Pathogenesis of crown-of-thorns starfish (*Acanthaster planci* L)

Thesis submitted by

Jairo A. Rivera Posada BVSc; DVSc

In March, 2012

For the degree of Doctor of Philosophy in Marine Biology and Microbiology, within the

ARC Centre of Excellence for Coral Reef Studies and the School of Veterinary and Biomedical Sciences, James Cook University

Statement of Access

I, the undersigned author of this thesis, understand that James Cook University will make it available for use within the University Library and, by microfilm or other photographic means, allow access to users in other approved libraries. All users consulting this thesis will have to sign the following statement:

> "In consulting this thesis I agree not to copy or closely paraphrase it in whole or in part without the written consent of the author; and to make proper written acknowledgment for any assistance which I have obtained from it."

Beyond this I do not wish to place any restriction on access to this thesis.

6th March, 2012

Jairo Rivera Posada

Abstract

Outbreaks of the crown-of-thorns starfish, *Acanthaster planci* (L.), represent one of the most significant biological disturbances on coral reefs, contributing greatly to widespread habitat degradation across the Indo-Pacific. While the cause(s) of outbreaks are still being debated, an equally important question is what causes population declines at the end of outbreaks. Like all echinoderms, *A. planci* appear to be susceptible to disease, which may explain sudden declines in abundance that have been observed in the wild, as well as providing a unique opportunity to potentially control starfish populations. The purpose of this thesis was to document potentially pathogenic organisms that are normally associated with *A. planci*, as well as describing the pathological progression of artifially-induced disease, following injection of Thiosulfate-citrate-bile-sucrose (TCBS).

Thiosulfate-citrate-bile-sucrose (TCBS) agar is a selective media culture that inhibits gram-positive organisms, suppresses coliforms, and allows selective growth of *Vibrio* bacteria. These bacteria constitute an important part of the bacterial microflora of numerous marine animals, and are also recognized as important pathogens of echinoderms and other estuarine animals. This study showed that injection of TCBS broth into *A. planci* organs induced disease characterized by skin lesions, loss of body turgor, matting of spines, and accumulation of mucus on spine tips. All starfish then died within 24 hours. TCBS broth promoted population growth of naturally occurring *Vibrio* spp., leading to an imbalance in natural symbiont communities. Moreover, diseased starfish often infected seemingly healthy *A. planci* that were either in direct contact or in very close proximity.

To identify potential causative agents of observed disease, and specifically identify all naturally occurring bacteria associated with *A. planci,* starfish were collected from two distinct locations in the western Pacific; Lizard Island (Great Barrier Reef, Australia) and Guam (USA, Western Pacific Ocean). A polyphasic approach, involving histology, scanning electron microscopy (SEM), biochemical profiling using the bacterial API identification system, PCR amplification and sequencing of 16S rRNA, *topA* (topoisomerase I) and *mreB* (rod shaping protein MreB) genes, was used to identify all bacterial isolated. The most significant bacteria isolated from *A. planci* were *V. rotiferianus, V. harveyi, V. owensii, Photobacterium eurosenbergii, V. fortis, V. natriegens* with sequences identities of 99%-100% for 16S rRNA, *topA*, and *mreB*. Specific bacteria isolated from infected tissues were *V. rotiferianus, V. owensii* and *V. harveyi*, which are considered as the most likely causative agents of observed disease.

Histological changes in tissues of *A. planci* following TCBS injection were assessed using conventional and scanning electron microscopy (SEM). Digestive glands were processed and stained with hematoxylin and eosin (H&E) to describe the histological architecture of the intestinal epithelium. Subsequent comparison of healthy versus infected tissues and Gram stains were carried out to confirm bacterial occurrence on infected tissues, characterize the structural changes induced by bacterial communities in COTS tissues and to determine if the histopathological changes of intestinal tissues were consistent with *Vibrio* infection. TCBS injections induced marked epithelial desquamation, hypertrophy and hypersecretion of glandular cells, epithelial cell destruction, pyknosis, reduction of thickness and disorganization of connective tissue and associated nerve plexus, presence of bacterial colonies, irregular eosinophilic foci in glandular cells, brush border disruption, atrophy and detachment of intestinal

microvilli and cell debris in the lumen. All these changes were attributed to a fulminating systemic dysbiosis and were consistent with *Vibrio* infections.

Standard histological procedures used to test for the presence of bacteria are often ineffective for marine organisms. As such, this study developed modified techniques to assess the presence of *Vibrio* bacteria and the preservation of *A. planci* delicate tissues. Detection of *Vibrio* bacteria was improved by the (1) use of short washes before fixation (2) the implementation of short cycles in the processing step; (3) embedding samples in agar prior to automated processing. The use of short cycles also decreased the amount of epithelial desquamation of COTS digestive glands. The study contributes to the standardization of histological techniques and biochemical test (API strips) for partial identification of marine bacteria, ensuring more accurate results, improving performance, enhancing reproducibility and increasing efficiency compared to standard operating procedures.

In order to reverse sustained and ongoing degradation of reef habitat, increasing attention is being given to management and control of *A. planci* outbreaks. Previous control methods, such as hand collecting individual starfish, are extremely labour intensive and often ineffective in either eradicating the coral-feeding starfish or preventing extensive coral loss. As a first step towards assessing whether injections of thiosulfate-citrate-bile-sucrose agar (TCBS) culture medium could be used to eradicate *A. planci*, especially during population outbreaks, we exposed a range of echinoderms to diseased starfish within a closed environment, and also compared naturally occurring bacteria across these echiinoderms. *Vibrio rotiferianus*, which was reported as a likely pathogen isolated from experimentally infected *A. planci*, was recovered from *Linckia guildingi*. Moreover, several *L. guildingi* exhibited skin lesions after several days of direct contact with sick *Acanthasther planci*. However, unlike infected *A. planci*, which all died within

48 hrs, all *L. guildingi* starfish fully recovered after 53 days. Further studies need to be carried out to test for cross-infection of *Vibrio* bacteria isolated from sick *A. planci* to corals, fishes and other echinoderms.

To better understand the specific effects of thiosulfate-citrate-bile-sucrose agar (TCBS) on *A. planci*, we tested responses of *A. planci* to individual components of TCBS culture medium. Four out of nine TCBS chemical ingredients tested induced allergic reactions and death in *A. planci*. Peptone 10 g l^{-1} and oxgall 8 g l^{-1} induced 100% mortality, while yeast extract and agar induced death in 40 % and 20% of starfish, respectively, 48 h after injection. Peptone was evaluated at three different concentrations (10g, 5g, and 1g l^{-1}). Peptone 10 g l^{-1} induced 100% mortality, peptone 5 g l^{-1} killed 60% of injected starfish, and peptone 1 g l^{-1} induced death in only 20% of starfish, indicating that toxicity of peptone is concentration-dependent. Sodium citrate induced moderate mucus production, but disease did not progress and all starfish completely recovered after 52 h. The remaining chemicals tested, sodium thiosulfate, ferric citrate, mix of sodium thiosulfate + ferric citrate, sucrose and sodium chloride did not produce any kind of clinical signs of disease. This study reported four new components that induced disease and death in *A. planci*. Peptone, oxgall, and yeast are potentially useful in controlling outbreaks because these simple protein extracts can be safer to use compared to previously used noxious chemicals. In addition, lowered concentrations are required to kill *A. planci*, potentially increasing efficiency and effectiveness of established control programs.

Acknowledgement

Foremost, I would like to thank my supervisors Prof. Morgan Pratchett and A/Prof. Leigh Owens for their unconditional support and assistance over the years and for the patience in checking my papers. I also wish to acknowledge Prof. Terry Hughes, Prof. David Yellowlees, Dr. Brenda Govan, Dr. Graham Burgess, Dr. Lyle Vail, Dr. Anne Hogget, Dr. David Bourne and Dr. Tracy Ainsworth which provided exceptionally valuable advice and feedback. I owe an immense debt of gratitude to Dr Ana Cano Gomez, who was generous with her time and knowledge. I would also like to express my sincere appreciation to Sue Reilly for her assistance in the histology area and to the administrative staff Olga Bazaka and Janet Swanson for being so accommodating and responsive to my needs.

Funding for this project was provided by ARC Centre of Excellence for Coral Reef Studies, the Australian Institute of Marine Science (AIMS) and Project AWARE Foundation.

I am deeply indebted to my volunteers: Laura Marin, Juan Arango, Ana Cano and Ciemon Caballes for their help, unfailing optimism and friendship.

Finally I would like to dedicate this work to the most important people in my life. They supported and encouraged me throughout this lengthy graduate career: my family – Las gordugas, Martha and Lola; my sisters Monica, Angela and Lina and my wife, Laura that work extensive hours and help me everyday during this years. Without their unconditional love, support, and encouragement, this work would not have been possible.

Statement on Sources

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.
Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

…….. 6th March, 2012

Jairo Rivera Posada

Table of Contents

List of Tables

CHAPTER 2: Injection of *Acanthaster planci* with thiosulfate-citrate-bile-sucrose agar (TCBS). I. Disease induction.

CHAPTER 3: Refined identification of *Vibrio* bacterial flora from *Acanthaster planci* based on biochemical profiling and analysis of housekeeping genes

CHAPTER 5: Modified techniques to improve tissue preservation, detection and characterization of *Vibrio* bacteria in marine organisms

List of Figures

CHAPTER 5: Modified techniques to improve tissue preservation, detection and characterization of *Vibrio* bacteria in marine organisms

CHAPTER 6: Interspecific transmission and recovery of TCBS-induced disease between *Acanthaster planci* and *Linckia guildingi*

CHAPTER 7: The Role of protein extracts in the induction of disease in *Acanthaster planci*

CHAPTER 1: General Introduction – *Vibrio* bacteria: pathogens of growing significance

1.1 Abstract

This review describes the historical, clinical, ecological and epidemiological features of the most important and emerging pathogenic *Vibrio*. We highlight the increased pathogenic potential of *Vibrio* bacteria toward humans and marine animals due to the resultant environmental conditions, which have bred an abundance of pathogens that are naturally present in the environment. The review illustrates the main adaptive response mechanisms that play important roles in the virulence of *Vibrio* species, which allow vibrios to persist in hostile environments and summarize the more reliable identification methods.

1.2 Introduction

Vibrio bacteria are generally associated with gastrointestinal diseases and septicemia in humans and are recognized as one of the most important pathogens of marine and estuarine animals, causing substantial losses in both aquaculture and wild populations (Kaysner *et al.,* 2004). *Vibrio* bacteria are naturally widespread, and occupy a variety of ecological niches, such as the human and animal gut, the mucus layer of corals and the surface of chitinous organisms, predominantly copepods, amphipods and other crustaceans (Sawabe *et al.,* 2007). During the last decade there has been significant improvements in *Vibrio* taxonomy, largely due to increased use of molecular techniques that have not only established the phylogenentic relationships among well-studied species, but also revealed several new species of *Vibrio* bacteria, mostly in marine environments (Gurtler & Mayall, 2001; Yoshizawa *et al.,* 2009; Cano-Gomez *et al.,* 2010).

Scientific interest in *Vibrio* bacteria has been further stimulated by a number of factors, including the growing problems of antibiotic resistance among pathogens (Zbinden, 2006; Vlieghe *et al.,* 2009), the emergence of numerous infectious diseases in the marine environment worldwide (Bossart, 2007; Schaefer *et al.,* 2009), the potential threat of bioterrorism (Horton *et al.,* 2002; Azad, 2007) and the effects of global climate change (Lipp *et al.,* 2002; Bossart, 2007; Sganga, 2009). The Center for Disease Control and Prevention estimates that average annual incidence of all *Vibrio* infections in the United States increased by 41 percent between 1996 and 2005 (CDC, 2006) and there are similar reports of increased incidence in Europe (Schets *et al.,* 2006; Wilkins, 2008). At least 30 new diseases have emerged during the last two decades in North and South America, Asia, Australia, Europe and the Middle East (Bossart, 2007; Igbinosa & Okoh, 2008), most of which have been linked to *Vibrio* bacteria.

With rapid growth of human populations, especially in coastal environments, exposure to pathogenic *Vibrio* is increasing. Coastal ecosystems are becoming important reservoirs of infectious organisms due to agricultural, animal, industrial and medical waste which coupled with higher temperatures due to global warming allowed proliferation and diffusion of opportunistic *Vibrio* pathogens to higher latitudes (Sganga *et al.,* 2009) and induced changes in host-pathogen relationships leading to susceptibility of animals to epidemic diseases (Rosenberg *et al.,* 2007). Early detection and treatment of *Vibrio* infections is particularly important as they can rapidly cause septic shock and death (Wilkins, 2008). Diagnosis may be difficult because special techniques are required for identification and sensitivity testing (Duthie *et al.,* 1990; Wilkins, 2008). However, rapid and accurate means of identification are essential in ensuring appropriate treatment (Duthie, 1990). Importantly, due to the increment in the trades of Seafood worldwide, *Vibrio* infections are not limited to endemic areas (CDC, 2004). Pathogenic vibrios,

in combination with a stressed population, habitat loss, elevated temperature due to climate change and anthropogenic pollution can result in severe economic loses and disease outbreaks which can ultimately affect human health and lead to the extinction of endangered species.

The objectives of this review are four-fold: 1) describe the historical, clinical, ecological and epidemiological features of the major emerging and reemerging pathogenic vibrios; 2) highlight the increased pathogenic potential of vibrios toward humans and marine animals due to the resultant environmental conditions which have bred an abundance of pathogens that are naturally present in the environment; 3) explore the main adaptive response mechanisms that play important roles in the virulence of *Vibrio* species which allow vibrios to persist in hostile environments; and 4) describe recent advances in the identification of *Vibrio* bacteria.

History of *Vibrio*

Human populations throughout history have been affected by devastating outbreaks of *Vibrio cholerae*. Hippocrates (460-377 BC), a surgeon of Roman gladiators, in his *Corpus Hippocraticum* and Galen of Pergamum (120–201 AD), described an illness that might well have been cholerae (Kohn, 1995; Cox, 1996). Epidemic *Vibrio cholerae* was described in 1563 at Goa, Southwestern India by Garcia de Orta (1501–1568), a Spanish physician pioneer of tropical medicine, who performed the first recorded autopsy of cholerae in India. The population at Goa was roughly 200,000 in 1543 and was reduced to only 1,500 by 1775 as a result of malaria and cholerae (Roddis, 1931; Boxer, 1963).

The modern history of cholerae began in 1817 when cholerae spread out of India in what is described as the first of seven pandemics: (1) pandemic 1817-1823; (2) pandemic 1829-1851; (3) pandemic 1852-1859; (4) pandemic 1863-1879; (5) pandemic 1881-1896; (6) pandemic 1899

to 1923 and (7) pandemic that began in Asia in the 1960s, and then spread westward in the next decade to the Middle East and Africa. The African continent is believed to have been struck harder at this time than ever before; In 1990, Africa was the origin of more than 90 percent of all cholerae cases reported to the World Health Organization. In July 2009, Zimbabwe reached 98,000 cases, including over 4000 deaths, and showed no signs of abating (WHO, 2010). A total of 19 African nations reported nearly 140,000 cases in 1991 and the outbreak also appeared unexpectedly in Peru, which suffered 3,000 deaths in the first year. The disease rapidly spread to Ecuador, Colombia, Brazil, and Chile and further northward to Central America. More than 396,000 cases were reported in the Americas during 1991 (PAHO, 2006). From January 2000 to December 2003 over 130,000 cases of cholerae were reported in South Africa (WHO, 2009). In 2005 cholerae had been reported in nearly 120 countries and continues today in many parts of the world (WHO, 2010).

During the cholerae epidemics in Florence (1854-1855), the Italian physicians Filippo Pacini (1812 -1883) and Francesco Magni (1828-1887), who were primarily interested in microscopical research, examined the blood and feces of cholerae victims as well as the changes of the intestinal mucosa of cholerae corpses. Their findings were published in a report, “Osservazioni microscopiche e deduzioni patalogiche sul cholera asiatico”. Pacini discovered that cholerae is a contagious disease characterized by the destruction of the intestinal epithelium, followed by an extreme loss of water from the blood. These investigations proved the presence of millions of rod-shaped living microorganisms, of which he called "vibrions", that were responsible for the intestinal injuries common to the disease and he also provided the first drawings of the vibrions that were observed microscopically in the intestine of cholerae victims (Pacini & Magni, 1854).

In 1849, John Snow (1813-1858) a founding member of the London Epidemiological Society, tracked the incidence and geographical distribution of cholerae in London and other cities and developed the first statistical mapping methods (Vinten-Johansen, 2003). In 1883, Robert Koch (1843-1910), a German physician considered as one of the founders of microbiology, successfully isolated *Vibrio cholerae* from the intestinal discharges of cholera patients. Koch and his colleagues developed and improved methods for staining bacteria, introduced the use of gelatin and agar as growing media for bacterial colonies, recommended the implementation of filters to remove bacteria from the water supply systems and also found that *Vibrio* bacteria were ubiquitous in aquatic environments (Thompson *et al*., 2004, 2006). Considerable advances were then made by Martinus Willem Beijerinck (1851–1931) and Sergei Winogradsky (1856–1953), who established the basic principles of virology. They formulated an enrichment media culture that allowed the cultivation of a wide range of microbes with different physiologies and successfully isolated the first nonpathogenic *Vibrio* species, i.e., *V. fischeri, V. splendidus*, and *Photobacterium phosphoreum* from the aquatic environment (Postgate, 2000; Thompson *et al*., 2004).

Definition and classification

Vibrio bacteria are gram-negative, non-sporing, straight or curved rods that can undergo both respiratory and fermentative metabolism (facultative anaerobes). Vibrios are heterotrophic organisms and obtain nutrients from their mutualistic, parasitic, or pathogenic relationships with other organisms and reproduce through asexual division (Kaysner *et al.,* 2004). Cells are generally 1 μm in width and 2 to 3 μm in length. They are motile organisms with monotrichous or peritrichous flagellum for travelling. They are largely halophilic but a few species are nonhalophilic, for example *V. cholerae* may be halophilic or nonhalophilic depending on their

sodium chloride requirements. Most *Vibrio* species have a broad temperature range from 18 to 37°C and most are chemo-organotrophic, oxidase positive and ferment glucose with the production of acid but no gas (Kaysner *et al*., 2004;Thompson *et al*., 2006). Recent taxonomic descriptions place *Vibrio* bacteria within the Kingdom: Bacteria; Phylum: Proteobacteria; Class: Gamma Proteobacteria; Order: Vibrionales; Family: Vibrionaceae with 80 species described until now.

Media cultures

Vibrio bateria can grow in a wide variety of culture media (Thompson *et al*., 2004; Sawabe *et al*., 2007). The concentration of salt for optimum growth and the range of salt tolerance differ with each species. In addition the concentration of salt for optimum growth may be influenced by the nature of the medium and the temperature of incubation. The majority of vibrios can grow over a pH range of 5.6 - 9.6, but the optimum pH is between 7.0- 9.0 (Barua & Grennough, 1992; Desmarchelier, 1997). *Vibrio* species associated with human diseases flourish at 30-37 ºC. Most *Vibrio* species show poor or no growth in nutrient broth that has less than 0.5% sodium chloride. Even for *V. cholerae* and *V. mimicus*, better growth is obtained in nutrient broth with 1% salt than in broth with lower concentrations (Barua & Greenough, 1992).

The predominant media used to culture *Vibrio* include: Thiosulfate-citrate-bile-sucrose (TCBS), HiCrome *Vibrio* agar, ZoBell's Marine Agar, MacConkey's agar, *Vibrio harveyi* agar, Marine broth, Heart infusion broth (HIB), Johnson's Marine Agar, *Vibrio* Recovery Medium, Colistin-polimyxin B-cellobiose agar, MicroKwik® culture. In addition, enriched media such sheep blood agar or brain heart infusion agar that usually support good development of vibrios without the need of additional salt, and Cary Blair Medium, which is routinely used as a

transport media (Kobayashi *et al*., 1963; Barua & Greenough, 1992; Harris *et al.,* 1996; Cerda-Cuellar *et al*., 2001; Kaysner *et al*., 2004).

1.3 Epidemiology and pathogenesis

Recent interest in the occurrence of potentially pathogenic *Vibrio* is very high, owing to major changes in environmental conditions that may promote increased incidence of disease. Terrestrial and aquatic habitats have been significantly altered, and often degraded, due to direct anthropogenic disturbances, the effects of which are now being further compounded by global climate change (Walther et al. 2002). These changing biological and environmental conditions have resulted in an increased abundance of naturally occurring but potentially pathogenic organisms (Lipp *et al*., 2002) affecting humans and marine animals (Lipp *et al*., 2002; Orozco-Noriega *et al*., 2007; Lee *et al*., 2008). *Vibrio* bacteria are responsible for the majority of non-viral infections in the USA, arising mostly from consumption of shellfish or exposure of open wounds to seawater. The incidence of *Vibrio* wound infections has increased 51% over six years in the USA (Forres, 2007) and also increased significant in several European countries (Schets *et al*., 2006). Natural Resources News Service survey (USA) showed that *Vibrio* wound infections have increased each year and are being reported further northward, probably because *Vibrio* bacteria thrive in warm waters of moderate salinity (Lipp *et al*., 2002; Forres, 2007). If so, the geographic range of these important pathogens will change in accordance with global warming, and there is a greater risk of increased exposure and infections. The ecology is changing not only due to anthropogenic influences, but also because of changes in plankton populations, and other hosts for which *Vibrio* are commensals or symbionts, which alter the ecology of these pathogens (Colwell, 1996; Lipp *et al*., 2002).

Adaptive response mechanisms

Vibrio bacteria are well equipped with a series of adaptive response mechanisms that allow them to persist in hostile environments. Chemotaxis is one phenomenon in which bacteria integrate environmental signaling to modulate behavior by biasing movements toward more favourable conditions or away from unfavourable environments. Motility and chemotaxis greatly influence the infectivity of *Vibrio* (Butler & Camilli, 2004; Larsen *et al*., 2004). Members of the genus *Vibrio* are brackish-water or marine organisms and many require higher concentrations of cations, particularly sodium (Na), potassium (K) and magnesium (Mg^2) that are usual for terrestrial organisms such as *Aeromonas, Pleasiomonas* and *Enterobacteriaceae* (Barua & Greenough, 1992). Concentration of these cations influences motility and growth in different ways. For example, low concentrations of magnesium decrease the number of polar flagella in *V. fisheri*, which affects swimming motility (Kawagishi *et al*., 1996; Muramoto *et al*., 1996; O'shea *et al*., 2005). The energy required to power flagellar rotation is derived from the transmembrane electrochemical potential of sodium ions (Imae *et al.*, 1986). Therefore, theses *Vibrio* require Na^+ for growth and movement (Barua & Greenough, 1992). Environmental conditions also influence the type of flagellation. For example, some species including *V.cholerae*, when grown planktonically display polar flagella while others such as *V. parahaemolyticus* when grown on solid medium produce both polar and lateral flagella (Thompson *et al*., 2006).

Chemotaxis to a variety of aminoacids and carbohydrates has been reported in numerous *Vibrio* species, including: (1) Serine (Sar *et al*., 1990); (2) N-acetylneuraminic acid (Deloney-Marino *et al*., 2003); (3) Chitin oligosaccharides, considered the second most abundant organic compound in nature that provides a food source for vibrios, enhances survival under starvation conditions, and also offers protection at low temperatures and under acidic conditions (Yu *et al*., 1993; Lipp *et al*., 2002); (4) algal products (Sjoblad & Mitchel, 1979); and (5) generally *Vibrio*

species display movement toward mucus. For example *V. anguillarum* and *V. alginolyticus* undergo chemotaxis to mucus collected from fish skin and intestines (Bordas *et al*., 1998). *V. shiloni*, a coral pathogen, migrates towards coral mucus (Banin *et al*., 2001); *V. coralliilyticus* exhibited chemotaxis towards the mucus of *Pocillopora damicornis* (Meron, 2009) and *V. cholerae* moves into intestinal mucus (Freter *et al*., 1981)

Horizontal gene transfer, genetic reassortment, clonal diversity and plasticity have also been key mechanisms involved in the pathogenicity and emergence of new virulent *Vibrio* strains (Simidu *et al*., 1971; Ochman *et al*., 2000; Lipp *et al*., 2002; Gomez-Gil *et al*., 2004). Moreover, *Vibrio* has a selective advantage in their ability to enter a dormant stage when environmental conditions are unfavorable for active growth and cell division. This stage known as the viable but non-culturable (VBNC) state occurs when metabolically active cells cannot be cultured on microbiological media. VBNC is induced by changes in environmental conditions, mainly a decrease in water temperature and salinity. These cells can remain viable in the environment for years and proliferate rapidly when conditions improve, subsequently continuing to pose a risk of causing disease (Colwell *et al*., 1985; Firth *et al*., 1994). Other mechanisms that play important roles in the virulence of *Vibrio* species are bacteriophage infection (Oakey & Owens, 2000); sucrose fermentation (Alavandi *et al*., 2006); and quorum sensing in which vibrios produce, release and detect signal molecules called autoinducers. Quorum sensing is responsible for controlling a plethora of virulence genes in several Gram-negative bacteria, by the N-acylhomoserine lactones (AHLs)-dependent quorum sensing (QS) mechanism (Fangfang *et al.,* 2008; Park *et al.,* 2008). AHL-based quorum-sensing systems ensure that certain phenotypic traits are expressed only when a particular population density has been attained. These regulatory systems control various functions, including bioluminescence, conjugative

plasmid transfer, biofilm formation that confer resistance to disinfectants, antibiotics and protect against detrimental environmental conditions (Karunasagar *et al*., 1994), antibiotic synthesis, motility and production of virulence factors in animal, plant and human pathogens (Peters *et al.,* 2003; Henke & Bassler, 2004). Most important vibrios as opportunistic species that can evolve and recombine under high microbial contact in animal guts or as part of aquatic biofilms transferring or inducing expression of virulence genes in less or non-pathogenic populations (Thompson *et al.,* 2004).

Microbes have developed a number of different strategies to utilize iron, which is a vital element for most pathogenic *Vibrio* bacteria (*V. cholerae, V. vulnificus, V. parahaemolyticus, V. anguillarum)* to establish infection. Nevertheless this element is not always readily available from the environment making these organisms heavily dependent on their ability to use the host-complexed iron (Koster, 2001). A key feature which enable pathogenic bacteria to survive and multiply within the host is the production of siderophores, iron-sequestering compounds, and the synthesis of their cognate transport systems, which are crucial in overcoming the defense mechanisms of the host (Neilands 1981, 1995; Crosa & Walsh, 2002). Moderate levels of iron increase the expression of the cholera toxin (CT) virulence factor. The introduction of iron into the environment by agricultural practices and industrial pollution may lead to increased survival and virulence of *Vibrio* bacteria (Patel & Isaacson, 1999).

Among the numerous physical factors the influence *Vibrio* bacteria, temperature has the most direct and significant effect on their biology and ecology. It is generally recognized that high densities of *Vibrio* organisms occur during the warmer months, a finding that is consistent with seasonal distribution of clinical cases and fatalities traced to the consumption of contaminated seafood (Levine & Griffin, 1993; Lipp *et al*., 2002). Warmer temperatures in

combination with elevated pH and plankton blooms, can also influence *Vibrio* attachment, growth, and multiplication in the aquatic environment. Sunlight and UV intensity increase the rates of induction and propagation of the CTX_ phage, and promote viability and culturability of vibrios. For instance, *V. cholerae* remains stable in full sunlight compared to enteric bacteria such as *E. coli*, which gives vibrios a clear advantage at tropical latitudes (Mezrioui *et al*., 1995).

In addition to temperature, elevated sunlight can stimulate the growth of indigenous heterotrophic bacteria within water bodies. For example, sunlight increases nutrient availability through the photochemical breakdown of complex polymers releasing organic metabolites (Eilers *et al*., 2000). Such sunlight induced nutrient enrichment in seawater samples and sediments have been correlated with increases in the relative abundance of *Vibrio* populations (La Rosa *et al*., 2001). Sea level rises following higher temperatures increase saltwater intrusion in coastal lands and exposes human population to vibrios. Sunlight, temperature and nutrients influence the growth of phytoplankton and aquatic plants, which consequently alter the dissolved oxygen (O_2) and carbon dioxide (CO_2) content of the water and therefore, the pH of the surrounding water. These direct and indirect effects on algal growth subsequently influence the population of vibrios in a given environment.

Vibrio spp are closely associated with aquatic invertebrates and can be found highly concentrated on zooplankton carapaces and in their gut, sediments and suspended detritus. Vibrios significantly affect nutrient cycling in these habitats and often comprise a major portion of aquatic animal's natural flora (Cano-Gomez *et al*., 2009; Lipp *et al.*, 2002). Generally non-fermenting bacteria such as *Pseudomonas* are found in association with phytoplankton, whereas *Vibrios* and *Aeromonas* are more likely to be associated with zooplankton (Simidu *et al*., 1971). High phytoplankton production increases food for zooplankton grazers. Once *Vibrio* is attached

to zooplankton, they are protected from the external environment and begin to proliferate and take advantage of the increased surface area and improved conditions of nutrition (Huq *et al.*, 1983). All these environmental triggers are epidemiologically important for the prevalence and virulence of *Vibrio* bacteria, which results in shorter onset times and lower infectious doses (Lipp *et al*., 2002).

1.4 Emerging and re-emerging *Vibrio* pathogens

Recent increases in the incidence of diseases, and emergence of new diseases, are linked with numerous factors, including declines in the physiological condition of potential hosts, habitat loss, climate change, pollution, and globalistion. In marine systems, declining water quality due to sedimentation, eutrophication and pollution causes localised outbreaks of disease that are then rapidly spread. Marine animals have few boundaries to dispersal and the spread of pathogenic organisms is futher enhanced by international shipping (Bogomolni *et al*., 2008). These factors coupled with adaptive mechanisms in *Vibrio* and potential new environmental conditions due to climate change, are predicted to have significant ramifications for marine systems and humans alike. The best known of these bacteria, along with their epidemiololgy and ecology, are described in turn.

1. *Vibrio cholerae* - *V. cholerae, along with V. parahaemolyticus* and *V. vulnificus* are the most significant human pathogens (Tantillo *et al*., 2004; Thompson *et al*., 2006) *V. cholerae* colonizes the small intestine and releases enterotoxins to produce an abrupt onset of vomiting and watery diarrhea which leads to profuse loss of fluids and electrolytes (sodium, potassium, bicarbonate), and subsequent cardiac arrhythmia and renal failure. Cholerae toxins block uptake of sodium and chloride from the lumen of the small intestine and produces death due to hypovolemic shock and metabolic acidosis. The mechanisms of transmission for cholerae include water, unwashed

contaminated food, and seafood from *Vibrio cholerae* estuaries (Griffit *et al*., 2006). Environmental reservoirs include oysters (Alexander *et al*., 1998); zooplankton and phytoplankton from various aquatic environments (Islam *et al*., 2002); brackish and salt waters (Forbes *et al.,* 2002); *Acanthamoeba castellanii* protozoa (Jain *et al*., 2006); and *Chironomids* generally known as the non-biting midges (*Diptera Chironomidae*) (Halpern *et al*., 2004).

V. cholerae display more than 200 serogroups based on somatic O antigen. O1 and O139 serogroups are responsible for the classic epidemic cholerae. The O1 serogroup is subdivided into two biotypes: El Tor and classical (or cholerae). There are three serotypes: Ogawa, Inaba, Hikojima. Some O1 strains do not produce cholerae enterotoxin (atypical or nontoxigenic O1 V. cholerae). Other strains are identical to O1 strains but do not agglutinate in O1 antiserum (non-cholera (NCV) or nonagglutinating (NAG) vibrios). Cholerae is endemic in regions of southern and south eastern Asia (the origin of pandemic cholerae outbreaks) (Mezrioui *et al*., 1995; Buttler & Camilli, 2004; Thompson *et al.*, 2006).

2. *Vibrio vulnificus* - *V. vulnificus* is a marine bacterium found as a contaminant in oysters as well as other seafood (Daniels & Shafaie, 2000; Igbinosa & Okoh, 2008). Brackish waters are considered as a natural reservoir of these bacteria. It is one of the most invasive and lethal human pathogens (Igbinosa & Okoh, 2008) and it shows different levels of pathogenicity. Even with prompt diagnosis and aggressive therapy, the case-fatality rate is more than 40 percent and the taste, appearance, and odor of seafood are not affected by *V. vulnificus* contamination (CDC, 2004). In people with pre-existing conditions, consumption of the pathogens can produce a rapidly fulminating primary septicemia. People who have liver disease, alcoholism, diabetes, hemochromatosis, or a compromised immune system are at particular risk of developing a septicemia that can be fatal within 48 hours in approximately 50% of the cases (Daniels &

Shafaie, 2000; Grau 2006). Males are more vulnerable because estrogens protect women against *V. vulnificus* endotoxin (Merkel *et al.,* 2001).

A second serious illness induced by this species is wound infections resulting from contact with contaminated seawater or shellfish. These infections can progress quickly and may require surgical debridement or amputation of the affected limb unless treatment is initiated promptly. *V. vulnificus* is responsible for 95% of seafood-related deaths (Oliver, 2005; Chatzidaki *et al*., 2006; Grau, 2006). The primary virulence factors are a capsular polysaccharide (CPS) that allows the bacteria to evade the host immune system and lipopolysacharidae (LPS), which is associated with shock and death (McPherson *et al*., 1991; Grau, 2006). Two phenotypic forms were previously known to exist, opaque (virulent) and translucent (avirulent), and have been shown to undergo spontaneous phase variation, each switching to the other phenotype. Researchers found a third phenotype, rugose, that is dry and wrinkled compared to the smooth colony (Grau, 2006). *V. vulnificus* has been recovered from recurrent outbreaks of diseased eels (*Anguilla anguilla*) in Spain and Denmark (Lise *et al*., 1998); from Japanese eels (*Anguilla japonica*) (Nishibuchi *et al*., 1980); and from fish and shrimps inducing a major outbreak of systemic *V. vulnificus* infections among Israeli fish market workers and fish consumers during the summer of 1996 (Bisharat *et al*., 1996). In addition, it was reported to cause illness in humans after handling of eels (*Mertens et al*., 1979).

3. *Vibrio parahaemolyticus* - Since the first report in 1950, *V. parahaemolyticus* has been recognized as the leading cause of human gastroenteritis associated with seafood consumption (Daniels & Shafaie, 2000; Cabrera-Garcia *et al*., 2004). It is also related with wound infections and septicemia (Fujino *et al*., 1953). Outbreak characteristics depend on the species of shellfish, the season, the location and the level of fecal pollution (Walkins & Cabelli, 1985). Clinical

symptoms include diarrhea, headache, vomiting, nausea, abdominal cramps and low grade fever, and the disease has an incubation period ranging from 4 to 96 hours (Daniels & Shafaie, 2000). *V. parahaemolyticus* pathogenicity is mediated by different mechanisms such as expressions of adhesins (Lijima *et al*., 1981; Robert-pillot *et al*., 2004) and production of a cell-associated Shiga-like cytotoxin (O'Brien *et al.,* 1984). *V. parahaemolyticus* display the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) as the main virulence factors (Cabrera-Garcia *et al*., 2004; Tyagi *et al*., 2009). Epidemiological studies have revealed an association between the Kanagawa phenomenon (KP) and gastroenteritis. KP is a type of beta-hemolysis induced by the thermostable direct hemolysin (TDH) in Wagatsuma agar (Cabrera- Garcia *et al*., 2004). *V. parahaemolyticus* has been recovered from an ample range of environmental sources. An estuarine neritid gastropod, *Clithon corona*, is recognized as a reservoir of thermostable direct hemolysin-produced by *V. parahaemolyticus* (Aiyamperumal *et al*., 2005). *V. parahaemolyticus* has been recovered from *Spartina alterniflora*, the dominant plant in salt marshes along the Atlantic and northern Gulf coasts of temperate North America (Criminger *et al*., 2007). It was also isolated from the lung of one of a group of stranded Pacific pilot whales (*Globicephala macrorhynchus = G. scammoni*) (Hall *et al*., 1971). This bacterium displays a very high prevalence in Asian warm coastal waters as well as fish and shellfish (Davies *et al*., 2001).

4. *Vibrio alginolyticus* - This species derives its name from its ability to utilize alginate. It is the most salt-tolerant among the pathogenic *Vibrio* species and can even grow in high salt concentrations up to 10% (Neill *et al*., 2005). Its virulence is related to its ability to produce hemolysis, hemagglutination, and proteolytic extracellular enzymes colagenasa and alkaline proteases (Reid *et al*., 1980; Zanetti *et al*., 2000). It forms large, yellow (sucrose fermenting)

colonies on TCBS medium. *V. alginolyticus* has been etiologically associated with cellulitis, wound infections, necrotizing soft tissue infection and acute otitis media or externa but also with gastroenteritis and bacteraemia in immunocompromised patients (Patterson *et al*., 1988; Schets *et al*., 2006). The route of infection is direct contact with contaminated seawater or ingestion of raw seafood. There are reports of septic shock in a cirrhotic patient after eating raw fish (Lee *et al*., 2008), necrotizing fasciitis following a soft tissue injury from a coral reef to the leg (Patterson *et al*., 1988), conjunctivitis (Lessner *et al*., 1985), articular infections in old patients (Schets *et al.*, 2006), myringitis (Lee & Choi, 1995), peritonitis associated with ambulatory peritoneal dialysis (Taylor, 1981), and intracranial infection by *Vibrio alginolyticus* following injury in salt water (Opals & Ramsey, 1986). *V. alginolyticus* is considered an occupational hazard for people who fish or are in constant contact with seawater like divers, and fishermen (Janda *et al*., 1987). Furthermore, this bacteria was reported as a causal agent of yellow band disease in corals *Montastraea spp* (Cervino *et al*., 2004); *V. alginolyticus* was isolated from necrotic stomatitis in a captive bottlenose dolphin *Tursiops truncates* (Colgrove *et al*., 1975); recovered from blowhole and gastric samples from a captive bottlenose dolphin with sinusitis (Wright *et al*., 1979) and was also reported on the successful treatment of skin disease caused by *V. alginolyticus* in a bottlenose dolphin held in an open ocean pool (Schroeder *et al*., 1985). *Vibrio alginolyticus* in specific association with copepods like *Tigropus filvus* and other plankton organisms are the most plausible reservoir from which epidemic, fully virulent strains could flourish (Carli *et al*., 1993).

5. *Vibrio mimicus* - This pathogen is a non-halophilic *Vibrio* named because of its similarity to *V. cholerae. V. mimicus* is a causative agent of gastroenteritis, food poisoning, wound and ear infections (Janda, 1987; Campos *et al*., 1996). It has been considered a secondary invader of red

claw crayfish (*Cherax quadricarinatus*) (Eaves & Ketterer, 1994). *V. mimicus* secretes a 63 kDa enterotoxic hemolysin as the most potent virulence factor. Enterotoxic hemolysin targets intestinal epithelial cells which affect ion transport to produce watery diarrhea, vomiting, and severe dehydration but no fever (Campos *et al*., 1996; Takahashi *et al.*, 2007). *V. mimicus* hemolysin (VMH) possess both hemolytic and enterotoxic activities (Sultan *et al*., 2007). *V. mimicus* has also been isolated from a number of environmental sources including oysters, fish, prawns, bivalves, rivers, and brackish waters. From April 1991 to December 1994, 33 cases of diarrhea caused by *V. mimicus* were reported by the emergency units of hospitals of the Costa Rican National Health Security System due to the consumption of raw turtle eggs (*Lepidochelys olivacea*) (Campos *et al*., 1996). Countries as far apart as Bangladesh, Calcutta (India), and the United States reported that between 10 to 35% of the *V. mimicus* found in clinical specimens produced Cholerae Toxin (CT). These data may explain why most of the patients studied (60 to 80%) presented a mild form of diarrhea, were treated as outpatients, with oral rehydration solutions only (Sanyal *et al*., 1983).

6. *Vibrio fluvialis* - This organism is a halophilic *Vibrio* first identified in 1975 in a patient with diarrhea in Bahrain (Furniss *et al*., 1977). The virulence factor is a Cytotoxin labile toxin and also can produce an enterotoxigenic El Tor-like cytotoxin (Kothary *et al*., 2001; Igbinosa & Okoh, 2008). It is biochemically similar to *Aeromonas hydrophila* but can be differentiated from this organism by its ability to grow well on media containing 7% sodium chloride. The two species can be identified by a salt tolerance test (Seidler *et al., 1980*). *V. fluvialis* causes wound infections or primary septicemia (Hlady & Klontz, 1996). The largest series of *V. fluvialis* infections involved 500 patients in Bangladesh; most of them were young children. Patients presented with diarrhea (75% bloody), vomiting (97%), abdominal pain (75%), dehydration

(67%), and fever (35%) (Huq *et al*., 1980; Daniels & Shafaie, 2000). *V. fluvialis* was also responsible for an outbreak of food poisoning in India which led to gastro-enteritis during 1981. The stools of 9/14 individuals revealed the presence of the organism (Thekdi *et al*., 1990). Also, it has been reported as a cause of wound infection leading to haemorrhagic cellulitis and cerebritis. The source of this infection was thought to be multiple fire-ant stings and brackish water (Huang & Hsu, 2005). Acute infectious peritonitis caused by *V. fluvialis* has also been reported (Lee Young *et al*., 2008). This organism is not only isolated from humans but also from marine and estuarine environments (Seidler *et al*., 1980; Morris & Black, 1985). *V. Fluvialis* have been implicated as the cause of limp lobster disease of the American lobster, *Homarus americanus* and pustule disease of the abalone (Tall *et al*., 2003) and also was isolated from a tail lesion in Gray whale *Esrhrichtius robustus* and from skin lesion, vagina, anus and blowhole of Belukha whales *Delphinapterus Leucas* (Buck *et al*., 1986).

7. *Photobacterium damselae* subsp. *Damselae* - This organism was first described in 1981 as a cause of granulomatous ulcerative dermatitis and death of saltwater damselfish - *Chromis punctipinnis* in California coastal waters (Love *et al*., 1981). It is similar to *V. vulnificus* but causes soft tissue infections following exposure of wounds to brackish water or injury by saltwater animals (Fraser *et al*., 1997; Barber & Swygert, 2000). *P. damsela* infections can be fulminating and are frequently fatal even in immunocompetent hosts. *P. damselae* display some special characteristics: (1) they have two hemolytic phenotypes, and (2) do not produce diarrhea (Kreger *et al*., 1987). Sixteen cases of *P. damsela* infection were reported between 1982 and 1996 of which four were fatal (Fraser *et al*., 1997).

P. damselae has been recovered from a wide range of other marine animals including sharks, turbot (*Scophthalmus maximus*), yellowtail (*Seriola quinqueradiata*) and red-banded sea

bream (*Pagrus auriga*) (Austin & Austin, 2007). The main virulence factors are: (1) Damselysin toxin, a phospholipase D with sphingomyelinase activity (Cutter & Kreger, 1990); (2) A neurotoxic acetylcholinesterase (Perez *et al*., 1998); (3) Extracellular products (ECPs) with low proteolytic activity (Fouz *et al*., 1993) and (4) A Siderophore-mediated iron sequestering system (Fouz *et al*., 1997).

8. *Vibrio metschnikovii* - This organism was first described in 1888 (Daniels & Shafaie, 2000). It is often isolated from the environment in a variety of farm animals showed cytotoxin production. *V. metschnikovii* have been cultivated from aborted cattle, swine, and horses as well as the brain tissue of ducks and geese in different parts of Germany (Kuehn, 2001; Stephan *et al*., 2002). This *Vibrio* has also been found in the intestines of chickens together with *E. coli* in cases of infectious hepatitis of fowl (Gerlach & Gylstorff, 1967a, b). *V. metschnikovii* is potentially pathogenic to larval giant clams, *Tridacna gigas* with infection leading to general disintegration of the tissues and total mortality (Sutton & Garrick, 1993). *V. metschnikovii* was isolated from 52.9% of fish samples, and from seven out of eight species of fish studied in Brazil; Pargo (*Snapper*), Sardinha (*Sardine*), Cherne (*Grouper*), Pescada (*Weakfish*), Betara (*Kingfish*), Carapau (*Mackere*l), and Bagre (*Sea catfish*) resulting in forty-eight isolates of *V. metschnikovii* recovered (Matte *et al*., 2007). Furthermore *V. metschnikovii* was found in raw fish and shellfish in markets and shops (Buck, 1991) *V. metschnikovii* is a zoonotic organism that can be transmitted to humans via the food chain. In humans *V. metschnikovii* was isolated from the blood of a diabetic woman with acute cholecystitis (Jean-Jacques *et al.,* 1981). *V. metschnikovii* was recovered from 5 infants with diarrhea during a cholerae surveillance program in Peru and from the stool of 6 diarrheal patients in Recife, Brazil (Daalsgard *et al.*, 1996; Magalhaes *et al*.,

1996). Also was found in a postoperative wound infection on the lower right leg of a patient after saphenectomy (Linde *et al*., 2004) and in a patient with pneumonia (Wallet *et al*., 2005).

Several virulence factors have been described reflecting the presence of hemolysins and verotoxins (Matte *et al*., 2007). A cytolysin was found from one strain of *V. metschnikovii* isolated from diarrheic feces (Miyake *et al*., 1988). The presence of plasmids also has been described (Dalsgaard *et al*., 1996). A gene encoding an extra cellular alkaline serine protease was cloned and characterized from *V. metschnikovii* strain RH530 (Kwon *et al*., 1995).

9. *Vibrio* (*Grimontia*) *hollisae* was first described in 1982 in patients with diarrhea. *V. hollisae* most commonly causes gastroenteritis but rare cases of septicemia and wound infections have been reported (Hickman *et al*., 1982; Rank *et al*., 1988; Hlady & Klontz, 1996; Hinestrosa *et al*., 2007). *V. hollisae* isolation from environmental samples is limited, since it grows poorly on selective TCBS media and MacConkey agar. It needs to be isolated from colonies on a blood agar plate and identification by biochemical test is also difficult because it is biochemically inert (Hickman *et al*., 1982). Four cases of septicemia associated with *V. hollisae* have been reported. Three cases occurred in patients with underlying liver disease (Rank *et al*., 1988; Gras-Rouzet *et al*., 1996; Hinestrosa *et al*., 2007). *V. hollisae* virulence is related to a hemolysin similar to the thermostable direct hemolysin (TDH) of *V. parahaemolyticus*. Nonetheless the hemolysin is heat-labile. *V. hollisae* also produces a heat labile factor that elongates CHO cells and induces intestinal fluid accumulation in suckling mice but it is different from cholerae toxin (Kothary *et al*., 1995).

10. *Vibrio cincinnatiensis* was isolated from the cerebrospinal fluid and blood in a 70 year old patient with confusion, showed positive results to biochemical tests included oxidase, sensitivity

to 0/129, O-nitrophenyl-I-D-galactopyranoside, lysine decarboxylase and fermentation of glucose, salicin, n-inositol, sucrose, L-mannose, L-arabinose, and arbutin. Negative to indole, ornithine decarboxylase, arginine dihydrolase fermentation of lactose, and production of gelatinase and urease; grew poorly on TCBS media culture (Brayton *et al.*, 1986). *V. cincinnatiensis* is a halophilic *Vibrio* that also has been isolated from diarrheal stool in a 67 year old patient suffering enteritis and from two aborted bovine fetuses of different herds. Nevertheless these strains grew on TCBS, CIN, MacConkey and XLD plates. Their biochemical activities were dependent on NaCl concentration, in particular the formation of indole, lysine and ornithine decarboxylases, arginine dihydrolase, the reduction of nitrate and behaviour in the Voges-Proskauer test. Moreover, lysine decarboxylase and nitrate reductase were temperature-dependent (Wuthe *et al.*, 1993). In addition *V. cincinnatiensis* were isolated from different fresh water environments as the Ohta River, Hiroshima, Japan, which demonstrated hemolytic and cytotoxic activities (Venkateswaran *et al*., 1989); in water samples collected from the Choptank River in Chesapeake Bay, USA in 1999. *V. cincinnatiensis* was present in much smaller numbers than other *Vibrio* species, with the highest counts being found in July and September (Heidelberg *et al*., 2002). *V. Cincinnatiensis* also was found in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea, Italy (Ripabelli *et al*., 1999)

11. *Vibrio furnissii* - This bacterium was originally considered as an aerogenic strain of *V. fluvialis.* In 1983, however, *V. furnissii* genetic analysis showed it to be a distinct species, which have lateral flagella*,* produce gas from glucose and are differentiated into various O serogroups for epidemiological studies (Brenner *et al.*, 1983; Shinoda *et al*., 1992). This organism has been isolated from the environment and from stool samples. *V. furnissii* was implicated with diarrheal disease in 16 patients in Brazil (Magalhaes *et al*., 1993); in an outbreak of gastroenteritis

occurring on an aircraft in 1969 (CDC, 1969). *V. furnissii* was also isolated in 14 patients, 6 with diarrhea and 8 without symptoms during a cholerae surveillance program in Peru during 1994 (Dalsgaard *et al.*, 1997). *V. furnissii* has been associated albeit tenuously with eel disease in Spain (Austin & Austin, 2007). The virulence factor is related to a hemolytic factor; ECPs damaged HeLa cells (Magalhaes *et al*., 1993); and with Phosphomannomutase (PPM) that induces intestinal damage within 3 days in mice after oral uptake (Kim *et al*., 2003).

12. *Vibrio harveyi* - This monotrichous bioluminescent *Vibrio* was originally named as *Achromobacter harveyi*. Then, at various times, it has been named as *Lucibacterium harveyi, Beneckea harveyi, Achromobacter harveyi, Pseudomonas harveyi, Photobacterium harveyi, Vibrio carchariae, Vibrio trachuri* (Austin & Zhang, 2006; Thompson, 2006). Its current taxonomic position as *V. harveyi* is due to the isolation from a shark that died in captivity in Baltimore, USA in 1982 (Grimes *et al*., 1984). It can be found globally in marine environments, either in a free-living state or in symbiosis with marine life. It is a common pathogen to many marine organisms. *V harveyi* has been isolated from necrotic enteric tissues of cultured flounder, *Paralichthys dentatus* in USA (Gauger *et al.*, 2006); from common snook, *Centropomus undecimalis* (Kraxberger-Beatty *et al*., 1990); black sea bream, *Acanthopagrus schlegeli*, yellowfin sea bream, *Acanthopagrus latus*, Japanese sea bass, *Lateolabrax japonicus* and red drum, *Sciaenops ocellatus* (Lee *et al.*, 2002); It is one of the main pathogenic bacteria recognized in crown-of-thorns starfish (Sutton *et al.*, 1988; Rivera-Posada *et al.,* 2011); cultured brown spotted grouper, *Epinephelus* tauvina in Kuwait (Saeed, 1995). In humans was reported from a leg wound resulting from a shark bite, USA (Pavia *et al.*, 1989). *Vibrio harveyi* pathogenicity mechanism are related with extracellular products like cysteine protease, phospholipase, haemolysins, ADP-ribosylating toxins (Liu *et al.*, 1996; Soto-Rodriguez *et al*., 2003);

lipopolysaccharide (Montero & Austin, 1999); Bacteriophage (Oakey & Owens, 2000); bacteriocin-like substance (Prasad *et al*., 2005); Quorum-sensing factors (Henke & Bassler, 2004); capacity to bind iron (Owens *et al*., 1996); ability to attach and form biofilms (Karunasagar *et al.*, 1994). Proteases and hemolysins are considered to play a major role in host tissue liquefaction (Deanne, 2005).

Coral reef pathogens

On coral reefs, *Vibrio* bacteria have been implicated in coral bleaching and coral diseases (Bourne *et al*., 2008). Reports on coral disease continue to rise with currently 29 reported syndromes in the Caribbean and 7 from the Indo-Pacific (Banin *et al*., 2000; Rosenberg & Loya, 2004; Sussman *et al*., 2008). Until now 5 *Vibrio* species have been directly related with coral diseases. Modern studies have demonstrated that host, pathogen and environment form a constantly evolving disease equilibrium contributing to a growing list of newly emerging infectious diseases (Sussman *et al*., 2008). Understanding the specific ways in which *Vibrios* influence coral diseases and how they affect reef function will allow a better prognosis of the conditions under which outbreaks and diseases flourish in the marine environment.

13. *Vibrio shiloi* - This bacterium was characterized as a new *Vibrio* species on the basis of 16S rDNA sequence, DNA–DNA hybridization and phenotypic properties which incorporate the cellular fatty acid profile (Kushmaro *et al*., 2001). *V. shiloi* is closely related to *V. mediterranei*. Nonetheless the major fatty acids found in *V. mediterranei* are hexadecane and octadecane, both of which are not present in *V. shiloi* AK1T. Another important difference is that *Vibrio mediterranei* does not infect corals while *V. shiloi* does (Kushmaro *et al.*, 2001). This coral-bleaching species with type strain AK1^{T} (=ATCC BAA-91^{T}=DSM 13774^{T}) was named *Vibrio*

shiloi because of the Russian microbiologist Moshe Shilo (1920-1990) pioneer of microbial ecology in Israel, which is also well known for his work in aquatic microbiology (Kushmaro *et al*., 2001; Keynan, 2008). This non-spore-forming rods measure 2.4 x 1.6 um, are motile by a single polar, displayed sheathed flagellum when grown on solid or liquid medium and their colonies are light yellow on marine agar after 48 h incubation at 30 °C. Furthermore they have slightly serrated edges, do not luminescence and cannot survive in corals at temperatures below 20 ºC requiring a fresh infection every year. The Bearded fireworm *Hermodice carunculata* is a vector and winter reservoir of *V. Shiloi*.

This *Vibrio* was the first bacterium recognized as a causative agent of coral bleaching in the Mediterranean coral *Oculina patagonica* (Kushmaro *et al.*, 1996, 1997, 1998; Banin *et al.*, 2000,). *V. shiloi* produces death of the endosymbiotic zooxanthellae and bleaching of corals at temperatures ranging from 20 to 32 °C. The virulence factors involved are related to extracellular P toxins that block photosynthesis, bleach and lyse zooxanthellae (Ben-haim *et al*., 1999; Banin *et al.*, 2001). This organism causes the obliteration or loss of algae, and produces both a heat-stable extracellular toxin that inhibits the photosynthesis of zooxanthellae and also heat-sensitive toxins that bleach and lyse algal cells isolated from corals (Banin *et al*., 2001). *V. shiloi* penetrates into the coral epidermis following adhesion to the coral surface, multiply in the tissue and enter a state in which they fail to form colonies on media that normally sustain the growth of *V. shiloi* (Banin *et al*., 2000).

14. *Vibrio coralliilyticus* (coral dissolving bacteria) is closely related to *Vibrio tubiashii* and *Vibrio shiloi*. It is a Gram-negative, motile, rod-shaped bacterium which measures 1.5 x 0.8 µm has a single polar, sheathed flagellum. Colonies are pale colored on marine agar and yellow on TCBS agar after 48 h incubation at 30 °C, have smooth edges and do not have luminescence.

The main cellular fatty acids are 16: 0 and 18: 1w7c. *Vibrio coralliilyticus* was reported as the causative agent of temperature induced bleaching of *Pocillopora damicornis* in the Indian Ocean and also was isolated from three different diseased *P. damicornis* colonies on the Eilat coral reef, Red Sea. In addition was isolated from diseased oyster larvae in Kent, United Kindom and from bivalve larvae in Brazil (Ben-Haim & Rosenberg, 2002; Ben-Haim *et al.*, 2003)

15. *Vibrio corallilyticus* was linked with white syndrome disease in Indo-Pacific corals. It was isolated from *Montipora aequituberculata* fragments collected in Nelly Bay, Great Barrier Reef; from *Acropora cytherea* corals in Marshall Islands and from *P. speciosa* sections of Nikko Bay Palau (Sussman *et al.*, 2008; Meron, 2009). This organism is present in different hosts, probably including bivalve larvae (Ben-Haim *et al.*, 2003). The virulence factor is related to zinc-metalloproteinases at temperatures > 26 ºC that caused quick photoinactivation of predisposed Symbiodinium endosymbionts followed by lesions in coral tissue (Ben-Haim *et al.*, 2003; Sussmann *et al.*, 2008).

16. *Vibrio tasmaniensis* was identified using fluorescent amplified fragment length polymorphism patterns. The type strain of this species is LMG 20012^sup T^ (CAIM 634^sup T^) isolated from Atlantic salmon (*Salmo salar*) in Tasmania. Cells are slightly curved and 1 um width by 2-3 um length. They appear as translucent, curved, non-swarming, smooth-rounded colonies with entire margin, beige in color and about 4 mm in diameter on TSA after 48 h incubation at 28 ºC. Strains formed green, translucent, smooth-rounded colonies of 4 mm on TCBS plates. *V tasmaniensis* have a facultative anaerobic metabolism and ferment glucose, mannitol and amygdalin, but not inositol, sorbitol, sucrose, melibiose and arabinose. Grow between 4 – 35 ºC but no growth is observed in the absence of NaCl or in media with > or = 8% (w/v) NaCl. Abundant growth occurs at 28 ºC in media containing 2.5% (w/v) NaCl. *Vibrio*

tasmaniensis is positive to oxidase, catalase, tryptophane deaminase, indole and Voges-Proskauer reaction. *Vibrio tasmaniensis* is a cold-water adapted organism which does not enter in the VBNC state at 4°C. This bacterium was recognized as part of the biota associated with marine organisms and also was isolated from necrotic tissue of the gorgonian octocoral *Eunicella verrucosa* from cool temperate waters with range temperatures of 7.5 to 17°C during an outbreak at Lundy Island, Devon, England in 2003 (Vattakaven *et al.*, 2006).

17. *Photobacterium eurosenbergii* - This bacterium was isolated from the mucus of the Caribbean elkhorn coral *Acropora palmate* (Ritchie, 2006). That study showed that antibiotic properties of coral mucus, and the mucus potential to select a set of commensal bacteria, were lost at increased temperatures during the 2005 bleaching event in Florida. These findings implied a temporal shift in the protective qualities of coral mucus, and a composition shift from beneficial bacteria to *Vibrio* dominance under conditions of increased temperature (Ritchie, 2006). *Photobacterium eurosenbergii* was also recovered from mucus and water surrounding bleached *Barabattoia amicorum* corals at Magnetic Island, GBR. *P. eurosenbergii* CC022 strain showed the highest superoxide dismutase activity displaying an important antioxidant defense (Colin *et al*., 2008).

1.6 Identification methods

The characterization of pathogenic isolates plays an essential role in the epidemiology of infectious diseases, producing the information necessary to identify, track, and potentially disease outbreaks (Urwin *et al.*, 2003). Biochemical bacterial identification test like API 20E and NE kits (BIOMÉRIEUX SA, Marcy-l'Etoile, France) identify *Vibrio* bacteria based on carbon source utilization patterns that can be compared to a database of known organisms to allocate the best match. These tests are easily performed, readily available, and relatively inexpensive.

However, the discriminatory power of this test is limited for environmental strains and the identification of *Vibrio* species is problematic, largely because of genetic plasticity (Cano-Gomez *et al*., 2009). Most members of the genus are halophilic and the addition of NaCl is often required for enzymatic activity; however the concentration of NaCl can affect (1) the biochemical profile (2) and lead to erroneous identification. In addition, the characteristics shared by *Aeromonas* and *Vibrio* resulted in the classification of isolates in the wrong genus (Ohara *et al.*, 2003). Identification is further complicated because of the color and size of the colonies in vary greatly in media cultures, depending on temperature, salinity, and food source.

Molecular techniques have significant advantages over biochemical techniques for identifying bacteria, providing data that are objectively scored to provide an unambiguous identification. Methods that utilize the polymerase chain reaction (PCR) can lead to identification of an isolate within hours as opposed to days, and can be used on small quantities of cells, including those that are not viable or are otherwise unculturable, and can be designed to be specific for genus, species, or allele. A range of different molecular methods have been used for identification and typing of *Vibrio* isolates such as Repetitive Extragenic Palindromic (REP-PCR), Multilocus sequence analyses (MLSA), DNA-DNA hybridization, Arbitrarily primed polymerase chain reaction (AP-PCR), Amplified fragment length polymorphism (AFLP), Random amplified polymorphic DNA (RAPD), Ribosomal restriction fragment length polymorphism (RFLP) (Thompson *et al*., 2007; Cano-Gomez *et al*., 2009). The highest resolution power and reproducibility are achieved with:

Multilocus sequence analysis (MLSA)

MLSA is a molecular technique for the typing of multiple loci that characterises isolates of bacterial species using the DNA sequences of internal fragments of multiple housekeeping

genes. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct genotype and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (Maiden *et al.,* 1998; Thompson *et al*., 2007; Cano- Gomez *et al.*, 2009). MLSA uses PCR amplification followed by DNA sequencing and the nucleotide differences between strains can be checked at a variable number of genes depending on the degree of discrimination desired. MLSA is highly clear, portable, reproducible and scalable with an electronic taxonomy database option (Sawabe *et al*., 2007; Thompson *et al.,* 2007). MLSA is becoming popular due to the ease of data analysis and has the advantage that it gives information about the clonal relationships of isolates that other technique as PFGE does not (Johnson *et al*., 2007). The application of MLSA is massive, and provides a resource for the scientific, public health, microbiologist, veterinary, epidemiology and food industry communities (Urwin *et al*., 2003).

DNA-DNA hybridization (DDH)

This technique has been used by bacterial taxonomists since the 1960's to determine relatedness between strains and is still the most important criterion in the delineation of bacterial species (Goris *et al.*, 2007). DNA Hybridization measures the degree of genetic similarity between pools of DNA sequences and it is one of the few universally applicable techniques available that could offer truly genome-wide comparisons between organisms. A value of 70% DDH is the gold standard for delineating species (Wayne *et al.*, 1987), although 80% DNA– DNA similarity is suggested for species definition within the family Vibrionaceae (Gomez-Gil *et al.*, 2004). Some disadvantages of this technique are the relatively large quantities of DNA required when compared with PCR techniques, it is labour intensive, time consuming, no incremental databases can be built, in contrast to sequence analysis (Cho &Tiedje, 2001; Gevers *et al*., 2005; Goris *et*

al., 2007). Because of these drawbacks and the decreasing cost of DNA sequencing it is likely that DNA-DNA hybridization will be replaced by sequence techniques like MLST in the near future.

Repetitive Extragenic Palindromic (REP-PCR)

This technique uses DNA primers complementary to naturally occurring, highly conserved repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative bacteria. Three families of repetitive sequences have been identified: (1) REP sequence; (2) ERIC sequence (Enterobacterial repetitive intergenic consensus); (3) BOX element (Versalovic *et al*., 1991). These sequences are located in distinct, intergenic positions around the genome. Appropriate PCR primers directed at these repeated sequences generates multiple amplification of distinct genomic regions located between these elements and are referred to as REP-PCR, ERIC PCR and BOX PCR respectively and as REP-PCR genomic fingerprinting collectively. Then, a gel matrix is created with the REP-PCR fingerprint and used to differentiate bacterial isolates at the species and strain level (Versalovic *et al.*, 1991, 1994). One advantage of Rep-PCR is that the primers work in a variety of Gram-negative and Gram-positive bacteria. This means that no previous knowledge of the genomic structure is necessary and by passes the need to identify suitable arbitrary primers by trial and error. In addition, the technique discriminated closely related vibrios such as *V. harveyi, V. alginolyticus, V. campbellii, V. parahaemolyticus*, and *V. rotiferianus*, and also suggested the existence of new *Vibrio* species (Cano-Gomez *et al.*, 2009). PCR with primers based on REP PCR sequences has been successful in differentiating bacterial strains from diverse species (De Bruijn, 1996; Bennasar *et al*., 2002; Bruant *et al.*, 2003; Hahm *et al*., 2003). BOX-PCR is quicker, cheaper, and in many cases more discriminatory than pulsed field gel electrophoresis (PFGE). Furthermore, patterns are not affected by the

culture age of the strain to be analyzed and fingerprinting output can be easily analyzed by computer-assisted methods. Nonetheless, it is generally less reproducible (Kang *et al*., 2003). These features make BOX PCR a frequently selected tool in biogeography studies in environmental microbiology. On the other hand, ERIC-PCR is the most widely adopted of the above three REP PCR typing methods and has been applied to the typing of many species, including *V. cholera* and *V. parahaemolyticus* (Wong & Lin, 2001). Following outbreaks on Canada's Pacific coast, 38 clinical strains of *V. parahaemolyticus* were analyzed by ERIC-PCR, ribotyping, PFGE, and restriction fragment length polymorphism. Six ERIC PCR patterns were identified by a single primer for the amplification, and showed that ERIC-PCR and ribotyping were helpful in evaluating genetic and epidemiological relationships among *V. parahaemolyticus* strains (Marshall *et al*., 1999). Nonetheless, REP-PCR is preferable to ERIC-PCR because of the greater reproducibility of its fingerprints (Wong & Lin, 2001.). Basically, all three REP-PCR typing methods described here could differentiate *Vibrio* from other species and effectively differentiate intraspecific strains.

Amplified fragment length polymorphism (AFLP)

It is a polymerase chain reaction (PCR) based genetic fingerprinting technique developed in the early 1990's by Keygene (Vos *et al.*, 1995). AFLP has high reproducibility, resolution, sensitivity and could amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification. As a result, AFLP has become beneficial in the study of new taxa including bacteria, fungi and plants and is widely accepted as an effective tool for identifying genomic differences among closely related species. Nonetheless it is time consuming and expensive. The procedure of this technique follow 3 steps: First there is a total cellular DNA digestion with one or more restriction enzymes and ligation of restriction half-site

specific adaptors to all restriction fragments. Then a selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences is produced and finally a electrophoretic separation and amplicons on a gel matrix, followed by visualization of the band pattern through autoradiographic or fluorescence methodologies (Vos *et al*., 1995; Mueller & Wolfenbarger, 1999; Thompson *et al*., 2001). The key feature of AFLP–PCR is its capacity for the simultaneous screening of many different DNA regions distributed randomly throughout the genome (Mueller & Wolfenbarger, 1999; Thompson *et al*., 2001).

1.7 Conclusions

Vibrio bacteria are a normal component of aquatic micro biota, common in water and a variety of different host organisms. *Vibrio* do not pose a risk to the health of most host, except when the organism possess nutritional, immunological or genetic deficiencies and/or when subject to significant environmental stress. *Vibrio* are opportunistic pathogens that can take advantage under ecological changes produced by factors like climate change, dense aggregations (culture environments), contaminated waters, high temperatures, light, salinity and others. *Vibrio* constitute an important element of the bacteria flora within the organism and their surrounding environment being a constant source of infections.

A better understanding of the ecology of infectious diseases of marine animals, which share marine resources and *Vibrio* pathogens, and knowledge about reservoirs and modes of transmission which have been useful in the past for developing technologies or mechanisms to control the spread of diseases and their cure, will improve the comprehension of marine diseases and the prediction of human health risks. Special awareness of the full-scale ocean iron fertilization scheme is required because iron could trigger the virulence factors of vibrios which

significantly affect nutrient cycling in these habitats, and comprise a major portion of aquatic animal's natural flora and the increase of incidences of emerging pathogens such as *Vibrio* species has been generally underestimated. Pathogens and environment are closely related and form a balance in nature. Disease and outbreaks are induced by multi-factorial mechanisms that need to be explored both independently and in conjunction with other factors before a decision could be considered. Studies of coral bleaching induced by *Vibrio* species could focus on the recognition and monitoring of vector species involved (i.e *hermodice carunculata-V shiloi*), and other fireworms to elucidate their infection mechanism and allow the development of possible biological control options.

CHAPTER 2: Injection of *Acanthaster planci* with thiosulfate-citrate-bile-sucrose agar (TCBS). I. Disease induction

2.1 Abstract

This is the first report of the successful induction of a transmissible disease in the coral-eating crown-of-thorns starfish *Acanthaster planci* (COTS). Injection of thiosulfate-citrate-bile-sucrose agar (TCBS) culture medium into COTS induced a disease characterized by discolored and necrotic skin, ulcerations, loss of body turgor, accumulation of colorless mucus on many spines especially at their tip, and loss of spines. Blisters on the dorsal integument broke through the skin surface and result in large, open sores that expose the internal organs. Oedema and reddened digestive tissues and destruction of connective fibers were common. Moreover healthy COTS in contact with these infected animals also displayed signs of disease and died within 24 h. TCBS induced 100% mortality in injected starfish. There was no introduction of new pathogens into the marine environment. TCBS promoted the growth of COTS' naturally occurring *Vibrionales* to high densities with subsequent symbiont imbalance followed by disease and death.

†This chapter was published (as written) in *Diseases of Aquatic Organisms*: Rivera-Posada JA, Pratchett M, Cano-Gomez A, Arango-Gomez JD, Owen L (2011) Injection of *Acanthaster planci* with thiosulfate-citrate-bile-sucrose agar (TCBS). I. Disease induction. Diseases of Aquatatic Organisms 97 (2): 85-94

2.2 Introduction

Outbreaks of crown-of-thorns starfishes (COTS) *Acansthater planci* L., defined as rapid increases in starfish densities to >1500 starfish km^{-2} (Moran & De'ath, 1992) represent the most significant biological disturbance on tropical coral reefs (Birkeland & Lucas, 1990), resulting in extensive (up to 90%) mortality among reef-building and habitat forming corals (Pratchett 2005;

Pratchett *et al.,* 2009). *A. planci* is the principal cause of long-term coral loss at many locations in the Pacific, including Palau, Guam and Fiji (Bruno & Selig, 2007). Extensive coral losses caused by outbreaks of *A. planci* have secondary effects on reef fishes (Sano *et al*., 1987).

Outbreaks of COTS starfish typically end with rapid population declines, though the causes of these rapid declines are largely unknown (Moran *et al*., 1985; Moran, 1986; Pratchett, 2005). In Fiji, the rapid disappearance of *A. planci* following an extensive outbreak in the 1980's was attributed to disease (Zann *et al.,* 1990). Disease has been implicated in the mass-mortalities of numerous other echinoderms (e.g. Dungan *et al.*, 1982; Lessios *et al.*, 1984; Williams *et al.*, 1986). Pratchett (1999) demonstrated that tissue removed from sick and dying COTS starfish, collected on the Great Barrier Reef towards the end of an outbreak, can be used to infect other seemingly healthy starfish, indicating that COTS starfish are susceptible to infectious diseases. While there was no attempt to isolate the pathogen involved, Sutton *et al*., (1988) isolated several potential pathogens, including *Vibrio harveyi, V. tubiashi, V. campbellii, Pseudomonas* and *Moraxella* bacteria in captive *A. planci* that were exhibiting conspicuous signs of disease. However, *Pseudomonas* and *Moraxella* were described as normal flora in coral reef seawater around healthy COTS in subtropical Japan (Okinawa) (Reed *et al.,* 1999). In another echinoderm, *Vibrio harveyi*, *V. natriegens* and *V. alginolyticus* were described as the causal agents of skin ulceration disease in cultivated juveniles of *Holothuria scabra* (Morgan, 2000; Becker, 2004). *V. anguillarum* and *Aeromonas salmonicida* also induced the bald sea urchin disease of *Strongylocentrotus purpuratus* (Gilles & Pearse, 1986). These pathogenic vibrios coordinate the expression of certain genes in response to their population density through a quorum sensing mechanism (QS), releasing and detecting signal molecules or autoinducers consisting of *N*-acyl homoserine lactones (AHLs). These regulatory systems control various

functions, including bioluminescence, conjugative plasmid transfer, biofilm formation, antibiotic synthesis, motility and production of virulence factors in animal, plant, and human pathogens (Peters *et al*., 2003; Fangfang *et al.,* 2008; Park *et al.*, 2008).

Vibrios are usually isolated and cultured in microbiology laboratories by the use of thiosulfate-citrate-bile-sucrose agar (TCBS), which is considered the primary plating medium universally used for the isolation of these bacteria (Kobayashi *et al.*, 1963). This highly selective agar meets the nutritional requirements of *Vibrio* spp., allowing vibrios to compete with intestinal flora (Baron *et al*., 1994). TCBS components include yeast extract and bacteriological peptone which provide the nitrogen, vitamins and amino acids. Sodium citrate, sodium thiosulfate and ox bile are selective agents, providing an alkaline pH to inhibit Gram-positive organisms and suppress coliforms. An increased pH is used to enhance growth of *Vibrios*, because these organisms are sensitive to acid environments. Sucrose is the fermentable carbohydrate. Sodium chloride stimulates the organism's growth and maintains the osmotic balance of the medium. Sodium thiosulfate is a sulfur source and together with ferric citrate acts as an indicator to detect hydrogen sulfide production. Bromothymol blue and thymol blue are pH indicators and agar is the solidifying agent.

The purpose of this study was to explore a novel method to control COTS through the injection of TCBS agar. This induces a fatal disease that spreads to in-contact COTS. A secondary aims was to identify naturally occurring bacteria in COTS before and after TCBS injections. Moreover, this study examines wheter these potential *Vibrio* pathogens would cause symptoms consistent with natural instances of disease observed previously.

2.3 Materials and methods

Study sites

The study was undertaken during February-December 2009 at 2 locations: (1) Lizard Island (14° 40 ´S, 145°27´E) located in the far northern section of the Great Barrier Reef (GBR; Australia) and (2) Haputo Point, northwest Guam, (13° 28 ´N, 144° 40' E) in the Western Pacific Ocean.

COTS collection and maintenance conditions

A total of 80 COTS were collected from a range of different depths (3-15 m) and from low- and high- density populations. Fifty starfish were from Lizard Island and 30 from Guam. A total of 58 starfish were placed in pairs in 68 L plastic aquariums (Nally bins; 64 cm long x 41 cm wide x 40 cm deep). The other 22 starfish were placed in a large tank (270 cm long x 160 cm wide x 50 cm deep) with constant water flow and an average temperature of 28 to 30°C.

Media cultures

Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS; Oxoid^{R}), Nutrient Agar (NA; Oxoid^{R}) supplemented with 2% NaCl and Marine Broth (MB; Difco^{TM}) were used to culture all organisms of potential significance from the starfish and to determine whether these culture media could induce disease in *Acanthasther planci.* A total of 88 grams of the TCBS agar were added to 1 l of reverse-osmosis purified water, heated with permanent agitation to promote the dissolution of the powder but were not excessively heated or autoclaved. This kept the TCBS media in a liquid state for subsequent injection in COTS. MB and NA were prepared following the manufacturer's instructions. All media were stored at 4°C.

Culture media experiments

Five different experiments were carried out.

Experiment 1. Nine COTS separated into 3 groups of 3 starfish were used in this experiment. Twelve ml of each medium were injected to individual COTS with a 21gauge syringe to determine which media culture induced disease. Nutrient agar was slightly heated to dissolve the agar and facilitate injection. COTS were individually placed in separate aquaria to observe their behaviour, reactions and the latency period of the disease and time to death.

Experiment 2. Fifteen COTS injected with TCBS were used in this experiment; separated in 3 groups of 5 starfish and placed in individual aquaria at three different temperatures (26, 28 and 30°C) to investigate wheter changes in temperature influenced the bacteria ecology, accelerated the presentation of clinical signs and affected time to death.

Experiment 3. Eighteen COTS were placed in pairs into Nally bins; one starfish of each tank was injected with 12 ml of TCBS solution to observe wheter the disease induced by TCBS injection could be transmitted to the healthy in-contact COTS.

Experiment 4. Sixteen COTS located in pairs in 8 aquariums were used in this experiment. Induction of disease through the injection of 12 ml of TCBS was followed by removal of sick COTS to investigate wheter diseases continued to progress without the presence of sick COTS. Once the exposed starfish showed the first clinical signs of mucus on the tip of spines and loss of vertical aspect of spines the injected COTS were removed and 1 ml of water with mucus was collected to measure bacterial density by the dilution and plating method.

Experiment 5. A total of 22 COTS were placed in a large tank at the Marine Laboratory University of Guam (UCLM) at 28.7 °C. Two were injected with the TCBS solution to (1) observe wheter Guam COTS were susceptible to the disease induced by TCBS injection (2) determine wheter healthy COTS at high densities and in contact would avoid the sick starfish;

and (3) investigate wheter healthy Guam COTS also become infected with the transmissible disease once they were exposed to a sick COTS (outbreak density simulation).

Sample collection and bacterial identification

Microbiology

In order to culture bacteria for later identification, sterile inoculation loops were used to swab the skin surface, dermal lesions and inside the anus of individual starfish. These samples were then streaked on TCBS and NA with 2% NaCl plates and grown for 24 h at 30-32 °C. Individual colonies from these plates were grown in MB at 30-32 °C with shaking. Bacterial stocks were stored in liquid nitrogen at -80 ºC in either MB with 30% (v/v) glycerol or in Microbank™ cryovials (Pro-Lab Diagnostics) for further identification using PCR.

DNA extraction and Polymerase Chain Reaction

Genomic DNA was extracted from overnight MB cultures of 19 COTS bacterial isolates using the Wizard Genomic DNA Purification Kit (Promega®) according to the manufacturer's instructions. Identification of isolates at the level of species was carried out on the base of 16S ribosomal RNA gene following PCR amplification and sequencing of this gene as described by Lane, (1991), using the primers 27f (AGA GTT TGA TCC TGG CTC AG) and 1492r (GGT TAC CTT GTT ACG ACT T). All PCRs were performed in a GENEAMP PCR System 9700 (Perkin Elmer, USA). PCR reactions (20 µl) contained approximately 20ng/µl of genomic DNA, 1X PCR buffer (Quiagen) with a final concentration of 1.5 mM MgCl2, 0.5 µM of each primer, 200 µM dNTPs and 0.5 U Taq Polymerase (Quiagen). A 5µl sample of each PCR product was resolved by electrophoresis at 100 V for 30 min in 1% agarose gel to detect amplicons of the expected sizes. PCR products were sequenced by Macrogen Ltd, Korea.

Phylogenetic analysis

DNA sequences were assembled using the computer software Sequencher 4.9 (Gene Codes). Sequences were manually corrected, trimmed, and finally compared with those of closest relatives available in the GenBank nucleotide database using the BLASTN search algorithm (Altschul *et al*., 1990). Subsequently, edited sequences were aligned by Clustal_X (Thompson *et al.,* 1997). Phylogenetic trees based on 16S rRNA sequences were constructed with PAUP v.4.0B10 (Swofford, 2003) under the neighbour-joining algorithm NJ (Saitou & Nei, 1987). A total of 19 sequence of the isolates under study and 24 publicly available sequences from type strains of species were used to build the trees. For the analysis, uncorrected "p" distance and the Kimura two–parameter correction (Kimura, 1980) were tested. Bootstrap support of phylogenies was calculated on 1000 replicates.

2.4 Results

Induction of disease

Experiment 1. TCBS was the only media culture that induced death of COTS ~ 11.5 h post-injection at 30°C. MB induced signs of disease 5 h post-injection but recovered after 10 h. NA broth did not lead to the display of significant signs of disease in healthy individuals.

The characteristic clinical signs of the disease showed by all COTS were discoloured and necrotic skin, ulcerations, loss of body turgor, accumulation of colourless mucus on many spines, especially at their tips and loss of spines. Blisters on the dorsal integument broke through the skin surface and resulted in large, open sores that exposed the internal organs. Oedema and reddened disgestive tissues, and lysis of connective fribers and the magnesium calcite skeleton were also common and were observed in infected COTS (Fig. 2.2).

Experiment 2. Changes in temperature significantly delayed the presentation of clinical signs of disease and time to death between 25 and 50%. COTS injected with TCBS and placed at 30 °C died within 10-14 h; at 28 °C died within 14-18 h and at 26 °C died within 18-24 h. A few starfish lasted longer than others (Table 2.2).

Experiment 3. Succesful transmission to healthy COTS was observed. COTS that were injected with TCBS displayed signs of disease and died within 24 h. In-contact COTS showed similar signs of disease and also died within 24 h. However, 2 in-contact COTS were exposed to high flow currents without direct mucus contact due to pump pressure problems; one displayed rapid recovery and the other was not infected.

Experiment 4. COTS exposed for short periods to sick COTS recovered normal spine positions, reduced mcusu production and recuperation was observed after 7 h of solitary confinement. In contrast, COTS with more advanced signs of disease (blisters, open sores) died even when sick TCBS-injected COTS were removed from the aquarium. Increases inbacterial densities ranging between 10^5 and 10^7 colony forming units (cfu) ml^{-1} were observed in these COTS, showing a direct relation with the presentation of disease.

Experiment 5. Guam COTS were susceptible to the infectious disease induced by injection of TCBS, and COTS at high densities with open sores did not avoid sick, in-contact starfish making them more susceptible to disease transmission. Succesful disease transmission was observed and infected starfish died in a similar period compared to those from the GBR (Fig. 2.3)

Isolates identification

The 16S rRNA gene was successfully amplified from 19 bacterial isolates. BLASTN searches allowed identification of isolates as *Vibrio* spp., *Photobacterium* spp and *Bacillus* sp. with 99-100% sequence identities. In the case of *Vibrio* isolates, phylogenetic analysis based on the 16S

rRNA sequences, and not only BLAST searches, was necessary for the identification at species level, due to the high similarities among species for this gene and the presence of multiple unclassified sequences in the databases (Gomez-Gil *et al.,* 2004; Cano-Gomez *et al.,* 2010). For isolates belonging to the *V. harveyi* species group (*V. harveyi, V. rotiferianus, V. campbellii* and *V. owensii*) the 16S rRNA gene sequence inter-species similarities ranged from 98.9 to 99.6%. The phylogenies offered a more precise identification although low bootstrap support was obtained in numerous nodes (Fig. 2.1). Strains isolated from sick *Acanthaster planci* were tentatively identified as *V. owensii, V. rotiferianus, V. harveyi, V. natriegens, V. fortis* and *Photobacterium eurosenbergii*. Strains of *Bacillus sp*., *V. fortis, V. rotiferianus, V. owensii* and *P. eurosenbergii* were found in wild healthy animals. Sequences of the 16S rRNA gene were submitted to GenBank (accession nos. HQ44999 to HQ449979; see table 2.1.). Accession numbers for *Bacillus* sp. (isolate: *Beige*, not shown) and *Pseudoalteromonas* sp. (isolate: Gumar, not shown) are HQ449959 and HQ449967, respectively.

ISOLATE	16 s rRNA
G2	HQ449968
Y2	HQ449979
HAEM	HQ449964
NA	HQ449960
CREAM TCBS	HQ449963
GREEN AGRESS	HQ449961
GREEN+	HQ449965
CREAM 12-13	HQ449966
GU2Y	HQ449969
GU5YEN	HQ449973
GU5DARKEN	HQ449971
GU4YEN	HQ449970
GUIGEN	HQ449975
GU5PALEEN	HQ449972
GUMAB	HQ449976
GU1.STAR	HQ449974
X1	HQ449977
X2	HQ449978
YG	HQ449962
BEIGE	HQ449959

Table 2.1. Accession numbers of Crown-of-thorns isolates deposited in GenBank for 16S rRNA gene.

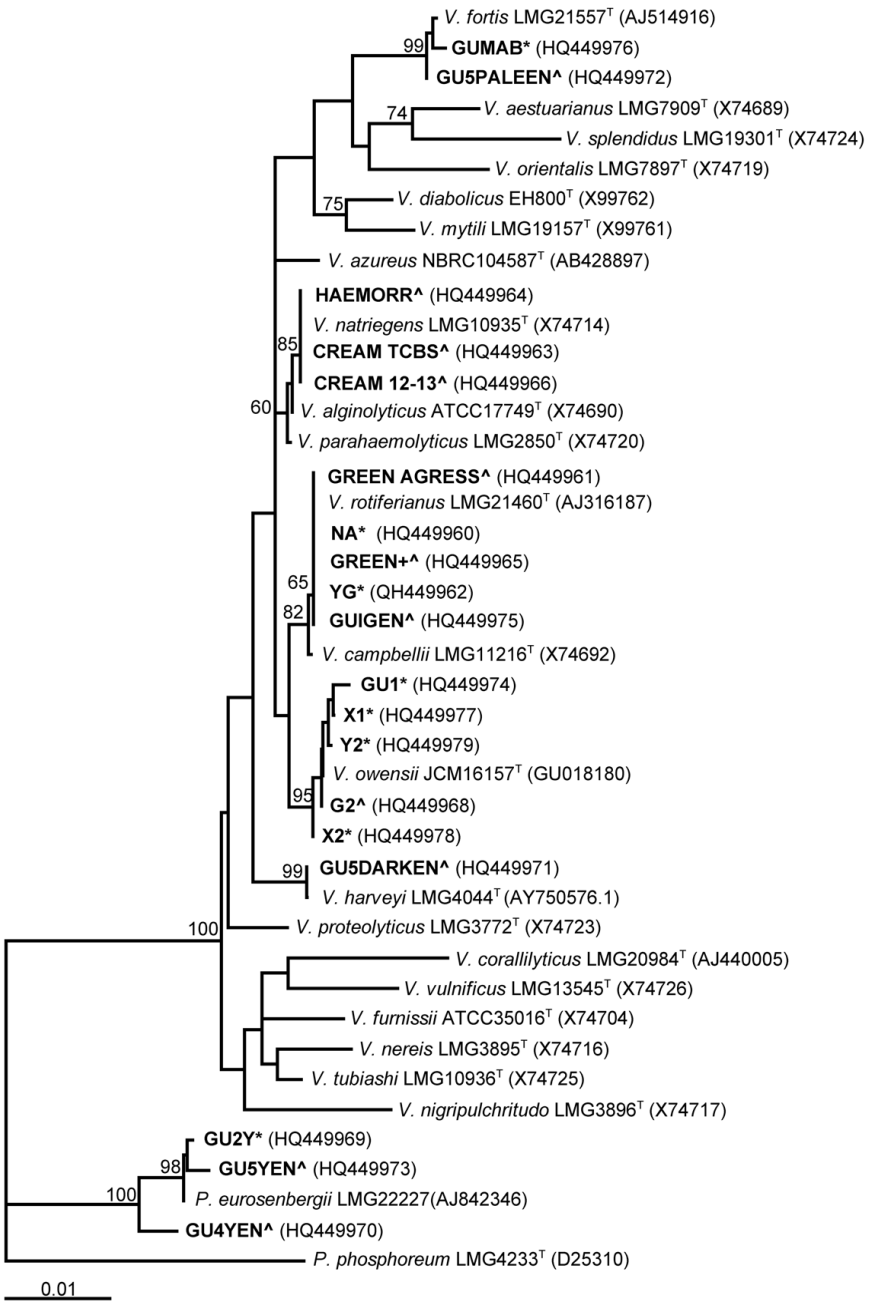

Figure 2.1 Phylogenetic analysis base on partial 16S rRNA gene sequences (1,302 nt), using the neighbour-joining algorithm and the Kimura-two-parameter correction, showing sequence clustering of crown-of-thorns (COTS) *Vibrio* isolates and related species. GenBank accession numbers are provided in parentheses. Photobacterium phosphoreum LMG 4233T was used as outgroup. Bootstrap support values after 1000 simulations are shown. Bar, 1% sequence divergence.
"GU" strains isolated from Guam (USA)
(*): strains isolated from healthy COTS.
(^): strains isolated from TCBS-injected moribund COTS.

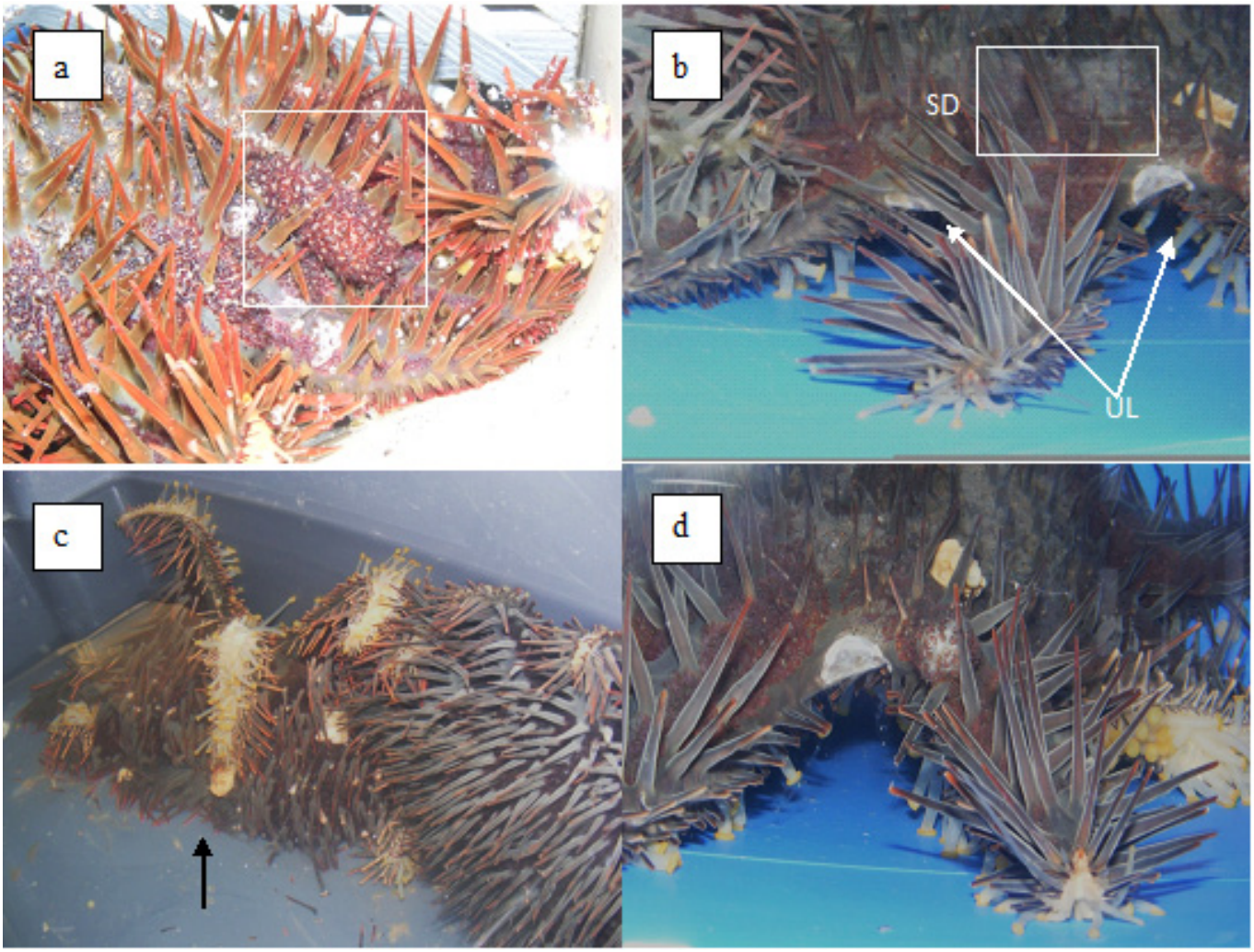

Figure 2.2. Clinical signs of COTS induced infection. (a) Loss of skin turgor, blister formation; (b) ulcerations (UL), changes in colour of skin (SD); (c) Loss of a straight orientation and fall of spines – black arrow; (d) blisters on dorsal surface, ulcerations and exposure of digestive glands.

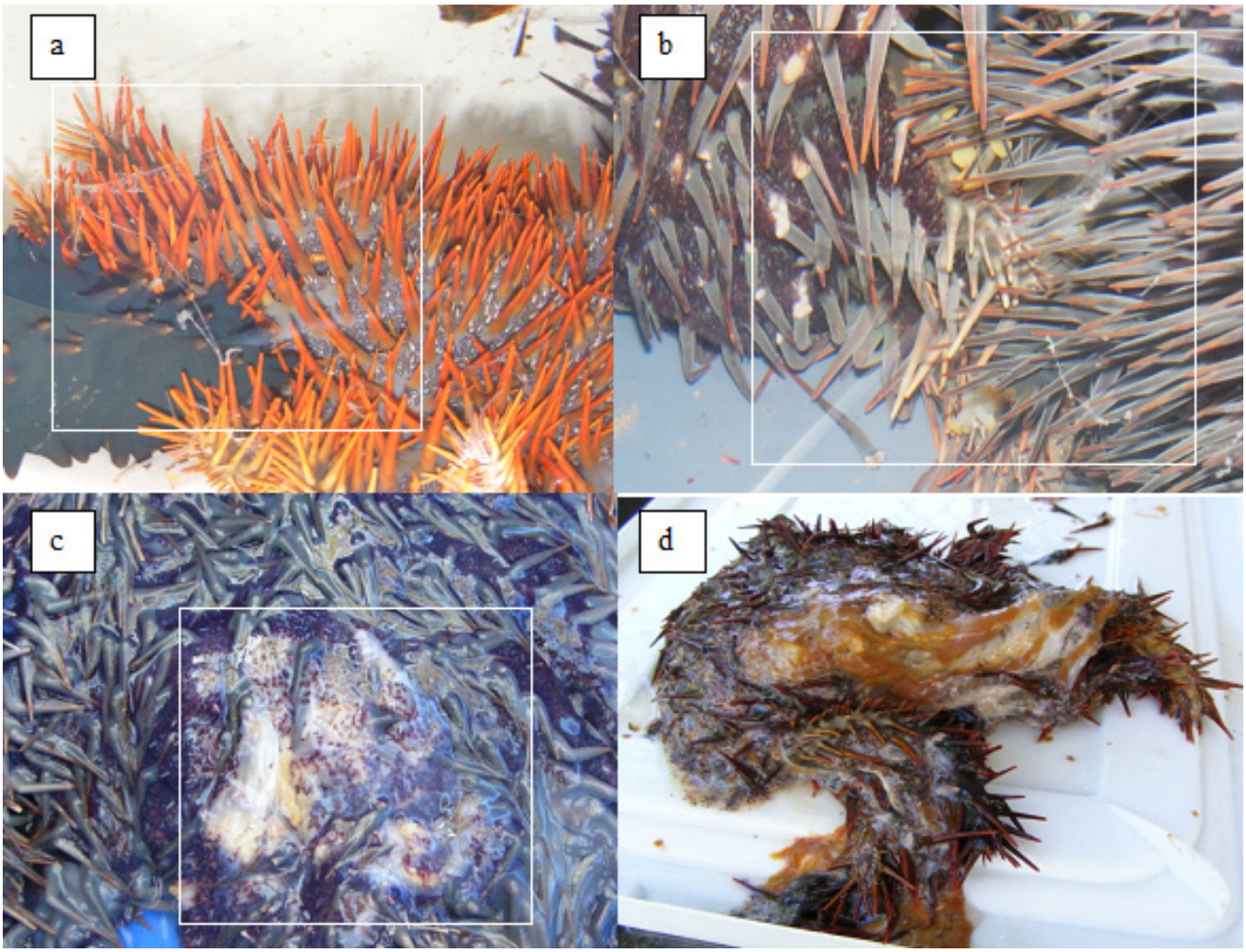

Figure 2.3. Suspected mechanism of disease transmission and advanced clinical signs of disease. (a, b) Colourless mucus at the top of the spines; (c, d) Destruction of collagen tissues (skin, digestive glands); (d) death.

Table 2.2. *Acanthaster planci.* Time to death of crown-of-thorns starfish (COTS) in relation to variations in water temperature (n = 5 COTS per temperature).

Exposed temperature		**Exposed temperature**		**Exposed temperature**	
26°C		28°C		30°C	
Number of COT'S used		**Number of COT'S used**		**Number of COT'S used**	
5		5		5	
Time to death		**Time to death**		**Time to death**	
COT No	Hours	COT No	Hours	COT No	Hours
1	19.25	1	16.1	1	11.5
2	24	2	16.4	2	12.43
3	20	3	19	3	14.5
4	25.5	4	12	4	9
5	17.4	5	11.5	5	10.3
Standard mean±		Standard mean±		Standard mean±	
	21.2		15		11.5

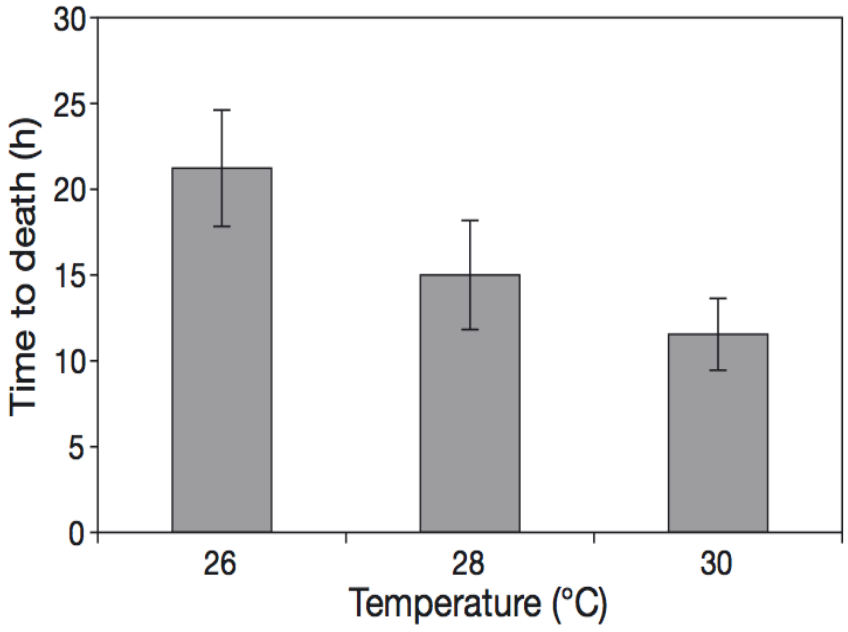

Figure 2.4. *Acanthaster planci*. Time to death of crown-of-thorns starfish (COTS) in relation to variations in water temperature (n = 5 COTS per temperature)

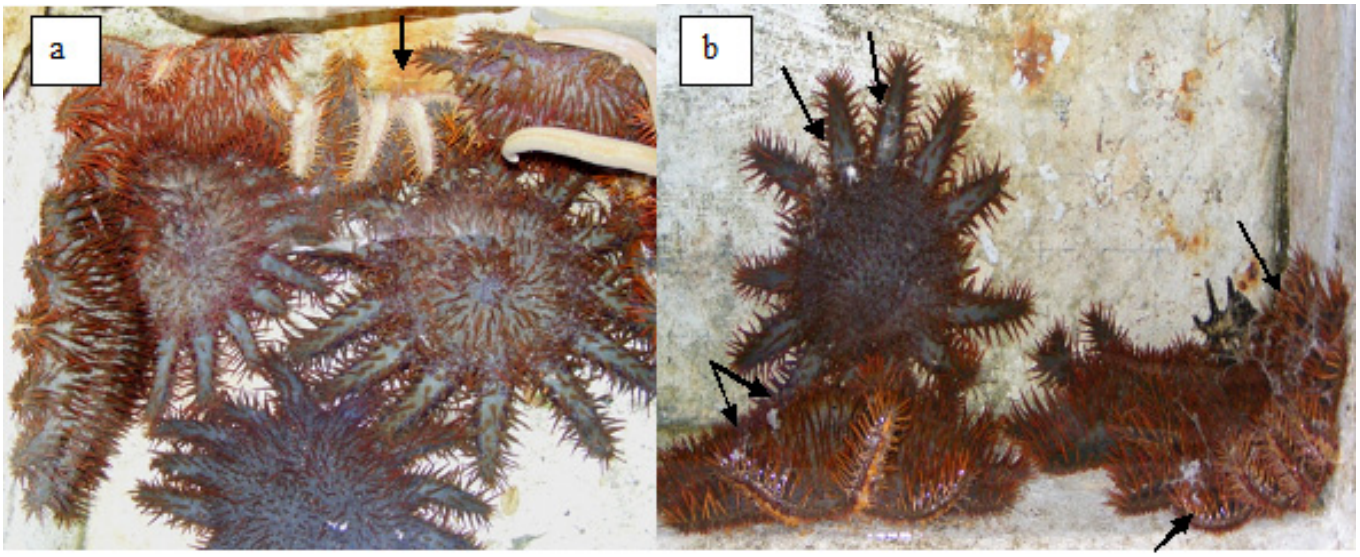

Figure 2.5. Disease transmission between in-contact COTS at high densities. (a) TCBS-injected COTS (black arrow) displaying initial signs of disease; there was no aviodance of sick starfish (b) Infected starfish covered by mucus (black arrows), indicating early signs of successful transmission of disease between COTS.

2.5 Discussion

Method of action

Intestinal flora plays an essential role in health, stimulating the immune system, protecting the host from invading pathogenic bacteria and viruses, supporting digestion and assimilating food (Orrhage & Nord, 2000; Guarner & Malagelada, 2003). Interactions of typical intestinal bacteria may also contribute to stabilization "Eubiosis" or destabilization "Dysbiosis" of the gastrointestinal tract affecting host homeostasis.

Different mechanisms are used by bacteria to maintain eubiosis within the gastrointestinal tract: (1) secretion of antibacterial substances like hydrogen peroxide, organic acids and bacteriocins to reduce the number of viable pathogenic organisms, or decrease bacterial metabolism and toxin production; (2) competition for nutrients necessary for pathogen survival; (3) competition with pathogens to prevent their adhesion to the intestine; (4) enhancement of production of defensive molecules in the host such as mucins; and (5) direct uptake or decomposition of organic matter or toxic material produced by pathogenic bacteria (Brook, 1999; Lievin *et al.*, 2000; Guarner & Malagelada, 2003). For instance Jiravanichpaisal *et al.*, (1997) inhibited growth of *Vibrio* spp., *Escherichia coli* and *Staphylococcus* sp. with *Lactobacillus* sp. in *Penaeus monodon* affected by white spot disease. Conversely blockage and disruption of beneficial bacteria can allow rapid growth of pathogens leading to infection. The greater the imbalance between beneficial and pathogenic bacteria the greater the symptoms. Under certain conditions gut microflora can be modified by the substrates which they receive, predominantly through the diet (Bird *et al.*, 2000).

The exact mechanisms that initiate disease with TCBS injection have not been identified. Non-bacterial agents such as viruses or allergic reactions cannot be totally excluded. However, observations using scanning electron microscopy (SEM) observations did not reveal any viruses (Becker *et al.,* 2004). An allergic reaction to chemicals in the TCBS formulation almost certainly does not initiate a transmissible disease that spreads to healthy in-contact COTS. TCBS, nutrient agar and marine broth formulations share most of their main ingredients. However, nutrient agar and marine broth do not induce death in healthy COTS (control). The main difference between these 3 media is that TCBS also contains ox bile, sodium thiosulfate, sodium citrate and sucrose (Table 2. 3).

Table 2.3. Typical formulas (g l^{-1}) of culture media and their uses

TCBS	**g l^{-1}**	**Nutrient Agar**	**g l^{-1}**	**Marine broth**	**g l^{-1}**	**Function of components**
Yeast Extract	5	Yeast Extract	2	Yeast Extract	1	Nitrogen, vitamins, amino acids.
Peptone	10	Peptone	5	Peptone	5	
Sodium Chloride	10	Sodium Chloride	5	Sodium Chloride	19.45	Stimulates growth
Agar	14	Agar	15			Food additive and solidifying agent
Ferric Citrate	1			Ferric Citrate	0.1	Indicator of hydrogen sulfide
Ox bile	8					Selective agents, inhibit Gram positive organisms and suppress coliforms
Sodium Thiosulfate	10					
Sodium Citrate	10					
Sucrose	20					Carbohydrate
Bromothymol Blue	0.04					pH indicators
Thymol Blue	0.04					
		Lab- Lemco P	1			Protein extract
pH 8.6 ± 0.2		pH 7.4 ± 0.2		pH 7.6 ± 0.2		

Note: Marine broth also contains magnesium chloride, magnesium sulfate, calcium chloride, potassium chloride, sodium bicarbonate, potassium bromide, strontium chloride, boric acid, sodium silicate, sodium fluoride, ammonium nitrate and disodium phosphate.

Ox bile, sodium thiosulfate and sodium citrate inhibit Gram-positive bacteria and suppress coliforms inducing dysbiosis of the gastrointestinal tract. Sucrose supplements the high metabolism of vibrios, promoting rapid growth. We hypothesize that inhibition of competitive bacteria and increase in *Vibrio* cell density activate the quorum sensing (QS) mechanism of the vibrios, turning on virulence factors (Peters *et al*., 2003) inducing rapid onset of symptoms followed by death.

Pathogenic *Vibrio rotiferianus* (isolates GREEN + and GREEN AGRESS) and *V. natriegenes* (isolates CREAM TCBS and CREAM 12-13) were recovered from mucus of infected COTS that spread over healthy in-contact *Acanthaster planci. Vibrio* species integrate environmental signalling to modulate behavior by biasing movements toward more favorable conditions or away from unfavourable environments. Motility and chemotaxis greatly influence the infectivity of vibrios (Butler & Camilli, 2004; Larsen *et al.*, 2004). For example *V. anguillarum* and *V. alginolyticus* undergo positive chemotaxis to mucus collected from fish skin and intestines (Bordas *et al.*, 1998). *V. shiloi*, a coral pathogen, migrates towards coral mucus (Banin *et al.*, 2001); *V. coralliilyticus* exhibits chemotaxis towards the mucus of *Pocillopora damicornis* (Meron, 2009) and *V. cholerae* moves into intestinal mucus (Freter *et al.*, 1981).

Under these conditions and the quorum sensing hypothesis, these migrating vibrios can cause disease in COTS populations by invading in-contact individuals as well as enhancing virulence of normal flora through quorum sensing signalling. *Vibrios* as opportunistic species can evolve and recombine genes under high microbial contact in animal guts or as part of aquatic

biofilms transferring or inducing expression of virulence genes in less or non-pathogenic populations (Thompson *et al*., 2004).

The features of TCBS coupled with an ideal water temperature, adequate salinity and alkaline pH stimulate *Vibrio* growth to high density, which activates their virulence factors via quorum sensing. Once the COTS immune system fails, skin, ulcerations and exposed organs are colonized by opportunistic bacteria.

Echinoderm body walls consist primarily of collagenous dermis and calcite ossicles. These tissues are generally known as mutable collagenous tissue (MCT) because they can change stiffness by over an order of magnitude within several seconds. MCT changes are accomplished by neurally mediated variation in the cation concentration of the proteoglycan matrix. This determines tensile strength, stiffness and viscosity of the body wall representing a key mechanism involved in many echinoderm functions like defense against predators, movement, feeding, immune protection against pathogenic bacteria and others (Oneill, 1989; Wilkie, 2002). Vibrios as pathogenic agents disrupt the hydroelectric transport of ions specially Na, K, Cl, HCO_3 inducing tissue structural changes and severe damage (Thompson *et al.*, 2006; Khemiss, 2009). Disruption of hydroelectric transport of ions affects the normal echinoderm body wall functions as well as the subcuticular bacteria symbionts (SCB) located between their ectoderm and surface cuticle. SCB were found in all 5 echinoderm classes and play important roles in defense against bacterial infestation in echinoderms (Burnett, 1997). Disruption of transport of ions induced by vibrios at body wall level explains the loss of body turgor and epidermal lesions described in COTS (Fig 2.2, 2.3)

Temperature enhancement

Among the numerous environmental factors that influence *Vibrio* spp, temperature has a significant effect on this study. A temperature drop of 4 degrees between experiments reduced the induction of disease and time to death between 25 and 50%, similar to findings of Thompson *et al.,* (2006). Neverheless, warmer temperatures in combination with other physical factors like alkaline pH, plankton blooms, sunlight and UV intensity can also influence bacterial attachment, growth and multiplication in the aquatic environment, increase the rates of induction and propagation of the CTX phage, and promote viability and culturability of virbios. For instance, *V. cholerae* remains stable in full sunlight in contrast to enteric bacteria such as *Escherichia coli*, which gives vibrios a clear advantage at tropical latitudes (Mezrioui *et al.,* 1995).

Isolates identification

For species belonging to the harveyi clade, identification based on 16S rRNA gene analysis is often imprecise (Vanderberghe *et al.,* 2003; Gomez-Gil *et al.,* 2004). Among the Harveyi clade, species of the *Vibrio harveyi* group (*V. harveyi, V. campbellii, V. rotiferianus* and *V. owensii*) have almost indistinguishable phenotypes and genotypes with cases of identical biochemical profiles and ~ 100% gene sequence identities among different species (Gomez-Gil *et al.,* 2004; Cano Gomez *et al.,* 2010).

Sequences analysis of the 16S rRNA gene is still essential for bacterial taxonomy (Cohan, 2001); however, the resolution power of this molecule is inappropriate in discriminating among closely related species (Kolbert & Persing, 1999, Janda & Abbott, 2007), such as those belonging to the Harveyi clade. In the present study, the resolution power of the 16S rRNA gene for discrimination of *Vibrio harveyi, V. rotiferianus* and *V. owensii* was low with low bootstrap supported tree branches and high inter-species sequence similarities (98.9 to 99.6%). Precise

identification was further obscured by the presence of multiple ambiguous nucleotide positions, scored following the degenerate base code. The effect of these positions was evident especially for the closely related clusters of *V. owensii* and *V. rotiferianus*. These ambiguities are related to the presence of 2 chromosomes in some *Vibrio* species (Tagamori *et al.,* 2002), responsible for the existence of multiple divergent copies of the 16S rRNA gene in the same bacterial genome. These arguments support ongoing studies performing biochemical test and analysis of additional genetic markers for a more precise identification of *V. harveyi*-related species associated with COTS.

Control methods

Attemtps to control *Acanthaster planci* outbreaks either by collection of adult individuals followed by burial ashore, by injections of starfish with toxic chemicals affecting other marine organisms or by placing underwater fences are all expensive and time consuming methods only feasible for small areas. Current control methods are ineffective in either eradicating the coral–feeding starfish or preventing further coral mortality. Biological control of populations through manipulation of predators, parasites or pathogens has not been fully investigated (Sutton *et al.*, 1988). Nevertheless, several authors state that predators do not limit the population density of COTS once they reach outbreak levels (McCallum, 1987; Moran, 1988). Moreover there are no reports about significant increases in the densities of the major fish predators of COTS (Williams, 1986).

Despite more than 3 decades of research and the commitment of millions of dollars, control methods have been unsuccessful as well as costly, time consuming and difficult to accomplish. For example, divers need to inject chemicals into several areas of each starfish to avoid the starfish simply rejecting the single, injected arm and regenerating. Furthermore, spines

are sharp and carry hemolytic toxins (saponins). A puncture wound from a spine is intensely painful, and causes oedema, erythema, and infection of the surrounding areas. Limiting the exposure of divers to spines by having a propagating epizootic would be a boom to safety. The susceptibility of *A. planci* to disease could provide an option for controlling population outbreaks. Injection of TCBS culture medium into *A. planci* L induces a rapid fulminating disease that is transmitted to in-contact COTS under favorable conditions. Additionally there is no introduction of new pathogens into the environment and it is a rapid and simple procedure with immense economic advantages. However, growth of vibrios enhanced by the TCBS might perhaps have detrimental effects on other reef organisms during a control program. Therefore, further disease dynamics and transmission experiments are warranted, especially in corals, carnivorous fishes, and benthic detritus feeders. This is the first part of a series of studies that evaluate wheter TCBS injection could be a new tool for management of COTS outbreaks.

2.6 Acknowledgements

Funding for this project was provided by the ARC Centre of Excellence for Coral Reef Studies, the Australian Institute of Marine Science (AIMS), and Project A.W.A.R.E. Field sampling was conducted with kind support and logistic assistance from Lizard Island Research Station and The Marine Laboratory University of Guam.

CHAPTER 3: Refined identification of *Vibrio* bacterial flora from *Acanthaster planci* based on biochemical profiling and analysis of housekeeping genes

3.1 Abstract:

A polyphasic approach was used for precise identification of bacterial flora (*Vibrionaceae*) isolated from crown-of-thorns starfish (COTS) from Lizard Island (Great Barrier Reef, Australia) and Guam (USA, Western Pacific Ocean). Previous 16S rRNA gene phylogenetic analysis was useful to allocate and identify isolates within the Photobacterium, Splendidus and Harveyi clades but failed in the identification of *V. harveyi*-like isolates. Species of the *V. harveyi* group have almost indistinguishable phenotypes and genotypes and thus, identification by standard biochemical tests and 16S rRNA gene analysis is commonly inaccurate. Biochemical profiling and sequence analysis of additional *topA* and *mreB* housekeeping genes were carried out for definitive identification of 19 bacterial isolates recovered from sick and wild COTS. For 8 isolates, biochemical profiles and *topA* and *mreB* gene sequence alignments with the closest relatives (GenBank) confirmed previous 16S rRNA-based identification: *V. fortis* and *Photobacterium eurosenbergii* species (from wild COTS), and *V. natriegens* (from diseased COTS). Further phylogenetic analysis based on *topA* and *mreB* concatenated sequences served to identify the remaining 11 *V. harveyi*-like isolates: *V. owensii* and *V. rotiferianus* (from wild COTS), and *V. owensii*, *V. rotiferianus* and *V. harveyi* (from diseased COTS). This study further confirms the reliability of *topA-mreB* gene sequence analysis for identification of these close species, and it reveals a wider distribution range of the potentially pathogenic *V. harveyi* group.

†This chapter was published (as written) in *Diseases of Aquatic Organisms*: Rivera-Posada J, Pratchett M, Owens L (2011) Refined identification of Vibrio bacterial flora from *Acanthasther planci* based on biochemical profiling and analysis of hosekeeping genes. *Diseases of Aquatic Organisms* 96:113-123

3.2 Introduction

*Vibrio*s constitute an important part of the bacterial microflora of numerous marine animals (Harris, 1993; Oxley *et al*., 2002). Some species are recognized as important pathogens of marine and estuarine animals, causing substantial losses in commercial production systems and natural waters throughout the world (Kaysner *et al*., 2004). Particularly within the Echinodermata, several species not only integrate *Vibrio* spp. as part of their natural microflora but also display susceptibility to this bacterium (Gilles & Pearse, 1986; Morgan, 2000; Becker *et al*., 2007). Moreover, remarkable similarities exist in the clinical signs, time to death, histology, scanning electron microscopy analysis and identification of vibrios as etiological agents (Morgan, 2000; Becker *et al*., 2004) with those described in experimentally-induced diseased in crown-of-thorns starfish (COTS; Rivera-Posada *et al*., 2011).

Resident bacteria constitute a critical barrier of resistance against colonization by exogenous microbes, preventing tissue invasion by pathogens (Guarner & Malagelada, 2003). This function is accomplished through different mechanisms, including (1) secretion of antibacterial substances to reduce the number of viable pathogens, decrease bacterial metabolism or inhibit toxin production; (2) competition for essential nutrients for pathogen survival; (3) supplementation with vitamins and short-chain fatty acids to the host tissues; (4) competition with pathogens preventing their adhesion to the intestine; (5) enhancement of the production of defensive molecules in the host; and (6) direct uptake or decomposition of organic matter or toxic material produced by pathogenic bacteria (Lievin *et al*., 2000; Guarner & Malagelada, 2003; Macfarlane & Macfarlane, 2003). Disrupting the bacterial ecological balance in healthy organisms allows overgrowth of pathogenic bacteria, and establishes a suitable environment for disease induction. Maladies and physiological manifestations such as intestinal inflammation,

digestive ulcers, and multiple organ failure have been associated with microflora imbalances (Lemaire, 1997; Tsuji *et al*., 1997; Zhang & Cheng, 2010).

Bacterial communities present in echinoderms and other closely related marine invertebrates must be compared to understand the role of bacterial balances in the health of these organisms. Bensoussan *et al.*, (1984) analyzed bacterial isolates of echinoderm digestive tracts in the asteroid *Solaster* sp. and the holothurians *Pseudostichopus villosus* in stable physico-chemical conditions (constant environment), and reported that the most common bacteria isolated from these echinoderms were *Vibrio*-like Gram-negative rods which constituted half of the total bacterial load; and that a clear separation exist between enteric bacterial communities and sediment microflora. Moreover, the authors suggested that microflora of echinoderms, and in particular of the Asteroidea have a more specialized tendency to the catabolism of organic compounds; and they found a less diversified microflora in echinoderms in comparison with sediment microflora.

Species of the Harveyi clade have been described as major pathogens to aquatic animals, causing disease outbreaks responsible for severe economic losses in the aquaculture industry worldwide. Increasing infections in marine vertebrates and invertebrates and even in humans have been linked to increased marine temperatures with diffusion of pathogens to higher latitudes (Bossart 2007, Igbinosa & Okoh 2008). According to current molecular taxonomy studies, the Harveyi clade (Sawabe *et al*., 2007) includes 10 *Vibrio* species: *Vibrio harveyi, V. campbellii, V. rotiferianus, V. alginolyticus, V. parahaemolyticus, V. mytili, V. natriegens*, the new described species *V. azureus* (Yoshizawa *et al*., 2009), *V. owensii* (Cano-Gomez *et al.,* 2010) and *V. sagamiensis* (Yoshizawa *et al.,* 2010). Among this clade, the *V. harveyi*-related species belonging to the so-called *V. harveyi* group (*V. harveyi, V. campbellii, V. rotiferianus* and

V. owensii) are phenotypically and genetically indistiguisable, sharing almost identical biochemical profiles and 16S rRNA gene sequences (Gomez-Gil *et al*., 2003, 2004; Owens & Busico-Salcedo, 2006). Therefore, conventional culture-based techniques, biochemical tests and 16S rRNA gene analysis frequently lead to misidentification of *V. harveyi*-like isolates (Pedersen *et al.,* 1998, Vanderberghe *et al.,* 2003). Previous analyses of 16S rRNA gene sequences allocated some *Vibrio* strains isolated from wild and sick COTS in Lizard Island (Great Barrier Reef [GBR], Australia) and Guam (USA) within the Harveyi clade, and some of these clustered within the *V. harveyi* group (Rivera-Posada *et al.,* 2011). However, due to the low resolution of 16S rRNA, we were prompted to perform additional biochemical test and sequence analysis of 2 additional housekeeping genes in order to precisely identify these isolates at the species level.

Multilocus Sequence Analysis (MLSA) is a recent approach that employs sequence analysis of several housekeeping genes in bacteria and subsequent phylogenetic analysis of their concatenated sequences to delineate species and infer genetic relationships (Gevers *et al*., 2005). For the Harveyi clade, this method has been described as an alternative to the expensive and labor-intensive DNA-DNA hybridization (Gomez Gil *et al.,* 2003, Cano-Gomez *et al.,* 2009), still considered the gold standard for bacterial spceies delineation (Stackebrandt *et al.,* 2002). However MLSA is costly, time consuming, and requires a considerable amount of experience to analyze concatenate, and construct phylogenies with DNA sequences of multiple loci. Recent efforst have focused on the design of a fast, practical, but still accurate identification method for *Vibrio harveyi*-related species and an alternative to 16S rRNA gene analysis and DNA-DNA hybridization. Short-term epidemiology studies or environmental surveys, for example, involve a high number of isolates to be identified rapidly and efficiently. In these cases, and under the evidences pointing to the Harveyi clade, the number of genes sequenced could be minimized for

a more practical identification (Thompson *et al.,* 2007, Cano-Gomez *et al.,* 2011). While more than 5 genes should be analyzed for taxonomic studies of cryptic bacterial species, the use of at least 2 independent housekeeping genes has been suggested for bacterial identification purposes (Martens *et al.,* 2008).

A recent MLSA study focused on the identification of *Vibrio harveyi*-related spceies by analysis of protein-coding genes (*rpoA, pyrH, topA, ftsZ, mreB*; Cano-Gomez *et al.,* 2011). Concatenation of only *topA* and *mreB* gene sequences offered similar resolution to that of full MLSA (5 genes) for identification of *V. harveyi*-related species. These authors suggested that initial allocation of *V. harveyi*-like isolates into the *V. harveyi* group (by biochemical or 16S rRNA gene analysis) and additional *topA*-*mreB* gene analysis offers a reliable identification of these close species, with resolution power comparable that that of a full MLSA analysis.

The purpose of this study was to analyse data from phenotypic characterization, concatenated sequences of *topA* and *mreB* genes, and previously obtained 16S rRNA gene-based phylogenies to: (1) identify bacterial strains isolated from COTS in the Pacific and discriminate between those naturally present from those inducing disease and death; (2) validate an identification approach for *V. harveyi*-related strains based on sequence analysis of *topA* and *mreB* protein-coding genes; and (3) provide baseline data for better understanding of the gut microbe role in the physiological processes of COTS.

3.3 Materials and methods

Samples

Samples consisted of DNA from 19 strains recovered from individual adult sick or wild COTS across two sample sites in the Pacific Ocean in 2009 (Rivera-Posada *et al.,* 2011). Eleven

of these strains were obtained at Lizard Island (GBR): 5 isolated from wild COTS and 6 sick COTS induced from thiosulfate-citrate-bile-sucrose agar (TCBS) injection (see Rivera-Posada *et al.*, 2011). The other 8 strains were obtained from COTS at Guam (USA, Mariana archipelago): 3 from wild COTS and 5 from diseased COTS after TCBS injection.

Biochemical tests

Phenotypic analysis for all 19 bacterial isolates was performed in triplicate by employing API 20NE commercial kits (bioMérieux) according to the manufacturer's instructions with the following modifications (1) 2% NaCl (w/v) solution was used to prepare the inocula, (2) the strips were incubated at 30°C for 48 h and (3) several colonies of pure cultures of a single organism were used when bacterial colonies were tiny to ensure that all tubes and cupules had enough bacteria for growth.

PCR amplification and sequencing

PCR amplification and sequencing was carried out with DNA from all 19 COTS bacterial isolates. Partial regions of the housekeeping genes *topA* (topoisomerase I) and *mreB* (rod shaping protein MreB) were amplified and sequenced as described by Sawabe *et al*., (2007) (Table 3.1). All PCR amplifications were performed in a Perkin Elmer Applied Biosystems GENEAMP PCR System 9700 (Perkin Elmer, USA) thermocycler. PCR reactions (20 µl) contained approximately 20 ng of genomic DNA, 1x PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$; pH 8.7; Qiagen), 0.5 µM of each primer, 200 µM dNTPs, and 0.5 units of *Taq* DNA Polymerase (Qiagen). Finally, PCR products were visually inspected in 1% agarose gels and subsequently sequenced by Macrogen Ltd, Korea, with appropriate primers.

Table 3.1. Amplification and sequencing primers.

Gene (gene product), length	Primer name	Primer sequence (5'-3')	Annealing temp (ºC)	Source
16S rRNA (16S ribosomal RNA) 1421nt	27F	AGAGTTTGATCCTGGCTCAG	54	Lane, 1991
	1492R	GGTTACCTTGTTACGACTT		
topA (topoisomerase I), 800 nt	VtopA400F	GAGATCATCGGTGGTGATG	50	Sawabe *et al.,* 2007
	VtopA1200 R	GAAGGACGAATCGCTTCGTG		
mreB (rod shaping protein MreB), 1000nt	VmreB12F	ACTTCGTGGCATGTTTTC	50	
	VmreB999R	CCGTGCATATCGATCATTTC		

Sequence and phylogenetic analysis

Electropherograms of *topA* and *mreB* sequences were assembled in Sequencher 4.9 (Gene Codes). Sequences were manually corrected and trimmed, and BLASTN searches were performed against public databases for preliminary identification and comparison with 16S rRNA-based identification. Subsequently, phylogenetic analysis was performed with those 11 isolates belonging to the *Vibrio harveyi* group as indicated by initial biochemical characterization and 16S rRNA gene sequence analysis. Protein-coding *mreB* and *topA* gene sequences were aligned employing Clustal_X (Thompson *et al*., 1997). These alignments also included publicly available sequences of 11 *Vibrio* species type strains and *Photobacterium phosporeum* LMG 423 ^{T} as outgroup. Finally, individual *topA* and *mreB* sequences from all 22 strains were concatenated and used to construct a multilocus phylogenetic tree for more accurate and supported identification. Phylogenies were obtained by the neighbor-joining (NJ) method (Saitou & Nei, 1987) in PAUP v.4.0B10 for Windows (Swofford, 2003). Bootstrap (BT) support values were calculated on 1000 replicates. For the NJ analysis, uncorrected 'p' distances and the Kimura 2-parameter correction (Kimura, 1980) were tested.

Accession numbers

The gene sequences were deposited in GenBank under accession numers HQ540692-HQ540710 and HQ540711-HQ540729 for the *topA* and *mreB* genes, respectively. (Table 3.2)

Table 3.2.Accession numbers deposited in GenBank for the *topA*, and *mreB* genes.

ISOLATE	*topA*	*mreB*
G2	HQ540692	HQ540729
Y2	HQ540693	HQ540728
HAEM	HQ540694	HQ540715
NA	HQ540695	HQ540711
CREAM TCBS	HQ540696	HQ540714
GREEN AGRESS	HQ540697	HQ540712
GREEN+	HQ540698	HQ540716
CREAM 12-13	HQ540699	HQ540717
GU2Y	HQ540700	HQ540723
GU5YEN	HQ540701	HQ540724
GU5DARKEN	HQ540702	HQ540725
GU4YEN	HQ540703	HQ540721
GUIGEN	HQ540704	HQ540722
GU5PALEEN	HQ540705	HQ540720
GUMAB	HQ540706	HQ540719
GU1.STAR	HQ540707	HQ540718
X1	HQ540708	HQ540726
X2	HQ540709	HQ540727
YG	HQ540710	HQ540713

3.4 Results

All PCR primer sets specifically amplified the target sequences of all 19 isolates. Sequence lengths of *topA* and *mreB* sequenced regions were 626 nt and 834 nt, respectively. After BLASTn alignments with closest relatives in GenBank, isolates were initially identified as *V. fortis, V. natriegens, V. harveyi*, *V. owensii*, *V. rotiferianus* and *Photobacterium eurosenbergii* with sequences identities of 99 to100% for both loci. However, for the 11 isolates belonging to the *V. harveyi* group (*V. harveyi*, *V. owensii* and *V. rotiferianus*), BLASTn-based identification was not achieved since high sequence similarities (99 to 100%) with 2 or more *V. harveyi*-related

spceies were obtained for each isolate. Phylogenetic reconstructions based on these 2 genes were consistent with previously obatained topologies based on the 16S rRNA gene (Rivera-Posada *et al*., 2011), but this time clades were supported by higher BT values (Fig 3.1). Isolates were allocated into 3 well supported clusters (100% BT support values), each containing the type strain of the identified species (*V. harveyi*, *V. owensii,* or *V. rotiferianus*). One *V. harveyi* strain was identified from sick COTS at Guam (GU5DARKEN), while *V. owensii* strains was found at both locations: 3 strains from wild (X1, X2 and Y2) and 1 from infected COTS (G2) in Lizard Island (G2), and 1 strain from wild COTS at Guam (GU1*). Finally, 4 strains of *V. rotiferianus* were found in both wild (NA and YG) and sick COTS (GREEN AGRESS and GREEN+) at Lizard Island and 1 strain from diseased specimens at Guam (GUIGEN). Distance matrix analysis of concatenated *topA-mreB* gene sequences revealed intra-species similarities of 98.6 to 99.5% for *V. harveyi*, *V. rotiferianus* and *V. owensii* clusters and inter-species similarities of only 90.7 to 92.3% (Table 3.3). These 3 species showed only 81.2 to 86.6% sequence similarity with the less related spceies identified, *V. natriegens* and *V. fortis.*

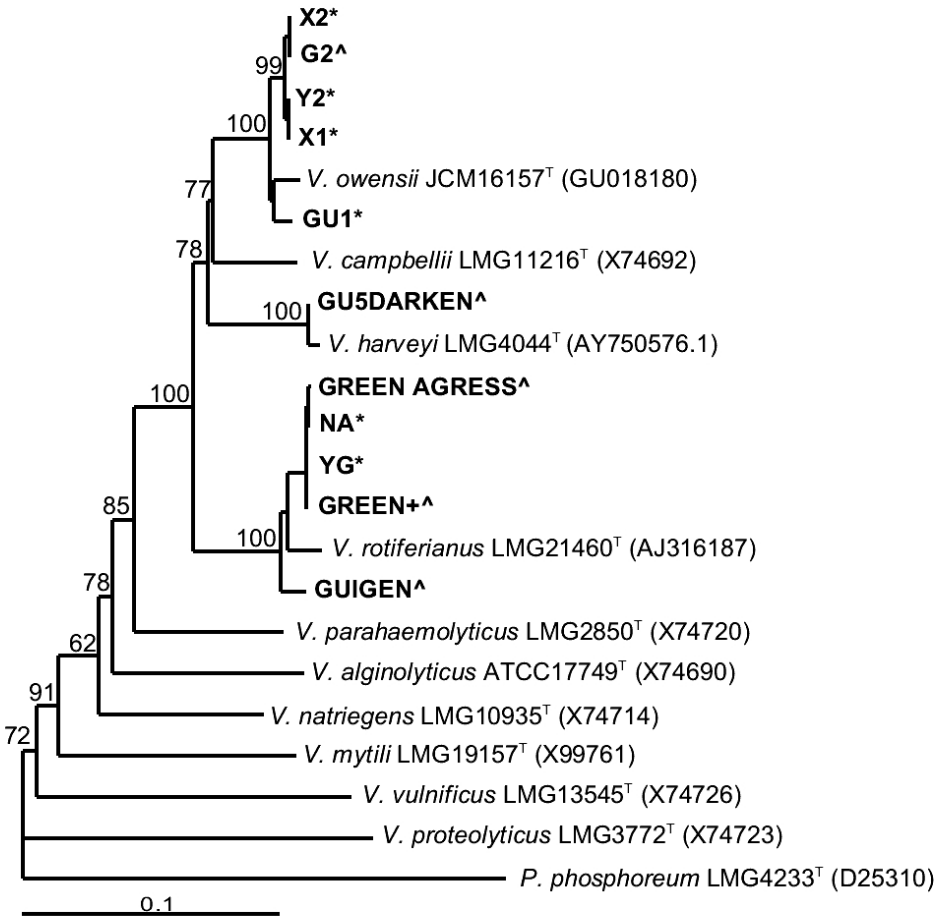

Figure 3.1. Phylogenetic analysis of isolates within the Harveyi clade based on *topA* and *mreB* concatenated gene sequences. Trees constructed using the neighbour-joining algorithm. *Photobacterium phosphoreum* LMG 4233T used as an outgroup. Bootstrap support values after 1000 simulations shown. Scale bar = 1% (0.01) or 10% (0.1) sequence divergence. X1, X2, Y2, YG, NA, GREEN+, GREEN AGRESS and G2 were found at Lizard Island - Great Barrier Reef. GUIGEN, GU5DARKEN, GU1*, were found at Guam-Mariana Archipelago. (*): strains isolated from healthy COTS. (^): strains isolated from TCBS-injected moribund COTS.

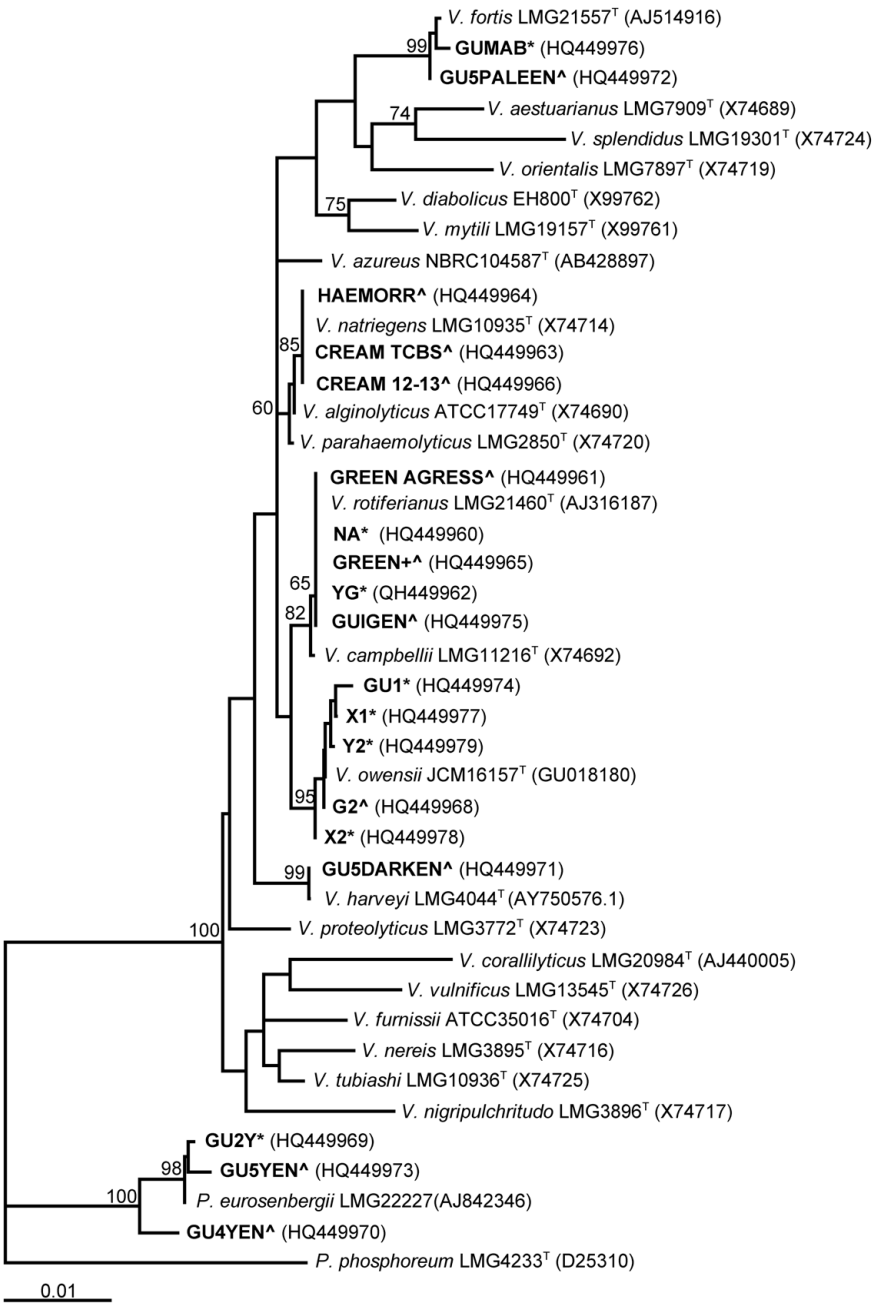

Figure 3.2. Phylogenetic analysis base on partial 16S rRNA gene sequences (1,302 nt), using the neighbour-joining algorithm and the Kimura-two-parameter correction, showing sequence clustering of crown-of-thorns (COTS) *Vibrio* isolates and related species. GenBank accession numbers are provided in parentheses. Photobacterium phosphoreum LMG 4233T was used as an outgroup. Bootstrap support values after 1000 simulations are shown. Bar, 1% sequence divergence.
"GU" strains isolated from Guam (USA)
(*): strains isolated from healthy COTS.
(^): strains isolated from TCBS-injected moribund COTS.

	16S rRNA	*topA*	*mreB*	2-locus MLS
Interspecies				
V. natriegens/V. fortis	98	73.6	86.5	81.1
V. natriegens/V. harveyi	98.8	80	90.8	86.4
V. natriegens/V. owensii	99.4	79.2	92.1	86.6
V. natriegens/V. rotiferianus	99.4	79	90	85.5
V. fortis/V. harveyi	97.3	75.1	87.1	82.2
V. fortis/V. owensii	98.3	74.3	86.6	81.4
V. fortis/V. rotiferianus	98.2	75.3	85.3	81.2
V. harveyi/V. owensii	99	90.1	93.9	92.3
V. harveyi/V. rotiferianus	99.1	88.6	93.2	90.7
V. owensii/V. rotiferianus	99.4	92.3	91.8	92.1
Intraspecies				
V. harveyi	99.9	99.3	99.6	99.5
V. owensii	99.9	99.3	98.1	98.6
V. rotiferianus	100	99.2	98.1	98.6

Table 3.3 *Vibrio* spp. Sequence analysis and statistics of single-gene and 2-locus (*topA*−*mreB*) sequence alignments. Values shown are % similarities. Intra- and interspecies similarities were deduced from the number of nt substitutions per site between populations: Dxy (K2P). Gene sequence lengths (nt) are as follows: 16S rRNA, 1298; *topA*, 626; *mreB*, 834; 2-locus multilocussequence (MLSA; i.e. *topA* and *mreB* concatenated sequences), 1460.

Results from the biochemical characterization are presented in Table 3.4. From the 21 tests contained in each API 20NE strip, *Vibrio* isolates were positive for potassium nitrate, L-tryptophane, D-glucose, esculine, gelatin, B-galactosidase, malate, glucose and oxidase and negative to L-arginine, urea, arabinose, adipate, caprate. N-acetylglucosamine was 50% positive and the other 50% showed weak results. Potassium gluconate test was negative only for a *P. eurosenbergii* strain. The remaining tests showed weak or variable results from *V. harveyi*-related species, and only citrate utilization was found as a discriminatory character between isolates identified as *V. harveyi*, and *V. owensii*.

3.5 Discussion

In this study, biochemical characterization, analysis of *topA* and *mreB* protein-coding sequences, and comparison with previous 16S rRNA gene-based phylogenies were used for identification of 19 *Vibrio* isolated from *A. planci* in the Pacific. Isolates were identified as *P. eurosenbergii*, *V. natriegens* and *V. fortis*, showing 100% *topA* and *mreB* sequence similarities by BLASTn alignments with type strains of these species. This identification was consistent with previous 16S rRNA based-phylogenies showing high bootstrap support values (85 to 100%) for these clusters. For the other 11 isolates belonging to the *V. harveyi* group, 16S rRNA gene (Rivera-Posada *et al.,* 2011a) and biochemical analysis profiles reported here failed in the identification at the species level. In contrast, the phylogenetic analysis of additional genetic markers (*topA* and *mreB*) offered a more definitive and discriminative identification of these closely related isolates. The 16S rRNA gene is still necessary for species delineation but its low resolution power for certain bacterial taxa, such as the *V. harveyi* group (Gomez Gil *et al.,* 2004; Thompson *et al.,* 2005; Cano-Gomez *et al.,* 2010), and the presence of multiple divergent gene copies in the same genome (Tagomori *et al.,* 2002) are clear disadvantages for a gene to be used as a sole identification marker. In a previous study to identify COTS isolates, the 16S rRNA gene was appropriate for allocation of several strains within the family *Vibrionaceae* and discrimination of distant species like *Bacillus* spp. and *Pseudoalteromonas* (Rivera-Posada *et al.,* 2011). However, low genetic distances and BT support values observed in 16S rRNA-based phylogenies (especially for *V. harveyi*-related isolates) prohibited precise identification at the species level. Compared to 16S rRNA gene analysis and expensive DNA-DNA hybridization, the MLSA is advantageous in terms of resolution power and reproducibility, respectively. Some loci have been recently described as highly discriminative among species of the Harveyi clade. Pascual *et*

al., (2010) developed an MLSA based on 7 housekeeping genes and found *rpoD*, *rctB*, and *toxR* protein-coding loci as the most discriminative and informative for differentiation of 6 species of the Harveyi clade. More specifically, in an MLSA with 7 different housekeeping genes, Thompson *et al.*, (2007) found *topA*, *mreB*, *ftsZ*, and *pyrH* to be the suitable protein-coding genes for differentiation of sister species *V. harveyi* and *V. campbellii*. Following these studies, a similar MLSA (*rpoA*, *pyrH*, *topA*, *ftsZ* and *mreB*) aimed at the description of the new species *V. owensii* within the *V. harveyi* species group (now consisting in *V. harveyi*, *V. campbellii*, *V. rotiferianus* and *V. owensii*). In addition, these authors reduced the number of genes in the analysis for more reliable and yet accurate identification of the 4 species. Results showed that concatenated sequence analysis of only *topA* and *mreB* genes produced consistent clustering of strains compared to full MLSA and 16S rRNA-based analysis. This combination of genes showed high phylogenetic signal, low inter-species sequence similarities, and thus high resolution for precise identification of the cryptic *V. harveyi* group.

The results in our study showed lower inter-species *topA-mreB* sequence similarities between strains identified as *V. harveyi*, *V. rotiferianus* and *V. owensii* (90.7 to 92.3 %) compared to those observed in previous 16S rRNA gene analysis (99 to 99.4 %). These values were higher when *V. harveyi*-related spp. were compared with the more distant *Vibrio* spp. identified as *V. natriegens* and *V. fortis* (81.2 to 86.6%) *topA-mreB* gene sequence similarity. These results and the congruence in phylogenies based on 16S rRNA and protein-coding genes support the usefulness of *topA* and *mreB* genes for *V. harveyi*-related species identification (Cano-Gomez *et al.,* 2011). Our study contributes to the databases with additional sequences from strains of different sources and geographic locations, and has revealed a wider distribution

range of the potentially pathogenic species *V. owensii*, recently described in diseased larvae of the Australian ornate spiny lobster *Panulirus ornatus*.

The species *V. harveyi* and *V. campbelli* are almost indistinguishable phenotypically, while *V. owensii* has been reported to differ from other species of the Harveyi clade in its ability to use citrate, and to produce acid from amygdalin, arabinose, and sucrose (API 20E) (Cano-Gomez *et al.,* 2010). Of these tests, the API 20NE used in this study only included citrate utilization and, being negative for all *V. owensii* strains, resulted as the only discriminatory character found between *V. owensii* and *V. harveyi* isolates (Table 3.3). The species *V. harveyi* and *V. rotiferianus* were characterized as positive for citrate use although 2 out of our 5 *V. rotiferianus* isolates had negative results. Aside from their high interspecies similarities, intraspecies variability of biochemical profiles within species of the Harveyi clade has previously been considered as a disadvantage of phenotypic methods for precise identification of *V. harveyi*-related strains (Gomez-Gil *et al*., 2004).

Vibrios constitute a natural and important part of the microflora in a wide array of echinoderms and numerous marine animals (Bensoussan *et al*., 1984; Harris, 1993; Oxley *et al*., 2002; Kaysner *et al.,* 2004). Not surprisingly, species belonging to the phylum Echinodermata also shows susceptibility to this bacterium (Table 3.5).

Table 3.4. Biochemical profiles of *Vibrio* isolates using API 20NE strips (bioMérieux ®).

Positive; negative; ± variable between strains; w weak reaction.

TEST	**Reaction**	***V. owensii*** *(X1,X2,Y2, G2,GU1)*	***V. harveyi*** *(GU5DARKEN)*	***V. rotiferianus*** *(GREEN AGRESS,NA,YG,G REEN+,GUIGEN)*	***V. natriegens*** *(CREAM TCBS, CREAM 12-13, HAEMORR)*	***V. fortis*** *(GUMAB, GU5PALEEN)*	***P. eurosenbergii*** *(GU2Y,GU5YEN,GU4 YEN)*
NO3	Potassium nitrate	Pos	Pos	Pos	Pos	Pos	Pos
TRP	L-tryptophane	Pos	Pos	Pos	Pos	Pos	Pos
GLU	D-glucose	Pos	Pos	Pos	Pos	Pos	Pos
ADH	L-arginine	Neg	Neg	Neg	Neg	Neg	Neg
URE	Uree	Neg	Neg	Neg	Neg	Neg	Neg
ESC	Esculine	Pos	Pos	Pos	Pos	Pos	Pos
GEL	Gelatin	±	Pos	Pos	Pos	Pos	Pos
PNPG	B galactosidase	Pos	Pos	Pos	±	Pos	Pos
GLU	Glucose	Pos	Pos	Pos	Pos	Pos	Pos
ARA	Arabinose	Neg	Neg	Neg	Neg	Neg	Neg
MNE	Manose	Pos	Pos	Pos	Neg	W	±
MAN	Manitol	w	Pos	±	Pos	W	Neg
NAG	n-acetyl-glucosamine	w	Pos	Pos	Pos	W	w
MAL	Maltose	w	±	Pos	Pos	W	Neg
GNT	Potassium gluconate	Pos	Pos	Pos	Pos	Pos	Neg
CAP	Caprique	Neg	Neg	Neg	±	Neg	Neg
ADI	Adipique	Neg	Neg	Neg	Neg	Neg	Neg
MLT	Malate	Pos	Pos	Pos	Pos	Pos	±
CIT	Citrate	Neg	Pos	±	Pos	Neg	Neg
PAC	Phenylacetique	Neg	±	±	Neg	Neg	w
OX	Oxidase	Pos	Pos	Pos	Pos	Pos	Pos

Table 3.5. List of Vibrios isolated from diseased echinoderms

Echinoderm	Area	Bacteria identified	Disease induced and site of isolation	References
Acanthasther planci	Australia, Guam	*V. owensii, V. rotiferianus, V. harveyi, V. natriegens V. fortis, Photobacterium eurosenbergii , V. tubiashi, V. campbellii*	Sick *Acanthasther planci*	Rivera *et al.,* 2011; Sutton *et al.,* 1988
Amphipholis gracillima	Georgetown USA	*V. parahaemolyticus*	Dermal tissue	Strahl *et al.,* 2002
Amphipholis squamata		*Vibrio* sp	Dermal tissue	Walker & Lesser 1989
Apostichopus japonicus	China	*V. harveyi, V. cyclitrophicus, V. splendidus, V. tasmaniensis, V. tapetis, V. lentus, V. pomeroyi, V. gigantis* and others *Vibrio* sp	Skin ulceration disease, Peristome edema disease, and infected dermal tissues	Ma *et al.,* 2006 a, b; Haiyan, 2009; Huang, 2009
Archaeopneustes hystrix and *Paleopneusteps cristatus*	Bahamas	*V. alginolyticus*	Epidermal vibriosis, isolated from gut gonads and infected lesions	Bauer, 2000
Astropecten jonstoni	Sardinia, Italia	*V. vulnificus, V. lentus, Vibrio* sp	Swabs of alive and dead animals	Staheli *et al.,* 2008
Holothuria scabra	Bribie Island-Australia, Toliara, Madagascar	*V. harveyi, V. alginolyticus, V. natriegens, other Vibrio sp*	Skin ulceration disease, infected tissues	Morgan, 2000; Becker *et al.,* 2004
Ophiactis balli	Unspecified	*V. harveyi*	Subcuticular bacteria of arms	Burnett, 1997
Paracentrotus lividus	France, Mediterranean and Atlantic Ocean, Brittany	*Vibrio* sp	Bald sea urchin disease, isolated from infected dermal tissues	Becker *et al.,* 2008; Maes & Jangoux, 1985
Strongylocentro-tus intermedius	Japan	*Vibrio* sp	Spotting disease	Tajima *et al.,*1997 a, b,; Takeuchi *et al.,* 1999
Strongylocentro-tus purpuratus	California (USA)	*V. anguillarum*	Infected epithelium	Gilles & Pearse, 1986
Strongylocentro-tus nudus and S. intermedius	Japan	*Vibrio* sp	Guts	Sawabe, 1995
Tripneustes gratilla	Toliara, Madagascar	*V. harveyi, V. parahaemolyticus, V. nigripulchritudo*	Body wall lesions	Becker *et al.,* 2007

The Harveyi clade can be considered as the most pathogenic *Vibrio* group for echinoderms followed by the Splendidus clade. Nearly all members belonging to the Harveyi group have been previously reported as etiological agents of echinoderm diseases except *V. mitili* (Table 3.5). Additionally, in this study, *V. owensii, V. rotiferianus, V. harveyi, V. natriegens* were also isolated from infected tissues of COTS, confirming the pathogenicity of the Harveyi clade. On the other hand, the Splendidus clade showed that 4 of its 8 members (*V. splendidus, V. lentus, V. pomeroyi, V. tasmaniensis*) were also identified as causal agents of echinoderm diseases (Table 3.5). Furthermore, our study includes a first-time report of *V. fortis* in COTS tissues.

COTS bacterial flora and physicochemical conditions like temperature and pH of the areas studied were comparable during the months of February at Lizard Island and November at Guam. In addition, Guam lies relatively close to the Indo-Pacific center of coral reef biodiversity (Veron, 2000). Over 150 species of scleractinian coral were documented at Haputo Environmental Reserve Area (Guam collection area), where coral cover was dominated by *Montipora* and *Porites* (Amesbury *et al*., 2001). However, the Guam COTS were collected during the late stages of a devastating outbreak after COTS shifted their diet towards non-preferred species like *Porites* and *Faviids* in the absence of *Acropora* and *Montipora*. These changes in food do not seem to have a major influence on bacterial microflora of COTS when compared with those from Lizard Island found feeding on their preferred corals, *Acropora* (Pratchett 2001, 2010). *V owensii, V. rotiferianus* were found at both locations. *V. fortis*, *P. eurosenbergii* and *V. harveyi* were found at Guam only. However, Sutton *et al*., (1988) previously isolated *V. harveyi, V. campbellii* and *V. tubiashi,* from *Acanthaster planci* and acknowledged these organisms as potential pathogens. The results of our study support those

reported by Sutton *et al.,* (1988) confirming that members of the Harveyi group are normal COTS inhabitants that play important roles in the induction of diseases in COTS and many other echinoderms (Table 3.5).

Furthermore our study suggests that host and digestive morphology may exert an influence over which bacterial species colonize the gut. These characteristics have been reported in other marine invertebrates and omnivorous animals (Oxley *et al*., 2002; Yasuda & Kitao, 1980; Harris, 1993; Egert *et al*., 2005; Brune & Friedrich, 2000).

Table 3.6. Similarities/differences in the microflora of the different COTS

Isolate	***Vibrio* identified**	**Healthy COTS**	**Sick COTS**	**Swab site**	**COTS Location**
X1, X2,Y2	*V. owensii*	X		Digestive tract	Lizard Island
G2	*V. owensii*		X	Open sores	Lizard Island
GU1	*V. owensii*	X		Dermal tissue	Guam
GU5DARKEN	*V. harveyi*		X	Open sores	Guam
GREEN+, GREEN AGRESS	*V. rotiferianus*		X	Digestive tract, mucus	Lizard Island
NA, YG	*V. rotiferianus*	X		Digestive tract	Lizard Island
GUIGEN	*V. rotiferianus*		X	Open sores	Guam
GUMAB	*V. fortis*	X		Digestive tract	Guam
GU5PALEEN	*V. fortis*		X	Opens sores	Guam
Cream TCBS, Cream 12-13	*V. natriegens*		X	Digestive tract, mucus	Lizard Island
Haemorr	*V. natriegens*		X	Open sores	Lizard Island
GU2Y	*P.eurosenbergii*	X		Digestive tract	Guam
GU5YEN, GU4YEN	*P.eurosenbergii*		X	Open sores	Guam

All COTS, irrespective of their site of collection (Lizard Island or Guam), had similar bacterial microflora and responded in the same way to the TCBS agar (Rivera-Posada *et al*., 2011), indicating that a common mechanism of disease induction affects their bacterial

communities and immune responses. Pathogenic and non-pathogenic *Vibrio* isolates identified as *V. owensii*, *V. rotiferianus*, *V. fortis*, *P. eurosenbergii* were recovered from sick and wild COTS at 2 different locations (Guam and Lizard Island), supporting the hypothesis that TCBS coupled with adequate environmental conditions could trigger *Vibrio* virulence factors turning normal vibrios pathogenic (Rivera-Posada *et al*., 2011).

The species *Photobacterium eurosenbergii* is of particular significance because it can infect both COTS and coral, although it was found only in COTS from Guam. This *Vibrio* is also recognized as a coral pathogen isolated from the mucus of the Caribbean elkhorn coral *Acropora palmata* (Ritchie, 2006). *P. eurosenbergii* was also recovered from mucus and water surrounding bleached *Barabattoia amicorum* corals at Magnetic Island, GBR (Colin *et al.,* 2008). Due to the feeding preferences of COTS, studying the transmission of bacteria between corals and COTS is necessary.

Studies of colonization sites within gut regions and growth patterns of different bacterial populations will allow a more focused approach to the types of associations within COTS. Density, production, and turnover estimation of gut microbes are also required to establish bacterial associations and to make comparisons of microbial processes between sick and wild COTS.

3.6 Acknowledgements

Funding for this project was provided by the ARC Centre of Excellence for Coral Reef Studies, the Australian Institute of Marine Science (AIMS), and Project A.W.A.R.E. Field sampling was conducted with kind support and logistic assistance from Lizard Island Research Station, the Australian Institute of Marine Science (AIMS) and the Marine Laboratory University of Guam.

CHAPTER 4: Injection of *Acanthaster planci* with Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS). II. Histopathological changes

4.1 Abstract

We assessed histological changes in the tissues of the crown-of-thorns starfish *Acanthaster planci* (COTS) after injection of thiosulfate-citrate-bile-sucrose agar (TCBS) which was used as a disease inducer (potential outbreak control method), by conventional and scanning electron microscopy. Digestive glands were processed and stained with hematoxylin and eosin (H&E) to describe the histological architecture of the intestinal epithelium. Subsequently comparison of healthy versus infected tissues and Gram stains were carried out to confirm bacterial occurrence on infected tissues, characterize the structural changes induced by bacterial communities in COTS tissues, and to determine if the histopathological changes of intestinal tissues were consistent with *Vibrio* infection. TCBS injections induced marked epithelial desquamation, hypertrophy and hypersecretion of glandular cells, epithelial cell destruction, pyknosis, reduction of thickness and disorganization of connective tissue and associated nerve plexus, presence of bacterial colonies, irregular eosinophilic foci in glandular cells, brush border disruption, atrophy and detachment of intestinal microvilli and cell debris in the lumen. All these changes were attributed to a fulminating systemic dysbiosis and were consistent with *Vibrio* infections

†This chapter was published (as written) in *Diseases of Aquatic Organisms*: Rivera-Posada JA, Pratchett M, Owens L (2011). Injection of *Acanthasther planci* with Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS). II. Histopathological changes. *Diseases of Aquatic Organisms* 97: 95-102

4.2 Introduction

The ecological impact of the corallivorous crown-of-thorns (COTS) starfish *Acanthaster planci* is enormous. Outbreaks of *A. planci* are the principal cause of long-term coral loss at many locations in the Pacific, including Palau, Guam and Fiji (Bruno & Selig, 2007). COTS represent the most significant biological disturbance to tropical coral reefs (Birkeland & Lucas, 1990), resulting in extensive (up to 90%) mortality among reef-building and habitat-forming corals (Pratchett 2005, 2009). Attempts to control the outbreaks, either by collection of adult individuals followed by burial ashore, injections of starfish with toxic chemicals or underwater fences are expensive, time consuming and only feasible for small areas. Current control methods are ineffective in either eradicating the coral-feeding starfish or preventing further coral mortality.

Thiosulfate-Citrate-Bile-Sucrose agar (TCBS) is a selective media culture that allows selective growth of vibrios while inhibiting Gram-positive organisms and suppressing coliforms and allows selective growth of vibrios. Rivera-Posada *et al*., (2011) explored an alternative control method for injecting TCBS into COTS in order to induce a fatal disease that is transmitted to healthy in-contact COTS under favorable conditions. They hypothesize that inhibition of competitive bacteria and increase in *Vibrio* cell densities (10^5 to 10^7 colony forming units [cfu] ml^{-1}) induced by TCBS activate the quorum sensing (QS) mechanism of the vibrios, turning on virulence factors inducing rapid onset of symptoms followed by death. Moreover, molecular analysis with 16S rRNA, *topA* and *mreB* of bacterial assemblages recovered from infected tissues of COTS identified several vibrios of the Harveyi and Splendidus clades that have been frequently described as etiological agents of echinoderm diseases (Rivera-Posada *et al.*, 2011 a, b).

The pathogen–host epithelium interactions can be broadly categorized as: (1) alterations in the structure and function of the tight junction barrier (erosion, ulceration, apoptosis etc); (2) induction of fluid and electrolyte secretion and; (3) activation of inflammatory cascades (Bock *et al.*, 2001; Sicherer *et al.*, 2001; Berkes *et al.*, 2003). For instance some pathogens use tight junction proteins as receptors for their attachment and subsequent internalization and others destroy the junctions providing an entrance to the underlying tissues (Guttman & Finley, 2009).

Vibrios as pathogenic agents disrupt the hydroelectric transport of ions, in particular Na, K, Cl and HCO_3, inducing tissue structural changes and severe damage through different mechanisms (Berkes *et al*., 2003; Thompson *et al*., 2006; Khemiss, 2009). For example *V. cholerae* alters tight junction permeability through the use of a surface protein called the zonula occludens toxin (ZOT), which is excreted upon contact with intestinal epithelial cells (Fasano *et al*., 1995; Uzzau *et al*., 1999). *V. parahaemolyticus* has a thermostable direct haemolysin (TDH) toxin that induces intestinal fluid secretion of Cl, cytotoxicity, and a dose-dependent increase in intracellular calcium (Raimondi *et al.*, 2000; Berkes *et al*., 2003).

Vibrio harveyi, which is one of the COTS pathogens previously reported by Rivera-Posada *et al.*, (2011a, b), possesses several key virulence factors including the ability to attach and form biofilms, quorum sensing, and secretion of various extracellular products (ECPs) such as proteases, phospholipases, hemolysins, cytotoxins and ADP-ribosylating toxins that are mediated by specific bacteriophages. Proteases and hemolysins are considered to play a major role in host tissue liquefaction (Deanne &Woo, 2005). These bacterial enzymes affect the physical properties of tissue matrices and intercellular spaces, thereby promoting the spread of the pathogen. For example, neuraminidase produced by *Vibrio cholerae* degrades neuraminic acid, an intercellular cement of the epithelial cells of the intestinal mucosa (Todar, 2011). All

pathogenic *Vibrio* species encompass extracellular chitinases as a virulent factor and have been described as the primary etiological agents of numerous diseases of marine invertebrates (Baumann *et al.,* 1980). The presence of chitinases aids the invasion of the pathogen through the chitinous cuticle of marine invertebrates as well as providing nutrients and amino acids through the enzymatic degradation of host tissues (Goday, 1990; Thompson *et al.,* 2006).

While there are several different contributing mechanisms by which the immune system fails allowing infection, the means by which these processes are carried out in echinoderms is intricate and scarcely understood. Knowledge in relation to the mechanisms involved in the induction of infectious diseases and the host tissue response in echinoderms is lacking.

The purpose of this study was to: (1) describe the anatomy and histology of COTS digestive glands; (2) compare healthy versus infected tissues and characterize the histopathological structural changes induced by TCBS injections in COTS tissues contributing to a better understanding of the pathogen-host interactions and physiological tissue responses involved in the induction of diseases in echinoderms; and (3) determine if the histopathological changes of intestinal tissues were consistent with *Vibrio* infection. This is a segment of on-going work to evaluate if TCBS injection could be a new tool for management of COTS outbreaks.

4.3 Materials and methods

COTS collection

Samples consisted of 16 digestive glands dissected from individual, adult sick COTS which were previously injected with TCBS to induce disease (n = 12), and healthy (n = 4) COTS collected at Lizard Island (14° 40 ´S, 145° 27´ E) located in the far northern section of the Great Barrier Reef (GBR) (Rivera-Posada *et al*., 2011a).

Histology

Four normal digestive glands were aseptically dissected after arrival at Lizard Island Research Station (LIRS) laboratory and immediately fixed in formalin 10% (10 parts of formalin to 1 part of tissue) (Bancroft & Stevens, 1996). Twelve digestive glands of infected COTS previously injected with TCBS (Rivera-Posada *et al*., 2011 a) were collected before death for better isolation of pathogens and to avoid the strong enzymatic activity in intestinal tissues after death that results in swelling of the villus, epithelial denudation and autolysis (McInnes, 2005). Processing of COTS pyloric caeca for hematoxylin and eosin (H&E) and Gram stains (GS) was performed as described in (Bancroft & Stevens, 1996). All tissues were sectioned at 5 µm. Histological changes were evaluated in terms of the appearance of microvilli, necrosis, piknosis, enterocyte detachment, cell debris, bacteria-like particles and epithelial integrity (appearance and detachment of layers).

Scanning Electron Microscopy

Twelve samples of infected tissues were collected for scanning electron microscopy (SEM) to confirm the presence of bacteria and to determine the tissue penetration of bacterial assemblages. All samples were fixed for 16 h in a 3% buffered glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.8). Then after dehydration in graded alcohol (50%, 70%, 90%, 100%), samples were critical-point dried, mounted on aluminum stubs, sputter-coated with gold and examined under a JEOL JSM-5410LV scanning microscope.

4.4 Results

Macroscopic observations

During the early stage of the disease, COTS displayed loss of body wall turgor, dropping of spines and production of mucus at the tip of the spines. As the disease progressed, blisters on the

dorsal integument broke through the skin surface and resulted in large, open sores that exposed the internal organs (Rivera-Posada *et al*., 2011 a). COTS tissues showed a progressive breakdown of muscle, disorganization of connective tissue, and degeneration and disruption of the epithelium. Muscle layers continued to fragment until there was little intact muscle remaining inserted on the collagenous connective tissues. Tissue colouration was occasionally darker in late stages of disease (Fig 4.1, 4.3 a, b).

Histology and Histopathological changes

Microscopically, COTS digestive glands consist of layers of connective tissue (CT), muscle (ME), brush border (BB), glandular cells (GC) which are involved in the control of digestive metabolism, and a basi-epithelial nerve plexus (NP) which form and extensive nervous layer adjacent to the tunica serosa (TS). The basi-epithelial plexus is an important part of the ectoneural nervous system division which provides functional connectivity between the viscera and other divisions of the nervous system. It is formed by abundant fibres associated with muscle layers (ME) and a few scattered neuronal cell bodies. Histopathological changes of affected COTS tissues after TCBS injection are detailed in Fig. 4.3. Histological appearance of affected tissues was mainly characterised by strong disorganization and destruction of connective tissue and muscle fibres, enterocyte detachment, marked epithelial desquamation, piknosis, hypertrophic glandular cells, moderate presence of bacterial colonies and disorganized basi-epithelial nerve plexus (Fig 4.3).

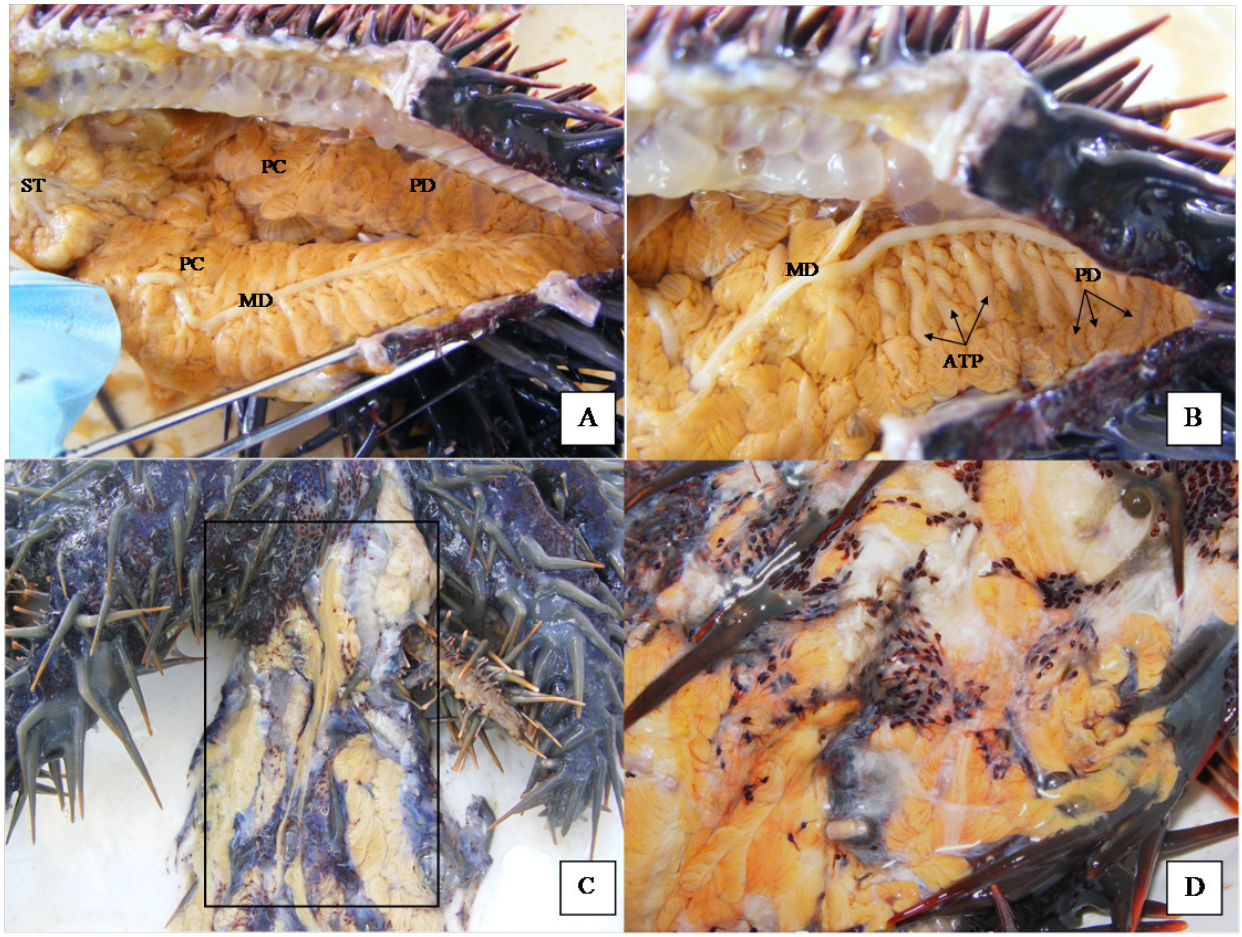

Figure 4.1. Macroscopic observations. (A, B) Normal appearance of COTS digestive glands. (A) Paired pyloric caeca (PC) present in the coelomic cavity of each arm. Each pyloric caecum is connected to the dorsal part of the stomach (ST) and consists of a long median duct (MD) on which are numerous branched diverticula (PD). (B) Aboral view of COTS digestive glands; accessory Tiedemann's pouch (ATP), median duct of pyloric caeca (MD) and pyloric diverticulum (PD). (C, D) infected COTS showing lyses of collagenous dermis and digestive glands with no observable pyloric caeca structure; (D) open lesions exposing internal organs; loss of epidermal tissue and lysis is marked.

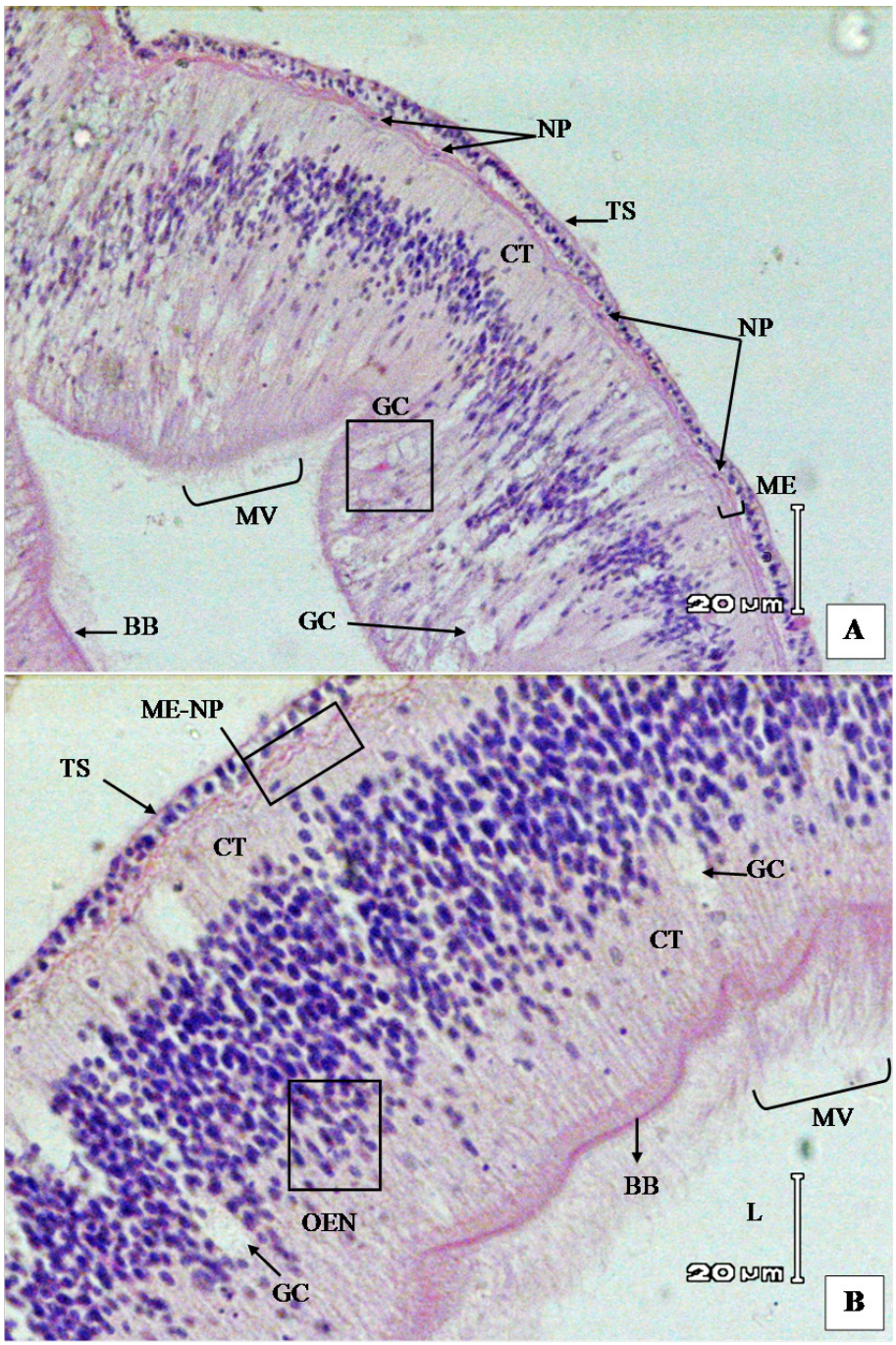

Figure 4.2. Histological appearance of normal COTS digestive glands. Hematoxylin & eosin stain. (A). 20x, (B). 40x. COTS intestinal wall from the lumen (L) to the periphery consist of a lamina of cylindrical simple ciliated epithelia (MV) with ovoid elongated nucleus (OEN) and thin brush border (BB); a moderate amount of glandular cells (GC) associated with a basi-epithelial nerve plexus (BNP); more externally there is a layer of loose connective tissue (CT), a muscular epithelium (ME) associated with a nerve plexus (NP) and a tunica serose (TS).

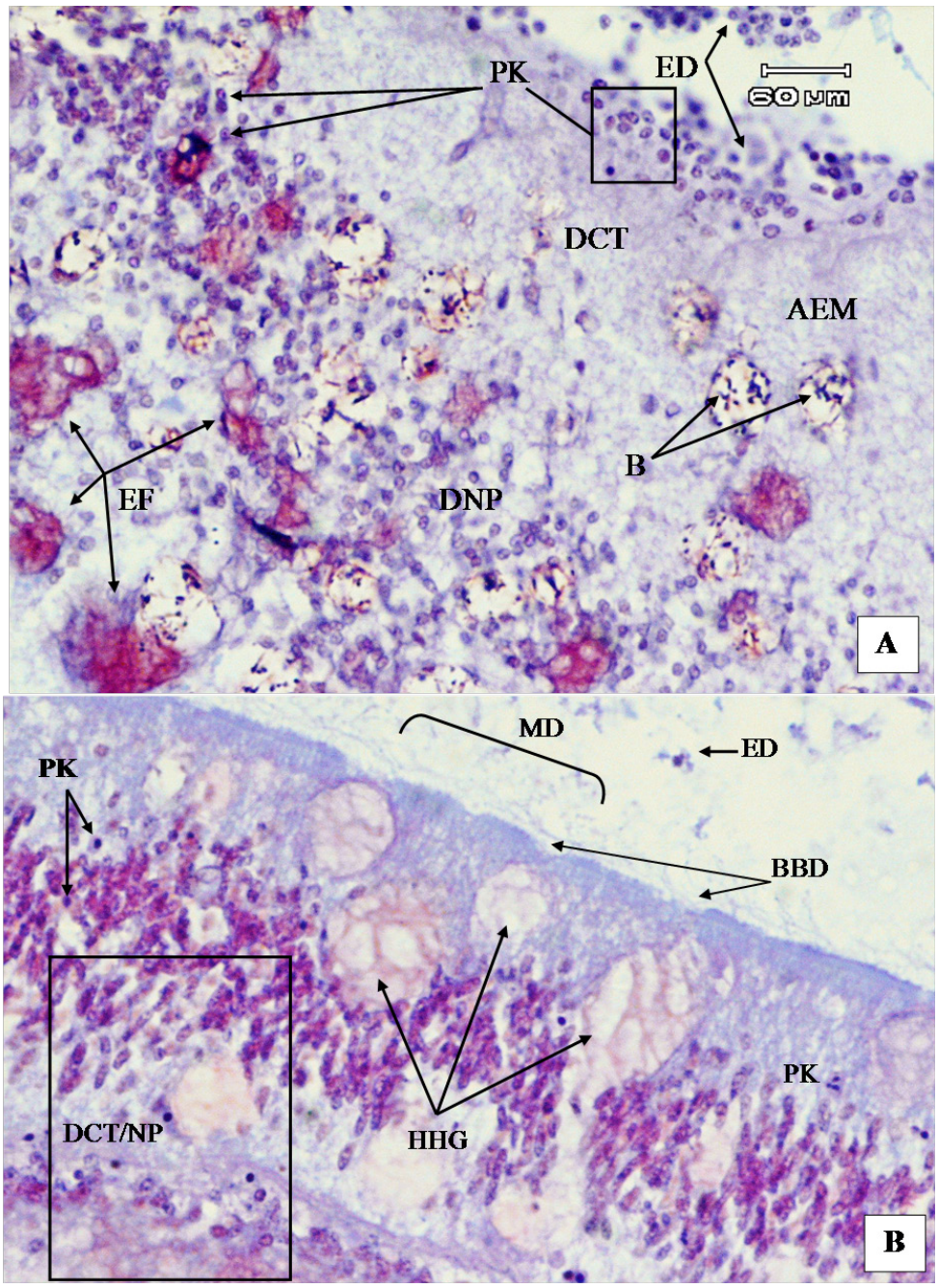

Figure 4.3. Histological appearance of affected tissues. A). 40 x, irregular eosinophilic foci in glandular cells (EF), enterocyte detachment (ED), moderate presence of bacterial colonies (B), disorganization of basi-epithelial nerve plexus (DNP), pyknosis (PK) in some cells, absence of epithelial muscle (AEM), reduction and disorganization of connective tissues (DCT). B). 40 x. Hypertrophic and hypersecreting glandular cells (HHG), marked epithelial desquamation (MD), brush border disruption (BBD), disorganized basi-epithelial nerve plexus (NP) and pyknosis (PK).

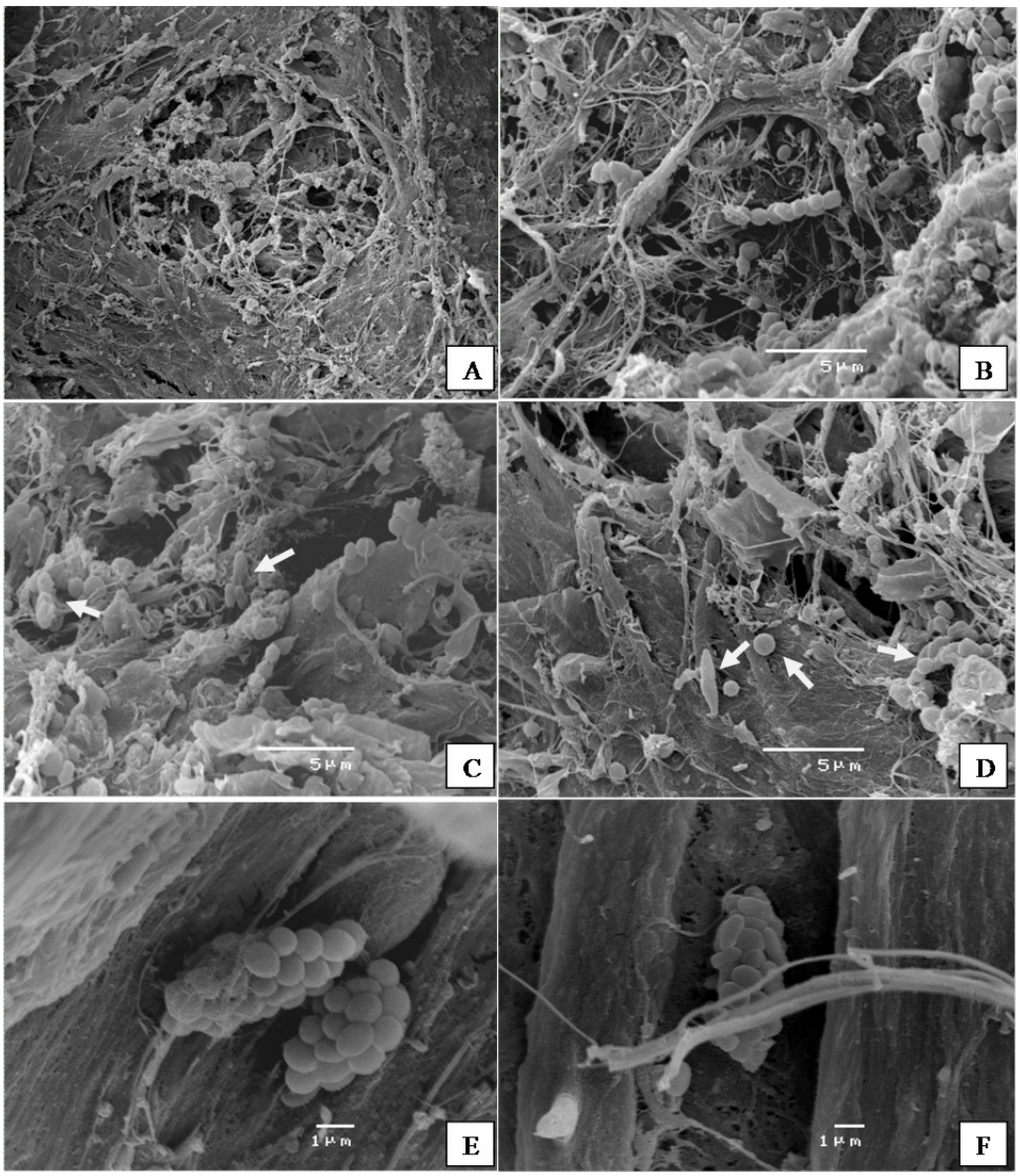

Figure 4.4. Scanning electron microscopy of tissues affected by bacterial assemblages. (A, B) disorganization of connective tissue, degeneration and disruption of epithelium and significant amounts of fibrin-like tissue over the samples; (B) magnified figure A. Bacterial cluster inside the lesion suspended between degraded tissue; (C, D, E, F) bacterial assemblages of diverse morphology and size – smooth, ovoid, rod shaped and clusters (arrows).

Scanning Electron Microscopy (SEM)

The epithelial surface was colonised by microorganisms in zones where patches of degrading epidermis were mixed with degrading, exposed connective tissue (Figs 4A, B). In the affected zone, collagen fibres were disorganized (Fig. 4C). Three bacterial morphotypes were observed in the areas affected: ovoid bacteria, rod-shaped bacteria and clusters of more than ten cells. Bacteria clusters were never observed at the surface of the healthy integument nor on ossicles. Bacteria were much more numerous at the border of the affected zone. Smooth ovoid bacteria (Fig. 4B, C, E), were abundant in comparison to rod shaped bacteria.

4.5 Discussion

The histopathological changes reported in this study are similar to those described in numerous animals affected by vibrios. The most significant histological change found in this study was the destruction, disorganization and significant reduction in the amount of COTS connective tissues (Fig. 4.1, 4.4). This finding was consistent with Owens *et al.,* (1992), who described a widespread colonisation of connective tissue by *Vibrio harveyi* in experimentally infected *Penaeus esculentus*. Interestingly *V. harveyi* was reported by Sutton *et al.,* (1988) as a potential COTS pathogen and is also one of the pathogenic vibrios previously isolated from COTS infected tissues (Rivera-Posada *et al.,* 2011 b). *Vibrio harveyi* ECPs are well known for their capacity to induce lysis of connective tissue. For example, Zorrilla *et al.,* (2003) evaluated the potential virulence role of *V. harveyi* and *V. parahemolyticus* extracellular products and reported that tissue lysis and ulcers were only seen on the surface of the fish after inoculation with ECPs from *V. harveyi*. Similarly, Zhang & Austin (2000) demonstrated that the most pathogenic isolate of *V. harveyi* to salmonids produced ECPs with a maximum effect on fish tissues. This bacterium

has also been reported as the etiological agent of body wall lesions in other echinoderms such as *Tripneustes gratilla* and *Apostichopus japonicus* (Ma *et al.*, 2006, Becker *et al.,* 2007). The clinical signs of the disease induced by TCBS injections in COTS, such as necrotic skin, open sores, and loss of body turgor (Rivera-Posada *et al.,* 2011a), have also been described in humans infected by vibrios as strong reactions in connective tissues characterized by necrosis and blisters (Chuang *et al.,* 1992). Marked pyknosis was observed in all samples analyzed in this study (2.5 over a scale of 0 to 3) (Fig.4.3). Not surprisingly, *V. harveyi* ECPs induced degeneration of *Penaeus monodon* intestinal matrix cells, characterized by prominent pyknosis (Intaraprasong *et al.,* 2009).

Moreover, our macroscopic and SEM analysis (Fig.4.1, 4.4) displayed large amounts of disorganized and degraded collagen tissues and abundant miscellaneous bacteria. Becker *et al.,* (2004) also used SEM analysis to describe skin ulceration disease in cultivated juveniles of *Holothuria scabra* (Holothuroidea, Echinodermata) and, similarly to our present study, found zones where the epidermis was totally destroyed, highly degraded collagen fibers and ossicles were exposed to the external medium, and areas with bacterial assemblages of various morphotypes were present. These remarkable similarities between studies coupled with the identification of *Vibrio sp., V. natriegens, V. harveyi and V. alginolyticus* as etiological agents of skin ulceration disease in *Holothuria scabra* (Becker *et al.,* 2004) and *V. natriegens*, *V. harveyi, V. rotiferianus, V. owensii*, and *V. fortis* in infected COTS tissues after injections with TCBS (Rivera-Posada *et al.,* 2011 a, b) clearly showed that members of the Harveyi group are major etiological agents in the induction of diseases in echinoderms. Furthermore, these findings confirmed that tissue structural changes in diseased echinoderms can be promoted by miscellaneous bacteria. Certainly, the marine environment is a rich source of opportunistic

microorganisms that normally do not cause disease but become pathogenic when the immune system is impaired and unable to fight off infection.

COTS digestive glands showed areas of epithelial cell destruction, microvilli atrophy, enterocyte detachment, absence of muscular epithelium, reduction of thickness and disorganization of connective tissue and their associated nerve plexus, presence of bacterial colonies, cell debris in the lumen and marked epithelial desquamation (Fig. 4.3) which is well known as an innate immune system defense mechanism that helps remove bacteria and other infectious agents that have adhered to the epithelial surfaces (Sturtinova *et al.,*1995). All these histological findings are also in agreement with other reports from marine invertebrates infected by vibrios. For example, histological evaluations of sea bream *Sparus aurata* intestines infected with *V. vulnificus* described changes such as detachment of enterocytes, disorganized intestinal layers, inflammation and alterations in the microvilli and brush border (Khemiss, 2009). *V. harveyi* caused a massive destruction of *P. monodon* digestive system where epithelial cells and basement membrane were damaged, necrotic and completely disappeared in advanced stages of the disease. Bacterial clusters among cell debris in the lumen were also described (Soonthornchai *et al.,* 2010). Furthermore, the hypersecreting intestinal glandular cells displayed in the present study (Fig. 4.3B) can be associated with the release of *Vibrio* enterotoxins which are recognized for their capacity to induce intestinal fluid secretion, inflammation and severe tissue damage (Raimondi, 2000; Thompson *et al.,* 2006).

There was good agreement between the histopathology findings, bacterial culture and identification (Rivera-Posada *et al.,* 2011 a, b, present study), suggesting that histopathological examination is a reliable tool that allowed identification of typical vibriosis lesions and could

contribute to the differentiation from other causes. Varello *et al.,* (2008) compared histologic techniques and bacteria cultures for the diagnosis of bovine tuberculosis reporting similar results.

The present study showed that bacteria assemblages found on affected COTS tissues played an important role in the development of disease supporting the hypothesis of activation of quorum sensing mechanism proposed by Rivera *et al.,* (2011 a). However, being opportunistic species, vibrios can evolve and recombine when reaching high densities in animal guts or as part of aquatic biofilms, transferring or inducing expression of virulence genes in less or non-pathogenic populations (Thompson *et al.,* 2004). Therefore, *Vibrio* quorum sensing studies are important to understand the pathways used to drive the presentation of the transmissible disease in COTS.

Knowledge of the signaling molecules involved in the QS process and their inter-relationship will allow a better understanding of the interaction between bacteria and host. It will also provide insight into the effect of transmissible disease on other marine animals that also have vibrios of the Harveyi and Splendidus clades as normal inhabitants. Disease dynamics and transmission experiments are warranted, especially in corals, carnivorous fishes, and benthic detritus feeders.

After several decades of research, current control methods are ineffective in either eradicating the coral-feeding starfish or preventing further coral mortality. The susceptibility of crown-of-thorns starfish *Acanthaster planci* to disease could provide an option for controlling population outbreaks. Injection of thiosulfate-citrate-bile-sucrose agar (TCBS) culture medium into *A. planci* induces a rapid fulminating disease that is transmitted to in-contact COTS under favorable conditions (Rivera-Posada *et al.,* 2011 a). Additionally there is no introduction of new

pathogens into the environment and it is a rapid and simple procedure with immense economic advantages.

4.6 Acknowledgements

Funding for this project was provided by the ARC Centre of Excellence for Coral Reef Studies, Queensland, Australia.

CHAPTER 5: Modified techniques to improve tissue preservation, detection and characterization of *Vibrio* bacteria in marine organisms

5.1 Abstract

Marine biologists typically follow standard histological protocols designed for human samples when processing and testing for the presence of bacteria in tissues of marine organisms. However, these procedures may reduce detection of bacteria and many specimens are lost during dehydration, clearing and/or paraffin infiltration. *Vibrio* bacteria are often concentrated on the surface of the tissues and likely to be washed away during long and repetitive washes of samples before fixation and with the use of 13 hours cycles in automated processors. These situations can be improved by using short washes (3 minutes immersion), reducing processing times (4:20 hrs) and embedding samples in agar. The use of short cycles also decreased the amount of epithelial desquamation in COTS samples. Furthermore, a simpler standardization of the API biochemical test for phenotypic bacterial identification of environmental strains was achieved by altering the incubation temperature, supplementation of NaCl 2% to the auxiliary media and the colony size. The present study contributes to the standardization of histological techniques and biochemical test (API strips) for partial identification of marine bacteria, ensuring more accurate results, improving performance, enhancing reproducibility and increasing time/money efficiency in comparison to extant procedures.

5.2 Introduction

Vibrio bacteria are highly diverse and commonly occur as normal microflora in a wide range of vertebrate taxa, or as free-living entities across various aqueous environments, such as coastal marine ecosystems. However, these Gram-negative microorganisms are also the etiological agents of many significant pathogenic disorders resulting in serious health related issues in human populations (Janda *et al.,* 1988; Faruque & Nair, 2002; Wilkins *et al.,* 2007). *Vibrio* bacteria also pose a serious problem for marine and freshwater aquaculture, whereby loss of production due to disease outbreaks and the costs of preventing diseases significantly affect economic viability (Bourne *et al.,* 2004; Cano-Gómez *et al.,* 2009).

The characterization of pathogenic isolates plays an essential role in the epidemiology of infectious disease, producing the information necessary to identify, track and intervene against disease outbreaks (Urwin *et al.,* 2003). However, *Vibrio* taxonomy and phylogenetics has been historically hampered by the high morphological similarity existing amongst several lineages and specific taxa. As such, identification of specific *Vibrio* bacteria is increasingly achieved through the application and development of novel bio-molecular techniques. Increased recognition of distinct *Vibrio* has been further motivated by the growing problems of antibiotic resistance among pathogens (Zbinden, 2006; Vlieghe *et al.,* 2009), as well as the emergence of numerous infectious diseases in the marine environment (Bossart, 2007; Schaefer *et al.,* 2009)

The aid of genetic methods, featuring an increasing number of molecular markers, together with biochemical test identification procedures, has greatly improved the resolution of *Vibrio* taxonomy (Cano-Gomez *et al.,* 2010). Currently 80 species of *Vibrio*, grouped into 14 evolutionary lineages have been identified (Sawabe *et al.,* 2007; Cano-Gomez *et al.,* 2011), several of which were previously misidentified and included within the same group or species

(Thompson *et al.,* 2007; Cano-Gomez *et al.,* 2010). In marine environments, the application of molecular techniques to identify bacteria is revealing many new species of *Vibrio* (Gurtler & Mayall, 2001; Van Belkum *et al.,* 2001; Yoshizawa *et al.,* 2009); in just the last 3 years, 3 new species, *V. azureus* (Yoshizawa *et al.,* 2009), *V. sagamiensis* (Yoshizawa *et al.,* 2010) and *V. owensii* (Cano-Gomez *et al.,* 2010) have been identified within the Harveyi group, which is known for causing highly virulent disease in marine organisms.

Current biochemical identification kits are mainly designed for use with bacteria that affect humans, or have industrial microbiological importance, causing significant bias in identifying *Vibrio* across different evolutionary lineages. Although these kits are useful when used according to their intended purpose, these kits fail to provide necessary resolution, efficiency and reproducibility when used in novel marine settings. Moreover, the standard procedures used to identify bacteria require modifications in order to enhance the identification of marine microorganisms. There has been considerable discussion on necessary changes to standard operating procedures to correctly process marine samples and to increase identification of aquatic organisms. (Thompson *et al.,* 2001; Fukui & Sawabe, 2008). For example, the addition of NaCL is used to adapt biochemical test for use with marine bacteria (MacDonell *et al.,* 1982; Martinez-Urtaza *et al.,* 2006). However, these modifications (such as addition of NaCL) are often implemented on a fairly ad hoc basis, failing to take into account of colony size or variation in environmental conditions across different aquatic habitats.

The purpose of this study was to develop systematic modifications to established histology techniques (H&E and Gram stains) and biochemical test for partial identification of marine Gram- negative bacteria, which have fundamental flaws in several aspects of their methodology. Here we will concentrate on *Vibrio* bacteria given their increasing importance in

marine ecosystems, aquaculture industry and public health. This study will focus on two instances of the procedures typically employed for partial identification of Gram-negative bacteria; (1) sampling and processing of marine tissues for histology and (2) standardization of biochemical test for environmental strains.

5.3 Materials and methods

Study organism and sampling

Samples consisted of 16 digestive glands dissected from individual adult sick (n = 12) and healthy (n = 4) COTS collected at Lizard Island (14° 40 ´S, 145°27´E) located in the far northern section of the Great Barrier Reef (GBR). Disease was experimentally induced by injecting each of the 12 specimens with 10ml of thosulate-citrate-bile-sucrose (TCBS) as described by Rivera-Posada *et al.,* (2011a).

Histology

Normal digestive glands were aseptically dissected after arrival at Lizard Island Research Station (LIRS) laboratory and immediately washed and fixed in phosphate buffered formalin 10% (10 parts of formalin to 1 part of tissue) (Bancroft & Stevens, 1996). Tissue samples of diseased COTS previously injected with TCBS (Rivera-Posada *et al.,* 2011) were collected before death for better isolation of pathogens and to avoid the strong enzymatic activity in intestinal tissues after death that results in swelling of villus, epithelial denudation and autolysis (McInnes, 2005). Six digestive glands (2 from healthy and 4 from diseased COTS) were immersed for 3 minutes in 0.9% saline, then embed in agar and processed using short cycle in the automatic tissue processor (Table 1). The other 10 digestive glands (2 from healthy and 8 from diseased COTS) were processed following the standard protocol of 13 h in the tissue processor. Five digestive glands (1 healthy and 4 from diseased COTS) were rinsed twice for 5 minutes and the other

samples (1 healthy and 4 from diseased COTS) immersed in 0.9 % saline for 10 minutes with subsequent 13 h processing. Staining of COTS pyloric caeca for hematoxylin and eosin (H&E) and Gram stains (GS) was performed as described in Bancroft & Stevens, (1996). Histological changes were evaluated in terms of the appearance of microvilli, necrosis, enterocyte detachment, cell debris, bacteria- like particles and epithelial integrity (appearance and detachment of layers).

Processing and embedding of tissue samples for histology

Standard processing of histological samples is usually performed overnight (13 hours), following which the tissue is ready for embedding in paraffin wax (Woods & Ellis, 1994). The purpose of the processing is to take fixed tissue and slowly dehydrate it using a series of ascending grades of ethanol. It then has a total of three changes of absolute ethanol. Before the tissue can be impregnated with paraffin it must be cleared in a solvent that is miscible with both ethanol and paraffin (Woods & Ellis, 1994). However, many marine tissues require special care during processing due to their small size (i.e larvae), delicate nature (i.e connective tissue, fresh nervous system), rapid degradation (i.e digestive glands of crown-of-thorns starfish) or stage of disease. Importantly, bacterial infections in many marine organisms are often restricted to the tissue surface (Becker *et al.,* 2004; Ringo *et al.,* 2007), and thus bacteria are prone to being washed away during long washes of samples previous to fixation and by the automated tissue processing that agitates the solutions over a prolonged period. In the final stages, the tissue is infiltrated under vacuum pressure with hot liquid paraffin wax (Paraplast®) at 60 ºC to assist in drawing the liquid paraffin into all the spaces in the tissue. This study used reduced processing times (Table 5.1); combined with additional changes to the preparing tissue samples (short washes before fixation and embedding of samples in agar).

Automated tissue processor (Short cycle)	
50% ethanol	20 minutes
70% ethanol	20 minutes
70% ethanol	20 minutes
80% ethanol	20 minutes
90% ethanol	20 minutes
95% ethanol	20 minutes
Absolute ethanol	20 minutes
Absolute ethanol	20 minutes
Absolute ethanol	20 minutes
Xylene	20 minutes
Xylene	20 minutes
Paraplast	20 minutes
Paraplast	20 minutes
Total time	**4.20 hours**

Table 5.1. Proposed short cycle processor times. Automated processor temperature 38 °C. Small tissue samples were used (0.2-0.5 cm) to ensure adequate penetration of formalin (0.05 mm/hour) and to decrease the time required for correct clearing.

Agar paraffin embedding

Prior to processing, samples were embed in agar. Agar 3% was prepared in an autoclave at 121°C for 15 minutes due to the tendency of untreated agar to promote bacterial and fungal growth (Lapage, 1970). The prepared agar was then stored in small Pyrex tubes, which were heated in a microwave to melt the agar prior to embedding. A small quantity of agar solution was poured onto the bottom of the mold. The tissue was then oriented using a stereomicroscope adjusting fragments with a metal pin. The remainder of the mold is filled with agar and placed on a plate cooled to 4ºC to ensure agar hardening and to prevent changes in tissue orientation. The solidified agar block was then placed in a histology cassette and processed in a Shandon Hypercentre XP® automated processor. Processed cassettes were then transferred to a Shandon

Histocentre 3, embedding centre. Here the cassette was opened and the agar plug was orientated face down in metal embedding molds. The back of the cassette containing the sample ID is then transferred to the back of the embedding mold and placed on an ice tray to cool. Ribbons of 5µ sections were then cut from the tissue block and floated out on a 47ºC waterbath and then mounted on a Menzel-Glaser Superfrost ®Plus slide. These sections were then stained using Gram-Twort staining method following (Bancroft & Stevens, 1996).

Scannin electron microscopy (SEM)

Tissue samples were fixed for 16 h in 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.8). Then, after dehydration in graded alcohol (50, 70, 90 and 100%) samples were critical point dried, mounted on aluminum stubs, sputter-coated with gold and examined under a JEOL JSM-5410LV scanning electron microscope.

Fluorescent in situ hybridization

Tissue samples were fixed in 4% paraformaldehyde suspended in phosphate buffered saline (PBS) for 24 h at 4°C and stored in 50% ethanol/PBS at – 20°C until processing. Then, tissue samples were placed in histology cassettes dehydrated through ethanol series and embed in paraffin. Tissue sections were cut to a thickness of 5 µ and transferred to adhesive glass slides (SuperFrost® Plus, Menzel-glaser, Germany). The bacterial specific probes EUB338, EUB338-II and EUB-III were labeled with indocarbocyanine fluorochrome Cy3 and combined to produce EUB +. Twenty-five ng/ µl of the solution was applied to the slides and incubated in 50 ml polypropylene tubes at 46°C for 3 h.

Biochemical test

Molecular methods are generally considered most accurate for identifying *Vibrio* bacteria, although biochemical bacterial profiles are widely used to complement molecular methods.

However, the discriminatory power of these test are limited for environmental strains of *Vibrio* spp, largely due to genotypic plasticity of *Vibrio* (Cano-Gomez *et al.,* 2009). Ohara *et al.,* (2003) compared and evaluated six commercial systems, based on the ability to distinguish 12 species of *Vibrio* usually found in clinical samples. The accuracy of these systems, which are widely used, ranged from 63.9% to 80.9%.

Phenotypic analysis of 19 bacterial isolates from crown-of-thorns starfish (COTS) collected from Lizard Island (Great Barrier Reef, Australia) and Guam (USA, Western Pacific Ocean) were performed in triplicate by employing API 20NE commercial kits (BIOMÉRIEUX SA, Marcy-I'Etoile, France) according to the manufacturer's instructions with the following modifications (1) 2% NaCl (w/v) solution was used to prepare the inocula, (2) API strips were incubated at 30 °C for 48 h and (3) several colonies of pure cultures of a single organism were used when bacterial colonies were tiny to ensure that all tubes and cupules have enough bacteria for growth. In order to culture bacteria for biochemical identification, sterile inoculation loops were used to swab the skin surface and dermal lesions of individual starfish. These samples were then streaked on TCBS and Nutrient Agar with 2% NaCl plates and grown for 24 h at 30°C. Further identification was achieved through 16 r RNA, *mreB* and *topA* (Rivera-Posada *et al.,* 2011).

5.4 Results and discussion

Modified processing methods used in this study were more effective in preserving delicate marine tissue samples and retain more bacteria when compared to standard histological methods. Moreover, a simpler standardization of the API biochemical test was achieved by altering the incubation temperature, supplementation of NaCl 2% to the auxiliary media and the colony size showing better and consistent results.

Histology

Using the modified techniques, all samples resulted in the visualization of more bacteria on the border or surface of the samples and less epithelial desquamtion. On the other hand, two tissue samples of sick spcecimens were lost during processing using standard protocols due to the delicate nature of the tissues; the other eight samples showed more epithelial desquamation, microvilli damage and reduction in the amount of bacteria, when compared to short cycle samples (Fig 5.1, 5.2). Thus, standardized procedures used for human samples are inappropriate when processing delicate tissues like digestive glands of Crown-of-thorns starfish that have a thin wall, are subject to rapid autolysis prior to fixation and tissues can be easily damaged during preparation. Other delicate tissues such as larvae, liver and brain of other marine organisms with different histological characteristics should also be evaluated.

Standard procedures followed by most histology laboratories recommend the use of two washes before the sample is immersed in the fixative solution for two main reasons: (1) to avoid dehydration, contraction, hardening and deformation of tissues because samples of terrestrial animals are collected from isotonic environments and then immersed in hypertonic media (fixative), with the exception of kidney and liver that are hypertonic in nature; and (2) to eliminate the excess of erythrocytes from the sample to avoid formation of formalin-heme pigment artifact which is most often observed in bloody tissues allowing better penetration of fixatives (Drury & Wallington, 1980). However, tissues of marine animals are already adapted to hypertonic seawater (Todd *et al.,* 2005). Additionally, many marine animals e.g., *A. planci* have a water vascular system and lack erythrocytes therefore formation of heme pigment is minimal. Consequently, the time of sample washing can be greatly decreased.

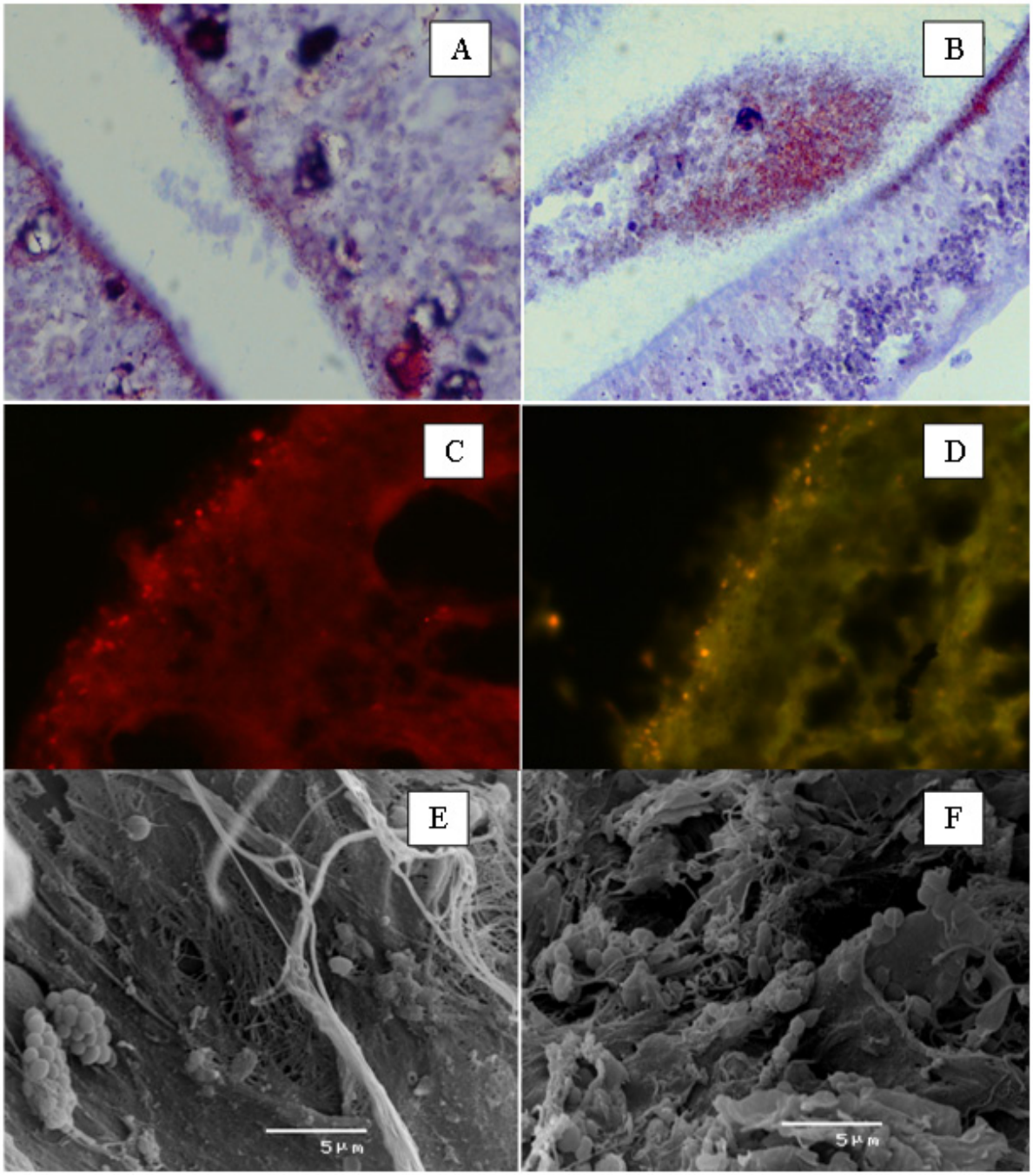

Figure 5.1. Bacterial assemblages in COTS infected tissues. A, B. (20x) Gram stain of digestive glands that were immersed for 3 minutes in 0.9% saline and processed with short cycle showing Gram-negative bacteria (pink, red) on the border of the tissues. B, C. Fluorescent in situ hybridization (FISH) displaying bacteria on border of infected digestive glands. E, F scanning electron microscopy (SEM) images showing bacterial assemblages of diverse morphology and size on the surface of affected COTS tissues.

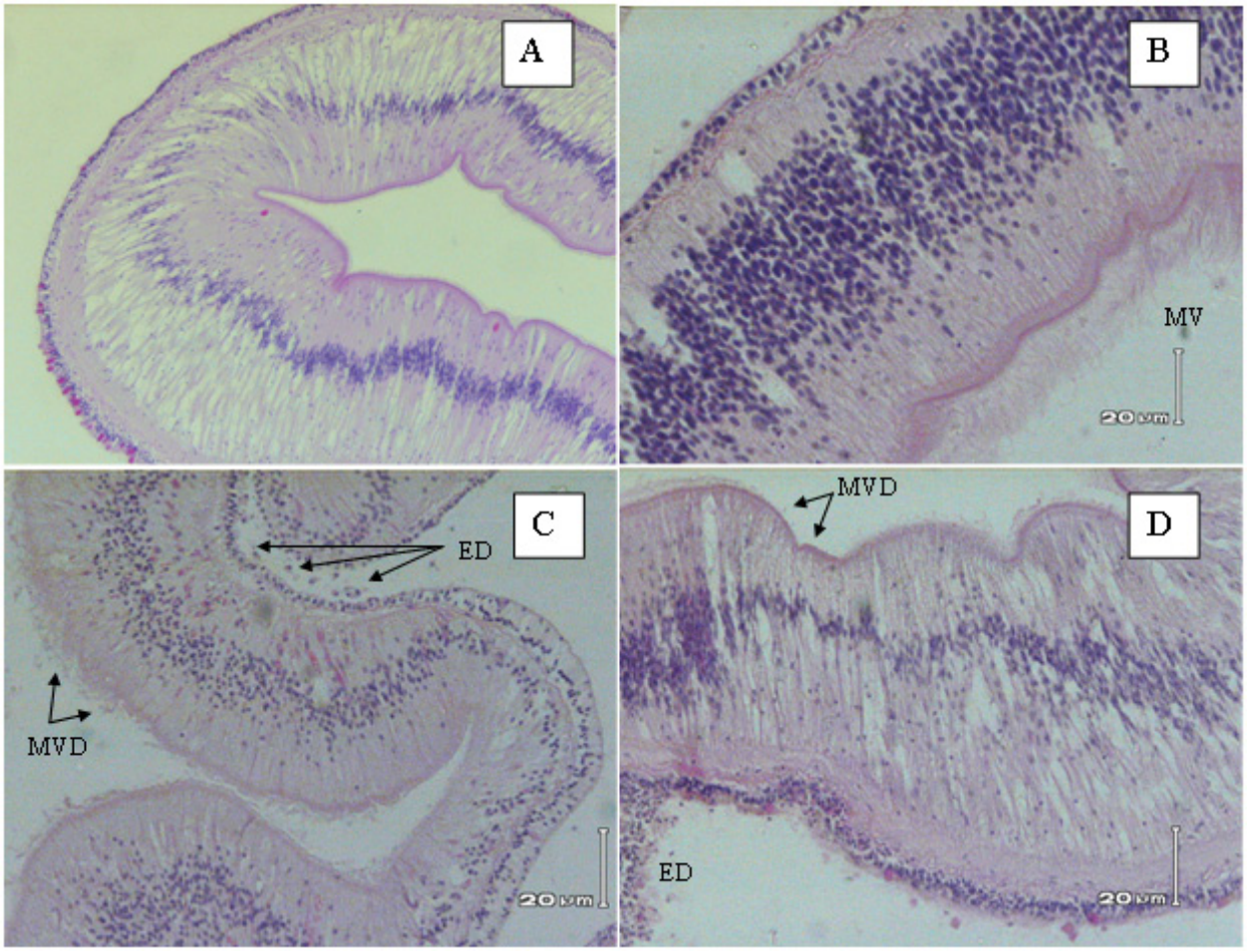

Figure 5.2. Comparison of short vs. long cycles employed for tissue processing of normal COTS pyloric caeca. A (20 x), B (40x) H&E stain of healthy COTS samples processed with short cycles, no epithelial desquamation was observed along with superior microvilli preservation (MV). C, D. COTS normal pyloric caeca processed with standard long cycles (13 hours), showing areas of epithelial desquamation (ED) and shortening and detachment of microvilli (MVD).

Generally, small pieces of tissue are cleared in 0.5-1 h; whereas larger (> 5 cm in thickness) are cleared in 2-4 h. The duration of impregnation depends on size and type of tissue and the clearing agents employed. Ethanol cause shrinkage, excessive hardening of tissues and disintegration of mitochondria and echinoderm skeleton. Additionally, echinoderm body walls and organs consist primarily of collagenous tissues that are dissolved by ethanol (Steadman, 1960; Wilkie, 2002). Thereupon, the use of short cycles is highly recommended. Slow dehydration of delicate tissues starting at 50% ethyl alcohol is also suggested to remove water gently allowing delicate tissues to slowly adjust to its removal. Rapid removal of water can distort the appearance of delicate cells and structures. Normal tissue specimens can be placed directly into 70% alcohol (Talukder, 2007).

Sample preparation is a critical step when processing marine samples because dissection and fixation of large pieces of tissues result in incomplete fixation and subsequently lysis of tissues. In addition, excessive rinsing of samples and inappropriate collection can result in lost of bacteria, thereby generating altered results. For example, Luter *et al.,* (2010) rinsed tissue samples from healthy and diseased sponges with 70% ethanol for subsequent bacteria culture in marine agar and conclude that bacteria, fungi, protozoa were not implicated in the development of brown spot lesions and necrosis of the elephant ear sponge, *Ianthella basta*. Ethanol is a biocide that instantly kills organisms by denaturation of proteins and breakdown of lipids and is effective against most bacteria, fungi, and viruses (McDonnell & Rusell, 1999). Additionally, there are numerous reports of echinoderm diseases that displayed bacterial pathogens on the surface of infected tissues and ethanol washing certainly can kill all epimural bacteria instantaneously (Becker *et al.,* 2004; Ringo *et al.,* 2007). Not surprisingly, numerous other studies in sponges and corals described increases in microbial diversity in response to disease

and/or environmental stress (Cooney *et al.,* 2002; Pantos *et al.,* 2003; Pantos & Bythell, 2006; Webster *et al.,* 2008). Increases in microbial diversity and density are expected findings in disease organisms because once the immune system fails the compromised individual is vulnerable to secondary infections and proliferation of opportunistic pathogens that are abundant in the marine environment.

As expected, most bacteria were located on the border or surface of tissue samples (Fig 5.1, 5.2) and might have otherwise, been washed away during more extensive processing. Similarly, cultivated juveniles of *Holothuria scabra* with skin ulceration disease induced by *Vibrio* sp., Bacteroides sp., and α-Proteobacterium showed bacterial assemblages on the borders of infected tissues (Becker *et al.,* 2004). Also, for intestinal lesions of Atlantic salmon (*Salmo salar* L.) following in vitro exposure to pathogenic *Vibrio anguillarum* and *aeromonas*, the bacteria were concentrated on the tissue surface (Ringo *et al.,* 2007). Our results indicate that long and repetitive washes or pressure rinsing of crown-of-thorns digestive glands before fixation in conjunction with the use of standard long cycles (13- 15 hours) in the automated tissue processor contribute to the lost of bacteria in all samples analyzed. Therefore, care must be taken in processing diseased tissues from marine organisms to maximize detection of pathogenic bacteria.

Vibrios are widespread naturally in the aquatic environment as free living entities or commensals and can be found highly concentrated on aquatic invertebrates, zooplankton carapaces, on the surface of chitinous organisms, the mucus layer of corals, sediments and suspended detritus (Lipp *et al.,* 2002; Sawabe *et al.,* 2007). Agar embedding coupled with collection of water, mucus and/or suspended detritus at the same time of tissue collection enhance the isolation of bacteria as well as allow a better observation of bacterial communities in

their natural spatial relationships. In addition do not interfere with stains for diagnostic pathology.

The results of our study showed less epithelial desquamation in all samples analyzed when 20 minutes cycles were used (Fig 5.2) and although shortening of the dehydration time with ethanol improve preservation of COTS tissues, it is important to take into account that excessively short times can lead to incomplete dehydration (less than 15 minutes), therefore the use of small samples (0.2-0.5 cm) is highly recommended for correct penetration and clearing (Bancroft & Stevens, 1996). Moreover, autolysis of digestive glands and/or other marine tissues can be reduced by trimming tissues to thin (2 to 5 mm) slices before immersing in fixative or by injecting fixative into the tissues. Short processing programs also require slices of tissue not exceeding 2 mm. This emphasizes the importance of accuracy during gross dissection of the specimens. Our results are in agreement with Shi-ming *et al.,* (2010) which reported that tissues fixed in 10% formaldehyde and dehydrated with gradient ethanol using 15 minutes cycle for each dehydration step showed less tissue damage and distortion of tissues in comparison to standard 15 hours processing time. Conventional tissue fixation and dehydration resulted in significant loss of peritoneal mesothelial cells. Moreover, our results are also in agreement with Ragazzini, 2005; Morales, 2008; and Mulkhom, 2008, which reported that histology of rapidly, processed tissues and their histochemical and immunohistochemical properties were comparable with those of the traditionally prepared material.

Biochemical API test standardization

Biochemical profile

From the 21 tests contained in each API 20NE strip, all *Vibrio* isolates were positive for potassium nitrate, L-tryptophane, D-glucose, esculine, gelatin, B-galactosidase, malate, glucose

and oxidase and negative to L-arginine, urea, arabinose, adipique, caprique. Citrate utilization was found as a discriminatory character between isolates identified as *V. harveyi*, and *V. owensii*. Potassium gluconate test was negative only for a *P. eurosenbergii* strain. N-acetylglucosamine was 50% positive and the other 50% showed weak results.

The species *V. harveyi* and *V. rotiferianus* were characterized as positive for citrate use although 2 out of our 5 *V. rotiferianus* isolates in this study resulted negative. Aside from their high interspecific similarities, intraspecific variability of biochemical profiles within species of the Harveyi group has been considered as an inconvenience of phenotypic methods for precise identification of *V. harveyi*-related strains requiring molecular techniques (Gomez-Gil *et al.,* 2004). However API strips make bacterial identification simpler, faster, reliable for many bacteria species and offer a clear advantage for marine biologist that can easily perform the test and partially identify their bacteria in remote locations (i.e research vessels and remote stations).

Table 5.2. API20NE Biochemical profile of *Vibrio* isolates of Crown-of-thorns starfish

TEST	**Reaction**	***V. owensii*** *(X1,X2,Y2,G 2,GU1)*	***V. harveyi*** *(GU5DARK EN)*	***V. rotiferianus*** *(GREEN AGRESS,NA,YG,GR EEN+,GUIGEN)*	***V. natriegens*** *(CREAM TCBS, CREAM 12-13, HAEMORR)*	***V. fortis*** *(GUMAB, GU5PALEE N)*	***P. eurosenbergii*** *(GU2Y,GU5YEN, GU4YEN)*
NO3	Potassium nitrate	Pos	Pos	Pos	Pos	Pos	Pos
TRP	L-tryptophane	Pos	Pos	Pos	Pos	Pos	Pos
GLU	D-glucose	Pos	Pos	Pos	Pos	Pos	Pos
ADH	L-arginine	Neg	Neg	Neg	Neg	Neg	Neg
URE	Uree	Neg	Neg	Neg	Neg	Neg	Neg
ESC	Esculine	Pos	Pos	Pos	Pos	Pos	Pos
GEL	Gelatin	±	Pos	Pos	Pos	Pos	Pos
PNPG	B galactosidase	Pos	Pos	Pos	±	Pos	Pos
GLU	Glucose	Pos	Pos	Pos	Pos	Pos	Pos
ARA	Arabinose	Neg	Neg	Neg	Neg	Neg	Neg
MNE	Manose	Pos	Pos	Pos	Neg	W	±
MAN	Manitol	W	Pos	±	Pos	W	Neg
NAG	n-acetyl-glucosamine	W	Pos	Pos	Pos	W	w
MAL	Maltose	W	±	Pos	Pos	W	Neg
GNT	Potassium gluconate	Pos	Pos	Pos	Pos	Pos	Neg
CAP	Caprique	Neg	Neg	Neg	±	Neg	Neg
ADI	Adipique	Neg	Neg	Neg	Neg	Neg	Neg
MLT	Malate	Pos	Pos	Pos	Pos	Pos	±
CIT	Citrate	Neg	Pos	±	Pos	Neg	Neg
PAC	Phenylacetique	Neg	±	±	Neg	Neg	w
OX	Oxidase	Pos	Pos	Pos	Pos	Pos	Pos

Temperature

Among the numerous physical factors that influence the ecology of *Vibrio* bacteria, temperature has the most direct and significant effect. It is generally recognized that high densities of *Vibrio* organisms occur during the warmer months, a finding that is consistent with seasonal distribution of clinical cases and fatalities traced to the consumption of contaminated seafood (Levine & Griffin, 1993; Lipp *et al.,* 2002). *Vibrio* species have a broad temperature range from 20 to 37°C (Kaysner *et al.,* 2004; Thompson *et al.,* 2006).

Vibrio species associated with human diseases flourish at 30-37 ºC although marine pathogenic *Vibrio* including those associated with corals have been found at much lower temperatures. For example *V. tasmaniensis* was recognized as part of the biota associated with marine organisms and also was isolated from necrotic tissue of the gorgonian octocoral Eunicella verrucosa from cool temperate waters with temperature ranges of 7.5 to 17°C during an outbreak at Lundy Island, Devon, England in 2003 (Vattakaven *et al.,* 2006). Moreover, lysine decarboxylase and nitrate reductase biochemical test are temperature-dependent (Wuthe *et al.,* 1993). Therefore drastic changes of incubation temperatures can lead to altered results. API 20 E and 20 NE instructions recommend incubation temperatures of 36 ºC and 29 +/-2 respectively. High temperatures potentially inhibit growth of some *Vibrio* bacteria, or lead to weak or erroneous results due to inability to induce bacterial response to the substrates in the strip. Environmental strains of *V. harveyi*, *V. rotiferianus, V. campbellii, V. owensii, V. alginolyticus, V. shilonii, V. corallyticus* recovered from marine echinoderms, aquaculture systems and corals grow well at 20 to 29 ºC. In the case of weak or inconsistent results, marine isolates should be incubated at a similar temperature to those of their natural habitats.

NaCl requirement

The optimum salinity for growth, and the range of salt tolerance differs between *Vibrio* species, mostly in accordance with their natural exposure (Colwell & Spira, 1992; Desmarchelier, 1997). *V. alginolyticus* for example, can grow in high salt concentrations up to 10% (Neill & Carpenter, 2005). Conversely, *V. mimicus* is considered to be a non-halophilic *Vibrio* (Janda, 1987; Thompson *et al.,* 2006). *Vibrio* species show poor or no growth in nutrient broth that has less than 0.5% sodium chloride. Even for *V. cholerae* and *V. mimicus*, better growth is obtained in nutrient broth with 1% salt than in broth with lower concentrations (Colwell & Spira, 1992). Members of the genus *Vibrio* from brackish-water or marine organisms require higher concentrations of cations, particularly Na, K, Mg^{2+} than are usual for terrestrial organisms such as *Aeromonas*, *Pleasiomonas* and *Enterobacteriaceae*. Concentrations of these cations are key factors that influence motility and growth in different ways (Colwell & Spira, 1992). Normally the conventional tests API 20 NE are inoculated with a 0.85% NaCl saline suspension that reconstitutes the media. At this concentration the media imitate the concentration of NaCl in human fluids that is 0.9% approximately. Then the assimilation tests are inoculated with a minimal amount of medium (200 µl) and the bacteria grow if they are capable of utilizing the corresponding substrate. Misidentifications have been reported in association with the use of this salt concentration for identification of marine bacteria (Seidler *et al.,* 1980; MacDonell *et al.,* 1982; Martinez-Urtaza *et al.,* 2006). In addition, biochemical test activities, in particular the formation of indole, lysine and ornithine decarboxylases, arginine dihydrolase, the reduction of nitrate and behaviour in the Voges-Proskauer test are also dependent of NaCl concentration (Wuthe *et al.,* 1993). Weak responses on the assimilation test of marine isolates were found in this study when concentrations of 0.85% NaCl were used. However, addition of NaCl to the

auxiliary media at concentrations of 2% provided much better and consistent results. *Vibrio* isolates used in this study enhanced their biochemical condition at salinities close to those existing in their marine habitat.

Colony size

Another aspect that should be considered is the size of the colony. Some marine bacteria form very small colonies and the instruction manual of the API 20E kit recommends collecting one colony that should be diluted in the media to inoculate the strips. In the case of very tiny colonies it is recommended to pick up several colonies from pure cultures of a single organism to ensure that all tubes and cupules have enough bacteria for growth. An easier standardization of the API test for marine environmental samples can be easily achieved taking into account the incubation temperature, the supplementation of NaCl 2% to the auxiliary media and the colony size.

5.5 Acknowledgements

Funding for this study was provided by the ARC Centre of Excellence for Coral Reef Studies.

CHAPTER 6: Interspecific transmission and recovery of TCBS-induced disease between *Acanthaster planci* and *Linckia guildingi*

6.1 Abstract

The susceptibility of the coral-feeding crown-of-thorns starfish (*Acanthaster planci*) to disease may provide an avenue with which to effectively control population outbreaks that have caused severe and widespread coral loss in the Indo-Pacific. It has been shown that injecting thiosulfate-citrate-bile-sucrose (TCBS) agar into *A. planci* tissues induced a disease characterized by dermal lesions, loss of skin turgor, collapsed spines, and accumulation of mucus on spine tips. Moreover, the symptoms (and presumably the agent) of this disease would spread rapidly intraspecifically, but interspecific transmission (to other species of equinoderms) is yet to be examined. *Vibrio rotiferianus*, which was previously reported as a pathogen isolated from lesions of experimentally-infected *A. planci*, was also recovered from *Linckia guildingi* lesions after several days of direct contact with diseased *A. planci* demonstrating disease transmission. However, all *L. guildingi* fully recovered after 31 ± 16 days. Further studies are in progress to understand the ecology of *Vibrio* infection in *A. planci* and the potential transmission risk to corals, fishes, and other echinoderms to evaluate if injections of TCBS could be a viable tool for controlling *A. planci* outbreaks.

† This research was conducted in collaboration with Ciemon Caballes (University of Guam) and was published (as written) in *Diseases of Aquatic Organisms*: Caballes CF, Schupp PJ, Pratchett MS, Rivera-Posada JA (2012). Interspecific transmission and recovery of TCBS-induced disease between *Acanthaster planci* and *Linckia guildingi.* doi: 10.3354/dao02480. This project was conceived as an extension of my own research, but Mr Caballes assisted with development and undertaking of experimental protocols, and also contributed to writing this paper.

6.2 Introduction

Outbreaks of the crown-of-thorns starfish, *Acanthaster planci* (L.) have had catastrophic effects on many reefs in the Indo-Pacific (Chesher, 1969), and remain one of the major causes of coral loss in this region (Bruno & Selig 2007, Pratchett *et al.,* 2011). During outbreaks, high densities of *A. planci* (up to 1 starfish per m2) can rapidly consume >90% of corals across vast tracts of reef habitat (e.g., Chesher, 1969). On Australia's Great Barrier Reef, outbreaks of *A. planci* account for 37% of recorded coral loss since 1995 (Osborne *et al.,* 2011). Similarly at Moorea, in the central Pacific, outbreaks of *A. planci* caused greater levels of coral loss than either cyclones or climate-induced coral bleaching (Trapon *et al.,* 2011). Control of outbreaks of *A. planci* may therefore, be the most immediate and effective mechanism by which to reverse sustained declines in the abundance of live coral cover on the Great Barrier Reef (e.g., Bellwood *et al.,* 2004) and throughout the Pacific (Bruno & Selig 2007).

Previous efforts to control outbreak populations of *A. planci* have been costly and ineffective. At Bootless Bay in Papua New Guinea for example, divers removed (hand-collected) or killed (injected with sodium bisulphate) almost 10,000 individual sea stars during very extensive control efforts, but were still unable to prevent extensive loss of live coral, nor restrict the spread of starfishes (Pratchett *et al.,* 2009). However, *A. planci* (like many echinoderms) are susceptible to infectious disease (Pratchett, 1999), which could provide an option for controlling outbreaking populations of *A. planci* (Rivera-Posada *et al.,* 2011a) or urchins (Miller, 1985). The close proximity between starfish during outbreaks would facilitate rapid transmission of potential pathogens, eliminating the need to directly kill or collect each individual starfish. Diseases have also been implicated in abrupt declines of *A. planci* numbers at the end of outbreak events (Zann *et al.,* 1990).

Thiosulfate-citrate-bile-sucrose (TCBS) agar is a selective culture medium that inhibits gram-positive organisms, suppresses coliforms, and allows selective growth of *Vibrio* spp. Rivera-Posada *et al.,* (2011a, 2011b, 2011c) showed that injection of TCBS broth into *A. planci* digestive organs could induce systemic dysbiosis and facilitate the growth of opportunistic *Vibrio* spp., leading to rapid mortality of individual starfishes. This disease is characterized by dermal lesions, loss of body turgor, matting of spines, and accumulation of mucus on spine tips (Fig. 1A, 1B). These signs are consistent with natural instances of disease previously observed in *A. planci* (Pratchett, 1999). The pathogenic *Vibrio* spp. can also be transmitted to healthy *A. planci* via direct contact with diseased individuals under certain environmental conditions (Rivera-Posada *et al.,* 2011a). Although the possibility of exploiting diseases in *A. planci* for developing biological control measures is promising, significant testing is needed to ensure that there is no chance of disease transmission to non-target species. There have been several exemplary biological control programs implemented in terrestrial systems, but little is known on the potential risks and effectiveness in marine systems (Secord, 2003). The purpose of this study was to explore the potential of interspecific transmission of putative disease agents from *A. planci* to other common and co-occurring echinoderm species, specifically *Linckia guildingi* (Gray starfish).

6.3 Materials and Methods

To test the possibility of interspecific transmission, seemingly healthy individuals of Guildingi's starfish (*Linckia guildingi*) were placed in direct contact with diseased individuals of crown-of-thorns starfish (*A. planci*). Disease in individual *A. planci* was induced through injection with TCBS, following Rivera-Posada *et al.,* (2011a), which causes allergic reaction and proliferation of naturally occurring bacteria (rather than directly introducing any new putative disease pathogens). A total of 5 *L. guildingi* were placed in individual containers with a diseased *A. planci*, while a further 5 *L. guildingi* were placed in

containers in direct contact with healthy *A. planci* to serve as controls. Experiments were performed at the University of Guam – Marine Laboratory with flow-through seawater at ambient conditions (mean temperature = 28°C, pH = 8.3, salinity = 34.5 ppt) for 60 days. All study organisms were collected at Haputo point, Guam, USA (13°34′14.16″ N, 144°49′18.12″ E).

Bacterial density was monitored every 8 hrs for two days by collecting water and mucus samples, which were appropriately diluted and plated on TCBS plates. Colony-forming units (CFUs) were counted after 24 hrs incubation at 28°C. Dead and decomposing *A. planci* were removed from the tanks after 4 days. To identify putative pathogens, lesions on *L. guildingui* were swabbed and plated on TCBS agar and DNA was extracted from four isolates. Comparable studies were also conducted for *A. planci* as described in Rivera-Posada *et al.,* 2011 b). All PCR amplifications were performed in a BIORAD 170-9701 PCR thermocycler (California, USA). PCR reactions (20 µl) contained approximately 20 ng of genomic DNA, 1x PCR buffer (Tris·Cl, KCl, $(NH_4)_2SO4$, 1.5 mM MgCl2; pH 8.7) (Qiagen), 0.5 µM of each primer, 200 µM dNTPs and 0.5 units of Taq DNA Polymerase (Qiagen). The first cycle was preceded by initial denaturation for 15 min at 95°C. Each cycle (30 cycles) consisted of denaturation for 1 min at 95°C, annealing for 1 min at 50°C, and extension for 1 min at 72°C. The last cycle was followed by a final extension step for 7 min at 72°C. Finally, PCR products were visually inspected in ethidium bromide-stained 1% agarose gels against known size standards to verify the presence of amplicons of the expected sizes. PCR products were thereafter purified and sequenced by Macrogen Ltd. (South Korea) using VtopA400F (GAG ATC ATC GGT GGT GAT G) and VtopA1200R (GAA GGA CGA ATC GCT TCG TG) primers (Sawabe *et al*., 2007).

6.3 Results and Discussion

There were no signs of disease in *L. guildingi* starfish collected from the field, nor were starfish placed together with healthy *A. planci* ever seen to exhibit symptoms of disease. In contrast, four out of five *L. guildigi* starfish placed with diseased *A. planci* developed skin lesions within 3 to 9 days (Fig 6.1). These lesions could be a result of the lysis of connective tissues induced by opportunistic bacteria as shown in histopathological analyses of diseased *A. planci* (Rivera-Posada *et al.,* 2011 c).

Bacterial growth on TCBS medium based on CFUs/ml of water sample peaked at 32 hours after injection, but decreased 8 hrs later (Fig 6.2A).. On the other hand, there was an exponential increase in bacterial growth in mucus samples after 24 h and continued to increase for 24 hrs thereafter (Fig 6.2B). The mucus secreted by *A. planci* after TCBS injection could be a potential pathway for intra- and interspecific transmission of pathogens and partly explains why this induced disease appears to be transmitted only through direct contact (Rivera-Posada *et al.,* 2011a).

Isolates from sampled lesions were identified as *Vibrio rotiferianus* using NCBI BLASTN database on the basis of 99% similarity to *topA* gene sequences. This was consistent with topologies based on 16S rRNA, *mreB* and *topA* analysisis previously reported by Rivera-Posada *et al.,* (2011 b), which described *V. rotiferianus* as a pathogen isolated from tissue lesions on infected *A. planci.* Sequences were deposited in GenBank under the accession numbers HQ591351, HQ591352, HQ591353, HQ591354. Nearly all *Vibrio* spp. under the Harveyi group have been reported as causative agents in echinoderm diseases (summarized in Rivera-Posada *et al.,* 2011b). Moreover, pathogenic *V. rotiferianus* was recovered from *A. planci* lesions, from mucus travelling from sick *A. planci* to *L. guildingi* and from *L. guildingi* lesions.

Vibrios are well equipped with a series of virulence factors and adaptive response mechanisms such as chemotaxis that greatly influence their infectivity (Butler & Camilli,

2004). For example, *V. anguillarum* and *V. alginolyticus* undergo positive chemotaxis to mucus collected from fish skin and intestines (Bordas *et al.,* 1998). *V. shiloi*, a coral pathogen, migrates towards coral mucus (Banin *et al.,* 2001); and *V. cholera* moves into intestinal mucus (Freter & O'Brian, 1981).

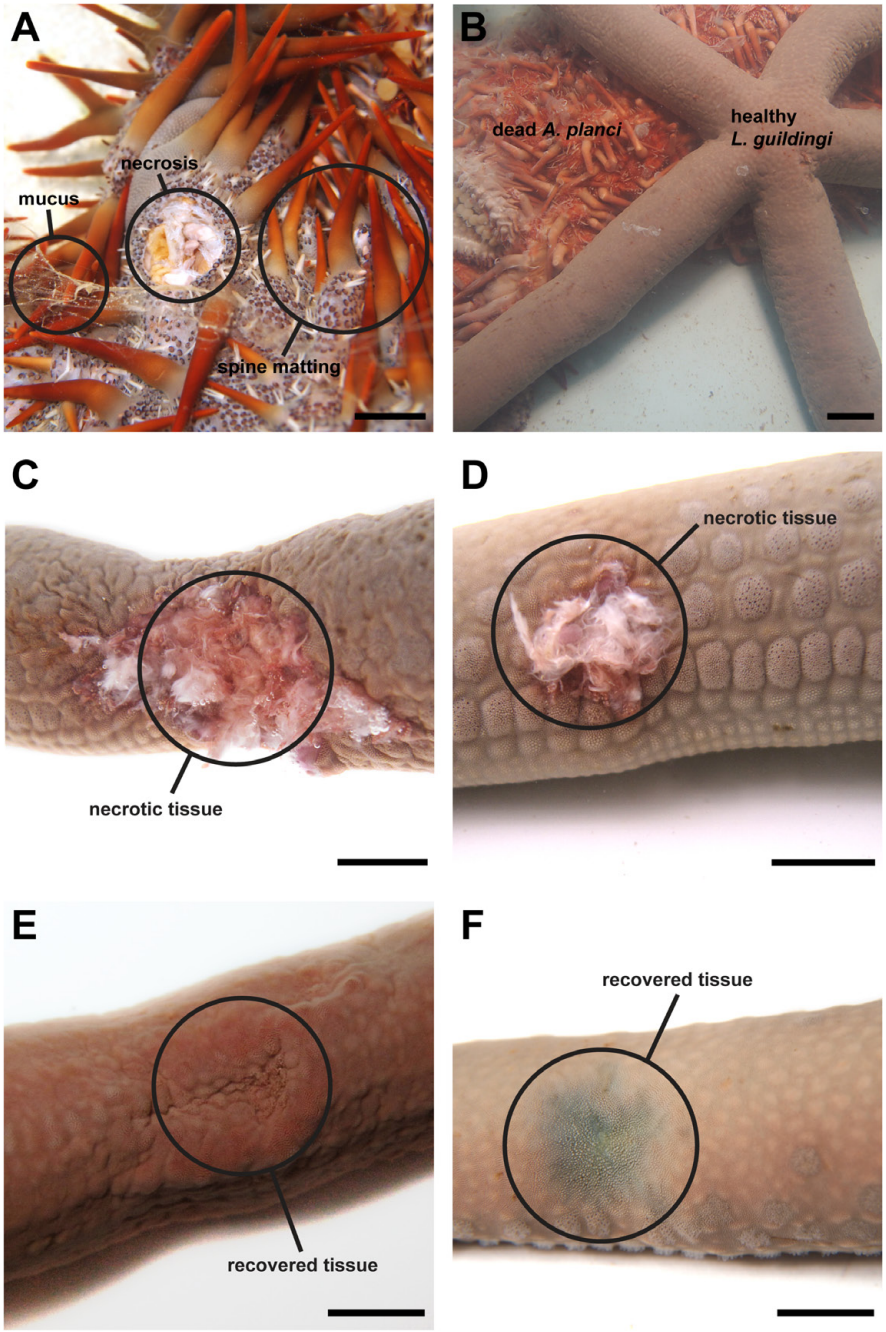

Figure 6.1. TCBS-induced disease in *A. planci*: signs, transmission and recovery. (A) diseased *A. planci* showing loss of body turgor, necrotic tissue, mucus, and matting of spines; (B) healthy *L. guildingi* in contact with diseased and dead *A. planci*; (C, D) lesions on *L. guildingi* afer direct contact with diseased *A. planci*; (E, F) recovered lesions on *L. guildingi*. Scale bar = 1cm.

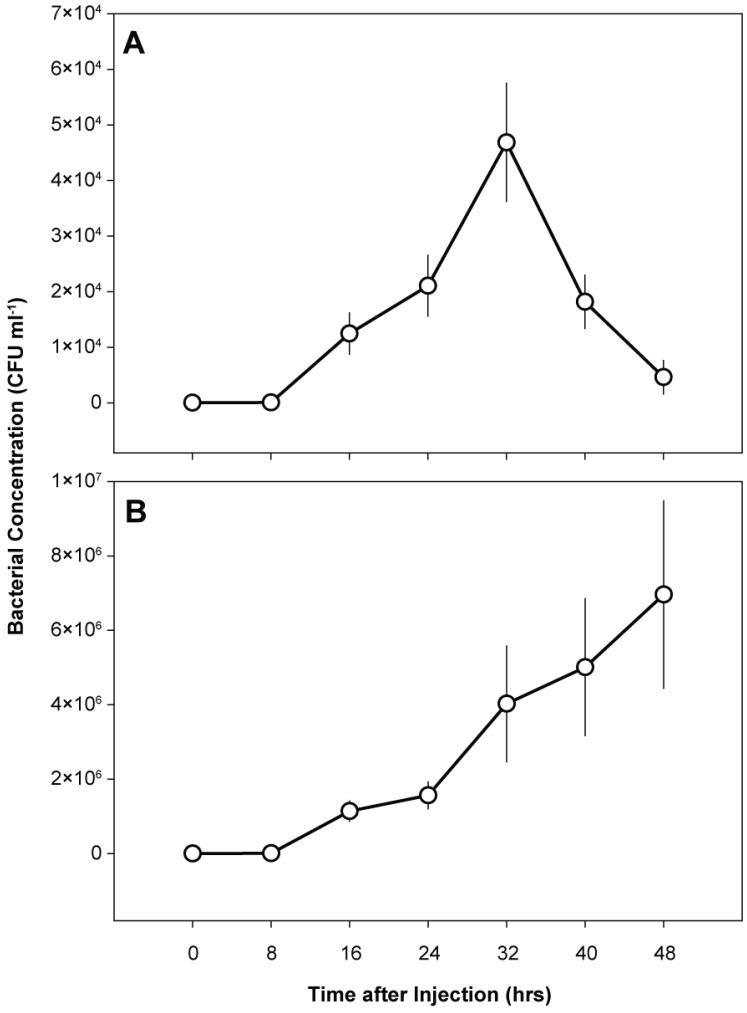

Figure 6.2. Bacteria growth after TCBS injections. (A) mean CFUs ml^{-1} ± SE in water samples; (B) mean CFUs ml ± SE in mucus samples.

Lesions in sick *A. planci* did not recover and disease progressed, leading to 100% mortality within 24 to 48 h. Rivera-Posada *et al.,* (2011a) also reported mortality in *A. planci* that were in contact with diseased individuals. On the other hand, all *L. guildingi* starfish

fully recovered (lesions healed) 31 ± 16 days after lesions were first observed (Fig 6.1). This disparity in resilence could be attributed to the lower antibiotic activity properties of *A. planci* compared to other asteroids –showing very weak activity against gram-negative bacteria (Burkholder, 1973). Although all starfish were collected in the same area, it is also difficult to determine the level of fitness of each individual, which could also be important in disease suceptibility and recovery. Individuals with higher levels of stress are more susceptible to facultative pathogens (Sutton *et al.,* 1988).

Disease induction through TCBS injection could be an effective option for controlling *A. planci* populations at a larger scale. Although this biological control mechanism does not involve the introduction of pathogens, further disease transmission experiments are warranted, especially in corals, carnivorous fishes, and benthic detritus feeders to verify host specificity and vulnerability of non-target species. This study showed a successful interspecific transmission of disease to *Linckia guildingi*, confirms the pathogenicity of *V. rotiferianus* to echinoderms and stress the high risk of applications of TCBS as a control method of *A. planci* outbreaks in the field without more detailed studies of the quorum sensing mechanisms involved in the induction of disease. It is crucial to take into account that *Vibrios* as opportunistic species can evolve and recombine genes under high microbial contact in animal guts or as part of aquatic biofilms, transferring or inducing expression of virulence genes in less or non-pathogenic populations (Thompson *et al.,* 2004). Furthermore, *Vibrios* are ubiquitous in the marine environment and experimental application of TCBS even in an isolated reef without more detailed studies could have disastrous results in marine ecosystems were no boundaries could limit the spread of disease. This is a portion of on-going work to evaluate if TCBS injection could be a new tool for management of COTS outbreaks.

6.4 Acknowledgments

We would like to thank Susanna Whitfield and Ana Cano-Gomez for their assistance. PJS and CFC acknowledge support from NOAA and US Dept. of Interior Grant CRIGU10 and funds for JARP were provided by the ARC Centre of Excellence for Coral Reef Studies, Australia.

CHAPTER 7: The Role of protein extracts in the induction of disease in *Acanthaster planci*

7.1 Abstract:

Thiosulfate-citrate-bile-sucrose agar (TCBS) has been shown to induce pathogenesis in the crown-of-thorns starfish (COTS), *Acanthaster planci*, providing potentially novel options to control COTS outbreaks, but the mechanistic basis for this effect is unknown. This study explores reactions of COTS to individual ingredients of TCBS, testing for allergic reactions versus pathogenesis. Four out of nine TCBS chemical ingredients tested induced allergic reactions and death in injected COTS. Peptone 10 g l^{-1} and oxgall 8 g l^{-1} induced 100% mortality, while yeast extract and agar induced death in 40 % and 20% of COTS, respectively, 48 h after injection. Peptone was evaluated at three different concentrations (10g, 5g, and 1g l^{-1}). Peptone 10 g l^{-1} induced 100% mortality, peptone 5 g l^{-1} killed 60% of injected COTS, and peptone 1 g l^{-1} induced death in only 20% of challenged COTS, indicating that toxicity of peptone is concentration-dependent. Sodium citrate induced moderate mucus production in all COTS challenged, but disease did not progress and COTS completely recovered after 52 h. The remaining ingredients in TCBS did not produce any kind of clinical signs of disease. Peptone, oxgall, and yeast are potentially useful in controlling outbreaks because these are protein extracts that can be safer to use compared to previously noxious chemicals. In addition, lowered concentrations are required to kill COTS, therefore increasing efficiency by saving time, money, and effort in COTS control programs.

†This chapter is in press (as written) in *Journal of Experimental Marine Biology and Ecology*: Rivera-Posada JA, Owens L, Caballes CF, Pratchett M (2012). The Role of protein extracts in the induction of disease in *Acanthaster planci*. *JEMBE* doi 10.1016/j.jembe.2012.06.008.

7.2 Introduction

Population outbreaks of the crown-of-thorns seastar (COTS), *Acanthaster planci*, are one of the most significant biological disturbances on tropical coral reefs. Importantly, localized outbreaks of COTS are the major contributor to coral loss and reef degradation on Indo-Pacific reefs (Bruno & Selig, 2007; Pratchett *et al.,* 2009). However, science for management of tropical coral reefs has been focused other treats (e.g, climate change; Hughes *et al.,* 2003), such that there has been limited progress in understanding the causes, cosequences, and potential options for managing COTS outbreaks. Control of COTS outbreaks may be the most immediate and effective mechanism by which to reverse sustained declines in the abundance of live coral cover, thereby maximizing opportunities for coral reef organisms to resisit and adapt to sustained and ongoing threats from climate change and other more direct anthropogenic disturbances.

The benefits of controlling outbreaks of COTS are well recognized (e.g., Yamaguchi, 1986) but previous control measures have been costly, largely ineffective and often involve dangerous side effects. One of these control methods involved injecting starfish with a variety of noxious chemicals that were known to be harmful to the marine environment, such as formaldehyde (CH_2O) and copper sulfate ($CuSO_4$). Copper sulfate is highly toxic to fish and many aquatic invertebrates, such as crabs, shrimps, and oysters (Yanong, 2010). Sodium hypochlorite (NaClO), ammonia (NH_3), ammonium hydroxide (NH_4OH), acetic acid (CH_3COOH), and sodium bisulfate ($NaHSO_4 \cdot H2O$) have also been used in past control efforts (Birkeland & Lucas, 1990; Harriott *et al.,* 2003). Sodium bisulfate is currently considered the best option to kill COTS *in situ*. However, this requires careful administration of solution into multiple areas of the oral disk, otherwise starfish experience only localized tissue damage and persist. Moreover, high concentrations (140 g l^{-1}) and volumes (25ml of solution per starfish) of

sodium bisulfate are used in controlling outbreak populations, which may comprise in excess of 53.750 sea stars/km^{-2} (Kayal *et al.,* 2011). Sodium bisulfite is a strong oxygen scavenger that is widely used to inhibit corrosion and remove traces of residual oxygen or chlorine in the brine recirculation systems of desalination plants at doses of just 0.5mg/l (Abuzinada *et al.,* 2008; Latteman *et al.,* 2008).

This, coupled with the fact that oxygen become less soluble in seawater with increasing temperature and salinity levels (as are predicted to occur with climate change), certainly can have detrimental effects on reef ecosystems when used on a large scale such as in control of population outbreaks of COTS (Romand & Gauzen, 1993). Low levels of oxygen in seawater can impair reef ecoystems resulting in marked decreases in marine life. Low oxygen levels recorded along the Gulf Coast of North America have also led to reproductive problems in fish involving decreased size of reproductive organs, low egg counts, and lack of spawning (Romand & Gauzen 1993; Diaz & Rosenberg, 2008).

Aside from injecting chemicals into individual starfish, there have been two methods used to attempts to minimize localized coral loss during outbreaks of COTS; (1) Hand collection and disposal of starfish on land and (2) Construction of physical barriers to restrict movement of COTS (Harriot *et al.,* 2003). Physical removal of starfish limits the potentially deleterious effects of poisoning, but is very labor intensive and ineffective. In southern Japan, for example, fishermen and divers collected and destroyed 13 million starfish from reefs in the Ryukyus from 1970 to 1983 (Yamaguchi, 1986). Despite this significant and prolonged effort, COTS still killed in excess of 90% of coral in areas where starfish were collected. Collection of starfish may be effective on very small-scales (e.g., to protect areas of high tourist visitation), but this requires considerable effort and vigilance. Similarly, installing low fences to prevent movement of

starfishes into tourism areas is only effective in small areas and for short periods (Harriot *et al.,* 2003). Developing more effective and less harmful methods to control COTS outbreaks is imperative (Nash *et al.,* 1988).

Like many echinoderms, COTS are very susceptible to disease (Pratchett, 1999), and this might provide novel opportunities to control population outbreaks. Primary sources of infectious disease are *Vibrio* bacteria and *Branchiomycetes* fungi (Sparks, 1981; Sutton *et al.,* 1988; Fenner, 1995). Rivera *et al.,* (2011) also explored an alternative control method by injecting TCBS into COTS in order to induce dysbiosis followed by a fatal disease that is transmitted to healthy in-contact COTS under favorable conditions. They isolated several *Vibrio* species from tissue lesions of injected and in-contact COTS and hypothesize that inhibition of competitive bacteria and increases in *Vibrio* cell densities promoted by TCBS activate the quorum sensing (QS) mechanism of vibrios, turning on virulence factors inducing rapid onset of symptoms followed by death. Sutton *et al.,* (1988) and Birkeland (1990) previously tested *Vibrios* as potential COTS pathogens state that only stressed animals were susceptible to disease. Moreover, an allergic reaction to TCBS ingredients has not been yet examined, which is essential to understand and elucidate the pathways, used to drive the presentation of the experimentally induced disease in COTS.

The aims of this study was to individually test TCBS components to assess if an allergic reaction is contributing to the breakdown of COTS immune system, allowing infection of opportunistic pathogens after TCBS injections. A second aim is to explore novel less toxic options (TCBS protein extracts) to control COTS outbreaks. This is a segment of on-going work to evaluate whether TCBS media culture and/or its' ingredients could be used as a new tool for management of COTS outbreaks.

Allergic reactions to some of the TCBS components have been previously documented. Harris & Owens, (1999) reported high mortality of *Penaeus monodon* larvae challenged with luminous broth. They concluded that protein digests such as peptone and yeast extract used in luminous broth were directly toxic to the larva. Yeast and peptone are also used as vaccine ingredients to deliver antigens and are the causes of numerous allergic reactions after vaccination (CDC, 2012).

7.3 Materials and methods

COTS collection and maintenance conditions

A total of 60 COTS were collected at Hospital Point, Guam, USA (13°30.154' N, 144°46.193' E) at 15 m depth, pH 8.3, salinity 34.5 ppt. Experiments were conducted at the University of Guam – Marine Laboratory and COTS were distributed in 12 groups of five starfish and individually placed in 68 L plastic containers with flow-through seawater at ambient conditions (mean temperature = 28°C, pH = 8.3, salinity = 34.5 ppt).

Media culture components

TCBS is a highly selective agar that meets the nutritional requirements of *Vibrio* spp., allowing vibrios to compete with intestinal flora (Baron *et al.,* 1994). TCBS components include yeast extract and bacteriological peptone that provide the nitrogen, vitamins and amino acids. Sodium citrate, sodium thiosulfate and ox bile are selective agents, providing an alkaline pH to inhibit Gram-positive organisms and suppress coliforms. An increased pH is used to enhance growth of vibrios, because these organisms are sensitive to acid environments. Sucrose is the fermentable carbohydrate. Sodium chloride stimulates the organism's growth and maintains the osmotic balance of the medium. Sodium thiosulfate is a sulfur source that acts with ferric citrate as an

indicator to detect hydrogen sulfide production. Bromothymol blue and thymol blue are pH indicators and agar is the solidifying agent.

Nine TCBS chemical components were used to challenge COTS. Ten ml of each chemical were injected to individual healthy COTS with a 21gauge syringe to examine which TCBS components induced disease and death. COTS were individually placed in separate aquaria to observe their behavior, clinical signs, latency period of the disease and time to death. The clinical signs of disease evaluated were evaluated and separated into those potentially related to an allergic, reversible response (hyperactivity, discolored skin, mucus accumulation, oedema/reddened tissues) and those related to disease (necrotic skin, ulcerations/blisters, loss of body turgor, matting/loss of spines, exposed internal organs, oedema/reddened tissues, lyses of connective tissues and time to death) realising there is considerable overlap and many signs are not mutually exclusive to one category. Severity indices of clinical signs of disease were assigned as 1 = low, 2 = moderate and 3 = strong reaction for each factor evaluated.

Table 7.1. List of TCBS Difco™ (MD, USA) chemical components tested and their concentrations. All chemicals were used at the same concentration of TCBS except for peptone that was evaluated at 1, 5 and 10 g l^{-1} to examine if peptone toxicity was concentration dependent.

Chemical Components Tested (g l^{-1})	**Commercial brand**
Sucrose (20g)	Difco, MD, USA
Oxgall (8g = 5g ox bile + 3g sodium cholate)	Difco, MD, USA
Peptone (1g, 5g, and 10g)	Difco, MD, USA
Yeast (5g)	Difco, MD, USA
Agar (15g)	Sigma, MO, USA
Sodium Thiosulfate (10g)	Sigma, MO, USA
Ferric Citrate (1g)	Sigma, MO, USA
Sodium Thiosulfate (10g) + Ferric Citrate (1g)	Sigma, MO, USA
Sodium Chloride (10g)	Sigma, MO, USA
Sodium Citrate (10 g)	Baker Chemical, NJ, USA

7.4 Results

Four out of nine TCBS chemicals tested in this study induced allergic reactions and death in injected COTS. Peptone 10 g l^{-1} and Oxgall 8 g l^{-1} induced 100% mortality. Yeast extract 5 g l^{-1} and agar 14g l^{-1} induced death in 40 % and 20% of injected COTS respectively. Sodium citrate 10g l^{-1} induced moderate mucus production in all COTS but all individuals recovered after 48 h. The remaining chemicals (sodium thiosulfate, ferric citrate, mix of sodium thiosulfate + ferric citrate, sucrose and sodium chloride) did not induce any kind of allergic reactions nor lead to symptoms of disease.

*Peptone***.**

Peptone 10 g l^{-1} induced death in all COTS injected. Four sea stars died 24 h post-injection and the remaining one at 48 h. There was a marked loss of body turgor in all COTS challenged that started at 4 h and reached the maximum intensity 24 h after injection (before death). COTS injected with peptone 10g l^{-1} also displayed moderate lysis of connective tissues and numerous open sores that expose the internal organs at 24 h. However, there was low mucus production. On the other hand peptone 5 g l^{-1} induced death in 60% of injected sea stars (3 out of 5) and time to death was 48 h requiring double amount of time to induce death when compared to COTS injected with peptone 10g l^{-1}. Loss of body turgor started just one hour post-injection and reach their maximum peak at 48 h. Changes in the color of skin were observed in all COTS injected with peptone 5 g l^{-1} between 24 and 48 h. Only one *A. planci* injected with peptone 1 g l^{-1} died 24 h after injection; the other four sea stars showed signs of disease such as loss of body turgor, production of mucus and mating of spines but recovered after 24 h (Fig 7. 2).

Oxgall.

Oxgall produced death in all injected sea stars. Four died in 24 h and the other one at 48 h after injection. COTS injected with oxgall showed a marked hyperactivity immediately after injection; moderate loss of body turgor and matting of spines was observed at 7 hours. There was minimum mucus production during the entire evaluation period (Fig 7.1, 7. 2).

Yeast.

Yeast caused death in 40% of COTS tested (2 out of 5). The signs included loss of body turgor and mucus production. Matting of spines was observed in 3 out of 5 COTS injected with yeast. Loss of body turgor was observed in 2 sea stars 10 hours post-injection. COTS injected with

yeast showed a slow induction of disease with 48h until mortality when compared to oxgall injected COTS (80% died in 24 h) (Fig 7. 1, 7.2).

Agar.

Agar induced death in just one sea star (1 out of 5) 48 post-injection. Three out of 5 sea stars displayed mucus production 4 h after agar injection and loss of body turgor after 10 h. The sea star that died showed ulcerations and exposure of internal organs at 48 hours and also moderate lysis of connective tissues.

Sodium Citrate.

Sodium citrate did not induce death in the five COTS challenged. Four out of five COTS injected showed a moderate mucus secretion in the first 24h after injection. However, COTS totally recovered after 48 h (Fig 7.2).

Sucrose

Sucrose did not induce any signs of disease.

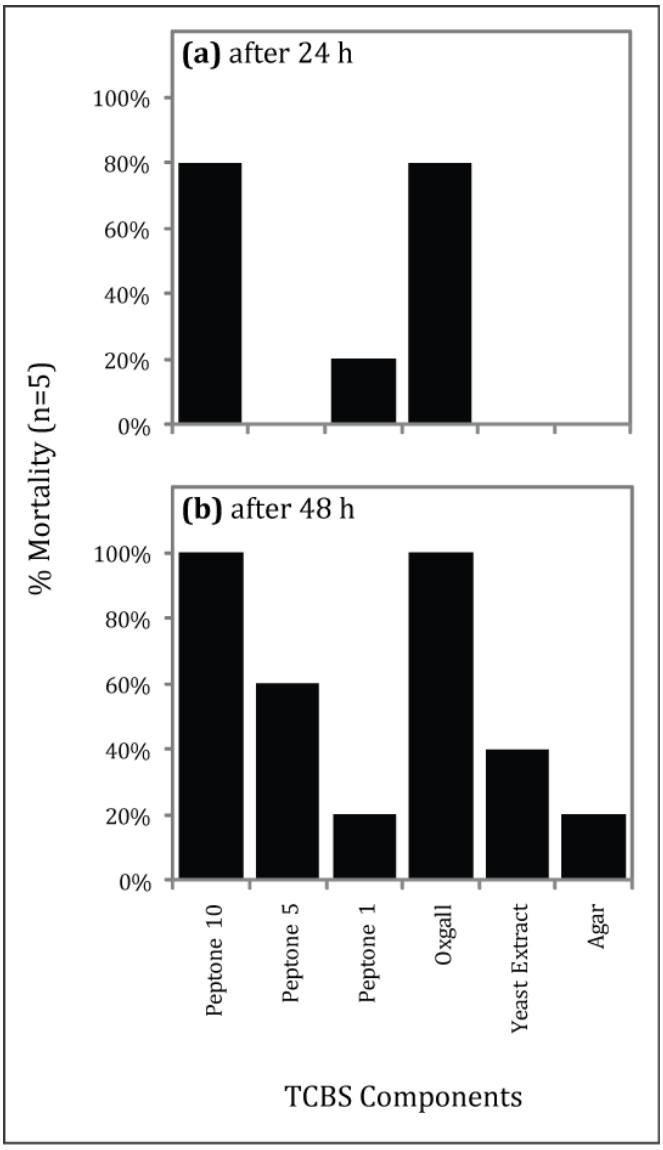

Figure 7.1. Mortality of COTS challenged with components of TCBS culture medium. (a) 24 h and (b) 48 h after injection. Peptone 10 = peptone 10 g l^{-1}, Peptone 5= peptone at 5g l^{-1}/, Peptone 1= peptone at 1 g l^{-1}.

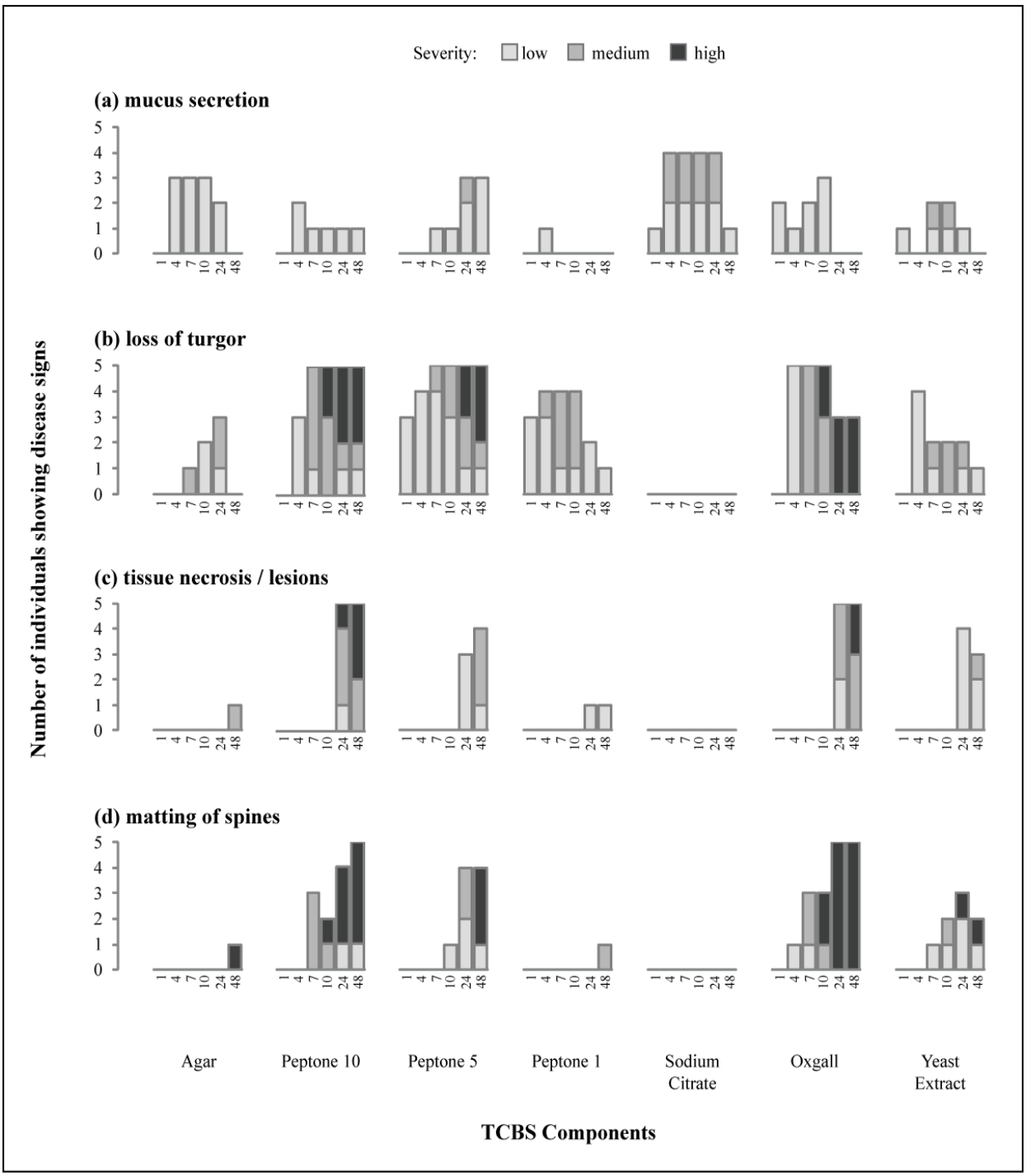

Figure 7.2. Time of appearance, severity, and number of individuals showing signs of disease. (a) mucus secretion, (b) loss of body turgor, (c) lesions and necrosis of dermal tissue, and (d) matting of spines. Levels of severity ranged from low to high (i.e. localized to widespread manifestation of disease sign)

7.5 Discussion

Toxigenesis is a common underlying mechanism by which many bacterial pathogens produce disease. Usually the best media for toxin production contains derivatives of animal products such as chopped or cooked meat, brain heart infusion, horse blood, or protease peptone, casein, trypticase and tryptone which are enzyme preparations obtained from animal pancreas or digests of dairy products (Al Saif & Brazier, 1996; Braun *et al.,* 2000; Fang *et al.,* 2009). Not surprisingly, all TCBS components that induced disease and death in injected *A. planci* (peptone, ox gall, yeast and agar) contained protein extracts. Peptone is an enzymatic digest of animal protein used as an organic nitrogen source in microbiological culture media for cultivation of a variety of bacteria and fungi and has been used extensively for the growth of bacteria in the production of toxins, vaccines and other biological products i.e Iwanaga *et al.,* (1986) used peptone for production of cholera toxin by *Vibrio cholerae* O1 El Tor. Rolfe & Finegold, (1971) also used peptone and yeast for growth and toxin formation of *Clostridium difficile*. Therefore peptone could have induced disease in injected COTS through production of toxins by indigenous microflora and/or direct allergic reaction.

It is possible that the method of preparation of the peptone might leave active proteases in the peptone that affected the COTS. Damage to proteins, vitamins and cofactors is a function of both heat and time. The degree of heat and the time for which the heat is applied can negatively affect yield. For this reason, many peptone manufacturers use sterile filtration instead of heat. The process used by Difco™ is hydrolysis/digestion, centrifugation, filtration, concentration and drying. Is it possible that the protease used to make the peptone is not totally inactivated by these methods. Therefore, more experiments with heat denatured or EDTA-treated peptone are necessary to determine if this mechanism is involved in the induction of the disease and to test if

there is association between the mode of action of oxgall that also contains proteases and induced 100% mortality.

Oxgall is a greenish-brown liquid mixture containing cholesterol, lecithin, taurocholic acid, and glycocholic acid that is widely used in medicine and in the Arts. In Medicine is employed at low doses in selective cultures such as TCBS to inhibit Gram-positive and coliforms and promote growth of vibrios. In the arts is mixed with alcohol and used as the wetting agent in marbling, engraving, lithography, and watercolor painting. Oxgall in combination with alcohol and/or at high doses can irritate mucus membranes in humans (Murray, 1995). Therefore more studies are required to determine if special safety procedures might be necessary for operators planning to use oxgall at low doses as a control compound. Nevertheless oxgall could induce disease in injected COTS through different mechanisms: 1). Inducing dysbiosis by inhibition of normal COTS microflora and promoting growth of vibrios (Rivera *et al.,* 2011a); 2). Generating an allergic reaction. It is well documented that antigenic peptides stimulate anaphylactic reactions and other undesirable immune reactions in injected hosts promoting tissue damage due to the release of histamine, cytokines, and inflammatory cells inducing more edema, congestion and inflammation of affected tissues (CDC, 2012); and 3). by toxin formation of indigenous microflora (Thompson, 2004).

The sucrose molecule is a disaccharide composed of 50% glucose and 50% fructose. Glucose and other rapidly used carbon sources down-regulate toxin production (Dinnen *et al.,* 2007). Karlsoon, (2008) reported that addition of 0.9% glucose to peptone-yeast cultures prior to entry into stationary phase (8 h of growth) strongly reduced toxin production of *Clostridium difficile*. Expression of promoters of the *tox* genes is repressed by rapidly used carbon sources

(Dupuy & Sonenshein, 1998). Synthesis of *ToxB* cytoxin was greatly reduced when glucose was used as the growth substrate (Osgood *et al.,* 1993). Interference with toxin formation was also observed when adding glucose to the fermentation medium (Rolfe & Finegold, 1979; Fang *et al.,* 2009). Rivera *et al.,* (2011a, b) previously used TCBS as a disease inducer in COTS. TCBS contains 20g l^{-1} of sucrose as the fermentable carbohydrate to provide energy and promote growth of vibrios. However this study individually tested the components of TCBS to determine if an allergic reaction is involved in the induction of disease in COTS. The absence of sucrose is important as it removes an inhibitor of the production of toxins leading to the rapid appearance of clinical signs of disease and death of COTS injected with animal protein extracts such as peptone and ox gall.

Another protein derivate that induced disease and death in injected COTS is yeast extract that is a concentrate of the water-soluble portion of autolyzed *Saccharomyces cerevisiae* cells. It is a non-animal product (herein, animal is defined as a vertebrate) used extensively for bacterial, fungal, mammalian and insect cell culture. Yeast extract is a mixture of peptides, amino acids, carbohydrates, as well as naturally occurring B vitamins. Its addition to many media or fermentation broths increases the yield of organisms and is recommended where rapid growth is required. Yeast has a low endotoxin level and is considered a safe product that does not contain any substances presenting a health hazard to animals within the meaning of the Dangerous Substances Directive 67/548/EEC. Additionally, Rolfe & Finegold, (1979) reported that when non-animal/non-dairy peptones were used, such as phytone or yeast extract, the toxin titers were markedly lower in comparison to animal protein extracts. Our study are in agreement with Rolfe & Finegold, (1979) because the animal protein compounds - peptone 10g l^{-1} and oxgall induced 100% mortality in just 24 hours which can be related to the fact that animal proteins induced

more toxin production, allergic reactions and tissue damage in comparison to yeast extract that just induced death in 40% of injected starfish and required double amount of time to produce death (48 h). This would suggest the mode of action of the yeast extract was to stimulate the growth of indigenous microflora rather than supply a toxin in itself or components that are easily incorporated into a toxin (i.e. not high in pro-toxin components).

Agar also known, as China grass is a mixture of polysaccharides extracted from species of the red algae known as agarophytes. Agar is used throughout the world to provide a solid surface-containing medium for the growth of bacteria and fungi. Microbial growth does not destroy the gel structure because most microorganisms are unable to digest agar. Therefore circulating agar, effectively a foreign body that cannot be digested or degraded could induce an allergic reaction in injected COTS.

Sodium citrate is used as food additive in energy drinks which improve performance of runners (Oopik *et al.,* 2003). It is also used as anticoagulant in blood transfusions. The citrate ion chelates calcium ions in the blood by forming calcium citrate complexes, disrupting the blood clotting mechanism. Swelling, fast heart rate, bloody stools, severe diarrhea, and seizures have been reported in allergic humans. Therefore the production of mucus observed after injection with sodium citrate may represent a defense mechanism to expunge noxious substances.

Sodium thiosulfate did not cause any visible reaction in COTS. Sodium thiosulfate is used for the neutralization of chlorine and/or iodine solutions at hatchery and aquaculture facilities. Though normally used intravenously, orally and transdermally it is also used for treating tap, distilled and reverse osmosis water for water detoxification, mineralization and other extended health benefits. Intravenous sodium thiosulfate is currently used as an antidote for the treatment

of cyanide poisoning and prevention of toxicities of cisplatin cancer therapies. It is used as a food and medicinal preservative and topically used as an antifungal medication. Therefore the possibilities to induce disease in injected COTS are minimal.

7.6 Conclusions

We believe that peptone, ox gall and yeast offer the possibilities for control and should be further tested as outbreak control methods because these protein extracts offer great advantages when compared to actual poisons used: (1) peptone and oxgall are animal protein extracts and not toxic chemicals (2) Low concentrations are required to induce death (8g l^{-1} and 10g l^{-1} for peptone and oxgall, respectively, compared to 140g l^{-1} for sodium bisulphate) and; (3) mortality rates are 100% with small, single doses (10ml per starfish, as opposed to 25ml of sodium bisulphate delivered into multiple locations within each starfish). Using the DuPont Veldspar Spot gun fitted with a 50 cm needle and 5-litre plastic bladder (setting dosage to 10ml) it would be possible to kill 500 COTS with one single injection per starfish (into either the arms or oral disk) greatly increasing efficiency and effectiveness compared to current best practice.

The individual testing of TCBS ingredients contribute to the elucidation of how disease is initiated by TCBS, what are the main components involved in the induction of disease and why previous attempts to induce disease in COTS through the injection of bacteria showed variable results.

This study shows that there are viable alternatives to injection of toxic substances in controlling outbreaks of COTS. However, toxin production and determination of DL dose of peptone, oxgall and yeast is still required to determine the minimal dose required to induce death in COTS and their possible use as an alternative COTS control method. More importantly, disease

transmission studies with peptone, oxgall, yeast also should be carried out to determine if there is any risk of inducing disease in other marine organisms, especially other echinoderms, in close proximity to infected COTS. Finally, Vibrio quorum sensing studies are also required to understand the pathways used to drive the presentation of the transmissible disease in COTS. Knowledge of the signaling molecules involved in the quorum sensing process and their inter-relationship will allow a better understanding of the interaction between bacteria, allergen and host.

7.7 Acknowledgements

Funding for this project was provided by the ARC Centre of Excellence for Coral Reef Studies. Field sampling was conducted with kind support and logistic assistance of the Marine Laboratory University of Guam.

CHAPTER 8: General Conclusions

8.1 Overview

This study has shown that crown-of-thorns starfish (*Acanthaster planci*) are highly susceptible to pathogenesis associated with allergic reactions and high densities of naturally occurring *Vibrionales* that induced dysbiosis (Rivera-Posada *et al.,* 2011, 2012 – Chapter 2 and 7). This research is important for two reasons. Firstly, naturally occurring diseases may be implicated in sudden declines in the abundance of *A. planci*, recorded following population outbreaks in the western Pacific (Moran *et al.,* 1985; Moran, 1986; Pratchett, 2005). Disease has been implicated in the mass-mortalities of numerous other echinoderms (Dungan *et al.,* 1982, Lessios *et al.,* 1984), but ongoing research is required to establish key factors (e.g., population density or individual condition) that initiate disease and/ or increase the probability of transmission. Secondly, the ability to artificially induce pathogenesis may provide a novel method for actively controlling outbreak populations of *A. planci*. In the first instance, injection of thiosulfate-citrate-bile-sucrose agar (TCBS) culture medium into *A. planci* was shown to induce a rapid fulminating disease that is transmitted to other adjacent starfishes under favorable conditions (Rivera-Posada *et al.,* 2011a – Chapter 2). Importantly, this technique does not involve any introduction of new pathogens into the environment and it is a rapid and simple procedure with immense economic advantages. However, significant ongoing research is required to ensure that artificial induction of pathogenesis in *A. planci* has no adverse affects on coral reefs or coral reef organisms, requiring improved understanding of the processes involved.

Many different factors may be involved in pathogenesis of *A. planci*, induced by the injection of TCBS. Intuitively, it would be expected that pathogenesis results mainly from an allergic reaction that induced dysbiosis of COTS immune sytem allowing increased abundance

and activity of *Vibrio* bacteria, given that TCBS is a selective media culture that allows selective growth of *Vibrios* while inhibiting Gram-positive organisms and suppressing coliforms (Rivera-Posada *et al.,* 2011c – Chapter 4). Indeed, TCBS injections induced marked epithelial desquamation, hypertrophy and hypersecretion of glandular cells, epithelial cell destruction, pyknosis, reduction of thickness and disorganization of connective tissue and associated nerve plexus, which are consistent with *Vibrio* infections (Rivera-Posada *et al.,* 2011c – Chapter 4). The question is however, to what extent the TCBS culture media contributed to these infections, though direct stimulation of quorum sensing, as opposed to antigenic reactions that compromise the immune response of *A. planci*.

All crown-of-thorns starfish collected from Lizard Island and Guam had similar bacterial microflora and responded in the similar fashion to the infection of TCBS agar, indicating that a common mechanism of disease induction affects their bacterial communities and immune responses. Pathogenic and non-pathogenic *Vibrio* isolates identified as *V. owensii*, *V. rotiferianus*, *V. fortis*, *P. eurosenbergii* were recovered from sick and healthy COTS at two different locations (Guam-Lizard Island) consistent with the hypothesis that TCBS coupled with adequate environmental conditions could trigger *Vibrio* virulence factors turning normal vibrios pathogenic (Rivera-Posada *et al.,* 2011b – Chapter 3). Moreover, allergic reactions to chemicals in the TCBS formulation are not likely to initiate a transmissible disease that spreads to healthy in-contact COTS, but certainly contribute to the breakdown of defense mechanisms as well as induce more tissue damage, accelerating pathogenesis and decomposition within infected individuals.

Biology of outbreaks

Our experiments indicate that bacteria found on affected tissues of *A. planci* were certainly involved, for artificially induced pathogenesis. This does not however, explain if, or why, high densities of *A. planci* would suddenly succomb to disease at the end of an outbreak. Previous instances of disease in *A. planci* have been observed towards the end of severe outbreaks on the Great Barrier Reef (Pratchett, 1999) and Fiji (Zann *et al.,* 1990) and among starfishes held in aquarium conditions (Sutton *et al.,* 1988). It is likely that these individuals had limited access to prey corals, and may have been in poor physiological condition. Preliminary experiments (Pratchett *et al.,* unpublished data) show that starfishes that were starved for up to 82 days exhibit increasing susceptibility to disease, providing a clear mechanism by which disease may become initiated at the end of outbreaks. Moreover *A. planci* at high densities invest more resources (high metabolic rate) into immune defense than individuals at lower densities as a counter-measure to density-dependent pathogen transmission rates (Mills 2012). Therefore COTS at high densities and under starvation conditions as those observed at the end of outbreaks spend high amounts of energy in an attempt to maintain their immune response to avoid pathogen transmission from in-contact COTS. Consequently there is a faster depletion of COTS energy reserves that eventually lead to a weaker individual with an increased susceptibility to opportunistic pathogens such as vibrios, sporozoans, etc.

Other environmental factors (especially temperature) are critical in natural incidence of disease; highest densities of *Vibrio* organisms and bacterial infections occur during the warmer months (Levine & Griffin, 1993; Lipp *et al*., 2002). Warmer temperatures, in combination with elevated pH and plankton blooms, can also influence *Vibrio* attachment, growth, and multiplication in the aquatic environment.

Vibrio bacteria are extremely opportunistic species that evolve and recombine under high microbial contact in animal guts or as part of aquatic biofilms transferring or inducing expression of virulence genes in less or non-pathogenic populations (Thompson *et al*., 2004). Therefore *Vibrio* quorum sensing studies are important to understand the pathways used to drive the natural presentation of transmissible disease in COTS. There is tremendous divergence in the signaling pathway systems that are activated not only by diverse pathogens, but also by a single organism. Knowledge of the signaling molecules involved in this process and their inter-relationship will allow a better understanding of the interaction between bacteria and host, as well as provide insight into the effect of transmissible disease on other marine animals that also have vibrios of the *V. harveyi* and *V. splendidus* clades as normal inhabitants.

Controlling outbreaks

Crown-of-thorns starfishes are one of the preodominant coral predators on tropical coral reefs (Rotjan & Lewis, 2008), and not only consume live corals, but like other corallivores (e.g., Raymundo *et al.,* 2009) may act as a vector for transmission of coral disease(s). Crown-of-thorns starfish have already been implicated in transmission of brown-band disease because many instances of this disease originate at the edges of large feeding scars caused by these starfishes (Nugues & Bak, 2009). If so, effective control of COTS populations will further serve to reduce and reverse rapid and extensive coral loss that is being observed throughout the world (e.g., Bellwood *et al.,* 2004). The causative agents of brown band disease are ciliate, recently identified as *Porpostoma guamense*, of the order Philasterida (Lobban *et al.,* 2011). It remains to be seen whether these ciliates are transmitted on or within *A. planci*, as infections may arise simply due to the feeding activities of *A. planci*, which provide an appropriate location for the initiation the disease (Chong-Seng *et al.,* 2011). *P. eurosenbergii* although it was found only in COTS from

Guam should also be studied because this *Vibrio* is recognized as a coral pathogen isolated from the mucus of the Caribbean elkhorn coral *Acropora palmate* (Ritchie, 2006). *P. eurosenbergii* was also recovered from mucus and water surrounding bleached *Barabattoia amicorum* corals at Magnetic Island, Great Barrier Reef (Colin *et al.,* 2008). Even so, there is good justification for further exploring the role of *Vibrio* and pathogenesis in potentially controlling populations of *A. planci*.

There are already many active programs that are attemptings to control *A. planci* outbreaks and minimize associate damage to reef environments, either by collection of adult individuals followed by burial ashore, by injections of starfish with toxic chemicals or by building underwater fences to prevent the movement of adult starfishes. Despite more than four decades of research, costing millions of dollars, current control methods are ineffective in either eradicating the coral-feeding starfish or preventing further coral mortality. Biological control of populations through manipulation of predators, parasites or pathogens has not been fully investigated (Sutton *et al.,* 1988). Nevertheless, several authors attest to the fact that predators do not and cannot limit the population density of COTS once they reach outbreak levels (McCallum, 1987). Although the possibility of exploiting susceptibility to diseases in *A. planci* for developing biological control measures is promising, significant testing is needed to ensure that there is no chance of disease transmission to non-target species (Caballes *et al.,* 2012 – Chapter 6).

There have been several exemplary biological control programs implemented in terrestrial systems, but little is known on the potential risk and effectiveness of biological controls for pest species in marine systems. Concerns over the potential for inter-specific transmission were raised during simultaneous infections in both *A. planci* and another common

reef echinoderm, *Linkia guildingi* (Caballes *et al.,* 2012 – Chapter 6). *Vibrio rotiferianus,* reported as a pathogen isolated from lesions of experimentally infected *A. planci*, was recovered from *L. guildingi* lesions after several days of direct contact with diseased *A. planci*, demonstrating disease transmission. Although, all *L. guildingi* fully recovered after 53 days, further studies are required to assess the ecology of *Vibrio* infection in corals, fishes and other echinoderms to evaluate if injections of TCBS could be a viable tool for controlling *A. planci* outbreaks.

Risks associated with inter-specific infection may be reduced by identifying the specific active ingredient in TCBS that causes pathogenesis in *A. planci* (Chapter 7). Peptone, ox gall and yeast all indiviudally caused rapid mortality in injected COTS. These TCBS components should be further tested as a possible outbreak control methods because these protein extracts offer great advantages when compared to actual poisons used: (1) peptone and oxgall are just animal protein extracts and not toxic chemicals; (2) less concentration is required to induce death and; and (3) many more COTS can be killed per litre of solution. Using DuPont Veldspar Spot gun fitted with a 50 cm needle and 5-litre plastic bladder, it is estimated approximately 500 COTS can be killed by diver with one single injection per starfish, greatly increasing efficiency over previous techniques. Nonetheless, toxin production and determination of DL dose of peptone, oxgall and yeast is still required to determine the minimal dose required to induce death in COTS and their possible use as an alternative COTS control method. Disease transmission studies with peptone, oxgall, yeast also should be carried out to determine if there is induction of disease in other marine organisms.

8.2 Future directions

This is the first study to develop appropriate and novel techniques to induce transmissible disease in the coral-eating crown-of-thorns starfish, or any other echinoderm. Echinoderms generally have a significant and often negative effects on the structure and resilience of coral reef environments (Rotjan & Lewis, 2008) and temperate rocky reefs (Ling, 2008). On coral reefs, extreme densities of specific urchin species (e.g., *Echinometra mathaei*) cause excessive bioerosion (McClanhan & Shafir, 1990), leading to structural collapse of reef frameworks. Elsewhere, range expansions of the sea urchin *Centrostephanus rodgersii*, to temperate locations have resulted in widespread loss of habitat-forming macroalgae, which have been replaced by urchin barrens that support far fewer species (Ling, 2008). There is therefore significant oppurtunities for extension of this research and application across a diverse range of marine ecosystems.

To date, management of marine ecosystems is focussed on minimising anthropogenic pressures (overfishing, sedimentation etc) and biodiversity loss, in the hope that the natural balance of species will preserve ecosystem function. It is clear however, that current and past management actions have failed to reduce or reverse widespread habitat degradation on coral reefs (Pandolfi *et al.,* 2005), or many other coastal ecosystems. As such, human intervention and active threat-based management are now considered critical to the long-term survival of coral reef ecosystems (e.g., McDermott, 2006). Outbreaks of the crown-of-thorns starfish not only represent one of the most significant biological disturbances on coral reefs, contributing greatly to widespread habitat degradation across the Indo-Pacific (Bruno & Selig, 2007; Pratchett *et al*., 2012), but are also one of the few major threats to corals reef ecosystems (unlike cyclones, and climate-induced coral bleaching) that may be amenable to direct and active management.

Global degradation of coral reefs environments and the impending threat of climate change provide greatly renewed incentive to control outbreaks of *A. planci*, regardless of whether these outbreask are caused by anthropogenic activities (e.g., Fabricius *et al.,* 2010) or not. Importantly, effective management and control of *A. planci* outbreaks will be a formative step in reducing and reversing sustained declines in coral cover on coral reefs in the Indo-Pacific, thereby maximising the capacity of corals, and other reef organisms, to cope with and potentially adapt to changing environmental conditions. Research presented in this thesis should be viewed as a proof of concept, and a starting point (not end point) for significant research on pathogenesis in crown-of-thorns starfish.

References

1. Abuzinada AH, Barth HJ, Krupp F, Boer B, Abdessalaam TZ. (2008). Protecting the Gulf's marine ecosystems from pollution. 1st edn. Birkhauser Verlag AG, PO Box 133, CH 4010 Basel, Switzerland, pp 301.
2. Aiyamperumal V, Mina K, Sayaka M, Megumi T, Norichika HK. (2005). An estuarine neritid gastropod, *Clithon corona*, a potential reservoir of Thermostable Direct Hemolysin-producing *Vibrio parahaemolyticus*. *J Vet Med Sci* **67** (8): 833-835.
3. Alavandi SV, Manoranjita V, Vijayan KK, Kalaimani N, Santiago TC. (2006). Phenotypic and molecular typing of *Vibrio harveyi* isolates and their pathogenicity to tiger shrimp larvae. *Lett Appl Microbiol* **43**: 566–570.
4. Alexander D, DePaola BA, Chirtelc S, Young RB. (1998). Detection of *Vibrio cholerae* in oyster (*Crassostrea virginica*) homogenate based on centrifugal removal of antimicrobial agents. *J Microbiol Methods* **33** (3): 237- 244.

5. Al Saif N, Brazier JS. (1996). The distribution of *Clostridium difficile* in the environment of South Wales. *J Med Microbiol* **45**: 133–137.
6. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.
7. Amesbury S, Bonito V, Chang R, Kirkendale L, Meyer C, Paulay G, Ritson-Williams R, Rongo T. (2001). *Marine biodiversity resource survey and baseline reef monitoring survey of the Haputo Ecological Reserve Area*, COMNAV-MARIANAS. US Dept of the Navy.
8. Austin B, Zhang XH. (2006). *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Let Appl Microbiol* **43**: 119–124.
9. Austin B, Austin DA. (2007). Bacterial Fish Pathogens, Disease of Farmed and Wild Fish, 4th edn. Springer Praxis, Godalming.
10. Azad AF. (2007). Pathogenic Rickettsiae as Bioterrorism Agents. *CID*, **45** (Suppl 1): 52-55.
11. Bancroft JD, Stevens A. (1996). Theory and Practice of Histological Techniques. Churchill Livingstone 4th edition. New York.
12. Banin E, Israely T, Kushmaro A, Loya Y, Orr E, Rosenberg E. (2000). Penetration of the coral-bleaching bacterium *Vibrio shiloi* into *Oculina patagonica. Appl Environ Microbiol*: 3031–3036.
13. Banin E, Israely T, Fine M, Loya Y, Rosenberg E. (2001). Role of endosymbiotic zooxanthellae and coral mucus in the adhesion of the coral bleaching pathogen *Vibrio shiloi* to its host. *FEMS Microbiol Lett* **199**: 33−37.

14. Barber GR, Swygert JS. (2000). Necrotizing fasciitis due to *Photobacterium damsela* in a man lashed by a stingray. *N Engl J Med* **342**:824.

15. Baron EJ, Peterson LR, Finegold SM. (1994). Vibrio and related species, *Aeromonas, Plesiomonas, Campylobacter, Helicobacter*, and others. In: Baron EJ, Peterson LR, Finegold SM (eds) Bailey & Scott's diagnostic microbiology, 9th edn. Mosby, St. Louis, MO, pp 429−434.

16. Barua D, Greenough WB. (1992). *Cholera*. Plenum Pub Corp Publisher. 392 p

17. Bauer JC, Young CM. (2000). Epidermal lesions and mortality caused by vibriosis in deep-sea Bahamian echinoids: a laboratory study. *Dis Aquat Org* **39**: 193-199.

18. Baumann P, Baumann L, Bang S, Woolkalis M. (1980). Reevaluation of the taxonomy of *vibrio Beneckea* and Photobacterium: Abolition of the genus Beneckea. *Curr Microbiol* **4**: 127-132.

19. Becker P, Gillan D, Lanterbecq D, Jangoux M, Rasolofonirin R, Rakotovao J, Eeckhaut I, (2004). The skin ulceration disease in cultivated juveniles of *Holothuria scabra* (Holothuroidea, Echinodermata). *Aquaculture* **242**: 13–30.

20. Becker P, Gillan DC, Eeckhaut I (2007). Microbiological study of the body wall lesions of the echinoid *Tripneustes gratilla*. *Dis Aquat Organ* **77**:73-82.

21. Becker PT, Egea E, Eeckhaut I. (2008). Characterization of the bacterial communities associated with the bald sea urchin disease of the echinoid *Paracentrotus lividus*. *J Invertebr Pathol* **98** (2): 136-147.

22. Bellwood DR, Hughes TP, Folke C, Nystrom M. (2004). Confronting the coral reef crisis. Nature **429**: 827-833.

23. Bennasar A, Luna G, Cabrer B, Lalucat J. (2002). Rapid identification of *Salmonella typhmurium*, *S. enteritidis* and *S. virchow* isolates by polymerase chain reaction based fingerprinting methods. *Int Microbiol* **3**: 31–38.

24. Ben-Haim Y, Banin E, Kushmaro A, Loya Y, Rosenberg E. (1999). Inhibition of photosynthesis and bleaching of zoozanthellae by the coral pathogen *Vibrio shiloi*. *Environ Microbiol* **1**: 223-229.

25. Ben-Hamin Y, Rosenberg E. (2002). A novel vibrio Sp. pathogen of the coral *Pocillopora damicornis*. *Mar Biol* **141**: 147-55.

26. Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, Rosenberg E. (2003). *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. *Int J Syst Evol Microbiol* **53**: 309-315.

27. Bensoussan MG, Scoditti PM, Bianchi AJM. (1984). Marine bacterial flora from echinoderm guts and associated sediment in the abyssal Vema Fault. *Marine Biology* **79**: 1-10.

28. Berkes J, ViswanathanVK, Savkovic SD, Hecht G. (2003). Intestinal epithelial responses to enteric ion transport, and inflammation pathogens: effects on the tight junction barrier. *Gut* **52**: 439-451.

29. Bird AR, Brown IL, Topping DL. (2000). Starches, Resistant Starches, the Gut Microflora and Human Health. *Curr Issues Intest Microbiol* **1** (1): 25-37.

30. Birkeland C, Lucas J. (1990). *Acanthasther Planci*: Major Management problem of Coral Reefs. CRC press, Inc. pp 257.

31. Bisharat N, Raz R. (1996). Vibrio infection in Israel due to changes in fish marketing. *Lancet* **348**: 1585–1586.

32. Bock SA, Munoz-Furlong A, Sampson HA. (2001). Fatalities due to anaphylactic reactions to foods. *JACI* **107**: 191–193.

33. Bogomoldi A, Gast RJ, Ellis JC, Dennett M, Pugliares KR, Lentell BJ, Moore MJ. (2008). Victims or vectors: a survey of marine vertebrate zoonoses from coastal waters of the Northwest Atlantic. *Dis Aquat Org* **81**: 13–38.

34. Bordas MA, Balebona MC, Rodriguez-Maroto JM, Borrego JJ, Morinigo MA. (1998). Chemotaxis of pathogenic vibrio strains towards mucus surfaces of gilt-head sea bream (*Sparus aurata*). *Appl Environ Microbiol* **64**:1573−1575.

35. Bossart G. (2007). Emerging diseases in marine mammals from dolphins to manatees. *Microbe* **2**, (11): 544-549.

36. Bourne DG, Young N, Webster N, Payne M, Salmon M, Demel S, Hall M. (2004). Microbial community dynamics in a larval aquaculture system of the tropical rock lobster, *Panulirus ornatus*. *Aquaculture* **242**: 31 – 51.

37. Bourne D, Lida Y, Uthicke S, Smith-Keune C. (2008). Changes in coral-associated microbial communities during a bleaching event. *ISME* **2**: 350–363.

38. Boxer CR. (1963). Two pioneers of tropical medicine: Garcia d'Orta and Nicolás Monardes. Welcome Historical Medical Library.

39. Brayton PR, Bode RB, Colwell RR, Russo FM, Bullock WE. (1986). A new Vibrio species. *Vibrio cincinnatiensis*, causing meningitis: Successful treatment in an adult. *Ann Intern Med* **104**: 55-56.

40. Braun M. (2000). Detection of the ADP-ribosyltransferase toxin gene (*cdt A*) and its activity in *Clostridium difficile* isolates from Equidae. *FEMS Microbiol Lett* **184**: 29–33.

41. Brenner DJ, Hickman-Brenner FW, Lee JV, Steirgerwalt AG, Fanning GR. (1983). *Vibrio furnissii* (formerly aerogenic biogroup of *Vibrio fluvialis*), a new species isolated from human feces and the environment. *J Clin Microbiol* **61**: 437-467.

42. Brook I. (1999). Bacterial interference. *Crit Rev Microbiol* **25**:155–72.

43. Bruant G, Watt S, Quentin R, Rosenau A. (2003). Typing of non-encapsulated *Haemophilus* strains by repetitive-element sequence-based PCR using intergenic dyad sequence. *J Clin Microbiol* **41**: 3473–3480.

44. Brune A, Friedrich M. (2000). Microecology of the termite gut: structure and function on a microscale. *Curr Opin Microbiol* **3**: 263–269.Bruno JF, Selig ER. (2007). Regional decline of coral cover in the Indo-Pacific: timing, extent, and subregional comparisons. *PLuS One* 2 (8): e711.

45. Bruno JF, Selig ER. (2007). Regional decline of coral cover in the Indo-Pacific: timing, extent, and subregional comparisons. *PLuS One*. **2** (8): e711.

46. Buck JD, Bubucis PM, Spotte S. (1986). Microbiological characterization of *belukha whales* from capture through extended captivity. 86th annual meeting, American Society for Microbiology: 248 p.

47. Buck JD. (1991). Recovery of *Vibrio metschnikovii* from market seafood. *J Food Safe* **12**: 73–78.

48. Burkholder PR. (1973). The ecology of marine antibiotics and coral reefs. In: Jones OA, Endean R (eds) Biology and geology of coral reefs, Vol 2. Academic Press, NY, pp 117–182.

49. Burnett WJ, Mckenzie JD. (1997). Subcuticular bacteria from the brittle star *Ophiactis balli* (Echinodermata: Ophiuroidea) represent a new lineage of extracellular marine symbionts in the subdivision of the class Proteobacteria. *Appl Environ Microbiol* **63** (5): 1721–1724.

50. Butler SM, Camilli A. (2004). Both chemotaxis and net motility greatly influence the infectivity of *Vibrio cholera*. *Proc Natl Acad Sci* USA **101**: 5018−5023.

51. Cabrera-Garcia M, Vazquez-Salinas C, Quinones-Ramirez E. (2004). Serologic and Molecular Characterization of *Vibrio parahaemolyticus* strains Isolated from Seawater and Fish Products of the Gulf of Mexico. *Appl Environ Microbiol*: 6401–6406.

52. Campos H, Bolanos H, Acuna M, Diaz G, Matamoros MC, Raventos S, Sanchez L, Sanchez O, Barquero C. (1996). *Vibrio mimicus* diarrhea following Ingestion of raw Turtle Eggs. *Appl Environ Microbiol* **62** (4): 1141–1144.

53. Cano-Gomez A, Bourne DG, Hall MR, Owens L, Høj L. (2009). Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: Current methods and future prospects. *Aquaculture* **287**: 1–10.

54. Cano-Gomez A, Goulden EF, Owens L & Høj L. (2010). *Vibrio owensii* sp. nov., isolated from cultured crustaceans in Australia. *FEMS Microbiol Lett* **302**: 175-181.

55. Cano-Gomez A, Owens L, Høj L, Andreakis N. (2011). Multilocus sequence analysis provides basis for fast and reliable identification of Vibrio harveyi-related

species and reveals previous misidentification of important marine pathogens. *Syst Appl Microbiol* **34** (8): 561-565.

56. Carli A, Pane L, Casareto L, Bertone S, Pruzzo C. (1993). Occurrence of *Vibrio alginolyticus* in Ligurian Coast Rock Pools (Tyrrhenian Sea, Italy) and its Association with the Copepod *Tigropus filvus* (Fisher 1860). *Appl Environ Microbiol* **59** (6): 1960-1962.
57. Centers for Disease Control. (1969). An outbreak of acute gastroenteritis during a tour of the orient -- Alaska. *MMWR* **18**: 150.
58. Centers for Disease Control and Prevention (2004). Summary of human Vibrio isolates reported to CDC. Accessed May 7, 2007, at: http://www.cdc.gov/foodborneoutbreaks/vibrio_sum/CSTEVibrio2004.pdf.
59. Centers for Disease Control and Prevention (2006). Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—10 states, United States, 2005. *MMWR Morb Mortal Wkly Rep* **55**: 392-395.
60. Centers for Disease Control and prevention (CDC). (2012). Vaccines and Immunizations. Depatment of Health and Human Services. Accesed January 17, 2012. http://www.cdc.gov/vaccines/
61. Cerda-Cuellar M, Permin L, Larsen JL, Blanch AR. (2001). Comparison of selective media for the detection of *Vibrio vulnificus* in environmental samples. *J Appl Microb* **91**: 322-327.
62. Cervino J, Hayes RL, Polson SW, Polson SC, Goreau TJ, Martinez RJ, Smith WG. (2004). Relationship of Vibrio Species Infection and elevated temperatures to

Yellow Blotch/Band Disease in Caribbean Corals. *Appl Environ Microbiol* **70** (11): 6855–6864.

63. Chatzidaki-Livanis M, Hubbard M, Gordon K, Harwood V, Wright A. (2006). Genetic Distinctions among Clinical and Environmental Strains of *Vibrio vulnificus*. *Appl Environ Microbiol* **72** (9): 6136–6141.
64. Chesher RH. (1969). Destruction of the Pacific corals by the sea star *Acanthaster planci*. *Science* **165**: 280–283.
65. Cho JC, Tiedje JM. (2001). Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays. *Appl Environ Microbiol* **67**: 3677–3682.
66. Chong-Seng KM, Cole AJ, Pratchett MS, Willis BL. (2011). Selective feeding by coral reef fishes on coral lesions associated with brown band and black band disease. *Coral Reefs* **30**: 473-481.
67. Chuang YC, Yuan CY, Liu CY, Lan CK, Huang AH. (1992). *Vibrio vulnificus* infection in Taiwan: Report of 28 cases and review of clinical manifestations and treatment. *Clin Infect Dis* **15**: 271-276.
68. Cohan FM. (2001). Bacterial species and speciation. *Syst Bio*. **50**: 513-524.
69. Colgrove GS, Sawa TR, Brown JT, Mcdowell PF, Nachtigall PE. (1975). Necrotic stomatitis in a dolphin. *J Wildlife Dis* **11**: 460-464.
70. Colin B, Munn Hannah K, Marchant A, Moody J. (2008). Defenses against oxidative stress in vibrios associated with corals. *FEMS Microbiol Lett* **281**: 58–63.
71. Colwell RR, Brayton PR, Grimes DJ, Roszak D, Huq S, Palmer L. (1985). Viable but non-culturable *Vibrio cholerae* and related environmental pathogens in the

environment: implication for release of genetically engineered microorganisms. *Bio/Tech* **3**: 817–820.

72. Colwell RR, Spira WM. (1992). The ecology of *Vibrio cholerae*. In: Cholera. Baura D, Greenough WB. New York, NY, United States of America: Plenum Publishers Co. pp. 107–127.
73. Colwell RR. (1996). Global climate and infectious disease: the cholera paradigm. *Science* **274**: 2025–2031.
74. Cooney RP, Pantos O, Le Tissier MD, Barer MR, O'Donnell AG, Bythell JC. (2002). Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. *Environ Microbiol* **4**:401–413.
75. Criminger JD, Hazen TH, Sobecky PA, Lovell CR. (2007). Nitrogen Fixation by *Vibrio parahaemolyticus* and its implications for a New Ecological Niche. *Appl Environ Microbiol* **73** (18): 5959-5961.
76. Crosa JH, Walsh CT. (2002). Genetics and assembly line enzymology of Siderophore Biosynthesis in Bacteria. *Microb Mol Biol Rev* **66** (2): 223–249.
77. Cutter DL, Kreger AS. (1990). Cloning and expression of the damselysin gene from *Vibrio damsela*. *Infect Immun* **58**: 266–268.
78. Cox FEG. (1996). The Welcome Trust Illustrated History of Tropical Diseases. London, hard bound: 452 p.
79. Dalsgaard A, Alarcon A, Lanata CF. (1996). Clinical manifestations and molecular epidemiology of five cases of diarrhea in children associated with *Vibrio metschnikovii* in Arequipa, Peru. *J Med Micriobiol* **45**: 494-500.

80. Dalsgaard A, Glerup P, Hoybye LL. (1997). *Vibrio furnissii* isolated from humans in Peru: a possible human pathogen?. *Epidemiol Infect* **119**: 143-149.

81. Daniels NA, Shafaie A. (2000). A Review of pathogenic Vibrio infections for clinicians. *Infect Med* **17**: 665-685.

82. Da Silva SG, Gillan DC, Dubilier N, Chantal De Ridder. (2006). Characterization by 16S rRNA gene analysis and in situ hybridization of bacteria living in the hindgut of a deposit-feeding echinoid (Echinodermata). *J Mar Biol Ass uk* **86**: 1209-1213.

83. Davies AR, Capell C, Jehanno D, Nychas GJE, Kirby RM. (2001). Incidence of foodborne pathogens on European fish. *Food Control* **12** (2): 67-71.

84. Deane EE, Woo NYS. (2005). Evidence for disruption of Na+-K+-ATPase and hsp70 during vibriosis of sea bream, *Sparus (=Rhabdosargus) sarba Forsskal. J Fish Dis* **28**: 239–251.

85. De Bruijn FJ, Rademaker J, Schneider M. (1996). Rep-PCR Genomic Fingerprinting of Plant-Associated Bacteria and Computer-Assisted Phylogenetic Analyses Biology of Plant-Microbe Interaction; Proceedings of the 8th International Congress of Molecular Plant-Microbe Interactions. APS Press: 497-502.

86. Deloney-Marino C, Wolfe AJ, Visik KL. (2003). Chemoattraction of *Vibrio fischeri* to serine, nucleosides and N-acetylneuraminic acid, a component of squid light-organ mucus. *Appl Environ Microbiol* **69**: 7527-7530.

87. Desmarchelier PM. (1997). Pathogenic vibrios. In: Foodborne microorganisms of public health importance, 5th Edition, (Eds) Hocking, A.D., Arnold, G., Jenson, I., Newton, K. and Sutherland: 285-312.

88. Diaz RJ, Rosenberg R. (2008). Spreading Dead Zones and Consequences for Marine Ecosystems. *Science* **321** (5891): 926–929.

89. Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL. (2007). Repression of *Clostridium difficile* toxin gene expression by CodY. *Mol Microbiol* **66**: 206-219.

90. Drury RAB, Wallington EA. (1980). Carleton's histological technique Ed. 5. Oxford University Press, Oxford, UK.

91. Dungan ML, Miller TE, Thompson DA. (1982). Catastrophic decline of a top carnivore in the Gulf of California rocky intertidal zone. *Science* **216**: 989–991.

92. Dupuy B, Sonenshein AL. (1998). Regulated transcription of *Clostridium difficile* toxin genes. *Mol Microbiol* **27**: 107–120.

93. Duthie R, Leung WC, Leung WY. (1990). Primary septicaemia due to Vibrio species: Three contrasting cases, aetiology, epidemiology and treatment. *J Hong Kong Med Assoc* **42**: 144

94. Eaves LE, Ketterer PJ. (1994). Mortalities in red claw crayfish *Cherax quadricarinatus* associated with systemic *Vibrio mimicus* infection. *Dis Aquat Org* **19**: 233–237.

95. Egert M, Stingl U, Dyhrberg Bruun L, Pommerenke B, Brune A, Friedrich MW. (2005). Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). *Appl Environ Microbiol*: 4556–4566.

96. Eilers H, Pernthaler J, Amann R. (2000). Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts. *Appl Environ Microbiol* **66**: 4634-4640.

97. Fabricius KE, Okaji K, De'ath G (2010) Three lines of evidence to link outbreaks of the crown-of-thorns seastar *Acanthaster planci* to the release of larval food limitation. *Coral Reefs* **29**: 593-605.

98. Fang A, Gerson DF, Demain AL. (2009). Production of *Clostridium difficile* toxin in a medium totally free of both animal and dairy proteins or digests. *PNAS* **106** (32): 13225–13229.

99. Fangfang Bai, Yin Han, Jixiang Chen, Xiao-Hua Zhang. (2008). Disruption of quorum sensing in *Vibrio harveyi* by the AiiA protein of *Bacillus thuringiensis*. *Aquaculture* **274**: 36–40.

100. Faruque SM, Nair GB. (2002). Molecular ecology of toxigenic *Vibrio cholera*. *Microbiol Immunol* **46** (2): 59-66.

101. Fasano A, Fiorentini C, Donelli G, Uzzau S, Kaper JB, Margaretten K, Ding X, Guandalini S, Comstock L, Goldblum SE. (1995). Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. *J Clin Invest* **96**: 710–720.

102. Fenner B. (1995). *Acanthaster*, Crown of Thorns Sea Stars. *FAMA* **1**: 95.

103. Firth JR, Diaper JP, Edwards C. (1994). Survival and viability of *Vibrio vulnificus* in seawater monitored by flow cytometry. *Lett Appl Microb* **18**: 268-271.

104. Forbes BA, Sahm DF, Weissfeld AS. (2002). *Vibrio, Aeromonas, Plesiomonas, Shigelloids,* and *Chromobacterium violaceum*. Bailey and Scott's Diagnostic Microbiology, 11th edition, Mosby, St. Louis. London. Philadelphia. Sydney. Toronto. 423 – 433.

105. Forres J. (2007). Vibrio bacteria a bigger threat to swimmers than sharks as Northern waters warm. Natural Resources News Service and the National Security News Service USA: 3p.

106. Fouz B, Barja JL, Amaro C, Rivas C, Toranzo AE. (1993). Toxicity of the extracellular products of *Vibrio damsela* isolated from diseased fish. *Curr Microbiol* **27**: 341–347.

107. Fouz B, Biosca EG, Amaro C. (1997). High affinity iron-uptake systems in *Vibrio damsela*: role in the acquisition of iron from transferrin. *J Appl Microbiol* **82**: 157–167.

108. Fraser SL, Purcell BK, Delgado B. (1997). Rapidly fatal infection due to *Photobacterium (Vibrio) damsela*. *Clin Infect Dis* **25**:935-936.

109. Freter R, O'brien PC. (1981). Role of chemotaxis in the association of motile bacteria with intestinal mucosa: chemotactic responses of *Vibrio cholera* and description of motile non-chemotactic mutants. *Infect Immun* **34**:215-221.

110. Fujino T, Okuno Y, Nakada D, Aoyama A, Fukai K, Mukai T, Ueho T. (1953). On the bacteriological examination of Shirasu food poisoning. *Med J Osaka University* **4**: 299–304.

111. Fukui Y, Sawabe T (2008). Rapid Detection of *Vibrio harveyi* in Seawater by Real-Time PCR. *Microbes Environ* **23**(2): 172-176.

112. Furniss AL, Lee JV, Donovan TJ. (1977). Group F, a new Vibrio?. *Lancet* **10**: 73-94.

113. Gauger E, Smolowitz R, Uhlinger K, Casey J, Gomez-Chiarri M. (2006). *Vibrio harveyi* and other bacterial pathogens in cultured summer flounder, *Paralichthys dentatus*. *Aquaculture* **260**: 10–20.

114. Gerlach H, Gylstorff I. (1967). Studies on biochemical properties, pathogencity and resistance spectrum against antibiotics in *Vibrio metschnikovi*. *Berl Muench Tieraerztl Wochenschr* **80**: 153–155.

115. Gerlach H, Gylstorff I. (1967). Tests made on biochemical properties, pathogenicity and resistance spectrum against antibiotics in *Vibrio metschnikovi*. *Berl Muench Tieraerztl Wochenschr* **80**: 161–164.

116. Gevers D, Cohan FM, Lawrence JG, Spratt BG, Coenye T, Feil EJ, Stackebrandt E, Van de Peer Y, Vandamme P. (2005). Re-evaluating prokaryotic species. *Nat Rev Microbiol* **3**: 733–739.

117. Gilles KW, Pearse JS. (1986). Disease in sea urchins *Strongylocentrotus purpuratus*: experimental infection and bacterial virulence. *Dis Aquat Org* **1**: 105– 114.

118. Goday G. (1990). The ecology of chitin degradation. *Adv Microb Ecol* **11**: 387-430.

119. Gomez-Gil B, Thompson FL, Thompson CC, Swings J. (2003). *Vibrio rotiferianus* sp. nov., isolated from cultures of the rotifer *Brachionus plicatilis*. *Int J Syst Evol Microbiol* **53**: 239–243.

120. Gomez-Gil B, Soto-Rodriguez S, García-Gasca A, Roque A, Vázquez-Juárez R, Thompson FL, Swings J. (2004). Molecular identification of Vibrio harveyi-related isolates associated with diseased aquatic organisms. *Microbiology* **150**:1769-1777.

121. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. (2007). DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microb* **57**: 81–91.

122. Gras-Rouzet S, Donnio PY, Juguet F, Plessis P, Minet J, Avril JH. (1996). First European case of gastroenteritis and bacteremia due to *Vibrio hollisae*. *Eur J Clin Microbiol Infect Dis* **15**: 864–866.

123. Grau BL. (2006). Molecular analysis of *Vibrio vulnificus* phase variants and characterization of a new biofilm-proficient rugose phenotype (PhD Thesis) Louisiana State University department of biological science. 200 p

124. Griffith DC, Kelly-Hope LA, Miller MA. (2006). Review of reported *cholera* outbreaks worldwide, 1995-2005. *Am J Trop Med Hyg* **75**(5): 973–977.

125. Grimes DJ, Stemmler J, Hada H, May EB, Maneval D, Hetrick FM, Jones RT, Stoskopf M, Colwell RR. (1984). Vibrio species associated with mortality of sharks held in captivity. *Microb Ecol* **10**: 271–282.

126. Guarner F, Malagelada JR. (2003). Gut flora in health and disease (review). *Lancet* (N Am Ed) **360**: 512-519.

127. Gurtler V, Mayall BC. (2001). Genomic approaches to typing, taxonomy and evolution of bacterial isolates. *Int J Syst Evol Microbiol* **51**: 3–16.

128. Guttman JA, Finlay BB. (2009). Tight junctions as targets of infectious agents. *BBA* **1788**: 832–841.

129. Hahm BK, Maldonado Y, Schreiber E, Bhunia AK, Nakatsu CH. (2003). Subtyping of foodborne and environmental isolates of *Escherichia coli* by multiplex-PCR, Rep-PCR, PFGE, ribotyping and AFLP. *J Microbiol Methods* **53**: 387–399.

130. Haiyan MA, Guoliang J, Zhiqiang WU, Xin W. (2009). 16S rRNA Gene Phylogenesis of Culturable Predominant Bacteria from Diseased *Apostichopus japonicus* (Holothuroidea, Echinodermata) *J Ocean Univ China* **8** (2): 166-170.

131. Hall JD, Gilmartin WG, Mattsson JL. (1971). Investigation of a Pacific pilot whale stranding on San Clemente Island. *J Wildlife Dis* **7**: 324- 327.

132. Halpern M, Broza YB, Mittler S, Arakawa E, Broza M. (2004). *Chironomid* egg masses as a natural reservoir of *Vibrio cholerae* non-O1 and non-O139 in freshwater habitats. *Microb Ecol* **47** (4): 341 – 349.

133. Harriott V, Goggin L, Sweatman H. (2003). Crown-of thorns starfish on the Great Barrier Reef. Current state of knowledge. Cooperative Research Centre (CRC) for the Great Barrier Reef World Heritage Area. Published by CRC Reef Research Centre Ltd, PO Box 772 Townsville, Queensland, 4810, Australia.

134. Harris JM. (1993). The Presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. *Microb Ecol* **25** (3): 195-231.

135. Harris L, Owens L, Smith S. (1996). A selective and differential medium for *Vibrio harveyi. Appl Environ Microbiol* **62** (9): 3548–3550.

136. Harris LJ, Owens L. (1999). Production of exotoxins by two luminous *Vibrio harveyi* strains known to be primary pathogens of *Penaeus monodon* larvae. *Dis Aquat Org* **38**: 11-22.

137. Heidelberg JF, Heidelberg KB, Colwell RR. (2002). Seasonality of Chesapeake Bay Bacterioplankton Species. *Appl Environ Microbiol* **68** (11): 5488–5497.

138. Henke JM, Bassler BL. (2004). Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. *J Bacteriol* **186**: 3794–3805.

139. Hickman FW, Farmer JJ III, Hollis DG, Fanning GR, Steigerwalt AG, Weaver RE, Brenner DJ. (1982). Identification of *Vibrio hollisae* spp from patients with diarrhea. *J Clin Microbiol* **15**: 395–401.

140. Hinestrosa F, Madeira RG, Bourbeau P. (2007). Severe Gastroenteritis and Hypovolemic Shock Caused by *Grimontia (Vibrio) hollisae* Infection. *J Clin Microb* **45** (10): 3462-3463.

141. Hlady WG, Klontz, KC. (1996). The epidemiology of Vibrio infections in Florida, 1981- 1993. *J Infect Dis* **173**:1176-1183.

142. Horton HH, Misrahi JJ, Matthews GW, Kocher PL. (2002). Critical Biological Agents: Disease Reporting as a Tool for Determining Bioterrorism Preparedness. *J Law, Medicine & Ethics*. **30** (2): 262-266.

143. Huan D, Chongbo H, Zunchun Z, Chang L, Kefei T, Nianbin W, Bei J, Xianggang G, Weidong L. (2009). Isolation and pathogenicity of pathogens from skin ulceration disease and viscera ejection syndrome of the sea cucumber *Apostichopus japonicus. Aquaculture* **287**:18–27.

144. Huang KC, HsuWei R. (2005). *Vibrio fluvialis* hemorrhagic cellulitis and cerebritis. *Clin Infect Dis* **40**: 75–77.

145. Hughes TP. (2003). Climate Change, Human Impacts, and the Resilience of Coral Reefs. *Science* **301**, 5635: 929-933.

146. Huq MI, Alam KMJ, Brenner DJ. (1980). Isolation of *Vibrio*-like group EF-6, from patients with diarrhea. *J Clin Microbiol* **11**: 621-624.

147. Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR. (1983). Ecological relationship between *Vibrio cholerae* and planktonic copepods. *Appl Environ Microbiol* **45**: 275–283.

148. Igbinosa EO, Okoh AI. (2008). Emerging Vibrio species: an unending threat to public health in developing countries. *Research Microbiology* **159**: 495-506.

149. Imae Y, Matsukura H, Kobayashi S. (1986). Sodium driven flagellar motors of alkalophilic bacillus. *Methods enzymol* **125**: 582-592.

150. Intaraprasong A, Khemayan K, Pasharawipas T, Flegel TW. (2009). Species-specific virulence of *Vibrio harveyi* for black tiger shrimp is associated with bacteriophage-mediated hemocyte agglutination. *Aquaculture* **296**: 185–192.

151. Islam MS, Goldar MM, Morshed MG, Khan MN, Islam MR, Sack RB. (2002). Involvement of the *hap gene (mucinase*) in the survival of *Vibrio cholerae* O1 in association with the blue-green alga, *Anabaena sp*. *Can J Microbiol* **48** (9): 793 – 800.

152. Iwanaga, Yamamoto, Higa, Ichinose, Nakasone, Tanube. (1986). Culture conditions for simulating cholera toxin production by *Vibrio cholera*e O1 El Tor. *Microbiol Immunol* **30**: 1075-1083.

153. Janda JM. (1987). Pathogenic *Vibrio* spp. An Organism Group of Increasing Medical Significance. *Clin Microbiol Newsl* **9** (7): 49-53.

154. Janda JM. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin Microbiol Rev* **1**: 245-267.

155. Janda JM, Abbott SL. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils and pitfalls. *J Clin Microbiol* **45** (9): 2671-2764.

156. Jain V, Dongre M, Raychaudhuri S. (2006). Interaction of *Vibrio cholerae* O139 with an intestinal parasite, *Entamoeba histolytica*. *J Med Microbiol* **55** (12): 1755 – 1756.

157. Jean-JacquesW, Rajashekaraiah KA, Farmer III JJ. (1981). *Vibrio metschnikovii* bacteremia in a patient with cholecystitis. *J Clin Microbiol* **13**: 711-712.

158. Jiravanichpaisal P, Chuaychuwong P, Menasveta P. (1997). The use of *Lactobacillus* sp. as the probiotic bacteria in the giant tiger shrimp (*Penaeus monodon Fabricius*) In:

Abstract of poster session of the 2nd Asian-Pacific Marine Biotechnology conference, Phuket thailand pp 16.

159. Johnson J, Arduino SM, Stine OC, Johnson JA, Harris AD. (2007). Multilocus Sequence Typing Compared to Pulsed-Field Gel Electrophoresis for Molecular Typing of *Pseudomonas aeruginosa*. *J Clin Microbiol* **45** (11): 3707–3712.

160. Kang HP, Dunne WM. (2003). Stability of repetitive-sequence PCR patterns with respect to culture age and subculture frequency. *J Clin Microbiol* **41**: 2694-2696.

161. Karlsson S, Burman LG, Akerlund T. (2008). Induction of toxins in *Clostridium difficile* is associated with dramatic changes of its metabolism. *Microbiology* **154**: 3430-3436.

162. Karunasagar I, Pai R, Malathi GR. (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture* **128**: 203-209.

163. Kawagishi I, Imagawa M, Imae Y, Macarter L, Homma M. (1996). The sodium driven polar flagellar motor of marine Vibrio as the mechanosensor that regulates lateral flagellar expression. *Mol microbial* **20**: 693-699.

164. Keynan A. (2008). Pioneers in Microbiology: Moshe Shilo. *FEMS*: 7p

165. Kaysner C, DePaola A. (2004). *Vibrios*. Bacteriological Analytical Manual Online, Chapter 9. US food and drug administration http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm.

166. Khemiss F, Ahmadi S, Massoudi R, Ghoul-Mazgar S, Safta S, Asghar Moshtaghie A, Saidane D. (2009). Effect of in vitro exposure to *Vibrio vulnificus* on hydroelectrolytic transport and structural changes of sea bream (*Sparus aurata L.*) intestine. *Fish Physiol Biochem* **35**: 541–549.

167. Kim SH, Ahn SH, Lee JH, Lee EM, Kim NH, Park KJ, Kong IS. (2003). Genetic analysis of Phosphomannomutase/phosphoglucomutase from *Vibrio furnissii* and characterization of its role in virulence. *Arch Microbiol* **180**: 240–250.

168. Kimura M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**: 111–120.

169. Kobayashi T, Enomoto S, Sakazaki R, Kuwahara S. (1963). A new selective medium for pathogenic vibrios: T.C.B.S. Agar (Modified Nakanishi.s Agar). *Jap J Bacteriol* **18**: 387-391.

170. Kohn GC. (1995). *Encyclopedia of Plague and Pestilence*. Facts on File, Inc. New York.

171. Kolbert CP, Persing DH. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Curr Opin Microbiol* **2**: 299-305.

172. Koster W. (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Res Microbiol* **152** (3-4): 291-301.

173. Kothary MH, Claverie EF, Miliotis MD, Madden JM, Richardson Sh. (1995). Purification and characterization of a Chinese hamster ovary cell elongation factor of *Vibrio hollisae. Infect Immun* **63**: 2418-2423.

174. Kothary MH, Delston RB, McCardell BA, Curtis SK, Tall BD. (2001). Purification and characterization of a vulnificolysin-like cytolysin produced by *Vibrio tubiashii*. *Appl Environ Microbiol* **67**: 3703-3711.

175. Kraxberger-Beatty T, McGarey DJ, Grier HJ, Lim DV. (1990). *Vibrio harveyi*, an opportunistic pathogen of common snook, *Centropomus undecimalis* (Block), held in captivity. *J Fish Dis* **13**: 557–560.

176. Kreger AS, Bernheimer AW, Etkin LA, Daniel LW. (1987). Phopholipase D activity of *vibrio damsel* cytolysin and its interaction with sheep erythrocytes. *Infect Immun* **55**: 3209-3212.

177. Kuehn T. (2001). Isolation procedure for halophilic vibrios from cattle. In Proceedings of the Xth International Symposium of Veterinary Laboratory Diagnosticians and OIE Seminar on Biotechnology, Salsomaggiore, Italy: 168–169.

178. Kuhnt-Lenz K, Krengel S, Fetscher S, Heer-Sonderhoff A, Solbach W. (2004). Sepsis with bullous necrotizing skin lesions due to Vibrio vulnificus acquired through recreational activities in the Baltic Sea. *Eur J Clinical Microb & Infect Dis* **23** (1): 49-52.

179. Kushmaro A, Loya Y, Fine M, Rosenberg E. (1996). Bacterial infection and coral bleaching. *Nature*: 380- 396.

180. Kushmaro A, Rosenberg E, Fine M, Loya Y. (1997). Bleaching of the coral *Oculina patagonica* by *Vibrio AK-1*. *Mar Ecol Prog Ser* **147**:159-165.

181. Kushmaro A, Rosenberg E, Fine M, Ben-Haim Y, Loya Y. (1998). Effect of temperature on bleaching of the coral *Oculina patagonica* by *Vibrio AK-1*. *Mar Ecol Prog Ser* **171**: 131-137.

182. Kushmaro A, Banin E, Loya Y, Stackebrandt E, Rosenberg E. (2001). *Vibrio shiloi sp*. nov., the causative agent of bleaching of the coral *Oculina patagonica*. *Int J Syst Evol Microbiol* **51**: 1383–1388.

183. Kwon Y, Kim J, Moon S, Yoo Y, Rho H. (1995). Cloning and characterization of the gene encoding an extracellular alkaline serine protease from *Vibrio metschnikovii* strain RH530. *Gene* **152**: 59–63.

184. Lane DJ. (1991). 16S/23S rRNA sequencing. Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt E & Goodfellow M, eds). John Wiley & Sons, New York, pp 115–175.

185. Lapage SP, Shelton JE, Mitchell TG. (1970). In Methods in Microbiology' Eds. Norris JR, Ribbons DW Vol 3A. p.116. Academic Press. London.

186. La Rosa T, Mirto S, Marino A, Alonzo V, Maugeri TL, Mazzola A. (2001). Heterotrophic bacteria community and pollution indicators of mussel farm impact in the Gulf of Gaeta (Tyrrhenian Sea). *Mar Environ Res* **52**: 301-321.

187. Larsen M, Blackburn N, Larsen JL, Olsen JE. (2004). Influences of temperature, salinity, starvation on the motility and chemotactic response of *Vibrio anquillarum*. *Microbiology* **150**: 1283-1290.

188. Lattemann S, Höpner T. (2008). Environmental impact and impact assessment of seawater desalination. *Desalination* **220**: 1–15.

189. Lee HJ, Choi JW. (1995). A case of *Vibrio alginolyticus* myringitis. *Korean J Clin Pathol* **15**:101-105.

190. Lee KK, Liu PC, Chuang WH. (2002). Pathogenesis of gastroenteritis caused by *Vibrio carchariae* in cultured marine fish. *Mar Biotechnol* **4**: 267–277.

191. Lee DY, Soo-Youn M, Sang-Oh L, Hee-Young Y, Hee-Joo L, Mi Suk L. (2008). Septic Shock due to *Vibrio alginolyticus* in a Cirrhotic Patient: The First Case in Korea. *Yonsei Med J* **49** (2): 329–332.

192. Lee Young J, Parkb J, Seung Hwan O, Hye Ran K, Jeong Nyeo L, Hwan Shin J. (2008). Acute infectious peritonitis caused by *Vibrio fluvialis*. *Diag Microbiol Infect Dis* **62** (2): 216-218.

193. Lemaire LC, van Lanschot JJ, Stoutenbeek CP, van Deventer SJ, Wells CL, Gouma DJ. (1997). Bacterial translocation in multiple organ failure: cause or epiphenomenon still unproven. *Br J Surg* **84**:1340–1350.

194. Lessios HA, Robertson DR, Cubit JD. (1984). Spread of Diadema mass mortality through the Caribbean. *Science* **226**: 335–337.

195. Lessner AM, Webb RM, Rabin B. (1985). *Vibrio alginolyticus* conjunctivitis. First reported case. *Arch Ophthalmol* **103**: 229-230.

196. Lipp EK, Huq A, Colwell R. (2002). Effects of Global Climate on Infectious Disease: the Cholera Model. *Clin Microbiol Rev* **15** (4): 757 -770.

197. Levine WC, Griffin PM. (1993). *Vibrio* infections on the Gulf Coast: results of the first year of regional surveillance. *J Infect Dis.* **167**: 479–483.

198. Lievin V, Peiffer I, Hudault S. (2000). *Bifidobacterium* strains from resident infant human gastrointestinal microflora exert antimicrobial activity. *Gut* **47**: 646–652.

199. Lijima Y, Tamada H, Shimoda S. (1981). Adherence of *Vibrio parahaemolyticus* and its relationship to pathogenicity. *Can J Microbio* **27**:1252-1259.

200. Linde HJ, Kobuch R, Jayasinghe S, Reischl U, Lehn N, Kaulfuss S, Beutin L. (2004). *Vibrio metschnikovii*, a Rare Cause of Wound Infection. *J Clin Microbiol* **42** (10): 4909–4911.

201. Ling SD (2008) Range expansion of a habitat-modifying species leads to loss of taxonomic diversity: a new and impoverished reef state. *Oecologia* 156: 883-894.

202. Lipp EK, Huq A, Colwell R. (2002). Effects of Global Climate on Infectious Disease: the Cholera Model. *Clin Microbiol Rev* **15** (4): 757–770.

203. Lise Høi, Dalsgaard I, Depaola A, Siebeling RJ, Dalsgaard A. (1998). Heterogeneity among Isolates of *Vibrio vulnificus* recovered from Eels (*Anguilla anguilla*) in Denmark. *Appl Envir Microbiol*: 4676–4682.

204. Liu PC, Lee KK, Chen SN. (1996). Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. *Lett Appl Microbiol* **22**: 413–416.

205. Lobban CS, Raymundo LM, Montagnes DJS (2011) *Porpostoma guamensis* n. sp., a philasterine scuticociliate associated with brown-band disease of corals. *J Eukaryotic Micriobiol* 58: 103-113.

206. Love M, Teebken-Fisher D, Hose JE, Farmer III JJ, Hickman FW, Fanning GR. (1981). *Vibrio damsela*, a marine bacterium, causes skin ulcers on the damselfish *Chromis punctipinnis*. *Science* **214**: 1139–1140.

207. Ludwig W, Strunk O, Westram R. (2004). ARB: A software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.

208. Luter HM, Whalan S, Webster NS. (2010). Exploring the Role of Microorganisms in the Disease-Like Syndrome Affecting the Sponge *Ianthella basta. Appl Environ Microbiol* **76** (17): 5736–5744.

209. Ma YX, Xu GR, Zhang EP, Wang PH, Chang YQ. (2006). The etiology of acute preistome edema disease in cultured juveniles of *Apostichopus japonicus*. *J Fish China* **30**: 377–382 (in Chinese with English abstract).

210. Ma YX, Xu GR, Chang YQ, Zhang EP, Zhou W, Song LS. (2006). Bacterial pathogens of skin ulceration disease in cultured sea cucumber *Apostichopus japonicus* (Selenka) juveniles. *J. Dalian Fish Univ* **21**:13–18 (in Chinese with English abstract).

211. MacDonell MT, Singleton FL, Hood MA. (1982). Diluent composition for use of API 20E in characterizing marine and estuarine bacteria. *Appl Environ Microbiol* **44**: 423–427.

212. MacDonell G, Rusell D. (1999). Antiseptics and Disinfectants: Activity, Action, and Resistance *Clin Microbiol Rev* **12** (1): 147–179.

213. Macfarlane S, Macfarlane GT. (2003). Regulation of short–chain fatty acid production. *P Nutr Soc* **62**: 67–72.

214. Maes P, Jangoux M. (1984). The bald-sea-urchin disease: a biopathological approach. *Helgol Meeresunters* **37**: 217–224.

215. Maes P, Jangoux M. (1985). The bald-sea-urchin disease: a bacterial infection. In: Keegan BF, O'Connor BD (ed.) Proc. intern. Echinoderm Conf, Galway. Balkema, Rotterdam, p. 313-314.

216. Magalhaes V, Castello A, Magalhaes M, Gomes TT. (1993). Laboratory evaluation on pathogenic potentialities of *Vibrio furnissii*. *Mem Inst Oswaldo Cruz* **88**: 593–597.

217. Magalhaes V, Branco A, Lima RA, Magalhaes M. (1996). *Vibrio metschnikovii* among diarrheal patients during cholera epidemic in Recife, Brazil. *Revista Instituto de Medicina Tropical de Sao Paulo* **38**: 1–3.

218. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA, Feavers IM, Achtman M, Spratt BG. (1998). Multilocus

sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci USA* **95**: 3140–3145.

219. Marshall SC, Clark G, Wang G, Mulvey M, Kelly MT, Johnson WM. (1999). Comparison of molecular methods for typing *Vibrio parahaemolyticus*. *J Clin Microbiol* **37**: 2473–2478.
220. Martens M, Dawyndt P, Coopman R, Gillis M, De Vos P, Willems A. (2008). Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus Ensifer (including former Sinorhizobium). Int J Syst Evol Microbiol **58**: 200−214.
221. Martinez-Urtaza J, Lozano-Leon A, Alejandro Vina-Feas A, de Novoa J, Garcia-Martin O. (2006). Differences in the API20E biochemical patterns of clinical and environmental *Vibrio parahaemolyticus* isolates. *FEMS* **255**: 75–81.
222. Matte MH, Baldassi L, Barbosa ML, Malucelli MIC, Nitrini Smoo, Matte GR. (2007). Virulence factors of *Vibrio metschnikovii* strains isolated from fish in Brazil. *Food Control* **18**: 747–751.
223. McCallum HI. (1987). Predator regulation of *Acanthaster planci*. *J Theor Biol* **127**:207-220.
224. McClanhan TR, Shafir SH (1990) Causes and consequences of sea urchin abundance and diversity in Kenyan coral reef lagoons. *Oecologia* **8**3: 362-370.
225. McDermott E (2006) Coral reefs: concerns, developments, and preservation efforts. Pages 111-120 In: Morhardt JE (Ed) Research in natural resources management. The Roberts Environmental Center Press, California.
226. McInnes E. (2005). Artifacts in histopathology. *Comp Clin Path* **13**: 100–108.
227. McPherson VL, Watts JA, Simpson LM, Oliver JD. (1991). Physiological effects of the lipopolysacharide of *vibrio vulnificus* on mice and rats. *Microbios* **67**: 272-273.

228. Merkel SM, Alexander S, Zufall E, Oliver JD, Huet-Hudson YM. (2001). Essential role for estrogen in protection against *Vibrio vulnificus*-induced endotoxic shock. *Infection and Immunity* **69**: 6119-6122.

229. Meron D, Efrony R, Wesley RJ, Schaefer AL, Morris PJ, Rosenberg E, Greenberg P, Banin E. (2009). Role of Flagella in Virulence of the Coral Pathogen *Vibrio coralliilyticus*. *Appl Environ Microbiol* doi: 10.1128/AEM.00198-09.

230. Mertens A, Nagler J, Hansen W, Gepts-Friedenreich E. (1979). Halophilic lactose-positive Vibrio in a case of fatal septicemia. *J Clin Microbiol* **9**: 233–235.

231. Mezrioui N, Oufdou K, Baleux B. (1995). Dynamics of non-O1 *Vibrio cholerae* and fecal coliforms in experimental stabilization ponds in the arid region of Marrakesh, Morocco, and the effect of sunlight on their experimental survival. *Can J Microbiol* **41**: 489–498.

232. Midturi J, Baker D, Winn R, Fader R. (2005). Tubo-ovarian abscess caused by *Vibrio vulnificus*. *Diagn Microbiol Infect Dis* **51**:131-133.

233. Miller RJ. (1985). Sea urchin pathogen: a possible tool for biological control. *Mar Ecol Prog Ser* **21**:169–174.

234. Mills SC (2012). Density-dependent prophylaxis in the coral-eating crown-of-thorns sea star, *Acanthaster planci. Coral Reefs* **31**: 603–612.

235. Miyake M, Honda T, Miwatani T. (1988). Purification and characterization of *Vibrio metschnikovii* cytolysin. *Infection and Immunity* **56**: 954–960.

236. Montero AB, Austin B. (1999). Characterization of extracellular products from an isolate of *Vibrio harveyi* recovered from diseased post-larval *Penaeus vannamei* (Bonne). *J Fish Dis* **22**: 377-386.

237. Morales AR, Nadji M, Livingstone AS. (2008). Rapid-Response, Molecular-Friendly Surgical Pathology: A Radical Departure from the Century-Old Routine Practice. *J Am Coll Surg* **207** (3): 320-325.

238. Moran PJ, Bradbury RH, Reichelt RE (1985) Mesoscale studies of the crown-of-thorns/coral interaction: a case history from the Great Barrier Reef. *Proc 5th Int Coral Reef Symp, Tahiti* **5**: 321–326.

239. Moran PJ. (1986). The Acanthaster phenomenon. Oceanogr Mar Biol Annu Rev **24**: 379-480.

240. Moran PJ. (1988). COTS starfish questions and answers. National Library of Australia's online catalogue http://catalogue.nla.gov.au/Record/2114412.

241. Moran PJ, De'ath G. (1992). Estimates of the abundance of the COTS starfish *Acanthaster planci* in outbreaking and non-outbreaking populations on reefs within the Great Barrier Reef. *Mar Biol* **113**: 509-515.

242. Morgan AD. (2000). Aspects de la gestion des stocks geniteurs d'holothuries de sable (*Echinoderme: Holothurides*). La Beche-de-mer Bulletin d'information **13**. Secretariat General de la Communaute´ du Pacifique: 2 –8.

243. Morris JG, Black RE. (1985). Cholera and other vibrios in the United States. *N Engl J Med* **312**: 343–350.

244. Mueller UG, Wolfenbarger LL. (1999). AFLP genotyping and fingerprinting. *TREE* **14** (10): 389-394.

245. Munkholm J, Talman ML, Hasselager T. (2008). Implementation of a new rapid tissue processing method–Advantages and challenges. *Pathology–Research & Practice* **204**: 899–904.

246. Muramoto K, Magariyama Y, Homma M, Kawagishi I, Sujiyama S, Imae Y, Kudo S. (1996). Rotational fluctuation of the sodium driven flagellar motor of *Vibrio algynolyticus* induced by binding of inhibitors *J Mol Biol* **259**:687-695.

247. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. (1995). Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, DC.

248. Nash WJ, Goddard M, Lucas JS. (1988). Population genetic studies of the crown-of-thorns starfish, *Acanthaster planci* (L.), in the Great Barrier Reef region. *Coral Reefs* **7** (7): 11-18.

249. Neilands JB. (1981). Microbial iron compounds. *Annu Rev Biochem* **50**:715–731.

250. Neilands JB. (1995). Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**: 26723–26726.

251. Neill MA, Carpenter CJ. (2005). Other pathogenic vibrios. In: Mandell, G.L., Bennett, J.E., Dolin, R., editors. Mandell, Douglas and Bennett's: principles and practice of infectious diseases. 6th ed. Philadelphia, PA: Elsevier Churchill Livingstone: 2544-2548.

252. Nishibuchi M, Muroga K, Jo M. (1980). Pathogenic Vibrio isolated from cultured eels. V. Serological studies. *Fish Pathol* **14**:117–124.

253. Ngues MM, Bak RPM. (2008). Brown-band syndrome on feeding scars of the crown-of-thorn starfish *Acanthaster planci*. *Coral Reefs* **28**: 507-510.

254. Oakey HJ, Owens L. (2000). A new bacteriophague, VHML, isolated from a toxin producing strain of *Vibrio harveyi* in tropical Australia. *J Appl Microbiol* **89**: 702–709.

255. O'brien AD, Chen ME, Holmes RK, Kaper J, Levine MM. (1984). Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (shiga-like cytotoxin). *Lancet* **1**:77-78.

256. Ochman H, Lawrence J, Groisman EA. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* **405**: 299–304.

257. Ohara C, Sowers E, Bopp C, Duda S, Strockbine N. (2003). Accuracy of six commercially available systems for identification of 15 members of the family *vibrionaceae*. *J Clin Microbiol* **41:** 5654-5659.

258. Ohwada K, Tabor PS, Colwell RR. (1980). Species composition and barotolerance of gut microflora of deep-sea benthic macrofauna collected at various depths in the Atlantic Ocean. *Appl Environ Microbiol* **40**: 746-755.

259. Oliver JD. (2005). Wound infections caused by *Vibrio vulnificus* and other marine bacteria. *Epidemiol Infect* **133**: 383-391.

260. O'neill P. (1989). Structure and mechanics of starfish body wall. *J Exp Biol* **147**: 53-89.

261. Oopik V, Saaremets I, Medijainen L, Karelson K, Jnason T, Timpmann S. (2003). Effects of sodium citrate ingestion before exercise on endurance performance in well-trained runners. *Brit J Sport Med* **37**: 485-489.

262. Opals Ramsey JS. (1986). Intracranial Infection by *Vibrio alginolyticus* following Injury in Salt Water. *J Clin Microbiol* **23** (2): 373-374.

263. Orrhage K, Nord CE. (2000). Bifidobacteria and lactobacilli in human health. *Drugs Exp Clin Res* **26** (3): 95-111.

264. Orozco-Noriega L, Acedo-Félix E, Higuera-Ciapar, Jiménez-Flores R, Cano R. (2007). Pathogenic and non-pathogenic Vibrio species in aquaculture shrimp ponds. *Rev Latinoamericana Microbiol* **49** (3-4): 60-67.

265. Osgood DP, Wood NP, Sperry JF. (1993). Nutritional aspects of cytotoxin production of *Clostridium difficile*. *Appl Env Microb* **59**: 3985- 3988.

266. O'shea TM, Deloney-Marino CR, Shibata S, Aisawa S, Wolfe AAJ, Visik KL. (2005). Magnesium promotes flagellation of *Vibrio Fisheri*. *J bacteriolol* **187**: 2058-2065.

267. Owen CR. (1946). Acetic acid inhibition of gram-negative bacilli in Culture media. *J Bacteriol* **52** (3): 353–356

268. Owens L, Muir P, Sutton D, Wingfields M. (1992). The pathology of microbial disease in tropical Australian Crustacea, In: Diseases of Asian Aquaculture, Asian Fisheries Society, Manila, Philippines, pp. 165-172.

269. Owens L, Austin DA, Austin B. (1996). Effect of siderophore production in *Vibrio harveyi* isolates. *Dis Aquat Org* **27**: 157-160.

270. Owens L, Busico-Salcedo N. (2006). *Vibrio harveyi*: pretty problems in paradise. In: Thompson FL, Austin B, Swings J. (Eds.). The Biology of Vibrios. pp. 266–280. ASM Press, Washington DC.

271. Oxley APA, Shipton W, Owens L, McKay D. (2002). Bacterial flora from the gut of the wild and cultured banana prawn, *Penaeus merguiensis*. *J Appl Microbiol* **93**: 214–223.

272. Pacini F, Magni F. (1854). Osservazioni microscopiche e deduzioni patalogiche sul cholera asiatico. Gazzetta medica italiana federativa toscana, Firenze, **4**, December. Reprinted in *Lo Sperimentale*, Firenze, 1924, **78**: 277-282.

273. Pan American Health Organization (2006). Number of cholera cases in the Americas (1990-2006).

274. http://www.paho.org/English/AD/DPC/CD/cholera-1990-2006.pdf

275. Pandolfi J, Jackson JBC, Baron N, Bradbury RH, Guzman HM, Hughes TP, Kappel CV, Micheli F, Ogden JC, Possingham HP, Sala E. (2005). Are U.S. coral reefs on the slippery slope to slime? *Science* **307**: 1725–1726.

276. Pantos O, Cooney RP, Le Tissier MDA, Barer MR, O'Donnell AG, Bythell JC (2003) The bacterial ecology of a plague-like disease affecting the Caribbean coral Montastrea annularis. *Environ Microbiol* **5**: 370-382.

277. Pantos O, Bythell JC. (2006). Bacterial community structure associated with white band disease in the elkhorn coral Acropora palmata determined using culture independent 16S rRNA techniques. *Dis Aquat Org* **69**: 79–88.

278. Park S, Sun-Yang P, Choong-Min R, Seung-Hwan P, Jung-Kee L. (2008). The Role of AiiA, a Quorum-Quenching Enzyme from *Bacillus thuringiensis* on the Rhizosphere Competence. *J Microbiol Biotechnol* **18** (9): 1518–1521.

279. Pascual J, Macian MC, Arahal DR, Garay E, Pujalte MJ (2010) Multilocus sequence analysis of the central clade of the genus Vibrio by using the 16S rRNA, *recA, pyrH, rpoD, gyrB, rctB* and *toxR* genes. *Int J Syst Evol Microbiol* **60** (1): 154 – 165.

280. Patel M, Isaacson M. (1999). The effect of iron on the toxigenicity of *Vibrio cholerae. Am J Trop Med Hyg* **60**: 392–396.

281. Patterson TF, Bell SR, Bia FJ. (1988). *Vibrio alginolyticus* cellulitis following coral injury. Yale *J Biol Med* **61**: 507-512.

282. Pavia AT, Bryan JA, Maher KL, Hester TR, Farmer JJ. (1989). *Vibrio carchariae* infection after a shark bite. *Ann Intern Med* **111**: 85–86.

283. Pedersen K, Verdonck L, Austin B, Austin DA and others. (1998). Taxonomic evidence that *Vibrio carchariae* Grimes et al. 1985 is a junior synonym of *Vibrio*

harveyi (Johnson and Shunk 1936) Baumann et al. 1981. *Int J Syst Bacteriol* **48**: 749−758.

284. Perez MJ, Rodriguez LA, Nieto TP. (1998). The acetylcholinesterase ichthyotoxin is a common component of the extracellular products of *Vibrionaceae* strains. *J Appl Microbiol* **84**: 47–52.
285. Peters L, Konig MG, Wright AD, Pukall R, Stackebrandt E, Eberl L, Riedel K. (2003). Secondary Metabolites of Flustra foliacea and their Influence on Bacteria. *Appl Environ Microbiol* **69** (6): 3469–3475.
286. Prasad S, Morris PC, Hansen R, Meaden PG, Austin B. (2005). A novel bacteriocin-like substance (BLIS) from a pathogenic strain of *Vibrio harveyi. Microbiology* **151**: 3051-3058.
287. Pratchett MS. (1999). An infectious disease in COTS starfish on the Great Barrier Reef. *Coral Reefs* **18**: 272.
288. Pratchett MS. (2001). Influence of coral symbionts on feeding preferences of crown-of-thorns starfish *Acanthaster planci* in the western Pacific. *Mar Ecol Prog Ser* **214**: 111–119.
289. Pratchett MS. (2005). Dynamics of an outbreak population of *Acanthaster planci* at Lizard Island, northern Great Barrier Reef (1995–1999). *Coral Reefs* **24**: 453–462.
290. Pratchett MS, Schenk TJ, Baine M, Syms C, Baird AH. (2009). Selective coral mortality associated with outbreaks of *Acanthaster planci* L. in Bootless Bay, Papua New Guinea. *Mar Environ Res* **67**: 230–236.

291. Pratchett MS. (2010). Changes in coral assemblages during an outbreak of *Acanthaster planci* at Lizard Island, northern Great Barrier Reef (1995–1999). *Coral Reefs* **29**: 717-725.

292. Pratchett MS, Bay LK, Gehrke PC, Koehn J, Osborne K, Pressey RL, Sweatman HPA, Wachenfeld D. (2011). Contribution of climate change to degradation and loss of critical fish habitats in Australian aquatic environments. *Marine and Freshwater Research* **62**: 1062–1081.

293. Posada D, Crandall KA. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

294. Postgate J. (2000). Microbes and Man. 4thed. Cambridge, Mass. Cambridge University Press.

295. Raimondi F, Kao J, Fiorentini C. (2000). Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable director Hemolysin in In Vitro systems. *Infect Immun* **68**: 3180–3185.

296. Ragazzani T, Magrini E, Cucchi MC, Foschini MP, Eusebi V. (2005). The fast track biopsy (FTB): Description of a rapid histology and immunohistochemistry method for evaluation of preoperative breast core biopsies. *Int J Clin Pathol* **13** (3): 247-252.

297. Rank EL, Smith IB, Langer M. (1988). Bacteremia caused by *Vibrio hollisae*. *J Clin Microbiol* **26**: 375-376.

298. Raymundo LJ, Halford AR, Maypa AP, Kerr AM (2009) Functionally diverse reef-fish communities ameliorate coral disease. *Proc Nat Acad Sci USA* **106**: 17067-17070.

299. Reed KC, Crowell MC, Castro MD, Sloan ML (1999) Skin and Soft-Tissue Infections after injury in the Ocean: Culture methods and antibiotic therapy for marine bacteria. *Mil Med* **164**: 198-201.

300. Reid GC, Woods DR, Robb FT. (1980). Peptone induction and rifampin-insensitive collagenase production by *Vibrio alginolyticus*. *J Bacteriol* **142**: 447-454.

301. Ringo E, Salinas I, Olsen RE, Nyhaug A, Myklebust R, Mayhew TM. (2007). Histological changes in intestine of Atlantic salmon (*Salmo salar L.*) following in vitro exposure to pathogenic and probiotic bacterial strains. *Cell Tissue Res* **328**: 109–116.

302. Ripabelli G, Sammarco ML, Grasso GM, Fanelli I, Caprioli A, Luzzi I. (1999). Occurrence of Vibrio and other pathogenic bacteria in *Mytilus galloprovincialis* (mussels) harvested from Adriatic Sea, Italy. *Int J Food Microbiol* **49**: 43-48.

303. Ritchie KB. (2006). Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar Ecol Prog Ser* **322**: 1–14.

304. Rivera-Posada JA, Pratchett M, Cano-Gomez A, Arango-Gomez JD, Owens L. (2011a). Injection of *Acanthaster planci* with thiosulfate-citrate-bile-sucrose agar (TCBS). I. Disease induction. *Dis Aquat Org* **97**: 85–94.

305. Rivera-Posada JA, Pratchett M, Owens L, Arango-Gomez JD, Cano-Gomez A. (2011b). Refined identification of bacterial flora from *Acanthasther planci* based on 16S rRNA, *mreB* and *topA* gene sequencing. *Dis Aquat Org* **96**: 113-126.

306. Rivera-Posada JA, Pratchett MS, Owens L. (2011). Injection of *Acanthaster planci* with thiosulfate-citrate-bile-sucrose agar (TCBS). II. Histopathological changes. *Dis Aquat Org* **97** (2): 95-102.

307. Robert-Pillot A, Guénolé A, Lesne J, Delesmont R, Fournier JM, Quilici ML. (2004). Occurrence of the *tdh* and *trh genes* in *Vibrio parahaemolyticus* isolates from waters and raw shellfish collected in two French coastal areas and from seafood imported into France. *Inter J Food Microbiol* **91**: 319–325.

308. Roddis L. (1931). Garcia da Orta, the first European writer on tropical medicine and a pioneer in pharmacognosy. *Annals of medical history.* 1 (2): 198-207.

309. Rolls G, Farmer N, Tarbet F. (2008). Assessing the Quality of Tissue Processing and the performance of Peloris^{TM} using the Leica Microsystems Scoring System. Leica Microsystems Rev. www.leica-microsystems.com.

310. Rolfe RD, Finegold SM. (1979). Purification and characterization of *Clostridium difficile* toxin. *Infect Immun* **25**: 191–201.

311. Roman MR, Gauzens AL. (1993). Effects of low oxygen waters on Chesapeake Bay zooplankton. *Limnol oceanograf* **38** (8): 1603-1614.

312. Rosenberg E, Loya Y. (2004). Coral Health and Disease. Berlin: Springer-Verlag Berlin Heidelberg New York.

313. Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I. (2007). The role of microorganisms in coral health, disease and evolution. *Nat Rev Microbiol* **5**: 355–362.

314. Rotjan RD, Lewis SM. (2008). Impact of coral predators on tropical reefs. *Mar Ecol Prog Ser* **367**: 73-91

315. Saeed MO. (1995). Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. *Aquaculture***136**: 21–29.

316. Saitou N, Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4** (4): 406–425.

317. Sano M, Shimizu M, Nose Y. (1987). Long-term effects of destruction of hermatypic corals by *Acanthaster planci* infestation on reef fish communities at Iriomote Island, Japan. *Mar Ecol Prog Ser* **37**: 191-199.

318. Sanyal SC, Huq MI, Neogi PKB, Alam L, Kabir MI, Rahaman AS. (1983). *Vibrio mimicus* as an aetiologic agent of diarrhoea and its pathogenesis. *Indian J Med Microbiol* **1**: 1–12.

319. Sar N, McCarter L, Simon M, Silverman M. (1990). Chemotactic control of the two flagellar systems of *Vibrio parahaemolyticus*. *J bacteriology* **172**: 334-341.

320. Sawabe T, Oda Y, Shiomi Y, Ezura Y. (1995). Alginate Degradation by Bacteria Isolated From the Gut of Sea Urchins and Abalones. *Microb Ecol* **30**: 193-202.

321. Sawabe T, Kita-Tsukamoto K, Thompson FL. (2007). Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *J Bacteriol* **189** (21): 7932-7936.

322. Schaefer A, Goldstein JD, Reif JS, Fair PA, Bossart GD. (2009). Antibiotic-Resistant Organisms Cultured from Atlantic Bottlenose Dolphins (*Tursiops truncatus*) Inhabiting Estuarine Waters of Charleston, SC and Indian River Lagoon, FL. *EcoHealth* **5**.

323. Schets FM, Van den Berg HH, Demeulmeester AA, Van Dijk E, Rutjes SA, Van Hooijdonk HJ, de Roda Husman AM. (2006). *Vibrio alginolyticus* infections in the Netherlands after swimming in the North Sea. *Euro Surveill* **11** (45): 3077.

324. Schroeder JP, Wallace WG, Cates MB, Greco SB, Moore WB. (1985). An infection by *Vibrio alginolyticus* in an Atlantic bottlenose dolphin housed in an open ocean pen. *J Wildlife Dis* **2** (1): 437- 438.

325. Secord D. (2003). Biological control of marine invasive species: cautionary tales and land-based lessons. *Biol Invasions* **5**: 117–131.

326. Seidler RJ, Allen DA, Colwell RR, Joseph SW, Daily OP. (1980). Biochemical characteristics and virulence of environmental group F bacteria isolated in the United States. *Appl Environ Microbiol* **40**: 715–720.

327. Sganga G. (2009). Global Climate Change and Wound Care: Case Study of an Off-season Vibrio alginolyticus Infection in a Healthy Man. *Ostomy wound management* **55** (4): 60-62.

328. Shi-ming l, Jun Li, Ying Wang, Ren-gao Ye, Bengt Lindholm, Tao Wang. (2001). Methods to Improve the Preservation of Peritoneal Tissues. *Adv Peritoneal Dialysis* **17**: 61-65.

329. Sicherer SH, Sampson HA, Bock SA, Munoz-Furlong A. (2001). Underrepresentation of the risk and incidence of anaphylaxis to foods. *Arch Intern Med* **161**: 2046–2047.

330. Simidu U, Ashino K, Kaneko E. (1971). Bacterial flora of phyto- and zoo-plankton in the inshore water of Japan. *Can J Microbiol* **17**: 1157– 1160.

331. Simidu U, Kaneko E, Taga N. (1977). Microbiological studies of Tokyo Bay. *Microb Ecol* **3**:173-191.

332. Sjoblad R, Mitchell R. (1979). Chemotactic responses of *Vibrio alginolyticus* to algal extracellular products. *Can J Microbiol* **25**: 964-967.

333. Soonthornchai W, Rungrassamee W, Karoonuthaisiri N, Jarayabhand P, Klinbunga S, Soderhall K, Jiravanichpaisal P. (2010). Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon* after an oral infection by *Vibrio harveyi*. *Dev Comp Immunol* **34**: 19–28.

334. Soto-Rodriguez SA, Roque A, Lizarraga-Partida ML, Guerra-Flores AL, Gomez-Gil B. (2003). Virulence of luminous vibrios to *Artemia franciscana* nauplii. *Dis Aquat Org* **53**: 231–240.

335. Sparks AK. (1981). Bacterial disease of invertebrates other than insects. Pathogenesis of Invertebrate Microbial Diseases, Davidson EW(Ed), Allanhed Osmus & Co, Totowa, NJ.

336. Stackebrandt E, FrederiksenW, Garrity GM. (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**: 1043−1047.

337. Staehli A, Schaerer R, Hoelzle K, Rib G. (2008). Temperature induced disease in the starfish *Astropecten jonstoni*. *JMBA*2 - Biodiversity records published on-line

338. Steedman HF. (1960). Section cutting in microscopy. Blackwell Scientific Publications, Oxford, UK.Shinoda S, Yakahima I, Yasui S, Kim YM, Ono B, Nakagami S. (1992). Lateral flagella of vibrios: serological classification and genetical similarity. *Microbiol Immunol* **36**: 303-309.

339. Stephan R, Taureck K, Knabner D, Shimada T, Larsen JL, Kruppe C. (2002). Zum Vorkommen von halophilen und human pathogenen Vibrionen in deutschen Nutztierbesta¨nden. Tiera¨rztl Prax **30**: 69–74.

340. Strahl ED, Dobson WE, Lundie LL. (2002). Isolation and Screening of Brittlestar-Associated Bacteria for Antibacterial Activity. *Current Microbiology* **44**: 450–459.

341. Stvrtinová V, Jakubovský J, Hulín I. (1995). Inflammation and Fever from Pathophysiology: Principles of Disease. Computing Centre, Slovak Academy of Sciences: Academic Electronic Press. http://nic.sav.sk/logos/books/scientific.

342. Sultan Z, Mizuno T, Sakurai A, Takata N, Okamoto K, Miyoshi S. (2007). Growth Phase dependent activation of the precursor of *Vibrio mimicus* hemolysin (Pro-VHM). *J Health Science* **53** (40): 430-437.

343. Sung HH, Hsu SF, Chen CK, Ting YY, Chao WL. (2001). Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of Vibrio communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture* **192**: 101–110.

344. Sussman M, Willis BL, Victor S, Bourne DG. (2008). Coral pathogens identified for White Syndrome (WS) epizootics in the Indo-Pacific. *PLoS ONE* **3** (6): e2393.

345. Sutton DC, Trott L, Reichelt JL, Lucas JS. (1988). Assessment of bacterial pathogenesis in Crown-of-thorns starfish, *Acanthaster planci*. Proceedings of the 6th International Coral Reef Symposium, Australia **2**: 171-176.

346. Sutton DC, Garrick R. (1993). Bacterial disease of cultured giant clam *Tridacna gigas* larvae. *Dis Aquat Org* **16**: 47–53.

347. Swofford DL. (2003). PAUP_: Phylogenetic Analysis Using Parsimony (_and Other Methods), version 4.0B10. Sinauer Associates, Sunderland, MA.

348. Tagomori K, Iida T, Honda T. (2002). Comparison of genome structures of vibrios, bacteria possessing two chromosomes. *J Bacteriol*. **184**: 4351–4358.

349. Tajima K, Hirano T, Shimizu M, Ezura Y. (1997 a). Isolation and pathogenicity of the causative bacterium of spotting disease of sea urchin *Strongylocentrotus intermedius*. *Fish Sci* **63** (2): 249–252.

350. Tajima K, Hirano T, Nakano K, Ezura Y. (1997 b). Taxonomical study on the causative bacterium of spotting disease of sea urchin *Strongylocentrotus intermedius*. *Fish Sci*. **63** (6): 897–900.

351. Takahashi A, Miyoshi S, Takata N, Nakano M, Hamamoto A, Mawatari K, Harada N, Shinoda S, Nakaya Y. (2007). Haemolysin produced by *Vibrio mimicus* activates two Cl-secretory pathways in cultured intestinal-like Caco-2 cells. *Cell Microbiol* **9**: 583–595.

352. Takeuchi K, Tajima K, Iqbal MM, Sawabe T, Ezura Y. (1999). Taxonomical and serological studies on the causative bacteria of the disease of sea urchin *Strongylocentrotus intermedius* occurring at low water temperatures. *Fish Sci* **65** (2): 264–268.

353. Tall BD, Fall S, Pereira MR, Ramos-Valle M, Curtis SK, Kothary MH, Chu DMT, Monday SR, Kornegay L, Donkar T, Prince D, Thunberg RL, Shangraw KA, Hanes DE, Khambaty FM, Lampel KA, Bier JW, Bayer RC. (2003). Characterization of *Vibrio fluvialis*-Like Strains Implicated in Limp Lobster Disease. *Appl Envir Microbiol* **69** (12): 7435-7446.

354. Talukder SI. (2007). Histopathology Techniques: Tissue Processing and Staining. www.talukderbd.com. Accessed, June 2011.

355. Tantillo GM, Fontanarosa M, Di Pinto A, Musti M. (2004). Updated perspectives on emerging vibrios associated with human infections. *Lett Appl Microbiol* **39**: 117–126.

356. Taylor R. (1981). *Vibrio alginolyticus* peritonitis associated with ambulatory 2peritoneal dialysis. *British Med Journal* **283**: 275.

357. Thekdi RJ, Lakhani AG, Rale VB, Panse MV. (1990). An outbreak of food poisoning suspected to be caused by *Vibrio fluvialis*. *J Diarrhoeal Dis Res* **8**: 163–165.

358. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**: 4876–4882.

359. Thompson FL, Hoste B, Vandemeulebroecke K, Swings J. (2001). Genomic diversity amongst Vibrio isolates from different sources determined by fluorescent amplified fragment length polymorphism. *Syst Appl Microbiol* **24**: 520–538.

360. Thompson FL, Iida T, Swings J. (2004). Biodiversity of vibrios. *Microbiol Mol Biol Rev* **68**: 403–431.

361. Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J. (2005). Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl Environ Microbiol* **71**: 5107-5115.

362. Thompson FL, Austin B, and Swings J. (2006). The Biology of Vibrios ASM Press Washington D.C: pp 325.

363. Thompson FL, Gómez-Gil B, Ribeiro Vasconcelos AT, Sawabe T. (2007). Multilocus Sequence Analysis reveals that *Vibrio harveyi* and *V. campbellii* are distinct species. *Appl Environ Microbiol* **73**: 4279–4285.

364. Todar K. (2010). Todar's Online Textbook of Bacteriology. http://www.textbookofbacteriology.net/

365. Todd LS, Strange K. (2005). Transcriptional targets of DAF-16 insulin signaling pathway protect *C. elegans* from extreme hypertonic stress. *Am J Physiol Cell Physiol* **288**: 467- 474.

366. Travers M-A, Basuyaux O, Le Goic N, Huchette S, Nicolas J-L, Koken M, Paillard C. (2009). Influence of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by Vibrio harveyi: an example of emerging vibriosis linked to global warming. *Global Change Biology* **15**: 1365-1376.

367. Tsuji S, Tsuji M, Sun WH, Gunawan ES, Murata H, Kawano S, Hori M. (1997). *Helicobacter pylori* and gastric carcinogenesis. *J Clin Gastroenterol* **25**: S186-197.

368. Tyagi A, Saravanan V, Karunasagar I, Karunasagar I. (2009). Detection of *Vibrio parahaemolyticus* in tropical shellfish by SYBR green real-time PCR and evaluation of three enrichment media. *Int J Food Microbiol* **129**: 124–130.

369. Urwin R, Maiden JC. (2003). Multi-locus sequence typing: a tool for global epidemiology. *TRENDS in Microbiology* **11** (10): 479-487.

370. Uzzau S, Cappuccinelli P, Fasano A. (1999). Expression of *Vibrio cholerae* zonula occludens toxin and analysis of its subcellular localization. *Microb Pathog* **27**: 377–385.

371. Van Belkum A, Struelens M, de Visser A, Verbrugh H, Tibayrenc M. (2001). Role of genomic typing in taxonomy, evolutionary genetics, and microbial epidemiology. *Clin Microbiol Rev* **14**: 547–560.

372. Vandenberghe J, Thompson FL, Gomez-Gil B, Swings J. (2003). Phenotypic diversity amongst Vibrio isolates from marine aquaculture systems. *Aquaculture* **219**: 9-20.

373. Varello K, Pezzolato M, Mascarino D, Ingravalle F, Caramelli M, Bozzetta E. (2008). Comparison of histologic techniques for the diagnosis of bovine tuberculosis in the framework of eradication programs. *J Vet Diagn Invest* **20**: 164–169.

374. Vattakaven T, Bond P, Bradley G, Munn CB. (2006). Differential Effects of Temperature and Starvation on Induction of the Viable-but-Nonculturable State in the Coral Pathogens *Vibrio shiloi* and *Vibrio tasmaniensis Appl Environ Microbiol* **72** (10): 6508–6513.

375. Venkateswaran K, Kiiyukia C, Takaki M, Nakano H, Matsuda H, Kawakami H, Hashimoto H. (1989). Characterization of toxigenic vibrios isolated from fresh water environment of Hiroshima, Japan. *Appl Environ Microbiol* **55**: 2613-2618.

376. Veron JEN. (2000). *Corals of the world, Vol. **3***, 490 pp. Australian Institute of Marine Science and CRR QLD Pty Ltd. Queensland, Australia.

377. Versalovic J, Koeuth T, Lupski JR. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**: 6823–6831.

378. Versalovic J, Schneider M, de Bruijn FJ, Lupski JR. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**: 25–40.

379. Vinten-Johansen P. (2003). Cholera, Chloroform, and the Science of Medicine: A Life of John Snow. OUP. ISBN 0-19-513544-X.

380. Vlieghe E, Phoba MF, Tamfun JJ, Jacobs J. (2009). Antibiotic resistance among bacterial pathogens in Central Africa: a review of the published literature between 1955 and 2008. *Int J Antimicrob Agents* **34** (4): 295-303.

381. Vos P, Hogers R, Bleeker M, Reijans M, Lee Th van der, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23** (21): 4407-4414.

382. Walker CW, Lesser MP. (1989). Nutrition and development of brooded embryos in the brittlestar *Amphipholis squamata*: do endosymbiotic bacteria play a role? *Mar Biol* **103**:519–530.

383. Walkins WD, Cabelli VJ. (1985). Effect of fecal pollution on *Vibrio parahaemolyticus* densities in an estuarine environment. *Appl Environ Microbiol* **49**:1307-1313.

384. Wallet F, Talchon M, Nseir S, Courcol RJ, Roussel-Delvallez M. (2005). *Vibrio metschnikovii* pneumonia. *Emerging Infect Dis* **11**: 1641–1642.

385. Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE. (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**: 463–464.

386. Webster NS, Xavier JR, Freckelton M, Motti CA, Cobb R. (2008). Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. *Environ Microbiol* **10**: 3366–3376.

387. Wilkie IC. (2002). Is muscle involved in the mechanical adaptability of echinoderm mutable collagenous tissue?. *J Exp Biol* **205**: 159–165.

388. Wilkins S, Millar M, Hemsworth S, Johnson G, Warwick S, Pizer B. (2008). *Vibrio harveyi* sepsis in a child with cancer. *Pediatric Blood and Cancer* **50**: 891-892.

389. Williams D. (1986). Temporal variation in the structure of reef slope fish communities (central Great Barrier Reef): short term effects of *Acanthaster planci* infestation. *Mar Ecol Prog Ser* **28**:157-164.

390. Woods AE, Ellis RC. (1994). Laboratory histopatology: a complete reference. Vols 1-2. Edinburgh: Churchill Livingstone.

391. World Health Organization, Garwood P. (2009). World health organization communicates. Global, national efforts must be urgently intensified to control Zimbabwe cholera outbreak.

392. World Health Organization (2010). Cholera http://www.who.int/immunization/topics/Cholera/en/index.html

393. Wright FH, Ott JE, Watts G. (1979). Parasitic, bacterial, and mycotic sinusitis in an Atlantic bottlenose dolphin (*Tursiops truncatus*): a case report. Annual Proceedings of the American Association of Zoo Veterinarians: 115-116.

394. Wong Hin-Chung, Chih-Hsueh L. (2001). Evaluation of Typing of *Vibrio parahaemolyticus* by Three PCR Methods Using Specific Primers. *J Clin Microbiol* **39**: 4233–4240.

395. Wuthe HH, Aleksic S, Hein W. (1993). Contribution to some phenotypical characteristics of *Vibrio cincinnatiensis*. Studies in one strain of a diarrhoeic human patient and in two isolates from aborted bovine fetuses. *Zentbl Bakteriol* **279**: 458-465.

396. Yamaguchi M. (1986). *Acanthaster planci* infestations of reefs and coral assembalges in Japan: a retrospective analysis of control efforts. *Coral Reefs* **5**: 23-30.

397. Yanong RP. (2010). Use of Copper in Marine Aquaculture and Aquarium Systems. Institute of Food and Agricultural Sciences (IFAS), University of Florida, Gainesville, FL. http://edis.ifas.ufl.edu/pdffiles/FA/FA16500.pdf

398. Yasuda K, Kitao T. (1980). Bacterial flora in the digestive tracts of prawns, *Penaeus japonicus* Bate. *Aquaculture* **19**: 229–234.

399. Yoshizawa S, Wada M, Kita-Tsukamoto K, Ikemoto E, Yokota A, Kogure K. (2009). *Vibrio azureus* sp. nov., a luminous marine bacterium isolated from seawater. *Int J Syst Evol Microbiol* **59**: 1645-1649.

400. Yoshizawa S, Wada M, Yokota A, Kazuhiro K. (2010). *Vibrio sagamiensis* sp. nov., luminous bacteria isolated from seawater. *J Gen Appl Microbiol* **56**: 499−507.

401. Yu C, Bassler L, Roseman S. (1993). Chemotaxis of the marine bacterium *Vibrio furnissi* to sugars. A potential mechanism for initiating the chitin catabolic cascade. *J Biol Chem* **268**: 9405-9409.

402. Zann L, Brodie J, Vuki V. (1990). History and population dynamics of the COTS starfish *Acanthaster planci* (L.) in the Suva area, Fiji. *Coral Reefs* **9**: 135–144.

403. Zanetti S, Deriu A, Volterra L, Falci MP, Molicotti P, Fadda G. (2000). Virulence factors in *Vibrio alginolyticus* strains isolated from aquatic environments. *Ann Ig* **12**: 487-91.

404. Zbinden R. (2006). Trends in antibiotic resistance among respiratory tract pathogens in children in two regions near France, a high level resistance country. *Eur J Pediatr* **165**: 1–2.

405. Zhang XH, Austin B. (2000). Pathogenicity of *Vibrio harveyi* to salmonids. *J Fish Dis* **23**: 93–102.

406. Zhang H, Chen L. (2010). Phylogenetic analysis of 16S rRNA gene sequences reveals distal gut bacterial diversity in wild wolves (*Canis lupus*). *Mol Biol Rep* DOI 10.1007/s11033-010-0060-z

407. Zorrilla I, Arijo S, Chabrillon M, Diaz P, Martinez-Manzanares E, Balebona MC, Morinigo MA (2003) Vibrio species isolated from diseased farmed sole, *Solea*

senegalensis (Kaup), and evaluation of the potential virulence role of their extracellular products. *J Fish Dis* **26**: 103–108.