periods at low proportions. Finally 303 –

304 bp fragments were detected in almost all the sampling period for CW and only at the initial stages (S1 to S3) in EW.

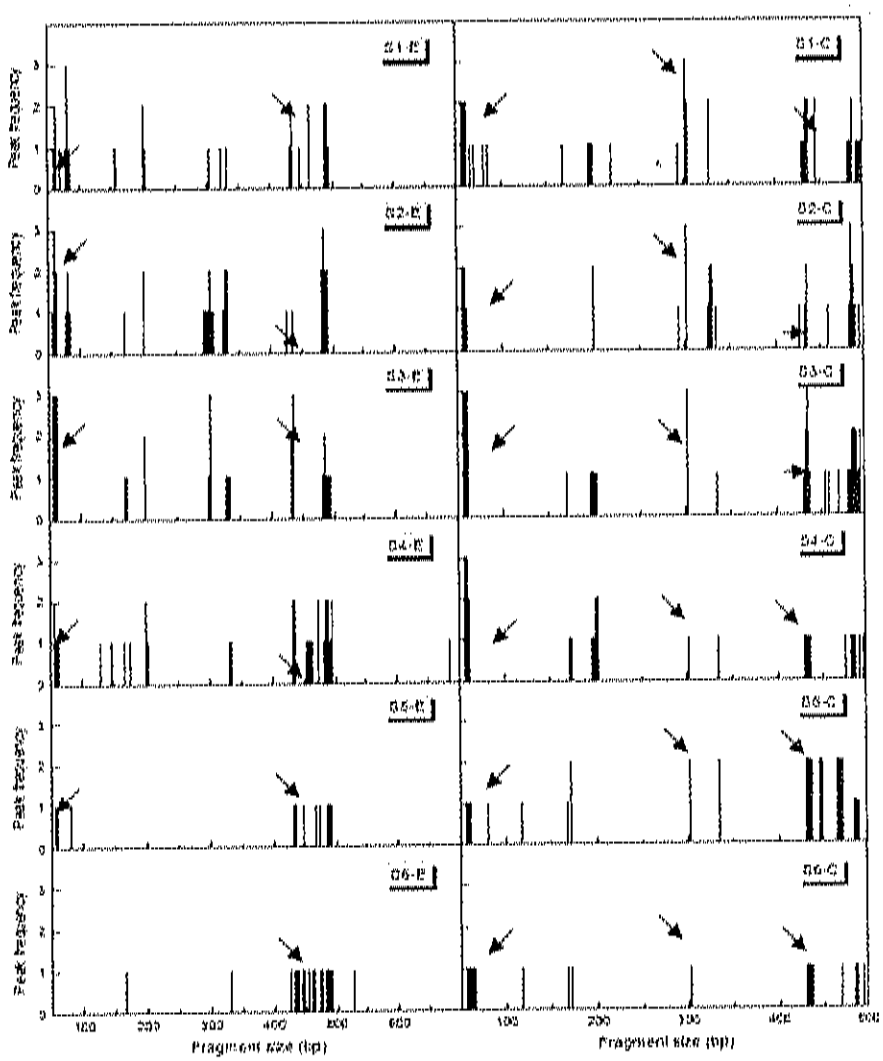

Figure 2.5. *HhaI* fragment size frequency for entrance (E) and composite (C) water by sampling period. Common fragment sizes for EW: 435 and 488-489 bp and 60bp for five sampling periods. Common fragments sizes for CW: 56, 60, 62, 304, 433-435, 485-486 and 489-490 bp.

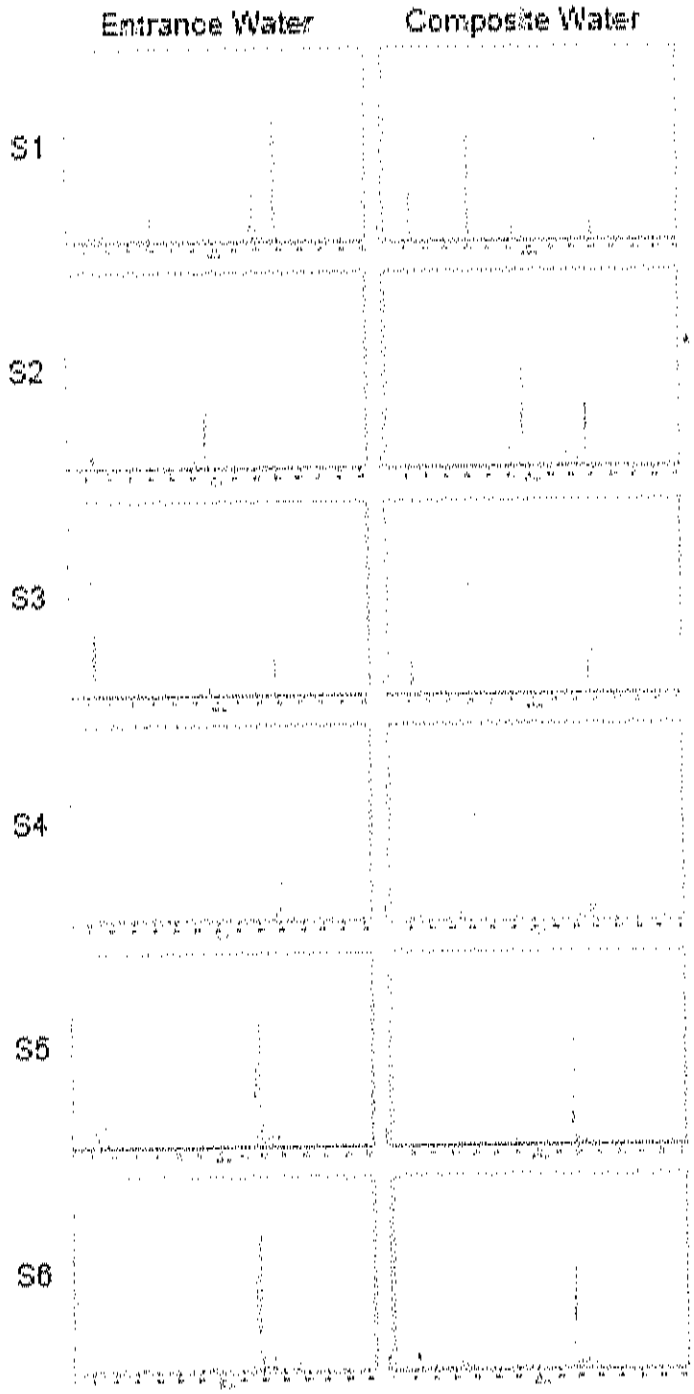

Figure 2.6. T-RFLP patterns found with *Hhal* enzyme at different sampling periods for entrance and composite water samples in pond A.

There were two group 433 and 435 - 437 bp fragment that were present in EW and CW along the sampling period, but they became significantly important part of the bacterial population from S4 to S6. Among other bacterial groups this fragments could represent some alpha – proteobacteria, Cyanobacteria, and *Vibrio* species when combined with the fragments sizes found with *HaeIII* in the same samples (Table 2.2). The importance of these fragments is that they seem to predominate in CW in S5 and S6, even when they are important for EW in S4. Since this DNA fragments could represent a mixture of bacterial groups (Table 2.2) more studies including more restriction enzymes and DNA sequencing clones should be conducted to give more information about their identity and the way they are affected by pond environmental conditions.

Table 2.1. Fragment size for *HhaI* and *HaeIII* present in entrance water (EW) but missing in composite water (CW) and vice versa.

BP	*TOTAL*	*HhaI –EW*	*HhaI – CW*	*BP*	*TOTAL*	*HaeIII-EW*	*HaeIII–CW*
65-66	3	0	3	87	3	3	0
69	1	0	1	90	1	1	0
79	2	2	0	149	5	0	5
118	2	0	2	151-153	5	0	5
128	1	1	0	166	2	0	2
145	1	1	0	171	1	0	1
157	1	1	0	186	1	0	1

BP	*TOTAL*	*HhaI –EW*	*HhaI – CW*	*BP*	*TOTAL*	*HaeIII-EW*	*HaeIII–CW*
164	2	2	0	212	1	0	1
167	1	0	1	216-217	4	4	0
195-196	3	0	3	224	1	0	1
220	1	0	1	237	1	0	1
299	1	1	1	241	1	0	1
309	1	1	0	244	1	1	0
323	1	1	0	246	1	1	0
333	2	2	0	249-250	2	0	2
440	1	1	0	266	4	0	4
444	1	1	0	286	2	0	2
446	2	0	0	351	1	1	0
463-464	4	4	0	365	1	1	0
476	1	1	0	367	1	1	0
478	1	0	1	373	2	0	2
527	1	1	0	376	1	1	0
684	1	1	0	424	1	1	0
---	---	---	---	427	1	1	0
---	---	---	---	448	1	1	0
---	---	---	---	458-459	2	2	0
---	---	---	---	464	2	2	0
---	---	---	---	469	1	1	0
---	---	---	---	474	1	1	0
Missing peacks		8	15			13	16

BP = Base pair.

Table 2.2. Fragment size for *HhaI* that were a major part of the population that also corresponds with a *HaeIII* fragment in the same sample and the possible identification using PAT (Phylogenetic Assignment Tool) program (Kent et al., 2003).

Size fragment (pb) – Pond: Sampling	Class	Possible Identification
58 – 62: **AEW**:2,3,5; **ACW**:3,4; **BEW**:1,2; **BCW**: 1,3,4,5; **CEW**: 2,3,4; **CCW**:1,2,3,5	*Flavobacteria*	*Flavobacterium* sp, Marinbacter sedimenticola
	Other	Marine bacterium, Uncultured bacterium
200 – 201. **AEW**:1, 3, 4; **ACW**:1, 3, 4; **BEW**:3, 4; **BCW**:3, 4; **CEW**:3; **CCW**:4	γ-proteobacteria	Uncultured γ-proteobacterium
	Clostridia	*Clostridium* sp.
303 -304 AEW: 2,3; ACW:1,2,3,5,6; BEW:1; BCW:1, 2,3,4,5; CEW:2,3; CCW:1,2,3,5	α -*proteobacteria*	α-methylotroph Hohenstaufen, marine α-proteobacteria, α-proteobacterium, *Agrobacterium* sp, *Afipia* sp, *Beijerinckia* sp, *Bradyrhizobium* sp, *Bosea* sp, *Chelatococcus asaccharovorans*, *Crassostrea virginica* symbiont, *Ketogulonigenium* sp *Loktanella salsilacus*, *Methylobacterium* sp, *Methylarcula* sp, *Nordella ligomobilis*, *Methylosinus* sp, *Nitrobacter* sp, *Oceanibulbus indolifex* type, *Pedomicrobium americanum*, *P. australiccus*, *Oleomonas sagaranensis*, *Oligothropa carboxidovorans*, *Reugeria* sp, *Rhizobiaceae* st, *Rhizobium* sp, *Rhodopseudomonas* sp, *Rhodobacter* sp, *Rhodoblastus acidophilus*, *Sulfitobacter* sp, *Roseovarius* sp, *Stappia* sp, *Roseobacter* sp, Type II methanotroph, Unc: *Rhodoplanes* sp, *Hyphomicrobium* sp, *Rhizobiales*, *Rhodobacteriaceae*
	γ-proteobacteria	*Moraxella* sp, Vestimentiferan symbiont and Uncultured type I methanotroph
	Clostridia	Unidentified and or uncultured eubacterium clone.

Size fragment (pb) – Pond: Sampling	Class	Possible identification
	Others	Bacterium, filamentous photosynthetic, furfural-degrading, Artic sea ice, glacial ice, metal-contaminated soil, petroleum-degrading bacterium; Unc: soil, earthworm cast, gold mine, sludge, yard-trimming-compost, Green Bay ferromangaous micronodule, proteobacterium, rape rhizosphere
330-331 AEW: 2, 3, 6; ACW: 2; BCW: 2; CEW: 4	*Actinobacteridae*	*Arthrobacter* sp, *Micrococcus* sp, *Rhodococcus* sp
	Clostridia	Uncultured eubacterium
	Others	Uncultured bacterium
433 AEW: S5, S6 ACW: S5, S5 BEW: S4 BCW: S4, S5 CEW: S4 CCW: S3	*α–proteobacteria*	α-proteobacterium, Marine α-proteobacterium, *Agromones oligotrophica, Albidovulum inexpectatum, Amaricoccus macauensis, A. veronensis, Afipia* sp, *Blastobacter denitrificans, Brevundimonas diminuta, Bradyrhizobium* sp, *Caulobacter* sp, *Maricaulis indicus, Kaistina koreensis, K. methylovorans, Stappia* sp, *Methylocystis* sp, *Methylobacterium* sp, *Paracoccus* sp, *Pedomicrobioum magnanicum, Rhodobaca bogoriensis, Photorhizobium thompsonianum Rhodopseudomonas* sp, *Rhodobacter* sp, *Rhodovulum* sp, *Rhizobiumium* sp, *Roseaobacter* sp, *Salipiger mucescens*, Type II methanotroph, Unc *Nitrobacter* sp
	γ-proteobacteria	*Vibrio* sp, *V. anguillarum, V. pomeroyi, V. splendidus*, Uncultured γ-proteobacterium
	Actinobacteria	*Actinobacteria, Arthorbacter* sp, *Brachybacterium* sp, *Cellulomonas* sp, *Micrococcus* sp, *Nesterenkonia aurantia, N. luteus*
	Cyanobacteria	*Anabaena* sp, *Cyanothece* sp, *Cylindrospermopsis raciborskii, Gloeothece* sp, *Synechococcus* sp, Uncultured: Antarctic cyanobacterium, *cyanobacterium*
	Clostridia	Uncultured *eubacterium*
	Other	Bacterium, Hydrothermal vent strain, Gram-positive, mucus and soil bacterium, Unc: Crater lake, earthworm cast, sludge, Green Bay ferromanganous micronodule

Size fragment (pb) – Pond: Sampling	Class	Possible Identification
435 – 437 AEW: S1, S3, S5, S6 ACW: S1, S2, S3, S5, S6 BEW: S1, S4 BCW: S2, S3, S4, S5 CEW: S2, S3, S4 CCW: S1, S3, S5	α –proteobacteria	α-proteobacteria, marine α-proteobacteria, *Amaricoccus macauensis*, *A.veronensis*, *Bradyrhizobium* sp, *Brevundimonas diminuta*, *Maricaulis indicus*, *Roseinatronobacter thiooxidans*, *Methylocystis* sp *Paracoccus* sp, *Pedomicrobium magnanicum*, *Stappia* sp *Rhizobium* sp *Rhodobacter* sp, *Rhodovulum* sp, *Roseobacter* sp, Type II methanotroph
	γ-proteobacteria	*Vibrio sp*, *V. pomeroyi*, *V. splendidus*, *V. tasmaniensis*
	ε-proteobacteria	Uncultured ε-*proteobacterium*
	Clostridia	Uncultured *eubacterium*
	Actinobacteria	*Arthorbacter* sp, *Brachybacterium* sp, *Micrococcus* sp, Uncultured actinobacterium
	Cyanobacteria	*Synechococcus* sp, Ucultured: cyanobacterium
	Other	Marine and mucus bacterium, Uncultured: proteobacterium, Crater lake, sludge, soil
484–485: **AEW**:2,3,4,5; **ACW**:2,3,4; **BEW**: 2,3,4; **BCW**:2,3,5; **CEW**:2,3; **CCW**:2,3	*Γ*-proteobacteria	*Vibrio salmonicida* Uncultured γ-proteobacterium
488–489: **AEW**: 2,5,6; **ACW**: 2,3,4; **BEW**: 1,2,3,4; **BCW**:2,3,5; **CEW**:3,4; **CCW**:1,3,4	*γ-proteobacteria*	*Vibrio salmonicida* (menos 19), Uncultured: Pseudomonas, γ-proteobacterium
	Other	Uncultured: bacterium, rumen bacterium
490 -492: **AEW**:1,2,3,4,5; **ACW**:3,4; **BEW**: 1,2,3,4; BCW:2,3,5; **CEW**:2,3; **CCW**:3	Other	Uncultured bacterium

Size fragment (pb) – Pond: Sampling	Class	Possible identification
492 – 494 **AEW**: 4, 5, 6; **ACW**: 3, 4; **BEW**: 3, 4; **BCW**: 3; **CEW**: 3; **CCW**: 4	*γ-proteobacteria*	*Vibno salmonicida, V. tasmanienesis*
	Other	Uncultured bacterium, Uncultured rumen bacterium
495 -496 **AEW**:4; **ACW**:2, 4; **BEW**:3, 4	Other	Uncultured bacterium

AEW: Pond A entrance water; ACW: Pond A composite water; BEW: Pond B entrance water, BCW: Pond B composite water; CEW: Pond C entrance water; CCW: Pond C composite water; Arabic numbers represent the sampling period.

Conclusions

Temperature and dissolved oxygen has an effect on pond general bacterial community diversity, although those variables might not affect *Vibrio* population. Total viable bacterial counts were stable along the sampling period, but diversity fluctuations were evident by T-RFLP patterns and *Vibrio* sp. counts probably associated with organic matter and the use of disinfectant lime (CaO). Bacterial community observed in entrance water (EW) was less diverse than pond water (CW), and it tends to stabilize after S4, where *Vibrio* sp. matches with predominant obtained fragments. Bacterial community at harvest was considered stable and probably composed by a mixture of *alpha-proteobacteria*, *Cyanobacteria* and *Vibrio* species.

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ARTÍCULO 3

Pathogenic *Vibrio* Associated to Aquaculture Shrimp and Harvest Operations

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Abstract

Human pathogenic *Vibrio* species present in pond water and in aquaculture shrimp shell-skeleton could survive the harvest operation and freezing process. The exposure to low processing temperatures will affect the quantity and diversity of *Vibrio* species found at the end product, but the potential health risk of these bacteria may not be eliminated. Therefore, the aim of this study was to detect those *Vibrio* species that are present in the shrimp pond and those that survive harvest and freezing. Samples (water, soil, ice and contact surfaces) were collected at harvest period and frozen product harvested from the same ponds was sampled at the processing plant. *Vibrio* spp. counts for frozen product were varying from 99 to 537 and 334 to 551 MPN of *Vibrio* spp./gr in the whole shrimp (edible portion and shell) and shell respectively. Pond water showed the highest values for *Vibrio* population and *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus* group were the most abundant. *Vibrio mimicus*, which was not the main pathogenic *Vibrio* specie found in pond water at harvest time, was detected at harvest operation and end-product (frozen shrimp) up to six months after processing.

Keyword: shrimp, *Vibrio*, Aquaculture

Introduction

Vibrio is a natural occurring bacterial genus in marine environments (Suñen et al, 1995) and has been associated as the most important pathogen in shrimp

aquaculture (Vandenberghe et al, 2003). Biochemical characterization of *Vibrio* species is difficult because the great diversity and similarity among species (Thompson et al, 2004). Most attention has been paid to those human and animal pathogenic species causing gastrointestinal infection (*V. cholerae*, *V. parahaemolyticus*, and *V. mimicus*) and septicemia (*V. vulnificus*, and *V. alginolyticus*) (Elhadi et al, 2004). *Vibrio* species have been associated to warm months, and reported prevalence is higher at higher water temperatures (Pfeffer et al, 2003). Its prevalence is also associated to organic matter and dry season in semi desert or tropical areas where temperature is high and more stable along the year (Deepanjali et al, 2005). Shrimp aquaculture in the northwest of Mexico presents those conditions, high water temperatures, high organic matter and low rain rate.

High levels of *Vibrio* spp. have also been reported associated to marine organisms. The prevalence of *Vibrio* species along the shrimp production chain has been studied. There are studies focused on infected (Goarant et al, 1999) or healthy shrimp (Gomez-Gil et al, 1998). Others studies reports different *Vibrio* species at retail level (Hosseini et al, 2004; Parisi et al, 2004) or associated to seafood handlers in the Southeast of Mexico (Monsreal et al, 1991).

The survival of pathogenic *Vibrio* species at frozen temperatures has been documented, for example recovery of *V. cholerae* in frozen seafood after more than 3 weeks (Reily and Hackney, 1985). *V. cholera* epidemiology has been explained in part with its attachment to copepods (Huq et al, 1984), and it has been suggested that chitin, a major component of crustacean exoskeleton, has cryoprotector role for *V. cholerae* (Platt et al, 1995). Most of *Vibrio* species has the capability to use chitin as carbon source, but the mechanism has not been completely elucidated, for all the *Vibrionaceae* family. The chitin utilization of *V. furnissii* has been explained by Meiborn et al (2004) in three steps: chitin sensing, attachment and degradation.

Shrimp ponds are a good source of chitin for the *Vibrio* community and the attachment to the shrimp shell with the consequent bacterial protection in further handling and processing could be expected. Therefore the aim of this study was

to determine those pathogenic *Vibrio* species that are present in the shrimp pond capable to survive the harvest and commercial process operation and could represent a potential risk health for consumers.

Materials and Methods

Pond sampling: Water and soil samples were collected previous to harvest operation, from a shrimp farm located in the coastal area of Hermosillo, Sonora, Mexico. A total of three ponds were chosen for sampling. Three samples were collected from each pond at different locations: a) incoming water, collected right after the filter in the intake water supply; b) composite water sample prepared from a mixture of bottom and surface water collected in the middle of the pond and water collected before the pond discharge outlet; and c) soil sample, collected in the wet portion of the pond side.

Harvest operations sampling: Six different points at harvest operations were sampled: a) ice and water mixture, used to kept shrimp after it is removed from pond; b) ice from plastic container boxes that will be in direct contact with shrimp, c) ice from transportation trucks (as it arrive to the farm); d) swabs from transportation truck, which was used to taken the shrimp to the process facility; e) swabs from plastic container boxes where the shrimp is transported to the process facility; and f) swabs from nets used for moving shrimp.

All samples were collected in sterile plastic containers and transported to the laboratory in insulated boxes to maintain low temperatures (below 5°C). Swab samples from a 25 cm^2 surface were collected in phosphate buffer solution and transported under the same conditions. Samples were analyzed the same day they were collected for viable total bacterial count and *Vibrio* spp, as described below. Harvested shrimp was tracked to the processing facility and shrimp frozen blocks (5 lb each) were collected, transported to the laboratory and keep at -20°C until it was analyzed. Triplicates of four shrimp production lots were collected at two different processing facilities three months after its arrival to the

establishment. Shrimp samples were analyzed for *Vibrio* spp in the shell portion and the "tail" (edible portion and shell).

Bacterial Count: Ten-fold dilutions were used in spread plates of modified Long and Hammer agar (Koutsoumanis and Nychas, 1999) incubated at 25 °C / 5 days, for total viable bacteria count. Medium composition was as follows (grams per liter of distilled water): Proteose Peptone, 20; gelatin, 40; K_2HPO_4, 1; NaCl, 10; agar, 15; ammonium ferric citrate, 0.25.

***Vibrio* spp**: Samples were analyzed based on the Bacteriological Analytical Manual for the identification of V. *cholerae, Vibrio parahaemolyticus, Vibrio vulnificus* and other *Vibrio* spp. (Elliot et al, 1992). Ten-fold dilutions in alkaline peptone water (APW) were prepared, partial (6-8 hr) and complete (18-24 hrs) incubation at 35-37°C was carried out before transferring to TCBS (thiosulfate-citrate-bile salts-sucrose, Difco) agar plates. All yellow, green, and blue green colonies were selected for biochemical characterization (0%, 3%, 6%, 8% and 10% NaCl growth, motility, indole production, lysine and ornithine descarboxilase, sucrose, glucose and lactose fermentation, arginine dihydrolase, gelatinase and oxidase), some isolated colonies were also analyzed using API® 20E strips. The following CAIM (Collection of Aquatic Important Microorganisms) strains were included in the analysis as control: CAIM320 - *V. parahaemolyticus;* CAIM512 - *V. harveyi;* CAIM516 - *V. alginolyticus;* CAIM593 - *V. fluvialis;* CAIM 602 -*V. mimicus;* and CAIM610 - *V. vulnificus*. Also the *V. cholerae* non O1 strain, provided by the Mexican Health Department was used.

Statistical analysis: Biochemical responses of isolated OTUs were used for cluster analysis (Simple Matching – Unweighted Pair Group Method with Arithmetic Mean or UPGMA). Dendrograms were obtained using Bionumerics® - Version 3.0 (Applied Maths).

Results

Thirty-two samples were collected and analyzed: 12 samples collected at pond farm (soil and water), 10 samples collected at harvest operation (water-ice, and contact surfaces) and 12 frozen shrimp blocks from processing facilities. Total viable bacterial counts and MPN of *Vibrio* spp for water, soil, shrimp tail and shrimp shell samples from sampled ponds are shown in Table 3.1. Results for ice and direct contact surfaces samples, collected at harvest time are shown re presented in Table 3.2.

A total of 607 OTUs (Operational Taxonomic Units) were obtained from analyzed samples; 51 recovered from soil, 125 from water samples (entrance and composite), 124 from harvest operation (ice and direct contact surfaces) and 307 from shrimp (whole tail and shell). A fully identification of all isolated OTUs was not possible with the performed biochemical test. Table 3.3 shows those *Vibrio* species identified using the Alsina and Blanch (1994) scheme by sample type. Control strains were used at individual cluster analysis in order to find similarities with the isolated OTUs (Table 3.4), separated dendrograms for soil, water, and shrimp and direct contact surfaces and ice samples are shown in Figures 3.1 to 3.4. Biochemical responses by sample type and those suspected non *Vibrio* species are shown in Table 3.5 and 3.6, respectively. The same analysis was completed for all OTUs, and the obtained dendrogram is shown in Figure 3.5, while major groups and the sample composition by group are presented in Table 3.7.

Discussion

Total viable bacterial counts (TBC) were higher for entrance water (EW) and soil samples. Similar values for EW collected in pond A and C were found, while pond B present lower counts. Major differences, up to 2 Log, were found in EW and composite water (CW) (Table 3.1). This bacterial depletion could be caused

by competition of the pond bacterial population, and pond conditions (low dissolved oxygen). Although, it seems to affect only the entire bacterial population and not the *Vibrio* counts. It seems that *Vibrio* species found in the ponds were already established as part of the major population.

As expected *Vibrio* species were higher at shrimp shell than the edible portion and shell. It has been reported that most *Vibrio* species can produce chitinase, (Shin et al, 2000) and some species posses a lectin-mediated systems which help to attach the bacteria to the crustaceans shell or other chitinosus material (Platt et al, 1995). Samples collected during harvest operation shown variable results for both TBC and MPN of *Vibrio* (Table 3.2). Highest values were found in ice water sample, which was used as a shrimp anesthetic. The water used in this operation, comes directly from the pond that has been harvested, then is mixed with the ice, and shrimp is placed in this mixture immediately after it is removed from the pond. The shrimp can stay in this mixture from several minutes to more than 1.5 hours before it is weighted and placed in plastic boxes of about 20 Kg. In addition, this mixture was not renewed frequently, but it was common the addition of ice to maintain low temperatures. Therefore this operation was the one that have more direct influence from the bacterial load present in the harvested ponds. Ice and direct contact surface samples were elements introduced to the farm at this stage. Although they were not properly sanitized, considering the high bacterial count found, they showed low levels of *Vibrio* population except for the ready-to-use ice sampled from a plastic container. Ice and truck used during shrimp transportation were showing the highest bacterial count; therefore cross contamination with non *Vibrio* species is possible from this surfaces to shrimp.

Table 3.1. Total viable bacterial count (ufc/gr or ml) and *Vibrio* spp (MPN/ml or gr).

	Total Viable Bacterial Count (ufc/ml or gr)			*Vibrio* spp (MPN/ml or gr)		
	Pond A	Pond B	Pond C	Pond A	Pond B	Pond C
Shrimp	ND	ND	ND	99	537	175.00
Shell	ND	ND	ND	334	551	502.00
EW	125,460	2,400	139,500	28 to >1100	>1100	>1100
CW	4,225	3,500	6,300	460 to 1100	1,100	>1100
Soil	267,500	90,000	950,000	<3 to >1100	9	>1100

ND: Not determined; Shrimp: shrimp tail; Shell: shrimp shell; EW: Entrance water; CW: composite water;

Table 3.2. TBC and *Vibrio* population for different harvest surfaces and used ice

	TBC - Pond A, B and C	*Vibrio* - Pond A, B and C
Ice – Water	2,220,000	290.00
Box – Surface	28,000	23.00
Ice from plastic box ready to use	2,355	>1100
Ice from transportation truck	34,123	3
Transportation truck surface	89,079	3 to 9
Net Surface	1,717	23

TBC: Total viable bacterial count (ufc/ml or cm^2); *Vibrio*: MPN/ml or cm^2 of *Vibrio* spp.

A complete *Vibrio* species identification was not possible with the used biochemical tests, nevertheless using the Aslina and Blanch (1994) scheme was possible to identify about 15% of the isolated OTUs (Table 3.3). *V. mimicus* and *V. vulnificus* B2 were the most common bacteria identified using this scheme,

and were present in all type of samples. Because of the low proportion of species identified at species level it is not possible to assume that those were the most frequent ones. Based on species variability direct contact surfaces were the most diverse and *V. vulnificus*, *V. mimicus* and *V. metschnikovii* were the most common identified. Cluster analysis do not group together those similar bacteria identified by Alsina and Blanch scheme (1994). This difference can only be explained with atypical strains or by method standardization. Ottaviani et al (2003) analyzed 159 strains isolated from frozen and fresh seafood and conclude that test method greatly influence in the results for an optimal *Vibrio* species identification.

Table 3.3. Identified strains using the Alsina and Blanch (1994) scheme.

Microorganism	EW	CW	Soil	Surface	Frozen Shrimp
Aeromonas	---	---	---	1	2
Photobacterium legionethi	---	---	---	1	---
Plesiomonas shigeloides	---	---	---	1	---
V. alginolyticus	---	---	---	2	---
V. carchariae	---	---	---	1	---
V. furnissii	---	---	---	---	3
V. fluvialis	1	---	---	4	1
V. harveyi	---	1	---	---	---
V. mediterranei	---	---	---	---	5
V. metschnikovii	2	---	---	---	15
V. mimicus	**2**	**2**	**10**	**1**	**3**
V. orientalis	---	1	---	---	---
V. vulnificus B2	**1**	**1**	**1**	**5**	**24**

EW = Entrance Water; CW = Composite water; Surface = contact surface; --- = not identified.

Biochemical responses of control strains were included in cluster analysis. Soil samples were the less diverse (Table 3.4), while harvest operation and end-product were the most diverse type of samples in terms of *Vibrio* population, according to Simpson's Index of Diversity. Identical biochemical responses for control strains and at least one OTU were found found only in water and end-product samples. Individual cluster analyses by sample type were performed showing differences in diversity and species distributions (Figure 3.1 to 3.4). About 51% of all OTUs were sucrose positive. Main biochemical differences were based in salt tolerance, for example, only 7.8% of OTUs recovered from soil samples growth at 0% NaCl, compared with 20.8, 55.2 and 55.4% of those isolated from water, harvest operation and end end-product respectively. End-product samples showed major biochemical differences when compared with other samples (Table 3.5), and they presented the lowest percentage of OTUs growing at 8%, 10% NaCl and motile bacteria. Most of marine bacteria including *Vibrio* species are motile (Madden et al, 1989) and it has been used as discriminatory test. Although, some non-motile species such *V. halioticoli* group has been reported (Thompson et al, 2004) therefore, the motility test itself can not be uses as determinative test for *Vibrio* spp.

Table 3.4. Diversity of *Vibrio* species isolated from different samples.

Sample	Group No.	Simpson's Index of Diversity	Biggest group	Control strains: OTUs (100% similarity)
Soil	10	78.77	VII–*V. alginolyticus*	Not found
Water	15	87.87	IX–*V. parahaemolyticus, V. harveyi* and *V. alginolyticus*	*V. parahaemolyticus* – *V. harveyi* : 2
Surface	18	91.11	V–any control strain	*V. mimicus*:1
Product	29	90.24	XXIII–any control strain	*V. mimicus*: 1

Most of the OTUs obtained from soil samples (Figure 3.1) cluster around control strain, showing a high similarity (≥80%). OTUs obtained from water samples also cluster around control strains (Figure 3.2). Even though it is possible to appreciate cluster XII that did not group with any control strain, but still was considered as *Vibrio* spp while all their units cluster with >70% similarity with its neighbor cluster (XI - *V. cholerae*, *V. mimicus* and *V. vulnificus group*).

Table 3.5. Percentage of positive biochemical responses for all isolated OTUs and by sample type.

Name	All Samples (%)	Soil (%)	Water (%)	Harvest (%)	End-product (%)
0% NaCl	44.22	7.84	20.80	55.28	55.37
3% NaCl	99.01	98.04	100.00	98.37	99.02
6% NaCl	74.14	84.31	66.40	64.52	79.48
8% NaCl	51.07	84.31	69.60	62.90	33.22
10% NaCl	32.34	62.75	41.13	46.77	17.92
Motility	77.19	94.12	93.60	89.52	62.62
Indole	48.09	60.78	53.66	47.58	43.93
Ornitine	43.87	37.25	52.00	41.13	42.76
Sucrose	51.67	39.22	58.68	46.77	52.98
Glu	95.88	100.00	97.60	97.58	93.81
GAS	24.71	47.06	12.80	25.81	25.41
Lactose	11.88	0.00	3.20	1.61	21.57
Arg	41.58	33.33	36.80	56.45	38.89
H_2S	13.84	15.69	14.40	16.13	12.38
Oxidase	74.45	96.00	91.80	87.50	59.28
Lys		91.30	78.13	68.57	27.36
Gel		94.74	92.42	81.40	40.53

Soil: soil samples; Water: entrance and composite water samples; Harvest: harvest operation samples; End Product: frozen shrimp samples. Indole: Indole production, Ornithine: Ornithine descarboxilase, Glu: Glucose fermentation, Gas: Glucose gas production, Lactose: Lactose fermentation, Arg: Arginine dihydrolase, Lys: Lysine descarboxilase, Gel: Gelatinase

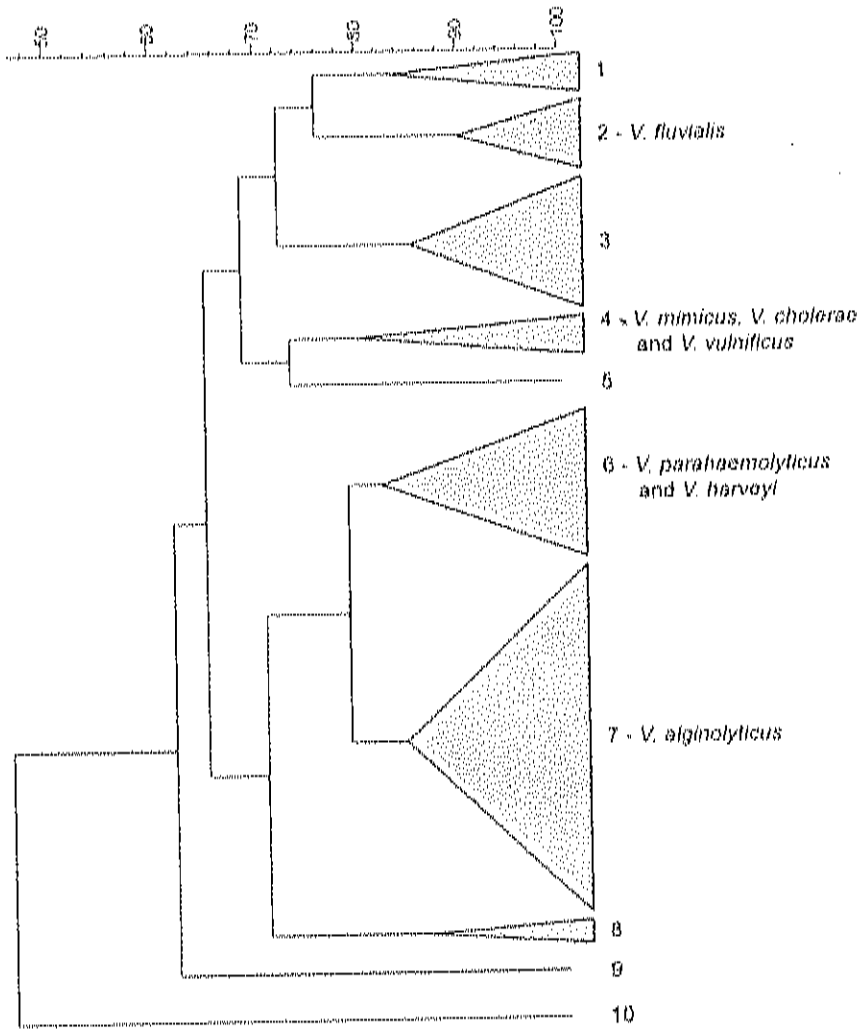

Figure 3.1. Dendrogram from OTUs obtained from soil samples.

Figure 3.3, shows those OTUs from harvest operation. The bigger group (Cluster 05) did not cluster with any control strain, but showed 68% similarity with *V. harvey – V. paraehamolyticus* group. Finally, OTUs obtained from end-product shown a completely different grouping pattern than control strains (Figure 3.4), but the main group still can be considered as *Vibrio* species (75.9%

similarity). End product samples shows 29 clusters, only seven of them (XXIII to XXIX) show similarities higher than 70% with control strains. Almost half of the isolated OTUs from the remaining clusters were non-motile (Table 3.6), and were not considered as *Vibrio* species. Pond water and harvest operation had and undoubted effect over end product samples (Table 3.7), but none of the major groups present in water samples (Figure 3.2 groups 07, 09 and 12) were predominant at the end end-product. For instance cluster I (*V. fluvialis* group) and cluster IX were represented by a large number of OTUs in water and harvest samples but were hardly detected in end end-product samples. On the other hand, cluster XXIV, XXVII, XXXIV, XXXV and XXXVI were practically not detected at water samples but well represented in end end-product samples. OTUs from end product samples that cluster within ≥70% of control strains were isolated from low dilutions or direct plating and low proportion of them are assumed. The *V. mimicus* control strain was the only one that clusters with an OTU obtained from end-product and harvest operation (ready to use ice from plastic box). Even though, *V. mimicus* was found at the lowest dilution used and its relative abundance was low, their importance is based on its pathogenic capacity and the similarity with other pathogenic *Vibrio* species such *V. cholerae*. *V. mimicus* that has also been associated as the causal agent of gastroenteritis but not epidemic diarrheas as *V. cholera*, and several toxins including CT –like toxin has been associated with both bacteria (Boyd, et al, 2000). It is important to note that V. mimicus does not report growth at 4°C (Alsina and Blanch, 1994), and temperature once harvest operation start could be the reason of the low incidence. The presence of this bacteria even at the end product, suggest that freeze process could not totally affect the availability of this bacteria. The chitin surface colonization of *V. cholerae* has been suggested by Castro-Rosas and Escartín (2002) as one of the major factor associated to survive under frozen conditions, therefore a similar mechanism might be expected for *V. mimicus* and other pathogenic or non pathogenic *Vibrio* species as a survival method.

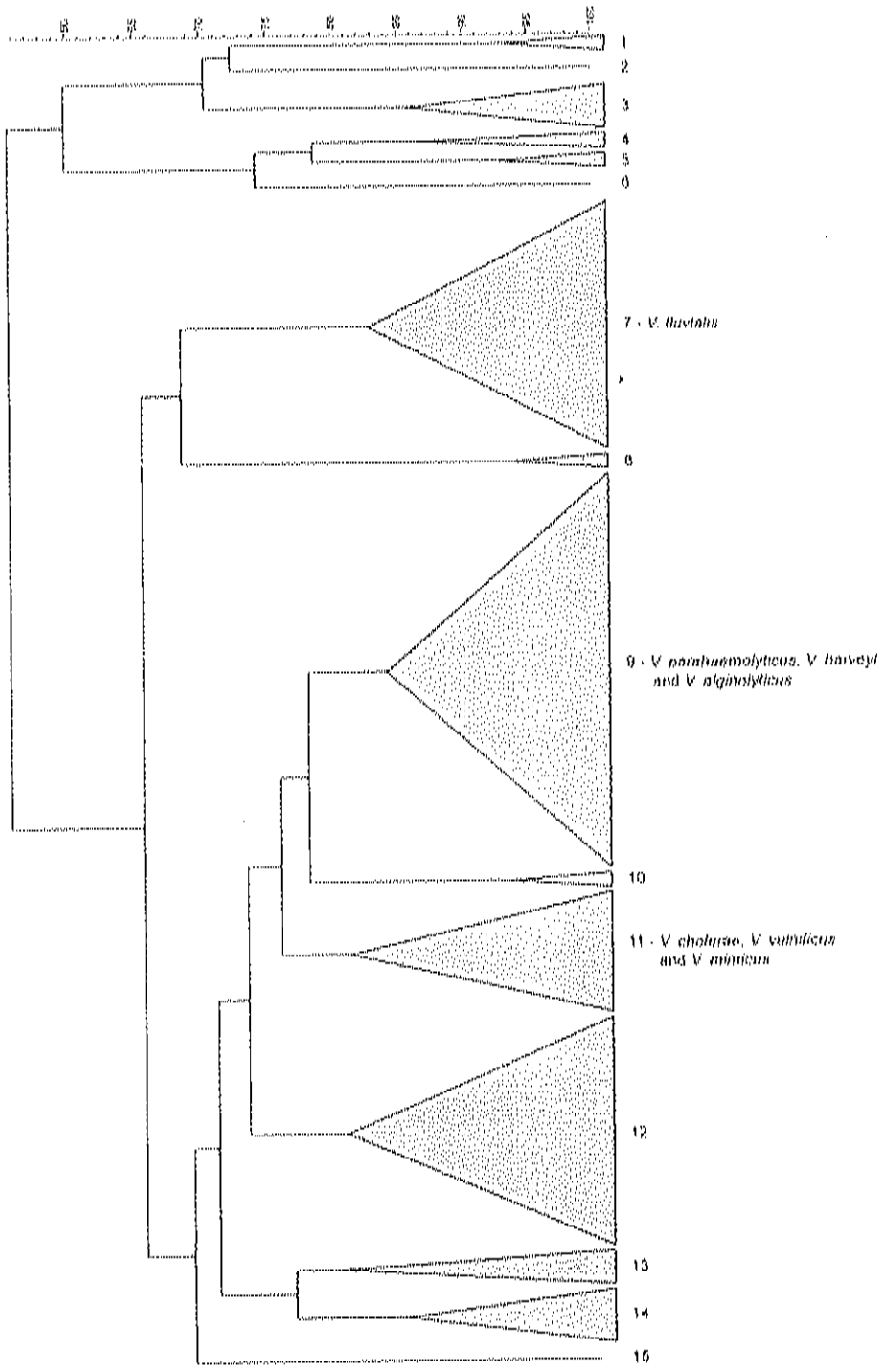

Figure 3.2. Dendrogram from OTUs obtained from water samples

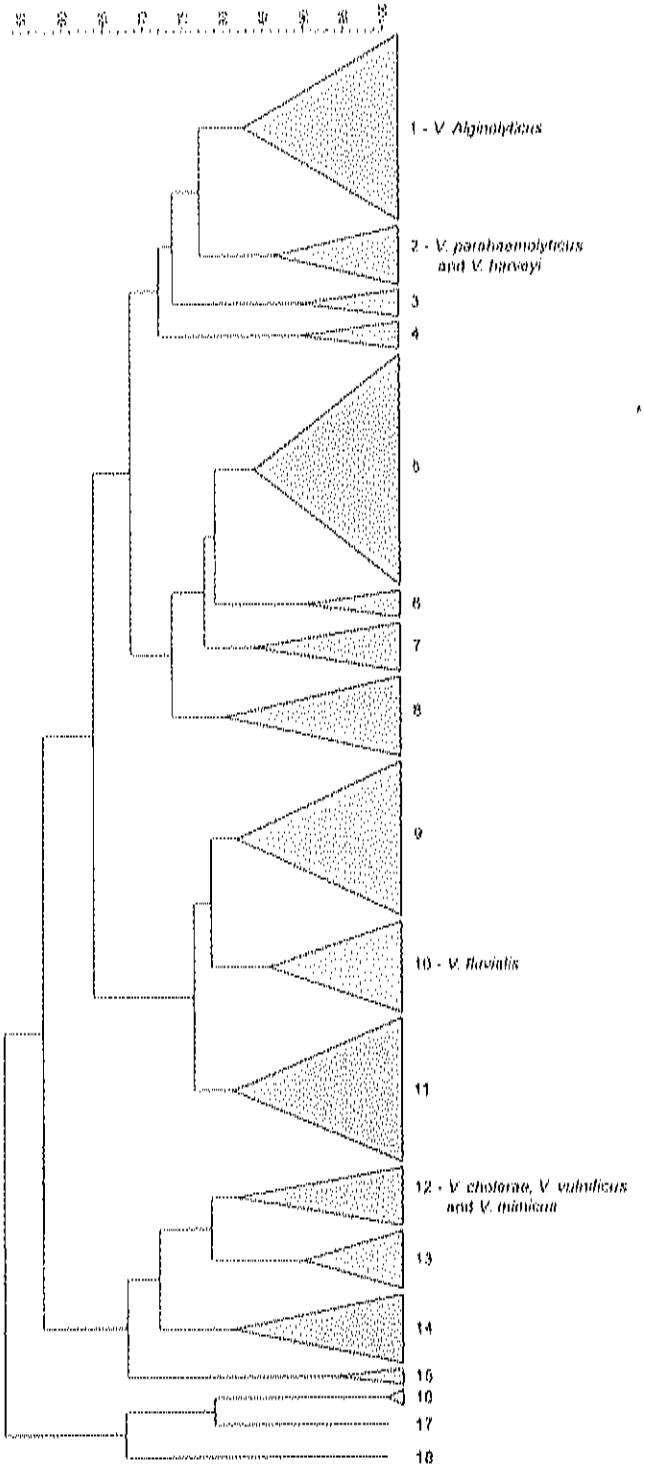

Figure 3.3. Dendrogram from OTUs obtained from harvest operation samples

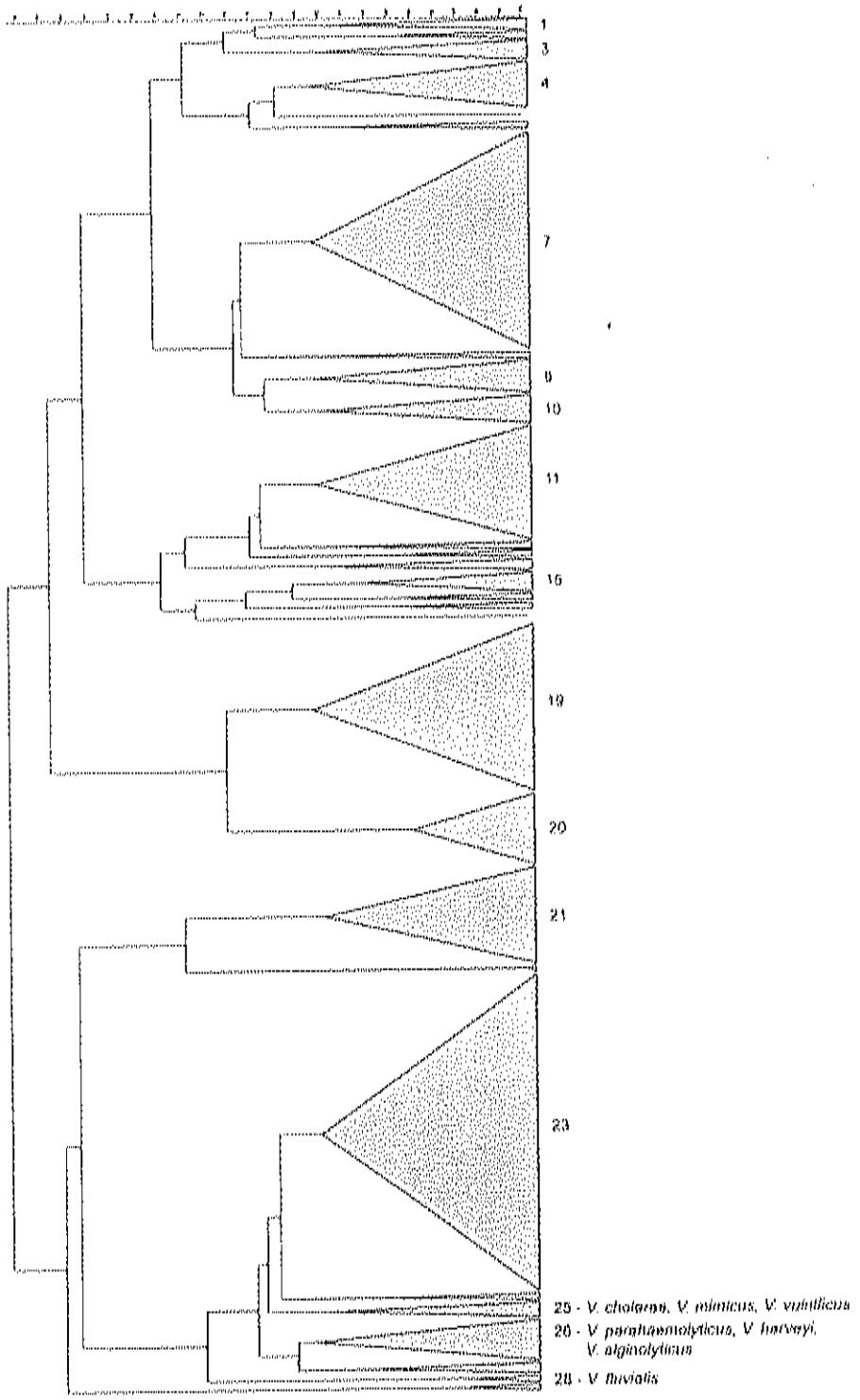

Figure 3.4. Dendrogram from OTUs obtained from end product samples

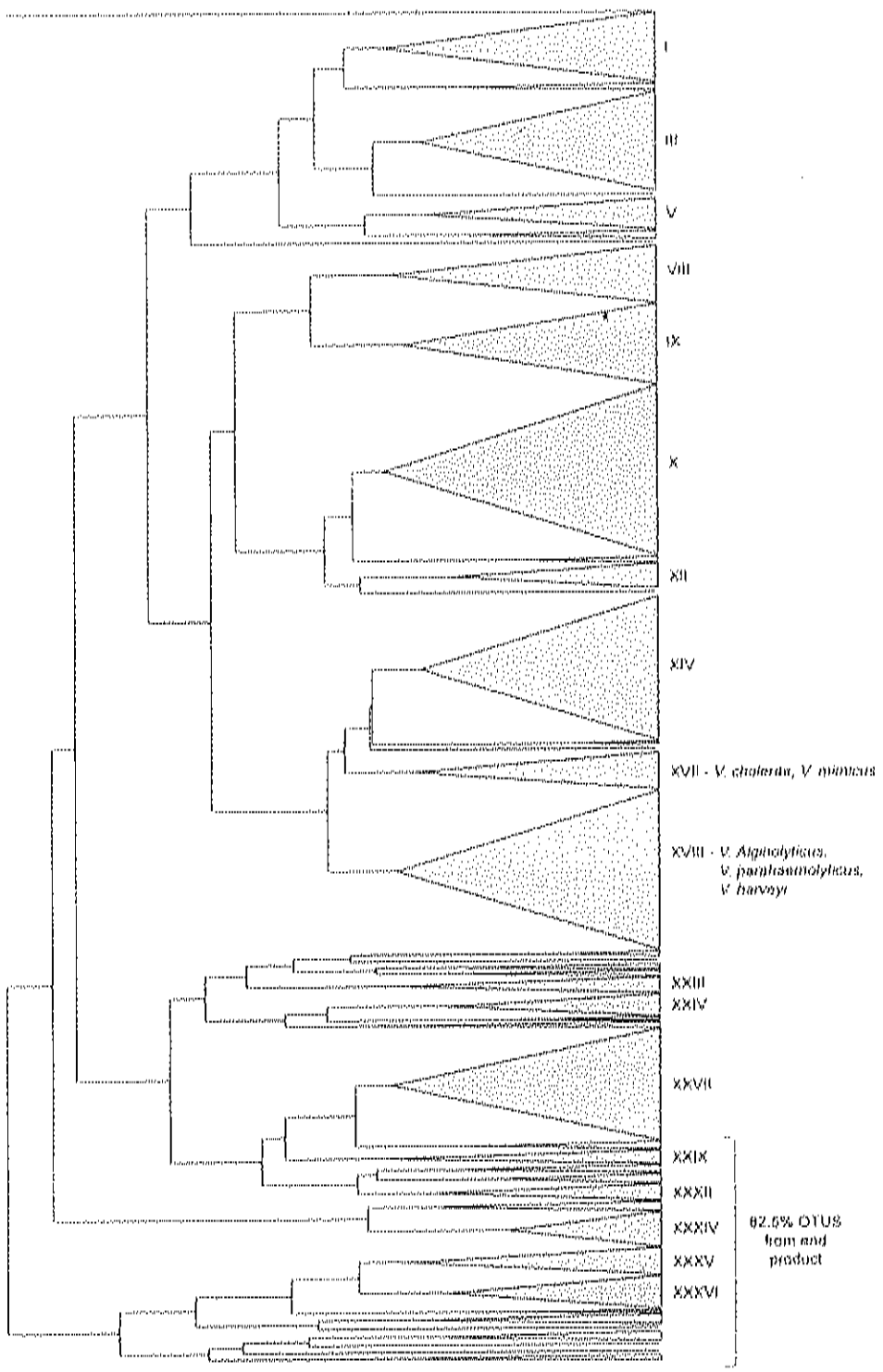

Figure 3.5. Dendrogram from OTUs obtained from all sample types.

Table 3.6. Biochemical responses from end-product OTUs from cluster I to XXII, considered not belonging to *Vibrio* species.

Biochemical	% of Positive
0 % NaCl growth	72.81
3 % NaCl growth	98.62
6 % NaCl growth	70.97
8 % NaCl growth	36.41
10 % NaCl growth	20.28
Motility	49.30
Indole production	34.42
Ornithine descarboxilase	54.67
Sucrose	37.26
Glucose fermentation	91.24
Glucose gas production	30.88
Lactose fermentation	25.81
Arginine dihydrolase	48.39
H_2S	16.59
Oxidase	42.40
Lysine descarboxilase	0.00
Gelatinase	17.29

Table 3.7. Clusters, OTUs number and its distributions depending of the sample type and control strain

Cluster No.	No. OTU	Control strain clustered in the group	Soil OTUs	Water OTUs	Harvest OTUs	End product OTUs
I	33	*V. fluvialis*	5	13	11	3
III	46	*V. vulnificus*	0	3	5	38
V	15	None	0	2	12	1
VIII	27	None	0	1	9	17
IX	38	None	1	9	18	10
IX	80	None	25	23	25	7
XII	12	None	2	4	3	3
XIV	68	None	2	11	1	54
XVII	16	*V. cholerae* and *V. mimicus**	0	8	3	5
XVIII	75	*V. alginolyticus*, *V. parahaemolyticus** and *V. harveyi**	9	38	9	15
XXIV	11	None	0	0	1	10
XXVII	53	None	1	0	8	44
XXXIV	17	None	0	1	0	16
XXXV	13	None	0	0	0	13
XXXVI	16	None	0	0	0	16

* Control strain and at least one OTU shown 100% similarity.

Therefore, the possibility to find any of the pathogenic *Vibrio* found in pond water can not be discharged, and probably will be affected by the bacterial concentration at harvest, among other factors. The adherence of V. mimicus or other pathogenic species might be of crucial importance from the food safety point of view. The surviving time for those bacteria under frozen conditions and its pathogenic effect should be studied

As it was mentioned above, most of the major groups founded at the end-product were not detected in water or soil samples, but match with clusters found in harvest operation samples, and cross contamination from not properly cleaned and disinfected surfaces might be assumed. The used isolation methodology, based on TCBS agar, does allow the growth of other marine and environmental bacteria such *Aeromonas, Photobacterium*, *Proteus*, *Enterococcus*, among others (Kaysner and DePaola, 2004, Ottaviani et al, 2003). The presence o possible non *Vibrio species* at end-product particularly those non motile that were found distributed between cluster I to XXII.

Conclusions

Total viable bacterial count presented more variable values in entrance water than composite water, while MPN of *Vibrio* sp shown similar values; those results indicate a stabilized pond bacterial population at harvest time. Therefore, pond environment represent a well established bacterial population at harvest time which was not greatly affected by the fresh seawater income at the analyzed ponds. An important part of the total viable bacterial population were composed by *Vibrio* species, as it can be appreciated by obtained values (MPN) of this bacterial group and TBC. Major *Vibrio* groups present at harvest time in shrimp ponds such do not necessarily survive until the end-product, it seems that those bacteria attached to the shrimp shell have major possibilities to

survive the stressed conditions, and it is possible to found them at low levels in the end-product. A high proportion of the isolates in TCBS agar at end-product belong to the *Vibrio* species, and those pathogenic species identified were present at low proportion. The public health concern should focus their attention in those areas where shrimp is eaten raw, or could be a vector of cross contamination to other foods.

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