This work is dedicated in memory of my father
Professor Zvi Sussman (1930-2006)

Coral disease pathogens of the Indo-Pacific Ocean

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Abstract

Since the identification of coral diseases in the Caribbean in the early 1970's, the number of reported coral disease syndromes, their prevalence and spread worldwide have rapidly increased. Despite increasing reports of coral epizootics resulting in mortalities, little is known about the direct causes of coral diseases. Currently, the study of coral pathogens, their natural reservoirs and possible vectors are still in their early infancy with only five causative agents identified and confirmed by fulfilling Henle-Koch's postulates. Uncertainty regarding the causes of disease has sparked a sharp debate about, whether coral diseases occurring in complex aquatic environments are only caused by primary pathogens, or by secondary pathogens in combinations with other factors, such as ocean warming or anthropogenic stress.

The aim of this study is to identify coral pathogens that are directly associated with the following Indo-Pacific scleractinian coral diseases: black band disease (BBD), red bands and white syndromes (WS's), and to clarify their role in disease onset.

Filamentous cyanobacteria forming red and black bands on three scleractinian corals from Palau were isolated, cultured and identified based on 16S rRNA gene identity as belonging to a single ribotype. Following trials of a range of specialized media and culture conditions, two media, Grund and ASN III, were identified as the best for successful isolation and culturing. Cultured cyanobacteria were examined under a light microscope to establish purity, color and morphological appearance. DNA extraction and partial sequencing of the 16S rRNA gene of both red and black cyanobacterial isolates demonstrated 100% sequence identity. These isolated strains were also found to have 99% sequence identity with an uncultured cyanobacterial strain previously identified by molecular techniques as belonging to a cyanobacterial ribotype associated with BBD infected corals in the Caribbean. Based on these findings, it is concluded that the classification of these two syndromes as separate coral diseases be postponed until further evidence is collected.

Coral pathogens from white syndrome (WS) epizootics in the Indo-Pacific were also investigated. Bacterial isolates were obtained from corals displaying disease signs at three WS outbreak sites: Nikko Bay in the Republic of Palau, Nelly Bay in the central Great Barrier Reef (GBR) and Majuro Atoll in the Republic of the

Marshall Islands, and used in laboratory-based infection trials involving exposure of healthy corals to putative bacterial pathogens in order to satisfy three separate criteria for establishing causality: Henle-Koch's postulates, Evan's rules and Hill's criteria.. Phylogenetic 16S rRNA gene analysis demonstrated that all six pathogens identified in this study were members of the γ-*Proteobacteria* family Vibrionacae, each with greater than 98% sequence identity with the previously characterized coral bleaching pathogen *Vibrio coralliilyticus*. Tests to determine the ability of putative coral pathogens to adhere to corals demonstrated that only the pathogenic strains could transit from aquaria seawater to coral mucus in less than 12 hours. Screening for proteolytic activity of more than 150 coral derived bacterial isolates by a biochemical assay and specific primers for a *Vibrio* family zinc-metalloprotease demonstrated a significant association between the presence of isolates capable of proteolytic activity and observed disease signs.

A *Vibrio* zinc-metalloprotease, derived from the supernatants of six identified WS pathogens, demonstrated rapid photoinactivation of susceptible *Symbiodinium* endosymbionts followed by lesions in coral tissue. *Symbiodinium* photosystem II inactivation was diagnosed by an imaging pulse amplitude modulation fluorometer in two bioassays, performed by exposing *Symbiodinium* cells and coral juveniles to non-inhibited and EDTA-inhibited supernatants.

Sequencing of protein bands (using nano LC/MS/MS) retrieved from pathogen supernatants (via protein electrophoresis) identified the *Vibrio* zinc-metalloprotease as a member of the thermolysin family and a potential virulence factor in the infection process. This virulence factor, which has been previously identified from numerous *Vibrio* pathogens of fish, mollusks and humans, showed highest activity when pathogen cultures were grown at 27ºC and inoculated into susceptible *Symbiodinium* cultures that were acclimatized to the same temperature.

This is the first study to identify coral pathogens on the GBR by fulfilling Henle-Koch's postulates, and the first study to investigate the phylogeny of cyanobacterial strains isolated from corals displaying both red band and black band disease signs. This study also presents novel findings on the aetiology of Indo-Pacific coral diseases, in particular the role of a bacterial virulence factor in causing WS disease signs and the potential effects of host and environmental conditions on its performance. Findings from this study will enable better monitoring of Indo-Pacific

coral diseases and their spread in the future, including better understanding of coral pathogen virulence mechanisms and coral disease aetiologies.

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Chapter 1
General Introduction

1.1 Coral disease: a novel field of research

The study of coral diseases is a novel field of research that has rapidly developed ever since the first coral disease signs were observed in the Caribbean by Anfred Antonius (1973, 1981a, 1981b) and others (Garrett and Ducklow 1975, Mitchell and Chet 1975), and a direct link between coral disease abundance and reef decline suggested (Dustan 1977, Dustan and Halas 1987, Porter and Meier 1992, Aronson and Precht 1997, Aronson et al. 1998, Hayes and Goreau 1998, Shinn et al. 2000, Nugues 2002). Currently, the number of reported disease signs associated with hard corals has increased to 29 in the Caribbean (Weil 2004) and to 7 in the Indo-Pacific Ocean (Willis et al. 2004). Many of these disease syndromes can be identified in the field by using traditional surveillance methods, such as viewing cards and underwater photography (Gefcoral.org). However, the direct cause(s) of the increased coral disease abundance on reefs is difficult to determine. This uncertainty has resulted in an on going debate among scholars regarding the question of whether coral diseases are caused by singular-specific agents, such as pathogens (Rosenberg and Falkovitz 2004), or by general disease-contributing factors and stressors, such as global warming and anthropogenic pollution (Lesser et al. 2007), or by various combinations of both specific and non-specific factors (Harvell et al. 2004).

From over 35 currently known coral diseases, 5 have been identified as infectious diseases and the respective causative agents characterized (Kushmaro et al. 1996, 1997a, Geiser et al. 1998, Patterson et al. 2002, Ben-Haim et al. 2002, 2003a, 2003b, Denner et al. 2003, Barash et al. 2005, Thompson et al. 2006). However, beyond the identification of visual disease signs, little is known about how coral diseases are caused or their aetiologies. Without this knowledge, little can be achieved in determining the actual health of coral reefs and even less in preventing epizootics from spreading across the oceans.

1.2 Diseases in the marine environment: an historical perspective

The study of diseases in the marine environment poses many challenges due to the remoteness of sites, the complex structure of reef habitats and the multitude of

species involved (Richardson and Poloczanska 2008). Diseases of the aquatic environment do not have a long historical record. In 1867 a typhus like disease; the first identified in fish, was reported in Lake Geneva in Switzerland (Woo and Bruno 1999). *Pestis salmonum* and *Pestis rubra angillarum*; later described as vibriosis and now known to be one of the most prevalent diseases of fish affecting more than 50 species (Woo and Bruno 1999), were the first diseases reported in salmon and eel. *Bacillus anguillarum* (1817), later identified as *Vibrio anguillarum* (1909), was found to be the causative agent of this disease. Furunculosis in cultured trout caused by *Aeromonas salamonicida* was identified by Emmerich and Weibel in 1888-1889 as *Bacterium salamonicida* (Lehmann and Neumann 1896), which led to the formation of a Furunculosis committee in Great Britain in 1928 to help combat this major disease. A systematic study of fish disease, which included disease agent characterization, followed from the 1930's (Woo and Bruno 1999). Viral diseases of fish were first observed in the 1960's as part of an improvement in diagnostic capability (Wolf 1988).

Based on this historical development, fish diseases were separated into categories based on the identification of causative agents: parasites, fungi, bacteria and viruses (OIE 2006). Not surprisingly, coral disease studies have followed a similar path with 5 bacterial coral disease agents and one fungal agent identified to date (Sutherland et al. 2004, Thompson et al. 2006). In both fish and coral disease studies, bacteria belonging to the γ–*Proteobacteria* family Vibrionaceae were among the first pathogens (causing summer infections) to be identified, although more than 175 years apart.

The recognition that international trade in fish and aquaculture products has contributed to the global spread of disease, has led to the signing of the Sanitary and Phytosanitary (SPS) agreement under the World Trade Organization (WTO). The need for standardization has produced the OIE (Office International Epizootics) diagnostic manual for aquatic animal disease (OIE 2006), which is updated once every 3 years and lists the current diseases that require notification. It is noteworthy that in the same manner in which the number of coral diseases has sharply increased in the past decades, so too have the number of fish, mollusc and crustacean diseases, with listings by the OIE increasing from only 14 in 1997 to 31 in 2003 and 33 in 2006 (http://www.oie.int/eng/normes/fmanual/A00004.htm). However, 19 out of the 33 notifiable aquaculture diseases listed by the OIE in 2006 were caused by viruses,

accounting for 58% in total (69% in fish, 88% in crustaceans and 13% in molluscs), compared with no viral diseases yet identified in corals (Davy et al. 2006).

Of concern is the fact that many aquaculture farms are in close proximity to coral reefs, and both rely on good health in order to support large human populations in developing countries. In 1995, global production of aquaculture was estimated at over US$42 billion growing to US$ 70.3 billion in 2004 (http://www.fao.org/docrep/009/a087e/a0874e00.htm), 91.5% of which came from developing countries in Asia. In order to protect the value of the world aquaculture market and trade, most aquaculture diseases listed by the OIE manual have been identified to a host-specific level and can be detected by using multiple diagnostic tools (OIE 2006). Coral reefs have also an intrinsic economic value to nations. Within Australia alone, much of the tourism industry, valued at AUS $85 billion in 2006/7 (Australian national accounts – 5249.0), promotes the diverse healthy reef systems on the East and West coasts. However, in contrast to aquaculture systems, most coral diseases can not be identified by any means other than the visual signs of disease, and most are still believed to affect multiple coral host species (Weil 2004, Willis et al. 2004) and be caused by general stress (Lesser et al. 2007). A general lack of knowledge on the fundamental factors causing coral diseases results in reefs being vunerable to devastating epizootics.

1.3 What constitutes a healthy coral?

The most general definition of disease is ***any alteration from the normal state of health*** (Lightner and Redman 1998). This definition might suit animals whose normal state of health can be expected. However, for corals there is currently no available information on what constitutes a ***normal state of health***, or whether such a ***normal*** fixed state of health actually exists. The Dorland medical dictionary's definition of disease, which describes disease as a ***definite morbid process, often with a characteristic train of symptoms*** (signs in corals; Dorland 2007), might therefore better apply to corals. In order to identify a characteristic train of signs in coral disease, knowledge of disease aetiology is required. Currently, very little is known about coral disease aetiologies with visible signs in the field only apparent at latter phases of disease progression (Ainsworth et al. 2007a). With no knowledge of disease aetiology or cause, the term ***syndrome*** is used to describe the often unfamiliar signs of coral diseases (Willis et al 2004).

A syndrome is defined as ***a combination of symptoms*** (or signs) ***resulting from a single cause or so commonly occurring together as to constitute a distinct clinical entity*** (Lightner and Redman 1998). However, many diseases, especially those known to occur in environmental settings, do not have a single cause or a distinct clinical entity. Such diseases have been named multi-factorial diseases (Thursfield 2005) and may have multiple causes set up in complex hierarchies that may result in various clinical entities, including diseases whose impacts are ***chronic*** or ***acute*** (Ainsworth et al., 2007a).

The period from 1884-1960, defined as the fourth period in the history of medicine and veterinary medicine advancement (Thrusfield 2005) included the acceptance of the ***microbial theory of disease***, as epitomised by the Henle Koch's postulates (Koch 1891). The use of these postulates defined infectious diseases by their single causative agent, and thus established medical control methods aimed at identifying and combating causative agents. During this period rigorous laboratory trials were developed to test putative agents that were isolated from infected hosts. Such putative agents had to fulfil 4 postulates before being identified as causative agents, namely:

1. That the putative agent can be isolated from infected hosts and is not found on non-infected hosts.
2. That the putative agent can be cultured as a pure culture in the laboratory.
3. That the pure culture can cause disease signs or symptoms identical with those observed in the field, when inoculated onto healthy hosts.
4. That the putative agent can then be re-isolated from laboratory infected hosts.

Most pathogens known to date have been identified by fulfilling Henle-Koch's postulates, including the identification of the HIV virus, which causes AIDS (Weiss and Duesberg 1990, Cohen 1994, Novembre et al. 1997). However, many infectious diseases, such as numerous enteric and respiratory diseases, have been found to be caused by multiple agents (Kaiser et al. 1999) or to be caused by singular agents but only under specific conditions (Israeli et al. 2001). Causes of some infectious diseases remain unknown, such as feline dysautonomia (Nunn et al. 2004) or equine grass sickness (Wlaschitz 2004). According to Thrusfield (2005), diseases which are identified by lesions (like most coral diseases) and not by aetiology or cause, tend to be caused by many agents or factors that may target multiple species. External disease

signs are often considered as “sub-optimal data” (Thrusfield 2005), which should only be considered the starting point for elucidating disease cause and aetiology.

The fifth and current period (1960-Present) in the history of medicine responded to the limitations of Henle-Koch’s postulates by introducing the ***multifactorial theory of disease*** (Thrusfield 2005) and a new understanding towards the risk factors involved in disease susceptibility, such as the association between smoking and the development of lung cancer (Doll and Hill 1994, Doll et al. 2004). Following these modern realizations, new disease control strategies have also been developed (Wilesmith et al. 1988), which focus on increased surveillance and monitoring of disease, including diseases in large eco-systems, such as forests (Desheng et al. 2006) and coral reefs (Weil 2004, Willis et al. 2004). A shift has been made towards the analysis of data and the management of health and disease in entire populations. Examples of such health control methods, which target populations in marine environments rather than individuals, are the establishment of marine protected parks (MPA’s; Agardy 1994, Dee-Boersma and Parrish 1999, Graham et al. 2008).

Currently, many complex disease aetiologies are being investigated by incorporating multiple paths, such as combining traditional methods based on fulfilling Henle-Koch’s postulates with modern methods that scan for non-culturable pathogens or virulence factors using molecular tools (Richardson et al. 2001, Wegley et al. 2007), in conjunction with pathological tools (Work and Aeby 2006) and an array of histopathological methods (Bythell et al. 2002). The advantage of obtaining pathogen isolates for the study of disease aetiology is obvious. However, it may not always be sufficient to understand why outbreaks are occurring at one environmental setting and not in another.

In order to understand the complexity of disease causation, the concept of a ***disease triad*** has been developed (Snieszko 1974), which identifies disease as a isosceles-triangle, in which the three apexes represent the pathogen, the host (susceptibility) and environmental factors contributing to disease (Fig. 1.1). In practice however, most coral diseases have been identified in the past three decades by looking at the coral host displaying signs of disease. More recent predator outbreaks of *Acanthaster planci* (Endean 1973) and coral bleaching (Hoegh-Guldberg 1999) have directed attention towards environmental factors, such as temperature and anthropogenic pollution. Only recently, the fear of emerging infectious diseases

spreading among reefs has focused attention further on coral pathogens, their reservoirs and vectors (Sussman et al. 2003, Rosenberg et al. 2007, 2008). Beyond the identification of complex disease causation, coral disease diagnostics still relies heavily on identifying macroscopic disease signs. In sharp contrast, the Office of International Epizootics (OIE) makes a clear standing on disease identification by stating that "The only dependable approach for diagnosis of fish diseases lies in the specific identification of the pathogens using laboratory methods" (OIE 2006).

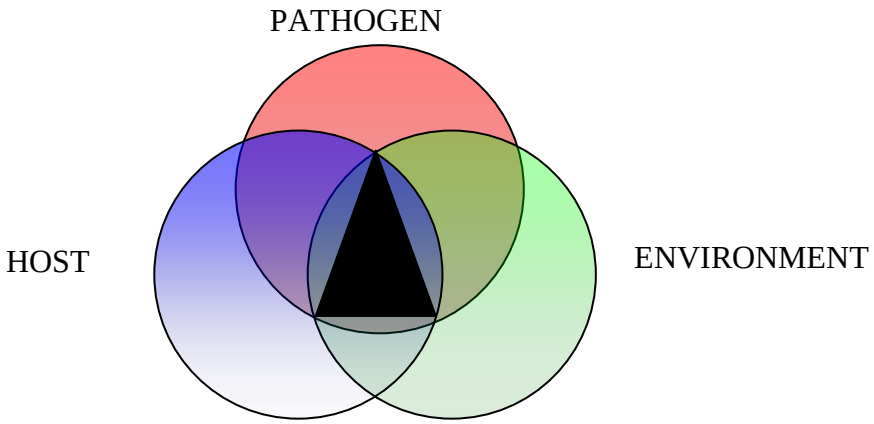

Fig. 1.1 The disease Triad (adapted from Snieszko 1974)

1.4 Climate change and anthropogenic disturbances

Reports on ocean warming due to climate change have been made since the 1960's (Domingues et al. 2008) and their association with emerging aquatic diseases has been demonstrated (Epstein et al. 1998, Harvell et al. 1999). Associations between ocean warming, growing anthropogenic stress on reefs and major declines in coral reef cover have produced alarming predictions about the future vitality of coral reefs due to higher stress levels, higher frequency of storms, acidity and higher abundance of disease including coral bleaching (Hughes 1994, Goreau et al. 1998, Harvell et al. 2002, Rosenberg and Ben-Haim 2002, Hughes et al. 2003, Hoegh-Guldberg 2004, Hoegh-Guldberg et al. 2007, Carpenter et al. 2008, Diaz and Rosenberg 2008). Since the mid 1980's at least three major bleaching events have struck coral reefs in the Caribbean (Glynn 1984, Goreau and Hayes 1994) and two mass bleaching events (1998, 2002) have devastated reefs on the Great Barrier Reef and in the Indo-Pacific Ocean (Wilkinson et al. 1999, 2003). All mass bleaching events correlated with increased seawater temperatures and exposure to increased radiation (Douglas 2003).

Thermal coral bleaching has also been replicated in experimental laboratory studies by exposing healthy corals to heat and light stress (Hoegh-Guldberg and Smith 1989, Glynn and D'Croz 1991, Iglesias-Prieto et al. 1992, Jones et al. 1998, Abrego et al. 2008). However, coral bleaching has also been shown to be caused by exposure of healthy corals to bacterial pathogens at elevated temperatures (Kushmaro et al. 1998, Banin 2000b, Israeli et al. 2001, Ben-Haim et al. 2003a) and to other stressors (Hoegh-Guldberg and Smith 1989, Muscatine et al. 1991). Laboratory experimens and modeling of seawater temperature data demonstrate that increased seawater temperatures have a direct influence on coral disease abundance and progression (Cervino 2004a, Boyett et al. 2007, Bruno et al. 2007). Bleached corals have also been shown to develop coral disease signs, suggesting that susceptibility to disease may be increased in compromised hosts (Muller et al. 2008). In addition, as seawater temperatures continue to rise, new emerging coral diseases have been reported and large scale epizootics have been documented in areas where no coral diseases have been previously observed (Korrubel and Riegl 1998, Aeby 2005, Dalton and Smith 2006, Haapkylä et al. 2007, Sato et al. 2009), including in temperate regions (Hall-Spencer et al. 2007). Nevertheless, lack of continuous data that document the impacts of climate change on various targets in the marine environment, such as pathogen abundance and spread, limits our understanding of how climate change may affect coral reef health in the future if seawater temperatures continue to increase. Current models forecast that by the end of the century, one third of reef building corals will become extinct by direct and indirect effects of climate change (Carpenter et al. 2008) including ocean acidification (Stone 2007).

In other more developed fields of disease research, such as human and terrestrial wildlife medicine, the ability to predict the impact of climate change on population health is supported by available data on pathogens and disease aetiology. In a recent report released at the IUCN World Conservation Congress, named "The Deadly Dozen: Wildlife Diseases in the Age of Climate Change" (The deadly dozen 2008), 12 infectious agents were listed by more than 60 scientists as contributing to growing potential threats to both wildlife and human populations due to climate change. According to the Wildlife Conservation Society (WCS), who published the report, increased monitoring of health in wildlife ecosystems will provide a new lens to document climate-related changes and will help governments, agencies, and communities to detect and mitigate threats before they become disasters.

The second major source of stress impacting on marine life arises from the discharge of anthropogenic effluents into nearshore environments. It has been demonstrated for both fish and corals that eutrophication of these environments represents a facilitating, predisposing and enabling factor for disease (Tomascik and Sander 1987, Moeller 1990, Vethaak and Rheinallt 1992, Richmond 1993, Arkoosh et al. 1998, Goreau et al. 1998, Richardson 1998a, Hughes and Connell 1999, Nyström et al. 2000, Knowlton 2001, Harvell et al. 2002, Szmant 2002, Letters 2003, Loya et al. 2004, Panek 2005, Kline et al. 2006, Smith et al. 2006, Voss and Richardson 2006, Baker et al. 2007, Johnson et al. 2007, Knowlton and Jackson 2008). On the GBR and throughout the Indo-Pacific, heavy rains results in large terrestrial flows into inshore and fringing reefs, which carry nutrients from agricultural lands that promote eutrophication and bacteria that directly impact on ecosystem health (Nairn 1993, Lafferty et al. 2004, Fabricius 2005). Terrestrial flows may also contain pesticides and sewage further deteriorating coral health (Grigg 1994, Wang et al. 2008). Fish exposed to polluted water in the laboratory developed skin haemorrhages, opaqueness of eyes and blindness (Grizzle et al. 1988), and *Aeromonas*, *Pseudomonas* and *Vibrio* cells were isolated from these diseased fish. In another study, infected fish were found as far away as 15 miles off-shore from the source of pollution (Mahoney et al. 1973). Corals exposed to eutrophic stress in the field showed increased progression rates and size of lesions caused by yellow band disease (Bruno et al. 2003), providing further corroborative evidence that nutrient stress is an aggravating factor in disease development.

1.5 Coral disease on the Great Barrier Reef and in the Indo-Pacific Ocean

Little research has been carried out on coral diseases on the GBR (Loya et al. 1984, Baird 2000). Some published notes on Indo-Pacific coral diseases relate to the GBR in general (Antonius, 1985a, 1988a, 1988b, Coles, 1994, Littler and Littler 1995, 1996, Antonius 1999, Antonius and Lipscomb 2000, Raymundo et al. 2003). Willis and collegues published an extensive review on coral diseases affecting GBR reefs in 2004 (Willis et al. 2004). Aeby (2006) and Kaczmarsky (2006) conducted similar studies in the Northwestern Hawaiian Islands and in the Philippines, respectively. In addition, reports on coral disease outbreaks from Hawaii (Aeby 2005, Friedlander et al. 2005), Indonesia (Haapkylä et al. 2007) and the Gulf of Eilat (Barash et al. 2005) have contributed to the acknowledgement that coral disease in the Indo-Pacific poses

a serious threat to coral populations. In contrast, studies of coral disease pathogens, possible reservoirs or vectors on the GBR have not been attempted, mainly due to the remoteness of outbreak sites, and the lack of expertise and funding needed for conducting medically oriented microbial surveys. The Australian Institute of Marine Science (AIMS) began to conduct coral disease surveillance in 1997 as part of its long term monitoring program (LTMP). However, without any diagnostics or sampling, little data have been collected beyond a visual inspection of disease abundance on reefs (Sweatman et al. 2001), which has limited usefulness for determining the causes and aetiologies of various coral diseases affecting corals of the GBR (Selig et al. 2006).

1.6 Black band disease (BBD)

Black band disease (BBD) was first reported from the Indo-Pacific in 1985 (Antonius 1985a) and from the GBR in 1994 (Dinsdale 1994, Littler and Littler 1996, Miller 1996, Dinsdale 2002). It has a world wide distribution and has been studied for the past 30 years demonstrating some of the early achievements in coral disease study, but also many of its short comings. As suggested by early Caribbean studies (Antonius 1981b, Rützler et al. 1983), BBD is a polymicrobial coral disease, which is caused by a consortium (Richardson et al. 1997a, Richardson and Kuta 2003) comprised of a dominant cyanobacterium (*Phormidium corallyticum*), which causes the black band (Rützler and Santavy 1983, Taylor 1983, Kuta 2000) and other bacteria, such as sulphur oxidizers and reducers (Carlton and Richardson 1995, Richardson 1996, Schnell et al. 1996, Viehman and Richardson 2002, Viehman et al. 2006). Although accepted as causative agents for BBD, this group of microorganisms has never been proven to actually cause the disease in laboratory experiments (Antonius 1985b). *P. corallyticum* has also been found on healthy corals in the field in contradiction to Henle-Koch's postulates (Richardson 1997b) and has only been diagnosed using light microscopy to detect specific physiological traits. Molecular studies undertaken in the Caribbean (Cooney et al. 2002, Frias-Lopez et al. 2002, 2004a) and in the Indo-Pacific (Frias-Lopez et al. 2003, Barneah et al. 2007) have all failed to identify the presence of the specific *P. corallyticum* strain in samples from BBD infected corals. In more recent studies conducted by the same authors, who identified the former putative pathogen *P. corallyticum*, a new group of putative BBD pathogens have been identified (Sekar et al. 2006, Myers et al. 2007, Voss et al. 2007),

suggesting that similar disease signs may be caused by different pathogens, or alternatively, that the causative agents of BBD may change with time, as some corals may develop resistance to infections, while certain cyanobacterial strains may become out-competed by others. It may also suggest that disease identification may be biased towards the tools and methods that are used for identification, suggesting that more rigid disease identification and classifications are currently required (Ainsworth et al. 2007a, Work et al. 2008). The failure to induce black band disease signs by conducting laboratory infections still prevents classifying this disease as an infectious-polymicrobial disease (Aeby and Santavy 2006).

The abundance of BBD on the GBR has been reported by Willis et al. (2004) and by Page and Willis (2006) and is typically low (under 1% prevalence) for the northern and southern sectors of the GBR. The AIMS long term monitoring programe (LTMP) reported less than 1 case of BBD infection per surveyed reef between the years 1997 to 2001, during which surveys for coral disease were conducted at 48 sites along the GBR (Sweatman et al. 2001).

The seemingly low prevalence of BBD from the GBR correlates well with current data from the Caribbean (Weil et al. 2002) that estimate the wider abundance of BBD on 19 different reefs to be under 0.5% (Weil 2004). Studies from the Caribbean (Edmunds 1991, Bythell and Sheppard 1993, Bythell et al. 1993, Kuta and Richardson 1997) demonstrate that coral mortalities caused by BBD outbreaks may contribute to a major decline in reef community structure. Currently, no reports have been made from the GBR regarding the phylogeny of cyanobacteria, or possibly other putative agents, found on BBD infected corals.

1.7 Red band disease (RBD)

Red band disease (RBD) was first reported by Rützler et al. (1983) and later by Richardson et al. in the Bahamas (1993, 1998a) and by Weil (2004) in the Caribbean. It is assumed to be a novel coral disease, although little is known about its cause or aetiology, but for the fact that bands progressing on diseased corals look red and not black, the typical appearance of black band disease (BBD).

1.8 White syndromes (WS's)

White syndrome (WS) is a general name given to patches or bands of bare white coral skeleton resulting from yet unknown causes, reported from the GBR

(Willis et al. 2004) and from the Indo-Pacific Ocean (Aeby 2005). This name has been adopted by the AIMS LTMP to differentiate between BBD infected corals, bleached corals, skeleton exposed by predation and all other white syndromes. The choice of this name follows on from experience in coral disease research gained in the Caribbean, where 7 "white" coral diseases and plagues have been identified including White Band I (Gladfelter 1982, Peters et al. 1983), White Band II (Ritchie and Smith 1998, Gil-Agudelo et al. 2006), White Plague I (Dustan 1977), White Plague II (Richardson et al. 1998b, Richardson et al. 1998c), White Plague III (Richardson and Aronson 2002) and additional white plague “like” diseases from the Caribbean (Rodriguez-Martinez et al. 2001, Pantos et al. 2003) and from the Red Sea in Eilat (Barash et al. 2005). The naming of all white plague (WP) and white band (WB) diseases in the Caribbean has followed the chronological sequence of documenting outbreaks, where different coral species have been shown to be susceptible to lesions of varying characteristics. However, a superficial disease identification process based on subjective observations has led to much confusion. Currently, causative agents have been verified only for Caribbean white plague II (Denner et al. 2003), but for none of the other syndromes. Lacking data on causative agents and disease aetiology for Caribbean white plague and white band diseases makes it very hard to adopt the Caribbean terminology for studies of similar disease signs found on corals in the Indo-Pacific region, as suggested by Willis et al. (2004). However, regardless of questions relating to either aetiology or cause, white diseases or syndromes both in the Caribbean and in the Indo-Pacific Ocean pose substantial threats to coral reefs.

White band (WB) disease in the Caribbean has eliminated *Acropora palmata* and *Acropora cervicornis* from reefs (Aronson and Precht 2001) leading to the listing of WB-susceptible corals on the endangered species list (Precht et al. 2004). WP II spread over 200 kilometer of reefs within 11 weeks during the 1995 Florida Key outbreak, infecting 17 species and causing 38% morality (Richardson and Aronson 2002). White band disease was reported on the GBR by Baird (2000) and by Antonious (1985a). A 20 fold increase in WS abundance was recorded between 1999 and 2003 by the AIMS LTMP on the GBR (almost 50 cases per reef) following the 2001/2 mass bleaching event (Willis et al. 2004). Willis et al. (2004) reported that WS affected over 5% of all corals at Heron Island following the mass bleaching event of 2002/3. Outbreaks of WS’s have been reported since 2002/3 from many Indo-Pacific locations, including the Northern Hawaiian Islands (Aeby 2005) and the Republic of

the Marshall Islands (Jacobson et al. 2006). Although WS's have been observed on more than 15 coral species (Willis et al. 2004), local outbreaks typically affect tabular coral species from the family *Acroporidae*, resulting in the naming of these disease signs as Acropora White Syndrome (AWS) when they occur on *Acropora* hosts (Aeby 2005, Roff et al. 2006). However, causes for WS outbreaks still remain ambiguous. Roff et al (2006) investigated diseased acroporid corals from Heron Island and suggested that AWS occurs following a "shut down reaction" response programmed by the coral. Other studies (Ainsworth et al. 2007b) detected DNA fragmentation of coral cells affected by AWS, suggesting that lesions were caused by a coral programmed cell death (PCD) initiated by the coral. Bruno et al. (2007) demonstrated a positive correlation between coral cover, temperature and disease abundance on GBR reefs, supporting the hypothesis that WS on the GBR may be an infectious disease. Without precise knowledge of WS disease aetiology and it's potential causes, little can be said about whether WS is one disease or many, and whether similar disease signs identified in the field are caused by one causative agent (or factor), or by many. Without insight into the aetiology of WS, little can be said about the association between WS disease abundance and environmental factors, such as ocean warming or anthropogenic pollution, or any other host-related factor. Such knowledge can aid in attempting to predict the future spread of WS's in the Indo-Pacific region.

1.9 *Atramentous necrosis,* a new coral disease outbreak at Magnetic Island, GBR

Following the mass bleaching event in the Austral summer of 2002, a new coral disease was observed on reefs fringing Magnetic Island (GBR). Jones et al. (2004) identified large lesions on diseased *Montipora aequituberculata* colonies that were later covered by sulphur deposits as the disease progressed. No causative agent has yet been identified for this seemingly newly emerging disease, named *Atramentous necrosis* (Jones et al. 2004). Recent work identified four distinct phases of the disease, with the intial signs being white tissue that dies back progressively to expose white skeleton and therefore fits the classification of a WS (Anthony et al. 2008). A study by Bourne (2005a) classified bacterial populations associated with diseased corals on Magnetic Island, suggesting that WS on Magnetic Island (*Atramentous necrosis)* may be bacterially caused. A coral disease affecting *Montipora spp*. in Hawaii displaying similar disease signs has been named Montipora White Syndrome – MWS (Jones 2007, Gochfeld and Aeby 2008).

1.10 Other coral diseases currently affecting the Indo-Pacific region

Greater surveillance efforts in the Indo-Pacific Ocean have produced numerous reports of newly emerging coral diseases with names based on their appearance, though characterization and establishment of either their cause or their aetiologies has not been performed. Assignment of these names is required to simplify any attempt to monitor outbreaks and follow their spread, though the current data collected on unfamiliar disease signs may only lead to temporary classifications (Work et al. 2008).

1.10.1 Skeletal eroding band (SEB)

The first report of this disease was made by Antonius and Lipscomb in 2001 and SEB is currently the most prevalent coral disease on the GBR affecting 2% of corals from 82 scleractinian coral species surveyed between 2003-2006 (Page and Willis 2008). Skeletal eroding band (SEB) manifests as dense aggregations of the ciliate *Halofolliculina corallasia* (Riegel and Antonius 2003), which form a distinct black band at the interface between recently exposed skeleton and apparently healthy looking coral tissue. Similar disease signs have also been observed in the wider Indo-Pacific (Winkler et al. 2004) and also in the Caribbean (Cróquer et al. 2006a, 2006b), although Caribbean infections are potentially caused by another species from the genus *Halofolliculina*. Currently, this disease is not classified as infectious to other corals.

1.10.2 Brown band disease (BrB)

Brown band disease (BrB) was first described by Willis and collegues, who observed macroscopic signs of the disease on three families of GBR corals (Willis et al. 2004). Macroscopic signs of the disease include a brown zone adjacent to healthy tissue on one side and to a zone of exposed white skeleton on the other. Occasionally, a white zone is also observed between the brown band and the healthy tissue, which may comprise bleached tissue and/or denuded skeleton. The brown zone contains a protozoan ciliate which accumulates zooxanthellae intracellulary, resulting in the characteristic brown colouring of the observed syndrome. The ciliates appear to ingest coral tissue at the lesion interface, accumulating symbiotic zooxanthellae (*Symbiodinium*) from coral endoderm (Bourne et al. 2008). Currently, no microbial

causative agents have been identified for this disease beyond coral infestation by ciliates (Ulstrup et al. 2007) and fungi (Yarden et al. 2007).

1.10.3 Porites ulcerative white spot disease (PUWS)

This emerging disease targets specific hosts from the family Poritidae (Raymundo et al. 2003). Novel PUWS disease signs are characterized by discrete bleached, round foci, 3 to 5 mm in diameter, that may either regress or progress to ulcerations the full thickness of the tissue layer that coalesce, occasionally resulting in colony mortality. Raymundo et al. (2003) found a positive correlation between host density and disease abundance. Currently, no causative agents have been identified for PUWS, although grafting experiments (performed by tying together diseased and healthy looking colonies in the field), demonstrated that this disease is communicable (i.e., transmissible by direct contact with an affected coral, the coral's discharges or by other indirect means) and possibly infectious (caused by the entrance into the body of organisms, such as bacteria, protozoans, fungi, or viruses; Raymundo et al. 2003). Until such evidence is provided, this disease may also be classified as WS.

1.10.4 Porites pink spots / Porites trematodiasis (TRM)

Porites trematodiasis is a coral disease caused by the infestation of coral tissue by the digenetic trematode and parasite *Podocotyloides stenometra* (Friedlander et al. 2005, Aeby 1998, 2007). Disease signs include 3-5mm, pink to pale, swollen nodules on the coral colony. This disease affects specific hosts from the family Poritidae. However, little is known about TRM aetiology and the conditions required for large scale outbreaks to occur. Similar disease signs have also been reported from the GBR (Willis et al. 2004), although no evidence of a trematode was found, and from Okinawa (Yamashiro 2000), where they were classified as growth anomalies (Domart-Coulon et al. 2006), which were previously described in the Indo-Pacific Ocean as neoplasia by Squires (1965) and as tumor formations on the GBR by Loya et al. (1984).

1.10.5 White plague “like” disease in the Gulf of Eilat

The causative agent of a disease affecting *Favia spp.* and *Goniastrea spp.* corals in the Gulf of Eilat was identified by fulfilling Henle-Koch’s postulates (Barash et al. 2005). Disease signs caused by the causative agent *Thalassomonas loyana*

(Thompson et al. 2006) include progressing tissue lesions which result in coral mortalities. These disease signs are similar to reported signs of white plague II from the Caribbean (Richardson et al. 1998c). However, the causative agent of this disease has not been identified elsewhere.

1.11 Coral disease pathogens: what is currently known?

Studies attempting to isolate putative bacterial coral pathogens commenced in the 1980's (Peters et al. 1983), however the first bacterial coral pathogens were not conclusively identified by fulfilling Henle-Koch's postulates until the 1990's (Kushmaro et al. 1996). Identified coral pathogens include the fungal agent *Aspergillus sydowii,* which causes the coenenchyme of sea fans to recede, exposing the axial skeleton in laboratory infection trials involving the gorgonian *Gorgonia ventalina* (Geiser et al. 1998) Identical disease signs were observed for the sea fan disease in the field (Smith et al. 1996) and the fungal agent was also implicated.

The bacterial causative agent, *Vibrio shiloi*, has been identified by Kushmaro et al. (1996) and shown to cause bleaching in laboratory exposure trials of the hard encrusting coral *Oculina patagonica*. These disease signs were identical with bleaching signs observed on infected colonies in the Mediterranean Sea. Although it is currently well accepted that less than 5% of all marine microorganisms are culturable on standard available media (Amann et al. 1995), the advantages of maintaining pure or axenic pathogen cultures is self evident. It is a perquisite for the fulfilment of Henle-Koch's postulates (Koch 1891), but also a necessity when attempting to study pathogen cell physiology, its virulence mechanisms and its function under varying environmental conditions. It is also a necessity, when attempting to design diagnostic tools of high validity that could be used for health screening purposes. Isolating pathogens on growth media is also a relatively cost efficient alternative to establish the identity of putative candidates. Such techniques can be accomplished at field stations, thereby removing the possibility of transmitting coral diseases by transporting infected corals or pathogens across reef regions. Nevertheless, other methods for screening diseased individuals are currently available, which are often used when direct isolation methods fail. These tools include cloning and denaturing gradient gel electrophoresis – DGGE (Bourne 2005), fluorescent *in situ* hybridization – FISH (Sussman et al. 2003), micro-arrays (Edge et al. 2005) and metagenomics (DeLong 2005, Edwards and Rohwer 2005, Yokouchi et al. 2006).

For establishing causation of multi-factorial diseases involving either multiple causative agents or enhanced host susceptibility as a consequence of environmental conditions, more modern conventions and criteria have been established (Hill 1965, Evans 1976) in order to overcome the many limitations arising when attempting to fulfil Henle-Koch's postulates in laboratory exposure trials (Casadevall and Pirofski 1999, Walker et al. 2006). New criteria rely on statistical significance for establishing causation, rather than on direct cause and effect (Thrusfield 2005). For some diseases, the maintenance of pathogen cultures may prove insufficient, for example when susceptible hosts develop resistance towards one primary agent and become susceptible towards another, such as in the case of *V. shiloi*, the causative agent for the bleaching of *O. patagonica* in the Mediterranean Sea (Reshef et al. 2006).

1.12 Pathogens from the *γ–Proteobacteria* family Vibrionacae

Vibrios are prime candidates for bacterial infections in the marine environment. *Vibrio* infections of fish (Egidius 1987), crustaceans (Brock 1992, Goarant et al. 1998) and clams (Paillard 2004) have been studied for several decades. From 21 diseases of shrimp reported from Asian aquaculture, all bacterial infections (three of a total 21) were associated with vibrios (Lightner and Redman 1998). A similar proportion (four *Vibrio* infections from a total of 24) was also reported from the Americas (Lightner and Redman 1998). However, many *Vibrio* infections have also been shown to be either opportunistic or secondary infections, which occur at a latter part of disease progression (Saulnier et al. 2000). The shrimp industry has provided significant evidence that many diseases are multifactorial, i.e., have mixed etiologies (Lightner 1996). Viral infections in highly compacted shrimp aquaculture facilities are often accompanied by bacterial and epicommensal infestations, which end up being the cause of death in already severely compromised populations (Beadling and Slifka 2004). In many cases, vibrios are among the dominant bacteria isolated from such mortalities (Johansen and Sommer 2001).

Corals have been shown to die following exposure to elevated levels of nutrients, which may cause a surge in bacterial abundance (including commensal vibrios) in coral mucus (Smith et al. 2006). Vibriosis, also known as saltwater furunculosis was first reported by Canestrini (1893). It took over 15 years until Bergman (1909) characterized the disease and its etiological agent *Vibrio anguillarum* (Snieszko 1975). The cost of *Vibrio* infections in closed aquaculture systems provided

the impetus to study of these pathogens, although they are also known to infect fish populations in the wild (Haastein and Holt 1972). Currently, more than half a dozen *Vibrio* species are listed as pathogens for invertebrates in the marine environment (Amaro and Biosca 1996, Lambert et al. 1998, Venkateswaran et al. 1998, Liu 1999, Villamil 2003, Wu 2004, Wang 2007), with pathogens, such as *Vibrio vulnificus* and *Vibrio parahaemoliticus*, known to infect human beings as opportunistic pathogens (Blake et al. 1980) causing high mortality rates (~60%) for compromised hosts (Oliver 1989).

Of all known *Vibrio* pathogens *Vibrio cholera* is one of the best studied due to its role as the causative agent of human Cholera epidemics for the past 1200 years, including 7 pandemics since 857 AD. (Karaolis et al. 1994, Wachsmuth et al. 1994, Colwell 1996). The study of *Vibrio cholera* contributed to current understanding of the potential threat of pathogens commonly found in the environment to human populations (Jiang et al. 2000, Codeco 2001). It also demonstrated the environmental factors contributing to the spread of disease (Colwell 1996), and the role of gene transfer in pathogen evolution (Waldor and Mekalanos 1996). Scientific discoveries accomplished through the study of *Vibrio cholera* provided a clear path to follow for the first studies of coral infectious diseases (Rosenberg 2005).

1.13 Coral pathogens from the *γ–Proteobacteria* family Vibrionacae

The most well characterized *Vibrio* infection of corals is the bleaching of the Mediterranean encrusting coral, *Oculina patagonica* by the coral bleaching agent *V. shiloi* (Kushmaro et al. 1996, 1997a, 2001). Although bacteria from the *γ–Proteobacteria* family *Vibrionacae* were previously identified on bleached corals in the Caribbean by Peters et al. (1983) and by Ritchie et al. (1994), the bleaching of *O. patagonica* in the Mediterranean Sea is extensive and unique because it occurs annually during the warm summer months (from May to September) and is followed by full recovery of infected colonies during the winter. Recovery occurs when seawater temperatures drop below 20°C (Kushmaro et al. 1997b, 1998), suggesting that *Vibrio* virulence is temperature regulated (Toren et al. 1998, Israeli et al. 2001). The pathogen, *V. shiloi*, has been shown to be capable of adhering to coral mucus (Banin et al. 2001a), and to have an ability to penetrate into coral cells, where it transforms into a viable but not culturable state - VBNC (Banin et al. 2000a, Oliver 2005). *V. shiloi* has also been shown to produce at least one specific toxin, a proline

rich peptide, which causes photoinhibition of *Symbiodinium* photosynthetic endosymbionts that are located within the coral host tissue (Banin et al. 2001b, Ben-Haim et al. 1999). The causative agent has been found to produce numerous other virulence factors, including an anti-oxidant (superoxide dismutase – SOD), which is critical for the pathogen's survival in an oxygen rich environment, such as when in close proximity to photosynthesizing cells (Banin et al. 2003, Reshef et al. 2008). Following the identification of the first *Vibrio* coral bleaching agent, another coral bleaching agent, *Vibrio coralliilyticus*, was characterized by Ben-Haim et al. (2002, 2003a) and shown to affect the Indo-Pacific coral *Pocillopra damicornis* by causing bleaching at lower temperatures ($\leq$28°C) and cell lysis resulting in tissue lesions and mortality at elevated temperatures (30°C; Ben-Haim et al. 2003a). This coral pathogen, which was isolated in Zanzibar, Tanzania from infected corals, was found to produce a proteolytic enzyme, which may cause photoinhibition of *Symbiodinium* cells (Ben-Haim et al. 2003a). *V. coralliilyticus* has a world wide distribution, including 3 strains causing coral bleaching isolated from infected corals in Eilat in the Red Sea, and a strain isolated from a diseased oyster larvae (*C. gigas*) in the UK (Ben-Haim et al. 2003a).

Recently, *V. coralliitycus* caused 100% mortality of Rainbow trout (*Oncorhychus mykiss*) exposed to the pathogen in laboratory experiments, suggesting a new hypothesis about its potential distribution in the wild (Austin 2005). Summer zoonotics devastating populations of the gorgonian coral *Paramuricea clavata* along western Mediterranean coasts in 1999 (Cerrano 2000) and in 2003 (Bally and Garrabou 2007) were demonstrated to have been caused by a novel strain of *V. coralliilyticus* isolated from infected gorgonian corals (Bally and Garrabou 2007).

In the Caribbean, isolates belonging to the *γ–Proteobacteria* family *Vibrionacae* where found to be associated with corals displaying White Band II disease signs (Ritchie and Smith 1995b). Recent experimental inoculation studies demonstrated that the causative agent for WB II in Puerto Rico is a *Vibrio harveyi* (Gil-Agudelo et al. 2006) and that infections are more likely to occur at elevated seawater temperatures during the summer than in winter months when infected corals were observed recovering (Gil-Agudelo et al. 2006). *Vibrio* isolates were also identified as the causative agents for the Caribbean coral disease named Yellow Blotch/Band – YB (Cervino et al. 2004a). Experimental exposure studies conducted by Cervino et al. (2004a) demonstrated that this disease might be caused by a

consortium of *Vibrio* strains and not by a single pathogen, and that Yellow Blotch/ Band pathogens target the coral's photosynthetic endosymbionts (Genus: *Symbiodinium*) rather than coral tissue itself. Microbiological surveys conducted in Zanzibar, Tanzania identified an elevated concentration of culturable *Vibrio* strains on corals displaying white syndrome (WS) disease signs compared to healthy looking corals (Piskorska et al. 2007). A significantly higher abundance of vibrios, particularly *Vibrio harveyi* (strain LB4), was also shown to be associated with rapid tissue necrosis (RTN) of *Pocillopora damicornis* (Luna et al. 2007). The presence of *Vibrio* strains on compromised hosts has recently been demonstrated for corals displaying growth anomalies (GA's; Breitbart et al. 2005), and in another study it has been demonstrated that GA's are transmissible (Kaczmarsky and Richardson 2007).

Investigation of coral disease pathogens and their hosts has led to the understanding that disease occurrence in eco-systems is a dynamic process, with pathogens, hosts and environmental factors subjected to change. Recently, it has been reported that the susceptible Mediterranean coral host, *Oculina patagonica*, can no longer be artificially infected by its bleaching causing agent, *V. shiloi* (Reshef et al. 2006). Such reports have led to the conclusion that the coral host has become resistant to its former pathogen (Reshef et al. 2006) and given rise to the establishment of the coral probiotic hypothesis (Reshef et al. 2006) as part of a hologenome theory (Rosenberg et al. 2007). The fact that currently coral bleaching in the Mediterranean waters still occurs annually (as in the past) may suggest that coral hosts have become susceptible to another 'competing' bacterium that causes similar bleaching signs, or that bleaching was not bacterially-caused in the first place (Ainsworth et al. 2008).

1.14 What makes *Vibrios* good candidates for virulence?

The adaptability of vibrios to various environmental conditions and changes is demonstrated by their ability to survive in multiple locations or hosts (Anderson and May 1982, Thompson et al. 2004, Higgins et al. 2007). Vibrios can survive long periods of dormancy in various forms including the viable but not culturable (VBNC) state (Oliver 2005), in which they were shown to maintain their virulence (Oliver and Bockian 1995). Some *Vibrio* strains have been shown to contain one chromosome and another chromosome-like plasmid, both of which contain identical genes which allow vibrios to switch "on" and "off" an entire set of expressional cascades directly triggered by environmental signals (Heidelberg et al. 2000, Chen et al. 2003, Makino

et al. 2003, Ruby et al. 2005). Three “languages”, or signaling systems, assist vibrios in direct communication among bacteria of the same species as well as in communication with different bacterial species and even eukaryotic organisms (Bassler 1999, Reading and Sperandio 2006). *Vibrio* “quorum sensing” (Dunlap 1999) was first discovered through the ability of *V. fisherei* cells to switch on luminescence at night when present at higher cell densities (Nealson et al. 1970) as mutalistic symbionts in light-emitting organs of certain squids and fishes (Haygood 1993, Ruby 1996). The study of *Vibrio* “quorum sensing” and its specific signaling molecules, which include acyl-homo-serine lactone (AHL) and autoinducers AI-I and AI-II (Bassler et al. 1997) identified the involvement of cell density regulated gene expression in the secretion of virulence factors. Such virulence factors within vibrios include the upregulation of HapR (zinc-metalloprotease) and repression of ToxR regulated Cholera toxin (CT) synthesis by *Vibrio cholera* (Zhu et al. 2002). The ability of vibrios to acquire genetic material through horizontal transfer of plasmids among bacteria (conjugation), via infection with bacteriophages (transduction) or by natural transformation (Dröge et al. 1999) allows these organisms to easily penetrate into new niches and quickly adapt to a rapidly changing environment (Thompson et al. 2004, Faruque et al. 2005). Some authors suggest that vibrios constantly migrate from a solitary existence in the environment to one in hosts (Higgins et al. 2007) requiring special genes for adhesion, penetration and for survival in hosts, compared to genes adapted for solitary survival. The cholera paradigm (Colwell 1996) demonstrates that *Vibrio cholera* cells can survive for long periods of time in the water column (Munro and Colwell 1996) under varying conditions of nutrients and salinity (Singleton et al. 1982). They can also digest mucin (Nelson et al. 1976) and chitin (Nalin et al. 1979) when adhering to copepods (Shukla et al. 1995) or plants (Shukla et al. 1995), respectively. Thus cholera pandemics correlate well with elevated seawater temperatures (Colwell 1996), which are associated with zooplankton and phytoplankton blooms (Lobitz et al. 2000, Lipp et al. 2002). In aquaculture facilities *Vibrio* virulence may be controlled by the use of antimicrobial peptides (Jia et al. 2000), yet in the marine environment, *Vibrio* prevalence and evolution is less studied and understood.

Few studies have investigated bacterial abundance on GBR reefs (Webster and Hill 2001). One study of *Vibrio* prevalence on a GBR reefs has identified multiple *Vibrio* species on both healthy and diseased corals (Bourne and Munn 2005),

suggesting that transition from a healthy state to a state of disease may not require an infection by an external pathogen, but simply the transition of healthy hosts into compromised-susceptible hosts. Another study by Bourne et al. (2008) demonstrated an elevated abundance of vibrios on corals prior to their bleaching during a mass bleaching event on Magnetic Island in 2001-2002, suggesting a possible elevated abundance due to heat stress. Studies by Ritchie and colleagues have demonstrated that healthy corals may harbour specific microbial populations (Ritchie and Smith 1997), which are capabale of utilizing specific coral-endosymbiont produced carbon sources (Ritchie and Smith 1995a, Ritchie et al. 1995, 1996), while potentially protecting corals against pathogen colonization (Ritchie 2006).

Vibrios have been shown to possess an array of potent virulence factors (Debra and Linkous 1999, Mekalanos 1992, Nishibuchi and Kaper 1995). Of over 200 *V. cholera* serotypes present in the environment (Yamai et al. 1997, Reidl and Klose 2002), only two are known to contain all the necessary genes to cause the Cholera pandemic; other less virulent *V. cholera* serotypes only cause minor infections (Singh et al. 2001). One of the most well studied functions of *Vibrio* pathogens is their ability to adhere to mucus, either mucus found on fish gills and fish skin (Bordas et al. 1998), or mucus along the digestive duct (O'Toole 1999). Comprehensive studies on the ability of pathogenic strains to adhere to their target host have demonstrated that only pathogens can successfully colonize the mucus of fish by attachment, whereas non-pathogens are only capable of displaying chemotaxis towards mucus, or mucus digestion, but not attachment (Bordas et al. 1996). Bordas et al. (1996) have also demonstrated that successful adhesion of pathogens to host mucus depends on environmental factors, mainly on temperature and salinity (Bordas et al. 1996). It has also been shown that specific compounds found in the host mucus (named: chemo-attractants) are required for the successful adhesion of bacteria (O'Toole 1999). It is therefore not surprising that *Vibrio* pathogens have also been shown to display chemotaxis and adherence to coral mucus (Banin et al. 2001a) by attachment to a β-D-galactopyranoside receptor present on the coral tissue. Banin et al. (2001a) also demonstrated that pathogen attachment was only successful for corals in which *Symbiodinium* endosymbionts were present. Sharon and Rosenberg (2008) concluded that coral pathogens may require special conditions to successfully out-compete bacterial populations that are the normal mucus-residents of healthy corals (Koren and Rosenberg 2006, Ritchie 2006).

The secretion of proteolytic enzymes by *Vibrio* pathogens has also been extensively studied, in particular the *V. cholera* HA/protease, a zinc-metalloprotease (Booth et al. 1983), which is closely related to other *Vibrio* pathogen zinc-metalloproteases (Norqvist et al. 1990, Milton et al. 1992, Hirono et al. 1996, Kim et al. 2002, Binesse et al. 2008, Hasegawa et al. 2008). This enzyme is involved in a number of functions, including digestion of connective tissue, attachment and detachment and cell perturbation. The *Vibrio* zinc-metalloprotease belongs to the thermolysin family of exocellular peptides and is defined as a pre-proenzyme containing five subunits, which have all been suggested to contribute to virulence (Marchler-Bauer et al. 2007, Varina et al. 2008). In addition, the fact that the synthesis of zinc-metalloproteases by *Vibrio* pathogens is regulated by cell density quorum sensing (Shao and Hor 2001, Kim et al. 2003, Milton 2006) supports placing this group of proteolytic enzymes at the core of *Vibrio* species virulence. The secretion of zinc-metalloprotease by *Vibrio* pathogens correlates with high cell densities during the late exponential growth phase and early stationary phase of these strains (Kreger and Lockwood 1981, Jeong et al. 2001, Denkin and Nelson 2004), suggesting that the secretion of this proteolytic enzyme is required when pathogens are invading, aggregating or swarming (Norqvist et al. 1990, Valiente et al. 2008). At these times zinc-metaloprotease synthesis suppresses the synthesis of other virulence genes, such as the cholera toxin – CT (Zhu et al. 2002), suggesting that all virulence factors are not secreted by *Vibrio* pathogens at once and that each pathogen (or serotype) may possess a unique set of virulence factors (Watanabe et al. 2004). However, since the zinc-metalloprotease gene has been found in both pathogenic and non-pathogenic *Vibrio* strains, it is not regarded as a major factor of virulence by all authors (Booth and Finkelstein 1986), and the exact role of this key enzyme is yet to be determined.

Vibrio coralliilyticus, the aetiological agent of bleaching of the Indo-Pacific coral *Pocillipora damicornis* (Ben-Haim et al. 2002, 2003b) has been shown to secrete a zinc-metalloprotease, whose partial protein sequence is closely related to the *V. cholera* HA/protease.(Ben-Haim et al. 2003a). The coral pathogen zinc-metalloprotease has been derived from a SDS gel sample containing the supernatant from a culture of coral pathogens, which caused instant photoinhibition of *Symbiodinium* cells in laboratory exposure trials (Ben-Haim et al. 2003a). *Vibrio shiloi* the aetiological agent of bleaching of the Mediterranean coral *Oculina*

patagonica has been recently shown to possess numerous virulence genes, which were also found to be absent from putative non-pathogenic strains (Reshef et al. 2008).

1.15 The aims of this study

To date, no attempts have been made to identify infectious diseases (i.e., diseases caused by micoorganisms) on the GBR, and only two coral pathogens have been successfully identified from the entire Indo-Pacific region (Ben-Haim and Rosenberg 2002, 2003b, Barash et al. 2005). This PhD study addresses this gap in knowledge through investigation that addresses the following specific aims:

1. To identify the causative agents of corals displaying black band disease signs on the Great Barrier Reef and red band disease signs in the Indo-Pacific Ocean by culturing cyanobacteria isolated from infected corals.
2. To identify the causative agents of white syndromes (WS's) currently affecting coral reefs in the Republic of the Marshall Islands, the Republic of Palau and on the Great Barrier Reef by culturing putative pathogens from infected corals.
3. To identify virulence factors that are associated with coral disease signs in order to investigate coral disease aetiology.
4. To determine the potential role of zinc-metalloproteases against coral and *Symbiodinium* hosts by developing novel bio-assays utilizing cultured *Symbiodinium* cells that have been isolated from disease-susceptible corals and non-susceptible corals, and coral juveniles infected with known *Symbiodinium* cells.

Chapter 2

A Single Cyanobacterial Ribotype is Associated with both Red and Black Bands on Diseased Corals from Palau

Sussman M, Bourne DG, Willis BL (2006) A single cyanobacterial ribotype is associated with both red and black bands on diseased corals from Palau. Dis Aqua Org 69: 111-118

2.1 Introduction

Cyanobacteria are associated with at least two band diseases that are differentiated macroscopically primarily based on color: black band disease (BBD), the first coral disease to be identified (Antonious 1973); and red band disease (RBD), first reported by Ruetzler et al. (1983) and later by Richardson in the Bahamas (Richardson 1993, 1998a). Black band disease is a band of filamentous cyanobacteria that progresses across live coral tissue, causing mortality of the coral along the advancing front (Antonius 1981b). It has been recorded world-wide and is found on 21 species in the Caribbean (Weil 2004) and 40 species on the Great Barrier Reef. (Page and Willis 2006). Red band disease (RBD) has been considered a separate disease category (Santavy and Peters 1997, Garzon-Ferreira et al. 2001) and has been recorded to affect 14 coral species in the Caribbean (Weil et al. 2002, Weil 2004). However, researchers have had little success in culturing the cyanobacteria associated with these putatively different coral diseases and thus it has not been possible to evaluate the significance of their color differences.

Early studies suggested that the causative agent of BBD is a consortium of microorganisms (reviewed in Richardson 2004). *Phormidium corallyticum*, a filamentous cyanobacterium from the genus *Oscillatoria*, was initially identified as the species forming the mat or band on infected corals (RÜtzler and Santavy 1983, Taylor 1983). The band's black appearance was suggested to be caused by a high concentration of cells containing the light-harvesting red photosynthetic pigment phycoerythrin (Richardson 1997a). Recent molecular analyses have failed to identify *P. corallyticum* and other proposed consortium-members, such as the sulfide-oxidizing bacteria *Beggiatoa* sp., in the black mat of BBD infected corals (Frias-Lopez et al. 2004a). Another ribotype of cyanobacteria from the genus *Oscillatoria* was shown to be associated with BBD infected corals from the Caribbean and Indo-Pacific (Frias-Lopez et al. 2002, Cooney et al. 2002, Frias-Lopez et al. 2003). These

conflicting findings have led to an uncertainty regarding the identity of the cyanobacteria associated with BBD and its role in causing infections (Frias-Lopez et al. 2004a, 2004b).

Morphological observations of the cyanobacteria associated with red bands identified two filamentous strains within the genus *Oscillatoria* (Richardson 1993, 1998a). These strains were reported as being visually distinct from *P. corallyticum*, yet never identified to a species level. Rützler et al. (1983) visually identified *Schizothrix calcicola* and *S. mexicana* on corals displaying red bands. Sutherland et al. (2004) claimed that due to lower prevalence in the field, red band might be grouped together with black band as a single classification. Harvell et al. (2004) was the first to publish a red band photo of an infected *Pachyseris speciosa* colony from Palau, emphasizing the need for further investigations. In recent surveys on the GBR, Willis et al. (2004) classified red bands and other cyanobacterial syndromes as part of a generalized cyanobacterial category due to uncertainty regarding their taxonomic identity. Needless to say, not all cyanobacteria found on corals constitute a threat to coral health (Lesser et al. 2004).

The aims of this study were to develop methods for culturing cyanobacteria associated with band diseases in corals and to determine if cyanobacteria isolated from red bands and BBD are genetically different. Isolation and culturing of cyanobacteria from one black and two red bands on corals sampled from reefs in Palau (Micronesia) tested whether pigmentation was: (1) independent of strain-host interaction, and (2) stable under identical growth conditions. Results of this study establish the first link between color, morphology and molecular-based taxonomic identification of cyanobacterial strains isolated from both red band and BBD infected corals.

2.2 Materials and methods

2.2.1 Sample collection

Samples from two corals infected with red bands (*Porites* sp. and *Pachyseris speciosa*) and one infected with BBD (*Montipora* sp.) were collected by SCUBA on Indo-Pacific reefs surrounding the island of Palau in January 2004. Samples were sealed in plastic bags immediately after collection and transported to a laboratory at the Palau International Coral Reef Center (PICRC). Samples were processed within two hours of collection. Red and black cyanobacteria associated with the microbial

mat of each disease were sampled from the corals with sterile forceps and transferred to agar plates.

2.2.2 Media and conditions of growth

Optimized conditions for growth of isolated cyanobacterial strains were determined by comparison of growth responses in 13 different media (see Table 2.1) under two light regimes. Media were chosen based on published recommendations for optimizing cyanobacterial growth in cultures (reviewed in McLachlan, 1973). Cultures were incubated at 26°C under either a 12:12 h fluorescent light regime or 24 hours of constant fluorescent illumination, both at 15µE m^{-2} s^{-1}. Half the plates used in this trial were scored (Vaara et al.1979). Scoring assists in purifying cultures from contaminants while selecting for motility, which is a key characteristic of cyanobacteria found on BBD and red band infected corals. Cultures were inspected daily and graded for growth on a scale from low growth (+) to highest growth (+++++) after a 7-day incubation period. The most successful media (Grund and ASN-III) and conditions (constant lighting/scored plates) (see Table 2.1 for summary of results) were chosen for studies of color expression under constant growth conditions. Grund media (von Stosch 1963, 1969) consisted of enriched seawater (enriched with 10 mgl^{-1} $CaCl_2$, 4 mgl^{-1} Na_2HPO_4, 1 gl^{-1} $NaNO_3$, 2mgl^{-1} Disodium EDTA, 20 mgl^{-1} Na_2CO_3) to which were added 1 ml micronutrient solution (A_5 + CO) and 1 ml vitamin solution (S-3) per liter of medium. The micronutrient solution A_5 + CO (Rippka et al. 1979) was made of an x 1000 stock solution and filter sterilized (2.86 gl^{-1} H_3BO_3 , 1.81 gl^{-1} $MnCl_2 \cdot 4H_2O$, 0.22 gl^{-1} $ZnSO_4 \cdot 7H_2O$, 0.39 gl^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.079 gl^{-1} $CuSO_4 \cdot 5H_2O$, 0.049 gl^{-1} $Co(No_3)_2 \cdot 6H_2O$). The vitamin solution S-3 (Provasoli 1963) was made as an x 1000 stock solution filter sterilized and kept in the dark (100 mgl^{-1} nicotinic acid, 500 mgl^{-1} thiamine · HCl, 100 mgl^{-1} Ca· pantothenate, 10 mgl^{-1} PABA, 1 mgl^{-1} biotin, 5 gl^{-1} *i*-inositol, 2 mgl^{-1} folic acid, 1 mgl^{-1} cyanocobalamin, 3 gl^{-1} thymine). In addition, 8 µM of Na_2-EDTA / $FeCl_3$ were added to each 1 liter of medium (Rippka et al. 2000). ASNIII, a synthetic media developed by Waterbury and Stanier (1981) was revised and made up of 1l double distilled water (DDW), 427 mM NaCl, 6.7 mM KCl, 3.4 mM $CaCl_2$, 14.2 mM $MgSO_4$, 9.8 mM $MgCl_2$, 0.09 mM K_2HPO_4, 1 mM $NaNO_3$, 0.19 mM Na_2CO_3. 8 µM of Na_2-EDTA / $FeCl_3$, 1 ml micronutrient solution (A_5 + CO) and 1 ml vitamin solution (S-3) were added to each 1 liter of medium. Agar plates were prepared using

washed agar (Waterbury and Willey 1988). A x1.33 concentration (in 750 ml) of liquid media (Grund or ASN-III) was filtered using Millipore 1000 ml Stericup®, preheated to 50ºC and added to 250 ml of autoclaved DDW with 6 g washed agar. Once dry, plates were scored with parallel lines using a flamed glass slide (Vaara et al. 1979) and incubated under unidirectional light. A liquid medium prepared from live coral tissue in seawater was also tested and found to enhance growth. This medium was autoclaved for 30 min and then sterile filtered (as above).

2.2.3 DNA extraction, PCR amplification and sequencing

DNA extraction was performed by a modified protocol from Wu et al. (2000). Following an initial growth phase of 10-14 days, filaments from liquid media flasks were inspected under light microscopy for strain purity, color and morphology. 200 mg of cyanobacteria culture RMS1 (*P. speciosa)*, RMS2 (*Porites* sp.) and BMS1 (*Montipora* sp.) were collected from each flask and placed in 2 ml tubes with 1 ml buffer A (100mM Tris, 50mM EDTA, 100mM NaCl at pH 8) for 10 min at room temperature. N-lauroylsarcosine (sarkosyl) was added to a final concentration of 0.1% and the samples incubated overnight at 4°C. Filaments were collected by centrifugation at 10,000 rpm for 10 min, washed in 2 ml of TES (50 mM Tris, 5 mM EDTA, 50mM NaCl at pH 8), centrifuged again for 5 min and resuspended in 1 ml TES. Lysozyme was added to a final concentration of 0.5 mg ml^{-1} and the sample incubated with RNase (20µg ml^{-1}) at 37ºC for 30-90 min with shaking. SDS was added to a final concentration of 1%, stirred thoroughly, and kept at 37°C for 10 min. 50µg ml^{-1} Proteinase K was added and the sample was incubated overnight at 4°C (or alternatively at 37°C for 1h). Extraction was performed by using the Promega Wizard Genomic DNA purification kit® starting with protein precipitation (step 4 of Gram negative protocol). After isopropanol and 70% ethanol precipitations, pellets were dried in a DNA110 Speed Vac (Savant) for 15 min and rehydrated overnight at 4°C. PCR amplification was performed following the protocol of Nübel et al. (1997). Cyanobacterial specific primers used were the CY106F forward primer (CGG ACG GGT GAG TAA CGC GTG A) and an equimolar mixture of CYA 781Ra (GAC TAC TGG GGT ATC TAA TCC CAT T) and CYA 781Rb (GAC TAC AGG GGT ATC TAA TCC CTT T) reverse primers. 50 µl reactions were made of 10 picomoles of each primer, 5 µl of 10x PCR buffer (*Taq* PCR Core Kit, Qiagen with 15mM $MgCl_2$),

50 nmol dNTP, 10 ng of template DNA (1:10 dilution of stock). 10 µl Q-Solution (Qiagen) was added to each tube and DDW to the volume of 50 µl. 1U of *Taq* was added at 80°C to minimize nonspecific annealing of primers following a 5 min hot start at 94°C. An Eppendorf Mastercycler® was used for template amplification with 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. A final 5 min extension step at 72 °C was performed. Reaction products were purified using DNAce Quick-Clean® and sequenced using ET-Terminators (Amersham Biosciences) on MegaBACE 1000 from Amersham Biosciences. Sequences were visualized using CHROMAS® (Conor McCarthy http://trihul.sci.gu.edu au/˜conor/chromas.html) and aligned (Altschul et al. 1990) with existing database using BLAST (http://www.ncbinih.nlm.gov/blast). Multiple alignments were performed using BCM - multiple sequence alignments (http://dot.imgen.bcm.tmc.edu/multi-align/multi-align.html). Partial 16S rRNA gene sequences were entered into the Genbank database under the following accession numbers: AY839639, AY839640, AY839641 (for further detail see table 2.2).

2.2.4 Light Microscopy

Cyanobacterial filaments were examined using the Zeiss Axioshop® 2 Mot Plus microscope equipped with an AxioCam® MRC5 digital camera using AxioVision® 4 software. Cyanobacterial filaments were photographed prior to the sampling for DNA extraction. Since this study aims to compare pigmentation among cynaobacterial strains, no further software-manipulation of AxioVision® 4 calibrated images has been undertaken and they appear as identical replications of originals.

2.3 Results

2.3.1 Media and conditions of growth

Thirteen different variations of solid and liquid media were evaluated in an attempt to isolate and grow filamentous cyanobacteria associated with infected corals. Cultures grew best in either Grund media (with seawater) or ASN III media (Table 2.1). Cyanobacteria did not grow on, or in, any of the media devoid of nitrogen (PCR-S8, PCR-S11, PCR-Tu2). This demonstrates that cyanobacteria found on red band and BBD infected corals do not fix nitrogen. Highest growth in liquid media (score: +++++) was achieved in a zooxanthellae broth consisting of coral tissue autoclaved in

seawater. At 26°C and using fluorescent lights (15μE m^{-2} s^{-1}), continuous (24 h) lighting provided better results than a 12:12 h light:dark regime.

Washing agar with ethanol and acetone, as suggested by Waterbury and Willey (1988), and scoring plates (Vaara et al. 1979) facilitated the motility of cyanobacteria towards a unidirectional light source placed at an angle away from the plates. Both red and black cyanobacterial filaments in scored plates progressed along groves at a speed of up to 5cm/day. Non-scored plates placed directly under light exhibited no growth. Supplementing the agar with 0.1% (wt/vol) Na_2S and raising pH to 9 (Vaara et al. 1979) did not result in enhanced motility. Nevertheless, strains isolated from infected corals demonstrated an ability to survive in higher concentrations of Na_2S (750mg $Na_2S{\cdot}9H_2O$ per liter of medium). In liquid media, filaments concentrated in clumps on the glass walls of flasks. They formed a straight line at the interphase between media and air while orienting towards the light. Placement of a sterile piece of coral skeleton in the flasks initiated settlement of the filaments onto the skeleton.

Table 2.1 Media and conditions of growth employed in this study to culture cyanobacteria associated with red band and BBD infected corals from Palauan reefs. Growth of cyanobacteria was scored qualitatively as follows. (-): no growth; (+): low growth; (+++++): highest growth.

Media	Seawater	DDW	scored plates *l*		non-scored plates		liquid media *m*	pH	Vitamins	Micronutrients
			12:12h light	24 h light	12:12h light	24 h light	24 h light			
Grund *a*	v		(++)	(+++)	(+)	(+)	(+++)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
ASN-III *b*		v	(++)	(++++)	(+)	(+)	(+++)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
f/2 c	v		(-)	(+)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
MN *d*	v		(-)	(+)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
SN *e*	v	v	(-)	(+)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
BG-11 Na2S *f*	v	v	(-)	(+)	(-)	(-)	(-)	9	S-3 *n*	A5 + Co *o, p*
Na2S *g*	v		(-)	(+)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
PCR-S8 *h*		v	(-)	(-)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
PCR-S11 *h*		v	(-)	(-)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
PCR-Tu2 *h*		v	(-)	(-)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
BG-11 *i*	v	v	(-)	(+)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
Erdschreiber *i*	v		(-)	(+)	(-)	(-)	(-)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*
Zoox. broth (liquid) *k*	v						(+++++)	8.1 - 8.3	S-3 *n*	A5 + Co *o, p*

a von Stosch 1963, 1969 supplemented with 1 gl^{-1} $NaNO_3$.

b Waterbury and Stanier 1981 supplemented with 1 gl^{-1} $NaNO_3$.

c Guillard and Ryther 1962.

d Waterbury and Stanier 1981.

e Waterbury and Willey 1988. 1 liter medium was prepared by mixing 750ml of filtered seawater with 250ml autoclaved DDW.

f BG-11 supplemented with 0.1% (wt/vol) $Na_2S \cdot 9H_2O$ (Vaara et al. 1979) and adjusted to pH 9.

g Cohen et al. 1975. Medium is prepared with filtered seawater, 750 mgl^{-1} $Na_2S \cdot 9H_2O$ at pH 8.1 - 8.3.

h Rippka et al. 2000. Seawater was filter sterilized.

i BG11 is a freshwater medium (Allen and Stanier 1968). For this study BG-11 was prepared by mixing 750 ml of filtered seawater with 250ml autoclaved Double distilled water (DDW).

j Schreiber 1927, Foyn 1934.

k Zooxanthelae broth was prepared by autoclaving coral tissue in seawater and filtering through a 0.22µm filter. Solution was supplemented with 1ml x1000 vitamin mix and 1ml micronutrient mix.

l Plates were scored (Vaara et al. 1979) with parallel lines using a flamed glass and kept under unidirectional light.

m Liquid media was prepared by filter-sterilization. Agar plates were prepared by autoclaving 250ml DDW with 6 gl^{-1} of washed agar (Waterbury and Willey 1988) mixed with 750ml of x1.33 liquid media kept at 50ºC.

n Provasoli 1963. x1000 S-3 solution consists of : 100 mgl^{-1} nicotinic acid, 500 mgl^{-1} thiamine · HCl , 100 mgl^{-1} Ca · pantothenate, 10 mgl^{-1} PABA, 1 mgl^{-1} biotin, 5 gl^{-1} *i*-inositol , 2 mgl^{-1} folic acid, 1 mgl^{-1} cyanocobalamin, 3 gl^{-1} thymine. X1000 solution is filter sterilized and kept in the dark. Volume added 1 ml^{-1} of medium.

o R.Ripka et al. 1979. x1000 A_5 + Co solution consists of: 2.86 gl^{-1} H_3BO_3, 1.81 gl^{-1} $MnCl_2 \cdot 4H_2O$, 0.22 gl^{-1} $ZnSO_4 \cdot 7H_2O$, 0.39 gl^{-1} $Na_2MoO_4 \cdot 2H_2O$, 0.079 gl^{-1} $CuSO_4 \cdot 5H_2O$, 0.049 gl^{-1} $Co(NO_3)_2$. 6H2O. Volume added: 1 ml^{-1} of medium.

p 8µM of Na_2-EDTA / $FeCl_3$ (Rippka et al. 2000) were added to each 1 liter of medium. 1 mM stock solution was prepared (Skulberg 1990) in 500ml volume and filter sterilized. Volume added: 4 ml^{-1} of medium.

2.3.2 Pigmentation and morphology of strains

Both BBD (Fig 2.1A) and red band (Fig 2.1B) infected corals sampled in Palau exhibited a clear band at the interface between coral tissue and skeleton. High light magnification (x1000) of cyanobacterial filaments derived from both BBD (Fig 2.2A) and red bands (Fig 2.2B) show clear segmentation between individual cells. Filaments appear to be similar in diameter, with rounded cells along the edges and at the ends of filaments. The length of individual cells within a filament is variable. Filaments isolated from BBD appear to gain pigmentation from black/brown granulation within each cell (see Fig 2.2A). Red pigmentation in filaments isolated from red band infected corals (Fig 2.2B) is more evenly distributed and it is therefore more difficult to tell whether it is caused by granulation.

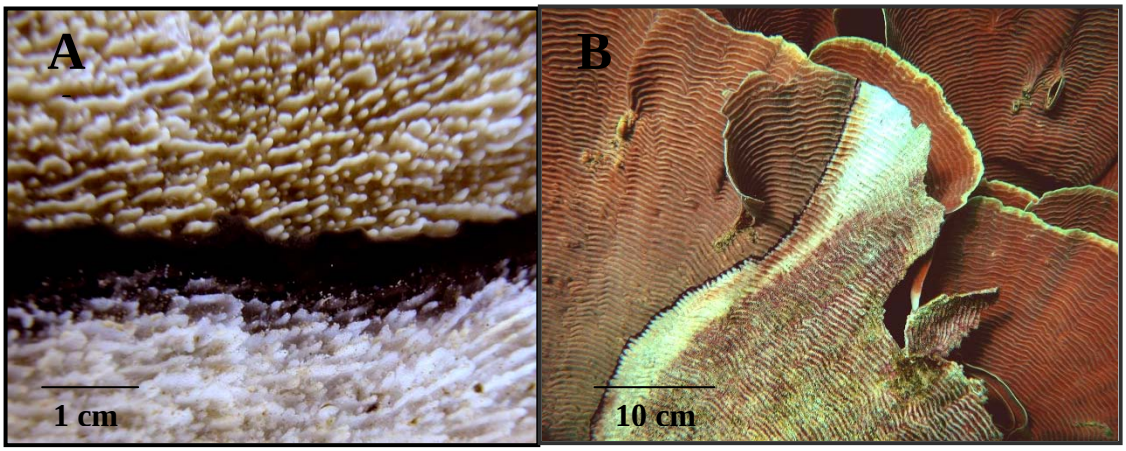

Figure 2.1 Cyanobacterial infections on corals collected from Palauan reefs.
A. *Montipora* sp. with black band disease (BBD). **B.** *Pachyseris speciosa* with red band (Photo by Dr. F. Cróquer).

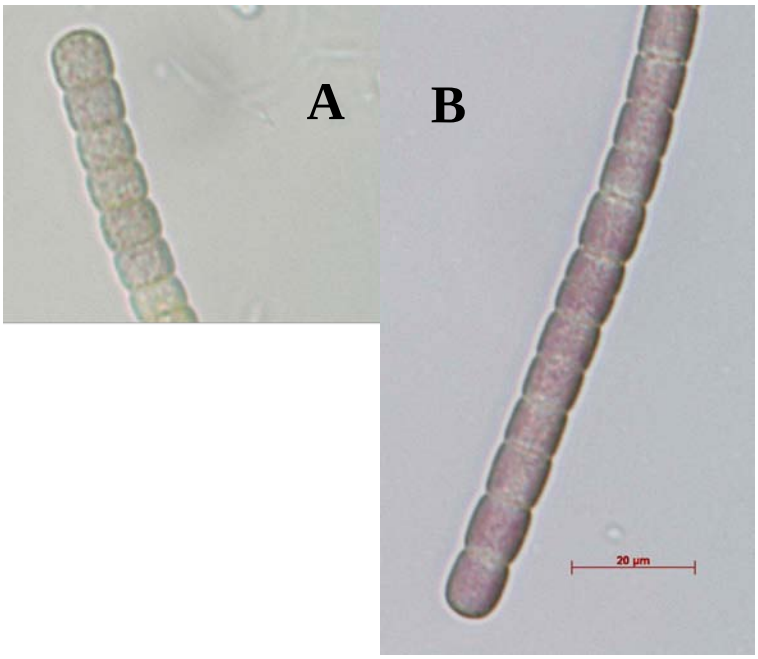

Figure 2.2 Cyanobacterial isolates
Filamentous cyanobacteria isolated and cultured from **A.** *Montipora* sp. with BBD and **B.** *Pachyseris speciosa* with a red band. The black pigmentation in 2A results from granulation observed in individual cells. Segmentation in both filaments is clear. The red pigmentation in 2B is maintained under identical growth conditions of light and nutrients as the black pigmentation in 2A. Red pigmentation is more evenly distributed along the filament. (x1000 magnification, scale bars 20 µm).

Isolates from red band infected corals remained red in liquid culture (ASN III, continuous illumination at 26°C) for 10-12 weeks until initial signs of chlorosis began to appear. At this point, red pigmentation paled to a light red color. Isolates from BBD infected coral maintained under the same growth conditions, remained dark in culture for a shorter period (4-6 weeks) before undergoing chlorosis. They became pale green before turning completely colorless and transparent.

2.3.3 Taxonomic identity of strains

All three cyanobacterial cultures isolated from corals in Palau produced identical partial 16S rRNA gene sequences (582bp) (Table 2.2). All three isolated cyanobacteria demonstrated 99% 16S rRNA gene sequence identity with an uncultured cyanobacterial strain (AF473936) associated with BBD infected corals in the Caribbean that has been previously identified by molecular analyses (Cooney et al. 2002).

Table 2.2 Properties of cyanobacterial strains associated with red band and BBD infected corals from Palau.

	Field observation		Isolated cultures		Taxonomic identity				
Coral species	Disease type	Color	Strain	Color	Accession #	Closest match	% match	Multiple alignment**	bp
Pachyseris speciosa	Red band	Red	RMS1	Red	AY839639	AF473936*	99	100%	582
Porites sp.	Red band	Red	RMS2	Red	AY839641	AF473936	99	100%	582
Montipora sp.	BBD	Black	BMS1	Black	AY839640	AF473936	99	100%	582

* Cooney et al. (2002)

** Multiple alignment of partial 16S rDNA gene sequences from RMS1, RMS2 and BMS1 was performed using BCM - multiple sequence alignments

2.4 Discussion

Results reported here represent the first successful isolation and culturing of filamentous cyanobacteria associated with both red band and BBD infected corals. Our molecular studies show that cyanobacterial filaments isolated from corals infected with red band and BBD share the same taxonomic identity. Observations of cyanobacteria in culture suggest that a single cyanobacterial ribotype associated with a coral disease can exhibit dual pigmentation under similar light and growth conditions. Previous studies of cyanobacterial pigmentation have demonstrated that complementary chromatic adaptation allows cyanobacteria to reversibly alter their pigmentation from green-blue to red in response to light (see reviews by Grossman et al. 1993a, 1993b). This has been demonstrated in the filamentous cyanobacterium, *Fremyella diplosiphon* (Kehoe and Grossman 1994). The color of cells can also be in response to other environmental factors, such as elevated or low nutrient levels (nitrogen, phosphorus and sulfur; Collier and Grossman 1992).

Consistency of color, either red or brown/black, of cyanobacteria cultured over the 12 weeks of our study suggests that complementary chromatic adaptation doesn't adequately explain our observations. Had light and growth conditions determined color expression, a complementary chromatic adaptation should have occurred and both red and brown/black cyanobacterial cultures would have converged to a similar pigmentation. The only shift in pigmentation suggestive of a complementary chromatic adaptation that we observed was from red to light green in some cultures growing on agar plates. This change was usually rapid and irreversible. Red cyanobacteria from red band infected corals never exhibited the dark brown/black pigmentation seen in the cultures originating from BBD infected corals. *Synechococcus* sp. strain SH-94-5 reacts to nitrogen depletion by upregulating the accumulation of beta-carotene, thus resulting in a pigmentation change to red. During exponential growth, this strain down regulates production of phycobiliproteins and becomes green (Miller et al. 2002). Whether such pigmentation changes occur in cyanobacteria infesting coral tissue in the field remains unknown. Currently, there are no reports of BBD infected corals transforming into red bands, or red bands transforming into black bands.

Two lines of evidence suggest that cyanobacterial growth in culture is optimized by the presence of substrate for settlement. First, cyanobacterial strains

grown in liquid cultures settled on the glass flasks along a line at the interface between the liquid media and air and initiated this growth characteristic on the side closest to the light. Secondly, addition of a sterile piece of coral skeleton to the media flasks initiated immediate settlement of the filaments. This preference for substrate might explain why shaking of cultures in liquid media has proven unsuccessful for growth. However, settlement of cyanobacterial filaments on coral skeletons in the laboratory was different from settlement observed in the field, where filaments concentrate only at the interface between coral tissue and skeleton leaving white exposed skeleton behind the moving front (as in Figs 2.1A-B). The settlement pattern observed in the laboratory is possibly explained by the distribution of nutrients inside media flasks. Cyanobacteria progressing along the border of degrading coral tissue are exposed to high nutrient levels (Taylor 1983). The high density of filaments forming the bands on infected corals also results in shading (Richardson et al. 2001), although there are conflicting reports of bands progressing towards the light or away from it (Antonius 1981b). Filament aggregation has been suggested by Castenholz (1982) as a mechanism adopted by cyanobacteria to increase shading in order to attain lower light levels needed for optimal photosynthetic activity. In our laboratory trials, in contrast, filaments tended to dissociate when placed in grooves on scored agar plates, with individual filaments separating and traveling up to 5 cm/day towards the light, eventually spreading over the entire agar surface rather than aggregating.

The speed with which cyanobacterial filaments isolated from both red band and BBD infected corals travel across agar indicates that band-progression in the field (only 0.3-1 cm/day) might be limited by other factors. The degree of motility of filaments in this study might explain how members of this taxonomic ribotype outcompete other cyanobacteria when settling on corals. Further study should be directed at determining the relationship between motility towards light and the propensity for aggregation and how these might be affected by variation in light and nutrient levels.

As in previous studies that have attempted to determine the identity of filamentous cyanobacteria associated with BBD (Cooney et al. 2002, Frias-Lopez et al. 2002, 2003), this study used partial sequencing of the 16S rDNA gene. Given that we sampled only two red bands from the field, our results do not imply that all red band infections carry the same cyanobacteria; further samples from red bands are required to test the generality of our results. Although we have shown that dual

pigmentation can be exhibited by a single ribotype, other less dominant cyanobacterial strains that we were unable to isolate and culture on the media used, may have been present in our original field samples. Our aim in this study was to isolate and culture the dominant color forming strains from infected corals in the field and to establish a reference point in the relation between cyanobacterial color, taxonomic identity and disease classification, rather than to identify the entire microbial complexity of cyanobacterial mats on infected corals. Cloning of extracted DNA from field samples using universal primers targeted at a larger region of the 16S rRNA gene would provide a more comprehensive analysis of cyanobacterial populations associated with both red band and BBD infected corals. If pathogenicity and virulence are to be studied, an attempt should be made to explore the genes linking identity and function, as has been recently demonstrated for BBD by Frias-Lopez et al. (2004b). In the case of *Vibrio cholera* it has already been shown that the 16S rRNA gene based identity of strains does not necessarily relate to virulence (Colwell and Huq 1994, Singh et al. 2001).

This study has identified dual pigmentation expressed by a single ribotype found on both red band and BBD infected corals but did not determine the mechanism involved. The fact that red bands are far less prevalent in the field than BBD might point towards a genetic variation that result in both a common and a rare phenotype.

The strains isolated and cultured by this study belong to a ribotype that has been previously identified by molecular tools on BBD infected corals in the Caribbean (Cooney et al. 2002, Frias-Lopez et al. 2002). The strains isolated from both red bands and BBD in this study were found by BLAST to share less than 90% match-identity with *P. corallyticum* (AF474001), previously suggested as the causative agent of BBD (Richardson and Kuta 2003), though later re-named as a *Geitlerinema sp.,* which has been shown to produce microcystin toxins (Richardson et al. 2007, Voss et al. 2007). The results of this study call for examination of the role of *P. corallyticum/Geitlerinema sp.* in BBD infections by comparing its ability to infect healthy corals with the newly isolated strains presented here (RMS1, RMS2 and BMS1).

The maintenance of new cultures associated with coral disease opens many doors for further research including examining the potential virulence and toxicity of these new strains isolated from red bands and BBD, their geographical distribution and the ways in which strains are transmitted. Based on the results of this study, we

strongly urge postponing the classification of red cyanobacterial bands as a separate coral disease until further evidence is collected.

Chapter 3

Coral pathogens identified for white syndrome (WS) epizootics in the Indo-Pacific

Sussman M, Willis BL, Victor S, Bourne DG (2008) Coral pathogens identified forWhite Syndrome (WS) epizootics in the Indo-Pacific. PLoSONE 3(6): e2393.doi:10.1371/journal.pone.0002393

3.1 Introduction

Reports on coral disease continue to rise (Harvell et al. 2002), however the causes for these diseases and the methods by which to investigate them are still heavily debated (Cervino et al. 2004a, Rosenberg and Falkovitz 2004, Lesser et al. 2007). Most efforts are directed towards traditional surveillance (Morens et al. 2004), with comparatively less research directed towards developing strategies for active engagement in coral reef health management, disease prevention and cure (Palumbi 2005, Pandolfi et al. 2005, Efrony et al. 2007). Unfortunately, a lack of knowledge of coral disease causative agents propels this debate to a stand still. To date, only five bacterial species and one fungal agent have been determined as causative agents for coral infectious diseases (Kushmaro et al. 1996, Geiser et al. 1998, Patterson et al. 2002, Ben-Haim et al. 2002, 2003a, Denner et al. 2003, Barash et al. 2005, Thompson et al. 2006), and currently no diagnostic tools or management efforts are able to validate these findings at a level required for active intervention (Hiney 1997, Hiney and Smith 1998).

The study of disease in complex environmental settings is often difficult. Modern studies have cast a shadow on traditional culturing methods that are required to satisfy Henle-Koch's postulates (Koch 1891), namely that a putative pathogen is first isolated on growth medium and then used in pure culture to duplicate disease signs in laboratory controlled infections. In many cases, more than 200 years after Henle-Koch's own revolution, these experiments often fail, requiring the introduction of modern rules and criteria in order to establish disease causation (Hill 1965, Evans 1976). These are often based on statistical associations rather than on "cause and effect".

Most microorganisms cannot be easily cultured (Amman et al. 1995) and other disease components, namely host susceptibility and environmental factors may jointly contribute to successful infections in what is known as the "disease triad" (Snieszko

1974). To this end, modern diagnostic tools have been developed that can be applied to enhance our knowledge of coral disease without targeting either a single or a cultivable agent. These tools include cloning and denaturing gradient gel electrophoresis (Bourne 2005a), fluorescent *in situ* hybridization (Sussman et al. 2003), microarrays (Edge et al. 2005) and metagenomics (DeLong 2005, Edwards and Rohwer 2005, Yokuchi et al. 2006), just to name a few, and are used to either detect new pathogens or validate their presence once detected. Nevertheless, the benefits from isolating and culturing pathogens are still many, especially when precise disease identification for health control purposes is needed (OIE 2006).

The study of epidemiology has revolutionized many concepts associated with disease studies (Thrusfield 2005) including some of the terminology used in infectious disease classifications. Traditional distinctions between primary vs. secondary, exogenous vs. endogenous and opportunistic agents (Dubos 1965, Stainer et al. 1971) are being replaced by schemes classifying the genes involved in infectivity (the ability to physically infect a host; Thomas and Elkinton 2004) and virulence (the severity of disease outcome inflicted by infection; Day 2002). Modern studies have demonstrated that host, pathogen and environment form a constantly evolving disease equilibrium (Osawa et al. 2000) contributing to a growing list of newly emerging infectious diseases (Cohen 2000). The hierarchy of causation has been translated into causal models and complex outbreaks are now considered as multi-factorial, comprised of an often-unknown range of component causes (Thrusfield 2005), which need to be explored both independently and in conjunction with other causes. Nevertheless it remains a paradox, that despite the growing complexity in our understanding of disease causation, it is often expected that emerging infectious outbreaks be successfully curtailed before causation is fully established (Wilesmith 1993), shifting the focus from cure of individuals to disease-prevention in entire populations.

The aims of this study were therefore twofold: firstly, to identify possible causative agents for white syndromes widespread throughout the Indo-Pacific by combining both traditional microbial tools such as culturing with biochemical and molecular methods, and secondly, to investigate the aetiology of WS in order to recommend the development of novel diagnostic tools that could be implemented and validated in an active coral reef health management plan targeted “to protect against disease in the framework of the concept of ecosystem management” (FAO 1995).

Since 2003, a variety of white syndromes have been reported from numerous locations throughout the Indo-Pacific and under various names (Jones et al. 2004, Willis et al. 2004, Aeby 2006, Roff et al. 2006, Haapkylä et al. 2007). Willis et al. (2004) suggested the use of a common term: white syndrome (WS), for Indo-Pacific scleractinian coral diseases displaying acute tissue loss exposing white skeleton in the absence of other disease signs or established causation. Three independent WS outbreaks were chosen for this three year study (2003-2006) in order to determine whether WS is one disease or possibly many, and whether a standard disease investigation protocol could be developed that could be used in future monitoring and management efforts.

3.2 Materials and Methods

3.2.1 Isolation and growth of bacteria from coral samples

For inoculation experiment I, ten fragments (2-10 g wet weight) from corals displaying WS disease signs and ten fragments (2-10 g wet weight) from corals lacking WS disease signs were collected from depths between 3-15 m at each of the following locations: 1) Nelly Bay fringing reef (S19 10' E 146 52') at Magnetic Island in the central section of the Great Barrier Reef (GBR) in September 2003; 2) Majuro Atoll the Republic of the Marshall Islands (N 9 00' E 168 00') in August 2004; and 3) Nikko Bay, an enclosed bay among rock islands in the Republic of Palau (N 7 30' E 134 30') in February 2005. WS mainly affected plate colonies of *Pachyseris speciosa* in Palau, tabular species of *Acropora* (*A. cytherea*, *A. hyacinthus* and *A. clathrata*) in the Marshall Islands and plate colonies of *Montipora aequituberculata* at Nelly Bay GBR. At each site, samples were transported from the reef to the laboratory in sterile containers.

For calculating the abundance of bacteria associated with diseased and non-diseased fragments, the following sub-samples were obtained at each site: healthy tissue from coral fragments with no disease (CON, n=3); tissue adjacent to lesions on coral fragments with WS disease signs (INF, n=3); healthy tissue on coral fragments displaying disease signs (H, n=3); lesion interface on coral fragments displaying disease signs (I, n=3); and exposed coral skeleton on coral fragments displaying disease signs (S, n=3).

Samples were crushed and diluted with 10 ml of 0.22 µm filtered seawater (Millipore, USA), and then vortexed for 3 min at maximum speed before being left to

settle for 3 min [26]. Supernatant (100µL) was streaked on agar plates containing a general heterotrophic bacterial medium (Marine Agar: 1.8% Marine Broth, Difco-2216, USA 0.9% NaCl, 1.8% Agar Bacto, Difco-214010, USA) and thiosulfate citrate bile salts sucrose (TCBS) agar, a *Vibrionacea* selective growth medium (Difco, USA). Plating was conducted in triplicates of serial dilutions ($1x10^{-1}$ – $1x10^{-6}$) followed by incubation overnight at 30ºC. Cultivable strains were quantified by counting colony forming units (CFU's) and the density of bacteria associated with corals was determined as mean CFU's per 1 ml of crush derived from 1 g (wet weight) of coral tissue (CFU's ml^{-1} g^{-1}). Single CFU's were picked from both Marine Agar (MA) and TCBS plates and transferred to fresh MA plates for further analyses.

Single isolates were grown in 250ml sterile flasks containing sterile marine broth (MB) media incubated at 30 ºC for 18h (i.e. to end of the logarithmic phase) with constant shaking (150rpm). Cell density in pure cultures was determined by plating triplicates of serial dilutions on MA and by measuring absorbance (595nm) in sterile microtitre well plates (n=6).

Additional bacterial isolates from fragments displaying signs of ongoing tissue loss in association with WS and from healthy fragments (controls) were retrieved for screening purposes from corals at Heron Island, GBR (March 2004) and at Dip Reef GBR (November 2004).

3.2.2 DNA extraction, PCR amplification and gene sequencing

Genomic DNA extraction from pure cultures of bacterial isolates retrieved by this study was performed using the Wizard genomic DNA purification kit® (Promega, USA) as per the manufacturer's instructions. The 16S rRNA gene was amplified by using universal primers 27F and 1492R. (Lane 1991). In addition, primers HA-F (5' – CATGAGGTCAGCCACGGTTTTACTGAGCAG) and HA-R (5' – CGCGCGGTTAAACACGCCACTCGAATGGTGAAC (Invitrogen, NZ) targeting a ~ 225 bp region including the zinc binding site of *Vibrio*-family zinc-metalloproteasses (Häse and Finkelstein 1991) were used to screen all bacterial genomic DNA. PCR reactions (50 µL) were run on an Eppendorf Mastercycler with the reaction mix consisting of 10 pmol of each primer, 5 µL of 10xPCR buffer with 15 mM $MgCl_2$, 50 nmol dNTP, 10 ng template DNA and 1U Taq (iTaq, Intron Biotechnology, Korea). DDW (Milli-Q, millipore) was added to the volume of 50 µL. Cycling conditions consisted of: 1) 27F/1492R - a 5 min denaturation step at 94ºC

followed by 30 cycles of 1 min at 94ºC, 1 min at 52ºC and 1 min at 72ºC and concluded by a 7 min extension step at 72ºC; and 2) HA-F/HA-R - a 5 min denaturation step at 94ºC followed by 30 cycles of 20 sec at 94ºC, 20 sec at 55ºC and 1 min at 72ºC, concluded by a 5 min extension step at 72ºC. Amplified bands of the correct size were confirmed on a 1% ethidium bromide stained TAE agarose gel and amplified gene products were sequenced at MACROGEN Inc. (Seoul, Korea) on an ABI PRISM 3730XL analyzer (96 capillary-Applied Biosystems, CA, USA) using the ABI PRISM BigDyeTM Terminator Cycle Sequencing Kit. Retrieved gene sequences were aligned for closest matches using BLAST (Altschul et al. 1997). In total, 152 partial sequences were retrieved from coral fragments displaying WS signs and from controls sampled at the three-infection sites.

3.2.3 Phylogenetic analyses

Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al. 1996). Sequence data were aligned to the most similar sequence using the BLAST database algorithm (Altschul et al. 1997), and then further analysed with the ARB software package (Ludwig et al. 2004). Tree topologies were evaluated by reconstructing phylogenies using maximum likelihood evolutionary distance analysis (Phylip Distance Method with Jukes and Cantor model) of aligned near full-length sequences (>1200 bp). Regions of ambiguous sequence (N) were removed from the analysis. Bootstrap values were obtained for branching patterns using the Phylip software package (version 3.65; Retief 2000) and values ≥50% were included for main nodes of the tree.

3.2.4 Infection experiments

Infection experiments were run as incurred matrices in 2 consecutive stages, described below as inoculation experiments I and II.

3.2.4.1 Inoculation experiment I: Testing for infectivity of bacterial isolates

To screen bacteria for infectivity, *i.e.*, the ability to initiate visual disease signs (lesions) regardless of their severity (Thomas and Elkinton 2004), 20 isolates retrieved from coral samples at each of the three sites (10 most abundant isolates on both MA and TCBS plates from both healthy and diseased colonies at each site) were grown to end logarithmic phase in MB (as described above) and inoculated

individually into 7L sterile aerated tanks (final inoculum concentration = 1 x 10^6 cells ml^{-1}) containing 4-6 healthy fragments of corals collected from sites without disease signs (*i.e*. healthy fragments of *Pachyseris speciosa* from a healthy Palau site, healthy *Acropora cytherea* fragments from a healthy Marshall Islands site, and healthy *Montipora aequituberculata* fragments from a healthy Nelly Bay site). Prior to bacterial inoculation, coral fragments were acclimatized for 5 days to allow recovery from handling and fragmentation following a protocol by Kushmaro et al. (1997a). Each of the 20 culture inoculations was tested in two tanks [n = 168-252 fragments per site, N = 21 inoculation treatments including 1 negative control treatment]. The negative control tanks contained coral fragments with no bacteria added. Seawater in the tanks was replaced every 48 h and tanks were observed and photographed for 140 h in order to detect developing disease signs. At the end of each experiment, infectivity was calculated as the proportion of exposed fragments per tank that became infected. Both infected and non-infected fragments were crushed and individual CFU's were picked and transferred to fresh MA plates for further analyses and DNA extraction, as previously described. Bacterial strains causing disease signs in this experiment were given the simplified names: P1-P7 (P1 from *M. aequituberculata* in Nelly Bay GBR; P2 and P7 from *A. cytherea* in Majuro Atoll the Republic of the Marshall Islands; and P3-P6 from *P. speciosa* in Nikko Bay Palau) and were inoculated as pure cultures in the following experiments under these names. Bacterial strain P6 was isolated from seawater above diseased *P. speciosa* colonies at Nikko Bay Palau. It caused infections in a separate experiment and was therefore added to the list of putative pathogens. Strain P7 from the Marshall Island caused partial disease signs on only one *A. cytherea* fragment (out of four fragments) when inoculated into two tanks (n = 4 fragments per tank). Inoculation experiment I was repeated using this isolate (n =12 fragments in each of 3 tanks) and it was removed from the putative pathogen list after failing to cause infections.

3.2.4.2 Inoculation experiment II: Replicated exposure trial to fulfil Henle-Koch's postulates and test for virulence

To fulfil Henle-Koch's postulates, a large multi-replicated exposure trial using successful putative pathogens that initiated disease signs in inoculation experiment I were grown as pure cultures and inoculated (final inoculum concentration = 1 x 10^6 cells ml^{-1}) into multiple tanks with colony fragments (of *P. speciosa*, *M.*

aequituberculata A. hyacinthus) collected from non-disease sites and acclimatized for 5 days. The number of fragments allocated to inoculation tanks at each site (n) was between 80-360, distributed as 4-6 fragments per tank, and the number of tanks per inoculation/control treatments (N) was between 4 and 12. At each site, four negative controls were run including: one treatment comprising tanks to which no bacteria were added, two treatments comprising tanks to which control bacterial strains were added at identical concentrations, and one treatment comprising tanks to which sterile bacterial media was added (1 ml MB per 1 L seawater) as potential "growth enhancer" for putative pathogens that might be already present on experimental coral fragments. Tanks were maintained at temperatures identical to those measured at infection sites. Fragments in each tank were observed and photographed for the entire length of the experiment and developing disease signs were recorded. The experiments were terminated following mortality in infection tanks. Case mortality rate, or virulence (the proportion of infected fragments in each tank that died; Day 2002) and the mean proportion of infected fragments per tank were calculated. Pathogenicity (the proportion of exposed fragments that died) was calculated following the formula of Thomas and Elkinton (2004): Pathogenicity = infectivity x virulence (where pathogenicity = # dying / # exposed, infectivity = # infected / # exposed and virulence = # dying / # infected). Finally, LT50 (the estimated time it takes to kill 50% of the infected fragments) was calculated as a temporal measure of virulence. Both infected and non-infected fragments were crushed and streaked on agar plates to determine mean CFU's, as previously described. Total DNA was extracted from retrieved isolates for elucidation of taxonomic identity (16S rRNA gene sequence). Complete alignment (100%) of the 16SrRNA gene sequences retrieved from bacteria re-isolated from infected fragments and the 16S rRNA gene sequences of inoculated bacteria (P1-P6) allowed the fulfilment of Henle-Koch's postulates (Koch 1891), namely, that:

1. An organism found only on infected corals could be isolated, taxonomically identified, and grown in pure culture.
2. The isolated organism reproduced disease signs when inoculated onto healthy corals.
3. An isolate retrieved from coral fragments that developed disease signs in inoculation experiments is demonstrated to be identical (by analysis of 16SrRNA partial gene sequences) with the organism used for inoculations.

3.2.5 Other rules and criteria for supporting causality used by this study

Results of experiments and screenings conducted in this study were used to evaluate compliance with Evans' rules (1976) and Hill's criteria (1965), defined as alternative requirements for establishing disease causation. Both Evans' rules and Hill's criteria are listed in the Index Section.

3.2.6 Adhesion of bacterial isolates to corals

To further test the physical ability of putative pathogens to migrate towards coral hosts, to adhere and to survive the initial contact with the coral host, before initial signs of infection are observed, mean bacterial CFU's were quantified from random sub samples of tank seawater (N=4 seawater sub-samples per treatment, each taken from a different tank) following inoculation with the six coral pathogens (P1-P6) identified in Inoculation Experiment I (final inoculum concentration = 1 x 10^6 cells ml^{-1}). One ml of tank seawater was collected at inoculation time (t=0 h), 1 h post inoculation (t=1 h) and then at 12 h intervals (t=12 h, t=24 h, t=36 h) from four infection tanks. 100 µL aliquots from each sample were spread in triplicates on agar plates containing Marine Agar and TCBS, as described previously. Mean CFU's ml^{-1} g^{-1} wet weight were determined from three crushed coral samples per treatment at corresponding times (Banin et al. 2001a). In addition, control bacterial strains were also tested to determine if they adhered to coral fragments following inoculation into tanks. Finally, both putative pathogens and control bacteria were inoculated into four seawater tanks (per bacterial treatment) lacking coral fragments to test their ability to survive and remain suspended in the water column for the experiment's duration. Seawater samples were collected from these tanks and plated in triplicate as described previously to determine mean bacterial density in seawater (CFU's ml^{-1}).

3.2.7 The asocasein proteolytic Assay

The proteolytic activities of supernatants derived from 152 isolated bacterial strains retrieved by this study were tested by the asocasein assay based on a protocol by Windle and Kelleher (1997). Briefly, 1 ml of bacterial cultures grown to end logarithmic phase were centrifuged at 10,000g (Eppendorf 5415D centrifuge) for 5 min. Supernatant was removed and filtered through a 0.22µm filter (Millipore, USA). 100µL supernatant was incubated for 30min at 30ºC with 5g ml^{-1} of asocasein as substrate (Sigma, USA) dissolved in Tris-Hcl (50 mM pH 8) containing 0.04% NaN_3

(wt vol^{-1}). The reaction was terminated by adding 10% (wt vol^{-1}) of trichloroacetic acid (TCA) to a final concentration of 6.7% (wt vol^{-1}) and incubating samples for 1 min. Samples were then centrifuged for 3 min at 10,000g and transferred to 700 µL of 525 mM NaOH. Absorbance of six replicates from triplicate culture samples was measured in 96 microtitre well plates at 450 nm using a Wallac spectrophotometer (Perkin Elmer, USA). Blank controls were prepared from supernatant derived from *E.coli* cultures boiled at 100ºC for 10 min, treated with 5mg ml^{-1} asocasein and directly thereafter by TCA. Protease activity was calculated as proteolytic units, when $1U = 1000 \times (OD_{450} \ CFU^{-1}) \times 10^{9}$ (Denkin and Nelson 1999). Isolates were divided into 3 groups based on their proteolytic activity: High activity (>3U), medium activity (1-3U) and no activity (<1U).

3.2.8 Statistical analysis

Means and Standard Errors (SE) for bacterial colony forming unit (CFU) counts and for the proportion of infected colonies were compared among treatments using One-Way ANOVA (Statistica, StatSoft, Inc. USA). Colony forming unit (CFU) counts are presented in this study using logarithmic scales.

The association between categorical values related to bacterial isolates retrieved independently from diseased and non-diseased corals and demonstrating positive or negative proteolytic activity was estimated using 2x2 contingency tables (Pearson Chi-square). Significant results were determined when $\alpha \leq 0.05$.

3.2.9 Coral pathogens

Six coral pathogen strains that were identified by this study were submitted to the public collection of BCCM/LMG at the Gehnt University, Belgium and are available for acquisition under the following accession numbers: LMG23691 - isolate P2 from a WS infected *Acropora cytherea* in Majuro Atoll the Republic of the Martshall Islands, LMG23692 - isolate P5 from a WS infected *Pachyseris speciosa* in Nikko Bay Palau, LMG 23693 - isolate P4 from a WS infected *P. speciosa* in Nikko Bay Palau, LMG 23694 - isolate P6 from seawater above a WS infected *P. speciosa* in Nikko Bay Palau, LMG 23695 - isolate P3 from a WS infected *P. speciosa* in Nikko Bay Palau and LMG 23696 - isolate P1 from a WS infected *Montipora. aequituberculata* in Nelly Bay the GBR. 16S rRNA gene sequences of all coral

pathogens identified by this study were submitted to Genbank (www.ncbi.nih.nlm.gov/Genbank/) under the following accession numbers: P1 (LMG23696) - EU372917, P2 (LMG23691) - EU372935, P3 (LMG23695) - EU372934, P4 (LMG23693) - EU372931, P5 (LMG23692) - EU372933, P6 (LMG23692) - EU372932. 16S rRNA gene sequences retrieved from isolates demonstrating positive results for proteolytic activity in both screening tests conducted by this study (asocasein assay and PCR amplification) were submitted to Genbank under the following accession numbers: EU372918-EU372930, EU372936-EU372939, and are presented in Fig. 3.8.

3.3 Results

3.3.1 Higher bacterial counts on WS corals

Densities of cultivable bacteria (measured as CFU's ml^{-1} g^{-1} wet weight) associated with corals sampled from each of the three Indo-Pacific outbreak sites examined in this study were significantly higher on corals displaying disease signs than on those lacking disease signs (Fig.3.1A-C). Mean CFU's from *Pachyseris speciosa* samples collected from Nikko Bay Palau (Fig. 3.1A) plated on a general heterotrophic Marine Agar (MA) were ~20 times higher for diseased corals (mean $1.50 \pm 0.42 \times 10^6$ CFU's ml^{-1} g^{-1}) than for corresponding samples lacking disease signs (mean $8.0 \pm 0.5 \times 10^4$ CFU's ml^{-1} g^{-1}). A ~200 fold difference was observed when the same samples were plated on TCBS agar selective for members of the family *Vibrionacae* (mean $4.42 \pm 1.84 \times 10^5$ and mean $2.0 \pm 0.1 \times 10^3$ CFU's ml^{-1} g^{-1}, respectively), suggesting higher *Vibrio* densities on diseased corals.

Cultivable bacterial densities were also found to be significantly higher on *Montipora aequituberculata* fragments (Nelly Bay GBR) displaying visual WS disease lesions, compared to coral fragments lacking lesions. Diseased fragments sampled from the interface (I) between lesions and healthy tissue (Fig 3.1 B), gave rise to ~7 times more *Vibrio* CFU's counts (mean $4.92 \pm 1.53 \times 10^2$ CFU's ml^{-1} g^{-1}) than the corresponding healthy fragments (H) from the same corals (mean $6.8 \pm 1.3 \times 10^1$ CFU's ml^{-1} g^{-1}). Fragments sampled from exposed coral skeleton (S) gave rise to ~50 times more CFU's (mean $3.42 \pm 0.77 \times 10^3$ CFU's ml^{-1} g^{-1}) than healthy fragments (H) from the corresponding corals.

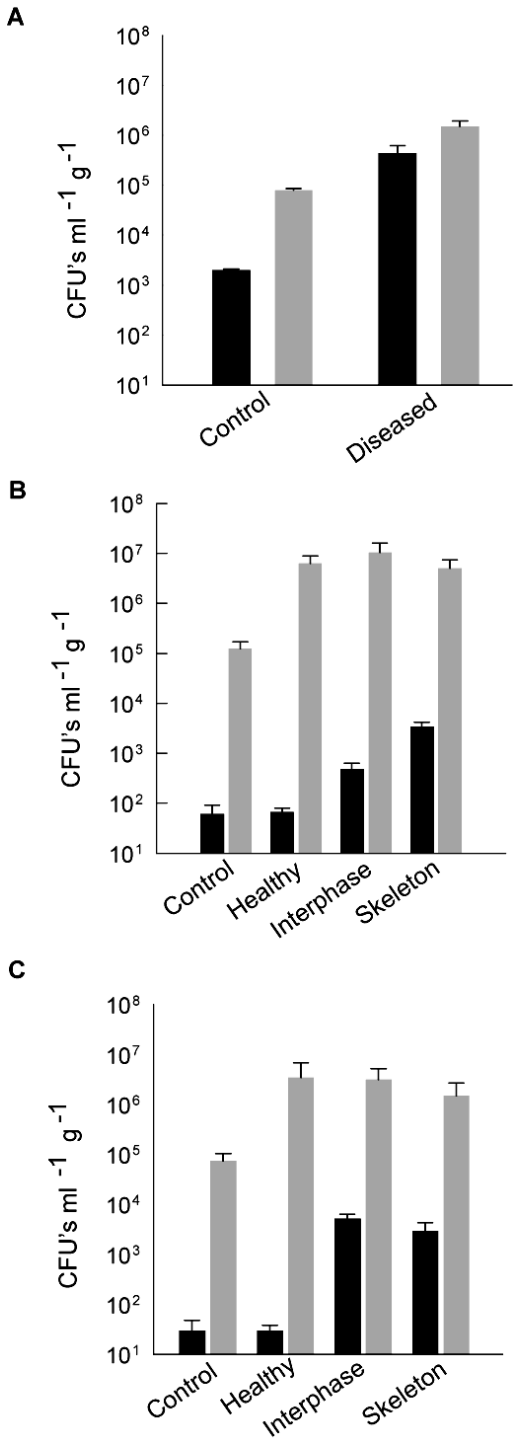

Figure 3.1 Bacterial density on corals sampled from the field
A. Mean CFU's ml^{-1} g^{-1} from crushed *Pachyseris speciosa* fragments sampled in Nikko Bay Palau. **B.** Mean CFU's ml^{-1} g^{-1} from crushed *Montipora aequituberculata* fragments sampled in Nelly Bay GBR **C.** Mean CFU's ml^{-1} g^{-1} from crushed *Acropora cytheria* fragments sampled in Majuro Atoll the Marshall Islands.
■ – Bacterial isolates streaked on TBCS agar. ■ – Bacterial isolates streaked on MA. **Control** – samples from coral fragments lacking disease signs. **Healthy** – Coral tissue lacking disease signs sampled from fragments displaying signs of disease**. Interface** – Coral tissue sampled at the border between exposed skeleton and healthy tissue. **Skeleton** – Exposed skeleton in areas of tissue lesions. CFU's ml^{-1} g^{-1} are presented in a logarithmic scale. Bars = Standard Errors.

Fragments sampled from *Acropora cytherea* corals (Marshall Islands) similarly had a significantly higher mean CFU's counts on TCBS for samples derived from the lesion interface (I) and skeleton (S) compared directly against healthy looking fragments (H) of the corresponding corals (Fig. 3.1C), suggesting an association between *Vibrio* densities and disease lesions within a coral colony. Laboratory exposure trials were subsequently designed to test for isolate infectivity and to satisfy Hill's criterion 4 (1965), namely that disease signs follow a "time sequence" with cause (bacterial presence) preceding effect (disease lesions).

3.3.2 Inoculation experiment I: Exposed colonies display disease signs

Bacterial strains isolated from corals displaying disease signs at each of the three outbreak sites (10 isolates from TCBS medium plates and 10 isolates from MA medium plates per site) were screened in infection trials with results from all inoculations presented in table 3.1.

Table 3.1 Inoculation experiment I

Outbreak	**Nikko Bay Palau**			**Nelly Bay GBR**	**Majuro Atoll Marshall Islands**	
Experiment Duration (h)	96			96	36	
Inoculation Dose	$1x10^6$ cells ml^{-1}			$1x10^6$ cells ml^{-1}	$1x10^6$ cells ml^{-1}	
Coral host	*Pachyseris speciosa*			*Montipora aequituberculata*	*Acropora cytherea*	
Exposed (# inoculum treatments)	20			20	20	
Infected (# treatments producing lesions) [1]	3			1	2	
Strains causing infections [2]	P3	P4	P5	P1	P2	P7 [3]
Infectivity (%) [4,5]	100	60	40	40	100	12

[1] The number of pure cultures in each experiment causing visible disease signs (lesions) on experimental fragments.
[2] Bacterial isolates causing disease signs were named Pathogen 1-7 (P1-P7).
[3] Inoculation experiment I was repeated for both isolates from the Marshall Islands (P2, P7) that demonstrated infectivity before ruling out isolate P7 as a possible putative pathogen.
[4] Infectivity represents the percent of fragments (# exposed / # infected) within each of the treatments displaying visible disease signs (lesions)
[5] No fragments in control treatments were infected

All five *P. speciosa* fragments (Nikko Bay Palau) inoculated with isolate P3 ($1x10^6$ bacteria ml^{-1}) developed disease signs following exposure for 96 h, while treatments with isolates P4 and P5 demonstrated lower infectivity (Fig. 3.2). Coral fragments in control treatments (n=17) including treatments with 7 other TCBS derived isolates and 10 isolates from MA plates remained unaffected for the duration of the experiment. Healthy fragments of *M. aequituberculata* (Nelly Bay GBR) were only infected by one strain (P1) of the 20 strains tested, with 40% of fragments displaying disease signs after a 96 h exposure to P1. 100% and 12% of healthy *A. cytherea* fragments (Majuro Atoll Marshall Islands) exposed to strains P2 and P7, respectively, displayed disease signs after 36 h. A repeat of the experiment with strain P7 resulted in no further positive results and therefore the strain was eliminated as a possible putative pathogen. Results from inoculation experiment I satisfied Hill's criterion 4 (1965) of "time sequence" (cause precedes effect) by demonstrating successful infectivity following putative pathogen inoculations.

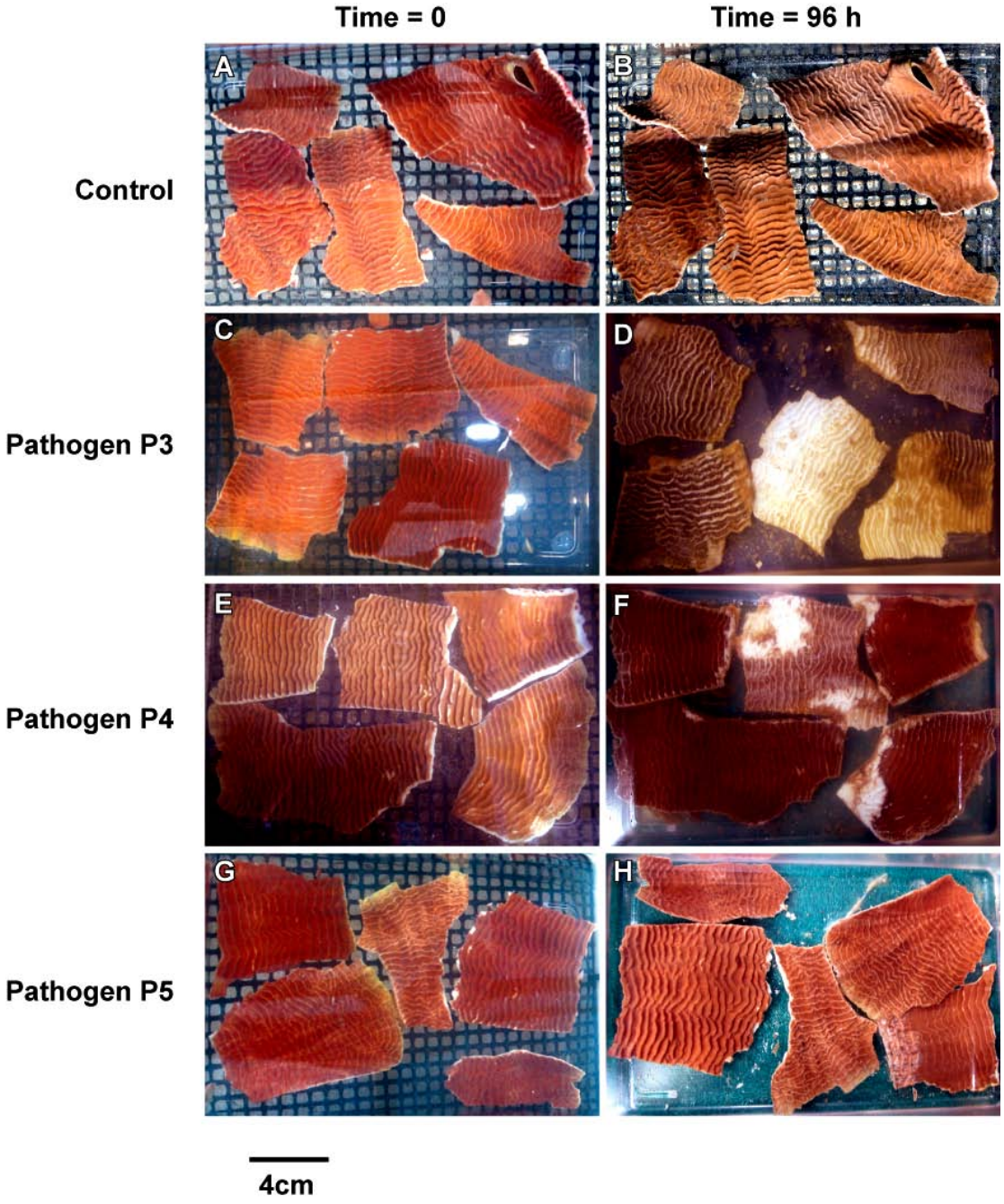

Figure 3.2 Inoculation experiment 1, Palau: **A-B.** *P. speciosa* coral fragments without inoculation (t=0h and t=96h). **C-D**. *P.speciosa* coral fragments inoculated with 1 x10^6 cells ml $^{-1}$ of pure culture P3 (t=0h and t=96h). **E-F**. *P.speciosa* coral fragments inoculated with 1 x10^6 cells ml $^{-1}$ of pure culture P4 (t=0h and t=96h). **G-H**. *P.speciosa* coral fragments inoculated with 1 x10^6 cells ml $^{-1}$ of pure culture P5 (t=0h and t=96h).

3.3.3 Inoculation experiment II: Fulfilling Henle-Koch's postulates

Results from three replicated experimental inoculation trials conducted to fulfil Henle-Koch's' postulates and determine the virulence of putative pathogens by causing mortality to infected corals are presented in table 3.2.

Table 3.2 Inoculation experiment II

Outbreak	**Nikko Bay Palau**	**Nelly Bay GBR**	**Majuro Atoll Marshall Islands**
Date	2005	2003	2004
Target host/s	*Pachyseris speciosa*	*Montipora aequituberculata*	*Acropora hyacinthus*
Putative pathogen	P3, P4, P5, P6 [1]	P1	P2
Effective concentration	1 x 10^6 cells ml $^{-1}$	1 x 10^6 cells ml $^{-1}$	1 x 10^6 cells ml $^{-1}$
Temperature in tanks	29.9°C	28°C	30°C
# fragments (n)	195	360	80
# inoculum treatments	11	3	3
# tanks per treatment (N)	3	12	4
# fragments per tank	5	6	4
Mean infected (Infectivity) [2]	0.88 ± 0.08	0.55 ± 0.13	0.94 ± 0.05
P value	P<0.00001	P=0.00005	P<0.00001
Mean mortality rate (Virulence) [3]	0.66 ± 0.05	0.87 ± 0.09	0.66 ± 0.11
Mean pathogenicity [4]	0.58 ± 0.11	0.48 ± 0.15	0.62 ± 0.24
Mean lesions on control fragments [5]	0.04 ± 0.02	0.08 ± 0.06	None
LT50 [6]	60h	180h	18h

[1] Data for separate inoculation experiments with isolates P3-P6 was pooled together.
[2] Mean infectivity was calculated as mean #infected / # exposed ± SE.
[3] Mean mortality rate, or virulence, was calculated as mean # dead / # infected ± SE.
[4] Mean pathogenicity was calculated as mean # dead/ #exposed ± SE.
[5] Mean lesions on control fragments were calculated as mean # lesions / # controls ± SE.
[6] LT50 is the time needed to cause 50% mortality of the exposed fragments.

Healthy colony fragments exposed to putative pathogens P1-P6 ($1x10^6$ cells ml^{-1}) displayed signs of disease similar to those observed in the field in all experiments (Fig. 3.3A-F). Exposure of *M. aequituberculata* to putative pathogen P1 resulted in lesions covered by a sulphurous deposit, which matched disease signs in the field (Fig 3.3A-B). Exposure of *P. speciosa* to putative pathogens P4 and P6 began by producing linear lesions resembling field observed lesions (Fig. 3.3C-D), while *P. speciosa* fragments exposed to P3 and P5 resulted in the development of larger lesions similar to a second, more common type of lesion observed at the site (Fig. 3.3E-F).

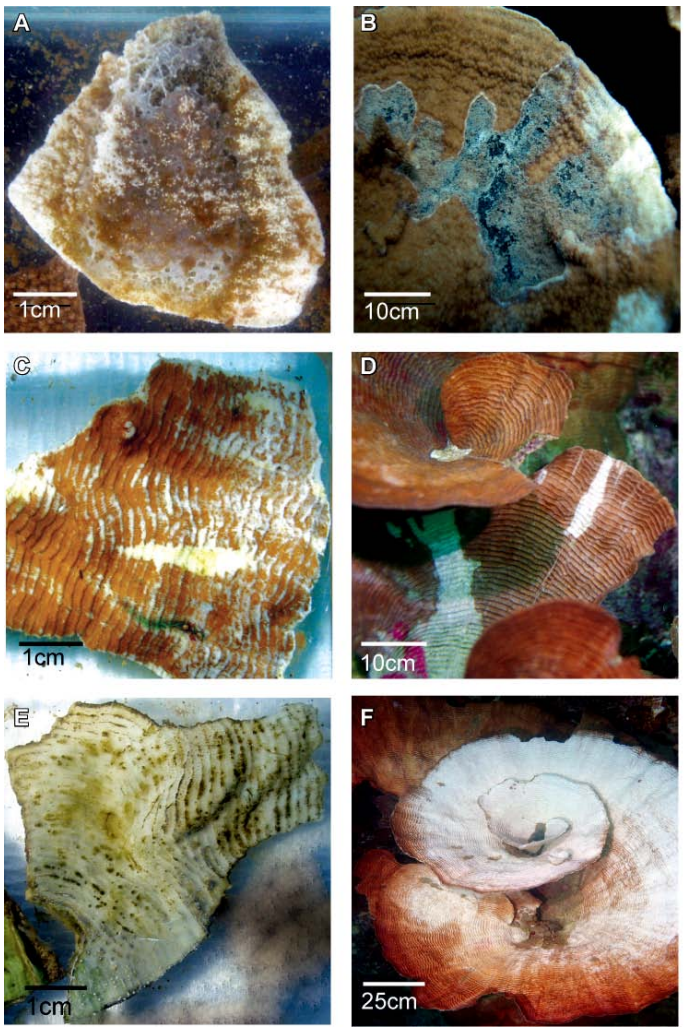

Figure 3.3 WS signs observed in the laboratory and in the field
A. *Montipora aequituberculata* exposed to pathogen P1 in laboratory inoculation experiment. **B**. *M. aequituberculata* with WS signs in the field (Nelly Bay GBR). **C.** *Pachyseris speciosa* exposed to pathogen P6 in laboratory inoculation experiment. **D.** *P. speciosa* with WS signs in the field (Nikko Bay Palau). **E.** *P. speciosa* exposed to pathogen P3 in laboratory inoculation experiment**. F.** *P. speciosa* with WS signs in the field (Nikko Bay Palau).

Coral fragments inoculated with control strains (non-pathogenic) and un-inoculated control fragments did not develop signs of WS lesions (Fig. 3.4 IA-B, 3.4 IIA-B) in contrast to lesion signs and mortality observed in all treatments with putative pathogens (Fig 3.4 IC-D, 3.4 IIC-J). Bacterial isolates from infected fragments retrieved at the conclusion of the experimental exposure, demonstrated 100% 16S rRNA gene sequence identity to inoculated strains. Recovery of inoculated strains from infected fragments fulfilled Henle-Koch's postulates for all six proposed agents examined in this study.

The proportion of exposed fragments per tank that became infected (infectivity) varied among the experiments, with 88% of fragments exposed to P3-P6, 55% of fragments exposed to P1 and 94% of fragments exposed to strain P2 becoming infected (Table 3.2). Pathogenicity (proportion of exposed fragments that died) measured 58%, 48% and 62%, and mortality rate, or virulence (proportion of infected fragments that died) equalled 66%, 87% and 66% for putative pathogens from Palau, Magnetic Island and the Marshall Islands, respectively (Table 3.2). Similarly, the times needed for 50% of the fragments to experience mortality (LT50) were 60h, 180h and 18h, respectively (Table 3.2).

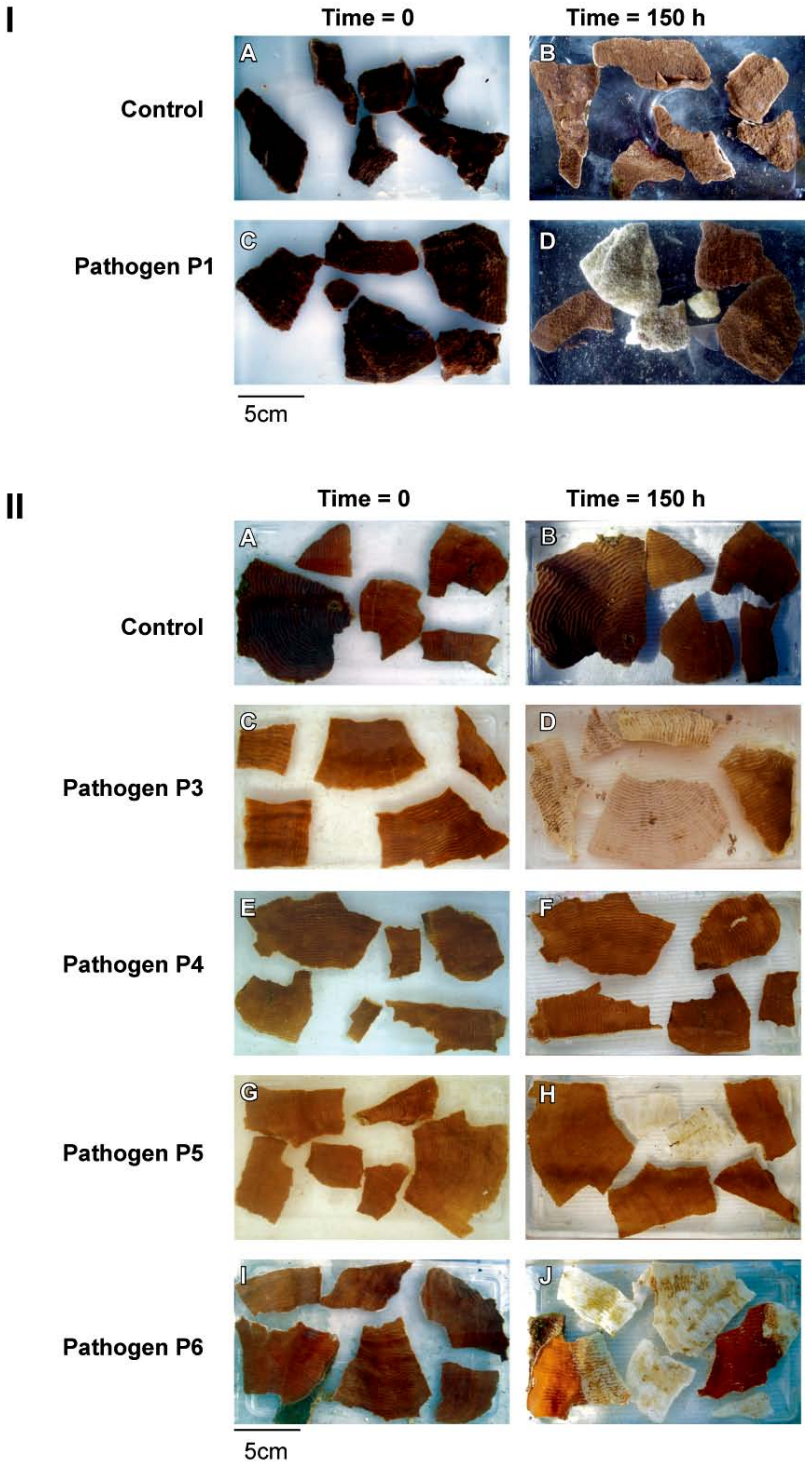

Figure 3.4 Inoculation experiment II

I A-B. *Montipora aequituberculata* coral fragments in un-inoculated control treatment (t=0h and t=150h). **I C-D**. *M. aequituberculata* coral fragments exposed to $1x10^6$ cells ml^{-1} of culture P1 (t=0h and t=150h). **II A-B**. *Pachyseris speciosa* coral fragments in un-inoculated control treatment (t=0h and t=150h). **II C-D.** *P. speciosa* coral fragments exposed to $1x10^6$ cells ml^{-1} of culture P3 (t=0h and t=150h). **II E-F.** *P. speciosa* coral fragments exposed to $1x10^6$ cells ml^{-1} of culture P4 (t=0h and t=150h). **II G-H.** *P. speciosa* coral fragments exposed to $1x10^6$ cells ml^{-1} of culture P5 (t=0h and t=150h). **II I-J.** *P. speciosa* coral fragments exposed to $1x10^6$ cells ml^{-1} of culture P6 (t=0h and t=150h).

3.3.4 Aetiology of WS

3.3.4.1 Adhesion of pathogens to coral tissue

Putative pathogen P1 (Nelly Bay GBR) demonstrated an 87% reduction in mean seawater CFU's (Fig 3.5A) within the first 12 h following inoculation into aquaria with fragments of *M. aequituberculata* (from mean 2.48 ± 0.37 x 10^5 cells ml^{-1} at t 0 to mean 3.17 ± 0.67 x 10^4 cells ml^{-1} at t 12). In comparison, only a 6% reduction was observed when the same corals were inoculated with control bacterial isolate MF1 (from mean 9.60 ± 1.81 x 10^5 cells ml^{-1} at t 0 to mean 9.07 ± 1.01 x 10^5 cells ml^{-1} at t 12). CFU's from un-inoculated control aquaria averaged 3.0 ± 0.69 x 10^2 cells ml^{-1} after 12 h. After 36 h, mean CFU counts from aquaria seawater treated with P1 dropped even further to 0.6% of the original inoculation concentration (mean 1.41 ± 0.27 x 10^3 cells ml^{-1}), which was similar to the density of cells in control tanks (mean 2.26 ± 0.49 x 10^3 cells ml^{-1}). In contrast, putative pathogens that were inoculated into sterile seawater without corals maintained a constant density of viable counts in suspension throughout the experiment (Fig. 3.5A) eliminating the possibility that bacteria died from the seawater itself or may have settled on the sides or bottom of aquaria. *Vibrio* density in aquaria containing *M. aequituberculata* fragments, which were inoculated with non-pathogen MF1 remained unchanged after 36 h, with mean 1.04 ± 0.15 x 10^6 cell ml^{-1} (100%) retrieved on TCBS agar plates.

CFU counts of crushed coral samples (CFU ml^{-1} g^{-1} wet weight) from aquaria inoculated with P1 reached a mean of 1.60 ± 0.78 x 10^5 ml^{-1} g^{-1} after 12 h (Fig 3.5B). In comparison, fragments from aquaria inoculated with control bacteria (MF1), or un-inoculated controls, resulted in CFU counts that were 94% and 97% lower after 12 h (mean 9.08 ± 2.82 x 10^3 ml^{-1} g^{-1} and mean 4.12 ± 2.45 x 10^3 ml^{-1} g^{-1}, respectively).

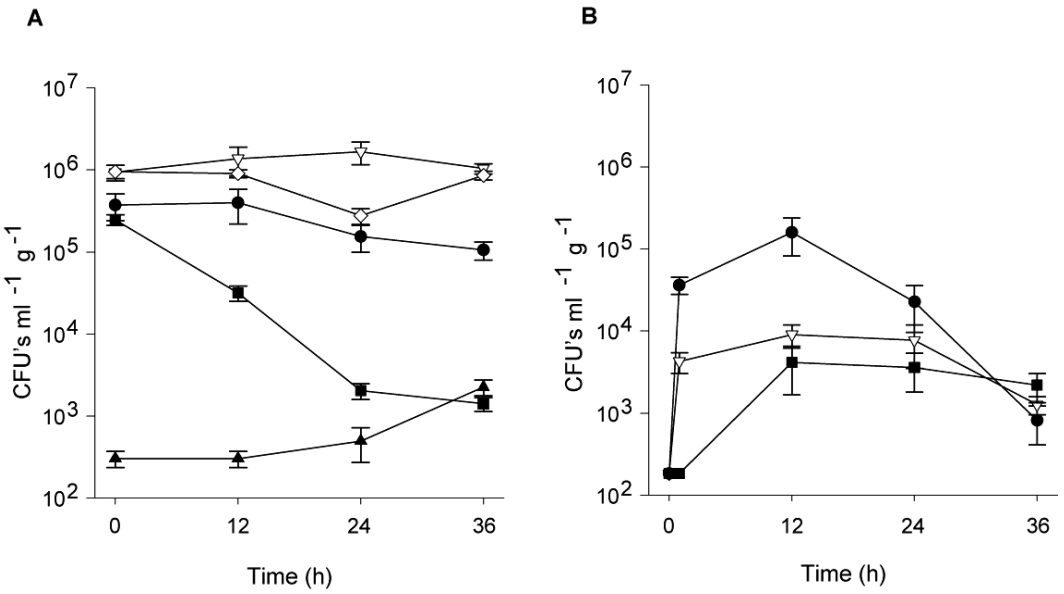

Figure 3.5 Adhesion experiment

A. CFU's counts (ml $^{-1}$) from SW samples. ● - 1x10^6 cells ml $^{-1}$ of culture P1 inoculated into SW without corals. ◇ - 1x10^6 cells ml $^{-1}$ of culture from control isolate MF1 inoculated into SW without corals. ■ - 1x10^6 cells ml $^{-1}$ of culture P1 inoculated into SW with *Montipora aequituberculata* coral fragments. ▽ - 1x10^6 cells ml $^{-1}$ of culture MF1 inoculated into SW with *M. aequituberculata* coral fragments. ▲ - SW with *M. aequituberculata* coral fragments without inoculation. **B.** CFU's counts (ml^{-1} g^{-1}) from crushed coral fragments. ● - 1x10^6 cells ml $^{-1}$ of culture P1 inoculated into SW with *M. aequituberculata* coral fragments. ▽- 1x10^6 cells ml $^{-1}$ of culture MF1 inoculated into SW with *M. aequituberculata* coral fragments. ■ - *M. aequituberculata* coral fragments without inoculation. Time represents hours (h) following exposure.CFU's ml^{-1} g^{-1} are presented in a logarithmic scale. Bars=Standard errors.

Table 3.3 summarizes the data from adhesion experiments conducted with putative pathogens and controls isolated from the three infection sites examined in this study.

Table 3.3 Adhesion experiment

Outbreak	**Nikko Bay, Palau**	**Nelly Bay, GBR**	**Majuro Atoll, Marshall Islands**
Cultivable putative pathogens in SW at t=12h (% from original inoculation at t= 0h) [1]	13.5	12.0	13.0
Cultivable putative pathogens on corals at t=12h (increase factor from CFU's at t=0h)	1049 x Increase	876 x Increase	484 x Increase
Cultivable putative pathogens on corals at t=12h (% from original inoculation at t=0h) [2]	0.9	0.7	0.8
% of cultivable putative pathogens missing from total original inoculation [3]	85.6	87.3	86.2

[1] The precent (%) of cultivable putative pathogens in seawater at t=12h was calculated by the formula: CFU's ml^{-1} [t=12h] / CFU's ml^{-1} [t=0h].
[2] The precent (%) of cultivable putative pathogen on corals was calculated by the formula: Total CFU's on coral fragments [t=12h] / Total original inoculation [t=0h].
[3] The precent (%) of missing cultivable cells was calculated by the formula: 100 – (% CFU's in sweater [t=12h] + % CFU's on coral fragments [t 12h]).

3.3.4.2 Loss of *Symbiodinium* followed by tissue lesions

Detailed photographs taken of *A. hyacinthus* fragments infected experimentally with P2 (Fig 3.6A-C) revealed 2 distinct disease-phases. An initial loss of *Symbiodinium*, visible as tissue paling was observed after 9-12 h of exposure (Fig 3.6 B-C) followed by developing tissue lesions. Similar patterns of paling were also observed when *P. speciosa* fragments were exposed to P3 (Fig 3.6D). Paling and loss of *Symbiodinium* commenced in coenosarc tissue (tissue between polyps) in distinct linear patterns starting 12 h post inoculation and corresponding with the peak in viable CFU counts retrieved from coral tissue. These early signs of disease then

developed into lesions that resembled those observed in the field (Fig 3.6E-F), suggesting that disease progression was consistent (Hill's criterion 2; Hill 1965) and followed measurable steps (Evans' Rule F; Evans 1976).

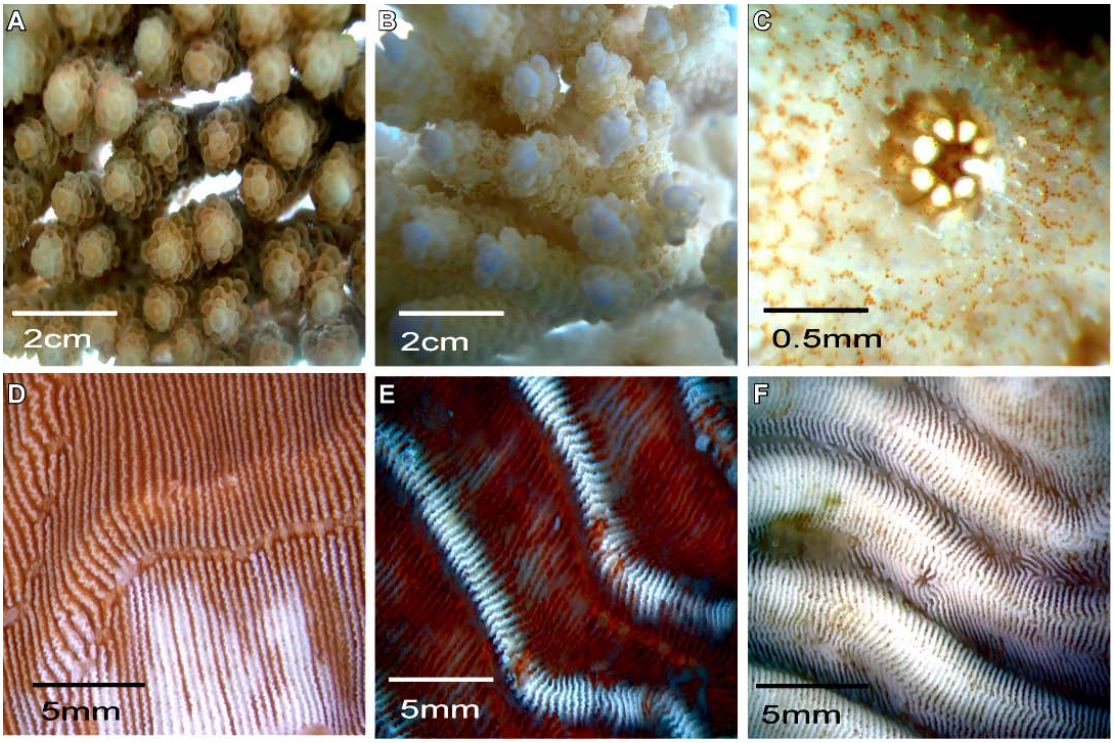

Figure 3.6 Disease progression
A. *Acropora hyacinthus* fragmennt inoculated with 1 x 10^6 cells ml $^{-1}$ of culture P2 (t=0h). **B.** Loss of *Symbiodinium* from *A. hyacinthus* inoculated with 1 x 10^6 cells ml $^{-1}$ of culture P2 (t=12h). **C.** Polyp and surrounding tissue - loss of *Symbiodinium* from *A. hyacinthus* inoculated with 1 x 10^6 cells ml $^{-1}$ of culture P2 (t=12h). **D.** Loss of *Symbiodinium* cells from *Pachyseris speciosa* inoculated with 1 x 10^6 cells ml $^{-1}$ of culture P3 (t=12h). **E.** Tissue lesions on *P. speciosa* inoculated with 1 x 10^6 cells ml $^{-1}$ of culture P3 (t=24h). **F.** Exposed skeleton on *P. speciosa* inoculated with 1 x 10^6 cells ml $^{-1}$ of culture P3 (t=60h).

In all experimental treatments inoculated with putative pathogens P1-P6, the proportion of fragments displaying acute disease signs (lesions) increased with time to between 55% and 94% of fragments per tank (Fig 3.7A-C) conforming with Evans' rules D and E (Evans 1976), namely that disease occurs, temporally, following specific incubation times and that the number of new cases and the severity of outcome should correlate positively with time. The proportion of *P. speciosa* fragments from Palau, *M. aequituberculata* fragments from Nelly Bay and *A.*

hyacinthus fragments from the Marshall Islands displaying acute disease signs increased consistently and significantly within the first 96 hours (Fig. 3.7A-B) and 12 hours (Fig. 3.7C) of the start of inoculation experiment II, at each site, respectively, resembling standard infection curves (Thrusfield 2005). In contrast, 0-8% of fragments in inoculated and un-inoculated control treatments developed disease signs (Fig 3.7A-C).

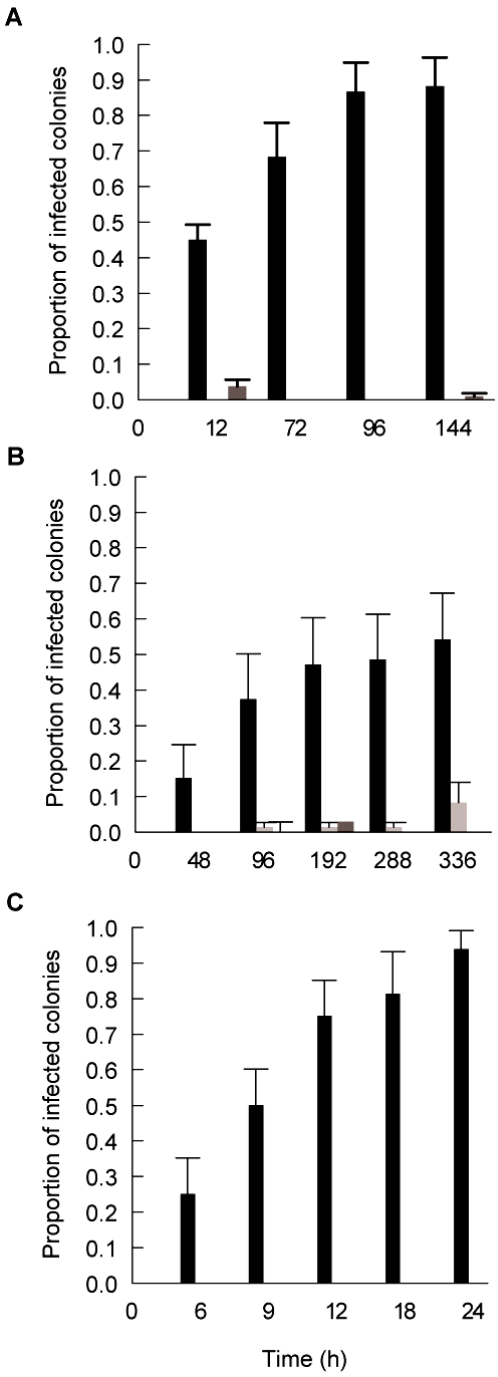

Figure 3.7 Disease transmission

A. Mean proportion of infected *Pachyseris speciosa* coral fragments displaying WS signs following exposure to cultures of P3-P6 in comparison to proportions in inoculated and un-inoculated control treatments. **B.** Mean proportion of infected *Montipora aequituberculata* coral fragments displaying WS signs following exposure to culture of P1 in comparison to proportions in inoculated and un-inoculated control treatments. **C.** Mean proportion of infected *Acropora cytherea* coral fragments displaying WS signs following exposure to culture P2 in comparison to proportions in inoculated and un-inoculated control treatments. ■ - Coral fragments inoculated with 1 x 10^6 cells ml $^{-1}$ of putative pathogen cultures. ■- Coral fragments inoculated with 1 x 10^6 cells ml $^{-1}$ culture of non-pathogen isolates. □ - Coral fragments without inoculation. Time represents hours (h) following exposure. Bars= Standard errors.

3.3.4.3 Isolates associated with disease signs are proteolytically active

Isolated bacteria (152 strains) recovered from both diseased and healthy corals were screened for proteolytic activity using the asocasein assay and specific PCR primers targeting the zinc-binding site of a *Vibrio* family zinc-metalloprotease. A total of 48% of strains (n=33 strains) retrieved from diseased *P. speciosa* in the field (Nikko Bay Palau) demonstrated high (≥3U) or medium (1-3U) proteolytic activity compared with 30% strains (n=23 strains) demonstrating high or medium activity that were retrieved from non-diseased colony fragments sampled in the field (Table 3.4). This difference, however, was not found to be statistically significant (Pearson's χ^2 = 1.825, DF = 1, p=0.177). In contrast, 11 positive PCR bands and derived partial sequences of the *Vibrio* zinc-metalloprotease gene were obtained from DNA of isolates retrieved from diseased *P. speciosa* sampled in the field compared with only one partial sequence from a non-diseased colony fragment. This difference was found to be significant by testing for Pearson's chi-square (χ^2 = 6.763, DF = 1, p= 0.0093).

Table 3.4 Proteolytic activity of bacterial isolates (Nikko Bay Palau)

	Bacterial isolates retrieved from field *Pachyseris speciosa* [1]		**Total**
	Diseased colonies	**Non-diseased colonies**	
+ ve PCR product [2]	11	1	12
- ve PCR product [2]	22	22	44
Total	33	23	56
High proteolytic activity [3]	6	4	10
Medium proteolytic activity [4]	10	3	13
No proteolytic activity [5]	17	16	33
Total	33	23	56

[1] Isolates retrieved from diseased and non-diseased *Pachyseris speciosa* colonies sampled in Nikko Bay Palau .
[2] Specific amplification of *Vibrio* zinc-metalloprotease active zinc binding site.
[3] High proteolytic activity >3U measured by the asocasein assay.
[4] Medium proteolytic activity 1-3U measured by the ascasein assay.
[5] No proteolytic activity <1U measured by the asocasein assay.

Similar results were obtained by screening field isolates from Nelly Bay GBR (Table 3.5). Bacteria demonstrating high and medium proteolytic activity by the asocasein assay made up 70% of all isolates retrieved from coral skeletons (S) exposed by WS disease at Nelly Bay GBR and 57% of all isolates from the lesion interfaces (I), compared with only 24% of all isolates obtained from healthy (H) tissue fragments on diseased colonies, demonstrating a significant difference in proteolytic activity between isolates associated with disease signs (I+S) and healthy (H) tissue (Pearson's $\chi^2 = 6.446$, DF = 1, p= 0.011). A significant difference was also obtained for the same 38 isolates when screened by the molecular method using PCR primers (Pearson's $\chi^2 = 12.518$, DF = 1, p<0.0001).

Table 3.5 Proteolytic activity of bacterial isolates

	Isolates retrieved from diseased *M. aquetuberculata* coral fragments collected in the field (Nelly Bay, GBR)			
	- ve Disease Signs[1]	**+ ve Disease Signs[1]**		
	Healthy on diseased	**Interphase**	**Skeleton**	**Total**
+ ve PCR product [2]	0	4	4	8
- ve PCR product [2]	21	3	6	30
Total	21	7	10	38
High proteolytic activity[3]	2	4	5	11
Medium Proteolytic activity[4]	3	0	2	5
No proteolytic activity[5]	16	3	3	22
Total	21	7	10	38

[1] Isolates retrieved from diseased field samples (H=healthy segment on diseased fragment, I=interphase between lesion and healthy tissue, S=exposed skeleton).
[2] Specific amplification of *Vibrio* zinc-metalloprotease active zinc binding site.
[3] High proteolytic activity >3U measured by the asocasein assay.
[4] Medium proteolytic activity 1-3U measured by the ascasein assay.
[5] No proteolytic activity <1U measured by the asocasein assay.

Finally, screenings by the molecular method performed on DNA extracted from 56 isolates retrieved from both infected and non-infected fragments at the conclusion of inoculation experiment II in Palau (Table 3.6), demonstrated that results obtained by screening field isolates were consistent with screening laboratory derived isolates (Pearson's $\chi^2 = 6.725$, DF = 1, p=0.010). Thus, in both field and laboratory

infections, the presence of a *Vibrio* family zinc-metalloprotease was associated with disease signs conforming to Evans' rules B, C and G (Evans 1976), suggesting that bacterial proteolytic activity may cause or contribute to observed WS lesions.

Table 3.6 Proteolytic activity of bacterial isolates

	Bacterial isolates retrieved from *P. speciosa* coral fragments[1]		
	Infected colonies	**Non-infected colonies**	**total**
+ ve PCR product [2]	27	2	29
- ve PCR product [2]	19	10	29
Total	46	12	58

[1] Isolates retrieved from laboratory infected and non-infected *P. speciosa* coral fragments
sampled following the termination of inoculation experiment II.
[2] Specific amplification of *Vibrio* zinc-metalloprotease active zinc binding site.

3.3.5 Pathogens identified by this study form a taxonomic cluster

Based on near complete 16S rRNA gene sequence comparisons, the six pathogens clustered in a tight taxonomic group and were found to share between 98-99% sequence identities with the previously characterized coral-bleaching pathogen *Vibrio coralliilyticus* (Ben-Haim et al. 2003a). All isolates which tested positive for the zinc-metalloprotease zinc-binding site and exhibited high proteolytic activity (when screened by the asocasein assay) were used to construct a maximum likelihood phylogenetic tree based on their 16S rRNA gene (Fig. 3.8). Our findings demonstrate that more isolates possess the genetic capacity to become proteolytically active than the six coral pathogens identified in this study, suggesting that successful infections require the expression of additional virulence genes, but also that other non-pathogens might be indirectly involved in enhancing infections.

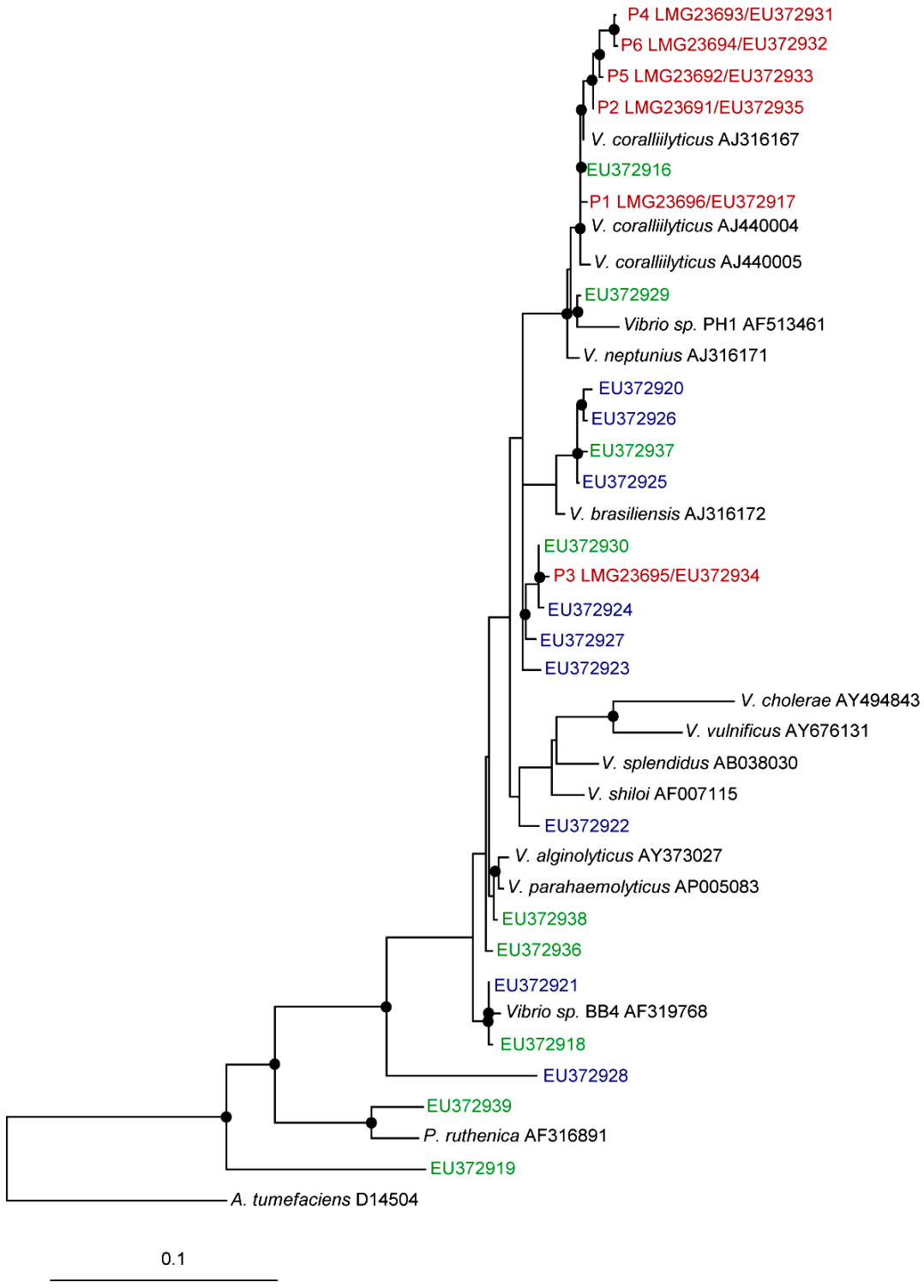

Figure 3.8 Phylogenetic tree of proteolitically-active isolates
Evolutionary distance maximum likelihood analysis based on 16S rRNA gene sequences of isolates obtained by this study. Coral pathogens are marked in red. Reference strains are marked in black. Isolates that demonstrated high proteolytic activity (asocasein assay) and tested positive for a zinc-metalloprotease gene are presented in blue (Palau isolates) and in green (Nelly Bay GBR isolates). Nodes represent bootstrap values≥50% based on 1000 re -samplings. Scale bar corresponds to 10% estimated sequence divergence.

3.4 Discussion

This study reports the successful isolation and identification of bacterial infectious agents implicated in a group of widespread Indo-Pacific coral diseases that affect numerous species at various geographical locations. Six coral pathogens were identified with close 16S rRNA gene phylogenetic affiliation with the previously identified coral pathogen *V. coralliilyticus* (Ben-Haim et al. 2003a). *Vibrio* pathogens have been previously demonstrated to cause fish, eel, shrimp and human mortalities (Milton et al. 1992, Amaro and Biosca 1996, Faruque et al. 1998, Lightner 1988, Linkous and Oliver 1999). Seasonal bleaching of the coral *Oculina patagonica* in the Mediterranean Sea has been shown to be caused by *V. shiloi* (Rosenberg and Falkovitz 2004, Kushmaro et al. 1996, 1997a, 2001) and *V. coralliilyticus* has been identified as the aetiological agent of *Pocillopora damicornis* bleaching in the Indian Ocean (Ben-Haim et al. 2002, 2003a, 2003b). Other coral diseases in the Caribbean, such as White Band Disease type II, Yellow Blotch/Band and Dark Spots Disease, were found to be associated with elevated *Vibrio spp*. prevalence (Ritchie and Smith 1998, Gil-Agudelo et al. 2006, 2007), suggesting the involvement of *Vibrio* strains in numerous coral diseases including coral bleaching (Ritchie et al. 1994, Rosenberg and Falkovitz 2004, Barneah et al. 2007, Bourne et al. 2008).

This study has found high prevalence of *Vibrio spp*. to be associated with WS signs in all diseased colonies sampled from three WS outbreaks. An association between bacterial presence and disease signs does not provide proof that bacteria actually cause the disease. However, such an association already fulfils requirements put forth by Evans' rule A (Evans 1976), namely that the "prevalence of the disease should be significantly higher in those exposed to the putative cause than in cases controls not so exposed". It also complies with the Read (1994) definition of virulence, which highlights an agent's contribution to reduction in host fitness caused by exploitation.

When bacterial strains were inoculated into tanks with healthy coral fragments, only putative pathogens adhered to coral tissue and a consistent peak in cultivable *Vibrio* abundance, 500-1000 fold greater than was found for control strains, was observed on fragments exposed to putative pathogens 12 h post inoculation. This experiment confirmed Hill's criterion of "time sequence" (Hill 1965), suggesting that following exposure and prior to the development of visual disease signs (lesions), putative pathogens were able to migrate towards the coral fragments, adhere to coral

tissue and survive initial contact in a viable state. In contrast, control strains were unable to perform this transmission, suggesting that motility towards corals and adhesion may be regarded as traits involved in pathogenicity. Initial visual signs of tissue paling and lesions were observed following a peak in cultivable bacterial abundance for all six putative pathogens, demonstrating a common aetiology of adhesion followed by disease progression. Despite this peak in cultivable *Vibrio* abundance 12 h post exposure, less than 1% of the original inoculation was retrieved by plating coral fragments, potentially indicating that *Vibrio* cells entered a viable but non-culturable (VBNC) state (Israeli et al. 2001), or alternatively, died.

The colonization of target hosts by *Vibrio* pathogens has been studied in detail, particularly the ability of *Vibrios* to adhere to mucus found either inside the gastro-internal track or externally on fish or corals (Bordas et al. 1996, Banin et al. 2001a). Denkin and Nelson (1999) have demonstrated that the transcription of zinc-metalloprotease by the fish pathogen *V. anguillarum* is regulated by mucus and can only occur after adhesion is completed. This duality in *Vibrio* function is often referred to as the "transmission-virulence trade-off" (Anderson and May 1982) and highlights the fact that the ultimate goal of *Vibrio* pathogenicity is not to kill a host, or to complete a necessary biological life-cycle within it, but to re-enter the environment in larger numbers and initiate a new cycle of infections (Higgins et al. 2007). It explains why *Vibrio* pathogens are commonly found in environmental reservoirs (Sussman et al. 2003), or transmitting through the water column, like pathogen P6 isolated in this study from seawater above infected corals at Nikko Bay Palau.

This study has demonstrated that 55%-94% of coral fragments exposed to pathogens cultured from diseased corals at their respective field sites become infected and that 66%-87% of those infected die, compared with significantly lower infection and mortality for fragments exposed to control bacterial strains (0-8%). These results conform to Evans' rule G (Evans 1976), requiring that experimental reproduction of the disease should occur in higher incidence in those exposed to the putative cause than in those not so exposed. However, a proportion of exposed fragments did not develop disease signs, demonstrating that the probability of becoming infected may not be equal among healthy colony fragments collected from the field, and that other host related factors potentially contribute to successful infections. Such unknown factors can be explored in future inoculation trials.

The presence of *Vibrio spp*. on both healthy and diseased corals has led to the conclusion by some authors (Bourne and Munn 2005, Ainsworth et al. 2007a) that *Vibrio* infections of corals may be opportunistic in nature. This assumption fits well into models of disease occurring in environmental settings, where multiple factors, such as host density (Bruno et al. 2007) and temperature (Colwell 1996) have been shown to influence the probability of successful infections. Combinations of virulent and a-virulent *Vibrio* strains are found readily in environmental samples (Stelma et al. 1992) with non-clinical *V. cholera* strains found to be capable of causing infections despite lacking the cholera toxin gene (Honda et al. 1989). Many *Vibrios* specialize in multiple host attachment and detachment (Nelson et al. 1976, Tamplin et al. 1990, Kirn et al. 2005), suggesting a broad scope for potential coral infections by *Vibrios* including possible host shifts due to fish depletion from coral reefs (Connolly et al. 2005). Amaro and Biosca (1996) have demonstrated that *Vibrio vulnificus* biotype 2 is both a primary pathogen for eels and an opportunistic pathogen for humans, indicating that the identification of opportunistic pathogens requires rigorous testing. Nevertheless, none of the claims to define *Vibrio* coral infections as opportunistic have so far provided conclusive evidence to show that suspects (identified by molecular screening methods) found on healthy corals are in fact pathogenic (whether opportunistic or not), or that only compromised hosts become infected. In addition, not all coral mortalities are caused by infectious agents, but rather by exposure to extreme conditions, such as pesticides or high nutrient levels (Negri et al. 2005, Kline et al. 2006), which may result in indirect shifts in microbial abundance. Infectious outbreaks can be distinguished from non-infectious ones by plotting infection curves (Thrusfield 2005) to demonstrate a bell-shape increase and decrease in incidence rate with time.

This study did not find evidence for the presence of coral pathogens on healthy corals in the field, nor evidence that exposed fragments might be successfully infected due to stress other than the direct exposure to the pathogens themselves. Control treatments in all inoculations remained healthy, including a proportion of those exposed to pathogens. Further studies are recommended to determine the prevalence of pathogens in field samples by developing diagnostic tools to target specific virulence genes in large scale screening efforts. These studies could then determine the proportion of exposed corals in the field that develop acute disease

signs and should become an integral part of establishing acute vs. chronic disease prevalence in environmental studies.

This is the first study to diagnose proteolytic activity as a possible component of the aetiology of WS through the screening of more than 150 isolates from both diseased and non-diseased corals. Zinc-metalloproteases have been characterized as virulence factors in many *Vibrio* family pathogens, such as *V. cholera* (Finkelstein and Hanne 1982), *V. vulnificus* (Miyoshi and Shinoda 1988), *V. harveyi* (Teo et al. 2003) and *V. anguillarum* (Denkin and Nelson 2004). *Vibrio* zinc-metalloproteases are involved in cleavage of connective tissue (Finkelstein et al. 1983), para-cellular perturbation (Wu et al. 1996), swarming and adhesion to mucus (Kim et al. 2007) and detachment (Finkelstein et al. 1992). The coral bleaching pathogens *V. shiloi* and *V. coralliilyticus* have been previously shown to harbour a zinc-metalloprotease (Ben-Haim et al. 1999, 2003a) along with other toxins that cause photosynthetic inhibition of coral *Symbiodinium* (Banin et al. 2001b). *Serratia marcescens*, the aetiological agent of acroporid serratiosis (coral White Pox disease; Patterson et al. 2002), resulting in acute tissue lesions, also possesses a virulent zinc-metalloprotease capable of connective tissue degradation (Kamata et al. 1985). However, it has been shown that both clinical and non-clinical strains possess zinc-metalloprotease genes (Booth and Finkelstein 1986), suggesting that it may not be the only virulence factor to cause successful infections. This study provided similar results, underlining the need to search for additional virulence factors in future studies.

Recent studies by Ainsworth et al. (2007b) did not detect bacteria associated with WS lesions of diseased corals sampled at Heron Island on the GBR, using direct microscopic techniques. In contrast, samples of WS corals obtained from Heron Island in this study for screening purposes demonstrated an abundance of *Vibrio* spp. isolates on WS lesions, including proposed putative pathogens that are proteolytically active and possess a zinc-metalloprotease gene. These contradicting findings underline the importance of 'comparative validation' (Dane 1990) in disease research and the need for standardized protocols for disease detection using better diagnostic tools.

Further histopathological studies by Ainsworth et al. (Ainsworth et al. 2007a, 2007b) utilizing commercial labelling kits have found that coral fragments displaying WS signs test positive for DNA fragmentation. These observations led to the hypothesis that WS is potentially the result of coral programmed cell death. However,

further proof is needed in order establish whether DNA fragmentation (or apoptosis) in corals is cause or effect. The induction of apoptosis by bacterial pathogens (*Salmonella Sp., E. coli, Shigella sp., C. difficile, L. monocytogenes, C. parvum* and others) has been previously demonstrated by many studies (Keenan et al. 1986, Fiorentini et al. 1998, Kim et al. 1998, Valenti et al. 1999, McCole et al. 2000), suggesting a possible link between bacterial infections and apoptosis. This link can be tested in future pathogen-exposure trials and used to design novel diagnostic protocols for WS, which would target bacterial enzymes causing DNA fragmentation.

In summary, this study demonstrated consistent results in applying cost effective culturing techniques combined with biochemical and molecular tools towards successful pathogen isolation, coral disease investigation and sample screening. Future research should be conducted to explore the virulence components of all six pathogens identified in this study and to test the contribution of multiple factors (pathogen, environment and host related) to the aetiology of WS. Enhanced monitoring and management of WS outbreaks will not only benefit coral health, but would also further validate results obtained in this study.

Chapter 4

Vibrio Zinc-metalloprotease causes photoinactivation of coral endosymbionts and coral tissue lesions

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4.1 Introduction

Of nine coral infectious diseases, whose pathogens have been characterized by fulfilling Henle-Koch's postulates (Koch 1891), six (including pathogens identified for Indo-Pacific WS's; previous chaper 3) are caused by agents from the family Vibrionacae (Kushmaro et al. 2001, Ben-Haim et al. 2003, Bally and Garrabou 2007, Sussman et al. 2008), adding to the many previously characterized *Vibrio* infections of shrimps (Goarant et al. 1998), clams (Paillard 2004) and fish (Egidius 1987), which date back to 1817 (Woo and Bruno 1999). Other coral disease signs in the Caribbean (Cervino et al. 2004a, Gil-Agudelo 2006) have also been associated with the presence of *Vibrio* agents. The study of coral disease signs in Zanzibar (Piskorska et al. 2007), bleached corals on the Great Barrier Reef (GBR; Bourne et al. 2008), black band disease signs on corals in the Gulf of Aquaba (the Red Sea; (Barneah et al. 2007) and even growth anomalies on Hawaiian corals (Breitbart et al. 2005) have all demonstrated significant correlation between disease signs and an elevated abundance of *Vibrio* strains. These newly emerging coral diseases, either caused or associated with members of the Vibrionacae family have sparked a debate on the origin of *Vibrio* pathogens and their role in the aetiology of coral diseases: Are *Vibrio* pathogens the primary causative agents of all these diseases, or are they opportunistic pathogens? Alternatively, are they secondary infections to other unknown causes? (Harvell et al. 2004, Bourne and Munn 2005, Smith et al. 2006, Lesser et al. 2007, Ainsworth et al. 2007, Rosenberg et al. 2007, 2008, Muller et al. 2008, Work et al. 2008)

In the previous chapter (Chapter 3; Sussman et al. 2008) we identified two novel *V. coralliilyticus* strains and four additional *Vibrio* pathogens as causative agents of three Indo-Pacific coral white syndromes (WS's). A link was demonstrated between WS disease signs on corals and the presence of *Vibrio* strains possessing a zinc-metalloprotease gene (Chapter 3; Sussman et al. 2008). Protein homologues of

this gene have been identified as key virulence factors of *Vibrio* pathogens of fish (Norqvist et al. 1990), shrimp (Aguirre-Guzmán et al. 2004), mollusks (Binesse et al. 2008) and humans (Crowther et al. 1987) acting to digest mucin and other connective tissue components, such as collagen IV (Miyoshi et al. 1998) and fibronectin (Finkelstein et al. 1983). These enzymes have also been shown to perturb paracellular barrier functions (Wu et al. 1996) and cause tissue necrosis (Milton et al. 1992) including pathogen detachment from epithelial mucus (Finkelstein et al. 1992). Ben-Haim et al. (2003a) suggested that *V. coralliilyticus*, the bleaching agent of the coral *Pocillopora damicornis*, expresses a *V. cholera*–like zinc-metalloprotease, which causes rapid photosystem II (PS II) inactivation of *Symbiodinium* endosymbionts. However, little is known about either the kinetics or the specificity of this reaction, and under which conditions it is likely to occur. Numerous studies have demonstrated that the zinc-metalloprotease gene is present in *Vibrio* pathogenic strains, but also in non-pathogenic strains (Booth and Finkelstein 1986, Sussman et al. 2008), suggesting that this gene may not be considered an essential virulence factor (Milton et al. 1992, Shao and Hor 2000).

In this chapter we test this hypothesis and the role of zinc-metalloprotease in the pathogenicity of coral WS's by developing two novel bioassays. *Symbiodinium* cells from four coral hosts at two locations on the GBR were isolated and grown in cultures (Z1-Z4; table 4.1) before being exposed in 96 well microtitre plates to bacterial supernatants derived from four coral pathogens (P1-P4; Table 4.2) that have been characterized in chapter 3 (Sussman et al. 2008) as the causative agents of coral WS's on Pacific reefs, *i.e.*, on the GBR (P1), in the Republic of the Marshall Islands (P2) and in Palau (P3-P4). In order to test the effects of pathogen supernatants on *Symbiodinium* cells living *in hospite*, a second bioassay is developed by rearing juveniles of *Acropora millepora* and infecting them with specific *Symbiodinium* isolates from clades C and D (Little et al. 2004).

Table 4.1: *Symbiodinium* cultures Z1-Z4

Culture	Clone names[1]	Isolated from	Location	Date	Genback Acc.[1]
Z1-1	***MAEQMI 12***	***Montipora aequituberculata***	**Nelly Bay**	**Jan. 2006**	**EU567151**
Z1-2	***MAEQMI 2***	***Montipora aequituberculata***	**Nelly Bay**	**Jan. 2006**	**EU567152**
Z2-1	***MAEQDR 38***	***Montipora aequituberculata***	**Davies Reef**	**Oct. 2005**	**EU567155**
Z2-2	***MAEQDR 37***	***Montipora aequituberculata***	**Davies Reef**	**Oct. 2005**	**EU567156**
Z2-2	***MAEQDR 2***	***Montipora aequituberculata***	**Davies Reef**	**Oct. 2005**	**EU567157**
Z3-1	**ATMI 21**	***Acropora tenius***	**Nelly Bay**	**Jan. 2006**	**EU567160**
Z3-2	**ATMI 23**	***Acropora tenius***	**Nelly Bay**	**Jan. 2006**	**EU567167**
Z3-3	**ATMI 48**	***Acropora tenius***	**Nelly Bay**	**Jan. 2006**	**EU567168**
Z4-1	**AMMI V6**	***Acropora millepora***	**Nelly Bay**	**Jan. 2006**	**EU567158**
Z4-2	**AMMI V24**	***Acropora millepora***	**Nelly Bay**	**Jan. 2006**	**EU567159**
Z4-3	**AMMI 18**	***Acropora millepora***	**Nelly Bay**	**Jan. 2006**	**EU567170**
Z4-4	**AMMI 12**	***Acropora millepora***	**Nelly Bay**	**Jan. 2006**	**EU567174**

[1] Sequences (~360bp) including the ITS-1 rRNA and its flanking regions were submitted to www.ncbi.nih.nlm.gov/Genbank and are identified by clone names and clone numbers.

Table 4.2 *Vibrio* white syndrome coral pathogens

	Isolated from	Location	Date	Genbank Acc.[1]	LMG Acc.[2]
P1	***Montipora aequituberculata***	**Nelly Bay, GBR**	**Sep. 2003**	**EU372917**	**LMG23696**
P2	***Acropora cytherea***	**Marshall Islands**	**Aug. 2004**	**EU372935**	**LMG23691**
P3	***Pachyseris speciosa***	**Nikko Bay, Palau**	**Feb. 2005**	**EU372934**	**LMG23695**
P4	***Pachyseris speciosa***	**Nikko Bay, Palau**	**Feb. 2005**	**EU372931**	**LMG23693**

[1] Near full-length 16S rRNA sequences (>1200 bp) were submitted to www.ncbi.nih.nlm.gov/Genbank

[2] Pathogen isolates were submitted to the public collection of BCCM/LMG at the Ghent University, Belgium under the following identifications: P1=MMS1, P2=RMI1, P3=MSP8, P4=MSP13.

To test PS II inactivation by pathogen supernatants, this study used an imaging pulse amplitude modulation (iPAM) fluorometer (Walz, Germany) to measure both dark adapted PS II quantum yields, $F_v/F_m = (F_m - F_o)/F_m$ (Havaux et al. 2003), and light adapted effective PS II quantum yields, $\triangle F/ F_m'$, which estimate *Symbiodinium* PS II activity in either a relaxed or active state, respectively (Genty et al. 1989, Ralph et al. 2005a, 2005b). Use of the iPAM system allowed up to 96 replicates per analysis of cultured *Symbiodinium* cells and up to 48 replicates per analysis of coral juveniles. From quantum yield values, PS II inactivation (I) was calculated as a proportion, where 1.0 represented 100% PS II inactivation following exposure to bacterial supernatants and four negative controls, including bacterial supernatants, whose proteolytic activity was inhibited by EDTA (Table 4.3).

Table 4.3 Bioassay of *Symbiodinium* cultures; treatment allocation

	1	2	3	4	5	6	7	8	9	10	11	12
	***Symbiodinium* culture**[1]				***Symbiodinium* culture**[1]				***Symbiodinium* culture**[1]			
A	**F2 dinoflagellate growth medium**											
B	**F2 growth medium + 50mM EDTA**[2]											
C	**Pathogen supernatant**											
D	**F2 dinoflagellate growth medium (same as A)**											
E	**Pathogen supernatant + 50mM EDTA**[2]											
F	**Pathogen supernatant (same as C)**											
G	**1:1 of F2 dinoflagellate growth medium + MB bacterial growth medium**											
H	**Pathogen supernatant + 50mM EDTA**[2] **(same as E)**											

[1] Each 96 well micro titre plate was loaded with equal aliquots from three *Symbiodinium* cultures (250 µL = 1 x 10^6 cells ml^{-1}). Treatments (250 µL per well) were added at experimental begin. Plates were rotated by 180º during the experiment in order to verify that PS II yield readings from the edges of the microtitre plates were identical to those obtained from its inner parts.

[2] Treatments with 50mM EDTA were incubated for 1 h at 30ºC before being used for exposure experiments. Treatments without EDTA were incubated under the same conditions (1 h, 30ºC).

4.2 Materials and Methods

4.2.1 Coral pathogens

Four coral pathogen strains (P1-P4; Table 4.2), identified in the previous chapter (Sussman et al. 2008) as causative agents for white syndrome diseases (WS's) affecting Indo-Pacific scleractinian corals by fulfilling Henle-Koch's postulates, were examined in this study. As highlighted previously, the 16SrRNA gene sequences of all four coral pathogen strains can be found in GenBank under accession numbers: EU372917,EU372931,EU372934,EU372935
(http://www.ncbi.nih.nlm.gov/Genebank). These isolates correspond to accession numbers LMG23691, LMG23693, LMG23695 and LMG23696, in the public culture collection of BCCM/LMG at Ghent University, Belgium (Table 4.2).

4.2.2 Growth curve and proteolytic activities of bacterial supernatants

Each of the four bacterial pathogens (P1-P4) was inoculated into a general heterotrophic bacterial medium, Marine Broth-2216 (Difco, USA) and grown to end logarithmic phase at 27ºC with shaking (150rpm). Tests performed to determine the optimal growth conditions for pathogens P1-P4, demonstrated that culture supernatants expressed the strongest proteolytic activity when incubated for 18 h to end logarithmic phase (Fig. 4.7A). Bacterial cell density was determined by colony forming unit counts (CFU; described in M&M chapter 3; Sussman et al. 2008) and by constructing a cell density calibration curve of absorbance (595nn) vs. CFU (Fig. 4.7B). Absorbance (595nm) of serial culture dilutions was measured in sterile microtitre 96 well plates (n=6) using a Wallac Victor 2 1420 multi label counter spectrophotometer (Perkin Elmer, USA). Bacterial supernatants used in exposure experiments were obtained by centrifugation (12,000 x g, 20 min, 4ºC) and serial filtration through 0.45µm and 0.22µm filters (Millipore, USA). These solutions were defined as bacterial supernatants P1-P4, and their protease activity was measured by the asocasein assay (Windle and Kelleher 1997) as proteolytic units (Denkin and Nelson 1999), where 1U = 1000 x (OD_{450} x CFU^{-1}) x 10^{9}. Protein concentrations in all bacterial supernatants (P1-P4) were determined by the Biorad protein assay (Biorad laboratories, USA). Bacterial supernatant aliquots were stored at -20ºC until used.

4.2.3 Inhibition by 1,10 Phenanthroline monohydrate (1,10 Pt) and phenyl methylsulfonyl fluoride (PMSF)

Inhibition of proteolytic activity by bacterial supernatants was tested by using three inhibitors: 1,10 Phenanthroline monohydrate (1,10 Pt),phenyl methylsulfonyl fluoride (PMSF) and EDTA. 1,10 Phenanthroline monohydrate (1, 10 Pt; SIGMA) was dissolved in DDW (Millipore). Pathogen supernatants were incubated for 1 h at 30ºC with 1,10 Pt in a final concentration of 5mM (Farrell and Crosa 1991). Proteolytic activity was measured by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999). PMSF (SIGMA), an alkaline serine protease inhibitor, was dissolved in ethanol and incubated for 1 h at 30ºC with pathogen supernatants in a final concentration of 5mM (Kuo-Kau et al. 1997). Following incubation, reactions were assayed for proteolytic activity by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999).

4.2.4 Inhibition of proteolytic activity by EDTA and reactivation with $ZnCl_2$

Bacterial supernatants (P1-P4) were exposed to treatments with four concentrations of EDTA (5mM, 10mm, 25mM and 50mM). Triplicate samples of each treatment were incubated for 1 h at 30ºC and then tested for proteolytic activity by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999). Control treatments included bacterial supernatant with no EDTA. Treatments of pathogen supernatants inhibited by adding 50mM EDTA and incubation (1h, 30ºC) were used as negative control treatments in all exposure experiments conducted in this study. The ability to reactivate the proteolytic activity of the P1 pathogen by adding divalent cations was tested by incubating P1 supernatant with 50mM EDTA (1 h at 30ºC) and adding five concentrations of $ZnCl_2$ (5mM, 10mM, 25mm, 50mM and 100mM). Samples were incubated for 1h at 30ºC and then tested for proteolytic activity by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999). Inhibition of pathogen P1 supernatant by EDTA reached 98% following exposure to 50mM EDTA. At lower concentrations, inhibition dropped to 87%, when P1 supernatant was exposed to a 5mM concentration and 91% and 96% when exposed to 10mM and 25mM, respectively. When 10mM $ZnCl_2$ were added to EDTA-inhibited samples, P1 supernatant recovered 77% of its proteolytic activity prior to EDTA inhibition. When treated with other $ZnCl_2$ concentrations, recovery was significantly lower, with 61% recovery following the addition of 5mM $ZnCl_2$ and 35% recovery following the

addition of 25mM $ZnCl_2$. Following the addition of higher $ZnCl_2$ concentrations (50mM and 100mM), no recovery was detected by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999), suggesting that the P1 harbors a zinc-metalloprotease that needs an optimal concentration of $ZnCl_2$ for activity. To test this, P1 supernatant was incubated with five concentration of $ZnCl_2$ (5mM, 10mM, 25mm, 50mM and 100mM) without prior inhibitory treatment with EDTA. Results demonstrated that the proteolytic activity of P1 supernatant was significantly affected by excess $ZnCl_2$ in all treatments, with a 95% loss of activity measured in the 10mM-100mM concentrations and 81% activity-loss when 5mM $ZnCl_2$ were added to the supernatant (data not shown). Similar inhibition was demonstrated when exposing the *H. pylori* zinc-metalloprotease to zinc concentrations above 1mM (Windle and Kelleher 1997). Other zinc peptides are also inhibited by excess zinc (competitive inhibition), which is proposed to result from the formation of zinc monohydroxide multidentate complexes which bridge the catalytic zinc to a side chain on the active site (Larsen and Auld 1991).

4.2.5 Isolation of *Symbiodinium* cultures from sampled corals

Colonies of *Montipora aequituberculata* (Z1), *Acropora tenius* (Z3) and *Acropora millepora* (Z4) were collected in sterile containers at Nelly Bay, Magnetic Island, GBR (S19 10' E 146 52'), an inshore fringing reef. Additional colonies of *Montipora aequituberculata* (Z2) were collected at Davies Reef, GBR (S18°81', E147°67'), a midshelf reef located less than 100 km away (Table 4.1). Coral tissue was removed by airbrush, centrifuged three times (3000 x g, 5 min) and resuspended in 0.22µm filtered SW (25ºC). Coral nematocysts were removed by two consecutive filtrations (20µm; Millipore, USA) using a vacuum pump.

4.2.6 *Symbiodinium* cultures

F2 dinoflagellate growth medium for *Symbiodinium* was prepared by modification of F2 and Erdschreiber media (Guilard and Ryther 1962). Briefly, seawater supplemented with 4mg l^{-1} Na_2HPO_4, 1g l^{-1} $NaNO_3$, 1ml l^{-1} from a x1000 concentrated A_5 + CO micronutrient solution (described in chapter 2; Sussman et al. 2006), 2.5 mg l^{-1} GeO_2, 80mg l^{-1} G-Penicillin, 80mg l^{-1} Streptomycin, 40mg l^{-1} Amphotericin, 0.4mg l^{-1} Thiamine-HCl, 2µg l^{-1} Biotin and 2µg l^{-1} Vitamin B_{12}

(cyanocobalamin). The growth medium was 0.22µm filtered and stored at 4ºC in the dark. Before use, 0.22µm filtration was repeated.

Symbiodinium cultures Z1-Z4 in F2 medium were inoculated into sterile 24 well plates (3ml per well), covered and sealed. Plates were incubated at 27ºC under 12h:12h light:dark irradiance (90 pmol photons m^{-2} s^{-1}). Cells were inspected daily and contaminated plates were discarded. Culture purity was enhanced by limiting dilution steps. Briefly, *Symbiodinium* cells were pipetted from wells into sterile 0.22µm filtered F2 medium in new 24 well plates and cultured for 7 days. This step was repeated for 4 months, during which samples were periodically obtained for molecular analysis (see 4.2.9) to ensure the taxonomic consistency of clades in each culture. Prior to experimental exposures, *Symbiodinium* cells were quantified (n=10) using a Neubauer haemocytometer and adjusted to one concentration ($1x10^6$ cells ml^{-1}) by adding F2 medium before transferring cultures into sterile 96 well microtitre plates (250 µL per well). An attempt was made to maintain the original *Symbiodinium* populations that were associated with the host coral at the time of isolation rather than to purify and maintain single axenic cultures (Santos et al. 2001), which would have less ecological relevance when tested for their susceptibility to pathogen supernatants. In order to confirm the taxonomic identity of *Symbiodinium* types in each culture, cloning of *Symbiodinium* DNA was performed at the time of isolation from corals and prior to using the incubated cultures for experimental procedures. Experiments exposing *Symbiodinium* cells to bacterial supernatants and controls were repeated twice to confirm the consistency of results. A full description of treatments is presented in table 4.3.

4.2.7 PS II dark adapted quantum yields (F_v/F_m) and PS II inactivation (I)

96 well microtitre plates containing *Symbiodinium* cells ($1x10^6$ cells ml^{-1}) were incubated in the dark (1 h) and centrifuged (5 min at 3000 x g). F2 medium was discarded and wells were loaded with treatment solutions. Plates were exposed in a Maxi imaging-pulse-amplitude-modulation (iPAM) fluorometer (Walz, Germany) to a saturation light pulse (Gain=1-2, Intensity=1-2, Saturation Pulse=7) at 5 min intervals and dark adapted PS II quantum yields were calculated by using the formula: Fv/Fm = (Fm – F_0) / Fm (Havaux et al. 2003), where Fm = maximal fluorescent yield, and F_0 = Dark fluorescent yield. From F_v/F_m values, PS II inactivation values (I) were calculated as proportions by using the formula: I (F_v/F_m) = (F_v/F_m at time 0 – F_v/F_m

at time n) / F_v/F_m at time 0**,** where 1.0 represented 100% PS II inactivation, following exposure to proteolytically-active and EDTA-inhibited bacterial supernatants and three additional controls (Table 4.3).

4.2.8 PS II effective light-adapted yields ($\triangle F/ F_m'$) and PS II inactivation (I)

The identical procedure for sample preparation before measurement of dark adapted yields was repeated before measuring effective light adapted yields. This step confirmed the results obtained from reading photosynthetic inactivation as a proportion of dark adapted yields. Some authors also consider it as a better estimate for photosynthetic function (Iglesias-Prieto et al. 2004), because quantum yields are measured when the cells are photosynthetically active. 96 well microtitre plates were prepared as described above. Each plate was dark adapted first and Fm, F1 and dark adapted quantum yields (Fv/Fm) were recorded at 5 min intervals for a period of 30 min, until consistent levels were obtained. Plates were then centrifuged as described above and returned to the imaging PAM chamber for initial light adapted measurements. An actinic light source of 90 pmol m^{-2} s^{-1} was switched on in the measuring chamber and cultures were exposed to a saturation light pulse at 5 min intervals for a period of 30 min until it was confirmed that readings of effective light adapted quantum yields were stable (Gain=1-2, Intensity=1-2, Saturation Pulse=7). Plates were then removed from the chamber and centrifuged. F2 medium was discarded from the plates and without further delay, plates were returned to the imaging PAM to be loaded with treatment solutions. Plates remained in the imaging PAM chamber under illumination for the entire duration of the experiment. The continuous measurement at 5 min intervals was preferred to the alternative of dark adapting the samples before each single light adapted reading, due to the nature of the experiment. Although photochemical quenching was not fully relaxed, this procedure allowed closer surveillance of the continuous effects of bacterial supernatant on PS II photosynthesis, as it might occur under environmentally relevant conditions, where corals are constantly exposed to light during the day and for longer periods during the summer compared to winter.

A similar protocol was used by Schreiber et al. (2006) to measure PS II photoinhibition caused by the toxic effects of diuron, suggesting that since quantum yields are calculated from the ratio of fluorescent values before (Ft) and after (Fm')

firing a constant saturation pulse, results are independent of signal amplitudes. According to Schreiber et al. (2006), 100 sec intervals between consequent saturation pulses (SP) were sufficient to allow complete reoxidation of Q_A and re-establishment of the original Ft levels. Light adapted effective quantum yields ($\triangle F/ F_m'$) were calculated by the formula: (Fm'-Ft) / Fm' (Genty 1989), where Fm' = maximal fluorescent yield under light conditions and Ft = fluorescence before a saturating pulse. PS II inactivation (I) was calculated (as a proportion) from light adapted effective quantum yields ($\triangle F/ F_m'$) as described above. An alternative method for calculating PS II inactivation by comparing PS II quantum yields of treatments with PS II quantum yields of negative controls at corresponding times (Schreiber et al. 2006) was tested and provided similar results.

4.2.9 Taxonomic identities of *Symbiodinium* cultures

DNA was extracted from *Symbiodinium* cultures incubated at 27ºC or directly from corals (Wilson et al. 2002) and amplified using primers targeting the ribosomal RNA (rRNA) internal transcribed spacer 1 region (ITS-1; (van Oppen et al. 2001). PCR products were cloned (pCR 2.1 TOPO kit, Invitrogen, CA) and inserts containing plasmid DNA were amplified with a 5'- tet fluorescently labelled ITS-1 forward primer and then screened on a single strand conformation polymorphism (SSCP) gel before sequencing (Ulstrup and van-Oppen 2003). Retrieved nucleotide sequences (~360 bp) including the ITS-1 rRNA and its flanking regions were edited using Chromas Lite software version 2.01 (Technelysium) and aligned using ClustalX version 1.83 (Thompson et al. 1994). Distance matrices were calculated using the DNADIST program in PHYLIP (Felsenstein 1989) and phylogenetic trees were generated from distance matrices using the neighbour-joining method (Saitou and Nei 1987) and Kimura substitution algorithm (Kimura 1983). Bootstrapping with 1000 replicates was performed using SeqBoot as integrated in PHYLIP (Retief 2000) and values ≥50% were included for main nodes of the tree. Ribosomal RNA sequences of *Symbiodinium microadriaticum* amplified with the ITS-1 primers and cloned were submitted to Genbank (http://ncbi.nih.nlm.gov/Genbank) under the accession numbers EU567151-567152, EU567155-567160, EU567167-567168, EU567170, EU567174 (Table 1). Reference *Symbiodinium* types for phylogenetic analyses were obtained

from the following authors: AJ311944 (Pochon et al. 2001), AF380532, AF380537, AF380543, AF380546 (van-Oppen et al. 2001), DQ238587 (Santiago-Vazquez et al. 2007), AY457958 (Fabricius et al. 2004), AF334660 (LaJeunesse 2001), AF396629 (Santos et al. 2003), EF455526, EF455528 (Magalon et al. 2007), out group *Heterocapsa sp*. FK6-D47 AB084097 (Yoshida et al. 2003).

4.2.10 Experimental coral juveniles

Rearing coral juveniles (*Acropora millepora*) and infecting them with *Symbiodinium* clades D and C1 was performed following the protocol of Little et al. (2004). Gametes were collected following spawning of eight (hermaphroditic) colonies of *Acropora millepora*, during the spawning event in October, and mixed together for fertilization. Fertilized larvae were raised in filtered seawater in two separate 500 L tanks in a temperature controlled (27° C) aquaria room. When the larvae were elongated and searching for settlement sites (four days after spawning), preconditioned and autoclaved terracotta tiles (preconditioned for six weeks at Magnetic Island, GBR) were placed on the bottom of the two 500 L tanks to provide settlement surfaces. *Symbiodinium* clade strains D and C1 were obtained from adult *A. millepora* and *A. tenuis* colonies from Magnetic Island, respectively, by airbrushing the coral tissue and isolating *Symbiodinium* cells out of the coral-*Symbiodinium* slurry by centrifugation (5min. 3500 x g). These *Symbiodinium* cells were offered at 10^8 cells $tank^{-1}$ three and five days after spawning and were acquired by newly settled juveniles. The terra-cotta tiles were attached to a fringing reef (Nelly Bay, Magnetic Island, GBR) in a zone where *Acropora spp.* were present and were then collected nine months after settlement (in July) for experimental exposure. Tile racks were removed from the reef and carried in large containers to the outdoor aquaria at the Australian Institute of Marine Science (AIMS). Individual tiles were separated and placed to recover in outdoor tanks with a flow-through seawater system. Following five days, individual juveniles were scraped from the tiles and placed in sterile 48 well plates. These plates were further suspended in flow-through tanks for recovery until juveniles attached to the bottom surface of the wells. In this setup, juveniles experimentally infected with either *Symbiodinium* D or C1 cultures originated from crosses involving the same parent corals, thereby minimizing potential host genetic differences that may influence the physiology of the holobiont (host-symbiont partnership). The rearing and infection of coral juveniles used in this chapter was

performed by PhD student Jos Mieog at the Australian Institute if Marine Sience (AIMS), as part of a collaborative effort.

Individual *A. millepora* juveniles infected with *Symbiodinium* clades D and C1 were placed in 48 well plates and exposed to the following treatments (n=4): 1. F2 dinoflagellate medium; 2. P1 supernatant diluted 1:1 with sterile seawater; 3. P3 supernatant diluted 1:1 with sterile seawater; 4. P1 supernatant diluted 1:1 with sterile F2 medium, treated with 50mM EDTA and incubated for 1 h at 30ºC; 5. P3 supernatant diluted 1:1 with sterile F2 medium, treated with 50mM EDTA and incubated for 1 h at 30ºC; 6. Bacterial medium (LB) mixed 1:1 with dinoflagellate medium (F2). All EDTA and non-EDTA treatments were incubated for 1 h at 30ºC prior to use. Plates were acclimatized for five days prior to exposure. Measurements and calculation of PS II dark and light adapted quantum yields and PS II inactivation were performed as described above. For measurements of PS II effective light adapted quantum yields ($\triangle F/ F_{m'}$), an actinic light source of 5 pmol m^{-2} s^{-1}, identical to light intensity in the field, was switched on in the measuring chamber of the imaging PAM. Well plates containing *A. millepora* juveniles identical to those exposed to pathogen supernatants and controls under the imaging PAM were exposed and photographed under identical conditions (5-pmol m^{-2} s^{-1}, 27ºC) at 30 min intervals using a dissecting microscope (x 1.6) and a digital camera.

4.2.11 Pathogen concentration experiment

Symbiodinium cultures (Z1 and Z4) were prepared as described above. Pathogen supernatant concentrations were prepared by diluting 0.22µm filtered P1 supernatant with modified F2 medium to end concentrations of 50%, 25%, 10%, 5%, 1% and 0.1% from original stock. Effective light adapted quantum yield ($\triangle F/ F_{m'}$) was measured under illumination as described above and PS II inactivation (I) was calculated. I of *Symbiodinium* culture Z1 and concentrations of P1, as proportions of 1.0, were plotted resulting in a parabolic curve. Reciprocating data for Z1 PS II inactivation (I) and P1 concentrations resulted in a Lineweaver-Burk (1934) - 'like' linear plot, commonly used to describe the relation between substrate concentration (S) and reaction velocity (V). The term 'like' is used in this study, since neither the substrate for bacterial supernatants nor the products of their catalytic activity were determined. It was thus assumed that both supernatant dose and PS II inactivation (I)

values are good estimates of S and V. The linear equation (y=ax+b) was used to determine 1/km', when y = 0 and, with km' defined as the concentration of P1 needed to cause a 50% PS II inactivation (I) of the susceptible *Symbiodinium* culture Z1 within 10 min following exposure.

4.2.12 PS-II photoinactivation as a function of temperature

For this experiment to be of environmental relevance, both bacterial pathogens and *Symbiodinium* cultures were required to be incubated and then mixed at the same temperatures. P1 was inoculated into MB media and grown to end logarithmic phase at 5 different temperatures (20ºC, 25ºC, 27ºC, 30ºC and 35ºC), which cover the range of seawater temperatures typical of sites where WS outbreaks occur. Pathogen supernatant P1 was derived from culture as previously described. *Symbiodinium* cultures Z1 and Z4 were acclimatized to the corresponding temperatures over a period of 5 days. Exposure of *Symbiodinium* cultures to pathogen supernatant P1 was conducted in a temperature controlled room and effective light adapted quantum yields were measured using the Maxi Imaging PAM. PS-II photoinactivation (I) was calculated as described previously. The following parameters were quantified prior to the commencement of the experiment in order to determine the direct effect of temperature on its components (bacteria and *Symbiodinium)*:

1. Bacterial cell density, reflecting pathogen P1 growth at 5 different temperatures. Cell density was quantified by plating serial dilutions of triplicate samples and by spectrophotometry (as described previously).
2. The relative level of proteolytic activities in bacterial supernatants filtered from cultures that were incubated at the various temperatures, as determined by the asocasein assay.
3. The physical state of *Symbiodinium* cells in cultures acclimatized to the five different temperatures prior to their exposure to bacterial supernatant, determined by measuring dark adapted maximum quantum yields (F_v/F_m), light adapted effective quantum yields ($\triangle F/ F_{m'}$) and calculating excitation pressure (Q) over photosystem II by the formula: $Q = 1- (\Delta F/Fm')/(Fv/Fm)$, when $\Delta F/Fm'$ is the light dependent reduction of the effective quantum yield of photosystem II (Iglesias-Prieto 2004).

Cultures of *Symbiodinium* Z1 ($1x10^6$ cells ml^{-1}) were exposed in 96 well micro-titre plates to 7 treatments (n=12 wells per treatment) consisting of four concentrations of P1 bacterial supernatant and three controls: P1 supernatant incubated with 50mM EDTA (1h 30ºC), *Symbiodinium* cultures in dinoflagellate growth medium (F2) and in a 1:1 mix of bacterial growth medium (LB) and dinoflagellate growth medium (F2). Pathogen P1 supernatant concentrations were prepared by diluting 0.22µm filtered P1 supernatant with modified F2 medium to concentrations of 100%, 50%, 10% and 1%. Each experiment was performed at each of the five selected temperatures (20ºC, 25ºC, 27ºC, 30ºC and 35ºC; 1 plate per temperature, n=2 repetitions), deriving five Lineweaver-Burk "like" plots, which demonstrated a gradient in enzymatic kinetics as a function of temperature. *Km'*, the apparent concentration needed to achieve a 50% photoinactivation of Z1 by P1 supernatant following a 10 min exposure was calculated from the linear regression equations obtained for the five temperatures.

4.2.13 Exposure of *Symbiodinium* cultures to 35ºC

96 well micro titre plates containing *Symbiodinium* cultures Z1 and Z4 ($1x10^6$ cells ml^{-1}) were incubated at 35ºC under a 12h:12h light:dark irradiance (90 pmol photons m^{-2} s^{-1}) for a period of 25 days to test *Symbiodinium* ability to withstand elevated temperatures. F2 media in wells was replaced every five days with fresh media (35ºC) and PS-II performance was estimated by measuring both dark adapted maximum quantum yields (F_v/F_m) and effective light adapted quantum yields ($\triangle F/F_m'$) at five day intervals (as described above). Following this experiment, sub-aliquots from each *Symbiodinium* culture were cloned (as described above) in order to determine whether a shift in the *Symbiodinium* populations in each culture had occurred following exposure to elevated temperatures (35ºC).

4.2.14 Temperature data logger Information

Seawater temperature data were obtained from *In situ* data loggers deployed on reefs. The Australian Institute of Marine Science (AIMS) and the Great Barrier Reef Marine Park Authority (GBRMPA) maintain the SeaTemps monitoring program, which is coordinated by Dr Ray Berkelmans (r.berkelmans@aims.gov.au). Data loggers instantaneously record sea temperatures every 30 minutes and are exchanged

and downloaded approximately every 12 months. Temperature loggers on the reef-flat are placed just below Lowest Astronomical Tide (LTD) level. They are also deployed on the upper reef slope (in depths of 5–9 m) and in deep reef-slopes (in depths of ~20 m). Loggers are calibrated against a certified reference thermometer after each deployment and are generally accurate to 0.1-0.2ºC. Mean Daily Seawater Temperature (MDST) was calculated by averaging data for all loggers at each site per one day. 38 data logger sites from which seawater temperatures have been recorded for at least five years (including the two major bleaching events on the GBR in the austral summers of 1997/8 and 2001/2), were selected and the number of days in which the mean daily seawater temperature (MDST) equalled or exceeded 27ºC and remained below or equalled 30ºC was calculated for each station per year, beginning in the austral winter (1 June-31 May). This temperature span (27ºC ≥X≤ 30ºC) corresponded best with the optimal temperature range suggested by this study for bacterial zinc-metalloprotease activity causing *Symbiodinium* photoinactivation. Annual seawater temperature data (number of days per year in which 27ºC ≥ MDST ≤ 30ºC) was analysed from 9 data-logger reef sites: Low Isles (S16º20', E145º30'), Myrmidon Reef (S18°26, E147°38'), Kelso reef (S18°45', E146°99'), Cattle Bay (S18°57', E146°48'), Davies Reef (S18°81', E147°67'), Geoffrey Bay, Magnetic Island (S19°16', 146°86'), Nelly Bay, Magnetic Island (S19°17', E146°85'), Turner Cay I (S21°7', E152°56') and Halfway Reef (S23°2', 150°97').

The daily span of sunlight (in min per day) was obtained for the city of Townsville, which corresponds to the latitude of Davies Reef.

4.2.15 Protein sequence retrieval

Bacterial cultures P1-P4 were grown (1.8 L) and crude extracts were derived by ammonium sulphate precipitation (Wu et al. 1996). Proteins in a total volume of 100 ml of supernatant derived from each of four pathogen cultures were precipitated by slowly adding ammonium sulphate to achieve the final % saturations of 20%, 40%, 60%, 80% and 100%. This procedure was undertaken to determine the optimal ammonium sulphate concentration needed to precipitate bacterial proteolytic enzymes from their corresponding supernatants without compromising proteolytic activity. Samples were stirred for 2 h and then centrifuged at 5000 x g for 1 h at 4ºC. Supernatants were discarded and the remaining precipitate was diluted by adding

200µl of 0.22µm-filtered phosphate buffered saline (PBS: 10mM Potassium Phosphate 150mM NaCl pH7.4). Samples were tested for proteolytic activity by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999) and the optimum % saturation of ammonium sulphate was chosen for the production of higher concentrations of active crude extracts from all pathogen supernatants. Pathogens P1-P4 measured the highest proteolytic activity by the asocasein assay when precipitated in a 20% saturation of ammonium sulphate.

For the production of proteolytic active crude extracts, 1.8 L volumes of bacterial cultures were incubated and crude extracts, diluted in 45ml 0.22µm-filtered PBS (7.4 pH), were derived (as described above). Each sample was then ultra-filtered using three Amicon 5,000M MWCO ultra centrifugal filter devices (Millipore, USA). Samples were centrifuged at 4000 x g in a swinging bucket Alegra X-15R centrifuge (Beckman Coulter, CA) at 4ºC for 45 min to derive a total final crude extract volume of 600µL from each pathogen supernatant, corresponding to a x3000 concentration of the original pathogen cultures. 1µL of each sample was then diluted in 99µL of 0.22µm-filtered DDW (Millipore, USA) and tested to confirm proteolytic activity by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999). Protein concentrations were measured with a protein assay kit from Bio-Rad and samples were frozen at -80ºC and sent to the Australian Proteome Analysis Facility (Macquarie University NSW, Australia) for fast protein liquid chromatography (FPLC) analysis. The samples were run on a Superdex 75 10/300 GL Column and the buffer used was PBS (pH7.4). The column was tested with low molecular weight standards (GE Biosciences) before the sample was injected (Albumin 67kDa, Ovalbumin 43kDa, Chymotrypsinogen A 25kDa and Ribonuclease A 13.7 kDa). The samples were prepared by filtering through a 0.45µm cellulose acetate microcentrifuge filter system unit at 10,000 x g for 1 min. The method flow rate was 0.5ml for 70 min and fraction size was 400uL (48 seconds). The sample was run on a Waters Alliance HT 2795 system with a PDA detector and Gilson FC204 fraction collector. 10µL from all 72 fractions from each sample were assayed for proteolytic activity by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999) and results were superimposed on the 280nm chromatograms generated for each sample for fraction selection. Selected samples were run on zymogen gels containing 0.1% Na-casein co-polymerized in gels as substrate revealing zones of hydrolysis. 15 active fractions from all four pathogens were re-run on a 12% SDS-PAGE following the method by

Laemmli (Laemmli 1970). 15 Bands were excised using a sterile blade and sent to the Australian Proteome Analysis Facility for nano-liquid chromatography peptide separation and mass spectrometry. Gel plugs were de-stained followed by tryptic digestion for 16h at 37ºC. Digested peptides were separated by nano-LC using a CapLC system (Agilent 1100 Series, Agilent Technologies, Germany). Sample (39µL) were injected onto a peptide trap (Michrome peptide Captrap) for pre-concentration and desalted with 0.1% formic acid at 10µL/min. The peptide trap was then switched into line with the analytical column containing C18 RP silica (SGE ProteCol C18, 300A, 3µm, 150µm x 10 cm). Peptides were eluted from the column using a linear solvent gradient, with steps, from H_2O:CH_3CN (95:5; + 0.1% formic acid) to H_2O:CH_3CN (20:80, + 0.1% formic acid) at 500 $nLmin^{-1}$ over a 45 min period. The LC eluent was subject to positive ion nano flow electrospray analysis on an Applied Biosystems QSTAR XL mass spectrometer (ABI, CA, USA). The QSTAR was operated in an information dependant acquisition mode (IDA). In IDA mode a TOFMS survey scan was acquired (m/z 400-2000, 1.0s), with the four largest multiple charged ions (counts >25) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 1 s (m/z 50-2000).

LC/MS/MS data were searched using Mascot (Matrix Science, London, UK) and bacterial entries in the NCBI non-redundant protein database (Altschul et al. 1997).

4.2.16 Statistical Analysis

Means and standard errors (SE) for bacterial colony forming unit (CFU) counts, for absorption readings (bacterial cell density and proteolytic activity), for PS II dark adapted quantum yields and light adapted effective quantum yields were compared among treatments using One-Way ANOVA (Statistica, StatSoft, Inc. USA). CFU counts are presented in this study using logarithmic scales. Means and standard errors (SE) for PS II inactivation (as a proportion of 1.0) in all exposure experiments (treatments and controls) were compared using multivariate repeated measures MANOVA (Statistica, StatSoft, Inc. USA), which does not rest on the assumption of sphericity and compound symetry (Huynh and Feldt 1970). Four multivariate tests of significance were applied (*Wilks' Lambda*, *Pillai-Bartlett Trace*, *Hotelling-Lawley Trace*, and *Roy's Largest Root)* with non-significant results used to overrule any previous assumptions of statistical significance. Significant results were determined when $\alpha \leq 0.05$.

4.3 Results

4.3.1 *Symbiodinium* culture Z1 is most susceptible to bacterial PS II inactivation

Symbiodinium culture Z1 isolated from the WS susceptible coral host *Montipora aequituberculata* at Nelly Bay, an inshore reef off Magnetic Island in the central GBR, was the most severely affected of the four *Symbiodinium* cultures tested when exposed to P1 supernatant under illumination ($p<0.01$; Fig. 4.1A). For *Symbiodinium* culture Z1, inactivation (I) of PS II (measured as light adapted quantum yields) was greater than 95% (mean I (Z1 $\triangle F/ F_m'$) = 0.968 ± 0.016) following exposure to P1 supernatant for 10 min in two independent experiments, and total PS II inactivation resulted after 20 min (Fig 4.1A). A significant (~40%; $p<0.0001$) difference in mean I was measured between this culture from Nelly Bay and culture Z2 isolated from the same coral species found at Davies Reef, a GBR midshelf reef located less than a 100 km away, where no signs of WS on *M. aequituberculata* have been observed [mean I (Z2 $\triangle F/ F_m'$) = 0.587 ± 0.021 following exposure to P1 supernatant for 10 min in two independent experiments]. The impact of P1 on culture Z3, which was isolated from the coral *Acropora tenuis* at Nelly Bay, where it has not been observed with signs of WS, was similar to its impact on *Symbiodinium* culture Z2 throughout the experiment ($p=0.426$). *Symbiodinium* culture Z4 isolated from the coral *Acropora millepora* at Nelly Bay, where it has not been observed with WS signs at this site, was the least affected (~3%; $p<0.01$) of all *Symbiodinium* cultures tested in this study [mean I (Z4 $\triangle F/ F_m'$) = 0.034 ± 0.019 following exposure to P1 supernatant for 10 min in two independent experiments]. Control treatments with dinoflagellate growth medium F2 (Fig. 4.1A) and bacterial growth medium (MB) resulted in limited or no PS II inactivation of the respective cultures [mean I (F2 $\triangle F/ F_m'$) = 0.001 ± 0.001; $p<0.01$, and mean I (MB $\triangle F/ F_m'$) = 0 following exposure for 10 min in two independent experiments].

Cloning and sequencing the ribosomal RNA (rRNA) internal transcribed spacer 1 (ITS-1) region of *Symbiodinium* from cultures Z1-Z4 identified Z1 and Z2 as two distinct types phylogenetically affiliated with *Symbiodinium* clade A (Fig. 4.2). Culture Z3 was phylogentically affiliated with *Symbiodinium* clade C, and Z4 contained two *Symbiodinium* types affiliated with *Symbiodinium* clades A and D (Fig 4.3).

4.3.2 Pathogen supernatants have a similar effect on *Symbiodinium* culture Z1

Exposure of the susceptible *Symbiodinium* culture Z1 to supernatants from pathogens P1-P4, resulted in total PS II inactivation (I) in all treatments after 20 minutes (Fig 4.1B; p=0.794). Comparisons of mean I among 16 pathogen-*Symbiodinium* culture combinations (P1-P4 and Z1-Z4) resulted in values ranging between 0 and 1.0 (Table 4.4).

Table 4.4 PS II inactivation (I) of *Symbiodinium* cultures Z1-Z4 by pathogen supernatants P1-P4

I; $\triangle F/ F_m' / 10min$[1]	P1[2]	P2[2]	P3[2]	P4[2]
Z1[3]	0.968 ± 0.016	1.0 ± 0	0.962 ± 0.012	1.0 ± 0
Z2[3]	0.587 ± 0.123	0.485 ± 0.048	0.027 ± 0.027	0.707 ± 0.042
Z3[3]	0.659 ± 0.021	0.763 ± 0.013	0.482 ± 0.021	0.675 ± 0.027
Z4[3]	0.034 ± 0.019	0.297 ± 0.036	0.011 ± 0.011	0 ± 0

[1] Mean PS II inactivation (I; $\triangle F/ F_m' \pm$ SE; n = 8) was calculated from light adapted effective quantum yields following 10 min of exposure to pathogen supernatants.

[2] Pathogen supernatants were obtained by growing pathogen cultures to end logarithmic phase (18 h, 27ºC) with shaking (150rpm).

[3] Wells in 96 microtitre plates were inoculated with 1 x 10^6 cells ml^{-1} of *Symbiodinium* cultures.

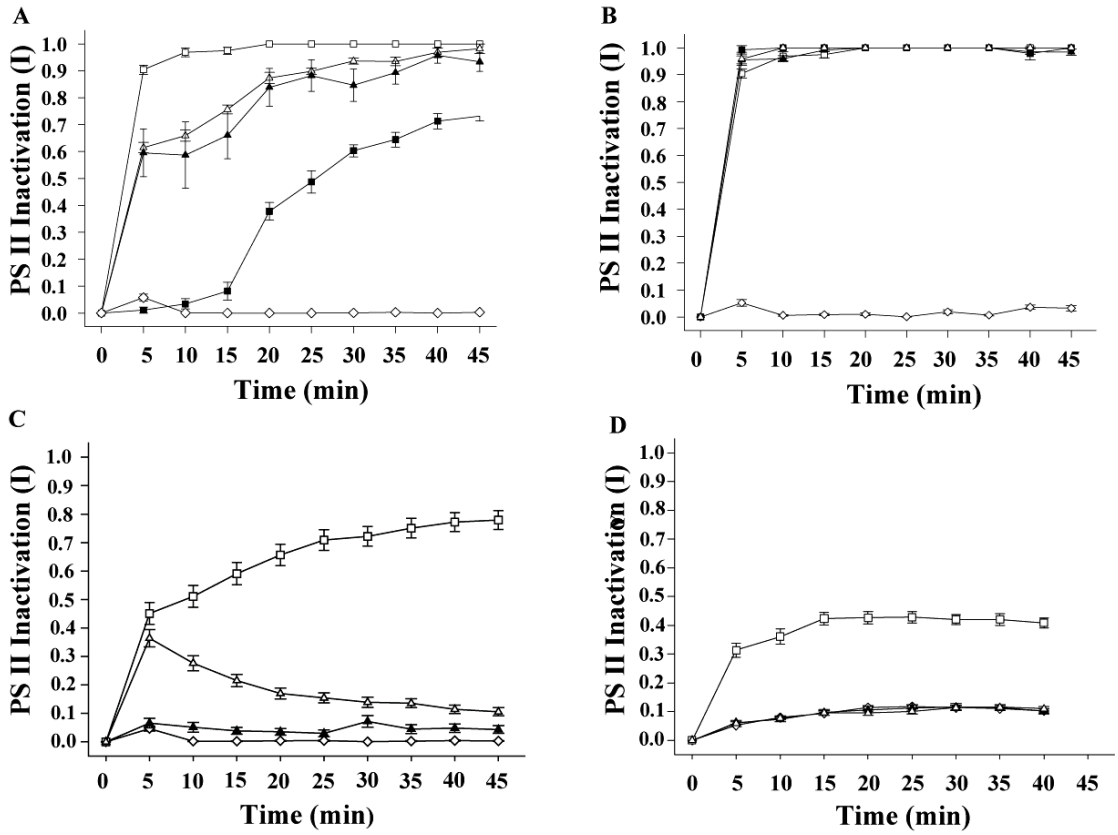

Figure 4.1 PS II inactivation of *Symbiodinium* cultures by bacterial supernatants

A. PS II inactivation **(**I; △F/ F_m') by P1 supernatant of *Symbiodinium* cultures Z1 □, Z2 ▲, Z3 △, Z4 ■ and pooled data for Z1-Z4 cultures exposed to dinoflagellate growth medium (F2) ◇. **B.** PS II inactivation **(**I; △F/ F_m') of *Symbiodinium* culture Z1 exposed to pathogen supernatants P1 □, P2 ■, P3 ▲, P4 △ and *Symbiodinium* culture Z1 exposed to dinoflagellate growth medium (F2) ◇ **C.** Pooled data for PS II inactivation (I; △F/ F_m') of *Symbiodinium* cultures Z1-Z4 exposed to: pathogen supernatants P1-P4 □, Pathogen supernatants P1-P4 inhibited by incubation with 50mM EDTA for 1h at 30°C △, a 1:1 mix of bacterial growth medium (MB) and dinoflagellate growth medium F2 ◇, Dinoflagellate growth medium (F2) ▲. **D.** Pooled data for PS II inactivation (I; F_v/F_m) of *Symbiodinium* cultures Z1-Z4 exposed to: pathogen supernatants P1-P4 □, Pathogen supernatants P1-P4 inhibited by incubation with 50mM EDTA for 1h at 30°C △, 1:1 mix of bacterial growth medium (MB) and dinoflagellate growth medium F2 ◇, Dinoflagellate growth medium (F2) ▲. 96 microtitre plates were loaded with 2.5 x 10^5 *Symbiodinium* cells well $^{-1}$. I; △F/ F_m' was based on measurements of effective light adapted quantum yields. I; F_v/F_m was based on measurements of dark adapted quantum yields. Bars = standard errors. n = 8 measurements per treatment.

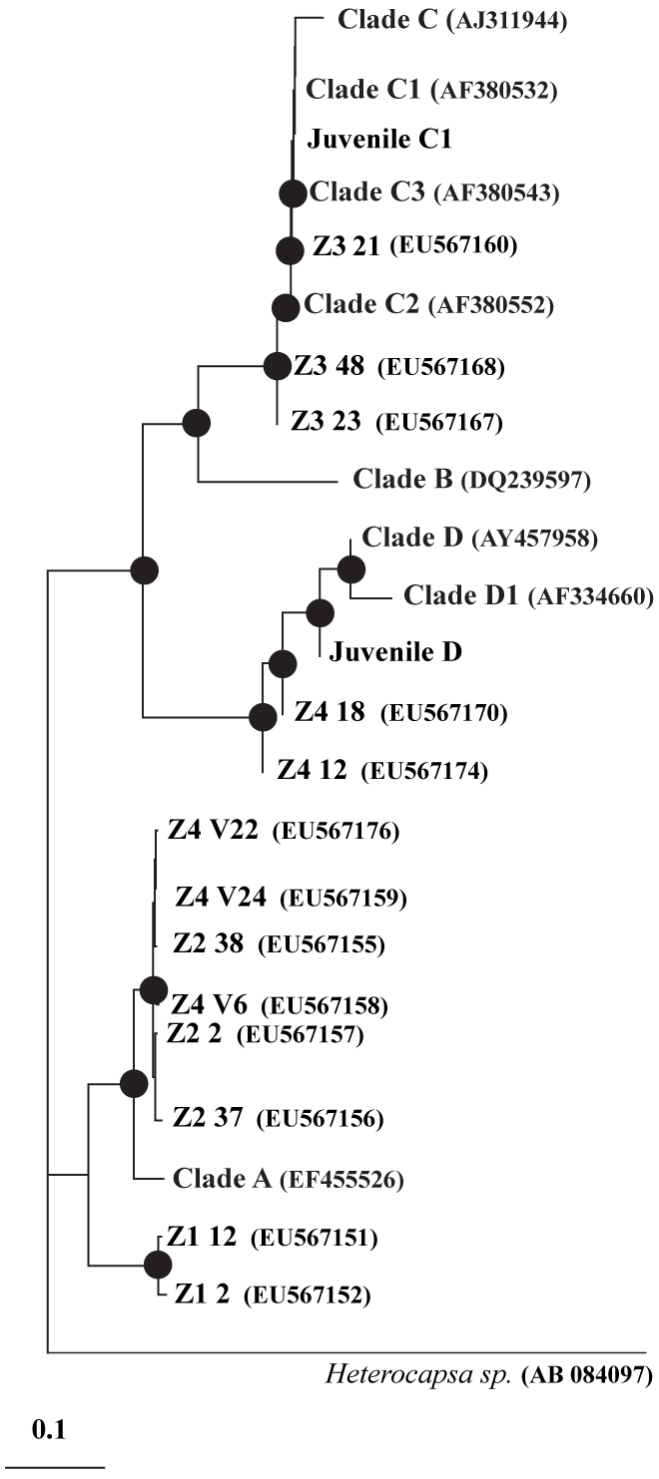

Figure 4.2 Neighbour joining phylogenetic tree of *Symbiodinium* cultures Z1-Z4
Symbiodinium sequences obtained via cloning of PCR products are presented by culture name (*i.e.*, Z1-Z4) followed by clone number and Genbank accession number (in brackets). Clones obtained from *Symbiodinium* cultures used to infect coral juveniles appear as Juvenile C1 and Juvenile D. Reference types representing *Symbiodinium* clades were obtained from authors listed in M&M. Bootstrapping with 1000 replicates was performed and values ≥50% were included for main nodes of the tree.

4.3.3 Pathogen proteolytic activity is inhibited by EDTA and reactivated by $ZnCl_2$

EDTA was the most potent inhibitor of proteolytic activity of bacterial supernatants P1-P4 when tested by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999) incorporating three standard protease inhibitors (EDTA, 1,10 Pt and PMSF; Fig. 4.3A). Proteolytic activity was reduced by 98% with the addition of 50mM EDTA to the supernatant of pathogen P1 (1h incubation at 30ºC; Fig. 4.3B) and by ~80%, on average, for pathogens P1-P4 (Fig. 4.3A). Addition of 10mM $ZnCl_2$ reversed the chelating effect of EDTA and reactivated the proteolytic activity of P1 supernatant to 77% of its original capacity (Fig 4.3C). This result combined with results from the screenings of the previous chapter (Chapter 3 section 3.3.4.3; Sussman et al. 2008), which detected the active zinc binding site of a metalloprotease using specific primers targeting the DNA in all pathogens (P1-P4), confirmed the presence of a zinc-metalloprotease. Following the addition of higher $ZnCl_2$ concentrations (50mM and 100mM), no recovery was detected by the asocasein assay, suggesting that the P1 zinc-metalloprotease requires an optimal concentration of $ZnCl_2$ for activity [for more information see M&M 4.2.4]. Other divalent cations ($NiCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$, $CuCl_2$, $HgCl_2$ and $FeCl_2$) failed to reactivate the P1 zinc-metalloprotease following inhibition by EDTA (data not shown).

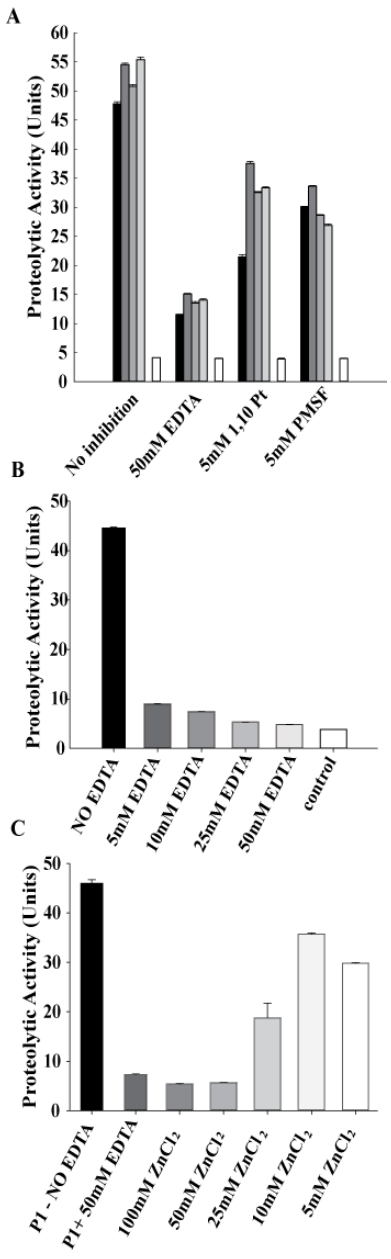

Figure 4.3 Inhibition of proteolytic activity of *Vibrio* pathogens
A. Mean proteolytic activity of pathogens P1-P4 supernatants determined by the asocasein assay and inhibited by 50mM EDTA, 5mM 1, 10 Pt and 5mM PMSF. Pathogen supernatant P1 ■; pathogen supernatant P2 ■; pathogen supernatant P3 ■; pathogen supernatant P4 ■. **B.** Mean proteolytic activity of pathogen P1 supernatant (Units) determined by the asocasein assay ■, and inhibited by incubation of P1 supernatants (1 h, 30ºC) in concentrations of EDTA: 5mM EDTA ■; 10mM EDTA ■; 25mM EDTA ■; 50mM EDTA. Negative control treatments were prepared by boiling *E. coli* supernatants (15 min 100ºC). **C.** Effect of $ZnCl_2$ on restoring the proteolytic activity of P1 supernatants inhibited by incubation (1 h, 30ºC) with 50mM EDTA. Mean proteolytic activity of P1 supernatant (Units) ■; Mean proteolytic activity of P1 supernatant incubated with 50mM EDTA (1 h, 30ºC) ■ and 100mM $ZnCl_2$ ■; 50mM $ZnCl_2$ ■; 25mM $ZnCl_2$ ■; 10mM $ZnCl_2$ ■; 5mM $ZnCl_2$ □**.** Negative control treatments were prepared by boiling *E. coli* supernatant (15 min, 100ºC) □**.** Bars = standard errors. n = 6 measurements per treatment.

4.3.4 *Symbiodinium* PS II inactivation by pathogen supernatants is inhibited by EDTA

Limited PS II inactivation was observed in all *Symbiodinium* cultures after 45 min when bacterial supernatant P1 was incubated with 50mM EDTA (Fig. 4.1C). This was in contrast to strong PS II inactivation when all cultures were exposed to non-chelated supernatants ($p<0.01$) [mean I (Z1, P1 EDTA, $\triangle F/ F_m'$) = 0.119 ± 0.017, mean I (Z2, P1 EDTA, $\triangle F/ F_m'$) = 0, mean I (Z3, P1 EDTA, $\triangle F/ F_m'$) = 0.267 ± 0.015 and mean I (Z4, P1 EDTA, $\triangle F/ F_m'$) = 0]. The EDTA inhibition of proteolytic activity was not significantly different among the four pathogen supernatants tested (P1-P4; $p=0.566$), supporting the hypothesis that they share a common virulence mechanism. Pooling all I ($\triangle F/ F_m'$) data for *Symbiodinium* cultures (Z1-Z4) exposed to four pathogen supernatants (P1-P4) clearly demonstrated that PS II inactivation (I) is caused by bacterial supernatants but was absent in controls in 16 experiments (Fig 4.1C; $p<0.001$). Addition of 50mM EDTA to pathogen supernatants resulted in significantly lower PS II inactivation ($p<0.01$). PS II inactivation was not eliminated completely, as shown by levels of I approaching non-EDTA treated supernatants in the first 5 min following exposure (Fig 4.1C). However, I in EDTA treatments diminished as time progressed, suggesting that the initial I values were due to EDTA not chelating all zinc cations available in supernatants and therefore preventing complete inhibition of the supernatant activity.

4.3.5 PS II inactivation is significantly greater when PS II centers are active

Pathogen supernatant-exposure experiments under illumination, equal to the light intensity in the culturing incubator (90 μmol photons m^{-2} s^{-1}), resulted in significantly higher I of all *Symbiodinium* cultures (Fig. 4.1C; $p<0.001$) in comparison to I calculated from identical control and supernatant exposure experiments that were conducted by measuring quantum yields (F_v/F_m) in the dark (Fig. 4.1D).

4.3.6 Pathogen supernatants cause *Symbiodinium* PS II inactivation *in hospite*

As *Symbiodinium* cells may function differently when free-living compared to when *in hospite*, a second bioassay system was developed, comprised of coral juveniles (*Acropora millepora*) harbouring *Symbiodinium* endosymbionts from clades

C or D. Juveniles harbouring clade D (JD) *Symbiodinium* and exposed to supernatant from pathogen P1 demonstrated PS II inactivation with mean I (JD, P1, $\triangle F/ F_m'$) = 0.219 ± 0.022 after 10 min and mean I (JD, P1, $\triangle F/ F_m'$) = 0.389 ± 0.030 after 45 min, significantly greater PS II inactivation than found in controls (Fig. 4.4A; $p<0.01$). I of JD exposed to P1 continued to increase reaching total inactivation after 7 h. When 50mM EDTA was added to bacterial supernatants, significantly lower I values were recorded (Fig 4.4A; $p<0.01$). Medium F2 to which 50mM EDTA were added to test the direct effect of EDTA on coral juveniles had no PS II inactivation effect on juveniles, with mean I (JD, F2+EDTA, $\triangle F/ F_m'$) = 0 after 4h. *A. millepora* juveniles infected with *Symbiodinium* from clade C1 and exposed to identical treatments demonstrated similar patterns (Fig. 4.4B).

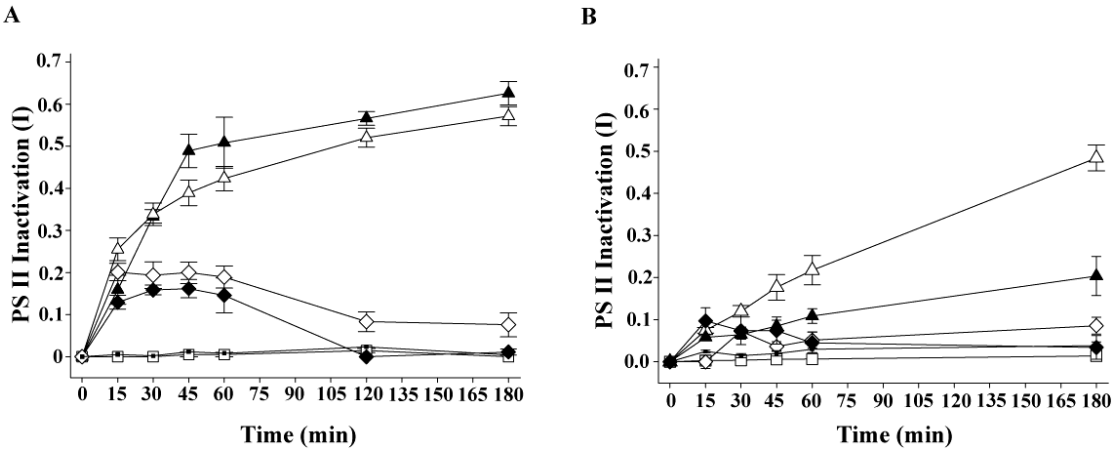

Figure 4.4 PS II inactivation (I) of *Symbiodinium* in coral juveniles

A. PS II inactivation (I; $\triangle F/ F_m'$) of the coral juvenile *Acropora millepora* hosting *Symbiodinium* clade D by the pathogen supernatants P1 and P3 and four control treatments: P1 supernatant ▲; P3 supernatant △; P1 supernatant incubated (1h 30ºC) with 50mM EDTA ◆; P3 supernatant incubated (1h 30ºC) with 50mM EDTA ◇; Dinoflagellate growth medium (F2) □; 1:1 mix of bacterial growth medium (MB) and dinoflagellate growth medium (F2) ■. **B.** PS II inactivation (I; $\triangle F/ F_m'$) of the coral juvenile *Acropora millepora* hosting *Symbiodinium* clade C1 by the pathogen supernatants P1 and P3 and four control treatments: P1 supernatant ▲; P3 supernatant △; P1 supernatant incubated (1h 30ºC) with 50mM EDTA ◆; P3 supernatant incubated (1h 30ºC) with 50mM EDTA ◇; Dinoflagellate growth medium (F2) □; 1:1 mix of bacterial growth medium (MB) and dinoflagellate growth medium (F2) ■. I; $\triangle F/ F_m'$ was based on measurements of effective light adapted quantum yields. Bars= standard errors. n = 8 measurements per treatment.

4.3.7 Tissue lesions and *Symbiodinium* loss caused by pathogen supernatant

A. millepora juveniles harboring *Symbiodinium* clade D and exposed to bacterial supernatant (P1 and P3) were observed to pale within minutes following exposure (Fig. 4.5A). Following addition of bacterial supernatants, juvenile polyps retracted and extended vigorously for a period of 30 sec before becoming irreversibly still. Degradation of the coenosarc tissue (tissue between polyps) was observed (Fig. 4.5B) and *Symbiodinium* cells were clearly seen separating from juvenile tissue and accumulating beside the host coral. Within 4 h, tissue lesions were observed (Fig. 4.5C) and by 8 h only skeleton remained (Fig. 4.5D), corresponding with total PS II inactivation registered by the imaging PAM. *A. millepora* juveniles harboring *Symbiodinium* clade C1 demonstrated similar results when exposed to both P1 and P3 supernatants, while *A. millepora* juveniles treated with supernatants P1 and P3, to which 50mM EDTA was added, did not show loss of *Symbiodinium* cells or any signs of tissue lesions.

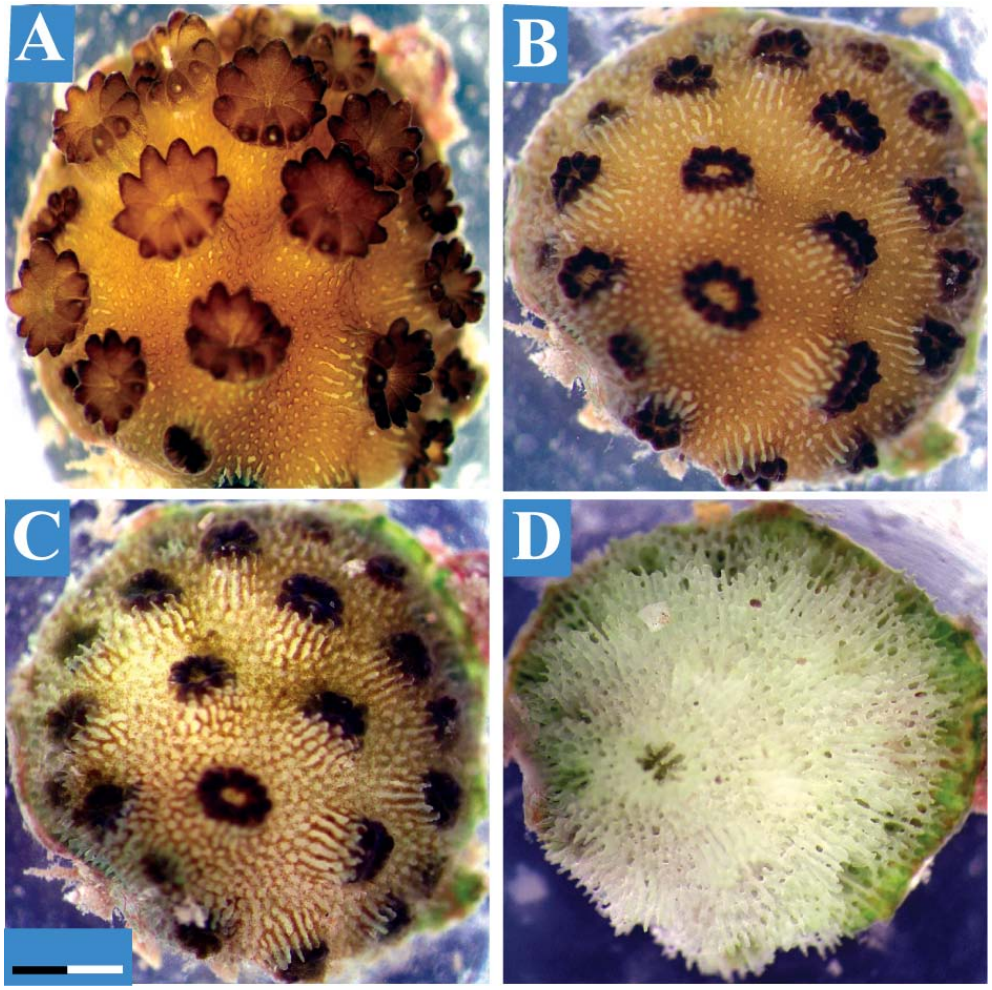

Figure 4.5 Effect of P1 supernatant on the juvenile coral host, *Acropora millepora*
A. millepora juvenile infected with *Symbiodinium* clade D exposed under a dissecting microscope to P1 supernatant. **A.** Before exposure. **B.** 2h following exposure**. C.** 4 h following exposure. **D.** 8 h following exposure. Bar = 2mm (x1.6 enlargement).

4.3.8 A biological dose response between P1 Supernatant and Z1 PS II inactivation

Significant PS II inactivation of Z1 was measured by exposure to P1 supernatant concentrations as low as 1% of the original supernatant stock (Fig. 4.6A; $p<0.001$), with mean I (Z1, P1, 1%, $\triangle F/ F_{m'}$) = 0.226 ± 0.028 following 10 min exposure. Total PS II inactivation of Z1 was measured in all P1 concentrations equal and above 25% following a 10 min exposure. In contrast, recovery of photosynthetic activity was detected in Z1 exposed to P1 concentrations of 5% and lower. Full recovery of photosynthetic activity was measured in Z1 cells exposed to 1% and 5% concentrations of P1 following 5 h and 24 h, respectively ($p>0.1$). In sharp contrast to the susceptible *Symbiodinium* culture Z1, *Symbiodinium* culture Z4 was only affected by higher P1 concentrations, with total PS II inactivation measured for P1 concentrations of 50% and 100% following 2.5 h and 45 min, respectively (Fig 4.6B). Proteolytic activity of bacterial supernatants, measured by the asocasein assay (Windle and Kelleher 1997, Denkin and Nelson 1999), was found in this study to correlate to culture cell density, with maximum activity measured when cultures reached their end logarithmic growth phase (18 h) and when cell density reached $1x10^9$ cells ml^{-1} (Fig. 4.7A-B).

4.3.9 Enzymatic kinetics supports PS II inactivation by P1 supernatant

To explain the high efficiency of P1 in causing PS II inactivation against in its susceptible Z1 target, we plotted Z1 I (Z1, $\triangle F/ F_{m'}$) as a factor of P1 dose and obtained a parabolic curve (Fig. 4.6C) often common in catalytic reactions. Taking the reciprocals of P1 dose and Z1 I resulted in a linear Lineweaver-Burk (1934) - 'like' plot (Fig. 4.6D; $R^2=0.9991$), where apparent km' represents the effective P1 supernatant concentration causing a 50% PS II inactivation of Z1 following 10 min of exposure.

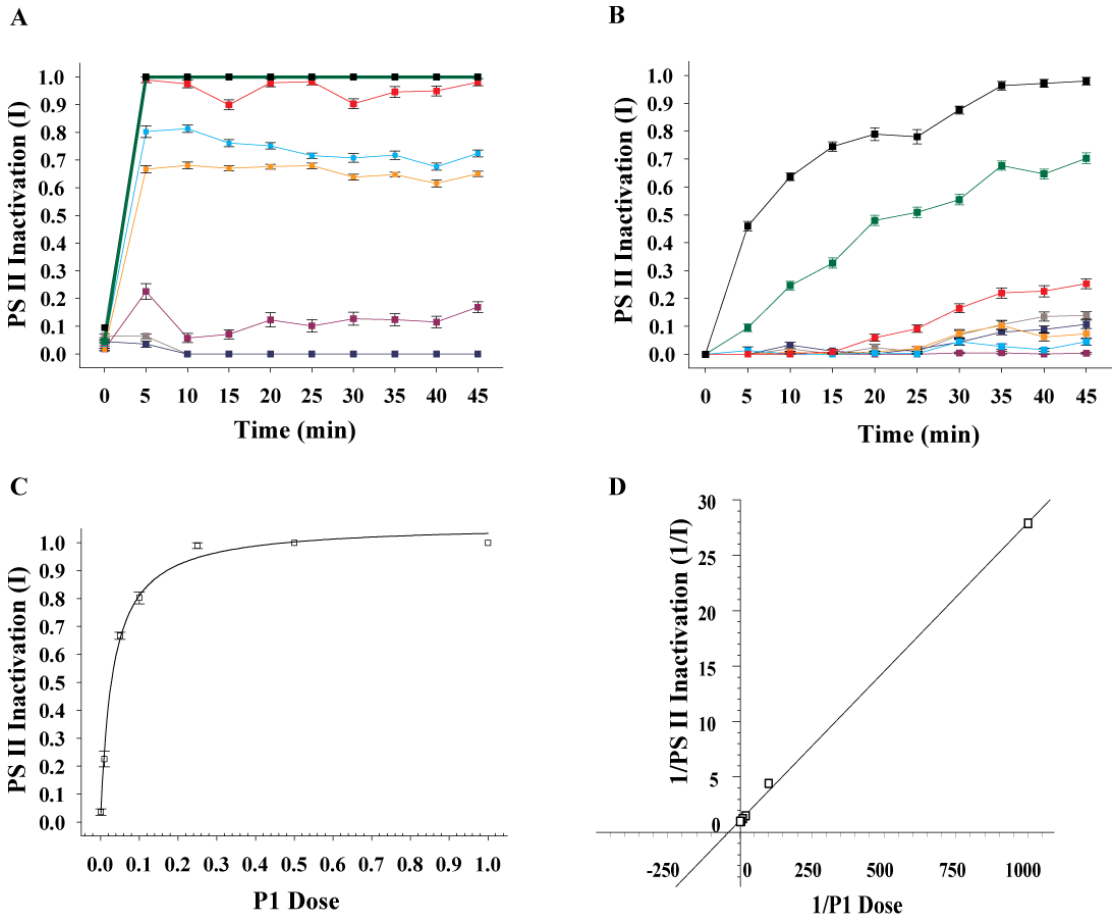

Figure 4.6 Dose response between P1 supernatant and Z1 PS II inactivation

A. Mean PS II inactivation (I; △F/ $F_{m'}$) of *Symbiodinium* culture Z1 (2.5 x 10^5 cells $well^{-1}$) exposed to dilutions from supernatant P1 stock: 1.0 (black line); 0.50 (green line); 0.25 (red line); 0.1 (azure line); 0.05 (orange line); 0.01 (purple line); 0.001 (blue line), and to control treatment with dinoflagellate growth mediun F2 (grey line). **B**. Mean PS II inactivation (I; △F/ $F_{m'}$) of *Symbiodinium* culture Z4 (2.5 x 10^5 cells $well^{-1}$) exposed to supernatant P1, to dilutions from P1 supernatant stock: 1.0 (black line); 0.50 (green line); 0.25 (red line); 0.1 (azure line); 0.05 (orange line); 0.01 (purple line); 0.001 (blue line), and to control treatment with dinoflagellate growth medium F2 (grey line). **C**. Parabolic curved plot for the correlation between P1 supernatant dose (0.001-1.0) vs. mean PS II inactivation (I; △F/ $F_{m'}$) of *Symbiodinium* culture Z1 following 10 min of exposure to P1. **D**. Lineweaver-Burk - "like" plot with linear regression for reciprocated P1 supernatant dose vs. reciprocated mean PS II inactivation of Z1 *Symbiodinium* culture following 10 min of exposure. 1/*I max'* is where the linear regression line crosses axis Y, and -1/*km' is* where regression line crosses axis X. *I max'* = maximum PS II inactivation, and *km'* = the P1 supernatant dilution needed to cause a 50% PS II inactivation (I; △F/ $F_{m'}$) of *Symbiodinium* culture Z1 following 10 min of exposure. The equation obtained from the linear regression is: Y= 0.027 X + 1.071; R^2 = 0.9991; *km'* = 2.52%. Bars = standard errors. n = 12 measurements per treatment.

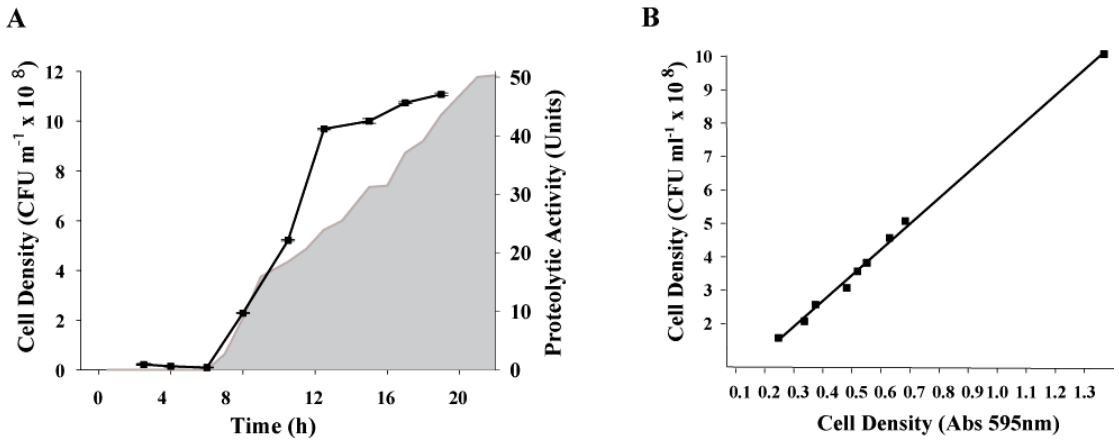

Figure 4.7 Pathogen P1 growth conditions
A. Bacterial cell density (absorbance 595nm) vs. incubation time (27ºC with shaking at 150rpm) ■, and proteolytic activity in Units determined by the asocasein assay (▬) vs. incubation time. **B.** Calibration curve for cultures of pathogen P1: bacterial cell standard errors. n = 6 measurements per treatment.

We were unable to determine an actual km and Vmax for P1 due to the fact that the exact concentrations of both the proteolytic peptide and its yet unknown Z1 substrate were not determined. An apparent km' for Z1 was calculated from the linear regression as km' = 0.0251, *i.e.*, a P1 supernatant concentration of 2.5% is needed to cause a 50% PS II inactivation of Z1 within 10 min. Taking the reciprocal for P1 dose and Z4 PS II inactivation failed to produce the same linear kinetics.

4.3.10 Temperature response of *Symbiodinium* cultures Z1 and Z4 to pathogen supernatant P1

To test the effects of temperature on *Symbiodinium* photoinactivation, P1 pathogen was cultured at five different reef-relevant temperatures (20 ºC, 25 ºC, 27 ºC, 30ºC, 35ºC) and bacterial cell density and the proteolytic activity of each culture determined following 18 h of growth (Fig. 4.8A). P1 bacterial cell density was highest in cultures incubated at 27ºC compared with all other incubation temperatures and protease activity (as measured by the asocasein assay) was greatest in cultures incubated at 27ºC-30ºC (Fig. 4.8A), suggesting a direct link between optimal growth conditions, high cell density and maximal proteolytic activity. In contrast, the direct effect of temperature on *Symbiodinium* cells prior to their exposure to bacterial supernatant (Fig. 4.9A-B) did not reveal significant results.

Symbiodinium cultures Z1 and Z4 were concurrently acclimatized to the same temperatures for 5 days and exposed to four dilutions of P1 supernatant (1%, 10%, 50% and 100%) with incubations at all five corresponding temperatures. The Z1 *Symbiodinium* culture exposed to non-diluted supernatant P1 for 10 min under illumination demonstrated the highest and most rapid photoinactivation at all five temperature treatments (Fig. 8B; $p<0.0001$) compared with culture Z4 exposed to P1 for 10 min. For Z1 incubations, the strongest photoinactivation was recorded for 27ºC acclimatized cells exposed at 27 ºC to P1 supernatant derived from a culture incubated at 27 ºC (mean I (Z1, P1, 27 ºC, $\triangle F/ F_m'$) = 0.889 ± 0.005 after 10 min). Photoinactivation of Z1 was significantly weaker after 10 min in the 30 ºC and 35 ºC treatments, with mean I (Z1, P1, 30 ºC, $\triangle F/ F_m'$) = 0.847 ± 0.007; $p=0.0019$ and mean I (Z1, P1, 35 ºC, $\triangle F/ F_m'$) = 0.706 ± 0.006; $p<0.0001$, respectively. It was also significantly weaker for cultures acclimatized to lower temperatures. The PS-II activity of *Symbiodinium* culture Z4 was not affected when exposed at 35 ºC to P1 cultures grown at 35 ºC (Fig. 4.8B).

In separate control treatments, *Symbiodinium* cultures Z1 and Z4 remained photosynthetically active following a 25 day exposure to 35ºC. Our findings suggest that maximum heat stress may not be an important facilitating factor for WS infections, which are more likely to initiate within the optimal 25ºC-30ºC temperature range. Apparent *Km'* values calculated for the various temperature treatments (Fig. 4.10) indicate that P1 supernatant concentrations of 2.44-3.32% (for temperatures ranging between 25ºC–30ºC) are needed to cause a 50% photoinactivation of Z1 after 10 min. Much higher concentrations of P1 (7.58% and over100%) were calculated to cause a 50% photoinactivation at 20ºC and 35ºC, respectively, suggesting that above 30ºC, poor performance by *Vibrio* pathogens would limit the probability of successful infections by an estimated factor of x 1/50, when all other conditions remain constant.

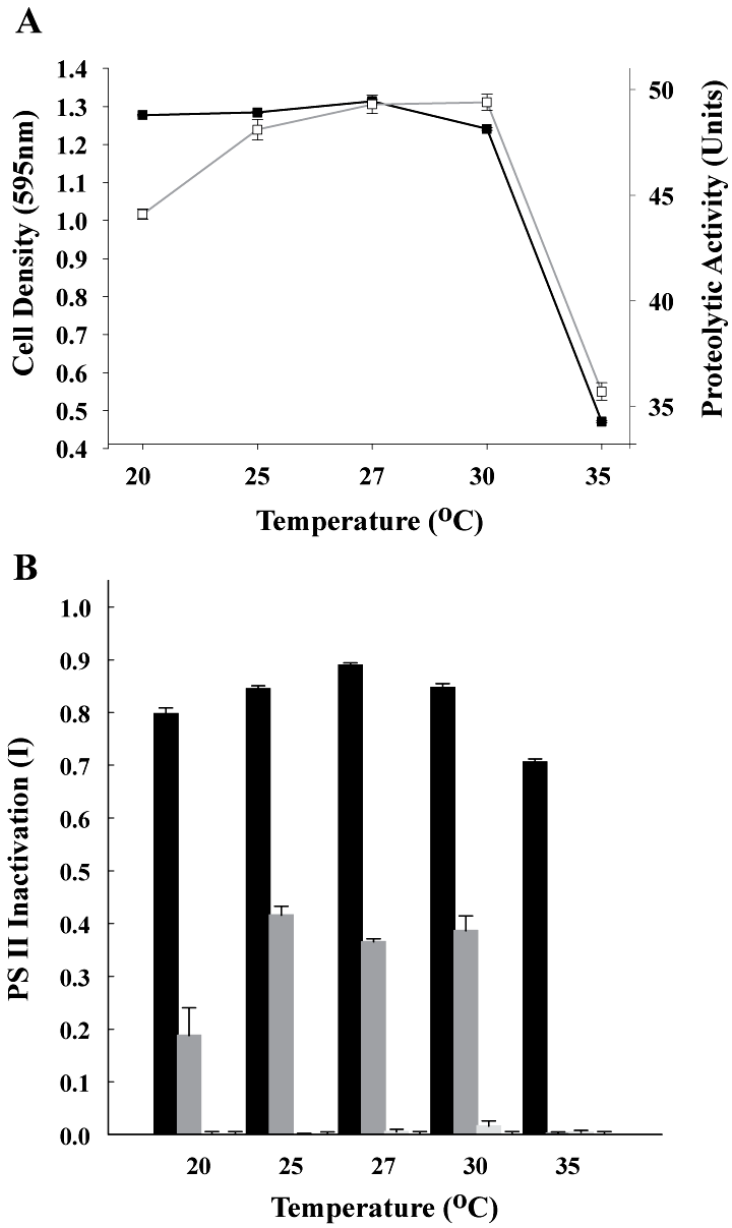

Figure 4.8 Effect of temperature on pathogen P1 and *Symbiodinium* photoinactivation.

A. Effect of temperature (20ºC-35ºC) on P1 growth (Cell density 595nm) ■ and P1 supernatant proteolytic activity (Units) determined by the asocasein assay □. Bacterial culture P1 was grown at each temperature (20ºC, 25ºC, 27ºC, 30ºC, 35ºC) for 18h with shaking (150rpm). n = 12 measurements per treatment. **B.** Mean light adapted ($\triangle F/ F_m'$) photoinactivation of *Symbiodinium* cultures Z1 ■ and Z4 ■ (2.5 x 10^5 cells $well^{-1}$) exposed to supernatant P1 for 10 min. Temperature treatments (20°C, 25°C, 27°C, 30°C, 35°C) indicate the temperatures in which P1 cultures were grown, the temperatures to which *Symbiodinium* cultures were acclimatized and the incubator temperatures at the time of *Symbiodinium* culture exposure to P1 supernatant. Control treatments included *Symbiodinium* cultures Z1 ■ and Z4 ■ (acclimatized to temperature treatments: 20°C, 25°C, 27°C, 30°C, 35°C) and exposed to dinoflagellate growth medium (F2) incubated at the corresponding temperatures. Bars = standard errors. n = 8 measurements per treatment.

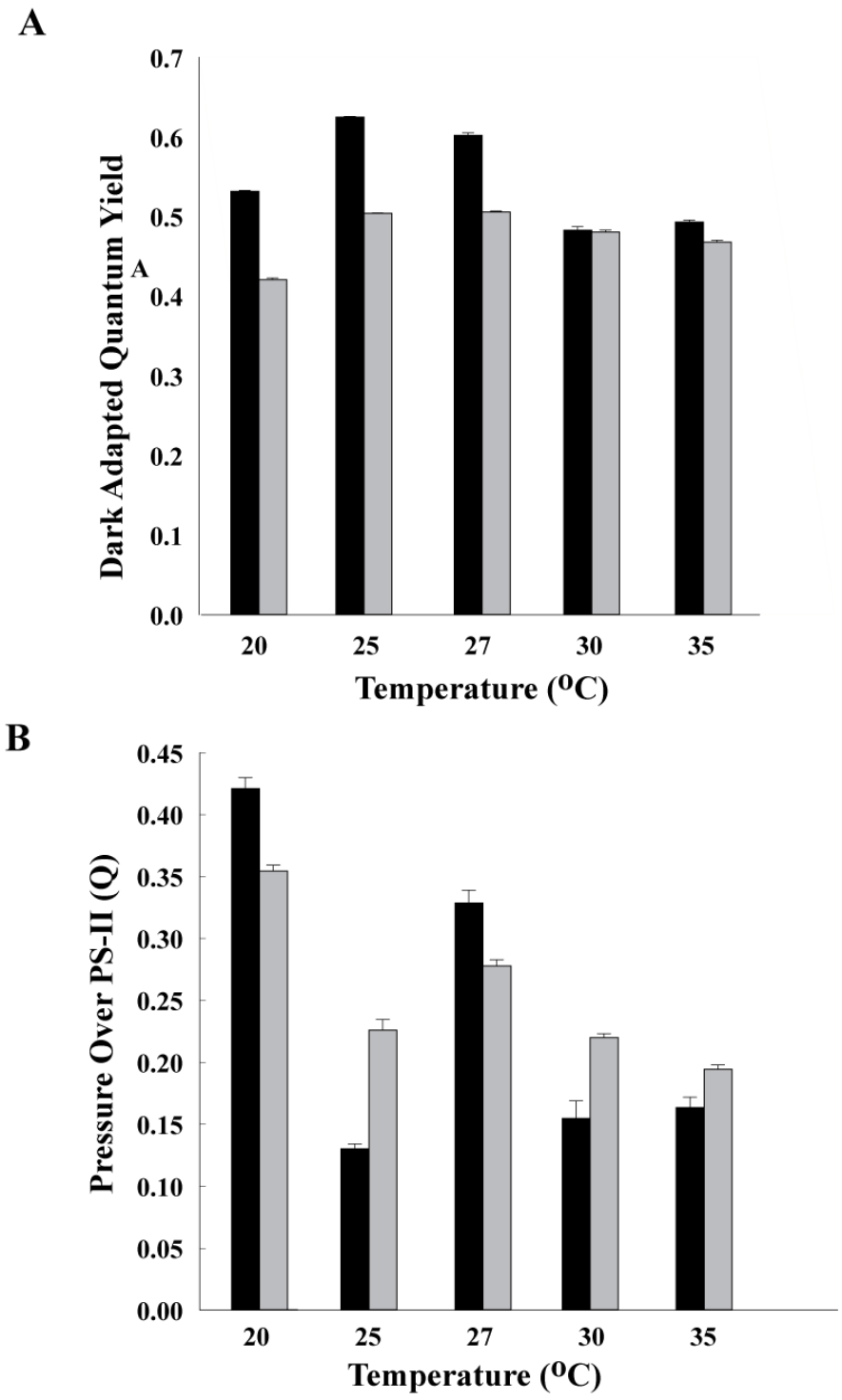

Figure 4.9 Effect of temperature on bacterial pathogens and *Symbiodinium* cultures

A. Effect of temperature on dark adapted maximum PS-II yields (F_v /F_m) of *Symbiodinium* cultures Z1■ and Z4 ■ acclimatized for 5 days to 5 temperatures (20ºC, 25ºC, 27ºC, 30ºC, 35ºC). **B.** Effect of temperature as pressure over PS-II (Q) on *Symbiodinium* cultures Z1■ and Z4 ■ acclimatized for 5 days to 5 temperatures (20ºC, 25ºC, 27ºC, 30ºC, 35ºC). Pressure over PS-II was calculated as: Q = 1-(ΔF/Fm')/(Fv/Fm). Bars=standard errors. n = 8 measurements per treatment.

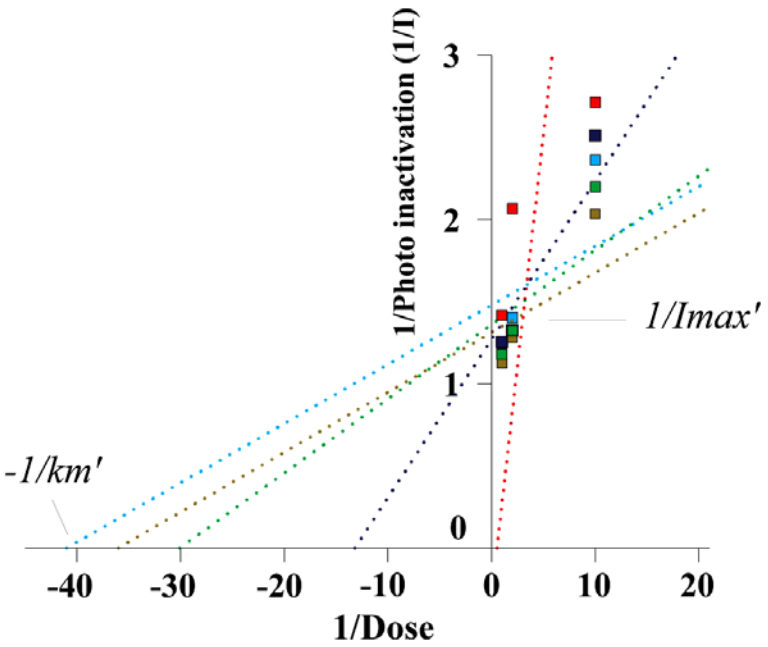

Figure 4.10 Effect of temperature on pathogen P1 supernatant photoinactivation of *Symbiodinium*
Lineweaver-Burk "like" plots with linear regressions (dotted lines) for reciprocated P1 supernatant dose (1/Dose) vs. reciprocated mean light adapted photoinactivation (1/I) of *Symbiodinium* culture Z1 following 10 min of exposure at one of the following temperatures: 20ºC ■; 25ºC ■ ; 27ºC ■; 30ºC ■; 35ºC ■. Lines point at 1/*I max'*, where the linear regression lines cross axis Y, and -1/*km'*, where regression lines crosses axis X. *I max'* = maximum photoinactivation, and *km'* = the P1 supernatant dilution needed to cause a 50% photoinactivation (I) of *Symbiodinium* culture Z1 following 10 min of exposure. Linear equations obtained from linear regressions are: 20ºC: Y= 0.096 X + 1.271; R^2 = 0.9983; *km'* = 7.58%; 25ºC: Y= 0.036 X + 1.478; R^2= 0.9566; *km'* = 2.44%; 27ºC: Y= 0.036 X + 1.311; R^2= 0.9798; *km'* = 2.78%; 30ºC: Y= 0.045 X + 1.360; R^2= 0.9848; *km'* = 3.32%; 35ºC: Y= 0.562 X – 0.295; R^2= 0.9956; *km'* > 100%.

4.3.11 What will happen if seawater temperatures continue to rise?

We investigated how a +2ºC rise in seawater temperature might affect the probability of WS infections by plotting light availability (length of day) and average daily seawater temperatures for the year 2002/3 at Davies Reef, GBR. The annual temperature/light cycle for 2002/3 is presented in Fig 4.11A. and demonstrates how each month of the year forms a unique combination of light and temperature, with WS infections likely to occur as days become longer and warmer crossing the 27ºC temperature-line in Spring. The temperature/light cycle for the year 2001/2 is presented in Fig 4.11B, a period during which mass-bleaching events occurred along the entire GBR. Average daily seawater temperatures reached the optimal temperature for *Vibrio* zinc-metalloprotease production (27ºC) a month earlier in 2001/2 (late in

October), compared to 2002/3 (late in November), and continued to rise beyond 30ºC in February (when mass-bleaching occurred).

However, if seawater temperatures continue to rise, +2ºC above the currently prevailing temperatures (Fig. 4.11C), the optimal temperature range for *Vibrio* pathogens is expected to shift towards the winter, with seawater temperatures remaining above 25ºC throughout the entire year. By calculating the seasonal length of optimal conditions for coral *Vibrio* pathogens (number of days per year with average seawater temperatures between 27ºC-30ºC; Fig. 4.11D), we found that currently longer and shorter seasons of optimal conditions for WS pathogens oscillate along the GBR, possibly contributing to increased WS transmission and abundance during longer seasons of optimal conditions, and coral host recovery during shorter seasons. Not surprisingly, the two mass bleaching events that have occurred along the GBR in recent decades (1997/8, 2001/2) correlate with peaks of longer warm seasons (Fig. 4.11D). For coral reefs in the southern GBR sectors (Halfway Island S23°2' and Turner Cay I S21°7', E152°56'), changes between warm and cold years have been dramatic. For example, less than 10 days with optimal mean temperatures for *Vibrio* pathogens were measured in 1999/2000 increasing to over 100 days in the mass bleaching year of 2001/2002 (Fig. 4.11D). Since 2003/2004, the length of optimal seasons for *Vibrio* pathogens has increased on those reefs with no colder years in between.

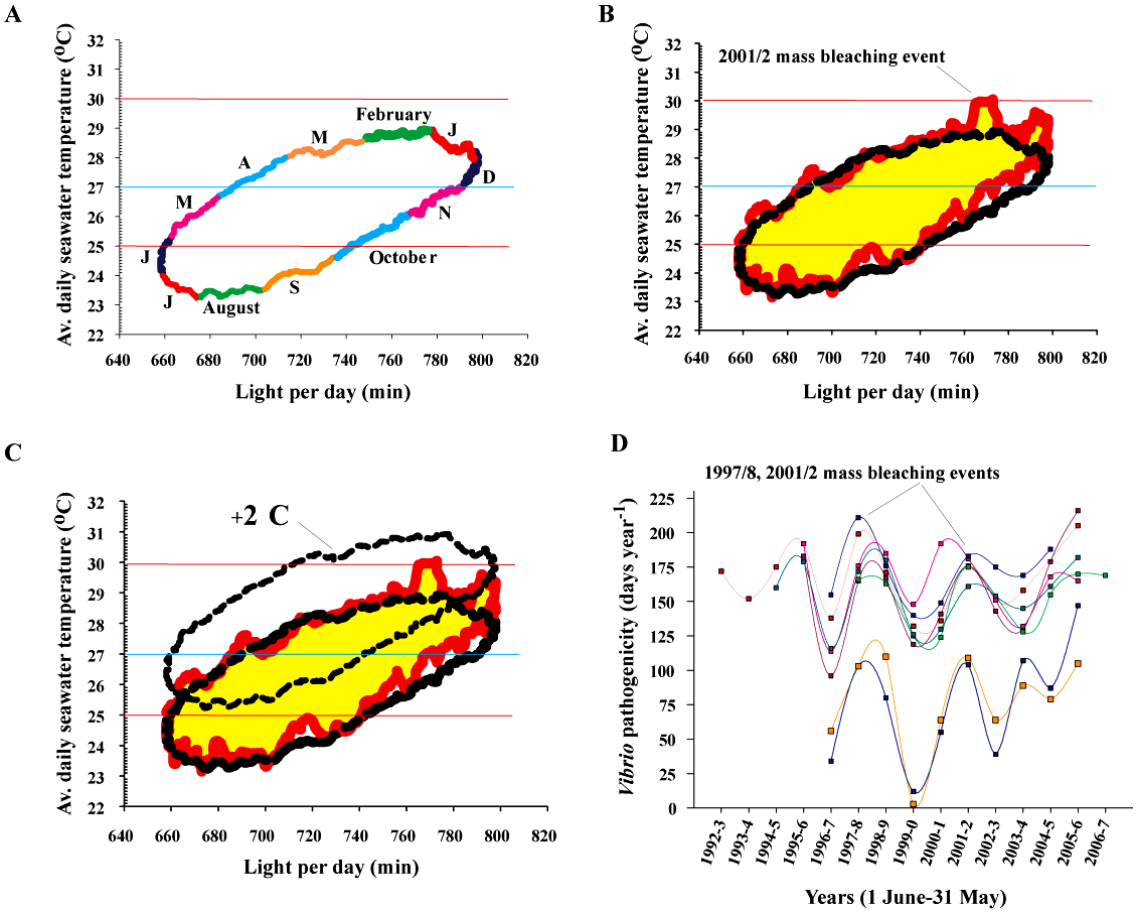

Figure 4.11 Current and future temperatures on the Great Barrier Reef.
A. Daily light availability (min day^{-1}) vs. average daily seawater temperatures (°C) at Davies Reef (S18°81', E147°67') for the year 2002-2003 (1st June – May 31st). Colored bars represent individual calendar months in the annual cycle. **B.** Daily light availability (min day^{-1}) vs. average daily seawater temperatures (°C) at Davies Reef for the year 2002-2003 (1st June – May 31st) , and for the coral mass bleaching year of 2001-2002 . **C.** Daily light availability (min day^{-1}) vs. average daily seawater temperatures (°C) at Davies Reef for the year 2002-2003 (1st June – May 31st) , the coral mass bleaching year of 2001-2002 and a predicted +2°C future seawater temperature rise above the average daily seawater temperatures recorded in 2002-2003 . Red horizontal lines and blue horizontal line represent the optimal temperature limits (25°C, 30°C) and temperature optimum (27°C) for *Vibrio* pathogen caused PS-II photoinactivation, respectively. **D.** Number of days year^{-1} (1st June - May 31st) with optimal mean daily temperature conditions (27°C-30°C) for *Vibrio* pathogen caused photoinactivation. Mean daily seawater temperatures were recorded at 8 reefs along the GBR: Halfway Island S23°2', 150°97' (1996/7-2005/6) ; Turner Cay I S21°7', E152°56' (1996/7-2005/6) ; Geoffrey Bay S19°16', 146°86' (1992/3-2005/6) ; Nelly Bay S19°17', E146°85' (1996/7-2004/5) ; Davis Reef S18°81', E147°67' (1997/8-2006/7) ; Kelso Reef S18°45', E146°99' (1995/6-2005/6) ; Cattle Bay S18°57', E146°48' (1994/5-2005/6) ; Myrmidon Reef S18°26', E147°38' (1995/6-2005/6) . Lines point at mass bleaching events that occurred in 1997/8 and 2001/2.

4.3.12 Virulent zinc-metalloprotease is a 86.141 kDa *Vibrio* thermolysin

The putative zinc-metalloprotease suspected to cause *Symbiodinium* PS II inactivation and coral tissue lesions was characterised using nano-liquid chromatography peptide separation and mass spectrometry (nano-LC/MS/MS) of proteolytically active bands derived from all pathogen supernatants (P1-P4). These analyses produced signature sequences consistent (by MASCOT and BLAST alignments) with one common bacterial 86.141 kDa pre-propeptide from the family of thermolysin (Fig. 4.12) that has been previously identified in other *Vibrio* pathogens, such as *V. cholera* (Booth et al. 1983) and *V. vulnificus* (Miyoshi et al. 1997). Partial protein sequence alignments matched four domains of the common zinc-metalloprotease (Marchler-Bauer et al. 2007): the N-terminal domain (PepSY propeptide and YPEB domain), the catalytic domain, the alpha helical domain and the C-terminal domain (Fig. 4.12).

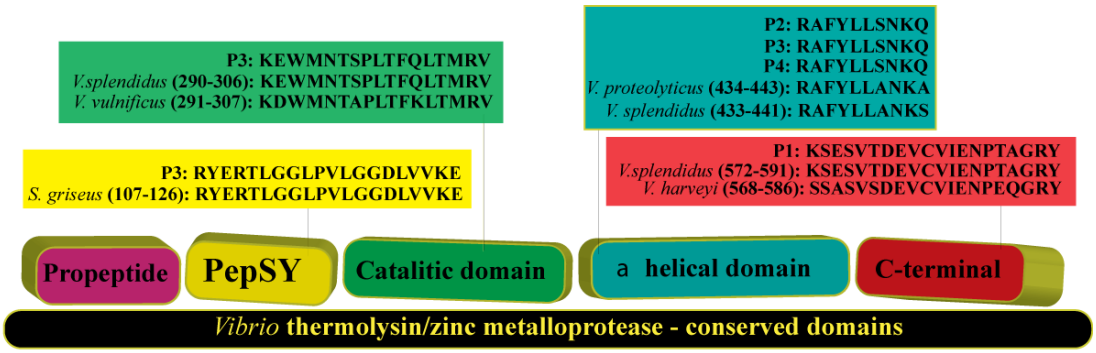

Figure 4.12 Zinc-metalloprotease - conserved domains
Domains of a 86.141 kDa pre-propeptide, a zinc-metalloprotease derived from coral pathogen supernatants (P1-P4). Propeptide (pink); PepSY (propeptide and YPEB domain; yellow); Catalitic domain (green); α-helical domain (azure); C-terminal domain (red). P1-P4 partial protein sequence alignments (BLAST/MASCOT) matched sequences of four conserved domains from previously identified *Vibrio spp.* zinc-metalloproteases: Pathogen supernatant P3: PepSY (propeptide and YPEB domain; yellow); Pathogen supernatant P3: Catalitic domain (green); Pathogen supernatant P3-P6: α-helical domain (blue); Pathogen supernatant P1: C-terminal domain (red).

4.4 Discussion

4.4.1 Bacterial caused PS II inactivation of *Symbiodinium* photosynthesis

Bacterial causative agents for coral diseases have been identified in previous studies (Kushmaro et al. 1996, Geiser et al. 1988, Ben-Haim and Rosenberg 2002, Patterson et al. 2002, Denner et al. 2003, Barash et al. 2005, Bally and Garrabou 2007, Sussman et al. 2008), including specific virulence mechanisms that enable coral colonization and disease progression (Banin et al. 2001a, 2001b, 2003), however, this study is the first to investigate the clinical effect of a virulence factor derived from multiple causative agents and applied to multiple targets. Our principal findings demonstrate that PS II inactivation of susceptible *Symbiodinium* cells (Z1) by pathogen supernatants is significantly higher than PS II inactivation of non-susceptible targets (Z2-Z4). Susceptibility of *Symbiodinium* cells to bacterial PS II inactivation was supported by demonstrating a biological dose response (Hill 1965). Partial peptide sequencing of proteolytically active fractions derived from WS pathogen supernatants identified a common 86.141 kDa zinc-metalloprotease from the family thermolysin, which is suspected of causing *Symbiodinium* PS II inactivation and coral tissue lesions. Nevertheless, our preliminary exposure trials could not determine the exact process by which bacterial zinc-metalloproteases affect *Symbiodinium* photosynthesis. A similar thermolysin derived from *Bacillus thermoproteolyticus rokko* has been reported to selectively cleave chloroplast outer envelope membrane (OEM) proteins causing PS II photoinhibition (Cline et al. 1984). Tests performed with *B. thermoproteolyticus rokko* thermolysin revealed that it can not penetrate through the chloroplast OEM (Cline et al. 1984), but can affect about 20 OEM polypeptides (Joyard et al. 1983), including components of a protein import apparatus (Tranel et al. 1995) that may indirectly influence PS II performance.

This study identified specific Lineweaver-Burk - ‘like’ kinetics between P1 supernatant and its susceptible Z1 target, suggesting that PS II inactivation of *Symbiodinium* Z1 is potentially the result of a specific bond between an enzyme and its target substrate. Failure to produce similar kinetics between P1 supernatant and a non-susceptible (Z4) target, supports the specificity of this bond and may possibly explain why *Acropora millepore* coral hosts harboring culture Z4 *Symbiodinium* at Nelly Bay (GBR), or *Montipora aequituberculata* colonies harboring Z2 *Symbiodinium* at Davies Reef (GBR) have not been observed with WS disease signs, whereas *M. aequituberculata* colonies from Nelly Bay, which harbor susceptible Z1

Symbiodinium, often display WS disease signs. Nevertheless, further studies are needed in order to determine the specific substrate of the zinc-metalloprotease identified in WS coral pathogens in this study. *Symbiodinium* PS II inactivation by low pathogen supernatant concentration (≤5%) was found to be reversible in this study. Recovery of full photosynthetic capacity of *Symbiodinium* cells within 24 h, after short-term PS II inactivation following low concentration exposures, suggests that zinc-metalloprotease damage to PS II might be repairable, as demonstrated for PS II repair by heat shock proteins (Schroda et al. 1999). Alternatively, PS II inactivation may be caused by enzymatic cleavage of *Symbiodinium* cell membranes, resulting in an irreparable cellular collapse and *Symbiodinium* mortality, as observed by Cervino et al. (2004b) for yellow blotch/band infections of *Montastraea spp*. corals in the Caribbean. Our study, however, could not find adequate support for this hypothesis, since total PS II inactivation occurred less than 20 sec following exposure to bacterial supernatants. Further studies are needed to examine the pathology of *Symbiodinium* exposed to coral pathogen zinc-metalloproteases. Damage to *Symbiodinium* PS II has been shown to be caused by a variety of factors including light and heat stress (Barber 1992, Lesser 1996, asada 1996, Warner 1999, Hill et al, 2004, Takahashi 2008) associated with mass coral bleaching (Hoegh-Guldberg and Smith 1989, Gates et al. 1992, Brown 1997, Hoegh-Guldberg 1999, Fitt et al. 2001), and by numerous bacterial toxins (Banin et al. 2001b, Ben-Haim et al. 2003a) suggesting that PS II damage may result from independent disease aetiologies. In order to test the hypothesis that these factors act in synergism, specific diagnostics must be designed, such as monoclonal antibodies that will register zinc-metalloprotease signals in the field.

In this study, *Symbiodinium* isolates affiliated with clade A were found to be both susceptible and non-susceptible to bacterial PS II inactivation, in contrast to findings by Stat et al. (2008), speculating that clade A *Symbiodinium* associations with diseased corals are closer to parasitism than to mutualism than similar associations of corals with clade C *Symbiodinium*. Better knowledge of *Symbiodinium* physiology and disease aetiology will assist in identifying why specific types are more susceptible to bacterial PS II inactivation. Further studies including the cloning and sequencing of the of zinc-metalloprotease genes from WS pathogens will enable validation of our current findings, potentially by utilizing mutant coral pathogen strains that lack the zinc-metalloprotease gene, or by utilizing differential expression

and coral pathogen zinc-metalloproteases expressed by a vector system in additional exposure trials (Foulongne et al. 2004) aimed at fulfilling Koch's molecular postulates (Falkow 1988).

4.4.2 Bacterial caused tissue lesions and *Symbiodinium* loss

In this study, visual observations and iPAM measurements of exposed coral juveniles revealed three distinct phases of disease: 1. *Symbiodinium* PS II inactivation; 2. paling of coral tissue through loss of *Symbiodinium* cells; and 3. spread of coral tissue lesions culminating in mortality. These signs, which were expressed by coral juveniles in response to bacterial supernatants, were identical to WS disease signs observed on adult corals in the field (Willis et al. 2004, Anthony et al. 2008) and during pathogen inoculation experiments (chapter 3; Sussman et al. 2008), and further support our findings of the previous chapter, which identified *Vibrio* pathogens as the primary causative agents of WS's (Sussman et al. 2008). This is the first study to successfully replicate WS disease signs by using cell free supernatants.

Vibrio zinc-metalloproteases are known to perform dual functions similar to the duality of function demonstrated by coral zinc-metalloproteases in this study, *i.e.*, in causing both *Symbiodinium* PS II inactivation and coral tissue lesions. For example, in the human pathogen *Vibrio cholera*, a virulent zinc-metalloprotease has been named hemagglutinin/protease because of its dual capacity to cause both hemagglutination and proteolytic cleavage (Finkelstein and Hanne 1982, Miyoshi et al. 1997). The *V. vulnificus* elastase/protease has also been shown to possess dual functions, enhancing vascular permeability and causing hemorrhagic damage (Kothary and Kreger 1985, Oliver et al. 1986). Numerous studies demonstrate that *Vibrio* zinc-metalloproteases are synthesized as inactive precursors that mature outside the bacterial cell following several processing stages which may alter their function (Miyoshi and Shinoda 2000), such as the cleavage of a C-terminal 10 kDa peptide from the *V. vulnificus* zinc-metalloprotease (VVP; Miyoshi et al. 2001) by a specific processing protease (Varina et al. 2008), which mediates effective binding of *V. vulnificus* VVP to its substrate. Future studies will determine whether coral pathogen zinc-metalloproteases undergo a similar maturing process.

4.4.3 White syndrome is a multifactorial coral disease

Findings from this current study support the classification of coral WS as a multifactorial disease with multiple component causes (Thrusfeld 2005). We found the expression of zinc-metalloprotease by WS coral pathogens to be cell density dependant, with greatest proteolytic activities measured at the end logarithmic phase, when bacterial cell density in cultures reached $1x10^9$ cells ml^{-1}. Based on dose response experiments, we calculated that the steady state concentration of coral pathogen derived zinc-metalloprotease required to cause a rapid and irreversible 50% PS II inactivation of susceptible *Symbiodinium* cells following 10 min of exposure is equal to the dose produced by a bacterial concentration of $\sim5x10^7$ cells ml^{-1}. This calculation, although preliminary, implies that WS disease signs are unlikely to occur in the field unless susceptible populations of *Symbiodinium* cells are exposed to a high concentration of pathogenic bacteria. Thus, the progressing band of exposed coral skeleton typical of WS signs in the field (Willis et al. 2004, Anthony et al. 2008), can be explained by the presence of high densities of pathogens at the interface between exposed skeleton and healthy looking tissue in progressing lesions. In the previous chapter, we found that *Vibrio* cell densities associated with a lesion interface were more than a 100 times higher than the cell densities found on healthy-looking tissue (Chapter 3 section 3.3.1; Sussman et al. 2008). In a field study, Roff et al. (2008) provided evidence that coral tissues remain photosynthetically active when they are less than 10 cm away from the interface of a progressing WS tissue lesion, suggesting that pathogen zinc-metalloprotease concentrations may be diluted to a non-effective dose away from a progressing tissue lesion interface. Reductions in zinc-metalloprotease concentrations when conditions for optimal pathogen growth are impared may also explain how corals can recover from WS infections.

4.4.4 The effect of seawater temperatures and global warming on *Symbiodinium* susceptibility to PS-II inactivation and pathogen virulence

The findings by Bruno et al. (2007), that both temperature and host density influence WS disease prevalence, support the definition of WS as a multifactorial disease. These two factors, plus the requirements for primary causative agents at elevated concentrations and for susceptible target *Symbiodinium* types, may all contribute to facilitating WS epizootics. Elevated seawater temperatures have been shown to be a major contributing factor to *Vibrio cholera* pandemics (Colwell 1996)

and a necessary factor for triggering the virulence of the coral pathogen *Vibrio shiloi* (Israeli et al. 2001).

Based on our findings, we postulate that optimal temperatures for *Vibrio* WS pathogen growth contribute directly to cell density dependant synthesis of zinc-metalloproteases that are required for infections. Our study suggests that optimal temperatures for *Vibrio* pathogens should be summed to the length of season in which optimal temperature conditions prevail, suggesting that both pathogen growth and pathogen transmission may be temperature and light dependant. Many *Vibrio* caused coral infections reported to date initiate and develop during spring and summer (Kushmaro et al. 1996, 1998, Rosenberg and Ben-Haim 2002, Ben-Haim et al. 2003a, Sussman et al. 2003), when days become longer and warmer. Many other *Vibrio* infections in the marine environment correlate with seawater temperatures within the optimal range of 25ºC–30ºC established in this study, including *V. cholera* outbreaks which have been shown to peak at 30ºC (Colwell 1996).

In sharp contrast, no *Vibrio* caused infectious diseases have been reported at elevated temperatures above 30ºC. This study tested the performance of WS pathogens at 20ºC, 25ºC, 27ºC, 30ºC and 35ºC and determined that the probability of WS infections occurring at elevated temperatures (above 30ºC) is limited. This study could not detect any temperature related stress affecting *Symbiodinium* cultures exposed to elevated temperatures (35ºC), however, it did detect slower growth rates of *Vibrio* pathogens and limited extra-cellular zinc-metalloprotease secretion at 35ºC. It is currently unknown how *Vibrio* pathogens might evolve as seawater temperatures increase, therefore making it very hard to predict the impact of global warming on the spread and prevalence of WS on Indo-Pacific reefs. Nevertheless, current seawater temperatures are probably optimal for the growth, virulence and spread of *Vibrio* WS pathogens, potentially explaining the large impact of this disease at present. Only precise monitoring of reefs using diagnostic tools that are able to detect *Vibrio* pathogens and their virulence factors could enable to determine whether global warming will remain a driving force enhancing the impact of WS on Indo-Pacific reefs in the future.

4.4.5 Are *Vibrio* WS pathogens primary pathogens, opportunistic pathogens, or secondary pathogens to other unknown causes?

Detection of *Vibrio* strains on both healthy and diseased populations of fish (Thune et al. 1993), shrimps (Karunasagar et al. 1994) and corals (Bourne and Munn 2005) has led to the conclusion that *Vibrio* infections are opportunistic in nature (Lightner 1993). The term 'opportunistic infection' was first defined by Utz in 1962 relating to fungal infections (Utz 1962), but has since been modified to include additional pathogen-host interactions, particulary those that can be represented by a 'damage-response framework' (Casadevall and Pirofski 2003), which defines pathogenicity and host-susceptibility as coupled variant traits. This study identified the variant traits of *Symbiodinium* hosts exposed to a common zinc-metalloprotease. However, it has also been found that the presence of a zinc-metalloprotease gene can be detected in DNA retrieved from non-pathogenic strains, which were unable to cause disease signs in controlled exposure trials (see chapter 3 section 3.3.4.3; Sussman et al. 2008). The presence of a zinc-metalloprotease gene in non-pathogenic *Vibrio* strains, suggests that the expression of other virulence genes is necessary for successful infections to occur. Thus we conclude that, although all WS pathogens identified possess a zinc-metalloprotease gene sufficient to cause rapid photoinactivation and coral tissue lesions, not all *Vibrio* strains possessing this gene can be classified as primary causative agents of WS. Genetic studies support the variant traits of *Vibrio* pathogens. From over 200 *V. cholera* serotypes, only a few have been shown to cause cholera pandemics, while others, possessing partial combinations of virulent genes, were shown to cause a gradient of attenuated disease symptoms (Singh et al. 2001, Reidl and Klose 2002). Work by Austin et al. (2005) demonstrated that the coral pathogen *V. coralliilyticus* (Ben-Haim et al. 2003a, Bally and Garrabou 2007), which was also identified in this study (Chapter 3 section 3.3.5; Sussman et al. 2008) also affects rainbow trout (*Oncorhynchus mykiss*) and *Artemia nauplii* by causing mortalities in animal models, suggesting it may target multiple species that are not necessarily compromised hosts by possessing broad pathogenicity. In contrast, the virulence of the coral bleaching agent *V. shiloii* (Kushmaro et al. 2001) was not adequate to infect *Oncorhynchus mykiss* and *Artemia nauplii* (Austin et al. 2005). In addition, *V. shiloi* has recently been reported to have stopped infecting its known coral host *Oculina patagonica* in the Mediterranean Sea (Reshef et al. 2006), suggesting a shift in the 'damage-response framework' (Casadevall and Pirofski

2003), defined by Rosenberg et al. (2007) as the 'hologenome theory of evolution', *i.e.*, the failure of variant pathogen-host traits to continue producing expected disease signs. Pathogen-coral interactions may be further complicated considering the fact that pathogens in the marine environment perform under different conditions than those confronted by terrestrial agents (McCallum et al. 2004, Murray 2008), and in particular, *Vibrio* coral agents, whose survival strategy might be aimed at specializing in 'adaptability' (Higgins et al. 2007), rather than in an obligatory 'selectivity' towards specific hosts.

In conclusion, our findings support classifying coral WS's as multifactorial diseases, which are caused by primary *Vibrio* pathogens. Based on findings from this study, *Vibrio* pathogens may be involved in numerous coral disease aetiologies as pathogens of variant traits, and may operate as primary, opportunistic, or as secondary agents. Their ubiquity and modes of action underline the need for further collaborative studies to explore the complexity of roles performed by *Vibrio* zinc-metalloproteases in both coral health and disease.

Chapter 5
General Discussion

5.1 Coral pathogens: global distributions with potentially multiple origins?

This study is the first to identify coral pathogens from the Great Barrier Reef and from remote locations in the Indo-Pacific Ocean, including the Republic of the Marshall Islands and the Republic of Palau. Principal findings from this study demonstrate that coral pathogens on the GBR and across the Indo-Pacific Ocean are closely related to bacterial pathogens identified from other reef locations, such as the Caribbean and the Mediterranean Sea. Based on 16S rRNA gene sequencing, cyanobacteria associated with corals displaying signs of black band disease (BBD) on the GBR and in Palau were closely affiliated with cyanobacterial strains associated with BBD infected corals in the Caribbean (Frias-Lopez et al. 2003) and in the Gulf of Aqauba (Barneah et al. 2007). Similarly, bacterial pathogens causing WS disease signs on the GBR (Magnetic Island) and in the Republic of the Marshall Islands were found to be closely affiliated with the coral bleaching pathogen *V. coralliilyticus* identified in Zanzibar and in the Gulf of Aquaba (Ben-Haim et al. 2002, 2003a). *V. coralliilyticus* has also been identified as the causative agent of gorgonian mortalities in the Mediterranean Sea (Bally and Garrabou, 2007), of fish and shrimp mortalities in laboratory infection trials (Austin et al. 2005) and of oyster disease in Kent, UK (Thompson et al. 2001). It is not clear, however, if such global distributions are the result of coral pathogen transmission across geographical regions, or the result of genetic similarity among multiple coral pathogens, each with a separate origin and a potentially highly varied arsenal of virulence genes resulting in niche specificity.

Many studies of infectious disease support the hypothesis that diseases emerge in one location and are then carried across regions by vectors. For example, the devastating effects of West Nile viral infections on avian wildlife populations in North America (Rappole and Hubálek, 2003) have been attributed to transport of the virus to New York by a human vector in 1999 (Lanciotti et al. 1999). Avian Flu virus infections have also been shown to spread across geographical regions by migrating birds (Olsen et al. 2006). In the marine environment, coral disease pathogens have been identified in ballast water brought into the Gulf of Mexico (Aguirre-Macedo et al. 2008). Modeling of disease spread and transmission based on the 'novel pathogen

hypothesis of infectious diseases' (Alford 2001), which assumes that a pathogen has recently spread into a new geographic area, has been a key factor in developing strategies to combat the spread of infectious diseases across geographical regions, for example in the development of strict isolation and quarantine measures (Fraser et al. 2004), as in the case of SARS (Seto et al. 2003), or quarantine and vaccination measures, as in the case of rabies (Perrin et al. 1999). The lack of rabies on inhabited islands, i.e., Great Britain, Hawaii and Australia (Johnson et al. 2002), provides corroborative evidence that the current distribution of the rabies pathogen is the result of spread across geographical regions. There are also records of numerous other spreading infectious diseases that have accumulated throughout the last 30 years (Cohen 2000). However, other infectious diseases may evolve from existing autochthonous pathogens found in environmental reservoirs that become virulent under numerous compounding conditions (Colwell and Huq 2001, Rachowicz et al. 2005). Although not fully established, *Vibrio* infections in general (Wittman et al. 1995) and *Vibrio cholera* infections in particular (Politzer 1959, Huq et al. 1990, Kaper et al. 1995, Rivera et al. 2001) may result from autochthonous pathogens, which were found in endemic reservoirs (Lipp et al. 2002).

Black band disease infections of corals were first identified in the Caribbean (Antonius 1973) and subsequently reported from the Indo-Pacific Ocean within the following decade (Antonius 1985a). The appearance of BBD in widely separated reef regions within such a time frame is consistent with the possibility that cyanobacterial strains are present normally within the environment. Detection of bacterial rRNA sequences in both healthy and diseased corals on the Great Barrier Reef (GBR), which were closely affiliated with rRNA sequences derived from corals from other geographical regions (Jones et al. 2004, Bourne and Munn 2005, Bourne 2005), supports the hypothesis that at least some coral pathogens may be autochthonous. In this study, the bacterial causative agent of white syndrome (WS) infections on reefs in the Republic of the Marshall Islands, a secluded Atoll in the Indo-Pacific where a major WS epizootic has been occurring since 2003 (Jacobson et al. 2006), was found to be phylogenetically related to similar *Vibrio* strains that were recovered from various locations, including diseased oysters in the English Channel (Thomspson et al. 2001). The Marshall Islands coral epizootic occurred on a site that is densely populated with large plating acroporid corals and was in proximity to the atoll's sewage outlet and waste disposal ground, two factors that have been shown

previously to be associated with the prevalence of coral diseases (Peters 1984, Bruno et al. 2003, Kaczmarsky et al. 2005, Klaus et al. 2005, 2007 Voss and Richardson 2006). In addition, the Marshall Islands epizootic was found to be more active during the warm months of the year, suggesting a direct correlation with elevated seawater temperatures (Jacobson et al. 2006), as has also been found on the GBR for WS abundance (Bruno et al. 2007) and for BBD abundance and rates of progression (Boyett et al. 2007; Sato et al. 2009).

In contrast, a recent expedition to the Northern Line Islands surveyed microbial communities along a gradient of increasing human population, which was inversely correlated with the number of sharks and other top predators, and found that the greatest abundance of coral diseases occurred on reefs surrounding populated and polluted atolls, where over-fishing and sewage disposal often occurs (Sandin et al 2008). They also demonstrated that *Vibrio* isolates could only be cultured from seawater samples obtained on populated reefs, where the abundances of virus-like particles and heterotroph-dominated bacteria in seawater samples were 10 times higher than on unpopulated atolls (Dinsdale et al. 2008), suggesting that *Vibrio* pathogens may not typically be members of healthy bacterial communities on corals. Nevertheless, the abundance of bacteria in seawater samples and the prevalence of disease cannot give clear indications as to the origin of coral pathogens. Currently the use of 16S rRNA gene sequencing for phylogenic determination is effective (due to the gene's variable structure and the availability of a large sequence-database). However, for the identification of pathogen-virulence among identical species, 16S rRNA resolution might not be sufficient. Thompson and colleagues (2005) demonstrated that *V. coralliilyticus* strains possess high variability for the rpoA, recA and pyrH genes, suggesting that 16S rRNA taxonomy can not indicate the origin and composition of virulence genes; whether these are the result of endemic speciation or a worldwide distribution. Additional tools must be developed in order to test the hypothesis that some coral pathogens are globally distributed and can be detected in endemic reservoirs, versus the alternative hypothesis that spread of coral pathogens across geographical regions is a major contributing factor facilitating coral disease outbreaks. Other tools should also be developed in order to estimate the evolutionary rate of coral pathogens, which can be significantly higher than previously estimated.

Although based on preliminary findings, results from this study are consistent with both BBD and WS outbreaks originating from endemic pathogens, which

become successful at causing coral infections under a complex series of compounding factors. However, further studies are needed in order to verify this hypothesis. Clarification of the origin of coral pathogens would determine future management strategies aimed at arresting the spread of coral disease on Indo-Pacific reefs and their relationship to other management tools, such as the construction of protected marine parks (Coelho and Manfrino 2007). Regardless of the origin of coral pathogens, it would be sensible to assume that the accumulation of stressors affecting reefs, such as elevated seawater temperatures caused by global warming and anthropogenic impacts, would positively enhance the susceptibility of corals to infectious diseases.

5.2 Closely affiliated pathogens cause various combinations of disease signs

Findings from this study described phylogenically consistent cyanobacterial isolates from two macroscopically different disease lesions: 1. a typical progressing BBD lesion; and 2. a progressing red band disease lesion. The cyanobacterial strains isolated from the classic BBD lesion and from the red band lesion both possessed almost identical (>99%) 16S rRNA gene sequences. Despite this close affiliation of isolates derived from diseased corals, this study could not verify whether both the red and black appearing cyanobacteria were part of identical disease aetiologies, causing similar tissue lesions on corals. However, results from this study clearly demonstrate that assigning disease names should be considered with great caution and should not be based on disease sign appearance alone, especially when signs are only observed in the field (Richardson 1993, Littler and Littler 1995, Cervino et al. 1998, Jones et al. 2004), to avoid misidentifications of disease.

Early studies performed in the Caribbean by Rützler and Santavy (1983) and by Rützler et al. (1983), as well as later studies performed by others (Richardson et al. 1991a, Richardson and Kuta 2003, Sekar et al. 2006, Myers et al. 2007), identified cyanobacterial strains associated with BBD that differ from those identified in more recent studies (Cooney et al. 2002, Frias-Lopez et al. 2002, 2003). In combination with the presence of BBD cyanobacteria on healthy corals (Richardson 1997b), these conflicting findings raise questions about whether BBD is, in fact, primarily caused by cyanobacterial strains. Recent work, however, established that tissue loss may be the result of the combined effects of cyanobacterial toxins (Sekar et al. 2006, Richardson et al. 2007) and anaerobic conditions resulting from sulfate production of microbial consortia (Viehman and Richardson 2002, Viehman et al. 2006). These

findings lead to the distinct possibility that BBD is a poly-microbial disease, *i.e.* the result of two or more microorganisms acting in consortia to cause the lesions. Further studies are needed in order to determine the exact role of cynobacteria in intiating BBD, and it may yet be concluded that cyanobacterial propagation on diseased corals follows other primary infections, infestations or predation (Aeby and Santavy 2006), such as the occurence of Caribbean rapid wasting disease (RWD) following parrotfish white spot biting (PWSB; Cervino et al. 1998).

Findings from this study also demonstrate that the coral disease WS, which is characterized by progressing tissue lesions culminating in coral mortality, is caused by *Vibrio* agents that secrete an extracellular thermolysin that causes rapid photoinactivation of *Symbiodinium* endosymbionts. This virulent effect cannot be observed in the field preceding other observed disease signs, including paling of coral tissue and the formation of lesions. In addition, not all WS disease signs caused by thermolysin-secreting *Vibrio* pathogens have the exact identical appearance in the field. Some lesions produced during the pathogen re-infection experiments appeared as patchy tissue loss on the host, while others formed the more typical line of tissue loss progressing across coral colonies. In some cases, tissue paling, which precedes the progressing band, is more apparent in lab experiments than in infections observed in the field, although Anthony et al. (2008) observed paling in the field for the coral disease atramentous necrosis, which has the same disease aetiology.

Currently, most coral diseases are identified in the field by visual observations and classified by variation in characteristics of progressing tissue lesions. Results from this study confirm that present coral disease identification is strongly biased towards macroscopic disease signs with little diagnostic capacity for distinguishing other aetiologies resulting in compromised coral health. For example, similar tissue lesions on corals in the Caribbean have been identified in the past as two distinct diseases, white band disease 1 (WBD1; Peters et al. 1983) and white band disease 2 (WBD2; Ritchie and Smith 1998), based on the fact that field observations could only distinguish paling tissues preceding the progressing line of tissue loss in the case of corals infected by WBD2 and not by WBD1. Based on findings from this study, which demonstrate that macroscopic signs of at least two coral diseases (BBD and WS) can vary in the field, the naming of the two Caribbean white band diseases as separate aetiological entities should be re-examined. Conversely, in a study of coral tissue lesions in the Gulf of Aquaba (Red Sea), Ainsworth et al. (2007a) detected

signs of DNA fragmentation in some cases of coral tissue lesions by using molecular markers, but not in others, suggesting separate aetiologies of disease signs that might mistakenly look identical.

Accumulating data on the pathogenicity of *Vibrio* isolates has demonstrated that presumably the same bacterial species, *i.e.*, *Vibrio coralliilyticus*, is involved in causing multiple diseases of multiple species. *V. coralliilyticus* was first identified as the causative agent of coral bleaching in Zanzibar (Ben-Haim et al. 2002, 2003b). It was later identified as the causative agent of gorgonian coral mortalities in temperate waters in the Mediterranean Sea (Bally and Garrabou 2007) and the causative agent of WS in the Pacific (chapter 3; Sussman et al. 2008). In addition, *V. coralliilyticus* strains tested positive as the causative agent of fish and shrimp mortalities in exposure experiments (Austin et al. 2005), although the virulent strains tested were not isolated from field infected animals. *V. coralliilyticus* is known to be associated with oyster disease (Thompson et al. 2001), and *Vibrio coralliilyticus* affiliated sequences have also been retrieved from healthy looking corals (Bourne and Munn 2005). These findings complicate the understanding of coral disease, and have resulted in a number of hypotheses to explain the onset of disease, including those that characterize *Vibrio* strains as primary causative agents of disease, and those that question the role of *Vibrio* pathogens in cases where bacteria cannot be found on diseased corals (Ainsworth et al. 2008). Findings from this study clearly demonstrate that infectious diseases caused by *Vibrio* pathogens can be classified as multi-factorial diseases (Thrusfeld 2005), with pathogen virulence influenced by temperature, light availability and the presence of susceptible target hosts, in addition to other environmental factors, such as host density, which have been demonstrated to play a role in disease occurrence by other studies (Bruno et al. 2007). It is therefore difficult to conclude if coral pathogens all perform the same roles in various disease aetiologies, highlighting the need for further studies to fill gaps in these areas. Among the novel tools that will help to determine the origin and function of multiple virulent strains are sequencing protocols of higher resolution than those currently used in taxonomic identification, which are based on ribosomal (rRNA) gene sequencing.

5.3 Traditional culturing techniques are still a necessary tool for biomedical research on corals

In this study, a protocol involving travel to the site of a coral disease outbreak to collect samples from both diseased and healthy colonies has been demonstrated to be a highly successful strategy for disease investigation. Since diseased corals are not brought to hospitals, as in the case of other wildlife species, the isolation of putative pathogens from corals displaying disease signs in the field is a cost effective approach that may potentially lead to the identification of causative agents. Growing these isolates and transporting them to appropriate laboratory facilities is of great value for further examinations of their role in potential disease outbreaks.

The advantages of this methodology are numerous, including:

1. It is much more likely to find pathogens on freshly infected samples.
2. General and selective growth media for bacteria are commercially available.
3. Identifying the real causative agent is much more useful than obtaining proof of its statistical existence by employing molecular diagnostic means.

However, the disadvantages of this methodology are also numerous:

1. Not all pathogens are culturable.
2. Specific growth media and/or methodology have to be developed in order to successfully isolate and grow potential microbial agents, including many bacteria, fungi and even viruses, and this process is time consuming.
3. Isolated putative agents may gain or lose virulence in association with artificial growth conditions, or preservation.
4. Diseases may be multifactorial (with environmental and host components), preventing the detection of causative agents in laboratory exposure trials.
5. Non infectious diseases have causes that need to be established by other means.

This study used protocols aimed at identifying causative agents of coral disease by fulfilling Henle-Koch's postulates (Koch 1891), and by satisfying other rules and criteria (Hill 1965, Evans 1976). The successful isolation of cyanobacteria associated with BBD and red bands from Palau and the GBR required testing numerous media and selecting the ones most suitable for optimal growth. Similar methodologies resulted in obtaining cultures of *Symbiodinium* endosymbionts from coral samples that were later used in exposure experiments. These experiments would not have been possible without the prior isolation of both pathogens and target *Symbiodinium* populations, including the ability to grow them in culture. Furthermore,

these cultures are available for continuing experimentation in the future. Since this study identified coral diseases which are multi-factorial in nature, and since protocols aiming to identify pathogens by fulfilling Henle-Koch's postulates are often criticized for failure to duplicate environmental conditions that might be the primary source of disease, it is recommended to include a validation step in procedures to identify coral pathogens. Validation will require the development of specific diagnostic protocols that would confirm (in large scale studies) the presence or absence of pathogens on infected and non-infected corals in the field. The justification for such a validation step is that it only takes one sample to be able to retrieve a pathogen, which may duplicate disease signs, but statistical validation is required to confirm that the identified pathogen is in fact the real cause of an epizootic affecting an entire population by demonstrating its abundance on a sub-sample of all infected corals and its absence from a sub-sample of healthy corals. This step can also be tuned to identify the presence and absence of specific virulence genes (and their expression) and can rely on modern molecular tools, such as metagenomics and transcriptomics (in particular those novel methods that can run multiplex analyses of numerous samples performed in single platform runs at lower costs). We recommend performing this validation step only once sufficient information is acquired on the pathogen in question, its virulence and the aetiology of disease. This study did not undertake this extensive validation step, which is planned for future studies. The improved Henle-Koch'e postulates would be defined as follows:

1. The agent is present in all cases of disease and absent (or at low background levels) in healthy cases.
2. The agent can be isolated in pure culture from infected cases.
3. The agent can replicate the same disease signs in a healthy candidate.
4. The same agent can then be re-isolated from a newly infected host.
5. ***The presence of pathogens on all infected corals and their absence (or low abundance) on healthy corals should be validated by developing specific diagnostic tools and by conducting sampling efforts which take into account the estimated prevalence of disease.***

5.4 Are all coral diseases multi-factorial diseases?

Results from this study suggest that a number of coral diseases are multi-factorial in origin and require the synchronization of multiple disease-enabling or

disease-causing factors for successful epizootics to occur in the field. This study failed to infect healthy corals with cyanobacteria isolated from BBD infected corals and kept in culture. Although BBD was first reported almost four decades ago, many attempts to test the pathogenicity of isolated cyanobacteria in controlled infection experiments during the intervening years have not produced conclusive results (Taylor 1983).

Some authors have claimed that an entire bacterial consortium is required to cause successful BBD infections (Richardson et al. 1997a, Richardson 2004), however, although the identities of consortium members have been suggested (Richardson 1996, 2004), no successful attempts to infect healthy corals with the full consortium have been reported. In addition, most proposed consortium members, such as sulfur oxidizers and reducers, are commonly found on both healthy (Frias-Lopez et al. 2002) and diseased corals (Viehman et al. 2006), including diseased corals from Bermuda displaying disease signs other than BBD (Garrett and Ducklow 1975). In many locations where BBD has been observed in the field during this study (2003-2006), BBD has been observed to be associated with the occurrence of other diseases, such as WS infections in Palau and on the GBR (Lizard Island), and following coral tissue infestation by ciliates associated with brown band disease (Willis et al. 2004, Bourne et al. 2008). These observations, although preliminary, might suggest that cyanobacteria comprising the progressing BBD band may not be primary causative agents of disease. Aeby and Santavy (2006) have reported that successful laboratory infections with cyanobacteria isolated from BBD infected corals only occurred following predation of healthy corals by fish, similar to observed fungal infections on Caribbean corals following parrot fish bites (Cervino et al. 1998). Another study by Ainsworth et al. (2007a), suggested that many healthy looking colonies in the Gulf of Aquaba (Red Sea) are chronically infected with various strains of cyanobacteria. It might, therefore, be conceivable that BBD infections do not require primary cyanobacterial agents or vectors to initiate disease signs, but only a combination of disease facilitating factors related to both host and environment. Records of BBD epizootics, mainly in the Caribbean, suggest that infected coral colonies are not clustered (Edmunds 1991, Porter et al. 2001), in contrast to other infectious diseases (Kulldorff and Nagarwalla 1995, Jolles et al. 2002). A similar report has been made for WP infections in St. Lucia by Nugues (2002). However, other studies report opposite observations on clustering (Rützler and Santavy 1983, Antonius 1985, Kuta

and Richardson 1996, Bruckner and Bruckner 1997a, Bruckner et al. 1997), suggesting that further studies are needed in order to determine the prevalence of BBD associated cyanobacteria in field reservoirs and the aetiological steps which are required for infections to develop (Plowright and Sokolow 2008) and spread (Whittemore et al. 1987).

Recent studies have also detected toxins in cyanobacterial strains that have been isolated from corals displaying signs of black band disease (Richardson et al. 2007). These studies emphasise the critical role of cyanobacteria in causing coral tissue mortality regardless of their exact role within the black band disease web of causation (McMahon et al. 1960). Such findings can contribute to the development of accurate diagnostic tools that could identify specific virulence factors in field samples, which would aid in gaining a better understanding of the aetiology of multi-factorial coral diseases, such as BBD, and enable future attempts at disease prevention (Richardson 1998a).

This study concentrated on identifying bacterial virulence factors that are involved in disease progression following successful infections. A zinc-metalloprotease from the family of themolysins was derived from pathogen supernatants, identified, and its capacity to cause *Symbiodinium* photosynthetic inactivation and coral tissue lesions was demonstrated in replicated exposure trials. The ability to inhibit zinc-metalloprotease activity by EDTA enabled the development of two novel bioassays; a bioassay based on cultures of *Symbiodinium* cells and one based on cultures of coral juveniles. Possessing a bacterial virulence factor involved in WS aetiology allowed testing the hypothesis that WS is a multi-factorial coral disease by varying the exposure conditions of corals and *Symbiodinium* cultures in the presence of EDTA-inhibited and non-inhibited pathogen supernatants. Following these experiments, this study identified the following disease contributing factors for WS:

1. **Host factors**

   Susceptible *Symbiodinium* strains are more vulnerable to photosystem inactivation than non-susceptible strains. Some corals may be resistant to pathogen infections, or to pathogen virulence. Host density may become a necessary factor to enable disease transmission through the water column.

2. **Pathogen factors**

   The pathogen virulence factor, *i.e.*, zinc-metalloprotease, is excreted at high

bacterial cell densities, requiring close to optimal growth conditions for disease transmission and progression.

3. **Environmental conditions**

   Light availability increases the effect of pathogen virulence on *Symbiodinium* PS II centers. Optimal temperatures for pathogen growth increase the virulence of pathogens. Optimal temperatures for pathogen growth (27ºC) on GBR reefs extend over more days in warmer years than in colder years, adding to the potential of disease transmission. An alternating cyclic occurrence of warmer years, with optimal conditions for pathogen growth and disease transmission, followed by colder years, allowing for coral recovery, may potentially support the persistent occurrence and spread of WS on GBR reefs.

Other studies have also identified WS as a multi-factorial disease with temperature and host densities contributing to disease prevalence observed in the field (Bruno et al. 2007). This study has identified potential disease contributing factors by conducting laboratory exposure trials. Nevertheless, the laboratory-based findings from this study require additional validation steps by identifying the contribution of pathogen strains, light, temperature and susceptible hosts in targeted field studies that would determine:

1. Pathogen abundance in the field.
2. Abundance of susceptible hosts in the field.
3. The direct influence of environmental factors on infections and on coral mortality vs. survivorship in the field.

5.5 The role of climate change in the aetiology of coral infectious diseases

The steep increase in reports on coral diseases worldwide (Harvell et al. 1999, 2002) and the devastating impact of diseases, such as white band and white plague, on Caribbean reefs (Gladfelter 1982, Aronson and Precht 2001, Gardner et al. 2003, Laferty et al. 2004, Weil 2004) have concided with a growing public awareness of climate change (McMichael et al. 2006) and the threats to coral reefs that may unfold in the future as seawater temperatures continue to rise and ocean acidification increases (Wilkinson 2004, Stone 2007, Hoegh-Guldberg et al. 2007, Diaz and Rosenberg 2008, Carpenter et al. 2008).

Growing awareness of the value and health of marine environments has contributed immensely to the volume of surveillance efforts on reefs (Ward and Laferty 2004) and to the scientific quality of reports on coral health and disease. Nevertheless, despite successful attempts to model the future of reefs in conjunction with ocean warming (Hoegh-Guldberdg 1999, Knowlton 2001, Kleypas et al. 2001) and ocean acidification (Hoegh-Guldberdg et al. 2007), little is known about the possible effects of environmental co-factors (such as global warming) on the spread of coral infectious diseases and the health of marine environments. However, this knowledge gap has, at least, been recognized (Harvell et al. 2004, Laferty et al. 2004, Coelho and Manfrino 2007, Richardson and Poloczanska 2008). Unlike non-infectious coral diseases such as coral bleaching, which has been demonstrated to be caused directly by sea warming (Brown 1997, Fitt et al. 2001), far less is known about the causes and aetiology of coral infectious diseases, other than that they are assumed to be complex in nature (Bruno et al. 2007, Plowright et al. 2008, Sussman et al. 2009) and mostly unpredictable.

Observations conducted during this four year study of numerous sites throughout the Indo-Pacific where coral disease epizootics have been recorded, suggest that the emergence of coral diseases cannot be attributed to climate change alone. On many reefs, coral epizootics occur in one bay or at one site, but not in adjacent bays or sites, which are presumably under the same influence of ocean warming. These observations also engender a measure of hope, suggesting that coral infectious diseases might potentially be prevented by controlling some of their more accessible causes, such as host density (Bruno et al. 2007), vectors of transmission (Sussman et al. 2003), or anthropogenic stressors (Pandolfi et al. 2005).

This study demonstrated that the optimal temperatures for WS pathogen growth, elevated virulence and zinc-metalloprotease production remain within the range of 25ºC-30ºC. Not surprisingly, this temperature range currently prevails on many Indo-Pacific reefs, possibly explaining the current rise in WS abundance and spread on the GBR (Willis et al. 2004), including its direct link to ocean warming (Bruno et al. 2007). Current seawater temperatures, as presented for the GBR in this study, demonstrate that longer seasons of optimal growth conditions for coral pathogens may result in increased disease transmission and spread, as reported for other infectious diseases (Lindgren and Gustafson 2001). However, unless *Vibrio* WS pathogens adapt to even higher seawater temperatures (above 30ºC), they may

become vulnerable (Webster and Hill 2007), making it unlikely that the prevalence of WS will continue to increase in the future.

In this study, it was suggested that both coral WS and BBD may possess complex aetiologies. It was also demonstrated that light and temperature conditions on reefs change daily throughout the year, potentially suggesting that the future effects of ocean warming on coral reef health may be more complex than initially anticipated (Rosenberg et al. 2007), or as suggested by other studies (Baker et al. 2004, Berkelmans and van Oppen 2006). The study of causal hierarchies among coral disease-facilitating factors may potentially indicate that ocean warming may not be the dominant driver of all coral diseases, such as demonstrated for autumn outbreaks of WP disease in Puerto Rico, presumably driven by hurricane occurrence (Bruckner and Bruckner 1997b). As the dominance of WS on Indo-Pacific reefs may subside, new coral infectious diseases may emerge, suggesting that continued collection of data on disease and disease aetiology will remaine a priority.

5.6 Suggestions for future research on coral disease

This study is the first to identify coral pathogens on the GBR and on reefs in Palau and in the Republic of the Marshall Islands. It is also the first study to identify the taxonomic identity of cyanobacteria associated with BBD infections on the GBR and in Palau, as well as the identity of cyanobacteria associated with red bands in Palau. For such vast territories of coral reefs, this preliminary study is only the beginning.

The main recommendation resulting from this study is that a monitoring system to document the health of reefs, which is based on microbiological characterization, is greatly needed, and that this monitoring system should be combined with existing and future efforts to monitor coral health. In addition, access to sites where epizootics are occurring should be prioritized in order to allow for data and sample collection. Based on findings from this study, which has identified the taxonomic identity of six WS pathogens plus one of their virulence factors, as well as strains associated with BBD and red bands on corals, novel diagnostic tools should be developed for specific detection of pathogen reservoirs and infected corals. If pathogens are transmitted onto reefs that have previously been free of these agents, attempts should be made to limit the spread of disease. If endemic pathogens are natural inhabitants of these coral reefs, attempts should be made to understand the

conditions which spark local epizootics, their virulence and the ability of diseased reefs to recover.

In conclusion, the documented decline in coral reef cover worldwide and the future threats to coral reef health from ocean warming, acidification and anthropogenic pollution, pose an imminent need to study the role of coral infectious diseases in coral reef deterioration, resilience and evolution. Due to the many factors involved, it is not an easy task, and beyond surveillance efforts of disease outbreaks, novel protocols for coral disease research must be developed and tested. To protect coral reefs from future threats and aid in reef restoration, parallel investigations are required in disease-related fields, which do not include the study of causative agents, but the study of coral host immunity, coral genomics, coral proteonomics and environmental factors that may facilitate disease. Results obtained in this study demonstrate that better data collection using novel diagnostic tools, combined with elaborate laboratory screening trials performed with novel bioassays, used in conjunction with both old and new microbiological protocols have the potential to fill knowledge gaps and advance the study of coral infectious diseases.

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Appendices

Appendix I: Hill's criteria (Hill 1965)

1. Time sequence: Cause must precede effect.
2. Strength of association: a strong positive statistical association between a factor and disease.
3. Biological gradient: a dose response relationship between a factor and disease.
4. Consistency: If an association exists in a number of different circumstances, then a causal relationship is probable
5. Compatibility with existing knowledge: It is more reasonable to infer that a factor causes a disease if a plausible biological mechanism has been identified.

Appendix II: Evans' Rules (Evans 1976)

A. The proportion of individuals with the disease should be significantly higher in those exposed to the supposed cause than in those that are not.
B. Exposure to the supposed cause should be present more commonly in those with than those without the disease, when all other risk factors are held constant.
C. The number of new cases of disease should be significantly higher in those exposed to the supposed cause than in those not so exposed, as shown in prospective studies.
D. Temporally, the disease should follow exposure to the supposed cause with a distribution of incubation periods on a bell shaped curve.
E. A spectrum of host responses, from mild to severe, should follow exposure to the supposed cause along a logical biological gradient.
F. A measurable host response (e.g., antibody, cancer cells) should appear regularly following exposure to the supposed cause in those lacking this response before exposure, or should increase in magnitude if present before exposure; this pattern should not occur in individuals not so exposed.
G. Experimental reproduction of the disease should occur with greater frequency in animals or man appropriately exposed to the supposed cause than in those not so exposed; this exposure may be deliberate in volunteers, experimentally induced in

the laboratory, or demonstrated in a controlled regulation of natural exposure.

H. Elimination (e.g., removal of a specific infectious agent) or modification (e.g., alteration of a deficient diet) of the supposed cause should decrease the frequency of occurrence of the disease.

I. Prevention or modification of the host's response (e.g., by immunization or use of specific lymphocyte transfer factor in cancer) should decrease or eliminate the disease that normally occurs on exposure to the supposed cause.

J. All relationships and associations should be biologically and epidemiologically credible.

Appendix III: Published Papers from this Study

Sussman M, Bourne DG, Willis BL (2006) A single cyanobacterial ribotype is associated with both red and black bands on diseased corals from Palau. Dis Aquat Organ 69: 111-118.

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