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Characterization of the coral disease '*Porites* bleaching with tissue loss' (PBTL) from Hawaii

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This thesis is dedicated to my parents

Gabriele and Volker Sudek

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Abstract

Coral reefs around the world are facing many threats and have sustained severe losses in coral cover over the past few decades. Coral bleaching and disease outbreaks have contributed substantially to this reef decline, however our understanding of factors contributing to the increase in coral disease prevalence are poorly understood. Information on the disease dynamics of different diseases affecting a reef system is essential for the development of effective management strategies.

The aim of this research was to characterise and build a case study of a bleaching response affecting *Porites compressa* in Kaneohoe Bay, Oahu, Hawaii. It manifests as a localised, discrete area on the coral colony with a bleached coenenchyme and pigmented polyps, giving the affected area a "speckled" appearance. A disease by definition is any interruption, cessation or disorder of body functions, systems or organs. Results of this study showed that this localised bleaching causes tissue loss and a reduction in the number of gametes, and hence harm to the host. It was therefore classified as a disease and named *Porites* bleaching with tissue loss (PBTL). In addition, PBTL does not appear to represent a common thermal bleaching response as it was present throughout the year during times when seawater temperature was well within the coral's thermal threshold.

Symbiodinium cell density in PBTL-affected areas of the coral colony was reduced by 65%, and examination of affected host tissue using light microscopy showed fragmentation and necrosis. However, no potential pathogen was observed. Transmission electron microscopy (TEM) revealed a high occurrence of potential apoptotic *Symbiodinium* cells and a potential increase in the abundance of virus-like particles (VLPs) in PBTL-affected tissue. However a causal relationship remains to be established.

Long-term monitoring showed spatio-temporal variations in PBTL prevalence. Temporal variations in prevalence reflected a seasonal trend with a peak during the summer months, linked to increasing seawater temperature. Spatial variations in disease prevalence were correlated with parrotfish density, turbidity and water motion. Of these, a negative correlation with variability (SD) in turbidity explained most of the variability in PBTL prevalence (12.8%). A positive correlation with water motion explained 9% and a positive correlation with the variability in parrotfish density explained 4.4%. Overall, only a relatively small

proportion of variability in PBTL prevalence could be explained by these three factors (26.2%), suggesting that other factors, not investigated in this study, play a more important role in explaining PBTL patterns or that temporal variation in temperature is the overall major driving force.

Monitoring of individually tagged *P. compressa* colonies showed that >80% of affected colonies sustained partial colony mortality (tissue loss) within two months; on average, one third of the colony is lost. The amount of tissue loss sustained was correlated to lesion size but not colony size. Case fatality (total mortality) was low (2.6%), however this disease can affect the same colonies repeatedly, suggesting a potential for progressive damage which could cause increased tissue loss over time. PBTL was not transmissible through direct contact or the water column in controlled aquaria experiments, suggesting that this disease might not be caused by a pathogen, is not highly infectious, or perhaps requires a vector for transmission. At present, PBTL has only been observed within Kaneohe Bay.

An investigation of the potential role of host and *Symbiodinium* genetics in disease susceptibility revealed the same *Symbiodinium* sub-clade (C15) in healthy and PBTL-affected colonies, suggesting no involvement of *Symbiodinium* type in disease etiology. Results regarding host genetics remained inconclusive; however a difference in allele frequency at one microsatellite locus was observed between healthy and diseased samples. This difference could, however, be due to a lower amplification of PBTL-affected samples at this locus and needs to be regarded with some caution.

The results of this study provide a case definition of PBTL which can be used as a baseline in further studies. *P. compressa* is the main framework building species in Kaneohe Bay, and the information gathered here on disease dynamics and virulence suggests that PBTL has the potential to negatively impact the resilience of reefs within the bay. Further research into the etiology of PBTL is necessary to fully understand the impact that this disease could have on coral reefs in Hawaii.

List of abbreviations

AIC	Akaike's Information Criterion
BBD	Black band disease
BrB	Brown band disease
Chl-*a*	Chlorophyll *a*
CIMR	Coconut Island Marine Reserve
CFU	Colony forming unit
DISTLM	Permutational distance-based linear model
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DSS	Dark sport syndrome
EDTA	Ethylenediaminetetraacetic acid
ENSO	El Niño-Southern Oscillation
GA	Growth anomaly
GASW	Glycerol artificial seawater media
GBR	Great Barrier Reef
HCl	Hydrochloric acid
HIMB	Hawaii Institute of Marine Biology
HWE	Hardy-Weinberg Equilibrium
ITS	Internal transcribed spacer region
M	Molarity (moles/L)

$MgCl_2$	Magnesium chloride
MHI	Main Hawaiian Islands
MWS	*Montipora* white syndrome
NOAA	National Oceanic and Atmospheric Administration
NWHI	Northwestern Hawaiian Islands
PBTL	*Porites* bleaching with tissue loss
PERMANOVA	Permutational multivariate analysis of variance
PCD	Programmed cell death
PCO	Principal Coordinates Analysis
PCR	Polymerase chain reaction
PO	Prophenoloxidase
PorTL	*Porites* tissue loss
PRIMER	Plymouth Routines in Multivariate Ecological Research
PUWS	*Porites* ulcerative white spot disease
RBD	Red band disease
rDNA	Ribosomal DNA
ROS	Reactive oxygen species
SD	Standard deviation
SE	Standard error
spp	Species
SPSS	Statistical Package for the Social Sciences
SST	Sea surface temperature

TCBS	Thiosulfate citrate bile salts sucrose agar
TEM	Transmission electron microscopy
UV	Ultraviolet
VLP	Virus-like particle
WBD	White band disease
WP	White plague
WPS	White patch syndrome
WS	White syndrome
YBD	Yellow band/blotch disease

Glossary

Allele: a variant segment of a gene that is located at a specific position on a chromosome (one pair for any particular gene in diploid organisms)

Allelochemical: a chemical that is produced by a living organism and released to exert a detrimental physiological effect on other organisms

Antimicrobial: destroying or inhibiting the growth of microorganisms, especially pathogenic microbes

Apoptosis: a process of programmed cell death that leads to cell shrinkage, nuclear fragmentation, chromatin condensation and formation of apoptotic bodies; can also be triggered by an external factor

Clade: monophyletic group of taxa

Codominant: expression of heterozygote phenotypes that differ from the homozygote phenotypes. Microsatellites are codominant genetic markers as heterozygotes (two bands) can be distinguished from homozygotes (one band)

Coenenchyme: the tissue (mesoglea) surrounding and uniting the polyps in colonial anthozoans

Cytopathology: the diagnosis of disease changes on a cellular level

Cytoplasm: all material within a living cell, excluding the nucleus

Diploid: a double complement of chromosomes (generally a maternal and a paternal set)

Disease: any interruption, cessation or disorder of body functions, systems or organs

Disease incidence: number of new disease cases within a population during a certain time period

Disease severity: amount of tissue loss

Disease prevalence: the number of disease cases in a given population at a specific point in time (usually expressed as percentage)

Disease progression: rate of spread of the disease on a coral colony

Electrophoresis: polarized gel (often agarose) in which DNA or proteins can be run to separate them by weight or polarity

Eosinophilia: cells or tissues that stain readily with eosin dyes and appear pink to red when using a hematoxylin and eosin staining procedure

Epizootic: disease outbreak in an animal population

Etiology: study of disease causation and its mode of operation

Flanking region: the stretches of DNA outside the sequence tandem repeats (microsatellites), which are used as primer binding sites

F-statistics: a measure of genetic structure:

$\mathbf{F_{ST}}$ = proportion of total genetic variance contained in a subpopulation relative to the total genetic variance (0 = identical, higher values imply a degree of differentiation)
$\mathbf{F_{IS}}$ (inbreeding coefficient) = proportion of the variance in the subpopulation contained in an individual (high values imply a degree of inbreeding)

Gene frequency: the number of a specific type of allele in comparison to the total number of alleles in the genepool

Genetic drift: the change in allele frequency in a population due to random sampling (some individuals have more offspring than others causing changes in allele frequencies over time; neutral alleles will eventually become fixed at 0 or 100% frequency if no other factors affect allele distributions)

Genotype: the genetic makeup of an organism

Growth anomaly: abnormalities of coral tissue and skeleton, for example tumours

Hardy-Weinberg equilibrium: allele frequencies remain constant unless disturbing influences such as non-random mating and mutations affect a population

Heterozygosity: possession of two different alleles of a particular gene (one inherited from each parent)

Heterozygosity (expected): also known as gene diversity, is an individual or population-level parameter and calculated as the proportion of loci expected to be heterozygous in an individual based on Hardy-Weinberg expectations

Histopathology: study of the microscopic manifestation of disease

Holobiont: an organism with all its associated microbial community

Homozygosity: possession of two identical alleles of a particular gene (one inherited from each parent)

Hyaline: a homogenous, structureless, fibrous, eosinophilic substance occurring in tissue degradation

Hypereosinophilia: an extreme degree of eosinophilia

Infectious disease: also known as transmissible disease; a disease that is caused by the infection of a transmissible disease agent, such as bacteria, viruses, fungi or parasites

Karyolysis: swelling of the nucleus and gradual loss of its chromatin

Karyorrhexis: rupture of the nucleus with the chromatin disintegrating into small pieces

Koch's Postulates: four criteria used to establish a causal relationship of a potential causative agent and a disease: 1) the organism has to be commonly found in association with the disease but not in healthy organisms; 2) isolation of the organism and growth in pure culture; 3) the cultured organism must cause disease when inoculated with an healthy organism; 4) the organism has to be re-isolated from the inoculated, diseased host and identified as the same organism as the original causative agent

Large allele drop out: preferential amplification of shorter alleles

Lesion: anatomic change associated with disease, also includes tissue injuries due to physical damage, for example fish bites or anchor damage

Light microscopy: a microscope which uses a combination of lenses and lights to magnify small objects held on a slide

Locus: a stretch of DNA on a particular chromosome – often used for gene in the broad sense (analyzed for variability, e.g. microsatellite marker)

Lysis: breakdown of a cell due to compromised cell integrity

Mesentery: internal longitudinal partition of tissue which provides structural support and an increased surface area

Mesogloea: connective tissue of cnidarians

Microsatellites: short tandem repeats of nucleotide sequences

Necrosis: process of cell death that causes cell swelling, chromatin digestion and disruption of the plasma and organelle membranes. Induced by external factors such as trauma or infection

Null allele: an allele that is present in a sample but not amplified. A mutation at the primer binding site can cause inhibition of the amplification of an allele resulting in a null allele. Therefore, a heterozygous individual with a primer binding site mutation for one of the alleles will be typed as homozygote.

Parasite: an organism that lives on or within a host and benefits at the expense of the host

Pathogen: a disease-causing agent/microorganism

Polymicrobial: the presence of several different microorganisms

Population: a group of individuals of the same species living in a given area that can interbreed

Programmed cell death: cell death mediated by a regular, intracellular program that is of advantage during an organism's life-cycle

Pyknosis: condensation or reduction in cell or nucleus size

Symbiont: an organism living in a symbiotic relationship

Symbiosis: a mutually beneficial relationship of two or more organisms

Syndrome: the signs and symptoms that comprise disease

Transmission: the passing of a disease from an infected individual host or group to another individual or group

Transmission electron microscopy: a microscopy technique using a beam of electrons which is transmitted through an ultra-thin section and forms a highly magnified image on a screen

Wahlund effect: a reduction in heterozygosity in a population due to a subpopulation structure

Vector: an agent that carries and transmits a pathogen to another organism

Virus-like particle: particles that resemble potential viruses

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Table 5.4 F_{ST}, $F_{IS,}$ R_{ST} and R_{IS} values for an estimation of genetic differentiation of healthy and PBTL-affected samples for all loci combined and individual loci comparisons. Values were computed using an AMOVA with both non-interpolated and interpolated missing data. Significance level for fixation indices: * = $p < 0.05$ and ** = $p < 0.005$. (A Bonferroni-correction for this sample size recommends a significance level of $p < 0.005$). 96

Table 5.5 Shannon index analyses based on a G-test using logbase 2 for individual loci comparisons. Significance level for fixation indices: * = $p < 0.05$ and ** = $p < 0.005$. (A Bonferroni-correction for this sample size recommends a significance level of $p < 0.005$). 96

Table 5.6 Number of samples showing a 100% and 99% match to C15. 97

Chapter 1

General Introduction

Coral reefs sustain an incredible biodiversity, with 32 of the 34 recognised animal phyla found in reef ecosystems compared to only nine phyla in tropical rainforests (Wilkinson 2008). Not surprisingly, coral reefs provide the livelihood for many coastal populations, especially in third-world countries, with over 30 million people being almost completely dependent on reefs as their source of animal protein (UNEP 2006; Wilkinson 2008). The extraordinary beauty and biodiversity of coral reefs also attract a thriving and valuable dive tourism and recreation industry which generates an estimated net benefit of US$30 billion each year (UNEP 2006). Marine tourism generates approximately US$800 million each year in Hawaii (Friedlander et al. 2008b) and over US$1.2 billion annually in the Florida Keys alone (UNEP 2006).

Despite their ecological and commercial importance, coral reefs have suatained severe degradation throughout the world (UNEP 2006). Most tropical reefs are found in developing countries near major urban centres, with over 30% of the world's population living within 50 km of a coral reef (UNEP 2006). Anthropogenic impacts such as pollution, over-fishing and sedimentation place high pressure on reef systems, with global warming being one of the biggest threats (Wilkinson 2002). It has been estimated that 19% of the world's coral reefs have already been lost with a further 35% critically threatened (Wilkinson 2008). Coral reefs in the Caribbean and Southeast Asia have experienced the highest rates of degradation and will most likely continue to be the most threatened (UNEP 2006). It is therefore essential that research targets areas of high importance that will contribute to the development of successful management plans. For example, coral bleaching, defined as the disruption of the symbiosis between the dinoflagellate symbionts and their coral host in response to stress (Hoegh-Guldberg & Smith 1989; Glynn & Croz 1990; Hoegh-Guldberg & Salvat 1995; Glynn 1996; Jones et al. 1998), has caused mass mortality of corals in numerous locations (Wilkinson et al. 1999). Another serious threat to the functioning and resilience of coral reef ecosystems is coral disease (Richardson 1998; Harvell et al. 1999). The prevalence and geographic range of coral diseases, as well as the number of coral species affected have all increased over the last few years (Sokolow 2009), however the field of coral disease research

is still in its early stages and our knowledge of the causes and effects of most diseases are still rudimentary.

1.1 Coral Bleaching

Cnidarian stony corals form a symbiosis with protozoan dinoflagellates from the genus *Symbiodinium* ('zooxanthellae'). This mutual symbiosis is based on nutritional exchange as the *Symbiodinium* cells can translocate > 90% of their photosynthetically-fixed carbon, sugars, amino acids, carbohydrates and perhaps lipids to the coral host (Muscatine & Porter 1977; Trench 1979; Muscatine 1990). The coral on the other hand supplies its unicellular symbionts with inorganic nitrogen, phosphorous and a stable environment (Muscatine et al. 1984; Trench 1979; Yellowlees et al. 2008) which appears to be essential in an environment that provides few inorganic nutrients (Hoegh-Guldberg 1999).

Photosynthesis by the *Symbiodinium* cells can be very efficient in the high-light environment of coral reefs. This process generates high concentrations of dissolved oxygen which can then form reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and superoxide (O_2^-) (Tchernov et al. 2004; Lesser 2006). ROS cause severe cellular damage, for example by denaturing proteins, oxidising membranes and damaging nucleic acids (Lesser and Farrell 2004; Richier et al. 2005; Lesser 2006). Both the host and the dinoflagellates have evolved adaptations to prevent cellular damage by expressing a broad array of ROS-handling antioxidant enzymes such as catalase, ascorbate, peroxidase and superoxide dismutase (Richier et al. 2005; Lesser 2006). These enzymes convert ROS back to oxygen and water (Weis 2008).

Environmental stressors however, can cause photoinhibition as well as damage to the chloroplast and photosynthetic apparatus in various ways (Weis 2008). Oxidative stress continues to build up and finally overwhelms the antioxidant defence system causing an accumulation of ROS which further damages photosynthetic membranes (Lesser 1996, 2006; Franklin 2004). In addition, ROS begin to diffuse into the host tissue where they cause further damage (Tchernov et al. 2004; Lesser 2006) and consequently lead to the symbiotic algae being either expelled or degraded (see Weis 2008 for different mechanisms of symbiont loss). This process is known as coral bleaching defined as the loss of the symbiotic dinoflagellates and/or their pigments from the coral host (Glynn 1993; Brown 1997).

Symbiodinium cells are golden brown due to photosynthetic pigments in their chloroplasts. Corals harbour millions of these algal cells in their tissues which contribute to most of their colouration (Weis 2008). When these symbiotic dinoflagellates are lost, the underlying white calcium carbonate skeleton is revealed, giving the coral a bleached appearance. Coral bleaching can be caused by several environmental stimuli such as high or low seawater temperatures (Glynn and D'Croz 1990; Gates et al. 1992; Winter et al. 1998), high UV radiation (Gleason and Wellington 1993; Drollet et al. 1995), changes in salinity (Fang et al. 1995), increased sedimentation (Wessling et al. 1999), pollutants (Jones and Steven 1997) and pathogens (Kushmaro et al. 2001).

Coral bleaching can range from local scales (Goreau 1964; Egna and DiSalvo 1982) to entire reef systems at geographical scales, when it is referred to as mass bleaching (Glynn 1984; Williams and Williams 1990; Hoegh-Guldberg and Salvat 1995; Kenyon et al. 2006). The most severe and geographically-extensive mass bleaching event occurred in 1998, coinciding with a particularly hot El Niño-Southern Oscillation (ENSO) event, and causing an estimated mortality of 16% of the world's corals (Reaser et al. 2000; Hughes et al. 2003). In 2010, another large-scale bleaching event was recorded in South-East Asia. Interestingly, bleaching was less severe at locations that experienced severe bleaching in 1998, suggesting a possible adaptation and/or acclimatization to thermal stress (Guest et al. 2012).

Elevated sea surface temperature appears to be the primary factor that triggers mass coral bleaching events (Glynn 1993; Hoegh-Guldberg 1999). Temperature increases of as little as 1-2^{o}C above the mean summer maximum persisting for several consecutive weeks can lead to coral bleaching (Jokiel and Coles 1990; Bruno et al. 2001; Cumming et al. 2002). Many coral species therefore live very close to their upper thermal limits (Jokiel and Coles 1990) demonstrating the severe threat that global warming can pose for the survival and resilience of coral reefs.

In 1996 it was shown for the first time that a bacterial infection could also invoke coral bleaching. Kushmaro et al. (1996) found that bleaching of the Mediterranean coral *Oculina patagonica* was caused by the *Vibrio* strain AK-1. The adhesion of AK-1 to the coral is thermally regulated and occurs only when seawater temperatures are elevated, as found during the summer (Kushmaro et al. 1997; Kushmaro et al. 1998; Banin et al. 2000). AK-1 then produces toxins that inhibit photosynthesis, and cause bleaching and lysis of the symbiotic dinoflagellates (Ben-Haim et al. 1999). DNA sequencing suggests that the AK-1

strain is a new species, *Vibrio shiloi* sp. nov., which is the first species of *Vibrio* shown to infect symbiotic dinoflagellates (Kushmaro et al. 2001). In the Red Sea, a similar case was observed. The widely distributed bacterium *Vibrio coralliilyticus* was shown to cause bleaching in the coral *Pocillopora damicornis*, with elevated temperature being the major driving force (Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003). These observations led to the Bacterial Bleaching Hypothesis (Rosenberg and Ben-Haim 2002; Rosenberg 2004; Rosenberg and Falkovitz 2004) which proposes that a microbial infection is a primary trigger of coral bleaching. However, Ainsworth et al. (2008a) found no evidence to support this hypothesis and strongly argued against its generalization. They suggested that the bacterial infection is opportunistic rather than a primary pathogenic cause of bleaching and that non-microbial environmental stressors trigger coral bleaching in *O. patagonica*.

Even though the role of bacteria in coral bleaching remains unclear it appears that a positive link between coral bleaching events and subsequent coral disease epizootics exists. In 2005, an unusual warm summer season caused extensive bleaching in the eastern Caribbean (Miller et al. 2006; Eakin et al. 2010). Eventually, coral colonies recovered their pigments but a subsequent infection with white plague disease caused significant coral mortality resulting in a 26-48% reduction of coral cover in this one event alone (Miller et al. 2006). Coral bleaching may compromise the health of corals and make them more susceptible to pathogenic infection. This hypothesis has been supported by reports of increased disease prevalence after bleaching events (Muller et al. 2008; Brandt and McManus 2009; Croquer and Weil 2009a; McClanahan et al. 2009). This synergistic effect is likely to be of significance as global warming will cause higher seawater temperatures and subsequently higher frequencies of coral bleaching events (Hoegh-Guldberg et al. 2007).

1.2 Coral disease

1.2.1 A short history

Coral disease research is a relatively young field with the first descriptive reports appearing between 1965 and the late 1970s. The earliest report was by Squires (1965), who described skeletal growth anomalies in the deep water coral *Madrepora kauaiensis.* The next diseases described were black band disease (BBD) (Antonius 1973), white band disease (WBD) of branching acroporid corals (Gladfelter et al. 1977) and white plague (WP) of massive and plate-forming corals (Dustan 1977). Although these diseases cause tissue-loss rates of several millimetres a day, only limited efforts were made to identify the pathogens (Richardson

1998). Through microscopic observations of diseased tissue the presence of various bacteria was revealed but no attempts to fulfil Koch's postulate were made (Richardson 1998).

In the 1980s, a disease that affected an entire colony at once and caused rapid tissue loss in scleractinian corals was discovered and named 'shut down reaction' (Antonius 1981). At the same time it was also seen for the first time that marine diseases can cause major population declines, resulting in community shifts and threatening biodiversity. This was demonstrated by the disease-induced mass mortalities of the Caribbean sea urchin *Diadema antillarum*, which in combination with two hurricanes and an outbreak of white-band disease resulted in a major decline in coral cover and ended in a phase shift from a coral to an algal dominated reef (Hughes 1994). The two main framework-building coral species in the Florida Keys, *Acropora palmata* and *A. cervicornis*, have sustained losses of up to 90% due to white band disease (Gladfelter 1982; Weil et al. 2006) and are now placed on the IUCN Red List of Threatened Species as being critically endangred.

In the 1990s, further research efforts led to the discovery of many more diseases including red band disease, yellow band/blotch disease (YBD), dark spot disease, white pox, sea fan disease (aspergillosis) and rapid wasting disease (Richardson 1998). However, most coral diseases have only been described on the basis of macroscopic features and are therefore unreliable for accurate disease diagnosis (Ainsworth et al. 2007a). An example is the case of rapid wasting disease; shortly after its discovery the cause of it was identified as fish bites rather than a pathogen (Bruckner and Bruckner 1998). Different names have also been given to different successive stages of the same disease, leading to confusion in the literature (Richardson 1998). To avoid further confusion, a consistent and standardized terminology is required for the gross description of coral lesions. Work & Aeby (2006) presented such a systematic approach for describing gross lesions in corals, and Ainsworth et al. (2007a) demanded an incorporation of microbial, cytological and physiological studies in coral disease research to provide a better understanding and diagnosis.

1.2.2 Prevalence

Coral diseases have been reported on reefs worldwide (Fig. 1), however the majority of coral disease research has focused on the Caribbean (e.g. Goreau et al. 1998; Kim et al. 2000a, 200b; Cervino et al. 2001; Patterson et al. 2002; Borger 2005; Croquer and Weil 2009a, 2009b) which has been labelled a 'disease hot spot' due to the fast emergence of new diseases (Weil et al. 2006). Other well-studied areas are the Florida Keys (Holden 1996; Kuta and

Richardson 1996; Richardson et al. 1998a, 1998b; Porter et al. 2001; Richardson and Voss 2005), the Red Sea (Winkler et al. 2004; Barneah et al. 2007), the Great Barrier Reef (GBR) (Willis et al. 2004; Ainsworth et al. 2007a; Boyett et al. 2007; Bourne et al. 2008), and Hawaii (Aeby 2005; 2007; Domart-Coulon et al. 2006; Aeby et al. 2010; Burns et al. 2011).

In contrast to the Caribbean where coral diseases have been reported for over 30 years, disease research in the Indo-Pacific has only been carried out over the last decade or so (Selig et al. 2006) and surveys in the remote Pacific have only very recently been conducted (Williams et al. 2008; 2011a; Vargas-Angel 2009). The steady rise in disease reports indicates an increase in emerging marine diseases (Harvell et al. 1999, 2002, 2004), however the lack of baseline studies makes it difficult to assess normal disease levels in the ocean (Harvell et al. 1999; Ward and Lafferty 2004). Disease prevalence is defined as the percentage of diseased individuals in a population. It can vary greatly depending on geographic location and coral species affected. For example, growth anomalies (GA) in *Porites* spp. in the Philippines can be as high as 39.1% (Kaczmarsky 2006), while they showed a prevalence of 21.7% at a particular site in Kaneohe Bay, Oahu (Domart-Coulon et al. 2006). In contrast, GA's in *Acropora* spp. showed a prevalence of 0% at Johnston Atoll and up to 3% in American Samoa (Work et al. 2008a).

1.2.3 Environmental factors

It is thought that degrading environmental conditions due to anthropogenic impacts and global warming have largely contributed to the increase in marine diseases, however the links are still poorly understood (Harvell et al. 2004; Sokolow et al. 2009).

It can be expected that a variety of factors simultaneously influence disease prevalence within a system, the relative importance of each varying among regions, scales and species (Bruno et al. 2003, 2007). Disease prevalence can be affected by temperature (Jones et al. 2004; Bruno et al. 2007), water quality (Bruno et al. 2003), coral cover (Bruno et al. 2007) and vector density (Aeby and Santavy 2006). To further our understanding of the potentially complex web of interactions between different environmental factors, Williams et al. (2010) proposed the use of predictive statistical modelling. Investigating coral disease-environment interactions is essential (Ainsworth and Hoegh-Guldberg 2009) for the development of future management and protections plans.

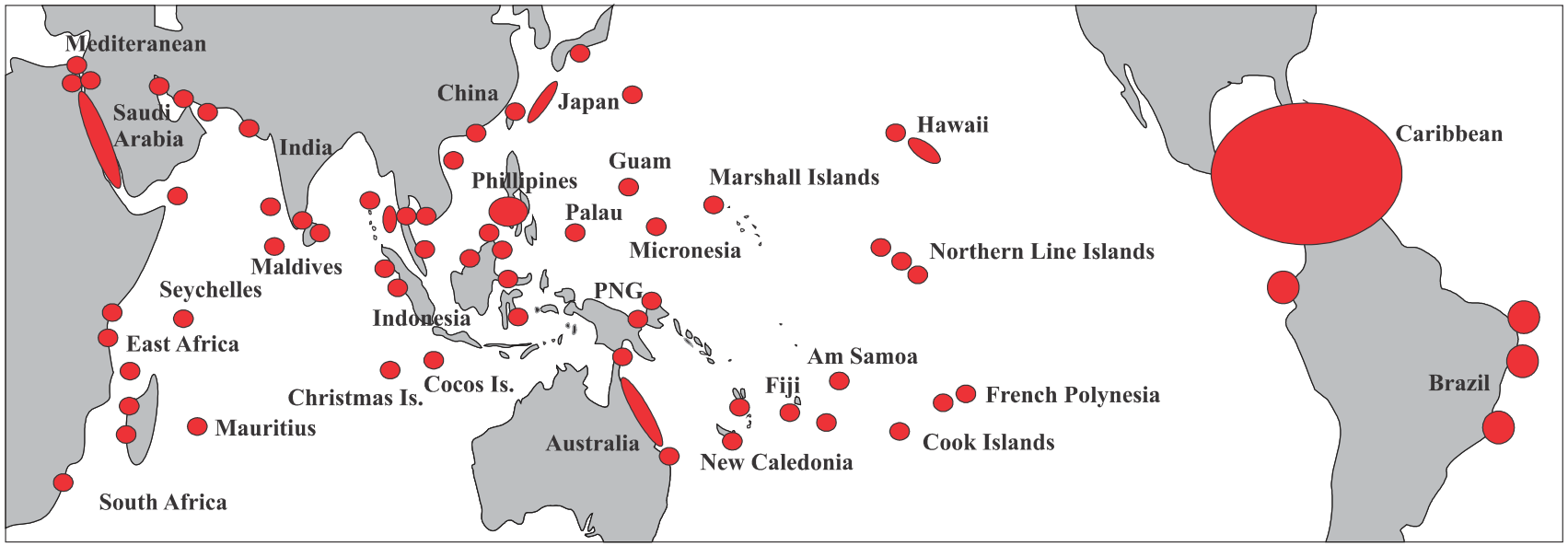


Fig. 1.1: Coral disease reports worldwide (red circles/ellipses only represent the geographical area from which diseases have been reported and are not representative of disease effects) (after Bourne et al. 2009).

1.2.3.1 Temperature

On land and in the ocean, a wide variety of species are affected by temperature sensitive disease outbreaks (Harvell et al. 2009). Coral reefs are particularly affected by small increases in temperature, which can cause coral bleaching and mortality, and can also facilitate disease outbreaks (Bruno et al. 2007; Hoegh-Guldberg et al. 2007; Harvell et al. 2009). The mechanisms of disease sensitivity to climate change are poorly understood (Harvell et al. 2002; 2007) but it has been hypothesised that elevated sea surface temperatures can increase pathogen virulence and host susceptibility (Harvell et al. 1999; 2002; Lesser et al. 2007). In at least three cases in which a coral pathogen was cultured (*Vibrio shiloi*, *Vibrio coralliilyticus*, *Aspergillus sydowii*), a positive relationship between pathogen growth and temperature was found (Banin et al. 2000; Alker et al. 2001; Ben-Haim et al. 2003).

In the Caribbean, the prevalence and virulence of YBD in *Montastraea* spp. has increased significantly between the 1990s and the mid 2000s, showing an interesting co-variation with the increase in the annual mean seawater temperature (Harvell et al. 2009). The once seasonal pattern of high lesion growth rates during the summer and low growth rates or complete disappearance of lesions during the winter has now been replaced by a significant increase of lesion growth rates throughout the year (Harvell et al. 2009). The observed significant positive relationship of lesion growth rate and mean water temperature provides evidence for a direct influence of temperature on YBD development (Harvell et al. 2009). Since 2002, winter average temperatures have not dropped below 26.5°C, which is 1°C above the average winter temperature of previous years causing YBD to remain common even during winter as growth and pathogenicity seem to be favoured by the warmer conditions (Harvell et al. 2009).

Increased coral disease prevalence and/or disease progression has been observed worldwide in connection with warm summer temperatures or during temperature anomalies, such as El Niño events (Kuta and Richardson 2002; Patterson et al. 2002; Riegl 2002; Jones et al. 2004; Boyett et al. 2007; Bruno et al. 2007). However, seasonal changes in disease severity are very likely to also be influenced by other factors that vary seasonally (Bruno et al. 2007). Irradiance for example, in combination with elevated sea surface temperatures, promotes the progression of BBD on the GBR (Boyett et al. 2007; Sato et al. 2011).

Interestingly, short term thermal stress increased host resistance to infection by *Aspergillus sydowii* (measured as the production of antifungal metabolites) in the sea fan coral *Gorgonia ventalina* (Ward et al. 2007). However, pathogen growth rate also increased with

temperature, emphasizing the importance of examining the dual effect of temperature on the host-pathogen interaction (Ward et al. 2007).

Evidence points towards a link between climate change and coral disease, however a lack of baseline studies in addition to incomplete disease time-series makes it difficult to distinguish between the effects of global warming and anthropogenic disturbance (Harvell et al. 2002). To better understand the potential links between temperature and disease outbreaks, large scale, longitudinal studies have to be carried out that combine long-term monitoring of several populations with precise temperature measurements, i.e. the variability of disease severity over years and locations (Bruno et al. 2007).

1.2.3.2 Water Quality

Another factor of concern is coastal marine eutrophication, or nutrient pollution, which is mainly related to an increase of phosphorus and nitrogen through terrestrial run-off and sewage (Nixon 1995; UNEP 2006). Even 'pristine' reefs could be under threat in the near future (Bruno et al. 2003) as the degree of nutrient pollution will increase with human population density and coastal development (Nixon 1995).

In the US Virgin Islands, disease prevalence of BBD and WP was positively correlated with exposure to sewage (Kaczmarsky et al. 2005). In the Caribbean, an experimental increase in nutrient concentration (nitrate, phosphorus and ammonium) increased the severity of both aspergillosis in sea fans and YBD in *Montastraea* spp. (Bruno et al. 2003). Black band disease progression in the Caribbean was also positively affected by nutrient enrichment (Voss and Richardson 2006) and the concentration of phosphorus was positively associated with *Porites* growth anomalies in Kenyan reef lagoons (McClanahan et al. 2009). Baker et al. (2007) found a significant relationship between aspergillosis in sea fans and nutrient levels, but interestingly disease severity (tissue loss) and prevalence were related to different nutrient parameters and timescales. Aspergillosis prevalence was only positively correlated to nitrogen if a multi-year average was used, whereas disease severity showed a positive link to the ratio of nitrogen and phosphorus during both short and long terms (Baker et al. 2007).

These findings lead to the hypothesis that nutrient enrichment could potentially enhance pathogen fitness and virulence (Bruno et al. 2003), however other environmental drivers with wider regional effects, such as elevated sea surface temperatures, might be more important

contributors to the observed increase in coral disease prevalence, especially in more pristine reef ecosystems (Voss and Richardson 2006).

1.2.3.3 Coral cover and host density

The influence of coral cover and host density on coral disease prevalence differs depending on geographic location, species and disease. *Porites* spp. are the dominant host on reefs in the Philippines but no correlation between disease prevalence and coral cover was found (Raymundo et al. 2005). Similarly, on the GBR, the spread of black band disease was not dependent on coral cover (Page and Willis 2006). In contrast, white syndrome (WS) on the GBR was most abundant on reefs with the highest percent hard coral cover (Willis et al. 2004) with a coral cover threshold of approximately 50% necessary for an outbreak of WS to occur (Selig et al. 2006; Bruno et al. 2007). In Guam, coral disease prevalence was strongly correlated to host abundance (Myers and Raymundo 2009) and the highest levels of *Porites* trematodiasis in Kaneohe Bay, Hawaii were found in areas with intermediate coral cover (Aeby 2007).

A link between high coral cover and disease prevalence is perhaps not surprising, as high host cover reduces the distance between infected and healthy hosts which increases the potential for horizontal disease transmission (Lafferty et al. 2004; Bruno et al. 2007). Moreover, competition among corals increases with coral cover (Connell et al. 2004) resulting in physiological stress and a reduction in fitness (Tanner 1997), which could contribute to a reduction in disease resistance (Bruno et al. 2007).

1.2.3.4 Vectors and reservoirs

Some infectious agents require vectors to transmit critical life-history stages from one host to another. This can be mechanical (simple transport from one host to the next) or biological (occurrence of a critical life-history stage of the pathogen within the vector) (Work et al. 2008c). Pathogens transmitted by vectors appear to be more common in terrestrial than in marine environments, although this could partly be due to the insufficient study of potential marine vectors (McCallum et al. 2004).

Several studies have suggested corallivorous fish as vectors for coral diseases. Chong-Seng et al. (2010) used *in situ* video observations to assess if and to what extend reef fish feed on coral disease lesions. They found that several different fish species actively feed on lesions highlighting their importance in potential disease transmission but also their role in the

removal of disease lesions which could result in a reduction of disease progression. In addition, certain reef fish (e.g. parrotfish, damselfish) as well as anchors and divers can cause significant injury to corals (Aeby and Santavy 2006) which could provide an entry point for a pathogen (Aeby and Santavy 2006; Page and Willis 2008).

In the Mediterranean, the marine fireworm *Hermodice carunculata* is a winter reservoir of the coral-bleaching pathogen *Vibrio shiloi* and can serve as a vector for the transmission of this disease (Sussman et al. 2003). In Hawaii, the butterflyfish *Chaetodon multicinctus* was found to be the primary vector for the coral disease *Porites* trematodiasis' (Aeby 2002), while the corallivorous snail *Coralliophila abbreviata* is capable of transmitting white band disease in the Florida Keys (Williams and Miller 2005). In addition, Aeby & Santavy (2006) demonstrated that, under laboratory conditions, the presence of the butterflyfish *Chaetodon capistratus* increased the spread of black band disease on the coral *Montastraea faveolata*, suggesting its involvement in the transmission of this disease. Nugues & Bak (2009) experimentally tested the involvement of the crown-of-thorn starfish *Acanthaster planci* in the transmission of brown band disease (BrB). They found that corals showing feeding scars had higher incidences of BrB, so providing preliminary evidence of a possible disease transmission by *A. planci*. The first evidence of an alga serving as a reservoir for coral disease has been suggested by Nugues et al. (2004). They revealed that physical contact with the macroalga *Halimeda opuntia* can trigger the outbreak of WP type II in the coral *Montastraea faveolata* in the Caribbean.

1.3 The coral holobiont

The coral with all its associated microorganisms has been termed the coral "holobiont" consisting of eucarya, bacteria, archaea, viruses, endolithic algae and fungi (Rohwer et al. 2002; Rosenberg et al. 2007a; 2007b) (Fig. 1.2). The associated eucarya include endosymbiotic dinoflagellates of the genus *Symbiodinium*, also referred to as zooxanthellae (see section 1). Culture-dependent as well as culture-independent microbial analyses have revealed a dynamic microbial community on the surface and within the tissues of scleractinian corals (Rohwer et al. 2002; Ainsworth et al. 2006; Kooperman et al. 2007; Littman et al. 2009; Shnit-Orland and Kushmaro 2009). The first culture-independent analyses of microbial 16S rDNA on corals showed that conventional culture-based studies had underestimated the diversity of coral microbes (Rohwer et al. 2001). Bacteria found in corals are very diverse, species specific and distinct from those found in the surrounding

water column (Rohwer and Kelley 2003); some bacteria might benefit their host by nitrogen fixation (Rohwer et al. 2002; Rosenberg et al. 2007a) and/or the production of antibiotics (Shnit-Orland and Kushmaro 2009).

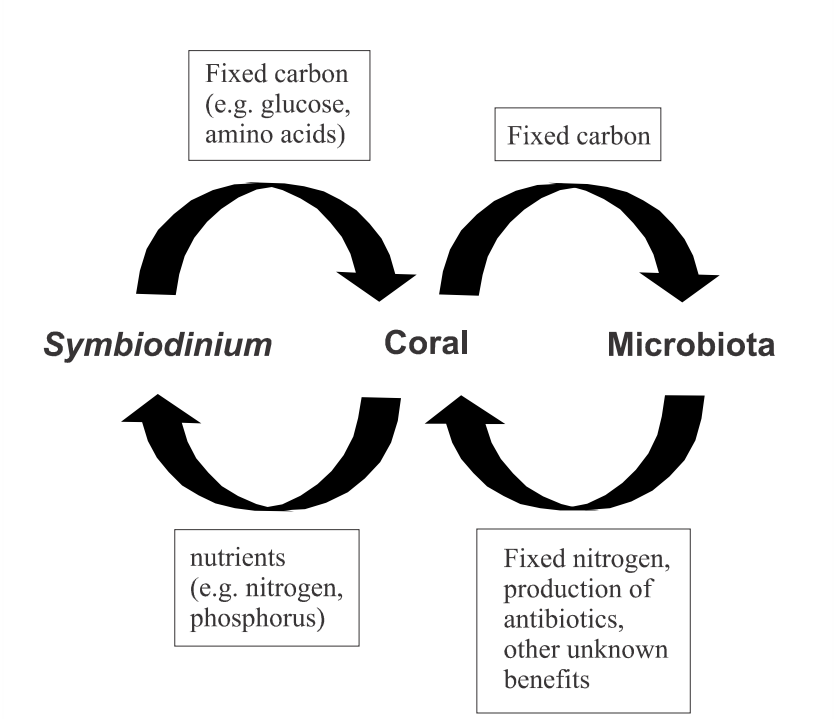


Fig. 1.2: Proposed model of the coral holobiont comprised of the coral animal, symbiotic dinoflagellates and all other associated microorganisms (protozoa, prokaryotes, endolithic algae, fungi, viruses and unknown organisms that have yet to be determined) (adapted from Rohwer et al. 2002).

Archaeal communities living in the coral are also distinct from those in the seawater and consist of many undescribed species (Rosenberg et al. 2007a). Archaea populations inhabiting the mucus are dominated by euryarchaeotes (Kellogg 2004) while those in the tissues are dominated by crenarchaeota (Wegley et al. 2004), but unlike bacteria, no species-specific associations have been found (Rosenberg et al. 2007a; 2007b). Endolithic fungi also live in close association with corals (Le Campion-Alsumard et al. 1995, Bentis et al. 2000, Ravindran et al. 2001) and might even play a symbiotic role in healthy corals (Le Campion-Alsumard et al. 1995). Environmental stress weakening the coral could disturb this host-parasite equilibrium, causing the fungi to become opportunistic pathogens (Le Campion-Alsumard et al. 1995, Bentis et al. 2000, Ravindran et al. 2001). Using a metagenomic approach, Vega-Thurber (2009) found a diverse assemblage of viruses in the reef coral

Porites compressa. However, our understanding of coral-associated viruses is still very limited and research uncovering the diversity and identity of coral viruses is still in its infancy (Davy et al. 2006; Vega-Thurber 2011).

The microbial community equilibrium of healthy corals appears to fluctuate during periods of environmental stress. For example, the microbial community of *Porites compressa* shifted to a community of microbes often found on diseased corals when stressed with temperature, pH and nutrients (Vega-Thurber et al. 2009). The natural bacterial community of corals can also change under experimental conditions, for example when corals are placed in an aquarium (Ainsworth and Hoegh-Guldberg 2009). Given this potential for change, an unresolved but important question is the potential change in pathogenicity of microbes normally found in corals (Teplitski and Ritchie 2009). Several *Vibrio* spp. have been linked with coral disease (Kushmaro et al. 1996; Ritchie and Smith 1998; Rosenberg and Falkovitz 2004; Cervino et al. 2008; Sussman et al. 2008), yet they can also have commensal functions (Criminger et al. 2007; Chimetto 2008; Shnit-Orland and Kushmaro 2009). It is unclear what, in some cases, triggers a switch from commensal or neutral to pathogenic interactions (Teplitski and Ritchie 2009).

It appears that the microbial equilibrium of healthy corals is very delicate and easily disrupted by changing environmental factors which could cause physiological changes that may lead to disease or death (Rohwer et al. 2002; Sokolow et al. 2009; Vega-Thurber et al. 2009). Our ability to understand microbial changes associated with disease is restricted by the limited research on normal microbial communities of healthy corals (Rohwer et al. 2002). However, research into the identification of the associated microbial communities in healthy as well as diseased corals has received more attention in recent years (Ritchie and Smith 2004; Rohwer and Kelley 2004; Klaus et al. 2005; Mouchka et al. 2010; Wilson et al. 2012) and the determination of which microbes are pathogenic and how changing environmental factors affect the symbiotic microbial community of healthy corals has become a critical area of research.

1.4 Coral pathogens

Our knowledge of coral disease pathogens is still rudimentary. To date, more than 35 coral diseases have been described (Lesser et al. 2007) but the causative agent for only a few of these diseases has been identified (Sussman et al. 2009). To prove that a microorganism causes a disease, Koch's postulates have to be fulfilled (Koch 1891). This proves to be

difficult for coral diseases due the intricate and highly sensitive associations of the coral holobiont, which is reflected in the low number of cases where researchers have actually managed to fulfil Koch's postulates to prove disease causation. The majority of identified pathogens are bacteria but causative agents can also include cyanobacteria, fungi, trematodes and ciliates (Table 1.1). A more recently proposed group of coral disease agents are viruses, but very little is known about their diversity and ecology (Davy et al. 2006; Vega-Thurber 2011). To date, no archaea have been characterized as coral disease pathogens (Rosenberg et al. 2007b).

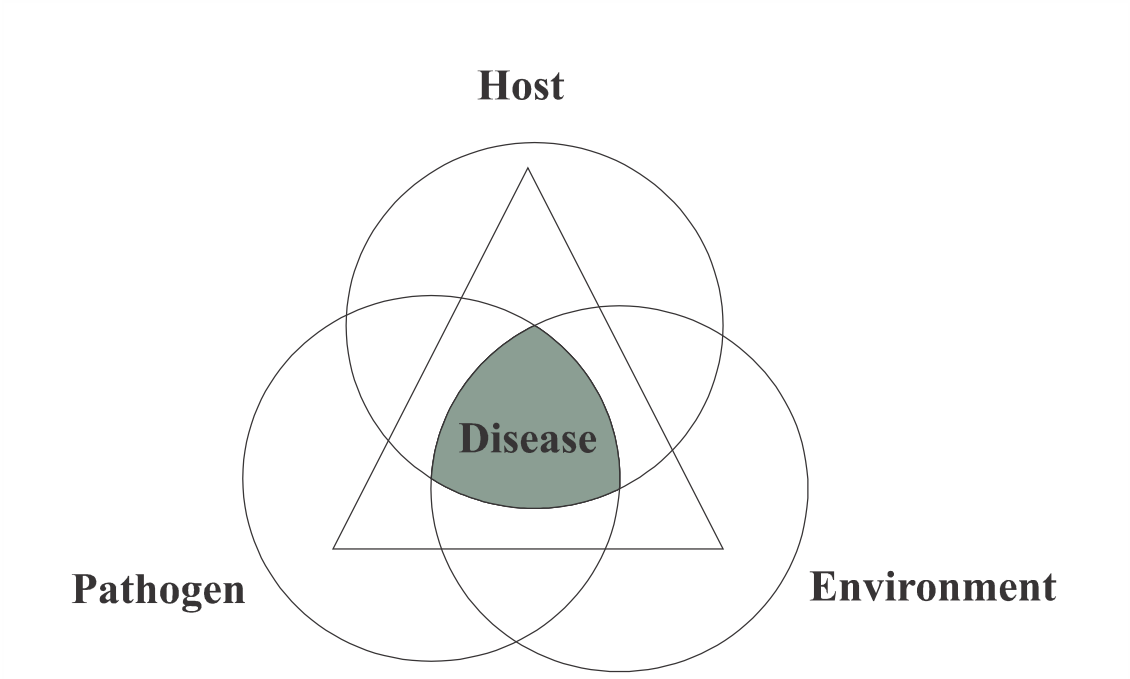


Fig. 1.3: The disease triangle (adapted after Snieszko 1974).

Determining the causative agent of a disease does however, not always help to explain why disease outbreaks occur in some areas and not in others. The concept of a disease triad has been developed (Snieszko 1974) to better understand the complexity of disease causation. Three factors contribute to disease causation: the host, the pathogen and the environment. Only if all three factors interact, creating the right conditions, will disease occur (Fig. 1.3). Coral disease diagnostics still rely predominantly on the identification of macroscopic disease signs and only recently has research been directed more towards the association of environmental factors, pathogens (including their vectors) and host susceptibility.

Table 1.1: Coral pathogens (revised after Rosenberg et al. 2007a). Different shades represent different groups of pathogens. *Presumed pathogen

Disease	**Acronym**	**Location**	**Pathogen**	**Pathogen group**
White plague-like	WP	Red Sea Caribbean	*Thalassomonas loyana*	Bacteria
White plague type II	WP II	Caribbean	*Aurantimonas coralicida*	Bacteria
White pox		Caribbean	*Serratia marcescens*	Bacteria
White band disease type I	WBD I	Caribbean	Genus *Rickettsia**	Bacteria*
White band disease type II	WBD II	Caribbean	*Vibrio charcharia*	Bacteria
White syndrome	WS	Indo-Pacific	6 pathogens from the family *Vibrionacae*	Bacteria
Porites ulcerative white spot syndrome	PUWS	Indo-Pacific	*Vibrio* sp.	Bacteria
Bacterial bleaching	BBL	Indo-Pacific Mediterranean	*Vibrio coralliilyticus* *Vibrio shiloi*	Bacteria
Yellow band disease	YBD	Indo-Pacific Caribbean	*Vibrio* spp. consortium	Bacteria
Black band disease	BBD	Indo-Pacific Caribbean	Microbial consortium	Dominated by cyanobacteria
Red band disease	RBD	Western Atlantic	*Oscillatoria* spp.*	Cyanobacteria*
Skeletal eroding band	SEB	Indo-Pacific	*Halofolliculina corallasia*	Ciliate*
Brown band disease	BrB	Indo-Pacific	Class Oligohymenophorea: Scuticociliatia*	Ciliate*
Aspergillosis	ASP	Caribbean	*Aspergillus sydowii*	Fungus
Porites Trematodiasis	PorTrem	Indo-Pacific	*Podocotyloides stenometra*	Digenetic tramatode
Growth anomalies	GA	Indo-Pacific Caribbean		Virus*

1.4.1 Bacteria

Corals provide habitats for bacteria in their surface mucus layer, tissue and calcium carbonate skeleton, each with a distinct, species-specific bacterial population (Rohwer et al. 2002; Bourne and Munn 2005; Rosenberg et al. 2007a, 2007b). The ecological role of these bacteria is however, poorly understood (Rohwer et al. 2002) and our knowledge of the normal bacterial associates of healthy corals is restricted (Rohwer et al. 2001; Klaus et al. 2005; Johnston & Rohwer 2007; Ainsworth & Hoegh-Guldberg 2009; Littman et al. 2009), so limiting our understanding of the changes associated with disease (Rohwer et al. 2002).

Examples of coral diseases caused by bacteria are WP, WBD and white pox (Table 1) which have all caused coral mortality in the Caribbean (Goreau et al. 1998). WP is divided into types I, II and II, and can affect multiple coral species (Dustan 1977; Richardson et al. 2001; Bythell et al. 2004). The causative agent was only identified for WP type II, as a novel alpha-proteobacterium *Aurantimonas coralicida* (Denner et al. 2003). WBD is divided into types I and II, and only affects branching *Acropora* species (Gladfelter et al. 1977; Ritchie & Smith 1998; Bythell et al. 2004). WBD type I is thought to be caused by a marine *Rickettsia* bacterium (Casas et al. 2004) and WBD type II was shown to be caused by *Vibrio charcharia* (Ritchie & Smith 1998). White pox disease exclusively affects *Acropora palmata* (Holden 1996; Rodriquez-Martinez et al. 2001; Porter et al. 2001; Patterson et al. 2002) and was found to be caused by the gram-negative bacterium *Serratia marcescens* (Patterson et al. 2002) which is also found in human intestines (Grimont & Grimont 1994). *S. marcescens* was the first coral pathogen identified with a possible link to human sewage pollution (Patterson et al. 2002), presenting the first evidence that a common member of the human gut microbiota can have the potential to be a marine invertebrate pathogen (Patterson-Sutherland & Ritchie 2004).

1.4.2 Cyanobacteria

Cyanobacteria have been reported in association with coral diseases but they have never been identified as the sole causative agent. BBD was first reported in 1973 (Antonius 1973) and is now one of the best studied coral diseases. It is widely distributed throughout coral reefs around the world (Page and Willis 2006) and it is caused by a pathogenic microbial consortium (Richardson et al. 1997; Frias-Lopez et al. 2004; Barneah et al. 2007). The pathogenic microbial community is dominated by the filamentous cyanobacterium *Phormidium corallyticum* (Ruetzler and Santavy 1983; Kuta and Richardson 1996;

Richardson & Aronson 2000) though numerous uncharacterized heterotrophic bacteria (Cooney et al. 2002), the sulphide-oxidising bacterium *Beggiatoa* (Richardson and Aronson 2000), sulphur-reducing bacteria dominated by *Desulfovibrio* spp. (Richardson and Aronson 2000; Cooney et al. 2002; Viehman and Richardson 2002) and marine fungi (Ramos-Flores 1983) are also present. Red band disease (RBD) closely resembles BBD except that it is bright red in colour (Richardson 1992). This disease has been observed in sea fans and massive corals, and is characterised by a narrow line of filamentous cyanobacteria from the genus *Oscillatoria* and other microorganisms that move over the coral leaving the bare skeleton behind (Richardson 1992).

1.4.3 Fungi

Corals respond to penetrating fungal hyphae by depositing new layers of aragonite resulting in the growth of pearl-like skeletal protrusions (Le Campion-Alsumard et al. 1995). Terrestrial fungi have been found to be common components of corals (Ravindran et al. 2001), and have possibly been introduced through terrestrial run-off (Raghukumar and Raghukumar 1991; Smith and Weil 2004) and/or African dust (Shinn et al. 2000; Garrison et al. 2003) before becoming adapted to the marine environment (Ravindran et al. 2001). However, very little is known about the diversity of fungi that inhabit corals (Ravindran et al. 2001). One example is aspergillosis of sea fans (Smith et al. 1996; Rypien et al. 2008) which is caused by the terrestrial fungus *Aspergillus sydowii* (Smith et al. 1996; Geiser et al. 1998), thought to be introduced through dust transported from deserts in Africa and Asia (Shinn et al. 2000; 2003; Garrison et al. 2003). Work et al. (2008b) observed coral colonies in Hawaii and American Samoa that showed a distinct dark colouration caused by an unidentified fungus which resembled dark spot disease found in the Caribbean.

1.4.4 Trematodes

To date, one trematode species has been found to cause disease in corals. This disease was named *Porites* trematodiasis and has been observed throughout the Hawaiian Islands and in other areas of the Pacific (Aeby 2003). *Porites* trematodiasis is caused by the digenetic trematode *Podocotyloides stenometra* (Aeby 1998) which has a complex life cycle including multiple intermediate hosts (Aeby 2003, Aeby & Santavy 2006). The first intermediate host has, as yet, not been identified but it is thought that a marine mollusc might serve as first host (Aeby 1998). The second intermediate host is coral of the genus *Porites*. The trematode borrows into the coral tissue causing the polyps to swell and develop a bright pink colour.

This colouration attracts coral-feeding fish, such as butterfly fish, which consume the swollen polyp containing the parasite. Within the fish the trematode completes its life-cycle, and parasite eggs are shed with the fish faeces and begin a new cycle (Aeby 1998). It has been proposed that parasites, such as trematodes, could be used as an indicator for a healthy ecosystem as they require a diverse and abundant host community and can be sensitive to environmental perturbations (Lafferty et al. 2004; Marcogliese 2005; Hudson et al. 2006).

1.4.5 Ciliates

Ciliates are common in marine environments but are rarely classified as pathogenic parasites (Lynn & Corliss 1991), especially in coral reef communities (Bourne et al. 2008). However, two coral diseases, namely brown band disease (Willis et al. 2004) and skeletal eroding band (Antonius 1999), have been associated with a band of ciliates feeding on the tissue. The relationship between their presence and coral mortality has however, not yet been firmly established (Rodriguez et al. 2009).

1.4.6 Viruses

Viruses have only recently been implicated as potential coral pathogens (Davy et al. 2006). The application of transmission electron microscopy (TEM) led to the discovery of virus-like particles (VLPs) in the temperate sea anemone *Anemonia viridis* (Wilson et al. 2001) and a few years later in tropical reef corals (Wilson et al. 2005; Davy et al. 2006).

Experiments have shown that three species of Indo-Pacific coral (*Pavona danai*, *Acropora formosa* and *Stylophora pistillata*) and one zoanthid species (*Zoanthus* sp.) produced numerous VLPs when thermally stressed (Davy et al. 2006). Cervino et al (2004) reported VLPs in isolated zooxanthellae from the Caribbean coral *Montastraea* sp. after exposure to elevated temperature or bacterial pathogens. These findings support the hypothesis that latent viruses are present within the host or its symbionts, which enter a lytic cycle when environmentally stressed (Davy et al. 2006). Recently, metagenomic analyses of the viral community of the Caribbean coral *Diploria strigosa* revealed an extraordinary viral diversity potentially infecting all members of the coral holobiont (Marhaver et al. 2008).

Viruses might also have a beneficial role in coral health (Bourne et al. 2009; van Oppen et al. 2009; Vega-Thurber 2011). Patten et al. (2008) observed VLPs within the gastrodermal and epidermal tissues of all *Acropora muricata* colonies sampled at Heron Island (GBR), Australia supporting the view that viruses represent an important component of the coral

holobiont. Bacteriophages potentially maintain the homeostasis of the holobiont by controlling bacterial population levels (Efrony et al. 2007, 2009; Bourne et al. 2009; Teplitski & Ritchie 2009). The exploitation of bacteriophages has even been investigated as a potential management tool in controlling infectious coral diseases (Bourne et al. 2009; Teplitski & Ritchie 2009).

1.5 Coral resistance to disease

Information about disease-resistance mechanisms in corals is limited in the literature, however their investigation and how changing environmental factors influence these mechanisms are essential in the light of global climate change (Palmer et al. 2008).

Corals contain an innate or natural immune system that provides a non-specific ability to react to different, potentially pathogenic organisms (eg. mechanical or physical barriers such as the epidermis and mucus, antimicrobial compounds and phagocytic cells) which are not altered with subsequent exposure (reviewed in Mullen et al. 2004). In invertebrate innate immunity, the prophenoloxidase (PO) pathway, which is also responsible for the production of melanin, is involved in wound healing, parasite and pathogen resistance, and general coordination of immune responses (Mydlarz et al. 2006). Recently, the presence of melanin has been identified in a variety of coral species (Palmer et al. 2008, Palmer et al. 2010), demonstrating that the PO pathway, widely recognized as a key component in invertebrate immunity, was active (Palmer et al. 2008). An elevated PO activity has also been observed in sea fans infected with the fungal pathogen *Aspergillus sydowii* (Mydlarz et al. 2008). In other invertebrates, the melanin-synthesis pathway aids in the deposition of encapsulating melanins (Nappi and Christensen 2005) and produces reactive oxygen species and quinines which provide antimicrobial defences (Nappi and Ottaviani 2000). The presence of melanin containing granular cells and PO activity in scleractinian corals suggests that they may play key roles in coral immunity (Palmer et al. 2010).

Corals do however, not produce antibodies and are therefore considered to lack an adaptive immune system (Mullen et al. 2004, Rosenberg et al. 2007b). There are certain cases though, that suggest that corals can develop a resistance to pathogens. For example, in the case of bacterial bleaching in the coral *Oculina patagonica* by the bacterium *Vibrio shiloi* (Kushmaro et al. 2001) it has been impossible to re-infect healthy corals taken from the field with laboratory stocks of *V. shiloi* that previously caused bleaching in controlled aquaria experiments (Reshef et al. 2006). Another example is the bacterium *Aurantimonas coralicida*

that caused WP type II in the Florida Keys in 1995 (Denner et al. 2003). Interestingly, healthy corals are no longer susceptible to this pathogen though it is unclear if this immunity was acquired during the original disease event or if it is a natural resistance of some of the surviving colonies (Richardson and Aronson 2000). Reshef et al. (2006) explains this potential immunity with the 'Probiotic Hypothesis'. It posits the existence of a dynamic relationship between the symbiotic microbiota and corals under different environmental conditions which favours the most advantageous coral holobiont. This would allow for a faster adaptation to changing environmental conditions than via mutation and selection (Reshef et al. 2006).

Recently, it has been discovered that corals, as well as their bacterial symbionts, possess antimicrobial-chemical defences against certain bacterial pathogens (Ritchie 2006; Shnit-Orland & Kushmaro 2009; Chen et al. 2012; Shnit-Orland et al. 2012). Most research on antimicrobial activity (reviewed in Kelman 2004) initially focused on sponges (e.g. Matsunaga et al. 1984; McCaffrey and Endean 1985; Newbold et al. 1999; Kelman et al. 2001) and gorgonian corals (e.g. Kim 1994; Jensen et al. 1996; Kelman et al. 1998; Kim et al. 2000b; Roussis et al. 2001; Koh et al. 2002; Harder et al. 2003), but there has been a recent increase of studies concentrating on scleractinian corals (Koh 1997; Geffen and Rosenberg 2005; Ritchie 2006; Gochfeld and Aeby 2008; Geffen et al. 2009; Shnit-Orland and Kushmaro 2009). These studies have demonstrated that extracts from a variety of corals exhibit antimicrobial activity, however; a high degree of variability down to the species and even population levels was observed (Jensen et al. 1996; Koh 1997; Mullen et al. 2004; Kelman et al. 2006, 2009; Gochfeld and Aeby 2008). These differences might enable certain populations, species or genotypes to have an advantage over others in resisting invasion by pathogens (Mullen et al. 2004; Gochfeld and Aeby 2008). Antimicrobial activity also displayed a high degree of selectivity for certain bacterial species, indicating that antimicrobial activity is species-specific rather than broad-spectrum, so enabling certain bacteria to live in symbiosis with their host while maintaining a chemical defence against microbial pathogens (Kelman 2004; Kelman et al. 2009).

The surface mucus layer protects the coral from UV damage, desiccation and smothering by sediment (Brown and Bythell 2005), but little is known about the role of mucus in coral disease resistance (Ritchie 2006). It has been proposed that it provides a physical barrier to the microbes in the surrounding environment, mucociliary transport of microbes to the polyp for digestion, and sloughing to avoid colonisation of pathogenic microbes, as well as serving

as a medium for allelochemicals with antimicrobial properties (reviewed in Brown and Bythell 2005). Ritchie (2006) demonstrated that mucus from healthy *Acropora palmata* inhibited the growth of potentially pathogenic bacteria by up to 10-fold. Rohwer et al. (2002) speculated that certain specialized microbiota found in mucus may protect the coral by producing secondary metabolites like antibiotics. Recently, it has been shown that several microorganisms occurring in the coral mucus layer indeed exhibit antimicrobial activity (Ritchie 2006; Shnit-Orland and Kushmaro 2009; Shnit-Orland et al. 2012) possibly acting as a first line of defence against pathogens (Shnit-Orland and Kushmaro 2009).

The involvement of host and symbiont genetics has also been explored as possible explanation of resistance/susceptibility to coral disease. Using microsatellite genotype information in combination with *in situ* transmission essays and field monitoring, Vollmer and Kline (2008) found that 6% of *Acropora cervicornis* colonies from Panama have a natural resistance to white band disease. This has important implications for coral reef conservation. Future research into the genetic basis of coral resistance to disease would enable us to identify resistant genotypes in disease-impacted coral species, providing the possibility of propagating and transplanting those genotypes into highly-degraded reefs (Vollmer and Kline 2008). Correa et al. (2009) tested the hypothesis that the resident *Symbiodinium* sp. genotype might contribute to the susceptibility or resistance of corals to disease. They found, however, that in all investigated diseases, the same *Symbiodinium* type was present in diseased and apparently healthy tissues. In contrast, Stat et al. (2008) found that some corals affected by white syndrome harboured clade A whereas healthy colonies had clade D. They proposed that clade A might be less beneficial to the coral as it provides less nutrition and so might make the coral more susceptible to disease.

1.6 Management of coral diseases

The successful management of coral diseases is hindered by difficulties in correctly identifying diseases and characterizing their etiology and epizootiology as well as an incomplete understanding of the interaction of diseases with environmental factors and anthropogenic stressors (Bruckner 2002; Raymundo et al. 2008). An interdisciplinary approach, combining ecological monitoring with biochemistry, histopathology, microbiology, toxicology and physical oceanography as well as the development of molecular identification tools is necessary to confidently identify, differentiate and characterize different coral diseases and their impacts on the reef system (Bruckner 2002).

Information on how different environmental factors, for example temperature and water quality, affect different diseases and which factors contribute to transmission and spread is vital for a development of early warning systems that would enable managers to predict potential disease outbreaks and develop strategies to minimize impacts (Bruckner 2002, Beeden et al. 2012). For coral bleaching, early warning systems have already been successfully established (Maynard et al. 2008, 2009). For example, the Coral Reef Watch Program operated by NOAA (noaa.gov) uses satellite remote sensing to assess data on sea surface temperature, light exposure and other environmental conditions, to quickly determine areas at risk of coral bleaching. These tools could also be useful in determining areas with a high likelihood of coral disease outbreaks (Maynard et al. 2011; Beeden et al. 2012), as certain coral diseases are positively correlated with increased seawater temperature (Bruno et al. 2007; Harvell et al. 2009) and there is evidence that bleaching increases disease susceptibility (Miller et al. 2006; Muller et al. 2008; Brandt and McManus 2009; Croquer and Weil 2009b). These high risk areas can then be monitored for disease (Beeden et al. 2012) with rapid assessments detecting disease outbreaks and targeted monitoring providing information on particular diseases and areas of interest (Bruckner 2002; Beeden et al. 2012).

The development of management strategies that reduce relevant anthropogenic stressors and improve habitat quality would promote reef resilience and likely minimize the effects of coral diseases (Bruckner 2002; Raymundo et al. 2008; Beeden et al. 2012). Other important research areas are the identification of reefs with greater relative resilience to climate change and high biodiversity (Beeden et al. 2012), as well as the re-introduction of disease-resistant genotypes and the development of treatments for coral diseases (Bruckner 2002).

1.6.1 Treatments of coral diseases

Direct treatment of coral diseases, including shading (Muller and Woesik 2009), removal of the microbial community and sealing of the lesion with putty or clay (Hudson 2000, Bruckner 2002) has been attempted in an effort to stop the progression of disease lesions. For example, the aspiration of the microbial mat that characterizes BBD with subsequent sealing of the lesion with modelling clay or putty (Hudson 2000, Bruckner 2002) has been quite effective to prevent further mortality in century-old colonies (Bruckner 2002). The application of putty and clay over disease lesions has also been attempted in YBD, WBD and WP showing various rates of success (Bruckner 2002, Raymundo et al. 2008). The mechanical removal of growth anomalies was attempted in Palmyra and Hawaii and appeared to be very successful

(Aeby and Williams, unpublished data), however more data needs to be collected to conclusively measure the rate of success of this technique. Direct or mechanical treatments are very time consuming and costly and so very impractical during a large-scale disease outbreak (Bruckner 2002). These tools might be most suitable on a small scale, only including the colonies that are of most value to the reef, such as large colonies of slow-growing massive species or rare species (Bruckner 2002, Raymundo et al. 2008).

The isolation and removal of diseased colonies has also been proposed to protect other colonies from the spread of disease (Hayes and Goreau 1998). However, this approach might actually promote dispersal and spread of the pathogen, as well as causing considerable habitat damage and is therefore very impractical and of minimal benefit (Bruckner 2002).

Recently, phage therapy (Teplitski and Ritchie 2009) and the use of probiotics (Ritchie 2006) have been proposed as possible tools for mitigating coral diseases. However, research is still in the early stages and the logistics, as well as the mechanics of these approaches have not yet been established (Teplitski and Ritchie 2009). In a study using lytic bacteriophages in controlled aquaria experiments, it was demonstrated that the coral pathogen *Vibrio coralliilyticus* (causing bleaching in *Pocillopora damicornis*) could be controlled by the pathogen-specific phage YB2 (Efrony et al. 2007). However, Efrony et al. (2009) demonstrated that the timing of the addition of the phage is crucial for disease prevention. They therefore speculated that phage therapy may be more valuable in preventing the spread of a disease rather than curing it. The proposed use of antibiotics could have severe long-term consequences as it may affect beneficial microbes or lead to the development of antibiotic-resistant pathogens (Bruckner 2002, Raymundo 2008). Adding antibiotics to putty or clay, so restricting the treatment to affected areas, might be a more plausible solution (Bruckner 2002).

1.7 Coral reefs of Hawaii

The Hawaiian archipelago is located in the Central Pacific Ocean and consists of the populated Main Hawaiian Islands (MHI) and the mostly unpopulated Northwestern Hawaiian Islands (NWHI). Hawaii's coral reefs stretch over 2500 km and are, due to their location, exposed to large open ocean swells and strong trade winds which have major impacts on the structure of the reef community (Friedlander et al. 2008a; 2008b). The geographic isolation of these reefs has resulted in one of the highest degrees of marine endemism found anywhere on Earth (Jokiel 1987).

Hawaii has 1.2 million inhabitants with over 70% located on Oahu and an additional 7 million tourists visiting the islands each year (Friedlander et al. 2008b). Marine tourism contributes largely to the state's economy, but it has also put high pressure on their coral reefs. Urban areas and popular tourist destinations have been affected in particular, with land-based pollution, recreational overuse, overfishing, coastal construction, and alien and invasive species being the main threats. The leading land-based pollutant is sediment as development for agriculture and urban growth increases. However, coral reefs in more remote areas are still in good condition (Friedlander et al. 2008a; 2008b).

Monitoring of Hawaii's coral reefs began in the 1960s and has since revealed several changes. Average annual sea surface temperatures (SSTs) have increased by 0.8˚C and three major bleaching events have been recorded (Friedlander et al. 2008b). The occurrence of various coral diseases (see Table 1.2) has been documented in the three main coral genera (*Porites, Montipora, Pocillopora*) (Friedlander et al. 2008b) with *Porites* having the highest prevalence of disease (Friedlander et al. 2008a). Nevertheless, broad-scale disease surveys have shown that general disease prevalence is widespread but currently occurring at low levels, providing the opportunity for managers to mitigate this emerging problem (Friedlander et al. 2008a; 2008b). The most common condition found on *Porites* colonies is growth anomalies but the causative agent(s) remains unknown (Friedlander et al. 2008b). To date, coral diseases have not caused mass mortalities in Hawaii but increasing anthropogenic pressure and global climate change leads to concern about the continuous health of Hawaiian coral reefs (Friedlander et al. 2008b).

In Kaneohe Bay, Oahu, *Porites compressa* is affected by *Porites* bleaching with tissue loss (PBTL) which manifests as diffuse areas of white discolouration of the coenenchyme and pigmented polyps, giving the coral a "speckled" appearance (Fig. 1.4B). PBTL has some similarity to *Porites* tissue loss (PorTL), another disease reported from these reefs (Williams et al. 2010) but because of variations in their gross morphology they were treated as separate diseases in this study. PorTL, in contrast to PBTL, does not show the "speckled" bleaching pattern but a narrow band of bleached tissue bordering patches of tissue loss (see Williams et al. 2010 for a full description of PorTL). In addition, polyps in the bleached band appear swollen (Fig. 1.4C) which is not observed in PBTL lesions. Identifying coral diseases at the gross level alone can be very difficult and diseases that show very similar gross lesions, such as 'white' diseases (spreading zone of tissue loss) reported from the Caribbean and Indo-Pacific, can have different causations (see Table 1.1). It is therefore important not to

prematurely combine disease signs, especially if the gross morphology of lesions shows a disparity. Until the causation of a lesion is determined, even similar disease lesions should be treated as separate diseases.

Table 1.2: Examples of coral diseases found in the Main Hawaiian Islands (MHI) and Northwestern Hawaiian Islands (NWHI). Photo credit: *1 Greta Aeby, *2 Maya Walton, *3 The Coral Reef Ecosystem Division (CRED): http://www.pifsc.noaa.gov/cred/coraldiseases.php

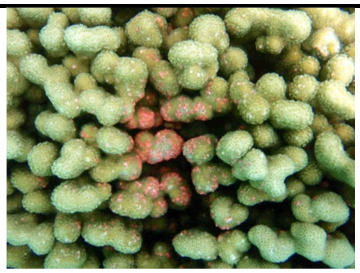
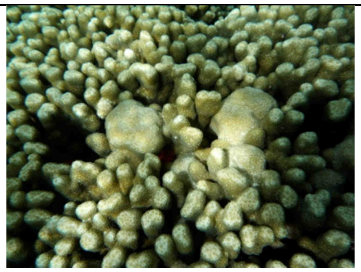
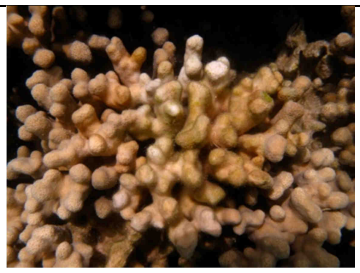
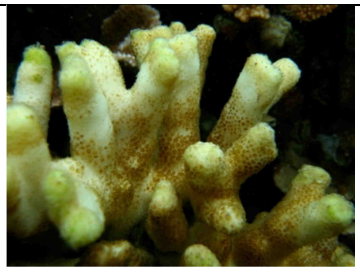
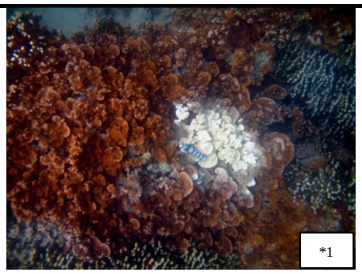
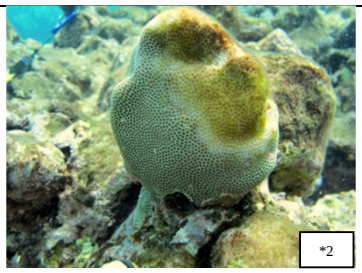
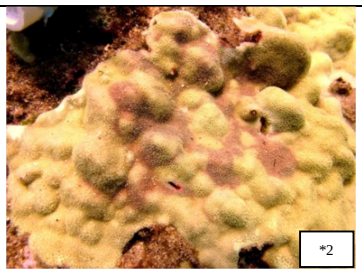
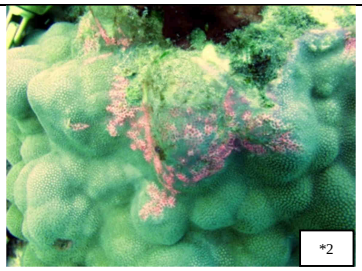
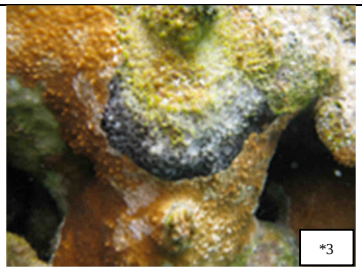
Disease	**Species affected**	**Photo**	**Occurrence**
Porites trematodiasis	*Porites* spp.		Occasional – abundant, MHI & NWHI
Growth anomalies	*Porites* spp., *Montipora capitata*, *Acropora cytheria*		Abundant, MHI & NWHI
Porites tissue loss	*Porites compressa*		Occasional, MHI
Porites bleaching with tissue loss	*Porites compressa*		Common, (Kaneohe Bay, Oahu)

Table 1.2 continued

Disease	Species affected	Photo	Occurrence
white syndrome	*Montipora capitata*, *Acropora spp.*	 *1	Occasional – abundant, MHI & NWHI
Sub-acute tissue loss	*Porites* spp.	 *2	Occasional, MHI & NWHI
Dark spot syndrome	*Porites* spp., *Pavona* spp.,	 *2	Rare – occasional, MHI & NWHI
Pigmentation response	*Porites* spp.	 *2	Common, MHI
Ciliate infection	*Montipora capitata*, *Pocillopora meandrina*	 *3	Rare, MHI

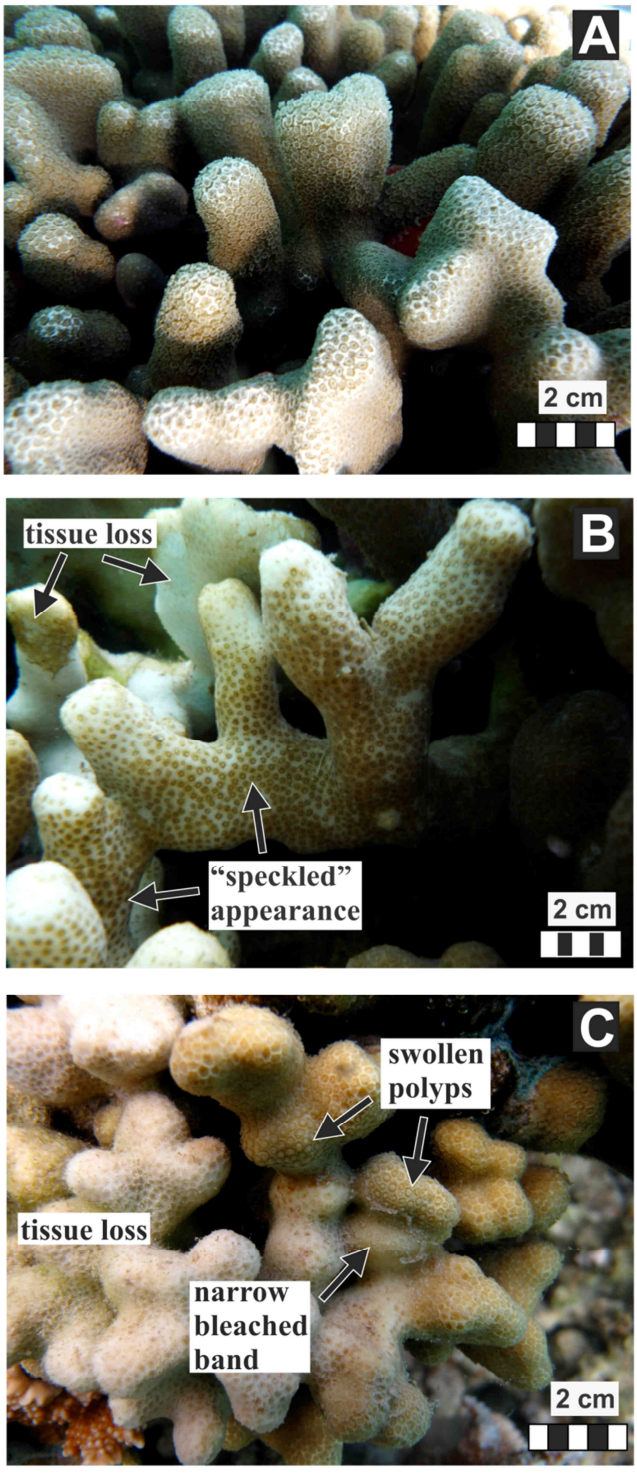


Fig. 1.4: *Porites compressa*. A: Healthy *P. compressa*. Note regular brown coloration. B: PBTL-affected *P. compressa*. Note pigmented polyps and bleached coenenchyme ("speckled appearance") with onset of tissue loss. C: PorTL-affected *P. compressa*. Note swollen polyps adjacent to a narrow bleached band surrounding an area with tissue loss.

1.8 Research objectives

The development of successful management strategies rely on high-quality characterizations of all diseases in a reef system, which provide information on disease dynamics including prevalence, virulence and environmental drivers.

Porites spp. are one of the main framework building corals in the Indo-Pacific and are often a dominant host for coral diseases, for example in Guam (Myers and Raymundo 2009), the Philippines (Raymundo 2005) and Hawaii (Domart-Coulon 2006; Aeby 2007; Aeby et al. 2011a). In Kaneohe Bay, Oahu, *P. compressa* is one of the main framework-building corals, so investigating diseases affecting this species is of great importance to assess the potential ecological impact that specific diseases could have on the reef.

This research thesis aimed to collect information on the gross and cellular manifestations, disease dynamics and possible pathogens of PBTL, to provide a baseline characterization of this disease. Multiple exploratory pathways, using a range of techniques, such as field surveys, histology, transmission electron microscopy, genetics and microbiology, were used to gather data that can be used as baseline for future studies.

Specifically this project was aimed to address the following questions:

- What are the gross signs of PBTL?
- Can PBTL be classified as a disease?
- What are the microscopic manifestations of PBTL?
- Is there evidence for the presence of a potential pathogen(s)?
- What is the prevalence and incidence of PBTL around Coconut Island Marine Reserve?
- Is there a seasonal trend in disease prevalence?
- What are the long-term effects of PBTL?
- Is PBTL transmissible?
- What is the geographic extent of PBTL?
- What is the relationship of disease prevalence and various environmental factors and what are specific environmental drivers?
- Does increased temperature affect the virulence of PBTL?
- Does *Symbiodinium* and/or host genetics affect susceptibility to PBTL?

The four data chapters in this thesis are written in paper format and are either published, submitted or in preparation. Hence there is a certain degree of overlap between chapters, particularly their introductions.

The majority of the work was conducted by the candidate. Any contributions of others are listed below each chapter in the following section.

Chapter 2 provides a systematic description of PBTL and investigates its virulence to determine if it can be classified as disease. The results were published as:

Sudek, M., Aeby, G.S., Davy, S.K. (2012) Localized bleaching in Hawaii causes tissue loss and a reduction in the number of gametes in *Porites compressa*. Coral Reefs 31:351-355.

Contributuion of others:
Simon Davy and Greta Aeby: editing and intellectual input
Alan Hovert: advice on histological techniques
Thierry Work: assistance with the interpretation of histological sections
Andrew Taylor: advice on statistical methods

Chapter 3 describes cytopahological observations in PBTL-affected colonies, using histopathology and transmission electron microscopy (TEM) techniques. The histopathological results were published as:

Sudek, M., Work, T. M., Aeby, G.S., Davy, S.K. (2012) Histological observations in the Hawaiian reef coral, *Porites compressa*, affected by *Porites* bleaching with tissue loss. Journal of Invertebrate Pathology 111:121-125

Contributuion of others:
Simon Davy, Greta Aeby and Thierry Work: editing and intellectual input
Thierry Work: assistance with the interpretation of histological sections
Alan Hovert: advice on histological techniques
Tina Carvalho: advice on TEM techniques and interpretation of sections

Chapter 4 describes changes in PBTL prevalence over the course of one year and investigates disease progression and transmission. In addition, the association of PBTL prevalence and eight environmental predictors is explored.

Sudek, M., Williams, G. J., Runyon, C., Aeby, G.S., Davy, S.K. (in review) Disease Dynamics of *Porites* Bleaching with Tissue Loss: prevalence, transmission and environmental drivers. MEPS

Contributuion of others:
Simon Davy, Greta Aeby and Gareth Williams: editing and intellectual input
Gareth Williams: advice on statistical methods
Jamie Sziklay: assistance in creating the survey site map in GIS
Christina Runyon: assistance with field work

Chapter 5 explores the possible involvement of host and *Symbiodinium* genetics in the susceptibility of *P. compressa* colonies to PBTL. The results of the *Symbiodinium* clade study are being prepared as Note:

Sudek, M., Stat, M., Gates, R. D., Aeby, G.S., Davy, S.K. (in prep) *Symbiodinium* type does not affect the susceptibility of *P. compressa* colonies to the coral disease *Porites* bleaching with tissue loss

Contributuion of others:
Simon Davy, Greta Aeby, Zac Forsman and Michael Stat: editing and intellectual input
Michael Stat: guidance on DNA extraction and sequencing
Kelsey Fee: microsatellite lab work
Robert Toonen and Ruth Gates: funding of the lab work

The versions of all published papers are provided under Appendix X.

Chapter 2

Localized bleaching in the reef coral *Porites compressa*: systematic description and virulence – can this lesion be classified as disease?

2.1 Introduction

A disease by definition is any interruption, cessation or disorder of body functions, systems or organs and can be caused by either biotic or abiotic factors (Webster 2011). Terrestrial diseases, especially in humans and other vertebrates, are well documented and of major concern for medical, veterinary and conservation biology authorities (Harvell et al. 2002; Lafferty and Gerber 2002; WHO 2004). Key examples of well studied human diseases include HIV/AIDS (Hladik and McElrath 2008), tuberculosis (DeBacker et al. 2006) and cholera (Ghose 2011). In contrast, our knowledge of diseases and disease outbreaks in freshwater (Johnson and Paull 2011) and marine (Harvell et al. 1999) ecosystems is lagging far behind.

Wildlife diseases can cause severe population declines which can further impact the ecosystem via "knock-on" effects (Daszak et al. 2001). For example, an unknown pathogen caused mass mortalities (98%) of the sea urchin *Diadema antillarum* in the Caribbean (Lessios 1988). The elimination of this important herbivore led to an increased growth of benthic algae causing catastrophic, widespread changes in the community structure on Caribbean coral reefs (Hughes 1994). However, many marine mortality events likely remain undetected due to the logistical difficulties in conducting marine monitoring and research (Harvell et al. 2004). The extent and ecological impact that diseases have in the ocean remains largely unknown, even in cases where economically and ecologically important species are affected (Harvell et al. 1999).

In vertebrates, diseases are expressed in various ways including external and behavioural signs of disease; for example, rabies (Niezgoda et al. 2002), foot-and-mouth-disease in cattle and swine (Meyer and Knudsen 2001), canine distemper (Deem et al. 2000) and gill disease in salmon (Mitchell and Rodger 2011). Invertebrates show a similar suite of disease responses, for example, shell disease in lobster (Mancuso et al. 2010), white spot syndrome in arthropods (Escobedo-Bonilla et al. 2008) and deformed wing syndrome in honeybees (Bowen-Walker et al. 1999). In the case of corals, diseases can only manifest in three main

ways: tissue loss, growth anomalies and/or discolouration (Work and Aeby 2006). Most coral diseases known to date can be placed within the tissue loss category. Well known examples are white syndromes, a general term used to describe coral diseases with acute signs of advancing tissue loss (Willis et al. 2004; Sussman et al. 2008) and "band diseases", such as BBD (Edmunds 1991; Kuta and Richardson 1996; Miller 1996) and WBD (Gladfelter 1982; Peters et al. 1983; Ritchie and Smith 1998; Aronson and Precht 2001). Growth anomalies have been observed in several different coral species around the world (Kaczmarsky 2006; Haapkylä et al. 2009; Myers and Raymundo 2009; Aeby et al. 2011a), while few diseases causing discolouration have been recorded to date (Kushmaro et al. 1996; Ravindran and Raghukumar 2002; Work and Rameyer 2005) (see Fig. 2.1 for examples).

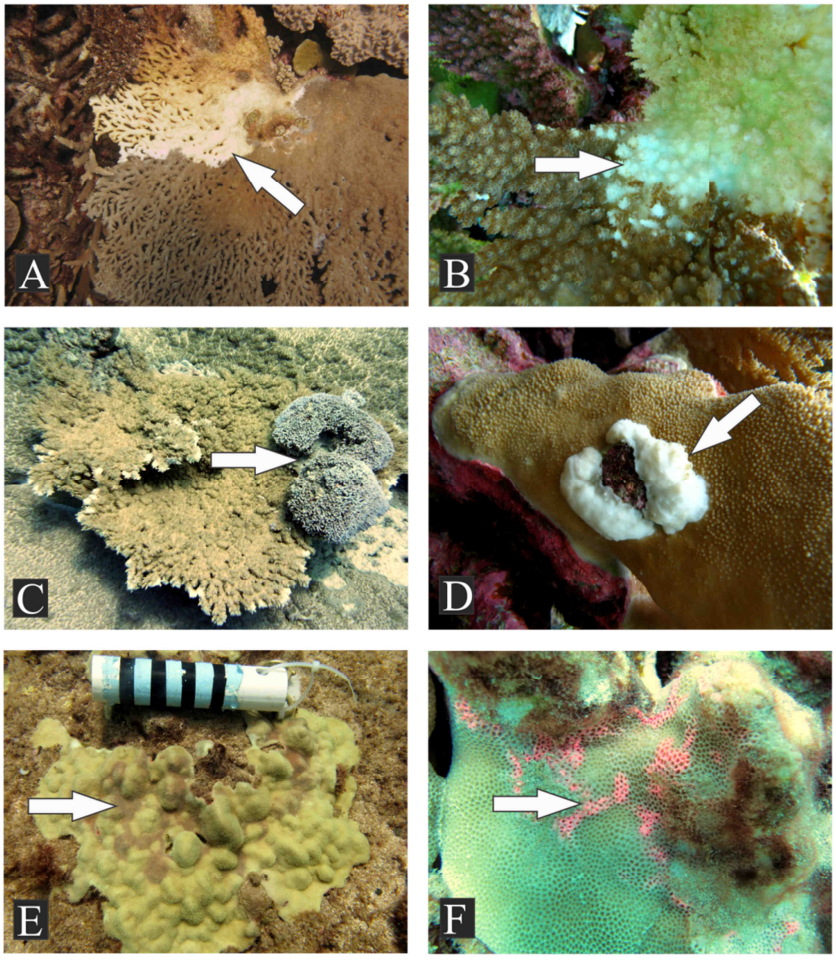


Fig. 2.1: Examples of coral diseases displaying tissue loss (A,B), growth anomalies (C,D) and discolouration (E,F). A: *Acropora* white syndrome, B: Close-up of *Acropora* white syndrome, C: *Acropora* growth anomaly, D: *Montipora* growth anomaly, E: *Porites* dark spot syndrome, F: *Porites* pigmentation response. Photo credit: A, B and D: Greta Aeby; E and F: Maya Walton.

The study of coral disease has been impeded by a lack of systematic nomenclature used in gross descriptions of coral lesions (Work and Aeby 2006) leading to misidentification of diseases, and in some cases the use of different names for different successive stages of the same disease (Richardson 1998). This has caused confusion in the literature about the true number of coral diseases described. By 2000, a total of 29 different diseases were recorded globally, however many of these disease descriptions were poor (Green and Bruckner 2000) and even to date there is no exact number on how many coral diseases occur worldwide. The majority of coral disease lesions are described primarily at the gross level (gross morphology), necessitating a standardized, systematic nomenclature for describing lesions in corals (Work and Rameyer 2005; Work and Aeby 2006). Work and Aeby (2006) provided such a standardized system (Fig. 2.2), which can be universally used and hence enable comparisons across geographical areas (Work and Aeby 2006).

Coral diseases have the potential to negatively impact coral functioning, such as reproduction and survival. It has been shown that disease can significantly reduce the reproductive output of affected colonies (Weil et al. 2009; Borger and Colley 2010) and cause widespread coral mortality leading to significant ecological changes in the functioning and structure of coral reefs (Aronson and Precht 2001a; Weil 2004). Investigating potential diseases that could pose a threat to the health and survival of coral reef ecosystems is therefore an important area of research.

In Kaneohe Bay, Oahu, a localised white discolouration affecting *Porites compressa*, one of the main framework building corals around Hawaii (Maragos 1972; Jokiel 1987), has been observed. Coral bleaching is defined as discolouration of the coral tissue due to the loss of the endosymbiotic dinoflagellates (*Symbiodinium* sp.) and/or their photosynthetic pigments (Glynn and D'Croz 1990; Hoegh-Guldberg and Salvat 1995). It can be caused by abiotic factors including high or low seawater temperatures (Glynn 1996; Mdodo and Obura 1998; Hoegh-Guldberg and Fine 2004), high light and excessive UV radiation (Drollet et al. 1995; Glynn 1996), or it can also be caused by biotic factors such as bacterial infections (Kushmaro et al. 1996; Ben-Haim et al. 1999). For example, in *Oculina patagonica* from the Mediterranean (Kushmaro et al. 1996) and *Pocillopora damicornis* from the Red Sea (Ben-Haim et al. 2003), a bacterial infection by *Vibrio* sp. causes bleaching and lysis of zooxanthellae (Ben-Haim et al. 1999). Coral bleaching could therefore be considered a sign of disease that can have abiotic or biotic causes (Jokiel 2004).

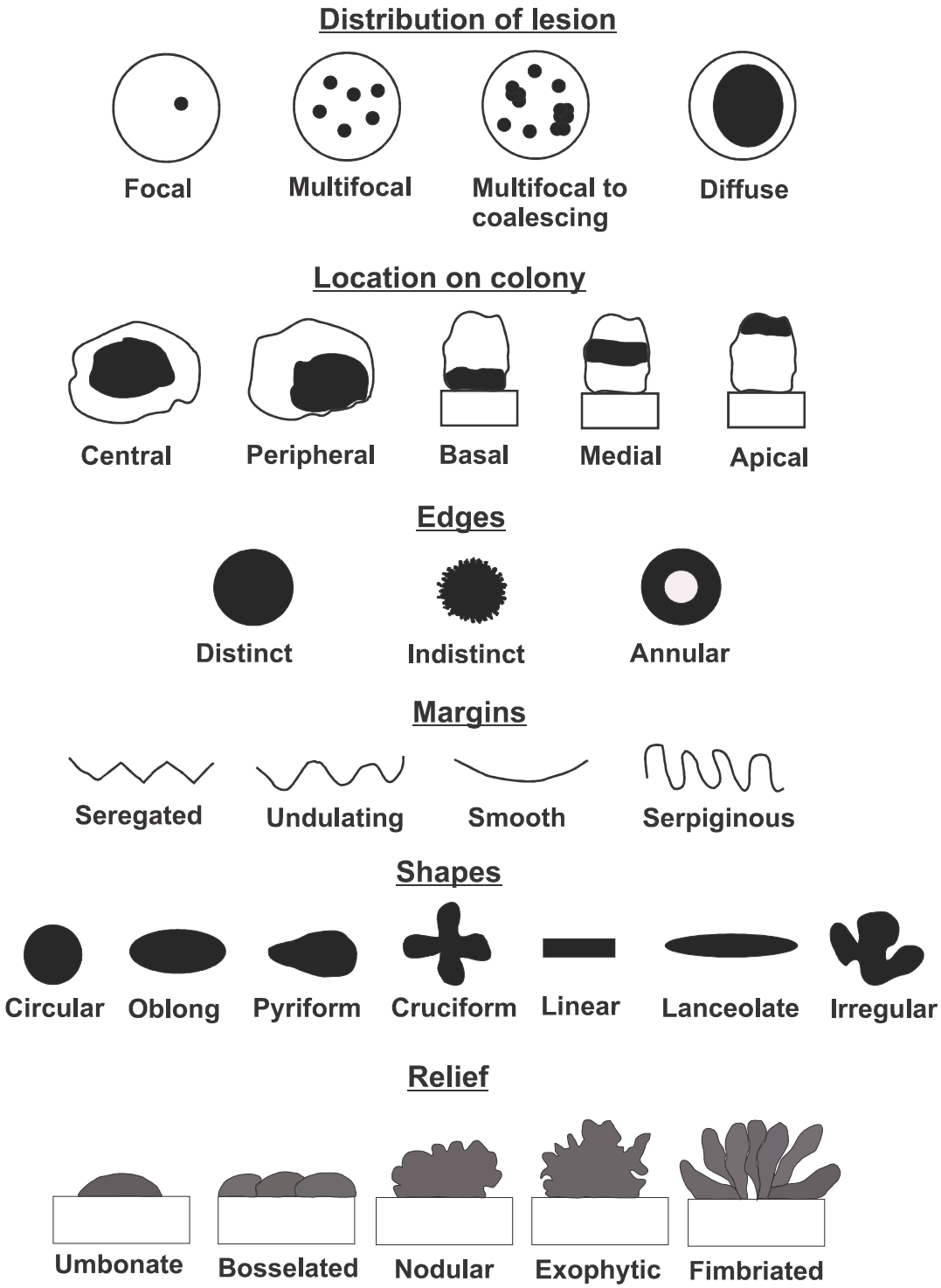


Fig. 2.2: Illustration of standardized terminology used for coral disease descriptions (after Work and Aeby 2006).

Mass coral bleaching has caused extensive coral mortality due to prolonged periods of increased sea surface temperatures often coinciding with strong El Niño events (Glynn and D'Croz 1990; Hoegh-Guldberg 1999). Entire reef systems can be affected on large geographical scales causing most corals to bleach (Hoegh-Guldberg 1999). In contrast, localized bleaching is a discrete area on a coral colony that shows white discolouration of the tissue. It has previously been recorded in poritid corals but little is known about this lesion (Work and Rameyer 2005).

The observed bleaching pattern in *P. compressa* appears different from thermally-induced coral bleaching as it occurs all year round during times when water temperatures are well within the coral's thermal tolerance thresholds. In addition, adjacent corals of the genus *Montipora* and *Pocillopora*, which are generally more susceptible to thermal bleaching (Jokiel and Coles 1990; Aeby et al. 2003; Kenyon et al. 2006), show no signs of bleaching at times when localized bleaching in *P. compressa* is observed.

The main objective of this study was to provide a systematic description of the localized bleaching based on the approach of Work and Aeby (2006) and determine the degree of virulence (harm to host) on the coral colony by measuring: 1) lesion progression, using individually tagged colonies; and 2) the effect on gametal development, using histological techniques.

2.2 Materials and methods

2.2.1 Systematic description

Multiple colonies affected by the localized bleaching were photographed and detailed notes were taken on the distribution of the lesion, its location on the colony, the shape of the lesion and its edges, margins, colour, size and structures affected. This information was then used to build a concise description of the lesion.

2.2.2 Tags

Forty-two colonies affected by localized bleaching were tagged around the perimeter of Coconut Island, Kaneohe Bay, Oahu, Hawaii (21°26.000'N, 157°47.000'W) in August 2010 and revisited in October 2010. All colonies were photographed and the percentages of healthy and affected tissue, as well as dead skeleton, were visually estimated *in situ*. The complex three-dimensional structure of *P. compressa* colonies and the often poor visibility in Kaneohe

Bay prevented an estimation of the affected area by digital analysis, necessitating the use of the semi-quantitative visual technique.

2.2.3 Reproductive output

P. compressa is a gonochoric broadcast spawner that spawns during full-moon periods between June and August (Neves 2000). Fragments ($3cm^2$) of *P. compressa* (22 fragments from healthy colonies and 31 fragments with evidence of localized bleaching) were collected from the reef crest around Coconut Island (please refer to Appendix I.1 for the sample design) in June 2010, two days before full moon and the first spawning of the season, so increasing the chance of finding well-developed gametes. Samples were fixed in zinc-formaldehyde solution, decalcified in HCl buffered with EDTA, and embedded in paraffin. The wax blocks were sectioned at 6 µm using a rotary microtome, stained with haematoxylin and eosin, and examined under a light microscope to determine the sex of the coral and assess the reproductive state of the gametes. The number of eggs was recorded within 5 haphazardly selected polyps from each reproductively active diseased and healthy female; this replication controlled for any errors arising from different planes of the histological sections. The maximum diameter of each egg within these polyps was measured using Image_J (http://rsbweb.nih.gov/ij/); volume could not be measured due to the limitations of the histological method used.

2.3 Data analysis

The data met the assumptions of normality but not equal variance. A two-sample t-test (equal variances not assumed) was carried out on replicate means using SPSS (PASW Statistics 18) to determine any differences in egg numbers and/or size between healthy colonies and those affected by localized bleaching.

2.4 Results

2.4.1 Systematic description

The observed bleaching generally manifests as focal or diffuse areas of white discolouration (tissue becomes translucent allowing the white skeleton to show through which gives the lesion a white appearance), with the polyps remaining brown giving the lesion a “speckled” pattern (Fig. 2.3 A). The lesion generally has a central or peripheral location on the colony (Fig. 2.3 B) which can, in smaller colonies, progress to envelop the entire colony (Fig. 2.3 C).

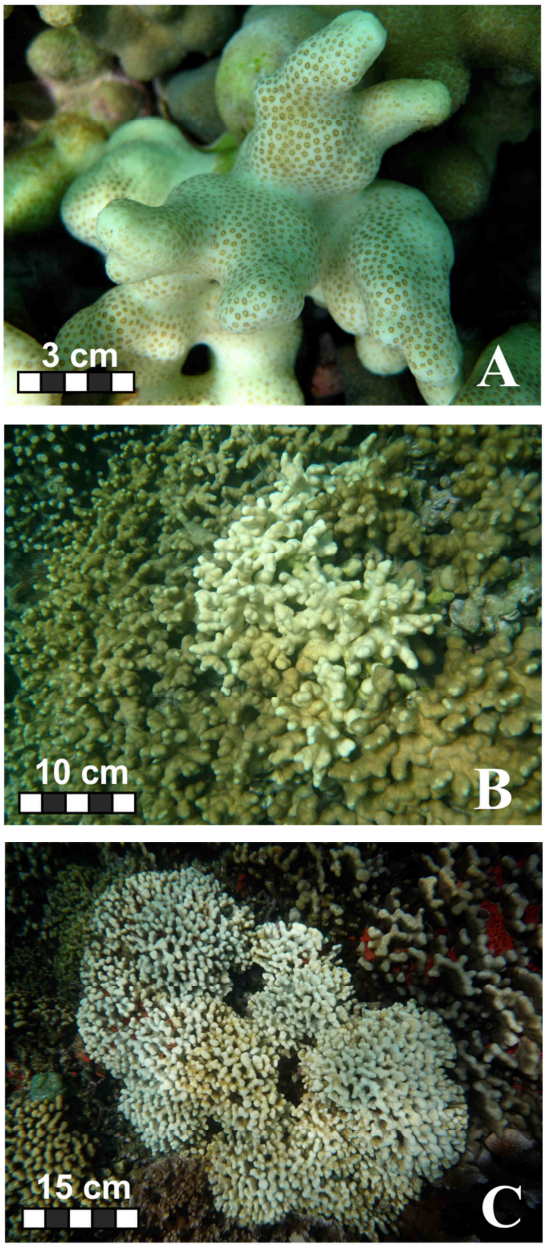


Fig. 2.3: *Porites compressa* colonies affected by localized bleaching. A: Localized bleaching. B: An extreme case with colony-wide bleaching. C: Close-up showing the discoloured coenenchyme and pigmented polyps. Photo credit: A and B Christina Runyon.

The size of the lesion is variable from a few branches affected to the entire colony showing signs of bleaching. The early stage of the lesion is characterized by the "speckled" appearance but the bleaching can progress to include the polyps as well. The lesion edge is distinct with undulating or smooth margins and the lesion can have a circular or oblong shape.

2.4.2 Tags and reproductive output

After 2 months, 35 out of 42 (83.3%) tagged colonies sustained an average partial colony mortality of 30.1% (range: 10-100%). However, partial re-pigmentation was also observed in 81% of cases.

All healthy *P. compressa* samples (n = 22) contained well-developed gametes (either oocytes or spermaries). In contrast, only 54.8% of colonies affected by localized bleaching (n = 17) had developed gametes (Fig. 2.4). Nine out of the 22 healthy samples and 6 out of the 17 samples affected by localized bleaching were females which were used for egg counts and measurements.

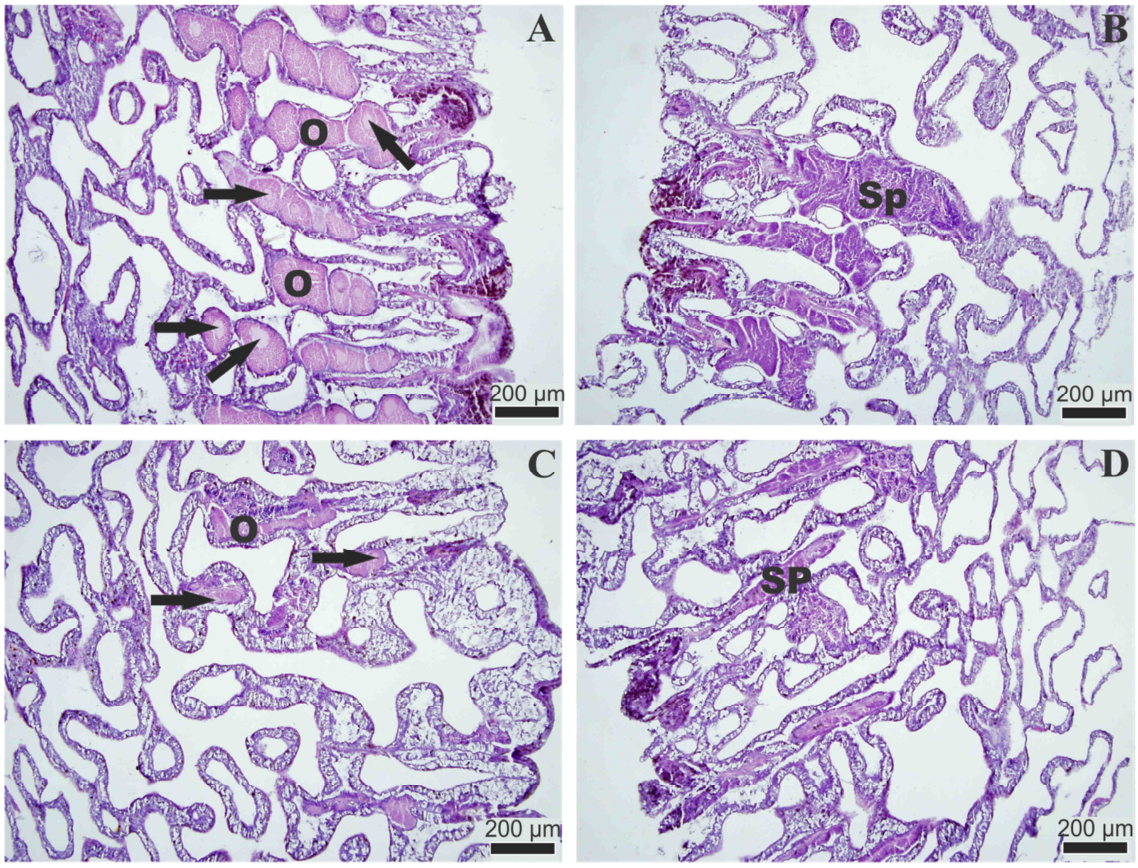


Fig. 2.4: *Porites compressa*. A: Longitudinal section of a healthy female showing well-developed oocytes containing eggs. B: Longitudinal section of a healthy male showing well-developed spermaries. C: Longitudinal section of a female affected by localized bleaching showing underdeveloped oocytes containing small eggs. D: Longitudinal section of a male affected by localized bleaching showing underdeveloped spermaries; O=oocytes, Sp=spermaries, ↑ eggs, all images 10x magnification. Scale bar = 200 µm.

A significant difference was found between both the number of eggs (df = 7.421, t = 5.031, p = 0.001) and the size of eggs (df = 8.141, t = 6.209, p < 0.001) in healthy *versus* diseased colonies. Healthy samples contained 4.9 ± 0.3 (mean ± SE) eggs per polyp with a maximum diameter of 44.6 ± 2.3 µm (mean ± SE) (n = 9). In contrast, samples affected by localized bleaching contained 1.6 ± 0.6 (mean ± SE) eggs per polyp with a maximum diameter of 23.9 ± 2.4 µm (mean ± SE) (n = 6). Healthy corals produced more and larger eggs (Fig. 2.4).

2.5 Discussion

This study showed that > 80% of colonies affected by localized bleaching sustained partial colony mortality but re-pigmentation was also observed, suggesting that the coral was, at least partially, able to recover. A similar pattern has been observed in other coral diseases. *Montipora* white syndrome (MWS) in Hawaii causes partial to total colony mortality but recovery occurs in approximately 30% of cases (Aeby et al. 2010). *Porites* ulcerative white spot disease (PUWS) from the Philippines causes small white lesions that can either regress or progress to tissue necrosis and cell death (Raymundo et al. 2003). Factors contributing to either recovery or mortality from a coral disease are still poorly understood.

Localized bleaching in *P. compressa* also causes a significant reduction in gamete development in both sexes. Almost 50% of the samples affected by localized bleaching developed no gametes, and all females that had gametes produced fewer and smaller eggs than did healthy colonies. Two female colonies affected by localized bleaching contained only under-developed oocytes with no eggs.

These results show that gametal development in *P. compressa* is significantly compromised in areas affected by localized bleaching. Similarly, thermal bleaching has also been shown to have a negative effect on the reproduction of many coral species (Szmant and Gassman 1990; Ward et al. 2000); though interestingly, Cox (2007) found no decrease in reproduction in thermally-bleached colonies of *Montipora capitata*, which has the ability to increase its heterotrophic feeding rates after bleaching (Grotolli et al. 2006). The impact of bleaching on coral reproduction is most likely linked to the loss of zooxanthellae, as these can contribute over 90% of the coral's energy requirements through photosynthesis, and support coral growth and reproduction (Muscatine et al. 1984). Alternatively, oocytes contain a very high concentration of lipids that can provide energy when resources are limited (Weil 2009) and could be re-absorbed, as has previously been recorded in stressed corals (Neves 2000; Okubo et al. 2009).

Sexual reproduction in corals is strongly influenced by biotic and abiotic stressors such as pollution (Guzman and Holst 1993; Cox and Ward 2002), sedimentation (Fabricius 2005) and competitive interactions (Rinkevich and Loya 1985; Tanner 1995). Diseases are a drain on energy resources to the host and are therefore very likely to impact reproduction, however comparatively few studies in the coral disease literature have measured the effect of disease on the reproductive output of corals. A decreased reproductive output was found in *Montastraea faveolata* from the Caribbean affected by yellow band disease (Weil et al. 2009) and *Montastraea faveolata* from the West Indies affected by white plague (Borger and Colley 2010). A localized suppression of reproduction has also been shown in Caribbean sea-fan octocorals affected by a fungus or protist infection (Petes et al. 2003).

The reproductive effort of an individual can be used as an evaluation of general fitness (Metz et al. 1992). For corals, fertilization success, larval dispersal, recruitment and survivorship drive population dynamics and coral reef regeneration (Hughes and Tanner 2000; Vermeij 2005, 2006) and even a small reduction in reproductive output can have the potential to cause severe negative impacts on recruitment (Hughes et al. 2000). The negative impact of diseases on the reproductive output of corals should therefore not be neglected. Indeed, in the case of disease outbreaks, reduced reproduction in addition to colony mortality could have major impacts on reef resilience and longevity.

2.6 Conclusion

The observed localized bleaching results in both partial colony mortality and reduced gametal development of the coral, causing harm to the host. It can therefore, by definition, be classified as disease and was termed *Porites* bleaching with tissue loss (PBTL). *P. compressa* is one of the main framework-building corals in Hawaii, and so PBTL has the potential to negatively impact the population structure and resilience of Hawaiian reefs warranting further investigation.

Chapter 3

Cytopathological observations in the Hawaiian reef coral, *Porites compressa*, affected by *Porites* bleaching with tissue loss

3.1 Introduction

In recent decades, coral diseases have increased in prevalence and geographical extent worldwide, threatening the health and survival of coral reefs (Harvell et al. 2004; Weil et al. 2006; Sokolow 2009). However, descriptions of most coral diseases have been based on field surveys, and many diseases lack systematic morphological descriptions at both the gross and cellular levels (Work et al. 2008c). Confounding the presence of lesions with causation of disease without appropriate laboratory confirmation has led to considerable confusion in the literature (Richardson 1998; Lesser et al. 2007; Work and Aeby 2011). The use of a standardized nomenclature that provides a systematic morphological description of coral disease lesions at the gross and cellular levels allows uncoupling of the description of the lesion from the inference of causation and comparisons across geographical areas (Work and Rameyer 2005; Work and Aeby 2006; Work and Aeby 2011). Systematic descriptions of lesions at the gross and cellular levels provide the initial step in the development of case definitions and may assist in identifying possible pathogens (Work and Rameyer 2005; Work et al. 2008c).

Corals harbour a diverse microbial assemblage including zooxanthellae, bacteria, cyanobacteria, archaea, fungi, protists, viruses and endolithic algae; the entire assemblage is named the coral holobiont (Rohwer 2002). Some microbes have been identified to be directly involved in coral disease manifestations (Kushmaro et al. 2001; Patterson et al. 2002; Denner et al. 2003; Sussman et al. 2008). In healthy corals, certain microbes may benefit the coral by nitrogen fixation (Rohwer 2002, Rosenberg 2007b), supply of supplemental nutrients (Kramarsky-Winter et al. 2006) and/or the production of antimicrobial compounds (Ritchie 2006). A disruption of the coral holobiont could cause physiological changes that may lead to a disease state (Rohwer 2002, Vega-Thurber 2009). Using metagenomic approaches, Vega-Thurber et al. (2009) demonstrated that with temperature, pH and nutrient stress the microbial community of *Porites compressa* shifted to a community of microbes often found on diseased corals. More research into the individual components of the coral holobiont is urgently

needed to better understand fluctuations caused by environmental factors that may lead to disease.

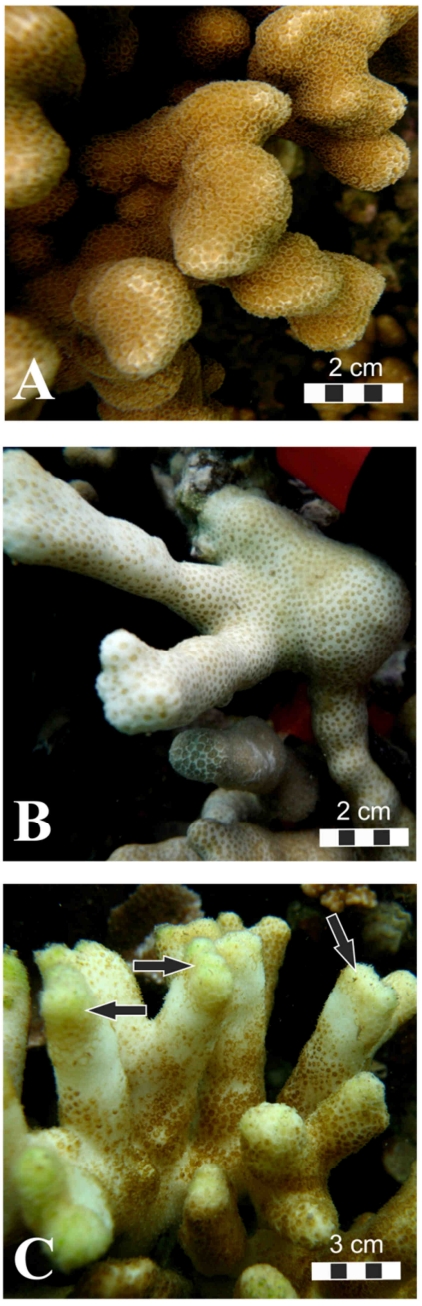


Fig. 3.1: *Porites compressa*. A: Healthy *P. compressa.* Note regular brown coloration. B: *P. compressa* affected by PBTL (early stage). Note white discoloration of coenenchyme and pigmented polyps but no signs of tissue loss. C: *P. compressa* affected by PBTL (progressive stage). Note beginning of tissue loss on the tips of the coral branches (arrowhead).

Porites compressa is one of the main framework building corals in Hawaii. In Kaneohe Bay, Oahu, this species is affected by *Porites* bleaching with tissue loss (PBTL) that manifests as diffuse areas of discolouration (secondary to translucence and visibility of skeleton through tissue) of the coenenchyme with pigmented polyps that are often retracted (Fig. 3.1). The lesion may be located in the centre or on the periphery of a colony or may be colony-wide (on smaller colonies). In most cases, PBTL results in partial tissue loss with subsequent algal colonisation of the dead skeleton (Chapter 2). PBTL does not appear to be a response to elevated sea surface temperatures as it occurs only in isolated colonies at times when water temperatures are well within the thermal threshold of this species.

Given the uncertain causes of this disease, I set out to characterize PBTL at the cellular level. Specifically, I measured 1) tissue thickness and *Symbiodinium* cell densities and described changes at the cellular level and 2) explored the potential presence of viruses in healthy and PBTL-affected coral fragments using transmission electron microscopy (TEM).

This study presents the first cytopathological information on PBTL, so providing a foundation for a case definition of this disease.

3.2 Materials and methods

3.2.1 Sample collection

Branches (2-3cm^2) of *Porites compressa* (36 fragments with signs of PBTL and 27 fragments from healthy control corals for histopathology and 4 fragments affected by PBTL and 4 fragments from healthy control colonies for TEM) were collected from the reef crest around Coconut Island, Kaneohe Bay, Oahu, Hawaii (21°26.000'N, 157°47.000'W) at a depth of 0.5-2 m in June 2010, June 2011 and November 2011 (please refer to Appendix I.1 for the sample design). Samples for histology were fixed in 20% zinc-formaldehyde solution (1 part Z-Fix concentrate [Z-Fix® Anatech, Battle Creek, MI, USA] in 4 parts 0.2 µm filtered seawater) and samples for TEM were fixed in Trumps fixative (4% formalin, 1% glutaraldehyde).

3.2.2 Sample preparation

3.2.2.1 Histopathology

Samples were decalcified in 2.5% HCl buffered with 0.1% EDTA, rinsed and stored in 70% ethanol until further processing. After decalcification, all samples collected in 2010 (n = 53)

were cut in half with a razor blade (tip to bottom) and laid open; measurements of tissue thickness were taken from the tip and the sides of the coral fragment using a Kincrome Digital Vernier Caliper. A 2 cm diameter core was then removed from one half of all control and diseased fragments with a cork borer placed at a distance of 1 cm from the tip of the fragment. The core was homogenized with 1 ml of 0.2 µm-filtered seawater in a tissue homogenizer and algal cells were counted on a haemocytometer (Improved Neubauer, Boeco Ltd. Germany), with 8 replicate counts per core. Cell densities were standardized to tissue volume of the core (π r^2 x thickness).

The other half of the decalcified coral fragment was trimmed and embedded in paraffin. Wax blocks were sectioned at a thickness of 6 µm using a rotary microtome, and the resulting sections stained with hematoxylin and eosin (H&E). Please refer to Appendix II for the histological protocol used. Sections were examined at the microscopic level and lesions classified according to the presence of: 1) necrosis characterized by cytoplasmic hypereosinophilia or fragmentation coupled with nuclear karyorrhexis, karyolysis or pyknosis; 2) tissue fragmentation characterized by loss of epidermis and exposure of the basal body wall and mesenterial filaments; 3) changes in *Symbiodinium* and melanin-containing granular cell densities and/or morphology; and 4) presence or absence of associated organisms.

I also observed putative bacterial aggregates (Peters, 1997) in the tissues of *P. compressa* in both healthy and diseased samples (Fig. 3.2A). All putative bacterial aggregates were enumerated in a standardized 1712 x 1289 µm area of coral tissue approximately 1 cm below the branch tip.

3.2.2.2 Transmission electron microscopy

Small pieces of the fragments were cut for decalcification in a 15% EDTA solution buffered with 2.5% glutaraldehyde, 0.1 M cacodylate and 0.44 M sucrose. After decalcification was complete, samples were washed in 0.1 M cacodylate buffer with 0.44 M sucrose for 2 changes, 20 minutes each. A cross section (about 1 mm^2) was cut and washed again for 20 minutes. These sections were then postfixed for 1 hour with 1% osmium tetroxide in 0.1 M cacodylate buffer. After postfixation, samples were dehydrated in an ethanol series (30%, 50%, 70%, 85%, 95%), with 3 changes per dilution for 5 minutes each. For the final dehydration, 100% ethanol was used for 3 changes, for 10 minutes each. Samples were then

substituted with propylene oxide during 3 changes for 10 minutes each. A 1:1 mixture of propylene oxide and epoxy resin (Ladd Industries: LX-112 4.6g, DDSA 2.04g, MMA 2.26g) was used to infiltrate the samples overnight. The next day, samples were immersed in freshly made 100% epoxy resin for 3 hours and then in another change of epoxy resin for 2 hours. Samples were then orientated in moulds, covered with fresh epoxy resin and polymerized at 60ºC for 3 days.

Ultrathin (60-80 nm) sections were obtained on a RMC Powertome ultramicrotome and double stained with uranyl acetate and lead citrate. The sections were viewed on a Hitachi HT7700 TEM at 100 kV, and photographed with an AMT XR41 four megapixel CCD camera.

3.3 Data analysis

The data for coral tissue thickness, *Symbiodinium* cell densities and bacterial aggregate counts were checked for normality and equal variance. For coral tissue thickness, these assumptions were met and a two-sample t-test was used to determine differences in tissue thickness between fragments affected by PBTL and control corals. Data for *Symbiodinium* cell and bacterial aggregate counts, did not meet assumptions of normality and equal variance, so a non-parametric Mann-Whitney U test was used for comparisons.

3.4 Results

3.4.1 Histopatholgy

Branches affected by PBTL had significantly thinner tissue on the tip ($df = 51$, $t = 6.887$, $p < 0.001$) and sides ($df = 51$, $t = 2.322$, $p = 0.024$) than healthy controls (Table 3.1). Corals affected by PBTL also showed a significant decrease in *Symbiodinium* cell density (Mann-Whitney $U = 25$, $n = 53$, $p < 0.001$) and in the abundance of putative bacterial aggregates (Mann-Whitney $U = 148.5$, $n = 53$, $p < 0.001$) (Table 3.1). Of the healthy coral samples, 77% had putative bacterial aggregates in the examined tissue section versus 26% of PBTL-affected samples. Putative aggregates were round to oblong and ranged from approximately 170 – 1520 μm^2 in PBTL-affected samples and 175 – 1914 μm^2 in healthy samples.

Histological examination showed reductions in both *Symbiodinium* and melanin-containing granular cell densities (please refer to Appendix III.1) that were more pronounced in the gastrodermis of the coenenchyme than in the polyps.

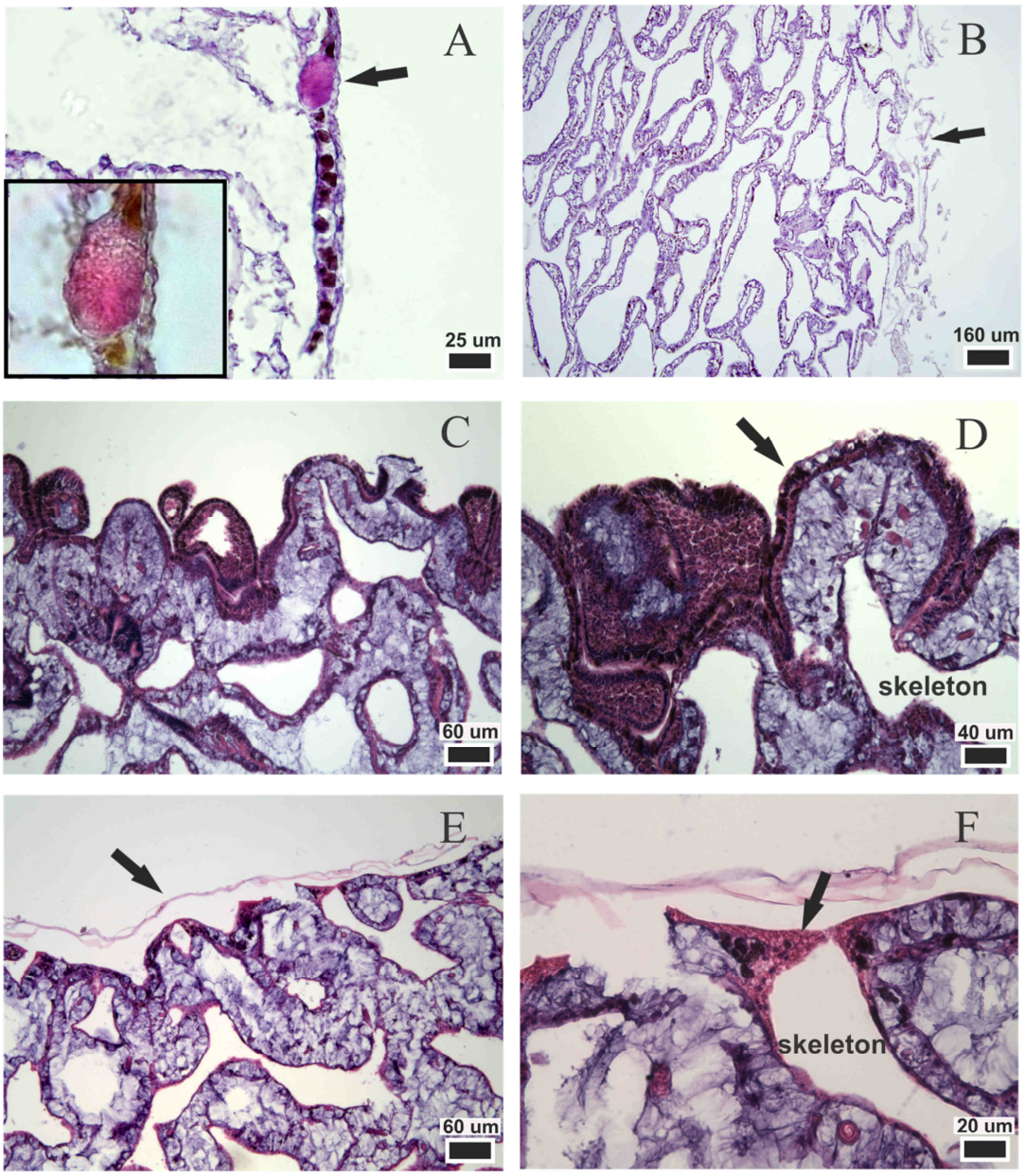


Fig. 3.2: *Porites compressa*. A: *P. compressa* with PBTL. Note putative bacterial aggregate in epidermis (arrowhead). Inset shows a close-up of the bacterial aggregate. B: *P. compressa* with PBTL. Note ablation of the epidermis (arrowhead). C and D: Normal *P. compressa.* Note regular columnar epidermis (arrowhead). E: *P. compressa* with PBTL. Note attenuation and ablation of epidermis with overlaying hyaline membrane (arrowhead). F: Close-up of E. Note hyaline membrane overlaying epidermis that manifests as cytoplasmic hypereosinophilia and karyorrhexis (arrowhead).

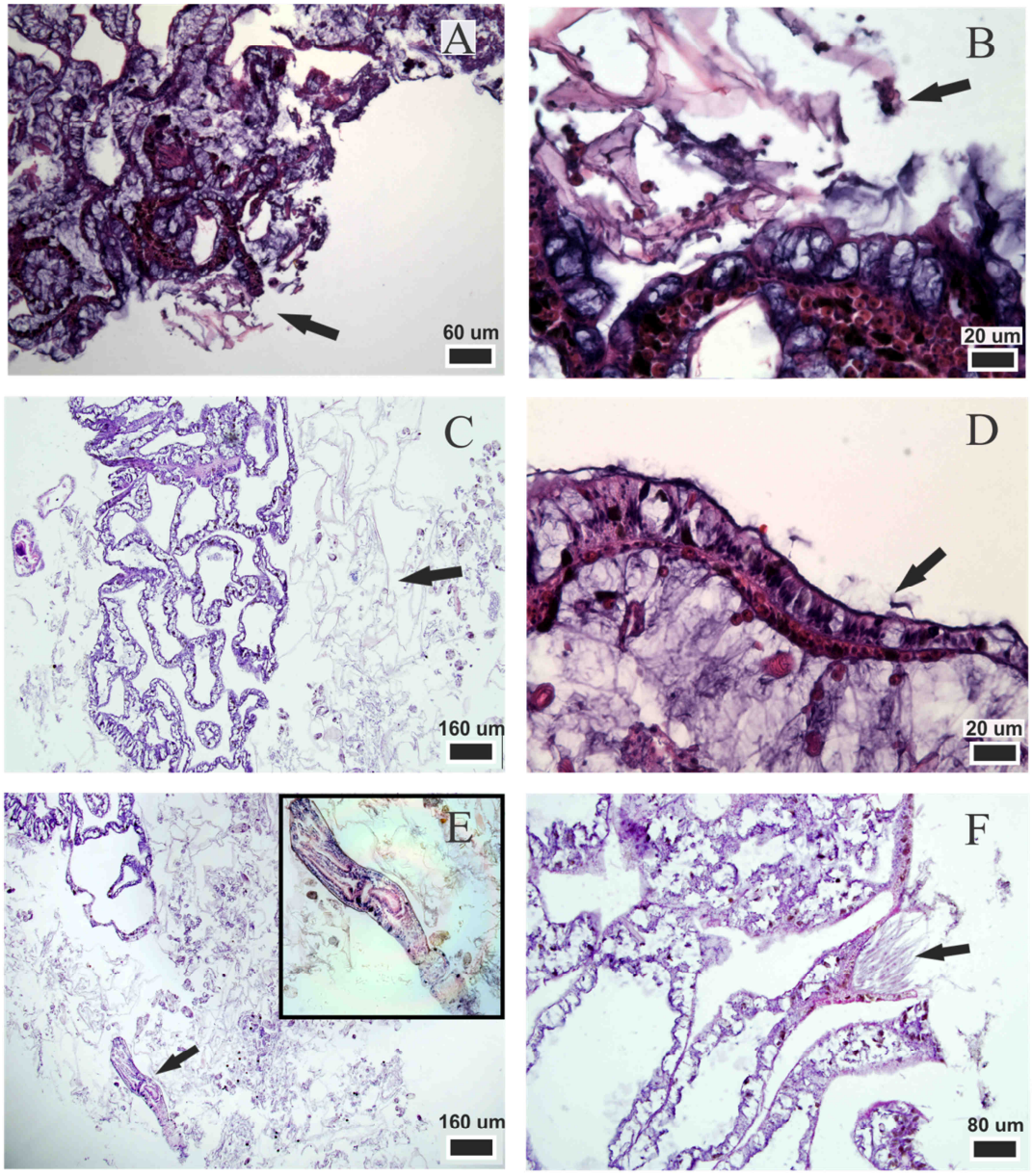


Fig. 3.3: *Porites compressa*. A: *P. compressa* with PBTL. Note tissue fragmentation and hyaline membranes effacing epidermis (arrowhead). B: Close-up of A. Note cell debris (arrowhead) mixed with hyaline membrane. C: *P. compressa* with PBTL. Note tissue fragmentation and cell debris (arrowhead). D: Normal *P. compressa*. Note regular columnar epidermis with remnants of mucus (arrowhead). E: *P. compressa* with PBTL. Note tissue fragmentation and helminths in the tissue debris (arrowhead). Inset shows a close-up of the helminths. F: *P. compressa* with PBTL. Note clump of diatoms on epidermis (arrowhead).

Ablation of the epidermis associated with algal overgrowth (Fig. 3.2 B) was observed in 39% of samples affected by PBTL. Necrosis manifested as cytoplasmic hypereosinophilia and karyorrhexis overlaid by a hyaline membrane (Fig. 3.2 E and F) and was observed in 19% of

samples examined. Tissue fragmentation (Fig. 3.3 A-C) was observed in 11% of PBTL-affected samples, and in two of these samples, helminths were observed in the degrading tissue (Fig. 3.3 E). Clumps of diatoms were also found on the epidermis of one diseased sample (Fig. 3.3 F). No other microbial or metazoan organisms were seen associated with diseased tissue for all remaining samples.

Table 3.1: Tissue thickness from the tip and side of the branches (mm), *Symbiodinium* cell densities (cells cm^3) and bacterial aggregate size (number/mm^2) in healthy control branches of *Porites compressa* and branches affected by PBTL. Values are mean ± SE and the range (minimum to maximum value). All pair-wise comparisons between healthy and PBTL-affected colonies were statistically significant ($p < 0.05$).

	Healthy	Range (min-max)	PBTL	Range (min-max)
Tissue thickness: tip (mm)	6.08 ± 0.18	4.69 – 8.21	4.38 ± 0.17	2.16 – 6.27
Tissue thickness: side (mm)	2.46 ± 0.07	1.84 – 3.19	2.21 ± 0.08	1.28 – 3.18
***Symbiodinium* density (cells cm^3)**	$1.4 \times 10^6 \pm 74533.1$	$4.7 \times 10^5 - 2.1 \times 10^6$	$4.8 \times 10^5 \pm 37027.8$	$1.5 \times 10^5 - 1.3 \times 10^6$
Bacterial aggregates (number/mm^2)	0.74 ± 0.14	0 – 2.3	0.19 ± 0.07	0 – 1.8

3.4.2 Transmission electron microscopy

TEM micrographs showed that most *Symbiodinium* cells in healthy fragments appeared healthy (Fig. 3.4 A & B) and degraded *Symbiodinium* cells were only occasionally observed (Fig. 3.4 C). In contrast, PBTL-affected samples had numerous *Symbiodinium* cells in a degraded state that were filled with black bodies and no nucleus was visible (Fig. 3.4 D); which was likely representing a late stage of apoptosis. Generally, these degraded cells were smaller than healthy *Symbiodinium* cells (Fig. 3.4 E) but were variable in size (Fig. 3.4 F). Healthy looking *Symbiodinium* cells were only rarely observed. Additionally, *Symbiodinium* cells in a stage of degradation without black bodies were also observed (Fig. 3.4 G & H); which was likely representing early stages of apoptosis.

VLPs were observed in the host tissue of both healthy (Fig. 3.5 A) and PBTL-affected samples (Fig. 3.5 C-F, 3.6 and 3.7) but were never very abundant. Nevertheless, VLP's were more often encountered in PBTL-affected samples (though this difference was not quantified). Most observed VLPs were hexagonal with a diameter of approximately 100 nm.

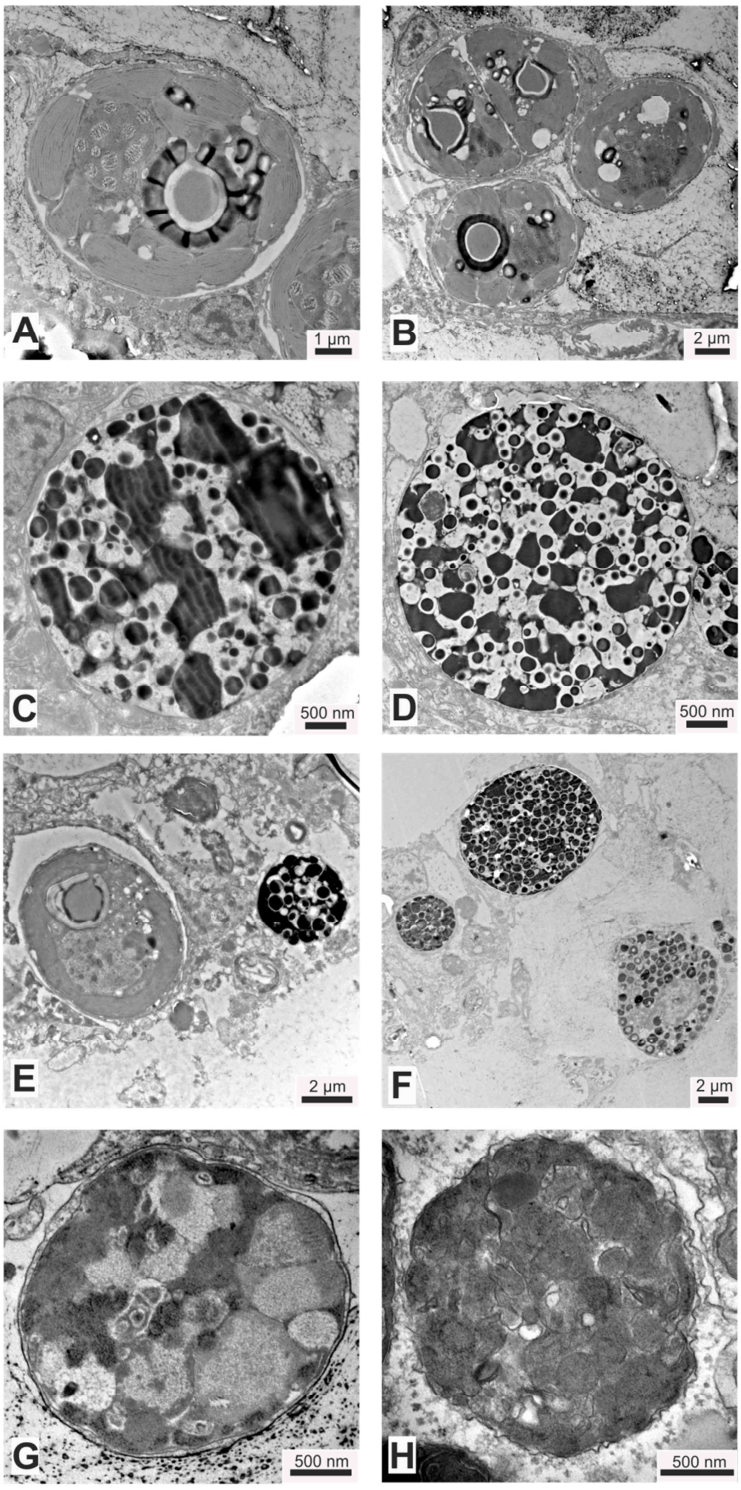


Fig 3.4: *Symbiodinium* cells in healthy and PBTL-affected *Porites compressa*. A: Healthy sample. B: Healthy sample with dividing *Symbiodinium* cell. C: Healthy sample with potential apoptotic cell. D: PBTL with potential apoptotic cell. E: PBTL sample with healthy and potential apoptotic cell. F: PBTL with potential apoptotic cells. G & H: PBTL with cell in potentially early stage of apoptosis.

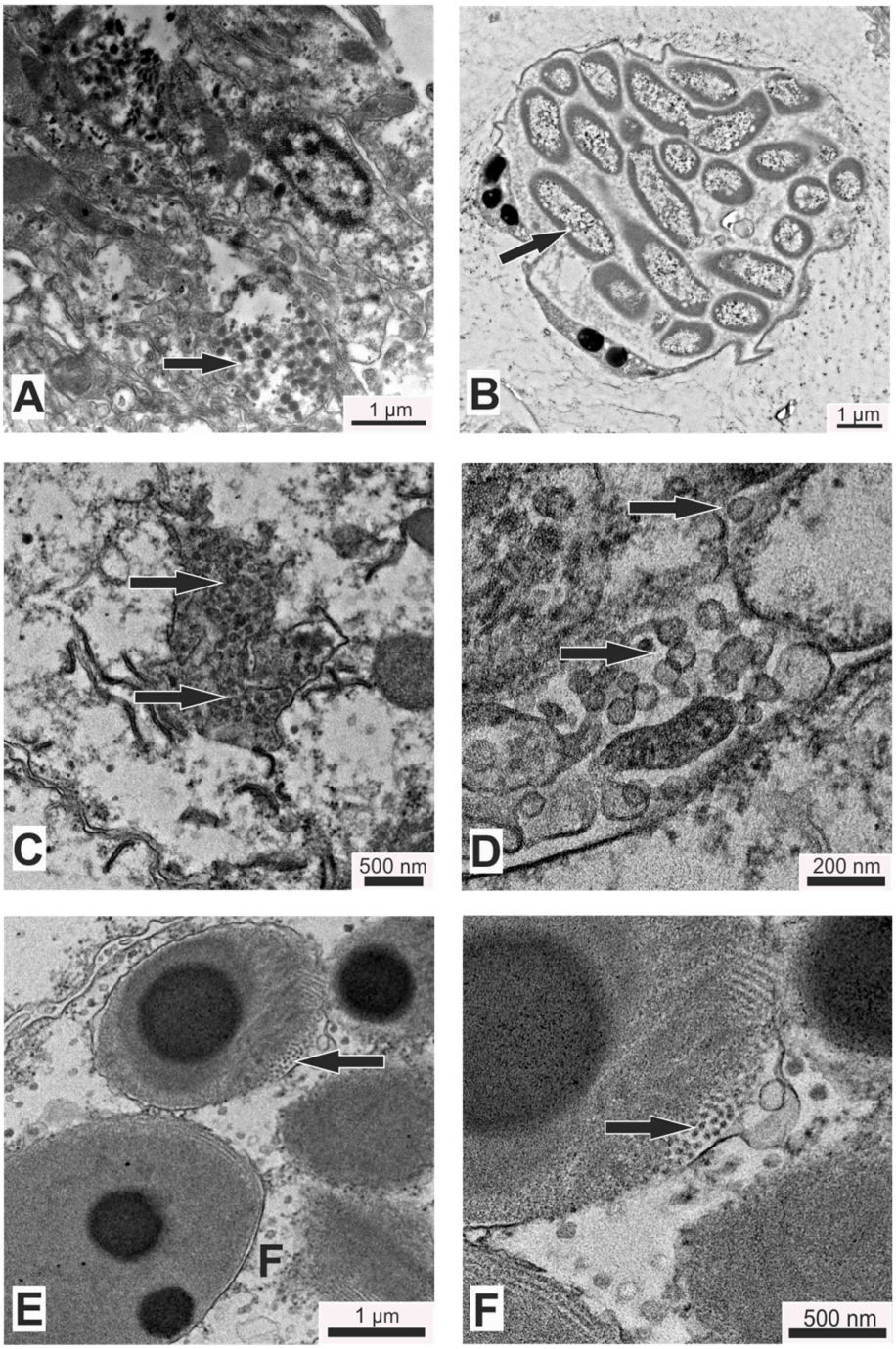


Fig. 3.5: VLPs in healthy and PBTL-affected *Porites compressa*. A: Healthy *P. compressa* with VLPs (arrowhead). B: Healthy *P. compressa* with bacterial aggregate. Arrowhead indicates individual bacterium. C-F: PBTL-affected *P. compressa* with VLPs (arrowhead). F is a close-up of E.

Herpes-like viruses encapsulated in a vacuole (Fig. 3.6 A and B) were observed in one particularly degraded section of a diseased sample but were not observed in any other

samples. A bacterial aggregate (Fig. 3.5 B) was also observed in a healthy sample. Most VLPs were found in aggregates and some aggregates were encapsulated by a vacuole (e.g. Fig. 3.6 C and D, Fig. 3.7 C and D).

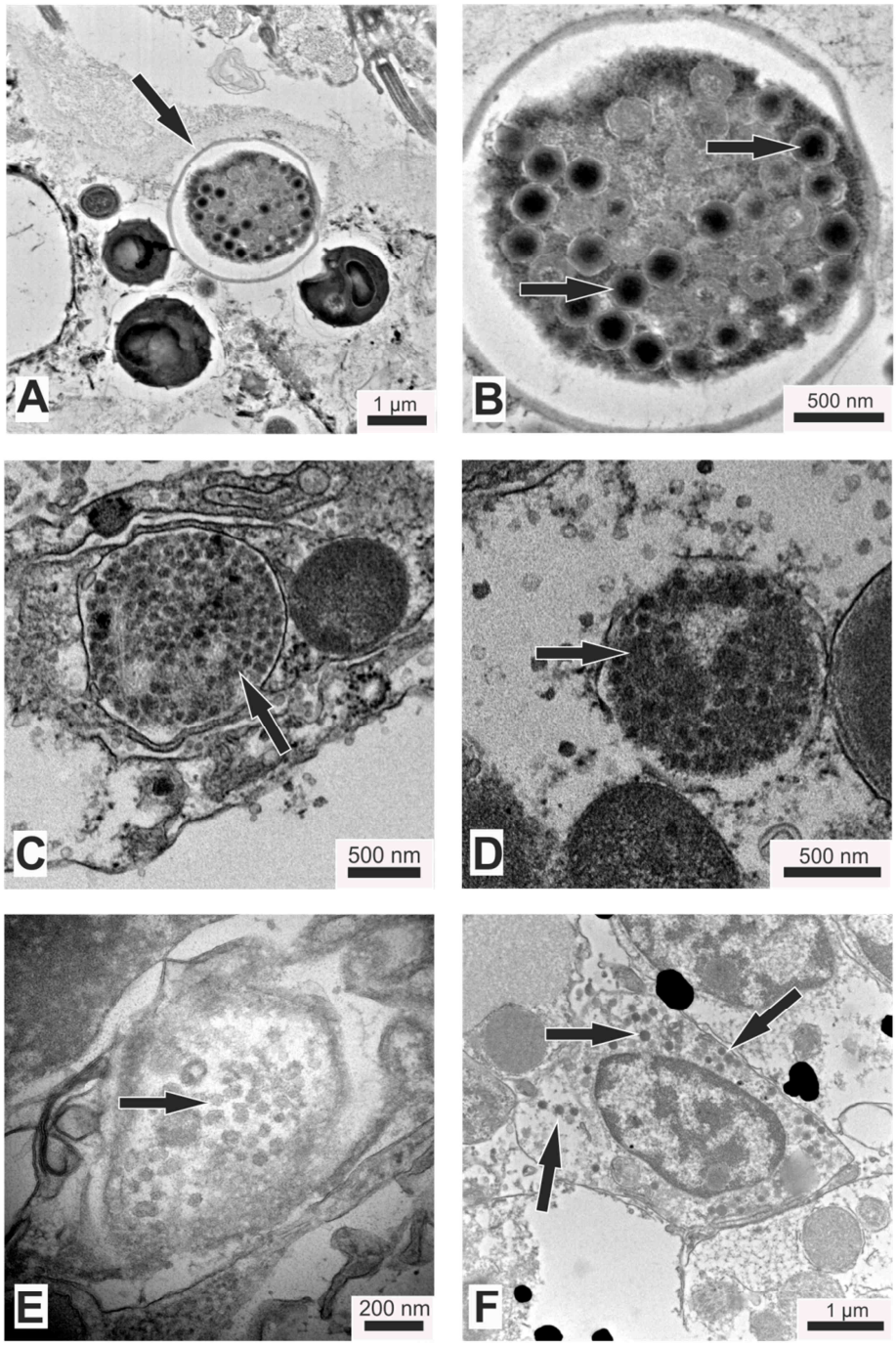


Fig. 3.6: PBTL-affected *Porites compressa*. A and B: Herpes-like viruses enclosed in vacuole. C – F: VLPs (arrowhead).

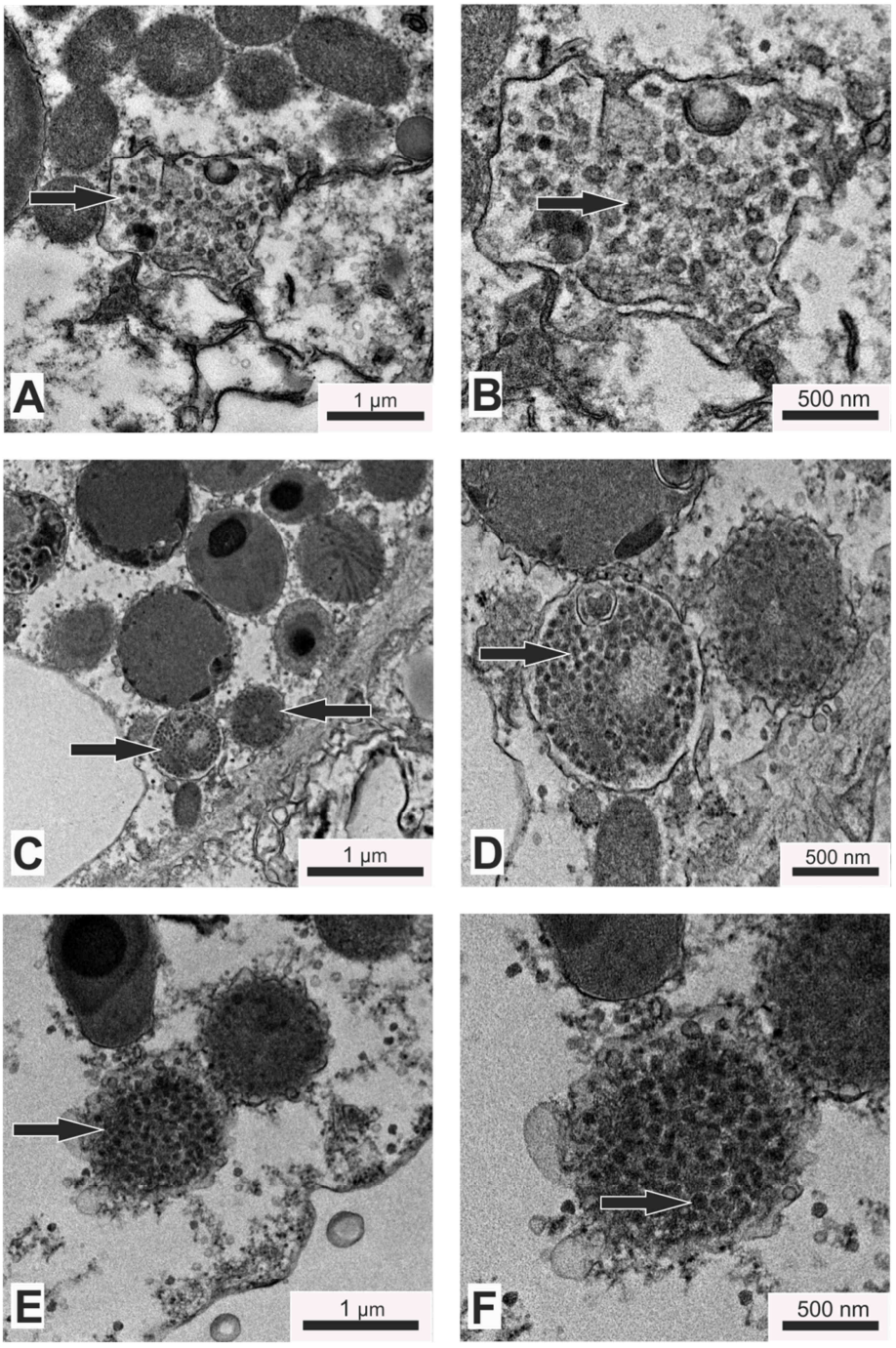


Fig. 3.7: PBTL-affected *Porites compressa*. A – H: VLPs (arrowhead). Image B, D and F are close-ups of previous image.

3.5 Discussion

3.5.1 Histopathology

Corals affected by PBTL show a significant loss of their symbiotic dinoflagellates (*Symbiodinium* spp.) and melanin-containing granular cells, mainly from the gastrodermis of the coenenchyme. This response was less pronounced in the polyps, thereby giving affected corals the typical "speckled" appearance (i.e. bleached coenenchyme and pigmented polyps). The extensive tissue loss of affected colonies observed in the field (Chapter 2) was found to result from tissue fragmentation and necrosis, leading to tissue mortality of affected areas.

PBTL-affected samples showed a 65% reduction in *Symbiodinium* cell density. This is similar to the loss seen in other corals that exhibit bleaching in response to disease. For example, bacterial bleaching in the reef coral *Pocillopora damicornis*, is characterized by a loss (>88%) and lysis of *Symbiodinium* cells due to an infection by *Vibrio* spp. (Ben-Haim et al. 2003). *Symbiodinium* cell loss (41-96.9%) is also seen in YBD, which affects *Montastraea* spp. from the Caribbean and starts as small blotches with reduced pigmentation, advancing over the colony and leaving dead skeleton behind (Cervino et al. 2001). PBTL, however, shows a very different and distinct bleaching pattern, with the coenenchyme bleaching first and the polyps remaining brown. In YBD, the observation that *Symbiodinium* cells degraded *in hospite* led to the assumption that the pathogen primarily targets the symbiotic dinoflagellates rather than the coral host (Cervino et al. 2004). In the case of PBTL, *Symbiodinium* cells appeared intact and did not differ morphologically from those seen in healthy control samples. However, the use of transmission electron microscopy rather than light microscopy is needed to clarify this issue.

The loss of *Symbiodinium* cells may have contributed to the observed atrophy in affected samples (tissue thinning of 28% on the tip and 11% on the sides), which is indicative of a stressed coral colony. *Symbiodinium* cells can contribute over 90% of the coral's energy requirements through photosynthesis (Muscatine et al. 1984); a loss of *Symbiodinium* therefore leads to less energy being available for growth and other life processes such as reproduction and repair. Moreover, to counteract the prolonged loss of nutrition, corals may reabsorb their tissues (Szmant and Gassman 1990). Atrophy can be observed in bleached corals (Glynn et al. 1985) and corals that are affected by sediment stress (Vargas-Angel et al. 2007) which can, if the stress is continuous, lead to tissue necrosis (Riegl and Bloomer 1995).

Tissue fragmentation and necrosis were only observed in a few samples affected by PBTL, probably because the sampled coral colonies were at different stages of the disease. During sampling, branches in earlier stages of the disease (i.e. with intact tissue) were targeted and only a few were collected with signs of tissue loss. Early stages of PBTL are mainly characterized by the loss of *Symbiodinium* cells, but it progresses to tissue fragmentation and necrosis in later stages. Cell death associated with tissue loss has been recorded in association with several coral diseases (McClanahan et al. 2004; Work and Rameyer 2005; Renegar et al. 2008; Williams et al. 2011c) and appears to be a common response to disease.

Ablation of the epidermis was associated with microalgal overgrowth, which may have contributed to tissue death. Other potentially opportunistic invaders, helminths and diatoms, were also observed in samples affected by PBTL. Helminths were found in the tissue debris associated with tissue fragmentation that has also been reported in other coral diseases (Work and Aeby 2011). Diatoms were observed on the epidermis of a diseased sample. Few studies have reported the occurrence of diatoms on the surface of corals (Rublee et al. 1980; Johnston and Rohwer 2007) likely due to healthy corals ability to protect themselves from settling organisms or sediment by mucus shedding (Brown and Bythell 2005). Sorting out whether organisms such as helminths, diatoms or other algae were primary invaders or sequelae to primary tissue loss will require longitudinal studies.

Using histology, bacterial aggregates have been observed in the tissues of corals (mostly *Acropora* spp.) affected by disease (Peters et al. 1983; Galloway et al. 2007), but no bacterial aggregates have been found in many other disease lesions (Glynn et al. 1985; Bythell et al. 2004; Ainsworth et al. 2007a). In this study, numerous clusters of putative bacterial aggregates were observed in both healthy and PBTL-affected corals but they were not associated with cell pathology, as seen in bacteria-induced diseases of vertebrates (Olsen et al. 2006; Magi et al. 2009), invertebrates (Johnson 1976) and plants (Nelson and Dickey 1970; Wallis and Truter 1978). Indeed, a 74% reduction of putative bacterial aggregates was observed in corals affected by PBTL versus healthy colonies suggesting a disruption of the coral holobiont. If the symbiotic relationship between the coral and its associated microbial community is disrupted for any reason (for example changes in environmental factors), the whole balance of the holobiont could be compromised, ultimately contributing to a disease state (Vega-Thurber et al. 2009). Identifying how these bacteria interact with the coral host and their role in coral defence is a potentially fruitful avenue of investigation.

Given the lack of consistency between lesions and a particular etiological agent, I do not suspect that PBTL is caused by metazoans, bacteria or protozoans (see also Appendix IV). However, smaller pathogens such as viruses, which are not easily detectable by light microscopy, cannot be ruled out and necessitate ultra-structural examination of tissues. Other possible causes to consider are toxins and/or environmental triggers.

3.5.2 Transmission electron microscopy

Cell counts revealed that PBTL reduced *Symbiodinium* densities by 65% but the resolution of the histology slides was too low to draw any conclusions on the health state of remaining algal cells. TEM imaging revealed that most of the remaining *Symbiodinium* cells showed severe degradation that resembled apoptotic *Symbiodinium* cells that have undergone thermal stress (Strychar et al. 2004a). However, to confirm that *Symbiodinium* cells in this study really were undergoing apoptosis, markers such as Annexin V have to be used.

Apoptosis is defined as active cell death where a specialized signalling pathway actively kills the cell (Häcker 2000), whereas necrosis is an accidental, passive and degenerative process that is triggered by gross injury/cell trauma (Darzynkiewicz et al. 1997). Programmed cell death (PCD) is often used as a synonym for apoptosis, however these terms should be used to describe different processes of active cell death (Strychar 2004a). PCD is induced by a genetic clock for eliminating the cell as part of a cell-death program, i.e this process is always going to take place (Darzynkiewicz et al. 1997; Strychar et al. 2004b). For example, the loss of webbing between the fingers of some mammals during embryo development is due to PCD, whereas the induction of apoptotic cell death of a virally infected cell is due to a stimulus (virus) (Strychar et al. 2004b). Typical apoptosis is characterized by cell shrinkage, with the plasma membrane staying structurally intact during initial phases of cell death (Darzynkiewicz et al. 1997; Häcker 2000; Strychar et al. 2004a). Protrusions then start to show on the plasma membrane, referred to as ‘blebs’ and the nucleus condenses and fragments (Häcker 2000). Cytoplasmic organelles remain initially intact but become involved in the later stages of apoptosis (Häcker 2000) when they start to fuse and eventually break up to form apoptotic bodies, with rupturing of the cell membrane (cell blebbing) often observed in the final stages of apoptosis (Darzynkiewicz et al. 1997; Strychar et al. 2004a). In contrast, necrosis is a slow disintegration of the cell characterized by swelling of the cell and its organelles, disintegration of organelles and rupture of the cell membrane, releasing

cytoplasmic constituents (Darzynkiewicz et al. 1997; Häcker 2000; Strychar et al. 2004a). Fig. 3.8 illustrates the processes involved in apoptosis and necrosis.

However, different organisms/cell types can differ in their pattern of apoptosis and not all features of typical apoptosis may be present (Häcker 2000). The degraded *Symbiodinium* cells observed in this study and in that of Strychar (2004a) did not show blebs protruding from the cell membrane, and apoptotic bodies formed within the cell. This was likely due to the cell wall persisting and providing structural support (Collazo et al. 2006).

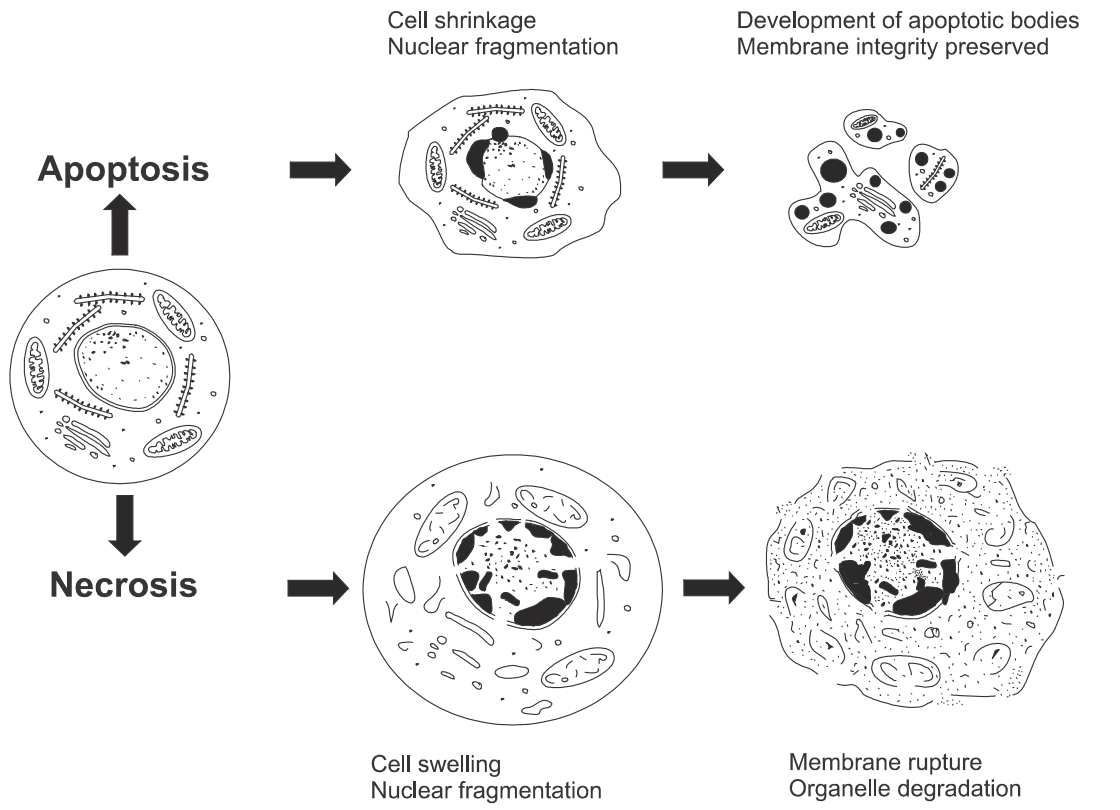


Fig 3.8: Typical processes of cell death involved in apoptosis and necrosis (adapted from Darzynkiewicz 1997 and Häcker 2000).

Dunn et al (2004) observed PCD in *Symbiodinium* cells of anemones undergoing heat stress. They also found condensation of organelles and cytoplasm, with aggregate bodies being the only well-defined cellular components in late stages of PCD. Most of the *Symbiodinium* cells observed in the micrographs in this study appeared to be in a late stage of apoptosis, as no organelles were visible. However, they differed visually from cells in Dunn et al (2004), in that the cell was rounder and had more defined apoptotic bodies. The observed degraded

Symbiodinium cells that did not show any form of apoptotic bodies could be in the early stages of apoptosis and resemble images in Dunn et al. (2004) and Strychar et al. (2004a). Fig.3.9 illustrates the proposed progression of apoptosis in *Symbiodinium* cells (based on the findings of Strychar et al. 2004a and this study).

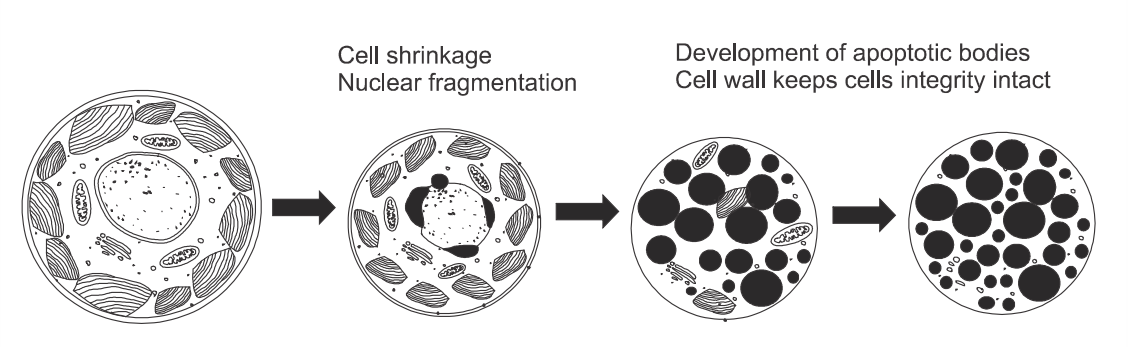


Fig 3.9: Diagram of proposed apoptotic processes in *Symbiodinium* cells. In contrast to apoptosis in animal cells, the cell wall keeps the cell integrity intact and prevents blebbing during late stages of apoptosis.

Few apoptotic cells were also observed in healthy samples, however the increased number of apoptotic *Symbiodinium* cells observed in PBTL-affected samples may suggest a link between the disease and cell lysis. Cell lysis was also described in connection to yellow-blotch disease (Cervino et al. 2004), however judging from the micrographs in this paper, *Symbiodinium* cells appeared to be in a stage of necrosis rather than apoptosis. Necrosis and apoptosis in *Symbiodinium* cells can be induced by temperature stress (Strychar et al. 2004a). However, coral fragments in the current study where sampled in November when water temperatures were around 24 ºC. Thermal stress can therefore be ruled out as cause of the apoptotic *Symbiodinium* cells observed in PBTL-affected samples. The trigger inducing apoptosis in these cells could be environmental or microbial (bacteria, virus). As I did not observe any bacteria or VLPs within *Symbiodinium* cells, an environmental cause may be more likely.

Because no pathogens were apparent in the histological sections, the possible involvement of viruses in the etiology of PBTL was also explored. Temperature (Edgar and Lielausis 1964), UV light (Jacquet and Bratbak 2003; Lohr et al. 2007) and nutrient availability (Scanlan and Wilson 1999) have been identified as important triggers in viral propagation and infection. All of these factors have also been implicated in affecting coral disease prevalence and/or lesion progression (Ben-Haim et al. 2003; Bruno et al. 2003; Bruno et al. 2007; Sato et al.

2011). In Kaneohe Bay, nutrient levels in the water column are generally low (Larned 1998; Stimson et al. 2001) but nutrient efflux from the reef sediments (Stimson et al. 2001) could increase nutrient levels close to the substrate (F. Cox, University of Hawaii, pers. comm.) and occasional sewage influx during times of high rainfall could also lead to increases in nutrient levels. In addition, the observed increase in disease prevalence during summer months when temperature and UV radiation are highest (see Chapter 4), would be consistent with an involvement of viruses in PBTL.

Viruses are highly diverse and abundant in the marine ecosystem (Fuhrman 1999; Breitbart et al. 2002) and have widespread ecological importance, playing key roles in various ecosystem processes, such as structuring planktonic communities (Middelboe et al. 2001; Jacquet et al. 2002; Wilson et al. 2002a, 2002b), biogeochemical cycling (Furhman 1999) and interceding horizontal gene transfer (Jiang and Paul 1998). It is likely that all organisms could potentially get infected by viruses on a regular basis (Wilson et al. 2005; Vega-Thurber and Correa 2011) and it would therefore not be surprising if viruses also play important roles in disease infection of reef corals and their symbiotic algae (Davy et al. 2006; Vega-Thurber and Correa 2011).

Several studies have confirmed the existence of VLPs in stony corals and *Symbiodinium* cells (Wilson et al. 2001, 2005; Davy et al. 2006; Davy and Patten 2007; Patten et al. 2008; Vega-Thurber et al. 2008). Heat shock treatments increased the number of VLPs observed, although they were also present in unstressed controls (Wilson et al. 2005, Davy et al. 2006). Davy and Patten (2007) examined the association of VLPs within the coral surface microlayer and found a wide diversity of VLPs belonging to several viral families. These studies provide evidence that the occurrence of viruses or VLPs does not necessarily indicate a disease state (Vega-Thurber and Correa 2011). Very little is actually known about the possible involvement of viruses in coral disease causation. Cervino et al. (2004) documented the degradation of *Symbiodinium* cells in corals affected by yellow blotch disease and reported virus-like particles (VLPs) within algal cytoplasm from freshly isolated samples exposed to bacteria and heat stress. However, the low resolution of the associated micrographs made an identification of these particles complicated (Vega-Thurber and Correa 2011). Patten et al. (2008) characterized VLPs within healthy and WS affected colonies from the Great Barrier Reef and observed VLPs in both healthy and diseased colonies with no differences in morphology or location. These studies support the hypothesis that viruses

represent an important component of the coral holobiont. It is therefore essential to deepen our knowledge of how these associations influence coral health.

In the current study, few VLP's were observed in both healthy and diseased host tissue but VLPs appeared to be more abundant in PBTL-affected samples. However, a quantitative study is needed to confirm this. VLPs resembled those observed in coral tissues of previous studies (Seymour et al. 2005; Davy et al. 2006). Viruses have been implicated as causative agents in coral disease (Davy et al. 2006, Vega-Thurber and Correa 2011) but have, as yet, not been proven to be involved in disease causation. Using a metagenomic approach, Vega-Thurber et al. (2008) identified various eukaryotic viruses associated with the reef coral *Porites compressa*. Interestingly, herpes-like viral sequences were rare in apparently healthy corals but increased considerably in environmentally stressed corals (Vega-Thurber et al. 2008). In humans, infections caused by the herpes simplex virus are also often stress related (Sainz et al. 2001). The observation of the vacuole filled with herpes-like viruses in a degraded portion of one PBTL-affected sample is interesting and could indicate a stress response to disease. However, no other herpes-like viruses were observed in the remaining samples; no conclusion can therefore be drawn regarding a link between herpes-like viruses and PBTL. The fact that VLPs appeared to be more abundant in PBTL-affected samples is very interesting and warrants further examination. However, at this time no definite link can be established between viruses and PBTL. The putative increase in VLPs may just be a result of a shift in the microbial community due to PBTL.

TEM is perhaps most effective as a confirmatory rather than exploratory tool. Once compelling morphological evidence is gained at the microscopic level that a virus may be involved in disease causation (e.g. inflammation indicative of viral infection, intracytoplasmic or intranuclear inclusions) then TEM is an excellent tool to confirm this suspicion. However, even then it can be difficult. For example, viruses have long been suspected to cause fibropapiloma tumours in the green sea turtle (*Chelonia mydas*) from Hawaii but it took over 10 years until herpes-like viruses were finally observed in "prickly cells" lining the tumor tissues (Work and Carvalho, pers. comm.). It is difficult to confirm that a certain microbial group (e.g. viruses or bacteria) is the causative agent, even if changes in their abundance and/or diversity have been observed in concurrence with disease (Vega-Thurber and Correa 2011). A microbial shift after disease onset could either mean an increase in virulence of certain microbes or fluctuating community dynamics due to physiological changes of the host (Lesser et al. 2007). To satisfactorily prove the involvement of a potential

pathogen in disease etiology, Koch's postulates (Koch 1891) have to be fulfilled. For coral diseases this has as yet only been achieved in very few cases (Kushmaro et al. 1996; Geiser et al. 1998; Sussman et al. 2008).

3.6 Conclusion

In conclusion, tissue affected by PBTL is thinner and shows a significant reduction in *Symbiodinium* densities with most remaining *Symbiodinium* cells appearing to be in a late stage of apoptosis. PBTL can result in necrosis and tissue fragmentation causing tissue mortality, but no increase in the number of bacterial aggregates was observed in diseased tissue. VLPs appeared to be more abundant in PBTL-affected samples, however a quantitative study is needed to confirm this hypothesis. Further research into the etiology of PBTL is needed to build a comprehensive case definition of this disease.

Chapter 4

Disease Dynamics of *Porites* bleaching with tissue loss: prevalence, transmission and environmental drivers

4.1 Introduction

Diseases have the potential to severely alter the structure and function of marine ecosystems (Ward and Lafferty 2004). In some species, infectious diseases have reduced population densities to such an extent that recovery is uncertain (Lafferty et al. 2004); for example the long-spined sea urchin in the Caribbean (Lessios 1988) and the abalone in California (Lafferty and Kuris 1993). The same is true for coral diseases. Coral disease outbreaks have caused declines in coral cover in many reef systems (Nugues 2002; McClanahan et al. 2004; Croquer et al. 2005; Bruckner and Hill 2009); however, the Caribbean has been particularly affected (Goreau et al. 1998; Porter et al. 2001) and is recognized as a disease hot spot. Disease outbreaks in the Caribbean have caused a severe reduction in the abundance of their two once most common hard corals, *Acropora palmata* and *A. cervicornis*, leading to an inclusion of these species on the IUCN Red List of Endangered Species and causing a shift in the reef community structure (Gladfelter 1982; Aronson and Precht 2001a; Patterson et al. 2002). Even though the Indo-Pacific appears to be less strongly affected by coral diseases than the Caribbean, an increasing amount of evidence suggests that coral diseases are also common throughout the Indo-Pacific (Sutherland et al. 2004; Willis et al. 2004; Raymundo et al. 2005), even at extremely remote, uninhabited islands (Williams et al. 2008, 2011a; Vargas-Angel 2009), with the types of diseases and their prevalence often varying across large spatial gradients (Aeby et al. 2011a, 2011b). In fact, the geographical extent, number of species affected and incidence of new diseases are increasing globally (Harvell et al. 1999; Ward and Lafferty 2004; Sokolow et al. 2009). Environmental stress, shifts in virulence of existing pathogens, introduction of novel pathogens from anthropogenic activities and global climate change are associated with this increase (Harvell et al. 1999; 2004; Sokolow et al. 2009).

Coral diseases can be expected to show intricate interactions with a variety of environmental factors (Williams et al. 2010). For example, an increase in temperature can lead to an increase in pathogen virulence or cause stress to the host which can increase its susceptibility to

disease (Harvell et al. 2002). Coral disease outbreaks, and increases in disease prevalence and progression have been linked to variations in temperature (Ben-Haim et al. 2003; Jones et al. 2004; Bruno et al. 2007; Sato et al. 2009), nutrients (Bruno et al. 2003), host density (Willis et al. 2004; Bruno et al. 2007; Myers and Raymundo 2009; Williams et al. 2010; Aeby et al. 2011a), vectors (Aeby and Santavy 2006) and coral bleaching (Muller et al. 2008; Brandt and McManus 2009; McClanahan et al. 2009). It is likely that several environmental factors simultaneously influence disease dynamics within a system, the relative importance of each varying among regions, spatial scales and species (Aeby et al. 2011a, 2011b). However, the complex web of interactions between environmental factors and disease prevalence remains largely unexplored in coral disease research (Williams et al. 2010), but some studies have begun to apply a multi-factor approach to studying coral disease dynamics (Bruno et al. 2007; Haapkylä et al. 2007; McClanahan et al. 2009; Williams et al. 2010; Aeby et al. 2011a, 2011b). For example, Williams et al. (2010) used predictive modelling to highlight the interaction of four Hawaiian coral diseases with a wide variety of environmental variables. They were able to identify a group of key predictor variables for each disease and stressed the importance of modelling diseases separately (i.e. not modelling overall disease prevalence on a reef as a single response variable) to ensure clear interpretation of disease-environment relationships. If we are to successfully manage our reef systems, it is vital to understand the often intricate disease-environment interactions that lead to complex temporal and spatial disease dynamics.

Different coral diseases show varying levels of ecological impact. For example, black band disease and white syndromes often cause severe colony mortality (Edmunds 1991; Bruckner et al. 1997; Willis et al. 2004; Roff et al. 2006; Aeby et al. 2010; Williams et al. 2011b) whereas corals with *Porites* ulcerative white spot syndrome (PUWS) in the Philippines often show complete recovery after infection (Kaczmarsky 2006). In addition, different coral species/taxa appear to vary in their susceptibility to disease infection. Haapkylä et al. (2007) found that 'bushy' *Acropora* colonies in Indonesia are a relatively rare growth form yet show a higher growth anomaly and white syndrome prevalence than any other taxa. On the Great Barrier Reef, black band disease affects 25 out of approximately 350 hard coral species, with branching *Acropora* spp. being most affected (Page and Willis 2006). The degree of damage to the ecosystem therefore depends on the suite of coral species and diseases that occur on the reef. The prospect of protecting our reef ecosystems will depend on our understanding of the nature and causes of the different diseases that affect a system (Ward and Lafferty 2004).

In this chapter I describe the disease dynamics of *Porites* bleaching with tissue loss (PBTL), a coral disease that affects *Porites compressa,* one of the main framework-building species in Hawaii. PBTL manifests as bleaching of the coenenchyme with the polyps remaining brown, giving the coral a 'speckled' appearance. This disease causes tissue loss due to necrosis and tissue fragmentation (Chapter 3) and a significant reduction in gamete development (Chapter 2). Preliminary observations of an apparent increase of PBTL prevalence during the summer months suggest a potential link to temperature (see Appendix VI) but overall little is known about the ecology of PBTL. The objectives of this study were therefore to: 1) examine the variability in disease prevalence (proportion of individuals affected) and incidence (appearance of new cases per unit time) over the course of one year; 2) examine virulence (degree of harm to the host) and determine the effect of temperature on disease progression and transmissibility; and 3) determine the environmental drivers of variations in disease prevalence.

4.2 Materials and methods

4.2.1 Study site and sample collection

Prevalence surveys were carried out at Coconut Island Marine Reserve (CIMR), Kaneohe Bay, Oahu, Hawaii (21°26.000'N, 157°47.000'W). The fringing reef around CIMR occurs at shallow depths and consists of a shallow reef flat (0.5-1 m) and often steep slopes (approximately 30º inclinations) that reach a sandy/muddy sediment bottom at around 5-7m. Generally, the slopes are covered with dense coral growth (Fig. 4.1), with *Porites compressa* and *Montipora capitata* being the dominant coral species (Maragos 1972) (Appendix V.1). Eight permanent sites (A-D and G-J) were established around CIMR (Fig. 4.2) at a depth of approximately 2 m. Sites were chosen to traverse known natural gradients in environmental conditions (Williams et al. 2010). Kaneohe Bay has a long history of impaired water quality due to sewage and non-point source influx (Hunter and Evans 1995) and these pollutants are still a problem during times of high rainfall or storm events (Jokiel 2008). Due to the long residence time, pollutants (mostly nutrients and sediments) remain in the bay for a relatively long time (approximately two weeks) in comparison to exposed coasts, where mixing of the water body is much greater (Friedlander et al. 2008a).

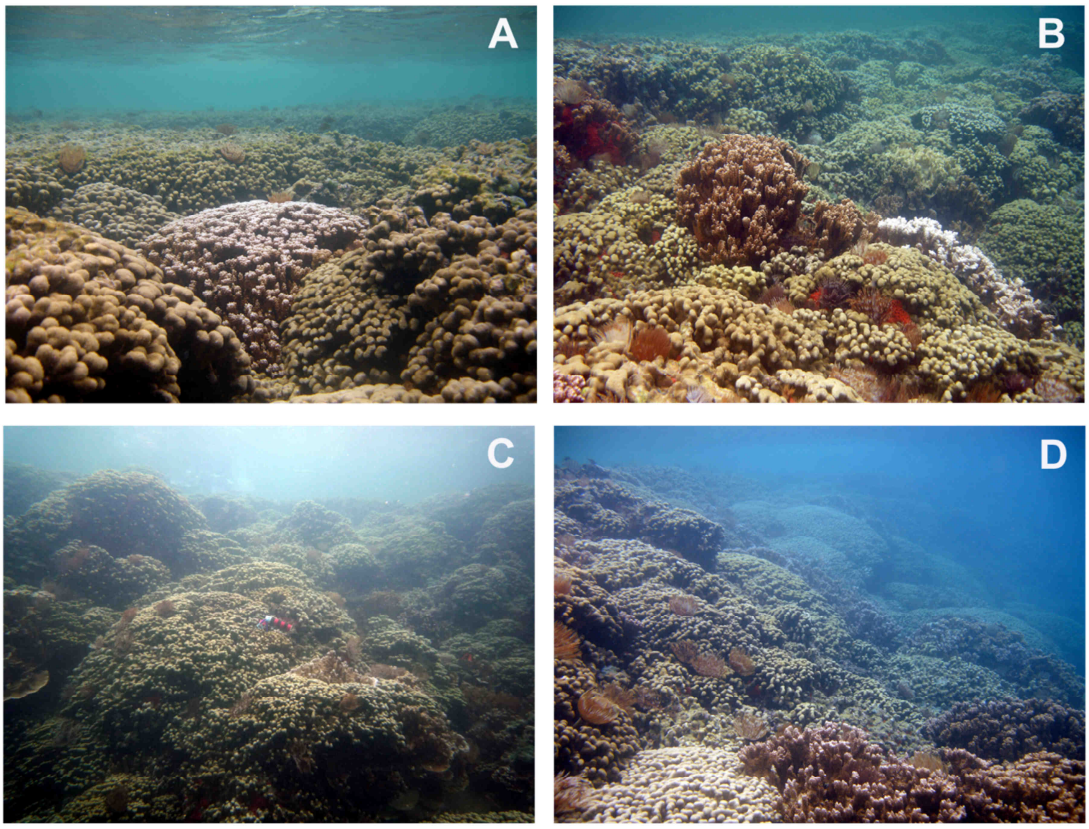


Fig. 4.1: Representative photos of the reef flat and reef slope at CIMR. A: Reef flat. B: Coral assemblage on the reef slope. C & D: Reef slope. Note the dense coral growth and relatively steep drop-off of the reef slope. Photo credit: Gareth Williams

All coral samples (about 3 cm^2) for the transmission and disease progression experiments (described below) were collected from the reef crest around CIMR (please refer to Appendix I.2 for sample design). Samples were returned to the lab in individual plastic bags to avoid any cross contamination.

4.2.2 Prevalence, incidence and spatial distribution

Prevalence surveys were conducted at the eight permanent sites around CIMR (Fig. 4.2) on an approximately monthly basis during 2011 (see also Appendix VI). Five 10 x 2 m belt transects were used in which every *P. compressa* colony was counted and examined for signs of PBTL. A 50 x 2 m transect was used to investigate disease incidence at each of the eight permanent sites. Every PBTL colony was marked and counted in March 2011, and then every newly-affected colony was documented and marked during each subsequent prevalence survey. Percentage cover of live *P. compressa* colonies was estimated using a point-intercept method at 50 cm increments along a 70 m transect line at each site (140 points per transect).

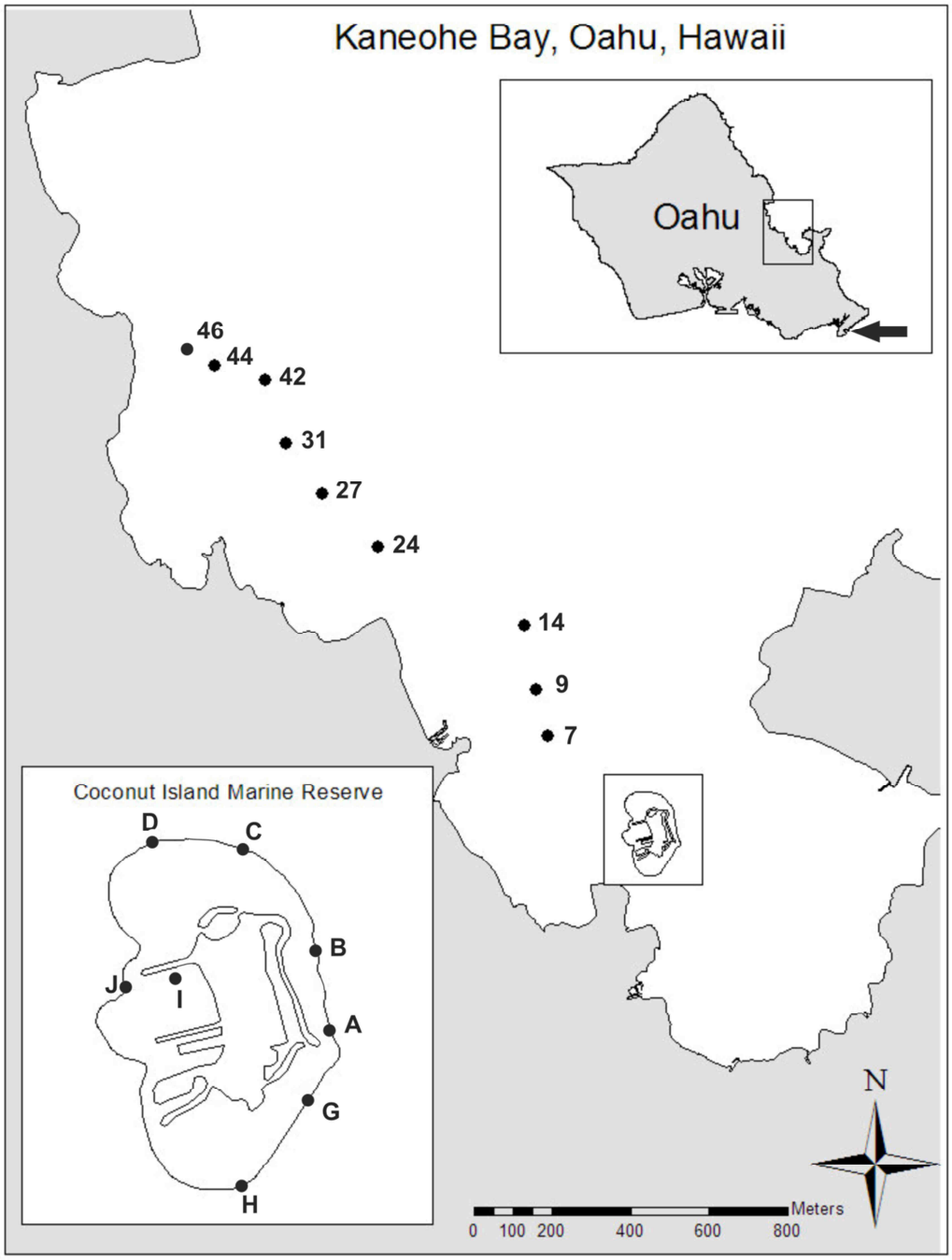


Fig. 4.2: Map of Kaneohe Bay showing the 9 rapid survey sites (7-46) numbered after Roy (1970) with an inset of Oahu showing the location of Kaneohe Bay and Hanauma Bay (arrow) and another inset of CIMR showing the 8 permanent sites (A-J).

To investigate the spatial extent of PBTL, rapid visual surveys were conducted on 9 reefs within Kaneohe Bay (covering the South, Mid and North Bay regions) (Fig. 4.2). In addition,

Hanuama Bay Marine Reserve (Fig. 4.2), a reef with high *P. compressa* cover located on the windward side of Oahu (approximately 20 km from Kaneohe Bay), was also surveyed for signs of PBTL. Within Kaneohe Bay, a snorkeler swam for 10 minutes at a speed of approximately 10 m min^{-1} along a haphazardly selected patch reef and recorded every PBTL-affected colony observed. In Hanauma Bay, two divers swam across the reef at approximately the same speed and recorded the number of PBTL-affected colonies encountered. All rapid surveys were conducted in October 2011.

4.2.3 Transmission, virulence and disease progression

To maintain water quality in all experimental aquaria, a bubbler was placed in the tanks to ensure water movement, and partial water changes, using 0.2-µm filtered seawater, were carried out every 5 days. Aquaria were kept outside under natural light conditions (a roof shaded the aquaria for most of the day).

To determine if PBTL is transmissible through the water column or via direct contact, a fragment showing signs of PBTL was placed touching a healthy fragment in an 8-L tank, with another fragment from the same healthy colony placed about 10 cm away from the PBTL-affected fragment. As a control, the same setup was used with fragments from the same healthy colony as those used in the transmission treatment, but the diseased fragment was replaced by a healthy fragment from a different colony to control for possible effects of intraspecific competition (see Appendix V.2 for experimental set-up). Healthy fragments were monitored for signs of PBTL over the course of at least 3 weeks or until the affected fragment died. The transmission experiment was carried out at ambient (25 ºC) and increased (28 ºC) water temperatures to determine if transmission would occur more readily in slightly stressed corals (n = 10/treatment, with each replicate in a separate tank). In Hawaii, the mean summer monthly water temperature reaches approx. 27 ºC (Jokiel and Coles 1977) and coral bleaching occurs with prolonged exposure to 29-30 ºC (Jokiel and Coles 1990). By choosing an increased water temperature of 28 ºC in this experiment, it was assumed that the coral fragments were slightly stressed but would not be affected by bleaching.

In 2010, 42 individual PBTL-affected colonies were tagged (Chapter 2), and an additional 36 PBTL-affected colonies were tagged in 2011 and followed over the course of > 6 months. The colonies that were tagged in 2010 were resurveyed and checked for new PBTL infections and/or signs of tissue recovery. Due to the three-dimensional structure of *P. compressa* and the often poor visibility in Kaneohe Bay, photographic surveys with subsequent *post hoc*

image analysis could not be relied upon. Instead, the percentage of healthy, dead and affected tissue was estimated visually *in situ*, in addition to photo documentation being performed.

To visualise disease progression and determine if temperature affects the speed of progression, paired samples affected by PBTL (bleached but not yet progressed to tissue loss) were collected from 6 coral colonies, photographed and placed individually in a tank at ambient (24-25 ºC) and increased (27 ºC) temperatures (one paired fragment per treatment). Coral samples in the elevated temperature treatment were acclimatised for 24 hours and then the temperature was slowly raised to the target temperature. Coral fragments were photographed once a week to document progression. PBTL starts as bleaching of the coenenchyme with polyps remaining brown. The tissue then starts to thin in multiple locations until only the bare skeleton remains, which makes it difficult to quantify the progression of tissue loss. Image analysis software could therefore not be used to measure the speed of tissue loss.

PBTL often only affects a localized area on the colony. To determine if areas that did not show any disease signs would develop signs of PBTL over time and if this was affected by temperature, fragments of six PBTL-affected colonies (2 paired fragments per colony from an apparently healthy area adjacent to the lesion and 2 paired fragments from an apparently healthy area furthest away from the lesion) as well as the same number of fragments from a healthy neighbouring colony (serving as a control) were sampled, photographed and placed into individual tanks using the same setup as described above. The samples were monitored for signs of PBTL over time (1-2 months) and photographed weekly.

4.2.4 Environmental drivers

Temperature data were collected at each site using HOBO®Pro data loggers (www.onsetcomp.com) with an accuracy of ± 0.2 ºC. The loggers were placed at the depth of the transect and recorded continuously every 30 min from late February to late December 2011. Turbidity, chlorophyll-*a* and salinity were measured at each site at the depth of the transect using a RBR® XR-420 data logger (www.rbr-global.com) recording every minute over a 36-48 h period on 4 – 6 different occasions in 2010 and 2011. The logger was moved randomly between sites to maximize spatial coverage over time. Water motion was estimated using the clod card technique (Jokiel and Morrissey 1993). Two clod cards were placed at the beginning of each survey site at the depth of the transect and left overnight (21-23 h). In addition, two clod cards were placed into a large bucket containing seawater (approx. 60 L)

to serve as a diffusion control. The exact time that the clod cards were immersed in water was recorded and the diffusion factor (DF, a dimensionless index of water motion) was calculated for each site (Jokiel and Morrissey 1993). Clod cards were deployed 4 times over the course of 6 months in 2011 and the average DF for each site was used in subsequent data analyses.

Corallivorous fish can be potential vectors of disease (Aeby and Santavy 2006) or a source of injury which can promote the spread of certain diseases (Page and Willis 2008; Raymundo et al. 2009). The densities of all corallivorous butterflyfish (facultative and obligate) and parrotfish were recorded over an area of 50 x 4 m at all eight sites. The observer swam at a speed of approximately 10 m per minute and recorded all butterflyfish to species level (*Chaetodon auriga, C. ephippium, C. lineolatus, C.lunulatus, C. multicinctus, C. ornatissimus, C. unimaculatus*). Due to difficulties with species-level identification, all parrotfish (adults and juveniles) were grouped. Fish counts were carried out during four different months in 2011 (July, August, September and December) and all sites were surveyed on the same day within 2 – 3 hours of each other. Mean numbers of fish were used in the subsequent data analyses.

4.3 Data analyses

4.3.1 Prevalence, incidence and spatial distribution

Prevalence data by transect did not display a normal distribution, even after transformation. A permutational repeated measures permanova analysis based on a binomial deviance matrix was therefore performed (the technique does not assume normality) in PERMANOVA+ (Anderson et al. 2008), to test the effect of two fixed factors (site, month) and their interaction with disease prevalence.

A chi-square analysis was used to compare the relative frequencies of colony size classes in the total population and PBTL-affected colonies. Analyses were performed in the R statistical program version 2.12.1 (R Development Core Team, http://www.r-project.org). Colony sizes were recorded as largest diameter within seven size classes: 1) <5 cm; 2) 5-10 cm; 3) 10-20 cm; 4) 20-40 cm; 5) 40-80 cm; 6) 80-160 cm; and 7) >160 cm. The largest size class was excluded from the analyses because of an insufficient number of colonies (relative frequency <1).

4.3.2 Transmission, virulence and disease progression

Only virulence data could be statistically analyzed. The data for 42 tagged PBTL colonies (Chapter 2) were combined with the data collected from an additional 38 tagged colonies in 2011, to more closely investigate the effect of lesion size (% initially affected area) and colony size on tissue mortality (% tissue loss). Lesion size (%), maximum colony diameter (cm) and mortality (%) were estimated *in situ*. The data did not meet the assumptions of normality. A non-parametric Spearman partial correlation was therefore carried out in R to test the effect of both lesion size and colony size on the mortality sustained while controlling for colony size and lesion size, respectively.

4.3.3 Environmental drivers

To investigate temporal variations in disease prevalence (potential seasonality of PBTL), the relationship of temperature and prevalence was explored over a period of 10 months using a General Linear Model (GLM) performed with SPSS (PASW 18). Prevalence was averaged for each site (over the 5 individual transects), and the data displayed a normal distribution. Temperature data were averaged over the 10-day period before each survey for every site.

To examine spatial variations in disease prevalence, eight environmental predictor variables were modelled against spatial variations in prevalence across sites. Measurements for predictor variables (apart from temperature) were not continuous through time and were therefore averaged for each site. To achieve the same resolution for temperature and prevalence data, all temperature data (10 days before each survey) and prevalence values (February – December) were averaged for each site. Predictor variables included biotic and abiotic factors that could potentially affect disease prevalence and help explain the observed differences in prevalence at the eight permanent sites. Predictor variables were: host cover; turbidity; temperature; chlorophyll-*a* (Chl-*a*); water motion; salinity; parrotfish density and butterflyfish density (Table 4.1). Because most butterflyfish species showed low abundances on the reef, all butterflyfish counts were grouped. The mean and one standard deviation (SD) of all predictor variables were initially examined to also account for the variability of factors at the individual sites. Inter-correlation of predictor variables was tested using Pearson's correlation, with predictors exceeding a correlation value of > 0.75 considered for removal and further examined using Principal Coordinates Analysis (PCO) plots (AppendixV.3: Fig. V.4). Variables chosen for inclusion in the model were mean values for host cover, temperature, Chl-*a* and water motion, and the variability (SD) in turbidity, salinity,

butterflyfish density and parrotfish density. A permutational distance-based linear model (DISTLM) was used (McArdle and Anderson 2001) to analyse the data. DISTLM is a multivariate multiple regression technique that quantifies the proportion of the variation in the response variable (in this case PBTL prevalence) explained by the predictor variables. Environmental data were normalized and the DISTLM routine was run using the 'best' selection procedure, based on 9999 permutations. Akaike's Information Criterion (Akaike 1973) with a second-order bias correction applied (AICc) (Hurvich and Tsai 1989; Burnham and Anderson 2004) was used for model selection. The most parsimonious model with the lowest AICc and highest R^2 value was selected. Modelling analyses were based on zero-adjusted Bray-Curtis similarity matrices (Clarke et al. 2006) and carried out using PRIMER v6 (Clarke and Gorley 2006) and PERMANOVA+ (Anderson et al. 2008).

Table 4.1: Predictor variables used in model analyses with their units and minimum and maximum values.

Variable	Description and units	Min	Max
water temperature	ºC	22.1	28.3
host cover	% *Porites compressa* cover	30.0	75.6
turbidity	Formazin turbidity unit (FTU)	0.3	22.6
chlorophyll-*a*	µg/L	0.02	2.1
water motion	Diffusion factor (DF)	1.26	7.95
salinity	ppt	33.0	35.7
parrotfish density	number per 200 m^2	0	68
butterflyfish density	number per 200 m^2	0	13

4.4 Results

4.4.1 Prevalence, incidence and spatial distribution

Overall, average PBTL prevalence at Coconut Island Marine Reserve was 1.5% (± 0.2% SE). There was a significant effect of site (Permanova: df = 7, Pseudo-F = 9.5969, $p < 0.001$) (see also Fig. 4.3A) and month (Permanova: df = 9, Pseudo-F = 8.5552, $p < 0.001$) (see also Fig. 4.3B) on prevalence, but no significant interaction between the two (Permanova: df = 63, Pseudo-F = 0.83471, p = 0.7473). P-values for individual interactions of months and sites are

shown in Tables 4.2 and 4.3, respectively. Average prevalence values throughout the year showed a peak during the summer months (Fig. 4.3B) with the highest average prevalence observed in June (2.5 ± 0.3% SE).

The highest disease incidence was found between April and August (1.2 – 1.5%); it then slowly dropped from September to November with a slight increase in December (Fig. 4.3B). Rapid surveys showed that PBTL was present in all of Kaneohe Bay (North, Central and South Bay regions) but that it was absent from Hanauma Bay (Table 4.4).

PBTL was most commonly observed in medium-sized colonies (20-80 cm), which made up the majority of the *P. compressa* community (Fig. 4.3C). However, a significant difference was found in the size-class frequency distributions between healthy populations and PBTL-affected colonies (Chi-square test: $\chi^2 = 24.5$, df = 5, $p < 0.001$). Smaller sized colonies (<20 cm) showed a lower PBTL frequency relative to their abundance on the reef than larger colonies (> 20 cm) (Fig. 4.3C).

Table 4.2: P-values for individual month interactions. Significant differences between two months are marked in bold ($p < 0.05$).

Month	Mar	Apr	May	Jun	Aug	Sept	Oct	Nov	Dec
Feb	0.927	0.197	0.058	**0.019**	0.427	0.099	0.86	**0.022**	**0.003**
Mar		0.137	**0.038**	**0.011**	0.359	0.073	0.834	**0.021**	**0.003**
Apr			0.478	0.239	0.557	0.351	0.078	**<0.001**	**<0.001**
May				0.510	0.184	0.701	**0.017**	**<0.001**	**<0.001**
Jun					0.068	0.332	**0.007**	**<0.001**	**<0.001**
Aug						0.310	0.328	**0.002**	**<0.001**
Sept							**0.043**	**<0.001**	**<0.001**
Oct								**0.006**	**<0.001**
Nov									0.353

Table 4.3: P-values for individual site interactions. Significant differences between two sites are marked in bold ($p < 0.05$).

Site	B	C	D	G	H	I	J
A	0.622	**0.027**	**0.007**	**<0.001**	0.062	0.251	0.257
B		0.086	**0.004**	**0.002**	0.158	0.475	0.162
C			**<0.001**	0.054	0.312	**0.008**	**0.001**
D				**<0.001**	**<0.001**	0.193	0.116
G					0.149	**<0.001**	**<0.001**
H						**0.041**	**0.003**
I							0.532

Table 4.4: The number of PBTL-affected colonies observed during rapid surveys within Kaneohe Bay and Hanauma Bay.

Area	Reef	Number of PBTL colonies
Kaneohe Bay:	46	16
North Bay	44	24
	43	19
Kaneohe Bay:	31	12
Central Bay	27	30
	24	21
Kaneohe Bay:	14	24
South Bay	9	25
	7	8
South-east corner of Oahu	Hanauma Bay	0

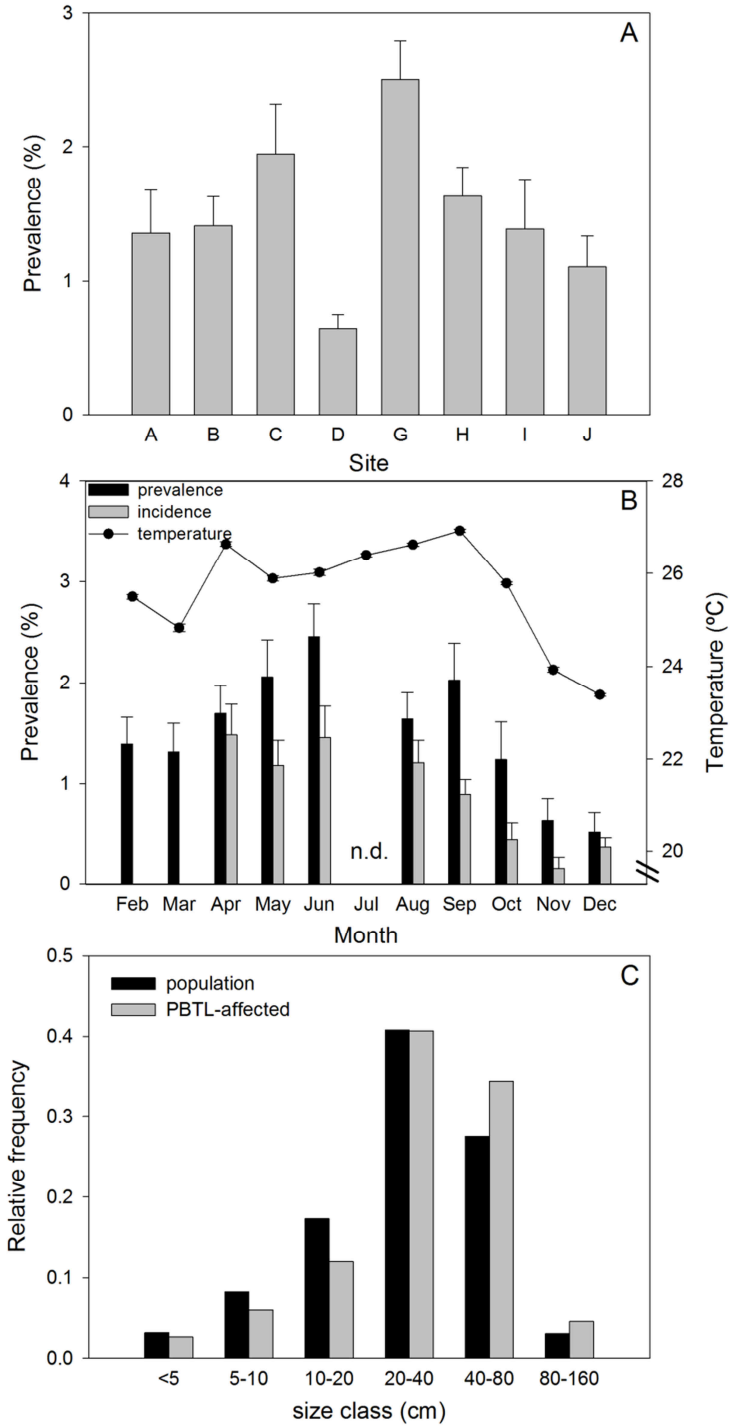


Fig. 4.3: PBTL prevalence, incidence, water temperature and size class distribution of *P. compressa* in Kaneohe Bay. A: Average prevalence (± SE) for each permanent site around CIMR. B: Average prevalence and incidence (± SE) for each month in 2011 with corresponding average temperature (the average over 10 days prior to surveying); n.d. = no prevalence/incidence data. C: Relative frequency of healthy and PBTL-affected colonies within each size class (summed over sites).

4.4.2 Transmission, virulence and disease progression

No disease transmission occurred between individuals via the water column or direct contact, in either the ambient or the increased temperature treatments.

PBTL affected individual colonies for an average of 2-3 months. Within this time, the majority of colonies (85%) showed colony mortality ranging from 5-100% (Fig. 4.4), with case fatality rate (total mortality) being 2.6%. On average, a colony lost a third (30.1%) of its tissue within two months. Of the 42 colonies tagged in 2010, 54.8% showed no signs of recovery in 2011 and 23.8% showed partial tissue re-growth.

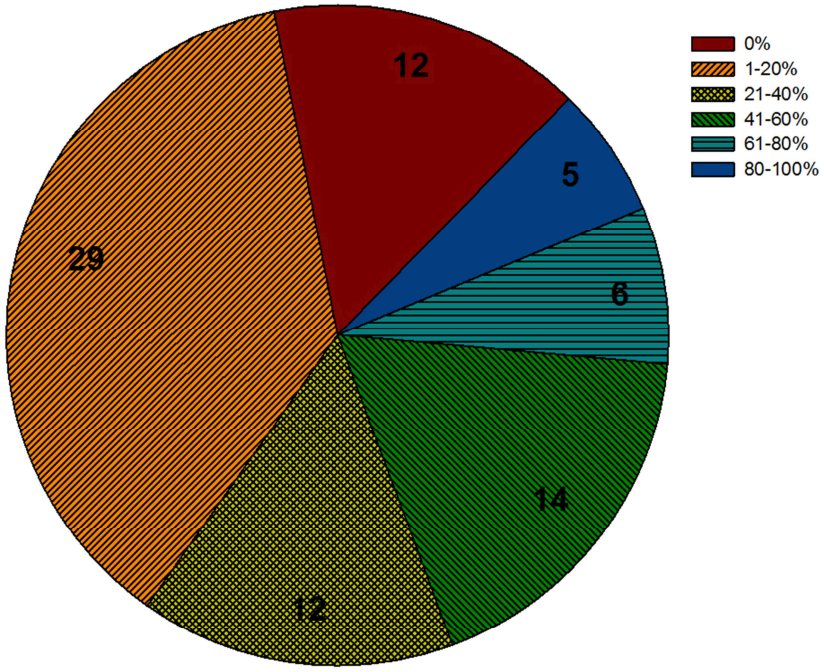


Fig. 4.4: The percentages of tagged colonies affected by PBTL that showed between 0-100% of tissue mortality (legend) after a period of two months (n = 78). Numbers within the pie graph represent the actual number of colonies in each tissue loss category.

In addition, 31% of these colonies became affected again with PBTL during 2011. The amount of tissue loss sustained was significantly correlated with lesion size (Spearman: $r = 4.735$, $n = 78$, $p < 0.001$) (Fig. 4.5A) but not with colony size (Spearman: $r = -0.187$, $n = 78$, $p = 0.852$). Even though Fig. 4.5B suggests that larger colonies sustained lower mortality, this relationship disappeared when lesion size was controlled for.

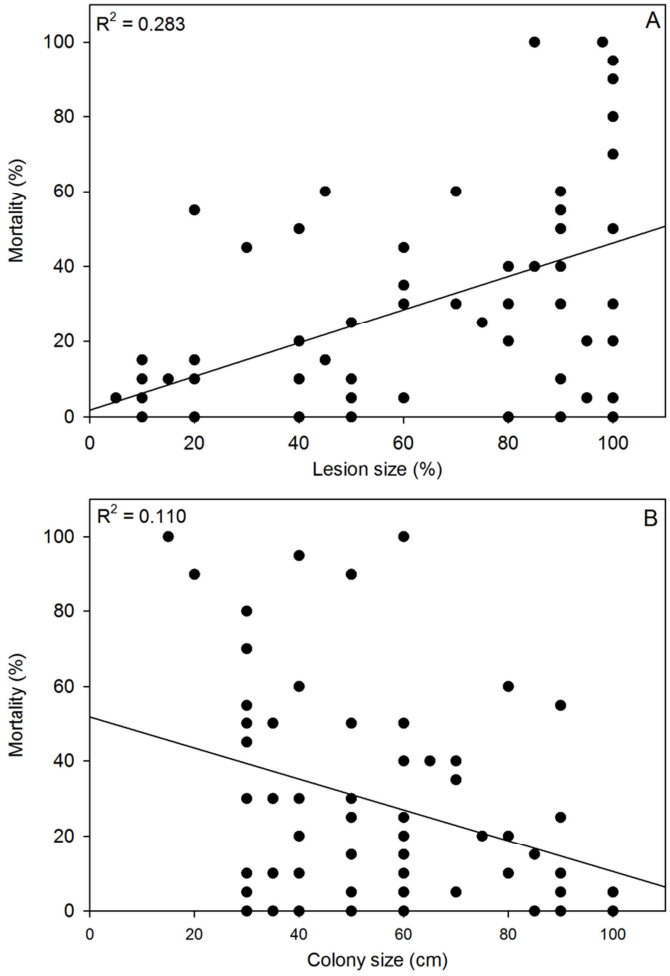


Fig 4.5: Tissue loss (mortality) in relation to lesion and colony size. A: Correlation of coral mortality and lesion size (area on the colony affected by PBTL). B: Correlation of coral mortality and colony size.

Although the speed of tissue loss could not be quantified directly, it was found that elevated temperature increased the rate of PBTL progression. All PBTL-affected samples kept at ambient temperature (25 ºC) showed a slower progression than their paired half at increased temperature (27 ºC) (Fig. 4.6). Total tissue loss (mortality) was observed in 100% (n = 6) of PBTL-affected samples kept at increased water temperature whereas only 33% (n = 2) of PBTL-affected samples kept at ambient temperature showed total mortality. Of the remaining four fragments, two started to show re-pigmentation (Fig. 4.6 and Appendix V.4) and the other two fragments showed only partial mortality by the end of the experiment (approximately 2 months). None of the apparently healthy samples collected adjacent to the lesion nor farthest away from the lesion developed any signs of PBTL during the course of the experiment.

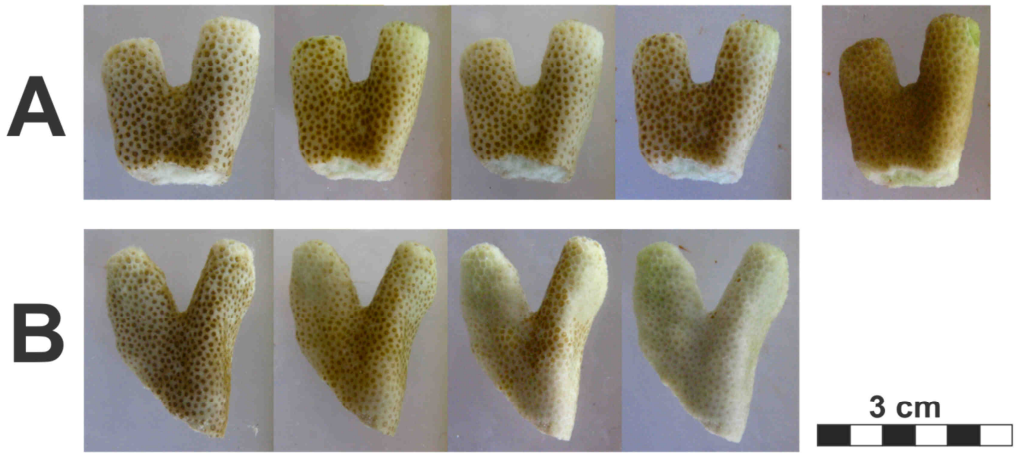


Fig. 4.6: PBTL progression at ambient (24-25 ºC) and elevated (27 ºC) water temperature. A: PBTL-affected fragment kept at ambient water temperature and photographed every week. Photo on the far right shows re-pigmentation at the end of the experiment (after about 2 months). B: PBTL-affected fragment kept at increased water temperature and photographed every week showing progressive tissue loss with total tissue loss (death) during week 4.

4.4.3 Environmental drivers

Seasonal temperature and disease prevalence showed a significant linear relationship (GLM: Wald $\chi^2 = 38.128$, df = 1, $p < 0.001$) (Fig. 4.7). With every degree increase in temperature, an increase of 0.29 – 0.56% in disease prevalence can be expected. However, there was some evidence that sites responded differently to temperature, with some sites showing a stronger linear relationship with temperature than others (Fig. 4.8).

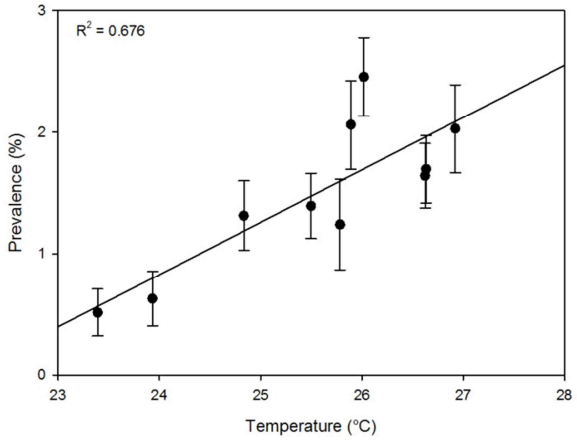


Fig. 4.7: Relationship of average prevalence ± SE for each month (Feb-Dec) with corresponding temperature (the average over 10 days prior to surveying).

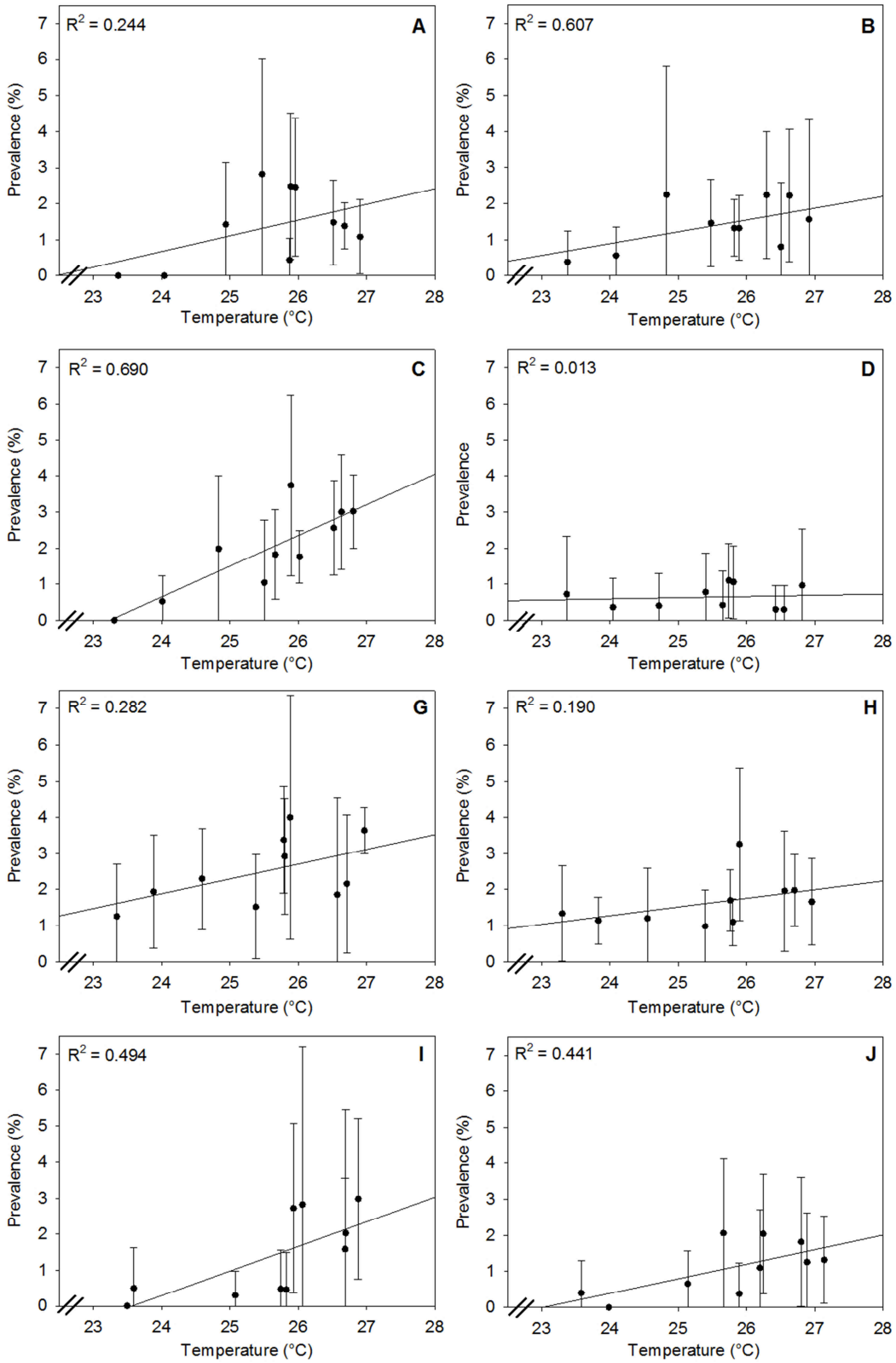


Fig. 4.8: Relationship of mean PBTL prevalence ± SD and temperature at the individual sites (A-G). Letters in the graphs represent the site name.

Modelling of the spatial variation in PBTL prevalence across sites identified water motion, and the variability in turbidity and parrotfish densities as the strongest predictors, with 26.2% of the total variability in PBTL prevalence across sites explained (Table 4.5 and Fig. 4.9). Water motion and parrotfish density showed a positive correlation to variations in PBTL prevalence whereas turbidity showed a weak negative correlation.

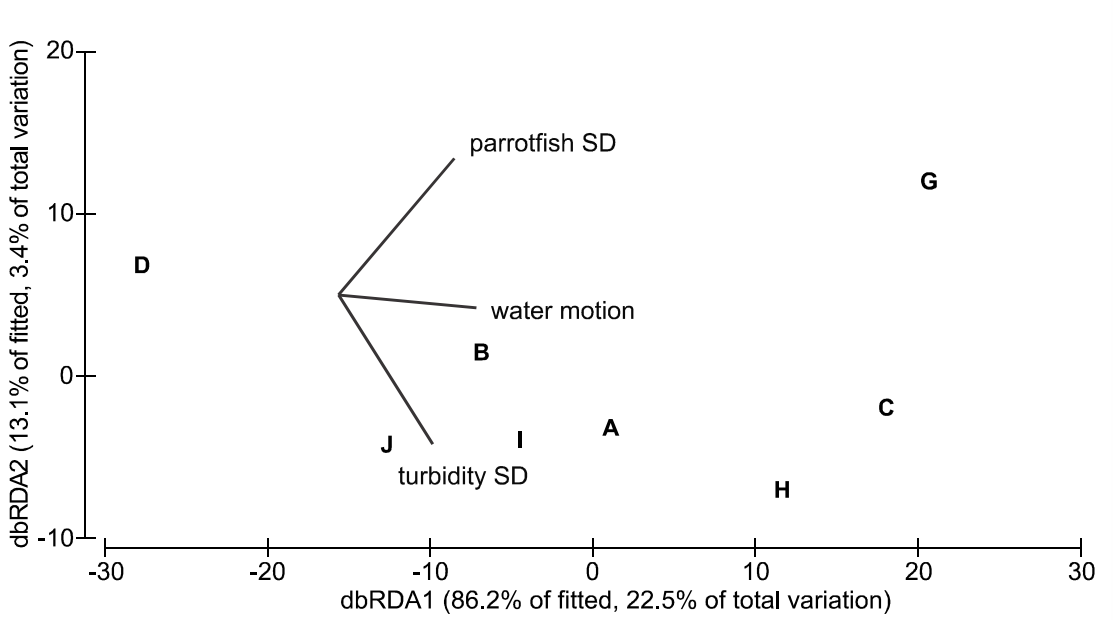


Fig. 4.9: Distance-based redundancy analysis (dbRDA) plot showing the predictors of the best model, visualizing the similarity of PBTL prevalence at the 8 different sites and the correlated change in environmental conditions.

Table 4.5: Summary results of a distance-based linear model (DISTLM) analysis showing the 'best' model with the lowest AICc, and highest amount of variability explained.

AICc	Predictor	Pseudo-F	p	% variability explained	Relationship with prevalence
274.94	turbidity SD	6.0364	0.0026	12.8	negative
	water motion	3.7671	0.0281	9.0	positive
	parrotfish density SD	2.1351	0.1148	4.4	positive
	Total			**26.2**	

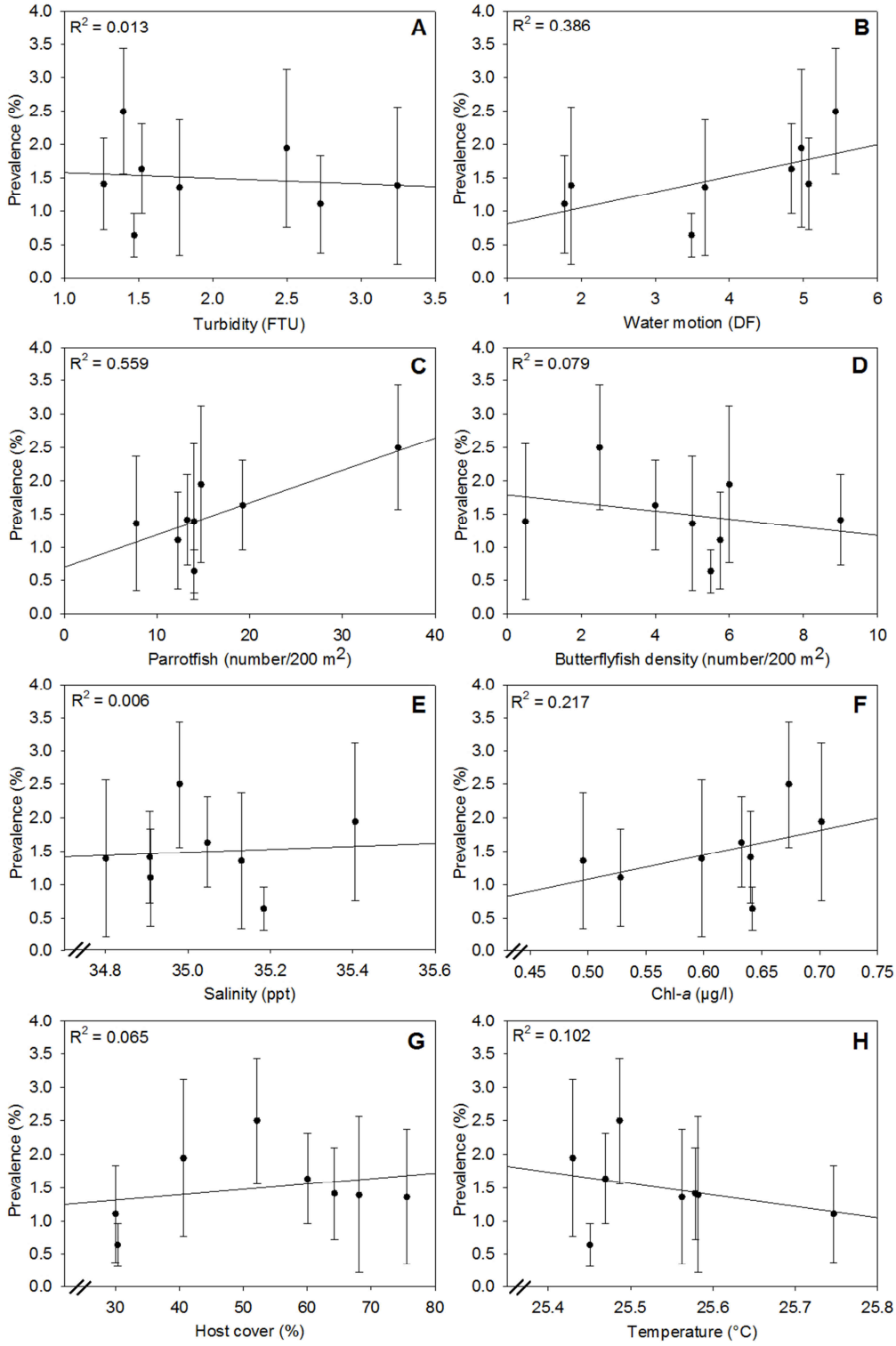


Fig. 4.10: Relationship of individual environmental predictors and mean PBTL prevalence ± SD used in the model to explain spatial variation in disease prevalence. A: turbidity; B: water motion; C: parrotfish density; D: butterflyfish density; E: salinity; F: Chl-*a*; G: host cover (*Porites compressa*); H: temperature.

Salinity (see also Appendix VII), butterflyfish density, Chl-*a*, temperature, and host cover were not found to be important predictors of PBTL prevalence across sites. See Fig. 4.10 for the relationship of individual predictors with disease prevalence (also refer to Appendix V.3: Table V.3 and Fig. V.5 for mean and SD values of environmental predictors at each site).

4.5 Discussion

Understanding disease dynamics is critical for the successful management of coral reef structure and function. Kaneohe Bay contains many fringing and patch reefs with high coral cover. Several coral diseases have been reported from Kaneohe Bay, of which *Porites* trematodiasis (Aeby 2007; Williams et al. 2010), *Porites* growth anomalies (Domart-Coulon et al. 2006; Williams et al. 2010; Stimson 2011) and *Montipora* white syndrome (MWS) (Aeby et al. 2010; Williams et al. 2010) are the most studied. In this chapter I introduce the disease dynamics of another prevalent disease in Kaneohe Bay, *Porites* bleaching with tissue loss (PBTL).

4.5.1 Prevalence, incidence and spatial distribution

Compared to other diseases found in Kaneohe Bay, PBTL prevalence is low to intermediate (average prevalence 1.5 ± 0.2% SE, range: 0 – 3.7%). *Montipora* white syndrome (MWS) can show a range in disease prevalence of 0 – 29% (Williams et al. 2010) and has an average prevalence of 0.23 ± 0.09% SE (Aeby et al. 2010), whereas *Porites* growth anomalies (Por GA's) can show a range of 1 – 56% (Williams et al. 2010) with an average prevalence of 21.7 ± 8.3% SE at a particular site (Domart-Coulon et al. 2006). Even though MWS has a much lower disease prevalence than Por GA's, MWS can cause extensive tissue loss and high colony mortality (Aeby et al. 2010) whereas Por GA's cause reduced growth rates and low tissue mortality but do not result in total colony mortality (Stimson 2011). This highlights the importance of long term studies examining the virulence of diseases as well as prevalence.

PBTL incidence closely tracked prevalence and was high from April to August, with a decrease from September onwards. A constant increase in incidence indicates the spread of a disease within a population and could point towards a disease outbreak (Raymundo et al. 2008). PBTL prevalence and incidence only increased during the summer, indicating strong seasonal dynamics. Sato et al. (2009) also found a strong positive correlation between black band disease incidence and seasonal changes in temperature and light, which appeared to be relatively stable over a 2.5-year period. To determine if prevalence levels of PBTL are

increasing through time, a multi-year study of prevalence and incidence is needed. In addition, a significant difference in the size-class frequency distributions of healthy and PBTL-affected colonies suggests that medium to large colonies (>20 cm) are most commonly affected by PBTL.

The fact that PBTL was not observed in Hanauma Bay but was abundant throughout Kaneohe Bay could indicate that PBTL might be restricted to Kaneohe Bay. However, further surveys are needed to support this hypothesis.

4.5.2 Transmission, virulence and disease progression

No disease transmission was observed in healthy fragments that were touching PBTL-affected fragments or healthy fragments a few centimetres away from the diseased fragments. This suggests that PBTL does not easily transmit via direct contact or the water column (at least over the period of approximately 1 – 2 months). It may be that the environmental conditions needed for successful transmission were not replicated by my experimental treatment. In contrast, other manipulative experiments have successfully shown disease transmission in aquaria. For example, *Montipora* white syndrome was shown to be transmissible through direct contact in aquarium conditions, with direct transmission also observed in the field (Aeby et al. 2010). In the case of PBTL, direct transmission was not observed in the field, an indication that this disease is either not highly infectious or that it is not caused by a pathogen (see also chapter 3). Alternatively, a vector may be needed for disease transmission.

Different coral diseases show various degrees of virulence. PBTL showed an average tissue mortality rate of 29.7% within two months and then ceased in most cases. In contrast, white band disease can progress up to 2 cm per day (Gladfelter 1982) and cause high colony mortality within a very short period of time. MWS was found to cause about 3% tissue loss per month (Aeby et al. 2010), whereas yellow band disease causes about 8% tissue loss per year (Bruckner and Bruckner 2006). Total colony mortality of PBTL-affected colonies was rare and in a few cases the disease regressed without causing any tissue death (15% of tagged colonies). It is unknown what factors contribute to full recovery of some corals. Of the PBTL-affected colonies tagged in 2010, 23.8% showed tissue re-growth but 54.8% showed no signs of recovery, suggesting that recovery rates are slow. In addition, 31% of colonies tagged in 2010 were affected again in 2011, with disease cessation between years. Recurrent infections of tagged colonies have been observed primarily for BBD. Sato et al. (2009) found

a 31% recurrent infection rate on the GBR, and Kuta and Richardson (1996) reported seasonal re-infection with BBD in the Florida Keys. Recurrent infections could cause cumulative tissue mortality which could lead to total colony mortality after multiple infections and cause increased damage to the reef system over time (Borger and Steiner 2005). A cumulative effect of PBTL could therefore have a potentially negative impact on *P. compressa*-dominated reefs in Hawaii.

All PBTL-affected samples maintained at elevated temperature sustained total tissue mortality whereas only 33% of samples kept at ambient temperature showed total mortality, suggesting that temperature negatively affects the ability of the host to fight the disease (i.e. a stressed coral is more susceptible) and/or increases the virulence of PBTL. The strong temporal relationship of temperature and prevalence observed in the field supports this hypothesis. In addition, two fragments kept at ambient temperature showed re-pigmentation (no mortality) when the temperature dropped below 25 ºC. Furthermore, none of the fragments sampled adjacent or furthest away from the lesion developed signs of PBTL during the course of the experiment, suggesting that the disease is localised and spread is limited.

4.5.3 Environmental drivers

Disease causation is dependent on intricate interactions between the host, environment and pathogen (Work et al. 2008c). One can therefore expect coral disease spatio-temporal dynamics to be highly complex and to be correlated with multiple, and possibly co-interacting environmental drivers (Williams et al. 2010).

The temporal variation in PBTL prevalence (peak prevalence during the summer months) was strongly correlated with water temperature. Increased coral disease prevalence often correlates with elevated seawater temperature, for example as seen for BBD (Boyett et al. 2007; Rodriguez and Croquer 2008) and WS (Selig et al. 2006; Bruno et al. 2007; Williams et al. 2010, 2011a). Increased temperature can lead to an increase in pathogen virulence and/or cause stress to the host making it more susceptible to disease (Harvell et al. 2007).

An increase in PBTL prevalence across sites was correlated with high water motion, lower turbidity and higher parrotfish densities. However, only 26.2% of the variability in PBTL prevalence could be explained by these factors, suggesting that other environmental factors that were not incorporated in the model may also be important drivers of PBTL patterns or that temporal variation in temperature is the main driving factor, hence reducing the

explanatory power of the model. Another explanation could be that variations in predictor variables across sites were not captured at an appropriate scale. In contrast to the temporal analysis, the spatial analysis did not identify temperature as a strong predictor. This was most likely due to the averaging of all temperature data across the year to achieve the same resolution as the other predictor variables which resulted in the loss of predictive power as it removed the seasonal fluctuations in temperature.

Variations in turbidity were identified as the overall strongest predictor, with 12.8% of the spatial variability in PBTL prevalence explained. Turbidity showed a weak negative relationship with PBTL prevalence, indicating that clearer waters may increase disease prevalence; this could suggest that increased light levels may contribute to PBTL prevalence. Sato et al. (2011) found that high light and elevated seawater temperature strongly drive the occurrence of black band disease on the GBR. They proposed that seasonally increasing light levels may be even more important in inducing new infections than increasing water temperature. A link to increased light levels could explain the spotty appearance of PBTL (bleached coenenchyme and pigmented polyps), as *Symbiodinium* cells may be more shielded in the polyps because they can retract into the skeleton. However, manipulative experiments are needed to clarify the link between light and PBTL. Interestingly, increased water temperature and low turbidity were also found to be strong predictors of PorTL (Williams et al. 2010) indicating that PBTL and PorTL either show similar interactions with the environment, or are caused by the same pathogen (if present).

Water motion explained 9% of the variability and showed a positive correlation with PBTL prevalence. High water motion may somehow enhance the conditions needed to induce PBTL in *P. compressa*. Non-random patterns of disease pathogen dispersal could be caused by local changes in water motion which could affect transmission mechanisms that operate on a small scale (Jolles et al. 2002). Aspergillosis of sea fans was found to be more prevalent in sheltered areas and was likely influenced by water currents (Nagelkerken et al. 1997; Jolles et al. 2002), while the spread of BBD was found to follow a down-current direction (Bruckner et al. 1997). However, in contrast to these two diseases, no concrete evidence has yet been found that PBTL is caused by a pathogen (see chapter 3).

A positive correlation to parrotfish density was incorporated in the best model predicting PBTL prevalence; however, parrotfish density only explained 4.4% of the variation in PBTL prevalence so the ecological importance of this factor is questionable. Butterflyfish density

did not appear to affect PBTL prevalence, highlighting the importance of investigating individual species interactions. Corallivorous fish, such as butterflyfish, have been implicated as coral disease vectors (Aeby and Santavy 2006; Raymundo et al. 2009) but they have also been suggested as a potential mechanism to reduce disease progression by removal of disease lesions (Cole et al. 2009; Chong-Seng et al. 2010). It is unclear if the positive association between parrotfish density and PBTL suggests a vector interaction. Manipulative experiments are required to investigate this possibility. Williams et al. (2010) found that a negative correlation to juvenile parrotfish most strongly predicted MWS prevalence. They speculated that a correlation to reef fish abundance could also be the result of an indirect interaction; for example factors that either negatively or positively affect fish may also favour disease prevalence (Williams et al. 2010).

Spatial variations in salinity, chlorophyll-*a* and *P. compressa* abundance were not found to be important predictors of PBTL prevalence. Chl-*a* can be an indicator of poorer water quality which has been suggested to increase prevalence of aspergillosis in sea fans (Bruno et al. 2003; Baker et al. 2007), black band disease (Kaczmarsky et al. 2005; Voss and Richardson 2006), white plague (Kaczmarsky et al. 2005), yellow-band disease (Bruno et al. 2003) and MWS (Williams et al. 2010). It was unexpected that *P. compressa* abundance was not found to be an important predictor of PBTL prevalence, as many other diseases show a strong relationship with host cover (Riegl 2002; Myers and Raymundo 2009; Aeby et al. 2010; Williams et al. 2010; Aeby et al. 2011a, 2011b). For example, white syndrome from the GBR was found to require a coral cover of >50% for an outbreak to occur (Selig et al. 2006). In the case of PBTL, sites with medium *P. compressa* cover (40-60%) showed the highest prevalence. Interestingly, the same was found for *Porites* trematodiasis in Kaneohe Bay (Aeby 2007; Williams et al. 2010), a coral disease that is caused by a digenetic trematode and has multiple hosts (fish, mollusc, coral) (Aeby 1998). It is unclear why medium coral cover positively affects prevalence in these cases. Highly infectious diseases benefit from high host density as it provides a higher chance of transmission. The weak association of host cover and prevalence is likely due to the fact that PBTL does not appear to be highly infectious.

4.6 Conclusion

The results of this study suggest that temperature is the major driving force of temporal variations in PBTL prevalence. The fact that only a very small amount of variability could be explained by other environmental factors supports this assumption. Warmer seawater

temperatures predicted by global warming could therefore lead to higher PBTL prevalence for longer periods of time, causing increased damage to corals. *P. compressa* is one of the main framework building corals in Kaneohe Bay, and so PBTL could have a potentially negative impact on the health and resilience of the reefs at this location.

Chapter 5

Are coral host and symbiont genetics involved in the disease etiology of *Porites* bleaching with tissue loss (PBTL) in *P. compressa*?

5.1 Introduction

Marine diseases are an emerging problem worldwide (Harvell et al. 1999) contributing to major changes in the structure and function of reef habitats (Hughes 1994; Aronson and Precht 2001). In addition to investigating disease etiology and ecosystem-level impacts, another critical factor is genetic differences that may contribute to disease susceptibility or resistance in individuals and populations (Lesser et al. 2007). In corals, these functional differences may not just occur within different coral genotypes but also in the presence of different clades of *Symbiodinium* (Correa et al. 2009), the endosymbiotic dinoflagellates that reside within the coral's tissues. However, our understanding of genetic disease resistance in corals and the relationship between *Symbiodinium* identity and coral disease is limited.

Genotypic resistance to coral disease has only been documented in a few cases. Gochfeld et al. (2006) investigated disease susceptibility of *Siderastrea siderea* to dark spot syndrome and found that certain individuals within a population appeared to have greater immunity. Another example is the white plague type II outbreak in the Florida Keys in 1995 (Richardson et al. 1998a) which was found to be caused by the bacterium *Aurantimonas coralicida* (Denner et al. 2003). Interestingly, healthy corals are no longer susceptible to this pathogen but it remains unclear if this immunity was acquired during the original disease event or if it is a natural, genetic resistance of the surviving colonies (Richardson and Aronson 2000). Using microsatellite genotype information in combination with *in situ* transmission assays and field monitoring, Vollmer and Kline (2008) found that 6% of *Acropora cervicornis* colonies from Panama have a natural resistance to white band disease.

Equally little is known about the involvement of *Symbiodinium* types in disease resistance or susceptibility of the coral host. The taxonomy of *Symbiodinium* is primarily based on molecular phylogenetics (LaJeunesse 2001). Using nuclear (28s rDNA) and chloroplast (cp23 rDNA) markers, the genus is currently divided into 9 clades named A-I (Pochon and Gates 2010), of which clades A-D commonly associate with scleractinian corals (Baker 2003; Goulet 2006). The ecological dominance of these clades differs in different oceans (Baker

2003). For example, in the Indo-Pacific approximately 95% of symbiotic invertebrate genera associate with clade C *Symbiodinium* (Baker 2003; LaJeunesse et al. 2004a). However, the cladal level does not provide a comprehensive account of the true genetic or functional diversity within the genus *Symbiodinium*. Genetic markers such as nuclear internal transcribed spacer regions (ITS1, ITS2) were developed to address this, resulting in the differentiation of numerous sub-clades or "types" (LaJeunesse 2001; van Oppen et al. 2001; Santos et al. 2004). In corals with horizontal symbiont transfer, juveniles often take up multiple *Symbiodinium* clades/sub-clades from the water column (Little et al. 2004; Abrego et al. 2009a, 2009b) with one type tending to become dominant through time (Goulet 2006); the other types may not be lost completely, however, but persist at low background levels (Mieog et al. 2007). Multiple symbiont types have been documented in a few coral species (Rowan and Powers 1991; Rowan and Knowlton 1995; Toller et al. 2001) and the potential for hosting multiple symbiont types appears to be common, even among scleractinians previously thought to be specialists (high specificity of the host for a particular symbiont type) (Baker 2003; Silverstein et al. 2012).

Functional differences between *Symbiodinium* clades/sub-clades influence the physiological properties of the host-symbiont assemblage. For example, corals that harbour clade D are less likely to bleach in response to increased temperature (Rowan 2004; Berkelmans and van Oppen 2006; Stat and Gates 2011). Differences in thermal tolerance can also be observed in some *Symbiodinium* sub-clades. For example, different symbiont types within clade C were correlated to bleaching susceptibility of the coral host (Sampayo et al. 2008; Howells et al. 2012). However, relatively little is known about the relationship between *Symbiodinium* clade/sub-clade and coral disease susceptibility. Correa et al. (2009) identified *Symbiodinium* types using ITS2 in multiple coral species affected by one of five different diseases and found no correlation of specific symbiont types with diseased tissue. However, few diseased colonies contained type D1a symbionts in comparison to their healthy conspecifics, indicating a possible contribution of this symbiont type to disease resistance (Correa et al. 2009). In contrast, Stat et al. (2008) found a correlation between the presence of clade A and health-compromised *Acropora cytheria*, in addition to a reduction in the amount of carbon fixed and released by clade A in comparison to clade C *Symbiodinium*. They therefore speculated that clade A *Symbiodinium* may be functionally less beneficial to corals; clade A was similarily found to be the least beneficial symbiont for *Acropora millepora* on the GBR (Mieog et al. 2009). Clade A symbionts may therefore not meet the host nutritional

requirements which could lead to a reduction in fitness and higher susceptibility to disease (Stat et al. 2008). Toller et al. (2009) also found clade A symbionts to be associated with coral disease; the tissue of *Montastraea* spp. affected by yellow blotch disease contained predominantly clade A symbionts whereas healthy tissue contained clade C symbionts, which are more typical for this species. The atypical symbionts found in diseased tissue might be "parasitic" and thrive at the expense of their host (Stat et al. 2008; Toller et al. 2009). Alternatively, the onset of disease might change the symbiont community composition, providing opportunities for usually cryptic and rare clades or sub-clades to become dominant (Baker 2003; Toller et al. 2009).

Porites bleaching with tissue loss (PBTL) is a coral disease that has recently been described from Hawaii. It manifests as bleaching of the coenenchyme with the polyps remaining brown and can lead to tissue mortality (Chapter 2). Field observations suggest a certain degree of clustering of PBTL-affected colonies, however direct contact does not lead to PBTL being transmitted (see Chapter 3). These observations lead to the hypothesis that host genetics may play a role in PBTL susceptibility. Because PBTL causes a bleaching response in the coral, I also investigated the potential involvement of *Symbiodinium* type in disease etiology. This study therefore aimed to: 1) determine if diseased coral colonies share the same genotype (i.e. are clones or genetically similar); and 2) identify the *Symbiodinium* ITS2 type associated with diseased and healthy tissue.

5.2 Material and methods

5.2.1 Sample collection

Fragments of *Porites compressa* (approximately 2 cm^2) were sampled from Coconut Island Marine Reserve, Oahu, Hawaii in June 2010. Samples were taken from disease lesions showing PBTL, a healthy area from the same coral and a healthy neighbouring colony (a total of 30 colonies affected by PBTL and 30 healthy colonies were sampled) (refer to Appendix I.3 for the sample design). Fragments were frozen for a few days at -20 ºC. A small piece (approximately 3 mm^2) was then removed and stored at 4 ºC in 400 µl of extraction buffer (50% (w/v) guanidinium isothiocyanate; 50 mM Tris ph 7.6; 10 µM EDTA; 4.2% (w/v) sarkosyl; 2.1% (v/W) β-mercaptoethanol) until further processing.

All samples were used for the determination of the symbiont sub-clade (n = 90 from 60 colonies) but only the samples from the disease lesion and the healthy neighbouring coral (n

= 60) were used for the host genotype investigation (refer to Appendix I.3 for the sample design).

5.2.2 DNA extraction

The coral samples stored in guanidinium buffer were incubated for 10 minutes at 72ºC and then centrifuged for 5 minutes at 13,200 x g. Supernatant (200 µl) was placed in a new tube and an equal volume of 100% isopropanol was added to precipitate the DNA. Precipitated DNA was stored overnight at -20 ºC. The DNA was pelleted by centrifugation at 13,200 x g for 15 minutes and all supernatant removed. The pellet was washed in 400 µl of 70% ethanol before re-suspension in 100 µl Tris buffer (0.1 M pH 8). The samples were then stored at -20ºC until further processing.

5.2.3 Host (microsatellites)

Eleven microsatellite regions, originally developed for *Porites lobata* (Concepcion et al. 2010) were screened but only nine loci were consistently amplified. Analysis was therefore restricted to these loci. Microsatellite loci were amplified using QIAGEN® Multiplex PCR kits (QIAGEN, Hilden, Germany) and nine fluorescently labelled primers (Table 5.1) at a starting concentration of 10 mM/µl. Primers with the same annealing temperature were combined into a primer mix (A or B). The total volume of each primer mix was 100 µl. Primer mix A contained 2.5 µl each of PL0340 forward and reverse primers, 1.25 µl each of PL0780 forward and reverse primers, 0.75 µl each of PL0905 forward and reverse primers, 2 µl each of PL1551 forward and reverse primers, 1.25 µl each of PL1629 forward and reverse primers, 1.25 µl each of PL1868 forward and reverse primers, and 82 µl of deionised sterile water. Primer mix B contained 3 µl each of the PL1370 forward and reverse primers, 1.75 µl each of the PL1483 primers, 3 µl each of the PL1556 forward and reverse primers, 2 µl each of the PL2258 forward and reverse primers, 2 µl each of the PL1490 forward and reverse primers, and 76.5 µl of deionised sterile water. PCR reactions contained 5 µl of QIAGEN® Multiplex PCR mastermix and 1 µl of sample DNA. PCR conditions were as follows: Primer mix A: 95 °C for 15 min, 27 cycles of 94 °C for 30 s, 55 °C for 30 min; and Primer mix B: 57 °C for 3 min, 72 °C for 30 s; 60 °C for 30 min. Fragments were run on a 3130 Genetic Analyzer at the Hawaii Institute of Marine Biology. Alleles were scored with GENEMAPPER 4.0 software (Applied Biosystems, Foster City, CA).

Table 5.1: Microsatellite loci for *Porites lobata* (used for *Porites compressa*) amplified in two multiplex reactions (A and B). The repeat type, fluorescent label, size range of alleles (bp) and the annealing temperature are provided.

Marker name	Primer sequence (5' – 3')	Repeat	Fluorescent label	Size range of alleles	Annealing temp (ºC)	Primer mix
PL0340	F: PET:GTTTGCCTCTCTTCTGTTCATT R:AACATTATGGCTAGTTCTTTGAACG	(ATCC)6 ATT (CGTT)4 TGTT (CATT)3	red	216 - 275	52	A
PL0780	F: VIC:GCCAGTAGGTGGATACACTGTT R: CAAGTACGTTGACGTCGTTG	(ATT)4 (GTT)7	green	136 - 163	52	A
PL0905	F: PET:GGTCCAAAGTCCACCATCA R: TGGTGGAAATAAGTGGTCGA	(ATC)9	red	135 - 160	52	A
PL1551	F: NED:TGTTTCTGAGTGGCTGTGCT R: GGTTGGAAAGGGTCCTTCAT	(GTT)8	yellow	170 - 196	52	A
PL1629	F: VIC:CCTTGGTTAATTTGCCCTTG R: ACCAGTCCGGAGTCAAGCTA	(GCT)8	green	168 - 180	52	A
PL1868	F: FAM:TAAGCCACAGCAGGTGTACG R: AAACGTTCCCTATCCCATCC	(AAGC)8	blue	180 - 205	52	A
PL1483	F: NED:AAACGT TCCCTATCCCATCC R: GCAAAGCTGCTACATTTCACTAA	(AAGC)8	yellow	145 - 175	54	B
PL1556	F: PET:CGTTGACGTAACCTTCACCA R: CACAGGGTAACCTTCCTTGC	(ATC)10	red	150 - 168	56	B
PL2258	F: PET:ATTAGCGGATGAAGCGAAGA R: TCCAATGTAACGCCAAATCA	(GAT)10	red	217 - 250	56	B

5.2.3 *Symbiodinium* (ITS2)

The *Symbiodinium* partial ITS2, 5.8S and 28S regions were amplified with the Polymerase Chain Reaction (PCR) using the forward ITS-DINO (5' GTGAATTGCAGAACTCCGTG 3') and reverse its2rev2 (5' CCTCCGCTTACTTATATGCTT 3') primers (Pochon et al. 2001). Each 25 µl PCR reaction contained 1 µl of DNA, 2.5 µl of 10x ImmoBuffer, 1 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM total dNTP's (2.5 mM each), 0.5 µl of each primer (5 pmol of each primer), 0.1 µl Immolase and deionized sterile water to volume. A BioRad iCycler™ using the following conditions was used to run the PCR: 7 min at 95 ºC, followed by 35 cycles of 45 s at 95 ºC, 45 s at 52 ºC, and 45 s at 72 ºC, with a final cycle for 5 min at 72 ºC (Stat et al. 2011). A gel was run to check if DNA was successfully amplified. The PCR product was digested using Fast APExo Mix for 30 min at 37 ºC followed by 20 min at 85ºC, and sequenced in the forward and reverse directions at the Hawaii Institute of Marine Biology using BigDye Terminators (PerkinElmer, MA) on an ABI-3100 automated sequencer.

5.3 Data analysis

5.3.1 Host (microsatellites)

P. compressa samples that only amplified at two or less microsatellite loci were excluded from the analyses (healthy = 2; PBTL-affected = 3), reducing the total sample size to 55 (healthy = 28; PBTL-affected = 27).

The program GeneAlEx 6.41 (Peakall and Smouse 2006) was used for subsequent analysis of genetic differentiation between healthy and PBTL-affected colonies. Descriptive statistics including allele frequencies and the number of private alleles were determined. Heterozygote deficiencies for each group were determined by calculating and comparing the observed and expected heterozygosities for deviations from the Hardy-Weinberg equilibrium. The function "find multilocus matches" was used to identify clones in the data set. To assess genetic differentiation, an analysis of molecular variance (AMOVA) based on 9999 permutations was used to calculate Wright's F_{ST} (1951) and Slatkin's (1995) R_{ST} with the significance-level of the estimators. Significance levels were adjusted for multiple tests by a Bonferroni-correction (setting the significance level at 0.005). F_{ST} (fixation index), R_{ST} (a form of F_{ST} designed specifically for microsatellites and based on allele sizes assuming a generalized step-wise mutation model) and F_{IS} (inbreeding coefficient), as well as the Shannon index (diversity

index), were calculated for overall genetic differences (all loci combined) and individual loci comparisons.

The data set contained 16% missing data which can be problematic for pairwise distance-based analyses, such as AMOVA (Peakall and Smouse 2006). GeneAlEx therefore offers an option to interpolate missing data in which the average genetic distance for each population-level pairwise contrast is inserted (Peakall and Smouse 2006). However, using this method, large numbers of missing data for individual-based distance analyses can cause bias (Peakall and Smouse 2006). Presented here are the AMOVA results (F_{ST}, R_{ST} and F_{IS}) for both interpolated and non-interpolated missing data, to provide a comparison and evaluation of the effect that the missing data may have on the outcome of the results.

The program Microchecker (van Oosterhout et al. 2004) was used to test for null alleles and large allele drop-out.

5.3.2 *Symbiodinium* (ITS2)

Symbiodinium sequences were identified to sub-clade level via the Basic Local Alignment Search Tool (BLAST) in the National Center for Biological Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/).

5.4 Results

5.4.1 Host (microsatellites)

Both diseased and healthy samples, showed a varying degree of amplification ranging from 17 to 27 samples that scored alleles (Tables 5.2 and 5.3). Missing data in the two groups therefore ranged from 0% to 37% (Tables 5.2 and 5.3), and overall 16% of the data was missing. However, individual loci appeared to amplify approximately equally well in healthy and PBTL-affected samples (Tables 5.2 and 5.3), with the largest difference in amplification observed at loci PL1629 and PL2258; in both cases, PBTL-affected samples showed lower amplification than healthy samples.

An exploration of the total number of alleles and individual allele frequencies at each locus showed variations between loci. Loci PL0780 and PL1629 exhibited the lowest number of alleles and locus PL1483 the highest (Tables 5.2 and 5.3).

Table 5.2: Healthy *Porites compressa* samples (n = 28): Number of samples that amplified at each locus with corresponding percentage of missing data; number of alleles and number of private alleles per locus; and observed and expected heterozygosity (Ho/He). Significance level: * = p <0.05 and ** = p <0.005.

Locus	amplification		Number of alleles	Number of private alleles	Heterozygosity Ho/He (observed/expected)
	Number of samples	Missing data (%)			
PL0340	21	29	4	0	0.286/0.634*
PL0780	27	4	2	0	0.037/0.431**
PL0905	19	32	5	0	0.474/0.717
PL1551	22	21	5	1	0.455/0.713
PL1629	27	4	2	0	0.185/0.168
PL1868	25	15	5	0	0.480/0.690*
PL1483	26	4	7	0	0.654/0.802*
PL1556	26	4	3	0	0.154/0.211*
PL2258	23	27	5	2	0.435/0.685**
Total over loci	216	7	38	3	Mean 0.351/0.561

Table 5.3: PBTL-affected *Porites compressa* samples (n = 27): Number of samples that amplified at each locus with corresponding percentage of missing data; number of alleles and number of private alleles per locus; and observed and expected heterozygosity (Ho/He). Significance level: * = p <0.05 and ** = p <0.005.

Locus	amplification		Number of alleles	Number of private alleles	Heterozygosity Ho/He (observed/expected)
	Number of samples	Missing data (%)			
PL0340	18	33	4	0	0.611/0.610**
PL0780	25	7	3	1	0.200/0.495*
PL0905	19	30	6	1	0.421/0.735
PL1551	21	22	5	1	0.714/0.722
PL1629	24	11	2	0	0.083/0.080
PL1868	22	19	5	0	0.727/0.778
PL1483	27	0	7	0	0.778/0.823
PL1556	27	0	3	0	0.111/0.106
PL2258	17	37	3	0	0.235/0.630**
Total over loci	200	9	38	3	Mean 0.431/0.553

The total number of alleles was similar between healthy and diseased corals at most loci (Tables 5.2 and 5.3), however loci PL1551, PL1868, PL1483 and PL2258 showed larger variations in allele frequencies (Fig. 5.1). For example, at locus PL1868 allele 193 and 200 had a higher frequency in PBTL-affected samples, and allele 185 had a higher frequency in healthy colonies (Fig. 5.1F). At locus PL2258, allele 232 showed a much higher frequency in PBTL-affected samples, and alleles 238 and 256 were only present in healthy samples (Fig. 5.1I). Private alleles were observed at some loci in both groups (healthy and diseased), ranging from 0 to 2 per locus.

Deviations from the expected Hardy-Weinberg equilibrium were observed in both groups for 6 loci in healthy samples (Table 5.2) and 3 loci in PBTL-affected samples (Table 5.3). The mean heterozygosity value across loci was 0.431/0.553 (Ho/He) for PBTL-affected and 0.351/0.561 (Ho/He) for healthy samples. Overall, the mean heterozygosity across diseased and healthy groups was 0.391/0.557 (Ho/He). No large allele drop-out was identified but possible null alleles were detected for all loci that deviated from the Hardy-Weinberg equilibrium (HWE). Allele frequencies for these loci were therefore regenerated using MicroChecker and adjusted genotype frequencies were calculated from these allele frequencies (described in van Oosterhout et al. 2004). The regenerated genotype frequencies were then used to re-calculate F_{ST} and R_{ST} values but it had little effect on the results. The results of the original analysis of genetic variation between healthy and PBTL-affected samples (non-modified data) are therefore presented below. (Results of the regenerated genotype frequencies analysis can be viewed in Appendix VIII).

No multilocus clones (matching at all nine loci) were detected in the data set with multilocus matches from <8 loci being a mix of healthy and PBTL-affected samples.

A test of genetic differentiation using AMOVA showed no genetic differences between healthy and diseased groups when all loci were combined (F_{ST} and R_{ST}) and for most individual loci (Table 5.4). Depending on whether missing data were interpolated, significant differences at the 5% level were detected at loci PL1868 and PL2258 (F_{ST}), and loci PL1483 and PL2258 (R_{ST}) (Table 5.4). However, only locus PL2258 was found to be significant by both F_{ST} and R_{ST} for both non-interpolated and interpolated missing data (Table 5.4). This difference was even detected using a strict Bonferroni-correction ($p < 0.005$), but only when missing data were interpolated for the calculation of F_{ST} for individual loci (Table 5.4).

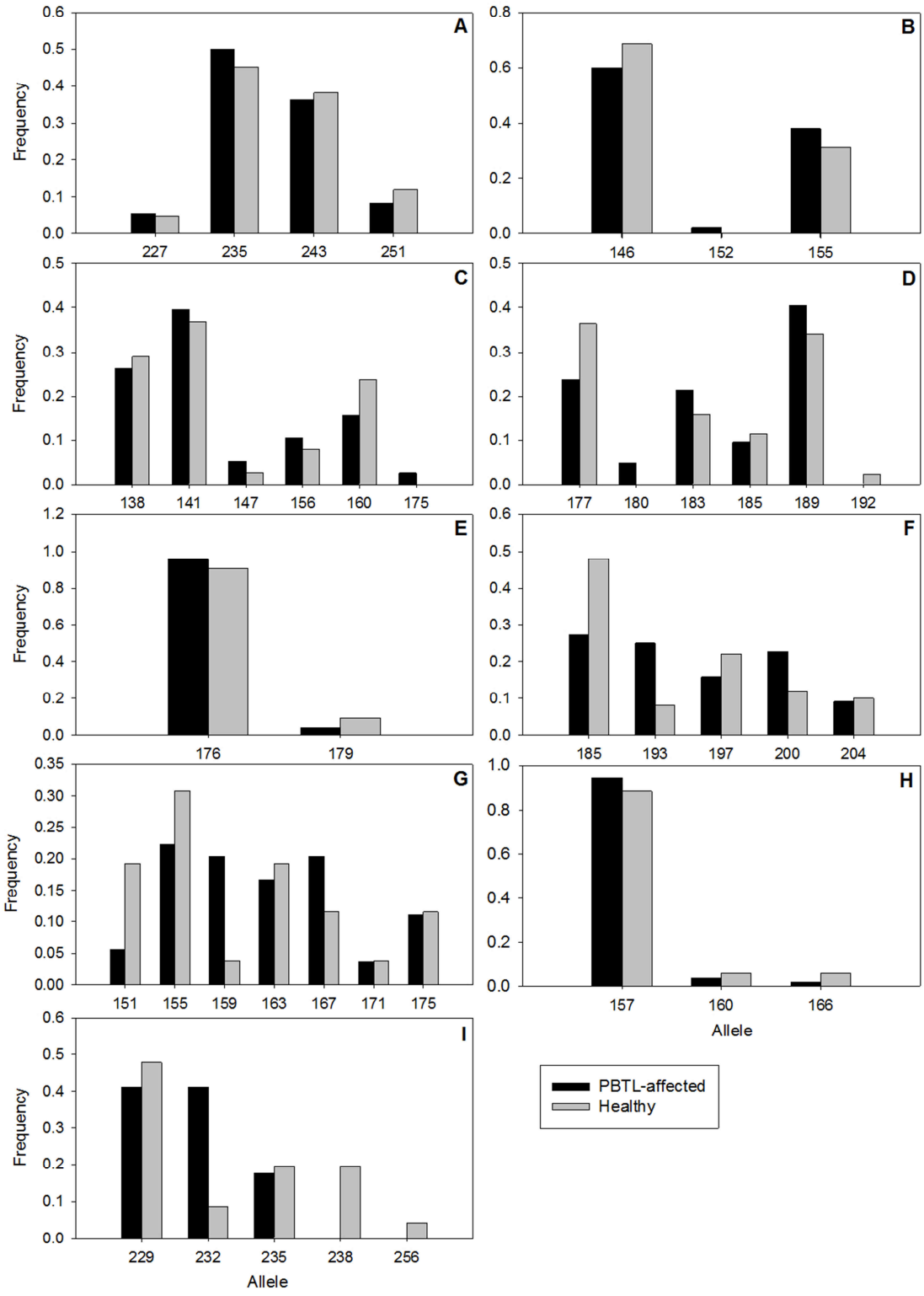


Fig 5.1: Allele frequencies at all nine loci. A: PL0340; B: PL0780; C: PL0905; D: PL1551; E: PL1629; F: PL1868; G: PL1483; H: PL1556; I: PL2258.

Table 5.4: F_{ST}, $F_{IS,}$ R_{ST} and R_{IS} values for an estimation of genetic differentiation of healthy and PBTL-affected samples for all loci combined and individual loci comparisons. Values were computed using an AMOVA with both non-interpolated and interpolated missing data. Significance level for fixation indices: * = p <0.05 and ** = p <0.005. (A Bonferroni-correction for this sample size recommends a significance level of p <0.005).

Locus	Non-interpolated missing data				Interpolated missing data			
	F_{ST}	**F_{IS}**	**R_{ST}**	**R_{IS}**	**F_{ST}**	**F_{IS}**	**R_{ST}**	**R_{IS}**
PL0340	-0.023	0.585**	-0.019	0.999**	-0.027	0.229**	-0.022	0.250**
PL0780	-0.019	0.795**	-0.026	0.997**	-0.020	0.717**	-0.020	0.745**
PL0905	-0.027	0.612**	-0.036	0.992**	-0.026	0.280**	-0.033	0.288**
PL1551	-0.016	0.432**	-0.038	0.998**	-0.009	0.166**	-0.018	0.293**
PL1629	-0.009	0.489**	0.006	1.000**	-0.002	-0.061	-0.002	-0.061
PL1868	0.023	0.363**	-0.018	0.994**	0.032*	0.176**	0.019	0.216*
PL1483	0.015	0.179**	0.056*	0.950**	0.016	0.132*	0.014	0.101
PL1556	0.024	0.416**	0.030	0.998**	-0.005	0.172	-0.001	0.386*
PL2258	0.046*	0.665**	0.060*	1.000**	**0.073****	0.358**	0.063*	0.562**
overall	0.002	0.497**	0.001	0.996**	0.008	0.257**	-0.004	0.287**

Table 5.5: Shannon index analyses based on a G-test using logbase 2 for individual loci comparisons. Significance level for fixation indices: * = p <0.05 and ** = p <0.005. (A Bonferroni-correction for this sample size recommends a significance level of p <0.005).

Locus	G	DF	p
PL0340	0.381	3	0.944
PL0780	2.076	2	0.354
PL0905	2.556	5	0.768
PL1551	5.997	5	0.307
PL1629	1.070	1	0.301
PL1868	9.110	4	0.058
PL1483	12.917	6	0.044*
PL1556	1.468	2	0.480
PL2258	21.723	4	**<0.001****

Values for inbreeding (F_{IS} and R_{IS}) were high and significantly different from zero in most cases (Tables 5.4).

A Shannon index analysis over loci for pair-wise population comparisons detected a significant difference at the 5% confidence level between healthy and diseased samples at locus PL1483 (G = 12.917, df = 6, p = 0.044), and using a strict Bonferroni-correction (p <0.005) at locus PL2258 (G = 21.723, df = 4, p <0.001) (Table 5.5).

5.4.2 *Symbiodinium* (ITS2)

Symbiodinium ITS2 type C15 was the dominant symbiont identified in 29 healthy colonies of *P. compressa*, 30 diseased colonies (fragments that were directly affected by PBTL) and 28 apparently healthy looking fragments of those diseased colonies. In most cases, a BLAST search showed that sequences were 100% identical to C15 (287bp). In a few cases, the BLAST search showed 99% similarity to C15 (Table 5.6) due to ambiguous nucleotides in the read. These ambiguities were mainly due to a few Ns (bp that could not be scored) and a few cases of intragenomic variation. However no other symbiont types were detected in the BLAST search.

Table 5.6: Number of samples showing a 100% and 99% match to C15.

	100% match		99% match	
	Number of samples	Percent %	Number of samples	Percent %
Healthy	18	62.1	11	37.9
PBTL-affected	20	66.7	10	33.3
Apparently healthy (from a PBTL-affected colony)	16	57.1	12	42.9

5.5 Discussion

No specific clone was associated with PBTL-affected samples, as determined by the screening of 9 microsatellite loci. However, a significant difference in allele frequency at one locus was observed between healthy and diseased samples. In addition, *Symbiodinium* sub-clade C15 was identified as the dominant symbiont type in both healthy and PBTL-affected *P. compressa*.

5.5.1 Host (microsatellites)

No clones were detected in the microsatellite data set, showing that PBTL-affected colonies are not of a clonal nature and so do not share the same genotype. Genetic homogeneity (random mating) between the two screened groups of *P. compressa* (healthy and diseased colonies) was confirmed by the low F_{ST} and R_{ST}-values and non-significant p-value of an overall comparison (all loci combined). *P. compressa* is a gonochoric broadcast spawner (Neves 2000) and the samples for this study were obtained from a relatively small-scale site (same reef), so it is not surprising that healthy and diseased samples belonged to the same population.

However, at the 5% confidence level, both F_{ST} and R_{ST} detected a significant difference between healthy and PBTL-affected colonies at one locus (PL2258). In addition, an exact G-test (Shannon_index), a very powerful test able to detect very fine differences in allele frequencies (Balloux and Lugon-Moulin 2002), also detected a difference at this locus. Although the complex interactions of disease infection are rarely exercised by a single gene, the expression of an allele at one locus could affect disease pathogenesis in individuals (Adams and Templeton 1998). Locus PL2258 could therefore potentially be associated with a gene that confers resistance/susceptibility to PBTL. However, as only 9 loci were screened, it seems somewhat unlikely that this study discovered a gene responsible for an increased susceptibility/resistance to PBTL.

In comparison to vertebrates and even plants, very little is known about genetic resistance to disease in reef corals. In humans and other vertebrates, it is widely accepted that the adaptive immune system in addition to complex genetic traits and environmental components contribute to disease susceptibility (Hill 1995; Adams and Templeton 1998; Morris 2007; Jovanovic et al. 2009; Baker and Antonovics 2012; Chapman and Hill 2012). Natural resistance to disease (disease resistance without prior exposure to pathogens) in vertebrates (Adams and Templeton 1998) is related to a chromosomal region known as the major histocompatibility complex (Lewin 1989). Genes involved in disease resistance have also been documented for plants (Hooker and Saxena 1971; Staskawicz et al. 1995; McDowell and Woffenden 2003; Wang et al. 2011). Plants, like corals, do not possess an adaptive immune system but they express R-genes (R = resistance) that encode for protein receptors responding to Avr genes (avirulence) expressed by a pathogen; these R-genes can provide effective resistance to particular pathogens (McDowell and Woffenden 2003). It is likely that

corals also possess R-genes, however very little research has been carried out on R-genes in corals, even though determining the involvement of genetic resistance or susceptibility to disease could have major implications for coral reef conservation. Vollmer and Kline (2008) identified genotypes of *Acropora cervicornis* that show a natural resistance to WBD; these genotypes could be used in transplantation efforts to restore damaged reef systems in the Caribbean (Vollmer and Kline 2008). Determining specific R-genes in *P. compressa* was beyond the scope of this study. However, future research into R-genes in corals could help explain why only certain individuals or species are affected by diseases and which coral species have more effective resistance to disease.

A reduction in disease resistance can also be caused by inbreeding (Keller and Waller 2002). In this study, a deficit in heterozygotes was observed in both groups of *P. compressa* (healthy and PBTL-affected), as well as in the overall population. In addition, F_{IS} values were relatively high and significantly different from zero in most cases. Heterozygote deficiencies are often found in marine invertebrates with low dispersal capabilities (Ayre and Duffy 1994; Brazeau and Harvell 1994; Ayre et al. 1997; Maier et al. 2001; Miller and Howard 2004) and may result from inbreeding or clonal reproduction, Wahlund effects (sampling individuals from more than one population), isolation and genetic drift, null alleles or large allele drop-out (Gutierrez-Rodriguez and Lasker 2004; Selkoe and Toonen 2006). As all samples in this study were collected from the same reef, isolation and genetic drift as well as a Wahlund effect seem unlikely in this case, because it would require a population structure on the scale of meters caused by some kind of geographic barrier. *P. compressa* planulae can survive for days to weeks in the water column (Hodgson 1985) and so have the ability to disperse further than on the scale of metres, making a Wahlund effect in the screened population improbable. Inbreeding on the other hand is a probable source of heterozygote deficiencies. Water in Kaneohe Bay has a relatively long residence time in comparison to more exposed shores and coral planulae may therefore settle closer to their parent colonies, which could increase the likelihood of mating between close relatives.

However, the interpretations of the data set have to be regarded with some caution as the presence of null alleles in microsatellite data can also create an apparent heterozygote deficiency (Selkoe and Toonen 2006) because heterozygotes that contain a null allele are scored as homozygotes. Null alleles were detected in most loci and so it seems likely that null alleles contributed to the observed heterozygote deficiency. Null alleles can also affect the estimation of population differentiation because it can cause a reduction of the genetic

diversity within populations, likely leading to an overestimation of population differentiation (i.e. increased F_{ST} value) (Chapuis and Estoup 2007). However that did not seem to be the case in this study as an analysis using adjusted genotype frequencies showed little qualitative change of the results. In addition, a relatively high proportion of the data was missing (16%) due to amplification problems. The primers used in this study were originally developed for *Porites lobata*. Flanking regions (binding site of the primers) are often highly conserved across taxa allowing cross-species amplification of microsatellite loci for closely related species (i.e. same genus or sometimes even family) (Selkoe and Toonen 2006). However, the success rate of primers is likely to decrease with genetic distance of the species (Selkoe and Toonen 2006), and even though *P. lobata* and *P. compressa* are genetically very similar (Forsman et al. 2009), the species difference could have contributed to the lower amplification observed in *P. compressa*. In addition, corals and other invertebrates are generally known for amplification problems (Hedgecock et al. 2004). Consequently, it is important to keep in mind that the relatively high percentage of missing data could have affected the analyses and therefore caused misinterpretations of the results.

5.5.2 *Symbiodinium* (ITS2)

Symbiodinium C15 was the dominant sub-clade in both healthy and diseased colonies, leading to the conclusion that there is no difference in the dominant symbiont type between healthy and PBTL-affected *P. compressa* in Kaneohe Bay. This finding was consistent with our existing knowledge of *Symbiodinium* C15, as *Porites* species associate with this subclade over the entire Pacific Ocean (LaJeunesse et al. 2003; LaJeunesse et al. 2004b); though, colonies below 10 m contain clade C15b (LaJeunesse et al. 2004b). Consequently, symbiont type does not appear to be involved in the etiology of PBTL.

Very few studies have investigated a potential link between symbiont identity and disease susceptibility in corals (Kirk et al. 2005; Stat et al. 2008; Correa et al. 2009; Toller et al. 2009). Associations of clade A *Symbiodinium* have been found with WS lesions in *Acropora cytherea* from the Northwestern Hawaiian Islands (Stat et al. 2008) and YBD lesions in *Montastraea* spp. from Panama (Toller et al. 2009). In contrast, Correa et al. (2009) did not detect clade A symbionts in YBD lesions in *Montastraea* spp. from the US Virgin Islands and the Florida Keys. It has, however, been suggested that clade A *Symbiodinium* may be parasitic in certain corals as it does not meet the host's energy requirements, which could

contribute to disease susceptibility of the coral host (Stat et al. 2008); this however awaits further investigation.

In addition, specific symbiont types were not associated with infected tissues of BBD, dark spot syndrome (DSS), WP (Correa et al. 2009) and aspergillosis (Kirk et al. 2005). However, Correa et al. (2009) found a negative correlation between *Symbiodinium* community diversity (harbouring multiple clades/sub-clades) and disease in *Siderastrea siderea* suggesting a potential link between symbiont diversity and disease susceptibility in this species.

Even though associations of a specific symbiont type have been found with certain diseases (Stat et al. 2008, Toller et al. 2009), the results remain inconclusive as not all diseased colonies always contained the same symbiont type. The hypotheses that 1) disease onset may cause a shift in the associated symbiont type, or that 2) the symbiont type directly contributes to disease resistance/susceptibility, await further investigation.

5.6 Conclusion

Host genetics are very likely to affect disease susceptibility in corals, however identifying the genes responsible for increased resistance or susceptibility is not an easy task. Hundreds of microsatellite primers would be needed for an increased chance of determining these genes. Next generation sequencing would provide data on the entire genome and hence make the search for R-genes more efficient.

Symbioses with certain types of *Symbiodinium* are more effective than others (Cantin et al. 2009; Mieog et al. 2009), which may contribute to the coral being more resistant or susceptible to disease infection. In the case where a disease directly affects the *Symbiodinium* cells rather than the host, differences in *Symbiodinium* physiology between different clades or subclades are likely to affect disease susceptibility. However, direct involvement of *Symbiodinium* in disease resistance or susceptibility has not yet been shown.

Chapter 6

General Discussion

This study aimed to explore and characterise various aspects of '*Porites* bleaching with tissue loss' (PBTL), a coral disease recently observed on reefs in Kaneohe Bay, Oahu, Hawaii. A range of techniques, including field surveys, histology, transmission electron microscopy, genetics and microbiology were used to establish a case definition of PBTL that can be used as a baseline for future studies.

6.1 Case definition of PBTL

PBTL affects *Porites compressa* and causes bleaching of the coenenchyme with polyps remaining pigmented. The lesion can manifest as focal or diffuse areas of discolouration with a central or peripheral location on the colony (Chapter 2). In the majority of cases PBTL leads to partial tissue mortality within two months (Chapter 4) caused by tissue fragmentation and necrosis of affected tissues (Chapter 3). Coral colonies can be affected by PBTL recurrently which can lead to increased damage over time (Chapter 4). In addition, PBTL causes a significant reduction in the reproductive output of the coral and so can negatively affect the resilience of affected reefs.

PBTL prevalence showed strong seasonal dynamics, represented by a peak prevalence during the warmer summer months, and this relationship appeared to be stable over two consecutive years (Chapter 4 and Appendix III). The temporal variability of disease prevalence can partially be explained by changes in water temperature. Field surveys and manipulative experiments established a link between elevated water temperature and disease prevalence and progression (Chapter 4). PBTL also showed spatial variation, with some sites showing higher disease prevalence than others. Of the environmental factors examined, parrotfish density, water motion and turbidity were found to explain 26.2% of the variability in PBTL prevalence (Chapter 4). Nevertheless, only a third of the variability could be explained by these predictors suggesting that other factors, not investigated in this study, also play an important role in PBTL prevalence patterns.

PBTL is not transmissible through the water column or direct contact (Chapter 4). It might require certain environmental conditions or a vector for successful transmission; or it is not caused by a pathogen and therefore not infectious. Other diseases have very specialised transmission pathways and are not transmitted via simple touch; for example HIV/AIDS,

which is nevertheless highly infectious. Further experiments are needed to establish if PBTL is infectious and how it is transmitted.

Symbiodinium clade has no effect on lesion development (all examined colonies showed C15 as their dominant subclade). In contrast, host genotype may contribute to PBTL susceptibility, as a difference in allele frequency at one locus was found between healthy and PBTL-affected samples. However, no conclusive results could be drawn from this study (Chapter 5).

The causative agent(s) of this disease (if any) remains elusive and warrants further investigation. Metazoans, bacteria and protozoans can likely be eliminated as potential etiological agents (Chapter 3), however a potential increase in the abundance of VLPs in PBTL-affected samples was observed (Chapter 3); further studies are needed to establish any link between viruses and disease causation.

6.2 *Porites* as disease host

Generally, *Porites* spp. are thought to be quite robust and more resistant to coral bleaching than other coral species, such as acroporids (Glynn 1984; Gleason 1993; Hoegh-Guldberg and Salvat 1995), but are they also more resistant to disease? Disease susceptibility appears to be species-specific but can also depend on geographic location. For example, *Acropora* on the GBR was the coral genus most affected by BBD (Page and Willis 2006). In contrast, in Indonesia BBD was not reported to affect Acroporidae even though it was observed in other species (Haapkylä et al. 2009). A variety of coral diseases affect *Porites* spp. around the world, including BBD (Miller 1996; Dinsdale 2000; Kaczmarsky 2006), GAs (Domart-Coulon et al. 2006; Kaczmarsky and Richardson 2007; Haapkylä et al. 2009; McClanahan et al. 2009) and WS (Willis et al. 2004; Haapkylä et al. 2009; Myers and Raymundo 2009). In the Philippines (Raymundo 2005), Hawaii (Friedlander et al. 2008a), Guam (Myers and Raymundo 2009) and Indonesia (Haapkylä et al. 2009), *Porites* spp. were found to be either the dominant or one of the main taxa affected by coral disease. In contrast, on the GBR, Poritidae was one of the taxa least affected by disease, with Pocilloporidae and Acroporidae showing much higher disease prevalence (Willis et al. 2004). This disparity can, in part, be explained by coral cover. Myers and Raymundo (2009) found a strong correlation between host cover and disease prevalence (i.e. *Porites* spp. were the most abundant corals on reefs in Guam and they also showed the highest disease prevalence). However, this relationship does not always hold true. In Indonesia, ‘bushy’ *Acropora* colonies are a relatively rare growth

form but they show a higher growth anomaly and WS prevalence than any other taxa (Haapkylä et al. 2007). Palmer et al. (2010) attributed the increased disease susceptibility of acroporids to their lower investment in immunity parameters, such as melanin, PO activity, and fluorescent proteins. Poritids show a very high investment in these immunity parameters which is reflected in their reduced disease susceptibility on the GBR (Palmer et al. 2010). In reality, disease susceptibility is most likely to arise from a complex interaction of multiple factors, such as host cover, coral cover, innate immunity mechanisms and environmental factors, as well as anthropogenic impacts and the coral's ability to cope with these stressors.

Various diseases/lesions that manifest as bleaching in *Porites* spp. have been reported previously (Raymundo et al. 2003; Work and Rameyer 2005; Davy 2007). One example is *Porites* ulcerative white spot disease (PUWS) from the Indo-Pacific (Raymundo et al. 2003). PUWS manifests as small (3-5 mm), discrete, round-to-ovoid bleached foci with intact tissue. The lesion can either regress or progress to complete tissue loss and in severe infections numerous lesions may coalescence causing high partial to total colony mortality (Raymundo et al. 2003). Disease prevalence varied greatly between different reefs (0-72%, average 22%) (Raymundo et al. 2003) and PUWS was found to be transmissible through direct physical contact (Raymundo et al. 2003). The causative agent of this disease was identified as *Vibrio* sp. (Arboleda and Reichardt 2010). Even though PUWS resembles PBTL lesions to a certain extent (i.e. it starts with bleaching and then progresses to tissue loss), the dynamics of these two diseases are very different. For example, PUWS affects various branching and massive *Porites* spp. and the onset of tissue loss is much slower (some colonies showed lesions for extended periods of time, i.e. several months, without onset of tissue loss) (Raymundo et al. 2003). A potentially common feature of these two diseases is a link to water temperature (increased water temperature increases prevalence), but in contrast to PBTL this link still remains to be verified for PUWS. This example illustrates that lesions with similar disease signs (e.g. bleaching) can have very different disease dynamics, and it therefore follows that combining different diseases in analyses of disease impacts or associations with environmental factors can lead to misinterpretations (Williams et al. 2010) which will likely reduce the effectiveness of management strategies that were based on these results. Even lesions that have nearly identical gross manifestations could be caused by different factors, such as environmental stressors or pathogens. More than six "white" diseases (spreading zone of tissue loss) have been described from the Caribbean alone, which are mostly differentiated by the speed of lesion progression (Sutherland et al. 2004). This highlights the difficulty of

reliably identifying different diseases in the field, and until evidence suggests that a lesion is caused by the same pathogen, disease signs should also be separated by host species to help achieve a higher degree of accuracy in disease analyses. Overall, there is a need for more robust coral disease diagnostic methods, using an interdisciplinary approach including both traditional and developing technologies (Pollock et al. 2011).

6.3 Baseline data – why are case definitions important?

It has been estimated that one third of coral species face the risk of extinction (Carpenter et al. 2008). Over the past 30 years, hard coral cover has declined by 80% in the Caribbean (Gardener et al. 2003) and 50% in the Indo-Pacific (Bruno and Selig 2007). This decline in coral cover on Caribbean reefs has been unprecedented over the past 3 millennia (Aronson and Precht 2001b). The causes of reef decline are diverse and include habitat destruction, pollution, invasive species, overfishing and climate change (Hoegh-Guldberg 1999; Hughes et al. 2003; Hughes et al. 2007; Burke et al. 2011). Recently, coral diseases were added to this list because reports of coral disease outbreaks have been increasing (Sokolow 2009) and coral diseases are known to have the potential to cause widespread coral mortality which can alter reef community structure (Aronson and Precht 2001a; Miller et al. 2009). The Indo-Pacific encompasses approximately 91% of the worlds coral reefs (Spalding and Grenfell 1997) so understanding the different coral diseases affecting these reefs is of great importance for the development of effective management plans. However, a lack of essential baseline data on the dynamics and etiology of coral diseases has hindered our understanding of the effect that these diseases have in this region (Willis et al 2004). For example, if no baseline data on 'natural' prevalence levels exist then it is very difficult to assess if diseases are increasing in the environment. In addition, insufficient descriptions of new diseases and possible but unverified links to environmental stressors highlight the need for standardised and quantitative disease assessments that elucidate the impact that diseases have on reefs and how this applies to reef management (Raymundo et al. 2003). High-quality characterisations of diseases should address important questions such as host and geographic range, prevalence, seasonality, environmental drivers and infectiousness. This information will help managers to identify diseases that have a higher potential to cause excessive reef damage; these diseases can then be classified as high management priority, warranting more targeted research and monitoring.

This study therefore aimed to provide a comprehensive characterisation of PBTL, as no data on this disease existed previously. The findings of this research indicate that PBTL could have negative effects on the reefs within Kaneohe Bay and so it warrants further investigation. The laboratory and field data collected on PBTL, referred to as the case definition of this disease (Hulley et al. 2001), can be used as the foundation upon which further studies regarding etiology and pathogenesis can build (Work et al. 2008c). Over time, a case definition should be continuously refined, which helps to more clearly differentiate different diseases from each other (Work et al. 2008c).

The onset of disease is linked intricately with the host, the pathogen and the environment (Work et al. 2008c). A comprehensive case definition of a coral disease should therefore provide information on all three of these factors. Hence, this study aimed to collect data on all three of these components. A multidisciplinary approach utilizing both traditional methods such as standardized ecological techniques and histology, as well as rapidly developing technologies such as microbiological and genetic tools, were used to achieve this; such interdisciplinary approaches are urgently needed in coral disease research (Bourne et al. 2009; Pollock et al. 2011). Comprehensive case definitions of coral diseases are currently rare; examples of a few, well-characterized diseases include BBD, aspergillosis and bacterial bleaching, but even in these cases many questions remain unanswered.

6.3.1 The host

Disease susceptibility of the host, in coral species as well as individuals, is likely an important aspect in explaining disease patterns and could have important implications for coral reef conservation (Vollmer and Kline 2008). In corals, resistance to disease is a factor of innate immunity pathways, antimicrobial properties and genetic traits. Certain coral species show higher levels of immunity parameters than others (Palmer et al. 2010) and corals display a high degree of variability in the production of antimicrobial compounds (Koh 1997; Gochfeld and Aeby 2008); however, research into genetic resistance to disease is limited. Identifying resistant coral genotypes, as well as determining if and to what extent a coral is able to defend itself, will help to predict future susceptibility to disease. This study investigated a potential genotypic difference between healthy and PBTL-affected corals but obtained only inconclusive results. Further studies into genetic resistance/susceptibility, as well as antimicrobial properties of *P. compressa* are therefore urgently needed.

The coral however, is not just comprised of the coral animal but also of all its associated microbial symbionts; together these are referred to as the coral holobiont. When building a case definition it is important to also investigate the other components of the coral holobiont, because the pathogen (if any is present) may not just affect the coral animal but may also directly infect other members of the holobiont such as the *Symbiodinium* cells or bacterial symbionts, or any combination of these (Bourne et al. 2009). For example, many diseases cause shifts in the bacterial community of affected tissue, such as WBD (Pantos and Bythell 2006), aspergillosis (Gil-Agudelo et al. 2006) and WS (Piskorska et al. 2007). In many cases it is, however, unknown if observed changes in the bacterial community arise as the cause or effect of infection (Mydlarz 2009). This study investigated both bacteria (Appendix IV) and *Symbiodinium* cells (Chapter 3 and 5), though because no increase in bacterial abundance in PBTL-affected tissue was detected, no further investigations of the bacterial community were conducted. However, only the traditional culture-based technique was used and so it is recommended for further studies to apply non-culture based techniques, such as metagenomics, to determine if and how bacterial communities shift in response to PBTL. *Symbiodinium* type does not affect the etiology of PBTL, however a lot of potentially apoptotic *Symbiodinium* cells were observed in diseased tissue suggesting that PBTL not only affects the host but also the *Symbiodinium* cells; this awaits further investigation.

Ecological monitoring can provide valuable information on disease dynamics and the degree of damage to the coral host. The information gathered through monitoring work can then be used to detect and evaluate disease outbreaks (Woodley et al. 2008). Monitoring should be conducted frequently to identify possible seasonal variations in prevalence and should consist of a large number of transects to ensure correct assessment of prevalence values (Bruckner 2002). Long-term studies of individual colonies that shed light on the duration of infection, the amount of mortality sustained and the frequency of re-infection (Bruckner 2002) are also important aspects of a case definition. This study therefore recorded PBTL prevalence monthly over the course of one year, with additional long-term monitoring of individually tagged colonies (Chapter 4, also see Appendix VI). These long-term studies not only provide reliable data on disease dynamics, but also characterise different progressive stages of a disease, so reducing the likelihood of misidentification. For example, atramentous necrosis was previously described as a black film covering lesions (Jones et al 2004). However, a long-term study by Anthony et al. (2008) found that the initial phases of atramentous necrosis manifest as bleached areas that progress to tissue loss, which is then followed by the black

film (final phase). Interestingly, the initial bleached patches closely resemble descriptions of white syndrome (Anthony et al. 2008) and so this disease might have been misidentified in the original short-term study. Anthony et al. (2008) also suspected that the microbial community associated with the black film likely contains secondary invaders associated with dying coral tissue, rather than containing the causative agent. The information gathered through long-term studies can therefore aid in the search of causative agent(s).

6.3.2 The pathogen

Many coral diseases are likely caused by intricate multi-factorial sources, with the pathogen(s) and the environment acting synergistically to cause disease (Work et al. 2008c; Sokolow 2009). These complex relationships currently impede our ability to determine causative agents and their interactions with the host and/or environment, hindering our understanding of coral disease dynamics (Bourne et al. 2009; Pollock et al. 2011). Even in the few cases where interactions of host and causative agent have been characterized at the gross, microscopic, molecular and biochemical levels, unexpected changes in virulence caused uncertainty about whether the initially isolated agent was still the primary pathogen (Bourne et al. 2009) or if the corals had developed resistance to this pathogen (Mydlarz et al. 2009). For example, during 1994 to 1997, an epizootic in sea fan corals (*Gorgonia ventalina*), caused by the fungus *Aspergillus sydowii*, led to mass mortalities in this coral species (Geiser et al. 1998; Kim and Harvell 2004). Since then, disease prevalence has declined (Kim and Harvell 2004) and now waned to low levels (Bourne et al. 2009; Mydlarz et al. 2009). Recently, *A. sydowii* was detected in healthy sea fans but not in colonies affected by aspergillosis, raising questions about the causation of this disease (Toledo-Hernandez et al. 2008). Another example is bacterial bleaching in *Oculina patagonica* from the Mediterranean. *O. patagonica* shows seasonal bleaching which was attributed to the bacterium *Vibrio shiloi* (Kushmaro et al. 2001; Rosenberg and Falkovitz 2004). However, since 2005 this pathogen can no longer be isolated from bleached corals and also no longer causes bleaching in experimental settings (Reshef et al. 2006; Rosenberg et al. 2007b), which has led to controversy of the actual cause of this bleaching (Lesser et al. 2007; Ainsworth et al. 2008a). Our limited understanding of the interactions between pathogens and the coral holobiont makes it unsurprising that we observe confusing disease dynamics at times (Bourne et al. 2009).

The current study was unsuccessful in determining the causative agent of PBTL. However, it is often very difficult to unravel the complex constitution of the holobiont and identify which organism is responsible for disease causation (if any). Microscopic investigation can provide evidence for the presence of certain microorganisms and experiments determining the transmissibility of a disease can give an indication of whether an infectious agent is involved (Work et al. 2008c). PBTL was not found to be infectious in controlled experimental settings, however that does not necessarily rule out an infectious agent because a variety of factors can affect disease transmission, such as host susceptibility, environmental conditions and vectors (Work et al. 2008c). Further investigation into the causation of PBTL is required, because only once the cause is determined can we characterise the pathogen's ecology and dynamics (if a pathogen is present), and fully understand complex disease patterns (Work et al. 2008c).

6.3.3 The environment

In order to be able to predict how coral diseases will react to global warming and increased human pressure we must understand how different environmental factors affect diseases. Different coral diseases can show very different interactions with a variety of environmental factors. Williams et al. (2010) found that four different Hawaiian coral diseases showed unique associations with abiotic and biotic factors. For example, MWS was best predicted by a negative correlation to juvenile parrotfish density and a positive correlation to chlorophyll-*a*, whereas *Porites* GAs was best predicted by a negative correlation to turbidity and depth (Williams et al. 2010). When diseases were combined, predictive performance of the model was reduced significantly, which highlights the importance of analysing and characterizing diseases separately, unless evidence suggest that they have etiologies that respond in the same manner to particular environmental factors (Williams et al. 2010). This study found spatio-temporal variation in PBTL prevalence that was associated with temperature, and to a certain extent turbidity, water motion and parrotfish density. Such multi-factor approaches to model coral disease dynamics have recently been utilized by various coral disease researchers (Bruno et al. 2007; McClanahan et al. 2009; Williams et al. 2010; Aeby et al. 2011a; 2011b) to uncover the intricate and often complex interactions of environment and disease, so increasing our understanding of disease dynamics worldwide. This information will provide a foundation upon which informed management decisions can be based. For example, diseases that show a strong correlation with chlorophyll-*a* (an indicator of water quality) are likely to show increased prevalence in areas with higher nutrient/sewage influx. In addition, diseases that show a strong association with temperature are likely to show an increase in disease

outbreaks with changing climate, which could cause increased coral mortality and so negatively impact the reef environment. Because PBTL was found to be strongly affected by temperature, it can be assumed that disease prevalence will increase with global warming. This kind of information can then be used to identify reefs that are expected to be more resilient to disease outbreaks and/or are expected to recover quicker if an outbreak occurs. Such reefs can then be considered for higher protection in the form of marine protected areas or no take zones.

6.3.4 The importance of spatial scale

Another important factor to consider is the effect of spatial scale. Factors driving coral disease patterns are likely to change over multiple spatial scales. A good example is *Porites* GAs in Hawaii. On a small spatial scale (a single reef), *Porites* GAs were best predicted by water quality and depth (Williams et al. 2010), whereas on a larger spatial scale (across islands) *Porites* GAs showed the strongest correlation with human population density (Aeby et al. 2011a). A small-scale study would not be expected to pick up on the importance of large-scale drivers, such as human population density. Nevertheless, these small-scale studies are important for detecting fine-scale drivers of coral disease patterns. In this study, only a third of the variation in PBTL prevalence could be explained by small-scale drivers. Water quality (chlorophyll-*a*) did not show a strong correlation with PBTL prevalence, however this disease has currently only been observed in Kaneohe Bay. On a larger scale, water quality within Kaneohe Bay could explain why PBTL has not been observed elsewhere. Kaneohe Bay has a long history of reduced water quality. In the early 1960s, raw sewage was discharged into the bay which had dramatic negative effects on the reef (Maragos 1972; Hunter and Evans 1995). In 1979, sewage outfalls were removed which greatly improved water quality, leading to a slow recovery of the reef system (Hunter and Evans 1995). However, occasional sewage spills during periods of high rainfall are still a recurring problem (Jokiel 2008) and could affect disease processes in the bay (e.g. introduction of potential pathogens, increased nutrient levels).

Even smaller scale differences in environmental conditions (below the single-reef scale) are likely to affect disease processes. Gorospe and Karl (2010) have shown that temperature in a small patch reef in Kaneohe Bay can vary by up to 1ºC on a scale of a few meters. Corals often live close to their maximum threshold temperature, especially during the summer. Temperature differences of up to 1ºC that affect only certain coral colonies could cause them

to be more stressed than neighbouring colonies, making them more susceptible to disease. Pathogen virulence could also be affected by these fine-scale temperature differences, making infection of colonies located in the thermally anomalous area more likely. These fine-scale differences in environmental conditions could help explain patchiness in disease occurrence. However, such fine-scale studies are very labour intensive and not feasible on a larger scale.

Investigating disease patterns at various spatial scales will provide important information on the factors driving these patterns. Management decisions can then be related to a spatial scale which will improve the effectiveness of management actions.

6.3.5 The need for a standardised database

In order to deepen our understanding of coral diseases and improve our ability to respond appropriately to disease outbreaks, it is important to share data on coral diseases with the management and research community (ICRI/UNEP-WCMC 2010). The Global Coral Reef Monitoring Network (GCRMN) has established a database – The Global Coral Disease Database (GCDD) (http://coraldisease.org) – with the aim of facilitating the visualization of coral disease data and its dissemination of descriptions of different coral diseases (ICRI/UNEP-WCMC 2010). The database is an online compilation of coral disease data derived from published literature, grey literature and contributions by researchers (ICRI/UNEP-WCMC 2010). Currently, 27 diseases are listed in the guide but the amount of information for each disease varies significantly. Even some well-characterised diseases, such as aspergillosis and YBD, have very limited to no information offered. Another example is "The Guide to Western Atlantic Coral Diseases and Other Causes of Coral Mortality" presented by UNEP and WCMC (http://www.masna.org/portals/0/NOAACoralDisease/index.htm). This site provides descriptions of several coral diseases in addition to a useful identification key for coral diseases and predation. However, both databases present the information mostly as text which makes it difficult to quickly and easily extrapolate information for disease comparisons. A standardised flow-chart with the key information displayed (Fig. 6.1 and 6.2) would facilitate a quick and easy comparison of diseases and aid in disease identification. Further information in addition to this flow-chart could be presented as text, to provide a deeper understanding and knowledge of the disease of concern.

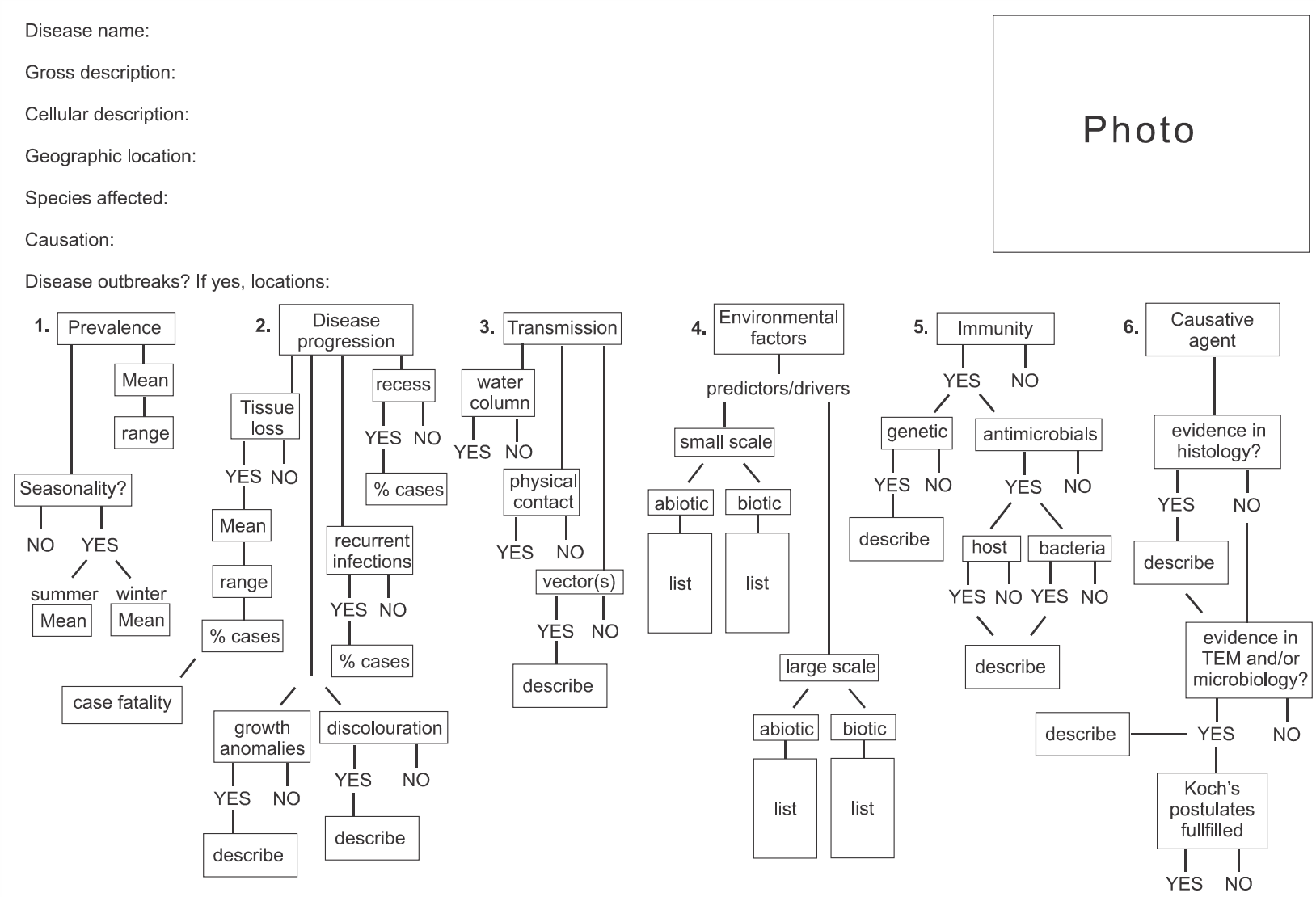


Fig. 6.1: Proposed flow-chart for disease descriptions. Further information and deeper explanations could be added underneath in the form of text.

Disease name: *Porites* bleaching with tissue loss (PBTL)

Gross description: Bleaching of the coenenchyme with polyps remaining pigmented; eventual tissue loss

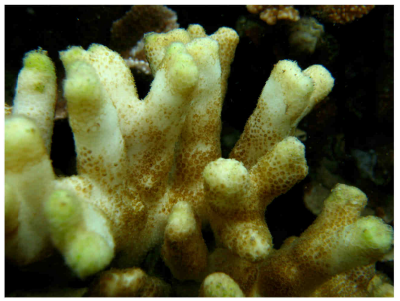
Cellular description: tissue thinning, necrosis, tissue fragmentation

Geographic location: Coconut Island Marine Reserve, Kaneohe Bay, Oahu, Hawaii

Species affected: *Porites compressa*

Causation: currently unknown

Disease outbreaks? If yes, locations: No



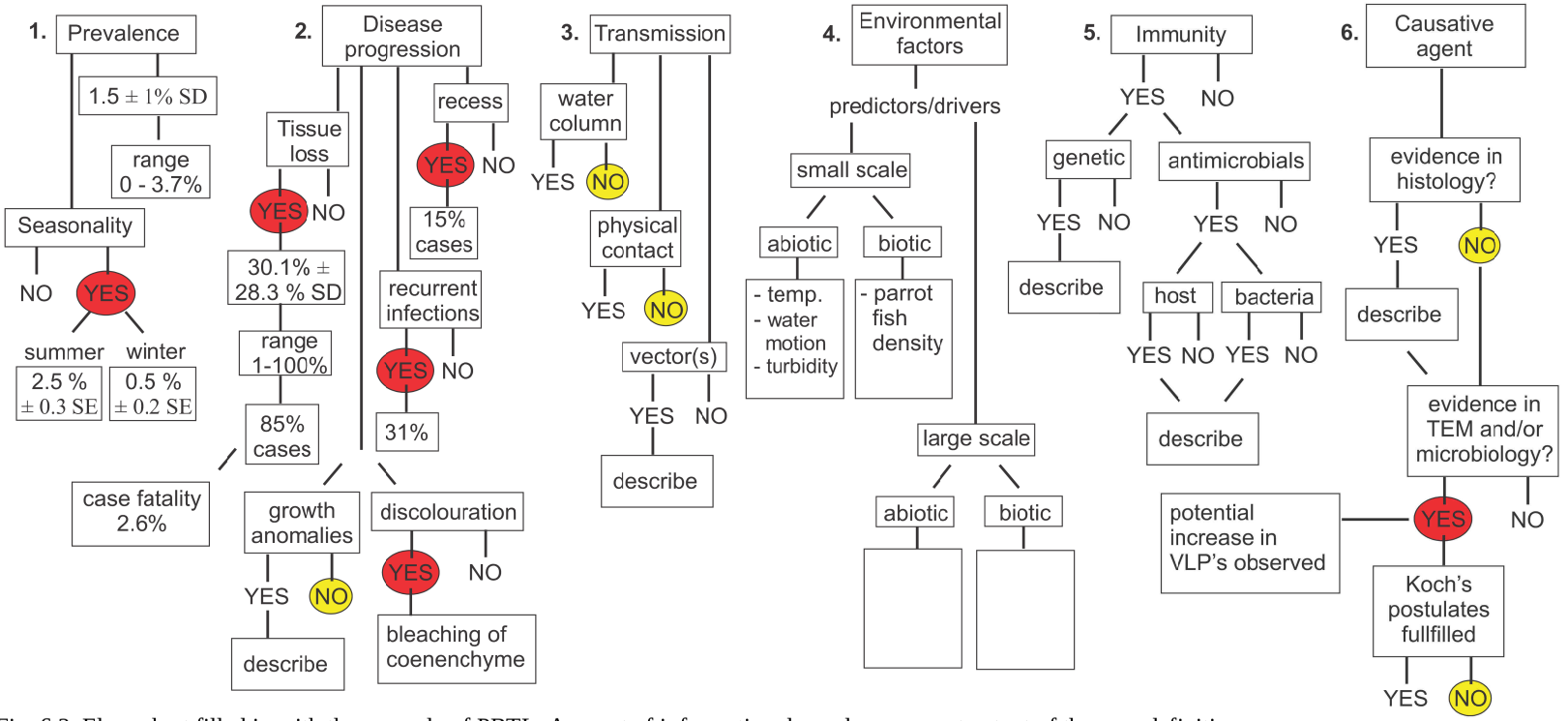


Fig. 6.2: Flow-chart filled in with the example of PBTL. Amount of information depends on current extent of the case definition.

6.4 Future research

The future of coral disease research lies within the exploration of rapidly developing molecular techniques for pathogen detection, such as quantitative PCR (qPCR) and antibodies (reviewed in Pollock et al. 2011). New-generation sequencing, such as metagenomics, has also proven to be a highly useful tool for elucidating disease and the complexity of the coral holobiont (Wegley et al. 2007; Vega-Thurber et al. 2008; Vega-Thurber et al. 2009; Littman et al. 2011). Further developing these techniques for the investigation of coral diseases is essential to move this field forward, and to reach the same standards and knowledge that have been achieved in biomedical and veterinary science.

However, a challenge in determining disease causality in corals is fulfilling Koch's postulates. Many marine microorganisms are very difficult to culture or are even regarded as unculturable (Rappe and Giovannoni 2003), so fulfilling Koch's postulates is only possible if putative coral pathogens can be isolated and cultured (Work et al. 2008c). In the cases where they cannot be cultured, modified versions of Koch's postulates that retain the same strict standards have to be developed (Work et al. 2008c).

In addition, further development of modelling techniques to unravel the complex interactions between the host, pathogen and environment is urgently needed to fully understand disease dynamics and implement efficient management tools.

The information on PBTL gathered in this study provides a baseline for future research on this disease, which can utilize newly developing techniques to further investigate promising areas of research. For example, the observation of a potential increase in VLPs in PBTL-affected tissues should be further investigated using metagenomics to increase the chance of detecting a potential shift in the viral community. The ultimate goal of any disease investigation is to determine the causative agent of a disease to be able to fully understand the ecology and spread of the disease; further research into the causation of PBTL is certainly needed to be able to fully unravel disease dynamics and provide constructive guidelines for reef managers.

6.5 Conclusion

In contrast to many other documented coral diseases PBTL manifests as a unique bleaching response of the coral colony followed by partial tissue loss. However it does not manifest as progressive disease lesion as shown in most other tissue loss diseases (e.g. black band

disease, white syndromes). In general, diseases that cause tissue loss should be regarded as potential threat to the reef system as outbreaks can result in major colony mortalities. At this point, PBTL may be restricted to Kaneohe Bay and no outbreaks have been documented so the potential effect this disease could have on Hawaiian reefs as a whole is likely negligible (at least at present). If however, PBTL is found in other areas, which will likely be areas that have similar conditions to Kaneohe Bay such as Hilo Bay, and/or if it will spread outside the bay, then PBTL could be a threat to *Porites compressa* populations in Hawaii due to its capacity to cause recurring tissue loss as well as a reduction in gamete development which could reduce the number of recruits on the reefs.

Additionally, decreasing anthropogenic stressors such as pollution, sedimentation and overfishing will increase reef resilience and help corals withstand disease outbreaks. During times of high rainfall, Kaneohe Bay still experiences occasional sewage influx as well as increased sedimentation and nutrient input from the land. If these stressors could be reduced or even eliminated, then the potential for disease outbreaks of PBTL and other diseases, such as MWS, would likely decrease.

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Appendix I

Sampling of *Porites compressa* colonies

I.1 Chapter 2 and Chapter 3

For the histological studies and TEM study, one sample was taken from the PBTL-affected area of a diseased colony and one sample was taken from a similar area of a healthy neighbouring colony of similar size (Fig. I.1).

I.2 Chapter 4

For the disease transmission study, one sample was taken from the PBTL-affected area of a diseased colony and four samples were taken from a healthy neighbouring colony of similar size. In addition, one sample was taken from another healthy neighbouring colony (Fig. I.2). See Appendix VII for experimental set-up.

For the disease progression study, two samples were taken from the PBTL-affected area of a diseased colony, four samples were taken from apparently healthy tissue of the same PBTL-affected colony and six samples were taken from a healthy neighbouring colony of similar size (Fig. I.3).

I.3 Chapter 5 and Appendix IV

For the host and symbiont genetics study, one sample was taken from the PBTL-affected area of a diseased colony, one sample was taken from apparently healthy tissue of the same PBTL-affected colony and one sample was taken from a healthy neighbouring colony of similar size (Fig. I.4). The same sampling design was used for the microbial study (Appendix IV), but instead of one sample, paired samples were taken from these three areas.

See Table I.1 for the number of samples taken for each study and the total number of colonies sampled. In some cases, the same samples were used for different studies (i.e. for histology: Chapter 2 and 3, and for host and symbiont genetics: Chapter 5).

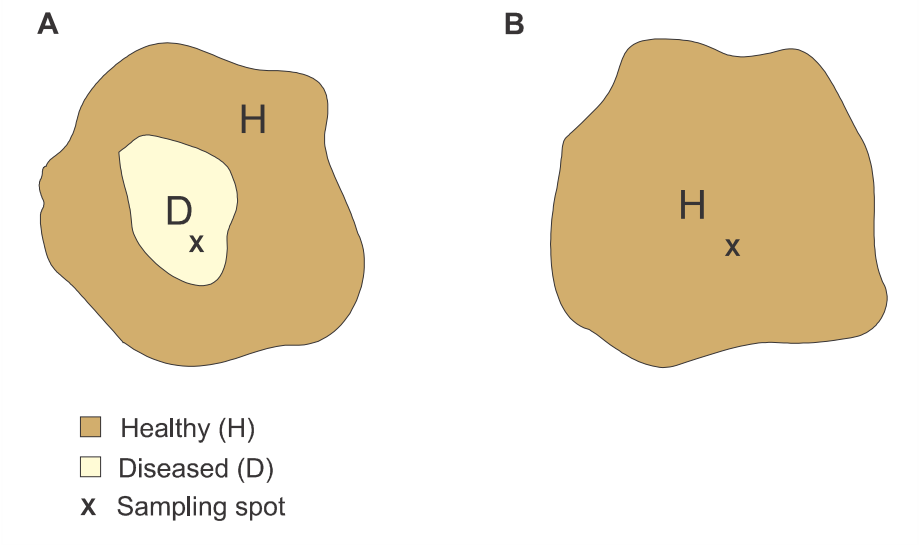

Fig. I.1: Sampling design for Chapter 2 (reproduction study) and Chapter 3 (histology and transmission electron microscopy). A: PBTL-affected *P. compressa*. B: healthy *P. compressa*.

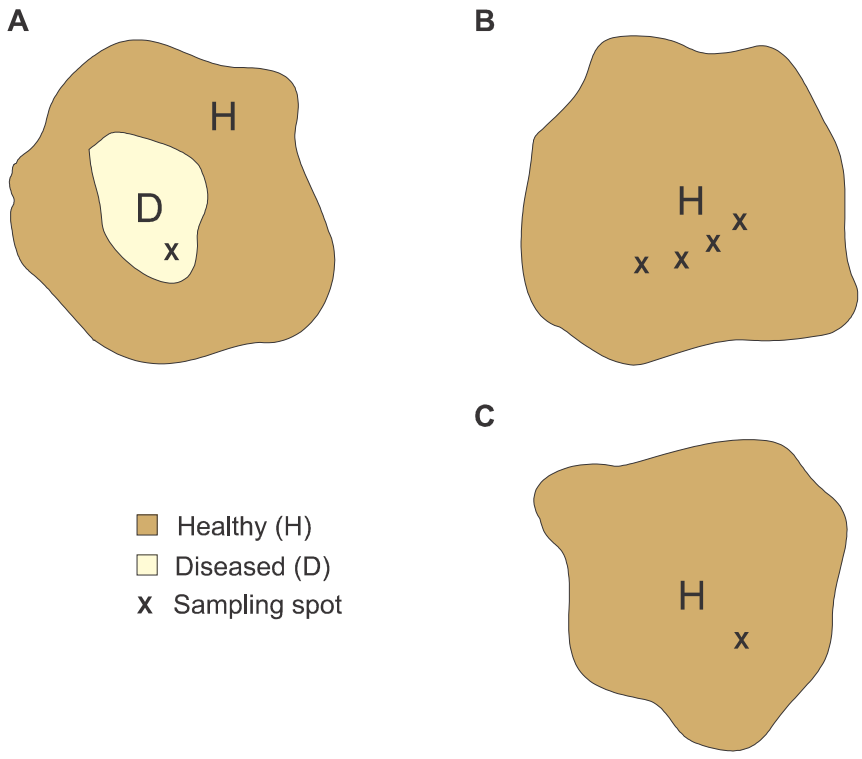

Fig. I.2: Sampling design for Chapter 4 (transmission). A: PBTL-affected *P. compressa*. B: healthy *P. compressa*. C: healthy *P. compressa*.

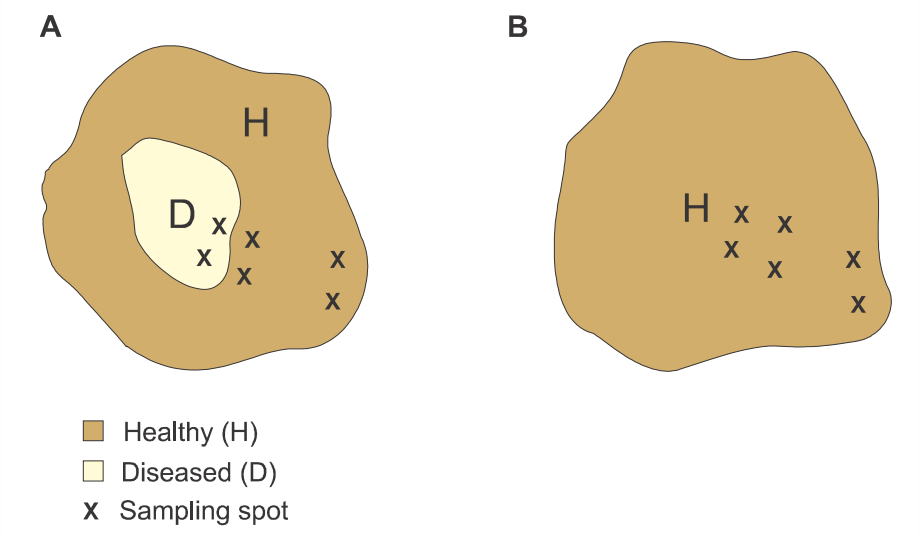

Fig. I.3: Sampling design for Chapter 4 (disease progression). A: PBTL-affected *P. compressa*. B: healthy *P. compressa*.

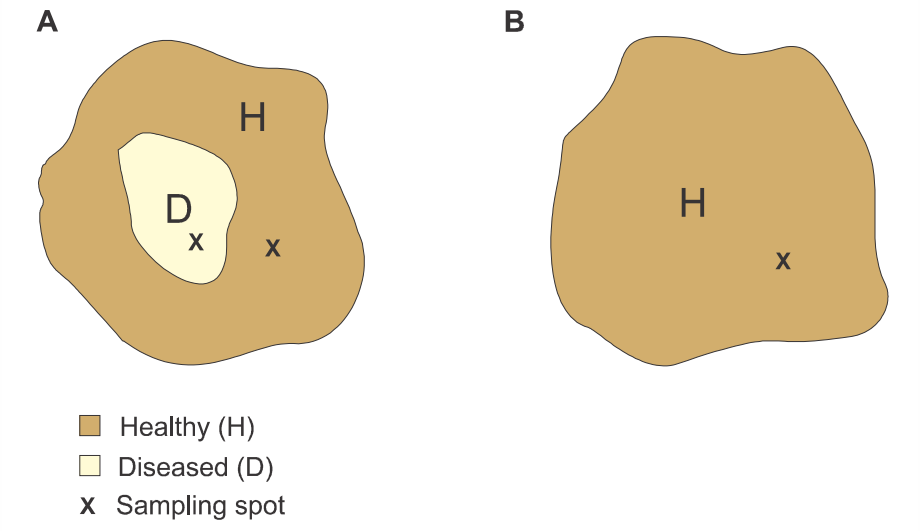

Fig. I.4: Sampling design for Chapter 5 (*Symbiodinium* and host genetics). A: PBTL-affected *P. compressa*. B: healthy *P. compressa*

Table I.1: The total number of samples taken from healthy and PBTL-affected colonies and the total number of colonies sampled for all manipulative experiments and other studies. All samples were <3 cm^2.

	Number of samples		Number of colonies	
	Healthy	PBTL-affected	Healthy	PBTL-affected
Histology/gametes (Chapter 2)	22	31	22	31
Histology (Chapter 3)	27	36	27	36
TEM (Chapter 3)	4	4	4	4
Transmission (Chapter 4)	100	20	40	20
Progression (Chapter 4)	36	12/ 24*	6	6
Symbiodinium genetics (Chapter 5)	30	30 / 30*	30	30
Host genetics (Chapter 5)	30	30	30	30
Bacterial abundance (Appendix IV)	46	46/46*	13	13

* from apparently healthy tissue of a PBTL-affected colony

Appendix II

Histopathology protocol

II.1. Solutions

II.1.1 Decalcification solution:

50 ml concentrated hydrochloric acid

1.95 L deionised water

2 g EDTA (tetrasodium salt)

2 g EDTA were dissolved in 1.95 L of deionised water and 50 ml of hydrochloric acid carefully added under a fume hood. The solution was then mixed gently. Solutions were made up fresh as needed.

II.1.2 Staining solutions:

II.1.2.1 Hermatoxilyn and eosin:

1% acid alcohol solution

380 ml of 80% ethanol

3.8 ml concentrated hydrochloric acid

Ammonium hydroxide solution

400 ml deionised water

0.5 ml concentrated ammonium hydroxide

Ehrlich's hematoxylin and eosin

These two solutions were prepared by Alan Hoverd and kindly made available to me.

II.1.2.2 Modified Heidenhain's azocarmine-aniline blue:

1% aniline-alcohol solution

1 ml aniline

100 ml 80% ethanol

1% acetic alcohol solution

1 ml glacial acetic acid

100 ml 95% ethanol

1% aqueous acetic acid solution

400 ml distilled water

4 ml glacial acetic acid

5% phosphotungstic acid solution

5 g phosphotungstic acid

distilled water to volume of 100 ml

aniline blue solution

0.5 g aniline blue, water soluble

2 g orange G

300 ml distilled water

8 ml glacial acetic acid

1 percent azocarmine G solution

1 g azocarmine G

100 ml distilled water

1 ml glacial acetic acid

The azocarmine G was mixed with the water and the solution was brought to boil, cooled to 56ºC and filtered through coarse filter paper (11 µm). After the solution had cooled completely, 1ml glacial acetic acid was added and the solution kept in the fridge until further use. Before each use, the solution was filtered through coarse filter paper (11 µm) and then heated to 60 ºC, and kept at this temperature until staining.

II.2 Decalcification:

Coral samples were placed into 50 ml Falcon tubes containing the decalcification solution with the bare skeleton facing upwards. In addition, a small piece of tissue was placed in the

top of the tube and the lid screwed on loosely to allow CO_2 bubbles to escape. The decalcification solution was changed twice daily until the skeleton was completely decalcified. This process could take up to two weeks, depending on the thickness/size of the sample. After decalcification was complete, the tissue was removed from the solution, briefly rinsed in tap water and placed in a 100 ml beaker containing deionised water for 24 hours to remove any remaining acid. The deionised water was changed at least once during the 24-hour period. The samples were then stored in 70% ethanol until further processing.

II.3 Embedding

The tissue samples were placed on a dissection board and trimmed into several pieces with a sharp scalpel. The sections were placed into perforated plastic processing cassettes and the tissue was dehydrated in several changes of ethanol and then cleared with xylene. After dehydration the embedding cassettes were placed in three changes of paraffin. For this step, a vacuum oven was used to aid the removal of xylene and any air bubbles trapped in the tissue. Please refer to Table II.1 for details.

Table II.1: Reagents and associated time used for tissue dehydration.

Beaker	Reagent	Time (in minutes)
1	80% Ethanol	30
2	95% Ethanol	30
3	100% Ethanol	30
4	100% Ethanol	30
5	½ Ethanol ½ Xylene	30
6	Xylene	30
7	Xylene	30
8	Xylene	30
9	Paraffin*	25 - 30
10	Paraffin*	25 - 30
11	Paraffin*	25 - 30

*Paraplast: melting point 56-60°C. Used for 5-10 mins with vacuum.

The embedding cassettes were placed into a dish filled with paraffin and kept warm on a hotplate. L-shaped brass moulds were fitted and filled with fresh molten paraffin. Using warm forceps, the tissue samples were transferred from the cassette into the mould. The mould was left overnight to ensure complete cooling and hardening of the paraffin block. Then the paraffin blocks were removed from the mould and mounted onto a wooden block by gently warming the underside of the paraffin block with a hot scalpel blade, gluing the two surfaces together.

II.4. Sectioning

A water bath was filled with deionised water and heated to about 30-40°C. The paraffin blocks were placed into the microtome and 6-µm thick tissue sections were cut using a stainless steel-knife. Suitable tissue sections were chosen from the resulting ribbon, gently placed onto the surface of the warm water bath to relax them and then carefully picked up using a 'superfrost' slide. Three to four tissue sections were placed onto one slide. The slides were briefly drained, then left to dry for two days and stored until staining.

II.5. Staining

The paraffin was removed from the tissue by placing the slides into three changes of xylene. To remove the xylene the slides were placed into three changes of absolute ethanol. To re-hydrate the tissue, the slides were placed in 95% and 70% ethanol (H&E) or 80% ethanol (aniline blue) followed by deionised water.

II.5.1 H&E

The slides were placed in Ehrlich's hematoxylin and the excess stain was washed out by placing the slides in a sink with flowing tap water. To differentiate the hematoxylin, the slides were dipped into 1% acid alcohol and subsequently placed into deionised water. Next, the slides were placed in ammonium hydroxide solution until the tissue turned blue. To remove excess solution, the slides were again placed into a sink with running tap water and then into deionised water. To prepare the slides for the eosin solution they were placed into 95% ethanol and then stained in eosin. To differentiate the acidophilic components and remove excess stain, the slides were placed in two changes of 95% ethanol. To stop the differentiation, the slides were placed in 100% ethanol then cleared with xylene. Finally, a coverslip was applied using DPX mounting medium. Refer to Table II.2 for details.

Table II.2: Staining protocol for hematoxylin and eosin.

Beaker	Reagent	Time (minutes each change)
1	Xylene (3 changes)	3
2	100% Ethanol (3 changes)	2
3	95% Ethanol	2
4	70 % Ethanol	2
5	Deionised water	2-5
6	Ehrlich's hematoxilyn	2
7	Running tap water	3
8	1% acid alcohol	1-2 seconds
9	Deionised water	2-5
10	Ammonium hydroxide	1
11	Running tap water	3
12	Deionised water	2-5
13	95% Ethanol	2
14	Eosin	1 ½
15	95% Ethanol	2
16	Fresh 95% Ethanol	2-5
17	100% Ethanol (3 changes)	3
18	Xylene (3 changes)	3

II.5.2 Aniline blue

The slides were placed in the preheated azocarmine G solution at 56 ºC and then rinsed in 1% aqueous acetic acid to remove the excess stain. After rinsing the slides in distilled water they were differentiated in aniline-alcohol solution and soaked in phosphotungstic acid solution, followed by another rinse in deionised water. The slides were then placed in aniline blue solution and rinsed again in deionised water to remove the excess stain. Tissues were dehydrated in ethanol, cleared with xylene and a coverslip applied using DPX mounting medium. See Table II.3 for details.

Table II.3: Protocol for aniline blue stain.

Beaker	Reagent	Time (minutes each change)
1	Xylene (3 changes)	3
2	100% Ethanol (3 changes)	2
3	95% Ethanol	2
4	80 % Ethanol	2
5	Deionised water	2-5
6	Ehrlich’s hematoxilyn	2
7	Azocamine G	15
8	Phosphotungstic acid	15
9	Deionised water	2
10	Aniline blue	15
11	Deionised water (3 changes)	2
12	95% Ethanol (2 changes)	2
17	100% Ethanol (3 changes)	3
18	Xylene (3 changes)	3

Appendix III

Supplementary histology images

III. 1 H&E

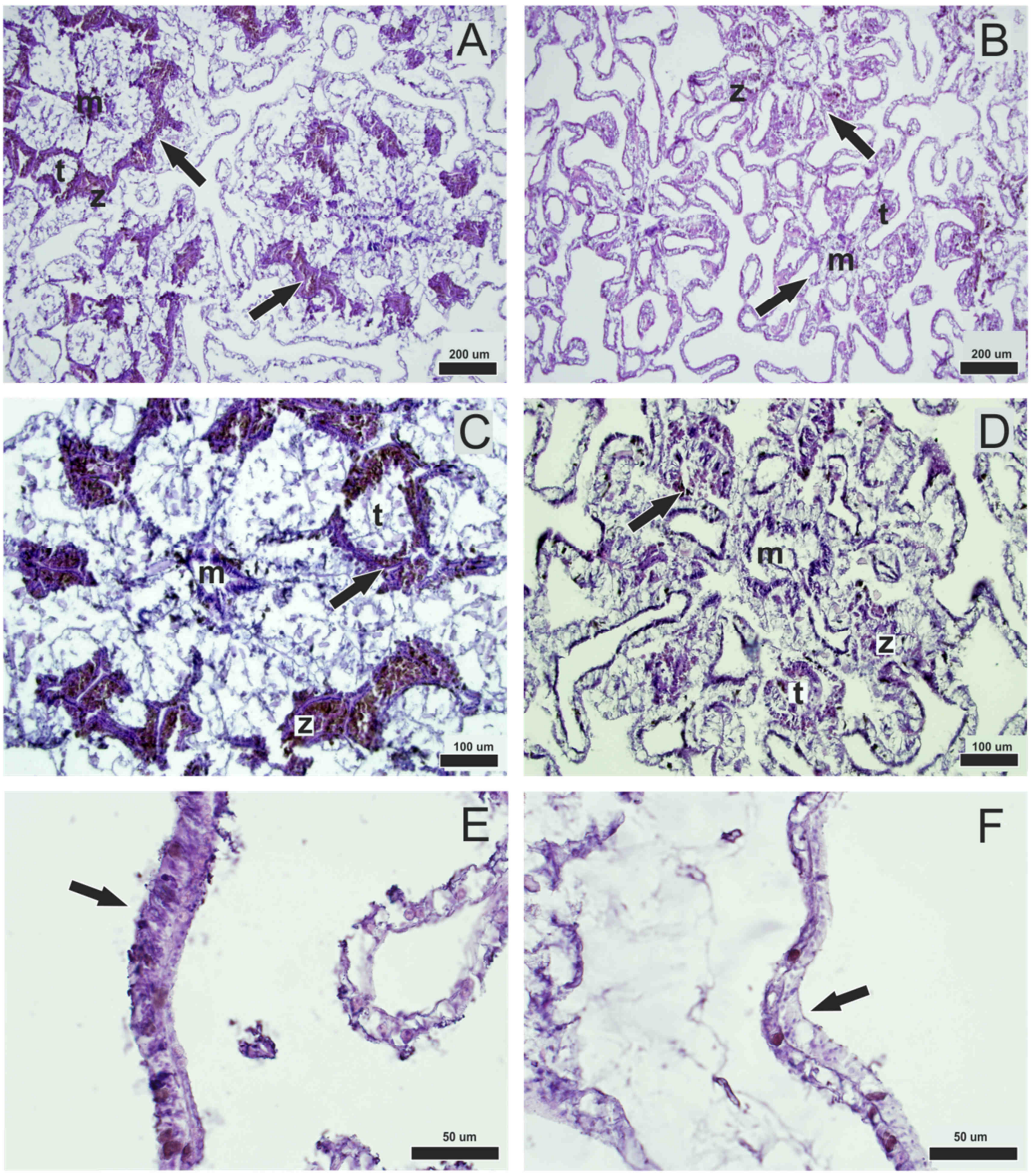

Fig III.1: *Porites compressa* stained with H&E. Oral orientation. A & C: Healthy polyp showing high densities of *Symbiodinium* cells (arrowhead). B & D: PBTL-affected polyp showing lower densities of *Symbiodinium* cells (arrowhead). E: epidermis of healthy sample showing high densities of *Symbiodinium* cells and melanin-containing granular cells. F: epidermis of PBTL-affected sample showing lower densities of *Symbiodinium* cells and melanin-containing granular cells. m = mouth, t = tentacle, z = zooxanthellae.

III.2. Aniline blue

III.2.1 Introduction

A modified version of Heidenhain's azocarmine-aniline blue method (see Appendix I.5.2) was attempted for a comparison of the condition of *Symbiodinium* cells. Healthy whole algal cells are supposed to stain a different colour than degrading cells or debris:

Mesogloea: stain bright blue (from the aniline blue)

Nuclei and granular cells: stain orange to red

Symbiodinium: healthy cells stain reddish; degrading cells or debris stain green

Mucus: often stains light blue

III.2.2 Methods

See Appendix II

III.2.3 Results

The stain did not appear to work accurately because *Symbiodinium* cells in both healthy and PBTL-affected samples either stained mostly green with occasional red or did not stain at all (brown). It is unknown why the stain did not work accurately because the methodology was used previously and described by experienced coral pathologists, such as Ester Peters (Histotechniques and Histology of the Anthozoans Workshop, HIMB 2004).

It was expected to find mostly red *Symbiodinium* cells in healthy samples, but because the majority did not stain at all (Fig. III.2 A-D) no conclusions could be drawn about possible differences in *Symbiodinium* cell integrity between healthy and PBTL-affected samples.

Samples stained with the aniline blue method also showed tissue debris in PBTL-affected samples (Fig. III.2 E & F) as observed with the H&E stain.

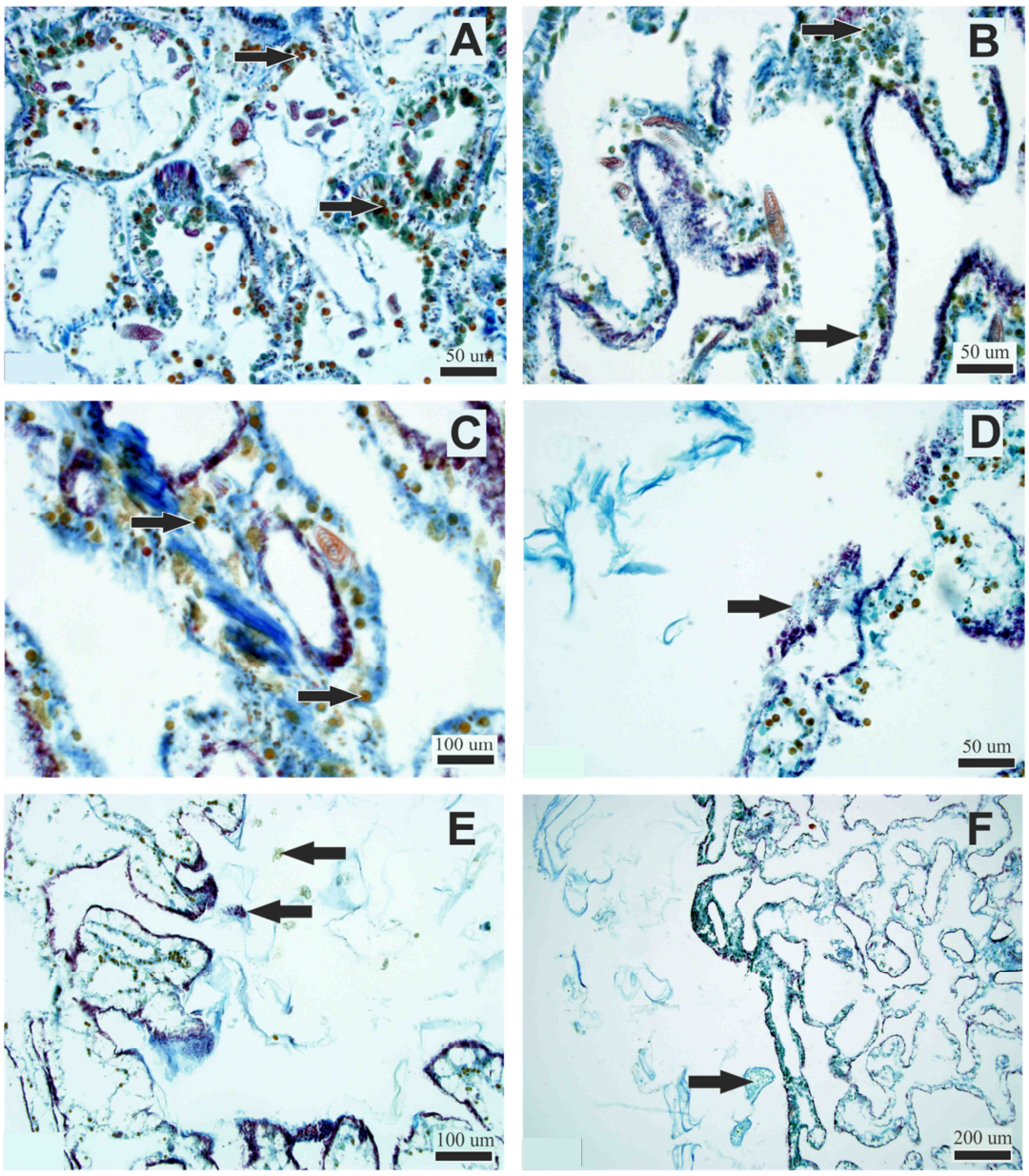

Fig. III.2: *Porites compressa* stained with aniline blue. A: Healthy *P. compressa*. Note unstained, brown *Symbiodinium* cells (arrowhead). B: PBTL-affected *P. compressa*. Note mostly green *Symbiodinium* cells with occasional brown, unstained cells (arrowhead). C: PBTL-affected *P. compressa*. Note mostly brown, unstained *Symbiodinium* cells (arrowhead). D: PBTL-affected *P. compressa*. Note effaced epidermis (arrowhead) and unstained (brown) *Symbiodinium* cells. E: PBTL-affected *P. compressa*. Note mucus with cell debris (arrowhead). F: PBTL-affected *P. compressa*. Note mucus and cell debris (arrowhead).

Appendix IV

Microbial comparison of healthy and PBTL-affected samples

IV.1 Introduction

If a microorganism can be isolated in pure culture, scientists can test a variety of physiological and biochemical traits to identify the organism (Amann et al. 1995). Even though only a fraction of the existing microorganisms are culturable species (Amann et al. 1995), maintaining pure pathogen cultures is of great advantage if one wants to fulfil Koch's postulates (Koch 1891) or study pathogen virulence under varying conditions. In addition, using growth media to isolate putative pathogens is a very cost-efficient method. Nevertheless, to fully characterize microbial assemblages and determine microbial changes due to disease, non-culture-based techniques, such as sequencing and metagenomics, are of great interest. As these techniques are very expensive, however, culture-based techniques often provide an adequate start in investigating potential microbial pathogens.

The causative agent of several coral diseases has been identified as bacteria (Kushmaro et al. 1996; Ritchie and Smith 1998; Patterson et al. 2002; Denner et al. 2003; Casas et al. 2004; Sussman et al. 2008), many of which belong to the *Vibrio* family (Ritchie and Smith 1998; Kushmaro et al. 2001; Sussman et al. 2008). *Vibrio* spp. have been shown to infect various marine organisms (Bullock 1977; Saulnier et al. 2000; Romalde and Barja 2010) causing primary or secondary/opportunistic infections (Saulnier et al. 2000).

To investigate if bacteria may be involved in the causation of PBTL, culture-based techniques were used to explore possible differences in the bacterial abundance of healthy and PBTL-affected colonies. Specifically I investigated: 1) general bacterial numbers; and 2) number of *Vibrio* spp.

IV.2 Methods

IV.2.1 Sampling

Small (2 cm^2), paired samples of healthy colonies and PBTL-affected colonies (areas showing disease signs and neighbouring apparently healthy areas) were sampled around CIMR in June 2010 (see Appendix I.3 for sample design). Samples were immediately returned to the lab in individual, re-sealable plastic bags and processed within one hour of collection.

IV.2.2 Mucus collection

One half of the paired fragments was used for mucus collection. Mucus was pipetted off the coral fragment and transferred into sterile 1 ml Eppendorf tubes. Approximately 0.5 – 1 ml mucus was collected from each fragment.

IV.2.3 Coral tissue slurry

The other half of the paired fragments was used to prepare a tissue slurry. The coral fragment was weighed and then crushed in a sterile mortar with 20 ml of sterile filtered seawater. The slurry was transferred into a sterile 50-ml Falcon tube and left to settle for 10 minutes, so that heavier particles (e.g. skeleton) could sink to the bottom of the tube. Because the tissue slurry was very thick, only the top layer was used for subsequent culture attempts.

IV.2.4 Plating

Dilutions (10^{-1}, 10^{-2} and 10^{-3}), using sterile filtered seawater, were prepared from the tissue slurry and mucus samples. Each dilution (undiluted, 10^{-1}, 10^{-2} and 10^{-3}) was plated on Glycerol Artificial Seawater media (GASW) and *Thiosulfate Citrate Bile Salts* sucrose agar (TCBS) (three replicates each) using 50 µl of sample and kept in a dark incubator (37 ºC) for 2-3 days. GASW is a growth medium that allows most bacteria to grow, whereas TCBS is highly selective for *Vibrio* spp.

The remaining samples of undiluted mucus and slurry were split in half, and one half was frozen with glycerol (to ensure survival of bacteria for possible experiments) and the other half was frozen without any additions (for possible sequencing).

IV.2.5 Bacterial counts

All colony forming units (CFUs) were counted on each plate. The average number of CFUs from the three replicate plates for each sample was used in statistical analyses.

From each plate, a number of CFUs were chosen haphazardly and transferred onto patch plates (GASW) to create a library of pure culture bacteria. The patch plates were placed in the incubator for 2 days and the individual CFU's were then transferred into tubes containing liquid GASW growth medium. The tubes were placed on the shaker for 2 days or until the medium turned cloudy. One half of the medium was frozen with glycerol and the other half

without additions for possible future experiments. In total >1000 pure culture bacteria were frozen.

IV.3 Statistical analyses

Because the dilutions, for both slurry and mucus, did not show much bacterial growth (CFUs), only the undiluted samples were used for analyses.

The assumptions of normality and equal variance were not met, therefore a Kruskal-Wallis test was performed using SPSS (PASW 18) to test for differences in numbers of CFUs between healthy, apparently healthy and diseased samples of mucus (n = 13) and slurry (n = 10).

IV.4 Results

Overall, tissue slurry had significantly higher bacterial numbers than mucus (χ^2 = 6.040, df = 1, p = 0.014) (Fig. IV.1). Fig IV.2A shows a slight increase in bacterial numbers in the mucus of diseased areas of the coral, however no significant difference was found in the number of CFUs between healthy, apparently healthy and diseased samples for mucus and tissue slurry (χ^2 = 1.753, df = 2, p = 0.416 and χ^2 = 0.637, df = 2, p = 0.727, respectively) (see also Fig. IV.2B).

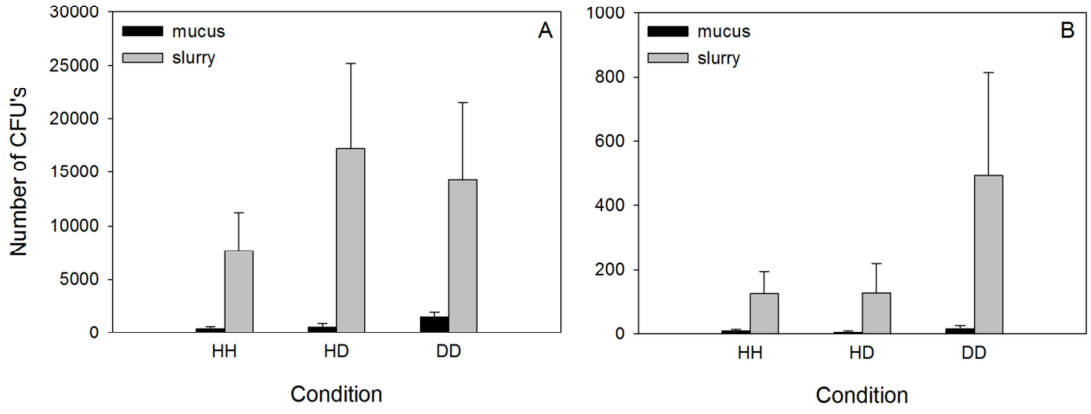

Fig. IV.1: Average number of colony forming units (CFU) (± SE) in mucus and tissue slurry from healthy (HH = healthy healthy), apparently healthy parts of PBTL-affected (HD = healthy diseased), and diseased parts of PBTL affected (DD = diseased diseased) colonies. A: GASW B: TCBS

Overall, there was no significant differences between the number of *Vibrio* cells in the mucus and slurry (χ^2 = 0.626, df = 1, p = 0.429) (Fig. IV.1B). There was also no significant differences between the number of *Vibrio* cells in healthy, apparently healthy and diseased samples, either in the mucus or slurry (χ^2 = 4.082, df = 2, p = 0.130 and χ^2 = 0.003, df = 2, p = 0.998, respectively) (see also Fig. IV.2C & D).

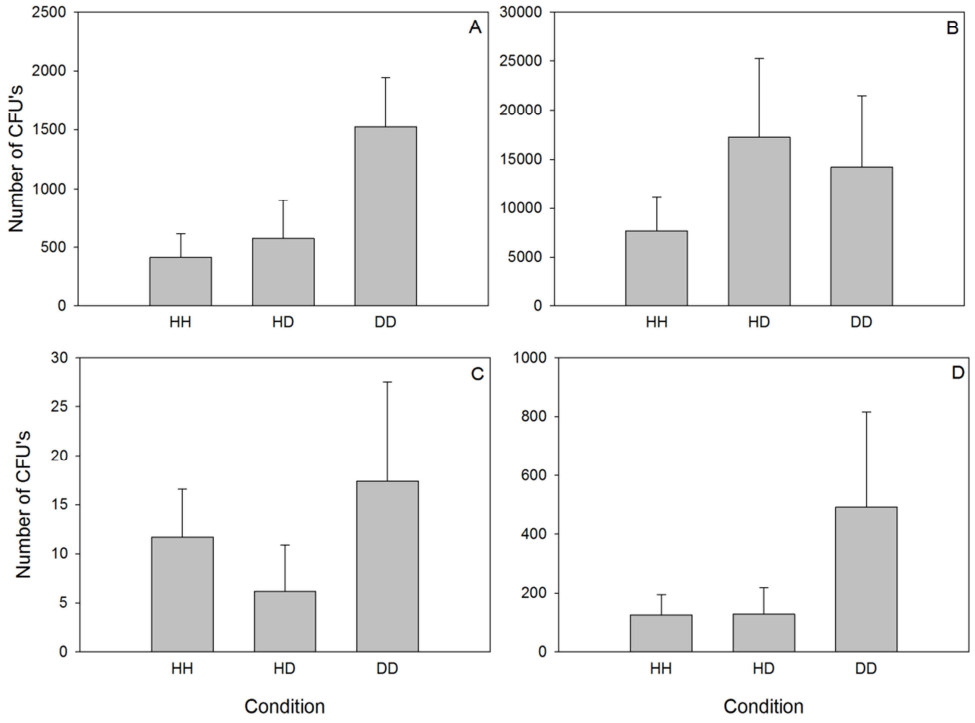

Fig. IV.2: Average number of colony forming units (CFU) (± SE) in mucus and tissue slurry from healthy (HH), apparently healthy parts of PBTL-affected (HD), and diseased parts of PBTL-affected (DD) colonies. A: mucus GASW B: slurry GASW C: mucus TCBS D: slurry TCBS

IV.5 Discussion

No significant difference was found in bacterial abundances (both broad-spectrum and *Vibrio* spp.) between healthy and PBTL-affected colonies, suggesting that bacteria are not the cause of PBTL. In contrast, an analysis of the microbial community associated with mucus from GAs in *P. compressa* from Hawaii revealed more culturable *Vibrio* spp. from GAs than healthy colonies (Breitbart et al. 2005). However, as many bacteria are not culturable, further investigations into the microbial consortium of healthy and PBTL-affected samples, using non-culture based techniques, are needed to clarify the role of bacteria in this disease.

Appendix V

Supplementary material for Chapter 4

V.1 Benthic cover

P. compressa is the dominant coral on the reefs around CIMR (52.6%), followed by *M. capitata* (21.2%); *P. damicornis* abundance is low (0.2%). Rubble and dead coral skeleton are low to medium at individual sites, and 8.2% and 9.4%, respectively, for CIMR overall. Both, algal and sponge cover are similar at the individual sites, and very low overall (<1%) (Table V.1). Individual sites differ, however, in their coral species composition. For example, at site D *M. capitata* cover is higher than *P. compressa* cover, and site J has roughly equal cover of both species. All other sites are dominated by *P. compressa* (Fig. V1).

Table V.1: Benthic cover (%) at all eight sites around CIMR and overall average cover for CIMR (± SE). PC = *Porites compressa*, MC = *Montipora capitata*, PD = *Pocillopora damicornis*, dead = dead coral skeleton, rub = coral skeleton rubble, sil = silt/sand, Sp = sponge, al = algae

site	PC	MC	PD	Dead	Rub	Sil	Sp	Al
A	75.6	7.6	0	8.8	4.6	2.6	0.1	0.3
B	64.3	13.9	0.2	12.6	6.4	2.0	0.1	0.3
C	40.6	29.6	0.5	9.9	12.5	6.7	0.1	0.1
D	30.3	47.1	0.1	10.0	8.1	4.1	0.2	0.2
G	52.1	12.8	0.1	8.4	12.4	12.9	0.9	0.5
H	60.1	17.4	0.4	7.2	5.9	8.9	0.1	0.2
I	68.1	11.8	0.1	7.6	3.1	8.2	0.2	0.9
J	30.0	29.6	0.5	10.5	12.3	15.4	0.7	0.8
CIMR	52.6 ± 6.1	21.2 ± 4.7	0.2 ± 0.1	9.4 ± 0.6	8.2 ± 1.3	7.6 ± 1.7	0.3 ± 0.1	0.4 ± 0.1

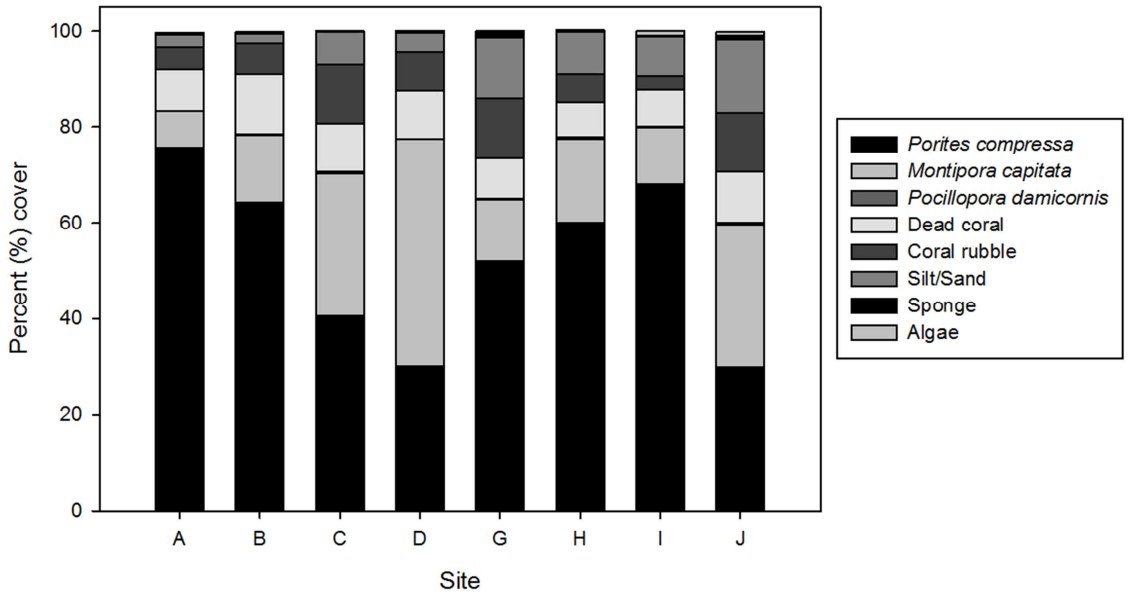

Fig. V1: Benthic composition (% cover) at all eight sites around CIMR.

Table V.2: Summary of Table V.1 showing overall coral cover (all species combined), overall amount of substrate (dead coral, rubble and silt combined) and other organisms (sponges and algae) for each site and overall average (± SE) for CIMR.

site	Coral cover	Substrate	Other organisms
A	83.14	16.01	0.42
B	78.43	21.01	0.39
C	70.76	29.06	0.21
D	77.45	22.12	0.42
G	65.02	33.64	1.35
H	77.83	21.88	0.28
I	80.01	18.86	1.13
J	60.13	38.24	1.48
CIMR	74.1 ± 2.8	25.1 ± 2.7	0.7 ± 0.2

Overall, coral cover is very high around CIMR (74%) and it varies between 60 – 83% at individual sites. Substrate, such as sand and rubble, makes up most of the rest of the reef, with other reef organisms (sponges and algae) represented with < 1% (Table V.2 and Fig. V2).

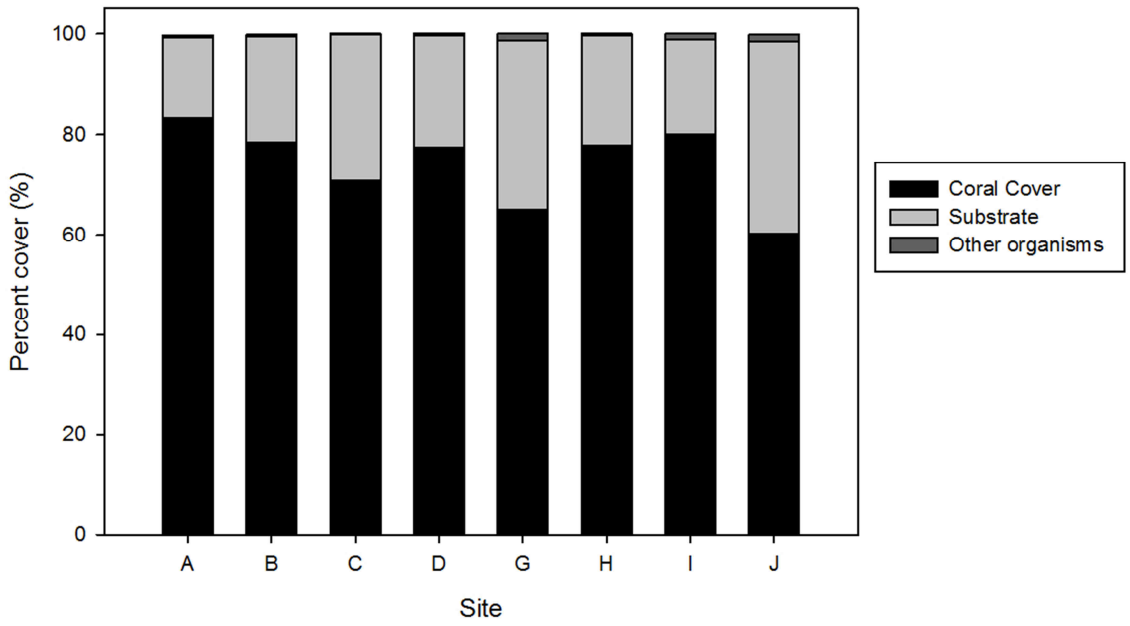

Fig. V2: Summary of Table V.1 showing overall coral cover (all species combined), overall amount of substrate (dead coral, rubble and silt combined) and other organisms (sponges and algae) for each site.

V.2 Transmission experiment

Fig. V.3 shows the treatment set-up for the transmission experiment described in Chapter 4.

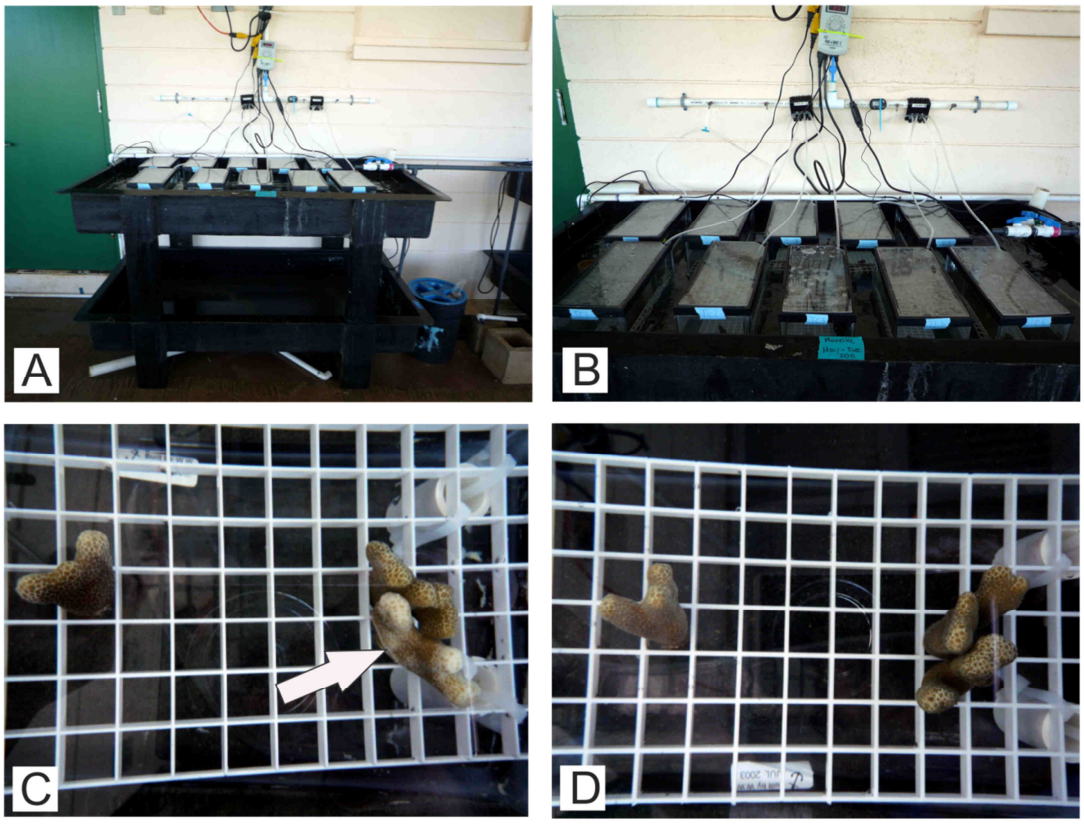

Fig. V.3: Set-up of transmission experiment. A&B: Individual aquaria placed inside a water bath (water table) of either ambient or increased water temperature. C: Treatment aquarium with 2 healthy *P. compressa* fragments from the same colony, one of which is touching a PBTL-affected fragment (arrowhead) from a different colony. D: Control aquarium with 2 healthy *P. compressa* fragments from the same colony, one of which is touching another healthy fragment from a different *P. compressa* colony.

V.3 Environmental predictors

Additional information on the environmental predictors used in Chapter 4:

Fig. V.4 shows the PCO plot used to choose either the mean or SD of the predictor, and Table V.3 and Fig. V.5 show mean and SD values of environmental predictors.

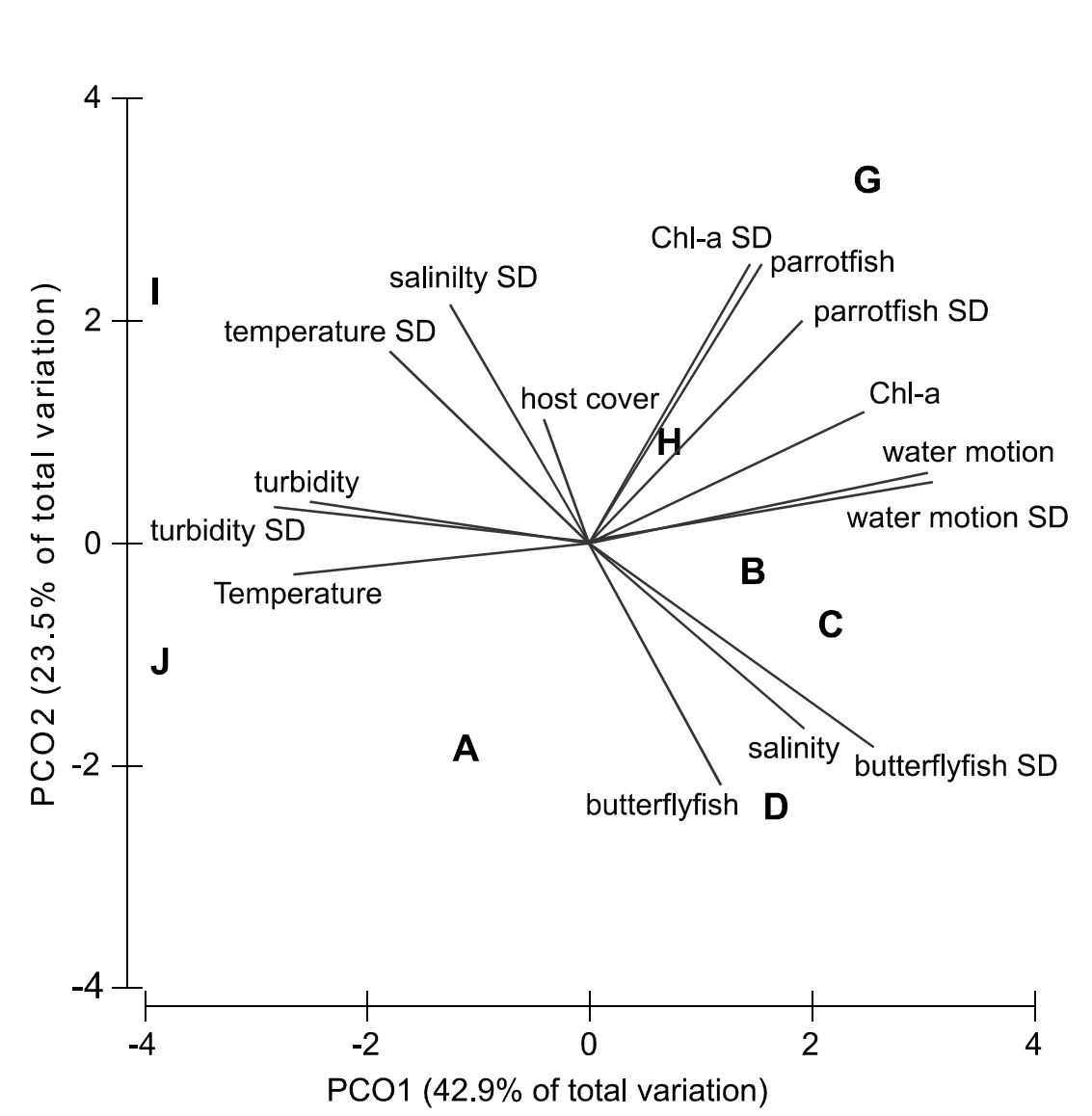

Fig V.4: Principal Coordinates (PCO) plot visualising the similarity of the different survey sites (A-J) and the correlated change in environmental conditions (means and SD).

Table V.3: Mean and SD for PBTL prevalence and the eight environmental variables collected for each site.

site	Prevalence (%)	Temperature (ºC)	Host cover (%)	Turbidity (FTU)	Chl-*a* (µg/l)	Water motion (DF)	Salinity (ppt)	Parrotfish density (200 m^2)	Butterflyfish density (200 m^2)
A	1.4 ± 1.1	25.6 ± 1.2	75.6	1.8 ± 1.6	0.5 ± 0.2	3.7 ± 1.2	35.1 ± 0.4	7.8 ± 11.0	5.0 ± 3.2
B	1.4 ± 0.7	25.6 ± 1.2	64.3	1.3 ± 0.8	0.6 ± 0.3	5.1 ± 1.6	34.9 ± 0.8	13.3 ± 11.0	9.0 ± 4.2
C	1.9 ± 1.2	25.4 ± 1.3	40.6	2.5 ± 1.4	0.7 ± 0.3	5.0 ± 1.6	35.4 ± 0.2	14.8 ± 17.5	6.0 ± 5.2
D	0.6 ± 0.3	25.5 ± 1.1	30.3	1.5 ± 0.7	0.6 ± 0.2	3.5 ± 1.2	35.2 ± 0.3	14.0 ± 10.2	5.5 ± 4.7
G	2.5 ± 0.9	25.5 ± 1.2	52.1	1.4 ± 0.9	0.7 ± 0.3	5.4 ± 1.9	35.0 ± 0.6	36.0 ± 32.9	2.5 ± 2.4
H	1.6 ± 1.4	25.5 ± 1.2	60.1	1.5 ± 1.5	0.6 ± 0.3	4.8 ± 1.7	35.1 ± 0.7	19.3 ± 9.7	4.0 ± 2.7
I	1.4 ± 1.2	25.6 ± 1.3	68.1	3.3 ± 2.0	0.6 ± 0.3	1.9 ± 0.5	34.8 ± 0.8	14.0 ± 10.0	0.5 ± 1.0
J	1.1 ± 0.7	25.8 ± 1.2	30.0	2.7 ± 1.9	0.5 ± 0.2	1.8 ± 0.4	34.9 ± 0.6	12.3 ± 6.6	5.8 ± 1.5

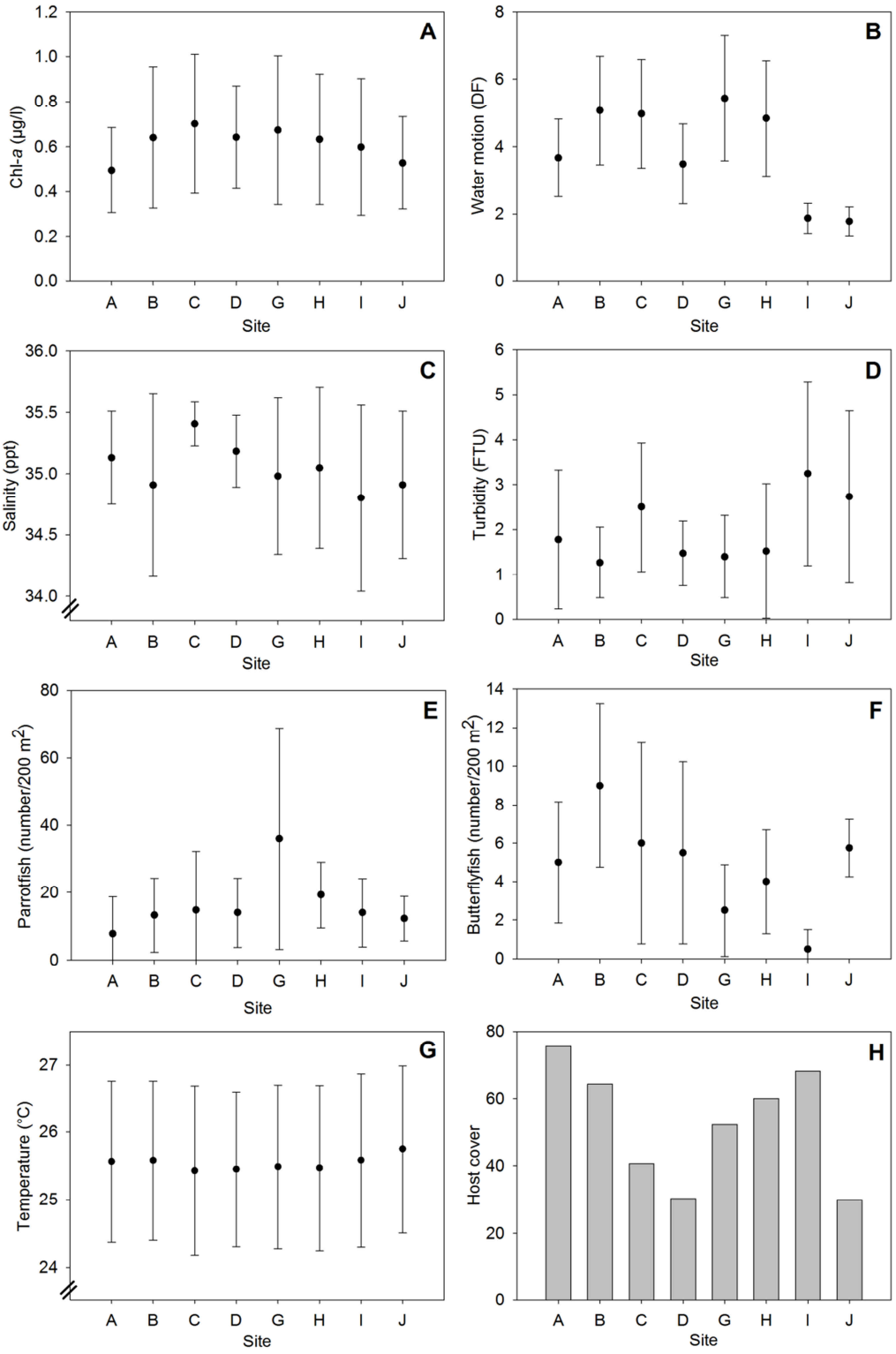

Fig. V.5: Means and SD for all spatial environmental predictors at each site. A: Chl-*a*; B: water motion; C: salinity; D: turbidity; E: parrotfish density; F: butterflyfish density; G: temperature; H: host cover (*Porites compressa*).

V.4 PBTL progression

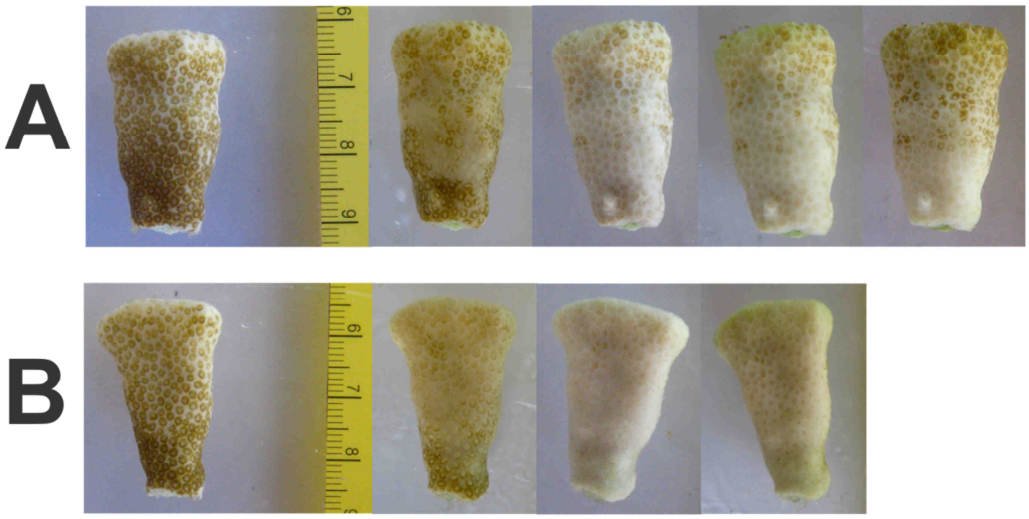

Fig V.6: Paired fragments of one PBTL-affected *P. compressa* colony. A: Fragment in ambient water temperature (24-25 ºC) photographed once a week after collection (first 4 photos) and the fifth photo shows the same fragment at the end of the experiment (approximately 2 months after collection). Note loss of pigmentation and tissue loss over time with the start of re-pigmentation at the end. B: Fragment in increased water temperature (27 ºC) photographed once a week after collection. Note the consistent and fast tissue loss with the fragment being completely dead by week 4.

Appendix VI

PBTL prevalence and temperature data from 2010

VI.1 Methods

Prevalence surveys were carried out around CIMR in May, August and October 2010. Eight permanent sites were surveyed using five 10 x 2 m belt transects with 5 m gaps in between transects. Every *P. compressa* colony that fell within the belt was counted and examined for signs of PBTL. Prevalence was calculated as: (number of PBTL affected colonies/total number of colonies) x 100, for each transect.

Temperature data were collected at each site using HOBO®Pro data loggers (www.onsetcomp.com) with an accuracy of ± 0.2 ºC. The loggers were placed at the depth of the transect and recorded continuously every 30 min from late May to early August and late October to early November 2010.

VI.2 Statistical analysis

As prevalence data did not meet the assumptions of normality and equal variance, a non-parametric repeated measures analysis (Friendman's test) was carried out in SPSS (PASW 18) to determine any differences in prevalence between survey sites (A-J) and survey months (May, August and October). A non-parametric Spearman correlation analysis was carried out in SPSS (PASW 18) to determine a possible correlation of temperature and prevalence.

VI.3 Results

PBTL prevalence differed at the individual sites (Fig VI.1A) and during different survey months (Fig VI.1B), with the highest prevalence observed in August. However, no significant difference in disease prevalence was found between the three months (χ^2 = 4.75, df = 2, p = 0.093) or the different survey sites (χ^2 = 12.778, df = 7, p = 0.078). The peak prevalence coincided with warmer water temperature during the summer (Fig VI.1B) and a significant correlation was found between water temperature and prevalence (Spearman r = 0.576, n = 24, p = 0.003) (Figure VI.2).

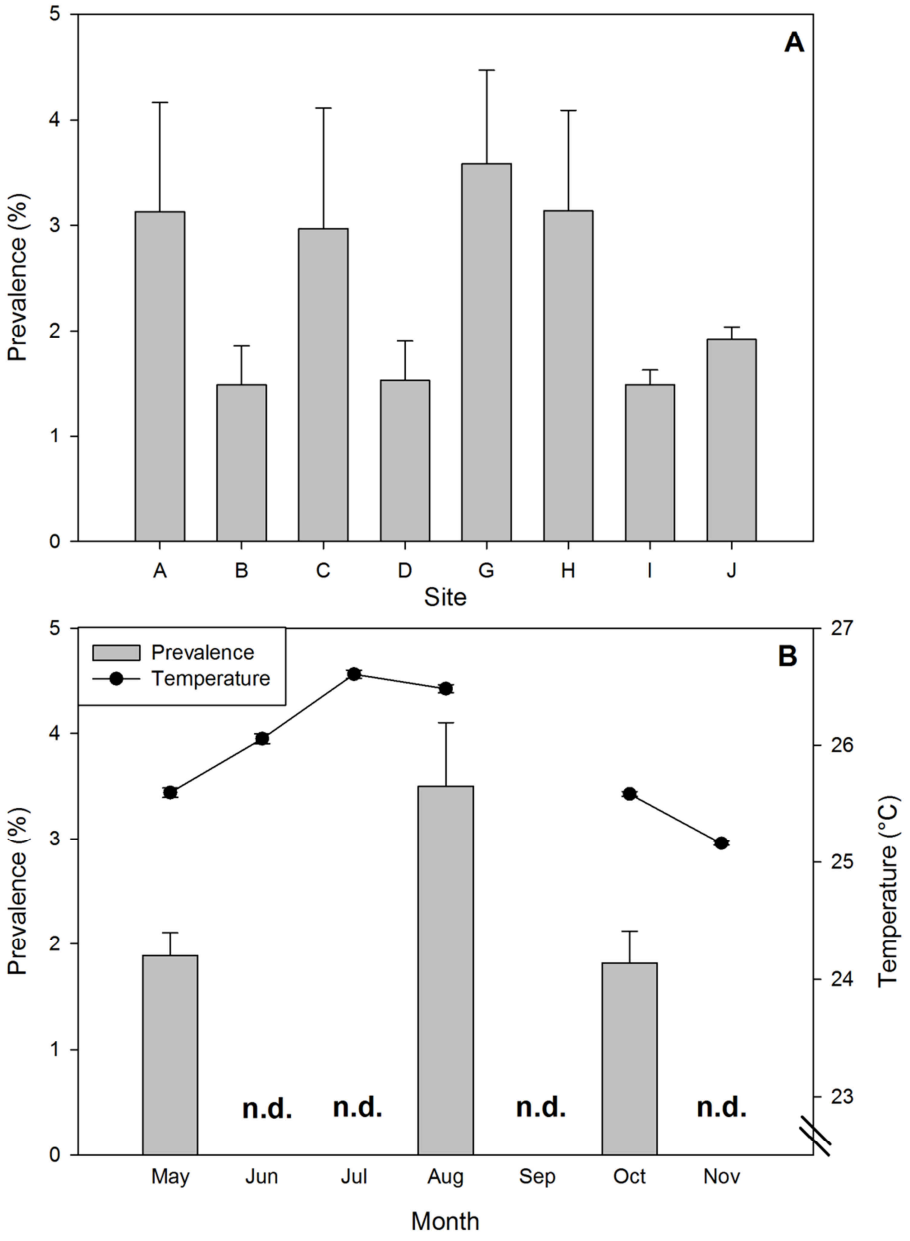

Fig. VI.1: PBTL prevalence data collected in 2010. A: Average prevalence (± SE) at each site around CIMR. B: Average prevalence and corresponding temperature for May, August and October 2010. n.d. = no data

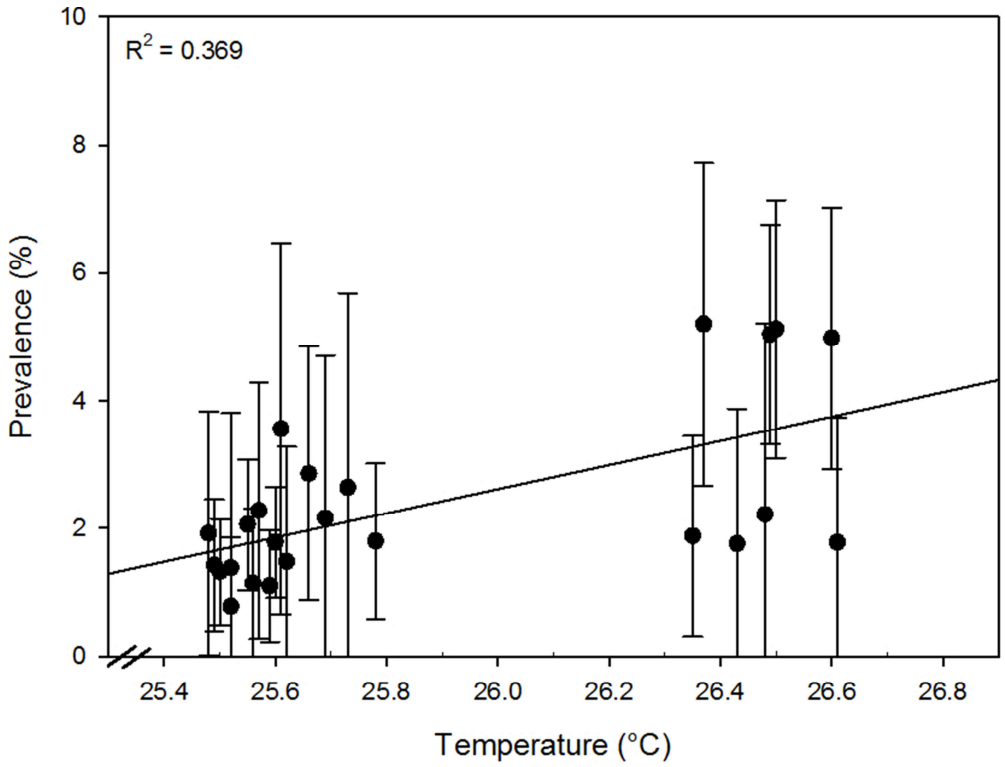

Fig VI.2: Relationship of temperature and mean PBTL prevalence ± SD for each site during May, August and October.

VI.4 Discussion

Higher water temperature appears to correlate with an increase in prevalence in 2010 (as it did in 2011). The non-significant results of differences in PBTL prevalence between survey sites and survey months were likely due to the small sample size and loss of power with the non-parametric test.

Appendix VII

Do changes in salinity cause the observed localized bleaching?

VII.1 Introduction and Method

Changes in salinity (due to high rainfall or freshwater seeps) can cause bleaching in corals (Coles 1992). To determine if changes in salinity (i.e. possible freshwater seeps) could be responsible for the observed bleaching (PBTL), water samples were taken with a 50 ml syringe from between the branches of PBTL-affected colonies (n = 15) and adjacent healthy neighbouring colonies (n = 15), and from the water column (n = 5). The samples were returned to the lab and immediately analysed for salinity using a refractometer (Aquatic Eco-System, Model RHS-10ATC). Three readings from each sample were taken.

VII.2 Data analysis

The three individual readings for each salinity sample were averaged and because the data did not meet the assumptions of normality, a Kruskal-Wallis test using SPSS (PASW Statistic 18) was carried out to test if salinity levels differed between water samples taken from the water column, and adjacent to healthy and diseased colonies.

VII.3 Results

No significant difference was found between salinity from water samples taken from the water column and adjacent to healthy and PBTL-affected colonies (χ^2 = 0.044, df = 2, p = 0.978). Water samples from the water column, adjacent to healthy colonies and PBTL-affected colonies had a mean (± SE) of 35.33±0.1‰, 35.36±0.1‰ and 35.36±0.1‰, respectively.

VII.4 Discussion

As no differences in salinity were found between water samples taken adjacent to healthy and diseased colonies, as well as from the water column it can be assumed that PBTL is not caused by freshwater seeps. A refractometer was used to measure salinity which is unlikely to have detected very small-scale changes in this parameter. However, Coles (1992) determined that *P. compressa* in Hawaii can withstand salinity levels of as low as 20 ppt (paling) but remains normally pigmented at 25 ppt. A drop in salinity to 20‰ would have been detected by this method.

Appendix VIII

Adjusted allele frequencies

Allele frequencies for the loci deviating from the HWE were regenerated (Fig. VIII.1) using MicroChecker and adjusted genotype frequencies were calculated from these allele frequencies. The regenerated genotype frequencies were then used to re-calculate F_{ST} and R_{ST} values with non-interpolated and interpolated missing data (Table VIII.1). Overall, the results did not change significantly. Locus PL2258 was still found to be significantly different between healthy and PBTL-affected samples by both F_{ST} and R_{ST} (with non-interpolated and interpolated missing data). However, most F_{IS} values for the interpolated missing data changed to non-significant differences from zero, indicating no interbreeding.

Table VIII.1: F_{ST}, $F_{IS,}$ R_{ST} and R_{IS} values for an estimation of genetic differentiation of healthy and PBTL-affected samples for all loci combined and individual loci comparisons using adjusted genotype frequencies. Values were calculated using an AMOVA with both non-interpolated and interpolated missing data. Significance level for fixation indices: * = p <0.05 and ** = p <0.005. (A Bonferroni-correction for this sample size recommends a significance level of p <0.005).

Locus	Non-interpolated missing data				Interpolated missing data			
	F_{ST}	**F_{IS}**	**R_{ST}**	**R_{IS}**	**F_{ST}**	**F_{IS}**	**R_{ST}**	**R_{IS}**
PL0340	-0.013	0.324**	-0.012	0.620**	-0.027	0.047	-0.025	0.072
PL0780	-0.011	-0.041	-0.005	-0.272	-0.017	0.144	-0.017	0.168*
PL0905	-0.022	0.306**	-0.026	0.611**	-0.029	0.064	-0.028	0.111
PL1551	-0.003	0.265**	0.004	0.691**	-0.008	0.043	-0.019	0.148
PL1629	-0.009	0.489**	0.006	1.000**	0.002	-0.061	0.002	-0.061
PL1868	0.005	0.219**	-0.027	0.680**	0.021*	0.057	0.004	0.107
PL1483	0.015	0.179**	0.056	0.950**	0.016	0.132*	0.014	0.101
PL1556	0.024	0.416**	0.030	0.998**	-0.005	0.172	-0.001	0.386*
PL2258	0.054*	0.170*	0.087*	0.307*	**0.068****	0.031	0.058*	0.193
overall	0.004	0.227**	0.016	0.504**	0.005	0.072**	-0.007	0.118*

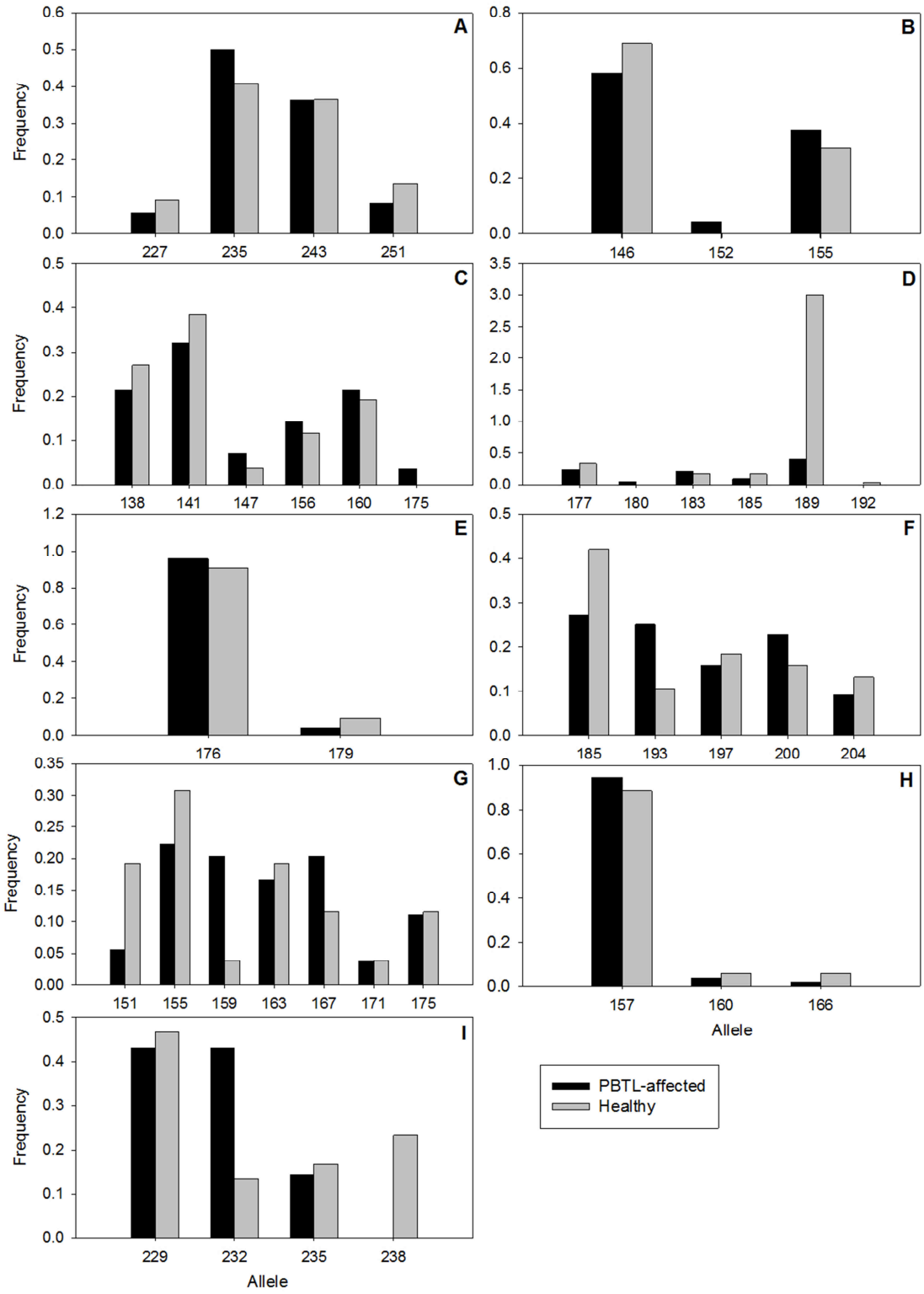

Fig. VIII.1: Adjusted allele frequencies at all nine loci. A: PL0340; B: PL0780; C: PL0905; D: PL1551; E: PL1629; F: PL1868; G: PL1483; H: PL1556; I: PL2258.

Appendix IX

Comparison of fluorescence in healthy and PBTL-affected tissue

IX.1 Methods

One sample of a healthy colony and one sample of a PBTL-affected colony were collected from CIMR and transferred into a glass dish filled with seawater. Shortly after collection, the samples were brought into the lab and a small tissue section was cut of the coral finger using a razor blade. The tissue section was then placed under the confocal microscope and imaged. A 3-D tile system using Z-stacks was used to create an image that shows multiple polyps. The coral tissue was not fixed before imaging and so shows fluorescence of living tissue.

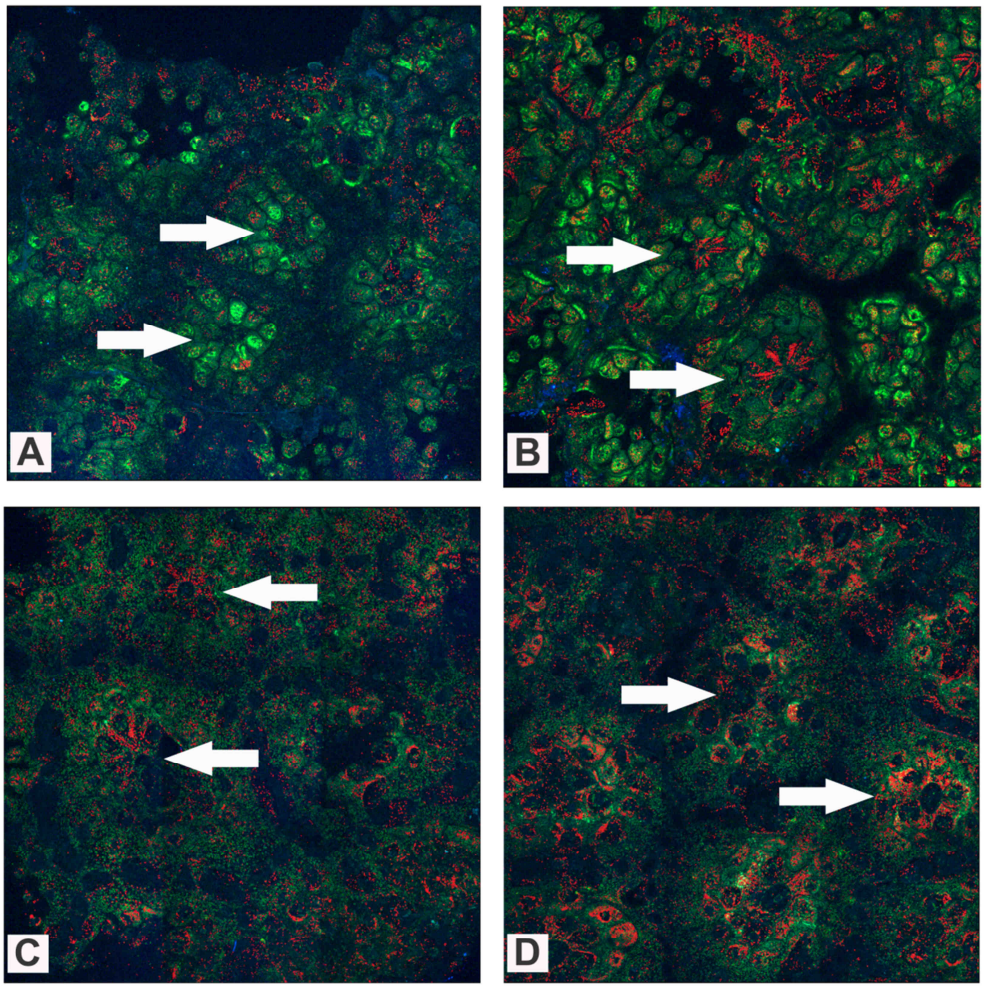

Fig. IX.1: Confocal image of *P. compressa*. A & B: healthy coral. C & D: PBTL-affected coral. Arrows point to individual polyps. Green: host tissue; red: *Symbiodinium*; blue: skeleton

IX.2 Results and Discussion

The PBTL-affected sample showed less intense green fluorescence in comparison to the healthy sample, which could indicate less dense tissue (Fig. IX.1). In Chapter 3, a reduction in tissue thickness of PBTL-affected samples was found. The reduced green fluorescence in the PBTL-affected sample may reflect tissue thinning.

In the healthy sample *Symbiodinium* cells appeared to be clustered in the tentacles, while in the PBTL-affected sample *Symbiodinium* cells appeared more scattered (Fig. IX.1). Overall, there was no apparent difference in *Symbiodinium* cell density. However, the thicker tissue (i.e. green fluorescence) may have masked some of the red fluorescence of the *Symbiodinium* cells in healthy samples.

Appendix X

Published Manuscripts

The versions of all published manuscripts are provided on the following pages.

X.1:

Sudek M, Aeby GS, Davy SK (2012) Localized bleaching in Hawaii causes tissue loss and a reduction in the number of gametes in *Porites compressa*. Coral Reefs 31:351-355

X.2:

Sudek M, Work TM, Aeby GS, Davy SK (2012) Histological observations in the Hawaiian reef coral, *Porites compressa*, affected by *Porites* bleaching with tissue loss (PBTL). Journal of Invertebrate Pathology 111:121-125

X.3:

The third manuscript is currently submitted and therefore not provided at this point.

Sudek M, Williams GJ, Runyon C, Aeby GS, Davy SK (in review) Disease dynamics of *Porites* bleaching with tissue loss: prevalence, transmission and environmental drivers.

Coral Reefs (2012) 31:351–355
DOI 10.1007/s00338-011-0844-1

NOTE

Localized bleaching in Hawaii causes tissue loss and a reduction in the number of gametes in *Porites compressa*

M. Sudek · G. S. Aeby · S. K. Davy

Received: 24 June 2011 / Accepted: 2 November 2011 / Published online: 17 November 2011

Abstract Localized bleaching (a discrete white area on the coral) was observed in one of the main framework-building corals in Hawaii, *Porites compressa*. This study aimed to determine the degree of virulence of the lesion. We investigated the whole-colony effects by following disease progression through time and examining the effect of localized bleaching on coral fecundity. After two months, 35 of 42 (83.3%) individually tagged colonies affected by localized bleaching showed tissue loss and partial colony mortality. Histological slides of healthy *P. compressa* and samples from colonies showing signs of localized bleaching were compared showing that affected colonies had a significant reduction (almost 50%) in gamete development, egg numbers, and egg size in the affected tissue. The observed localized bleaching results in both partial colony mortality and a reduced number of gametes and was termed *Porites* Bleaching with Tissue Loss (PBTL).

Keywords Coral disease · Coral bleaching · Reproduction · *Porites compressa* · Histology

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Introduction

A disease by definition is any interruption, cessation or disorder of body functions, systems or organs and can be caused by either biotic or abiotic factors (Webster 2011). In the case of corals, diseases manifest in three main ways: tissue loss, growth anomalies, or discoloration (Work and Aeby 2006). Coral bleaching is a discoloration of the coral tissue due to the loss of the endosymbiotic dinoflagellates (*Symbiodinium* sp.) and/or their photosynthetic pigments (Glynn and D'Croz 1990; Hoegh-Guldberg and Salvat 1995). It can be caused by abiotic factors including high or low seawater temperatures (Glynn 1996; Mdodo and Obura 1998; Hoegh-Guldberg and Fine 2004), high light and excessive UV radiation (Drollet et al. 1995; Glynn 1996), or it can also be caused by biotic factors such as bacterial infections (Kushmaro et al. 1996; Ben-Haim et al. 1999). For example, in *Oculina patagonica* from the Mediterranean (Kushmaro et al. 1996) and *Pocillopora damicornis* from the Red Sea (Ben-Haim et al. 2003), a bacterial infection by *Vibrio* spp. causes bleaching and lysis of zooxanthellae (Ben-Haim et al. 1999). Coral bleaching could therefore be considered a sign of disease that can have abiotic or biotic causes (Jokiel 2004).

Mass coral bleaching has caused extensive coral mortality due to prolonged periods of increased sea surface temperatures often coinciding with strong El Niño events (Glynn and D'Croz 1990; Hoegh-Guldberg 1999). Entire reef systems can be affected on large geographical scales causing most corals to bleach (Hoegh-Guldberg 1999). In contrast, localized bleaching is a discrete area on a coral colony that shows white discoloration of the tissue. It has previously been recorded in poritid corals but little is known about this lesion (Work and Rameyer 2005). We observed localized bleaching in Kaneohe Bay, Oahu,

affecting *Porites compressa*, one of the main framework-building corals around Hawaii (Maragos 1972; Jokiel 1987). It usually manifests as a diffuse area of white discoloration, with a central or peripheral location on the colony (Fig. 1a) which can, in smaller colonies, progress to envelop the entire colony (Fig. 1b). The bleaching typically displays a speckled pattern with polyps remaining brown, while the coenosarc appears bleached (Fig. 1c); the bleaching can progress to include the polyps as well. The observed bleaching pattern, however, appears different from thermally induced coral bleaching as it occurs all year round during times when water temperatures are well within the coral's thermal tolerance thresholds. In addition, adjacent corals of the genus *Montipora* and *Pocillopora*, which in general are more susceptible to thermal bleaching (Jokiel and Coles 1990; Aeby et al. 2003; Kenyon et al. 2006), show no signs of bleaching at times when localized bleaching in *P. compressa* is observed.

Our main objective was to determine the degree of virulence (harm to host) of localized bleaching on the coral colony by measuring the following: (1) disease progression, using individually tagged colonies; and (2) the effect on gametal development, using histological techniques.

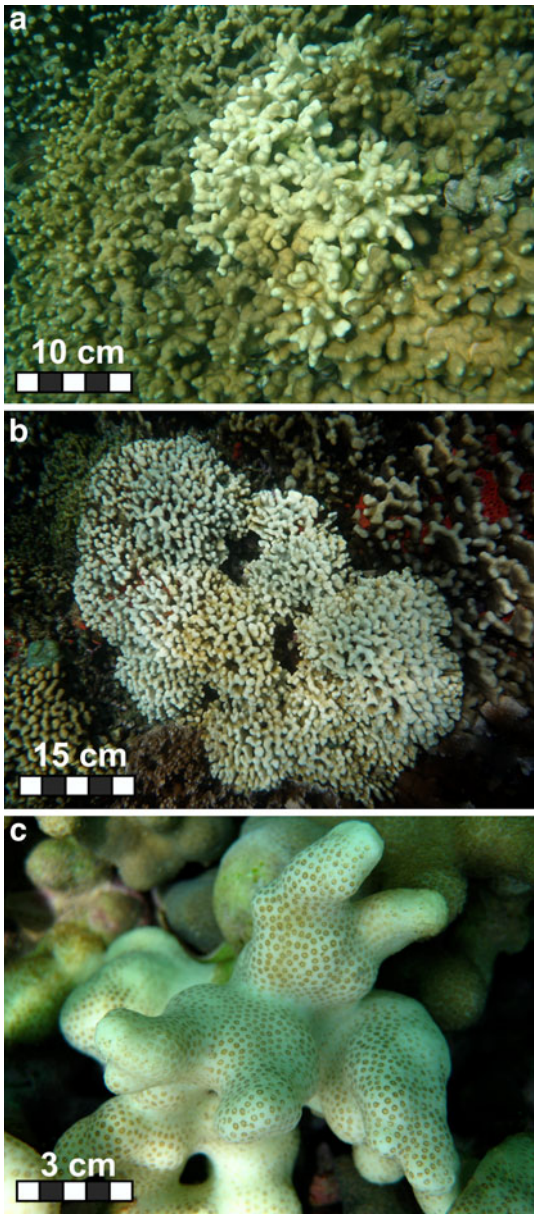

Fig. 1 *Porites compressa* colonies affected by localized bleaching. **a** Localized bleaching. **b** An extreme case with colony-wide bleaching. **c** Close-up showing the discolored coenosarc and pigmented polyps. Photo credit: Christina Runyon

Materials and methods

Tags

Forty-two colonies affected by localized bleaching were tagged around the perimeter of Coconut Island, Kaneohe Bay, Oahu, Hawaii (21°26.000′N, 157°47.000′W) in August 2010 and revisited in October 2010. All colonies were photographed and the percentages of healthy and affected tissue, as well as dead skeleton, were visually estimated in situ. The complex three-dimensional structure of *P. compressa* colonies prevented an estimation of the affected area by digital analysis, necessitating the use of the semi-quantitative visual technique.

Reproductive output

Porites compressa is a gonochoric broadcast spawner that spawns during full-moon periods between June and August (Neves 2000). Fragments (3 cm^2) of *P. compressa* (22 fragments from healthy colonies and 31 fragments with evidence of localized bleaching) were collected from the reef crest around Coconut Island in June 2010, 2 days before full moon and the first spawning of the season, so increasing the chance of finding well-developed gametes. Samples were fixed in zinc-formaldehyde solution, decalcified in HCl buffered with EDTA, and embedded in paraffin. The wax blocks were sectioned at 6 μm using a rotary microtome, stained with hematoxylin and eosin, and examined under a light microscope to determine the sex of the coral and assess the reproductive state of the gametes. The number of eggs was recorded within 5 haphazardly selected polyps from each reproductively active diseased and healthy female; this replication controlled for any errors arising from different planes of the histological sections. The maximum diameter of each egg within these polyps was measured using Image_J; volume could not be measured due to the limitations of the histological method used.

Data analyses

The data met the assumptions of normality but not equal variance. A two-sample *t*-test (equal variances not assumed) was carried out on replicate means using SPSS (PASW Statistics 18) to determine any differences in egg numbers and/or size between healthy colonies and those affected by localized bleaching.

Results and discussion

After two months, 35 out of 42 (83.3%) tagged colonies suffered an average partial colony mortality of 30.1% (range: 10–100%). However, partial re-pigmentation was also observed in 81% of cases, suggesting that the coral was, at least partially, able to recover from the disease. A similar pattern has been observed in other coral diseases. *Montipora* white syndrome (MWS) in Hawaii causes partial to total colony mortality but recovery occurs in approximately 30% of cases (Aeby et al. 2010). *Porites* ulcerative white spot disease (PUWS) from the Philippines causes small white lesions that can either regress or progress to tissue necrosis and cell death (Raymundo et al. 2003). Factors contributing to either recovery or mortality from a coral disease are still poorly understood.

All healthy *P. compressa* samples ($n = 22$) contained well-developed gametes (either oocytes or spermaries). In contrast, only 54.8% of colonies affected by localized bleaching had developed gametes. Nine out of the 22 healthy samples and 6 out of the 17 samples affected by localized bleaching were females, which were used for egg counts and measurements. A significant difference was found between both the number of eggs ($df = 7.421$, $t = 5.031$, $p = 0.001$) and the size of eggs ($df = 8.141$, $t = 6.209$, $p < 0.001$) in healthy versus diseased colonies. Healthy samples contained 4.9 ± 0.3 (mean ± SE) eggs per polyp with a maximum diameter of 44.6 ± 2.3 µm (mean ± SE) ($n = 9$). In contrast, samples affected by localized bleaching contained 1.6 ± 0.6 (mean ± SE) eggs per polyp with a maximum diameter of 23.9 ± 2.4 µm (mean ± SE) ($n = 6$). Healthy corals produced more and larger eggs (Fig. 2).

This study showed that localized bleaching in *P. compressa* causes a significant reduction in gamete development in both sexes. Almost 50% of the samples affected by localized bleaching developed no gametes, and all females that had gametes produced fewer and smaller eggs than did healthy colonies. Two female colonies affected by localized bleaching contained only under-developed oocytes with no eggs.

These results show that gametal development in *P. compressa* is significantly compromised in areas affected by localized bleaching. Similarly, thermal bleaching has also been shown to have a negative effect on the reproduction of many coral species (Szmant and Gassman 1990; Ward et al. 2000), though interestingly, Cox (2007) found no decrease in reproduction in thermally bleached colonies of *Montipora capitata*, which has the ability to increase its heterotrophic feeding rates after bleaching

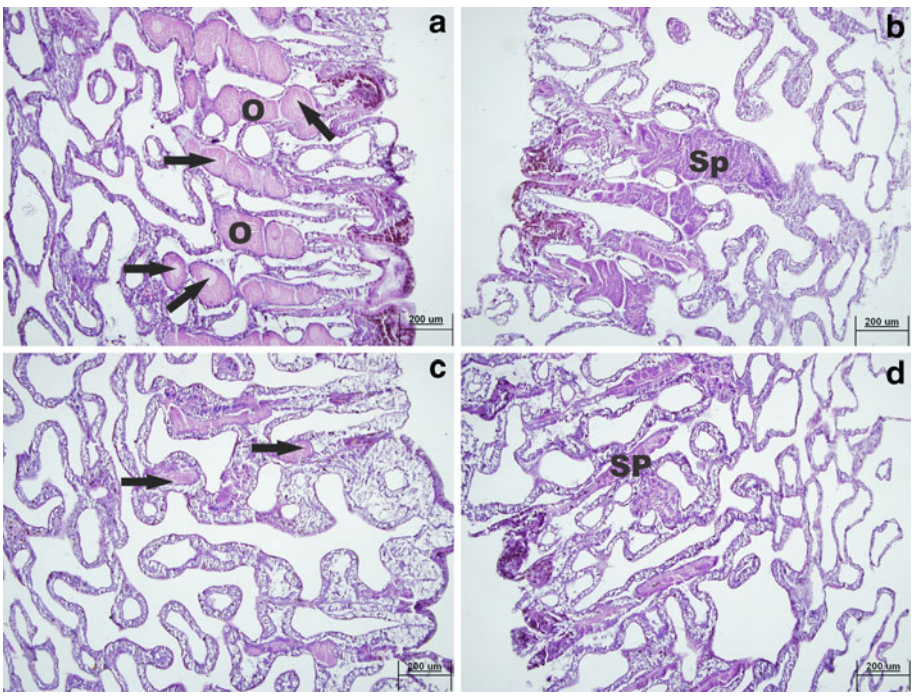

Fig. 2 *Porites compressa*. **a** Longitudinal section of a healthy female showing well-developed oocytes containing eggs. **b** Longitudinal section of a healthy male showing well-developed spermaries. **c** Longitudinal section of a female affected by localized bleaching showing underdeveloped oocytes containing small eggs. **d** Longitudinal section of a male affected by localized bleaching showing underdeveloped spermaries; O = oocytes, Sp = spermaries, ↑ eggs, all images ×10 magnification. *Scale bar* = 200 µm

(Grotolli et al. 2006). The impact of bleaching on coral reproduction is most likely linked to the loss of zooxanthellae, as these contribute over 90% of the coral's energy requirements through photosynthesis, and support coral growth and reproduction (Muscatine et al. 1984). Alternatively, oocytes contain a very high concentration of lipids that can provide energy when resources are limited (Weil et al. 2009) and could be re-absorbed, as has previously been recorded in stressed corals (Neves 2000; Okubo et al. 2009).

Sexual reproduction in corals is strongly influenced by biotic and abiotic stressors such as pollution (Guzman and Holst 1993; Cox and Ward 2002), sedimentation (Fabricius 2005) and competitive interactions (Rinkevich and Loya 1985; Tanner 1995). Diseases are a drain on energy resources to the host and are therefore very likely to impact reproduction, however, comparatively few studies in the coral disease literature have measured the effect of disease on the reproductive output of corals. In the Caribbean, a decreased reproductive output was found in *Montastraea faveolata* affected by yellow band disease (Weil et al. 2009) and by white plague (Borger and Colley 2010). A localized suppression of reproduction has also been shown in Caribbean sea-fan octocorals suffering a fungus or protist infection (Petes et al. 2003).

The reproductive effort of an individual can be used as an evaluation of general fitness (Metz et al. 1992). For corals, fertilization success, larval dispersal, recruitment, and survivorship drive population dynamics and coral reef regeneration (Hughes and Tanner 2000; Vermeij 2005, 2006), and even a small reduction in reproductive output can have the potential to cause severe negative impacts on recruitment (Hughes et al. 2000). The negative impact of diseases on the reproductive output of corals should therefore not be neglected. Indeed, in the case of disease outbreaks, reduced reproduction in addition to colony mortality could have major impacts on reef resilience and longevity.

The observed localized bleaching results in both partial colony mortality and reduced gametal development of the coral, and the disease was termed *Porites* Bleaching with Tissue Loss (PBTL). *P. compressa* is one of the main framework-building corals in Hawaii, and so PBTL has the potential to impact the population structure and resilience of Hawaiian reefs. This study is part of an ongoing project investigating the pathogenesis of PBTL.

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Histological observations in the Hawaiian reef coral, *Porites compressa*, affected by *Porites* bleaching with tissue loss

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ABSTRACT

The scleractinian finger coral *Porites compressa* is affected by the coral disease *Porites* bleaching with tissue loss (PBTL). This disease initially manifests as bleaching of the coenenchyme (tissue between polyps) while the polyps remain brown with eventual tissue loss and subsequent algal overgrowth of the bare skeleton. Histopathological investigation showed a loss of symbiont and melanin-containing granular cells which was more pronounced in the coenenchyme than the polyps. Cell counts confirmed a 65% reduction in symbiont density. Tissue loss was due to tissue fragmentation and necrosis in affected areas. In addition, a reduction in putative bacterial aggregate densities was found in diseased samples but no potential pathogens were observed.

1. Introduction

In recent decades, coral diseases have increased in prevalence and geographical extent worldwide, threatening the health and survival of coral reefs (Harvell et al., 2004; Sokolow, 2009). However, descriptions of most coral diseases have been based on field surveys, and many diseases lack systematic morphological descriptions at both the gross and cellular levels (Work et al., 2008). Confounding the presence of lesions with causation of disease without appropriate laboratory confirmation has led to considerable confusion in the literature (Richardson, 1998; Work and Aeby, 2011). The use of a standardized nomenclature that provides a systematic morphological description of coral disease lesions at the gross and cellular levels allows uncoupling of the description of the lesion from the inference of causation and comparisons across geographical areas (Work and Aeby, 2006, 2011; Work and Rameyer, 2005). Systematic descriptions of lesions at the gross and cellular levels provide the initial step in the development of case definitions and may assist in identifying possible pathogens (Work and Rameyer, 2005; Work et al., 2008).

Porites compressa is one of the main framework building corals in Hawaii. In Kaneohe Bay, Oahu, this species is affected by *Porites* bleaching with tissue loss (PBTL) that manifests as diffuse areas of white discoloration (secondary to translucence and visibility of skeleton through tissue) of the coenenchyme with pigmented polyps that are often retracted (Fig. 1). The lesion may be located in the center or on the periphery of a colony or may be colony-wide (on smaller colonies). In most cases, PBTL results in partial tissue loss with subsequent algal colonization of the dead skeleton (Sudek et al., 2012).

Coral bleaching is defined as a de-pigmentation of the coral's tissues due to a disruption of the symbiosis between the endosymbiotic dinoflagellates (*Symbiodinium* spp.) and the coral host; it is typically characterized by the loss of the symbiotic dinoflagellates (Glynn, 1996; Glynn and D'Croz, 1990; Hoegh-Guldberg and Smith, 1989). Environmental stimuli such as high or low seawater temperatures (Glynn, 1996; Hoegh-Guldberg and Fine, 2004), high light or UV radiation (Drollet et al., 1995; Glynn, 1996), or bacterial infections (Kushmaro et al., 2001) can trigger this process. PBTL does not appear to be a response to elevated sea surface temperatures as it occurs only in isolated colonies at times when water temperatures are well within the thermal threshold of this species. Given the uncertain causes of this disease, we set out to characterize PBTL at the cellular level. Specifically, we measured tissue thickness and *Symbiodinium* cell densities and described changes at the cellular level. This study presents the first histological information on PBTL, so providing a foundation for a case definition of this disease.

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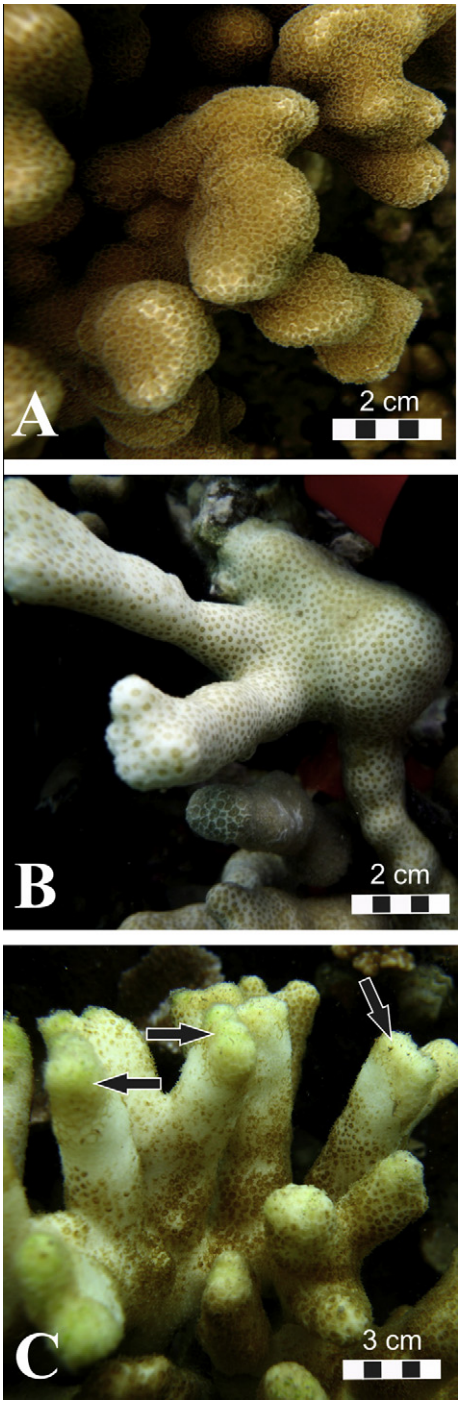

Fig. 1. (A) Healthy *Porites compressa*. Note regular brown coloration. (B) *P. compressa* affected by PBTL (early stage). Note white discoloration of coenenchyme and pigmented polyps but no signs of tissue loss. (C) *P. compressa* affected by PBTL (progressive stage). Note beginning of tissue sloughing on the tips of the coral branches (arrowhead).

2. Methods

2.1. Sample collection

Branches (2–3 cm^2) of *P. compressa* (36 fragments with signs of PBTL and 27 fragments from healthy control corals) were collected from the reef crest around Coconut Island, Kaneohe Bay, Oahu, Hawaii (21°26.000′N, 157°47.000′W) at a depth of 0.5–2 m in June 2010 and June 2011. The samples were fixed in 20% zinc-formaldehyde solution (1 part Z-Fix concentrate (Z-Fix® Anatech, Battle Creek, MI, USA) in four parts filtered seawater) immediately after collection.

2.2. Sample preparation

Samples were decalcified in 2.5% HCl buffered with 0.1% EDTA, rinsed and stored in 70% ethanol until further processing. After decalcification, all samples collected in 2010 ($n = 53$) were cut in half with a razor blade (tip to bottom) and laid open; measurements of tissue thickness were taken from the tip and the sides of the coral fragment using a Kincrome© Digital Vernier Caliper. A 2 cm diameter core was then removed from one half of all control and diseased fragments with a cork borer placed at a distance of 1 cm from the tip of the fragment. The core was homogenized with 1 ml of 0.2 μm-filtered seawater in a tissue homogenizer and algal cells were counted on a haemocytometer (Improved Neubauer, Boeco Ltd., Germany), with eight replicate counts per core. Cell densities were standardized to tissue volume of the core ($\pi r^2 \times$ thickness).

The other half of the decalcified coral fragment was trimmed and embedded in paraffin. Wax blocks were sectioned at a thickness of 6 μm using a rotary microtome, and the resulting sections stained with hematoxylin and eosin (H&E). Sections were examined at the microscopic level and lesions classified according to the presence of: (1) necrosis characterized by cytoplasmic hypereosinophilia or fragmentation coupled with nuclear karyorrhexis, karyolysis or pyknosis; (2) tissue fragmentation characterized by loss of epidermis and exposure of the basal body wall and mesenterial filaments; (3) changes in *Symbiodinium* and melanin-containing granular cell densities and/or morphology; and (4) presence or absence of associated organisms.

We also observed putative bacterial aggregates (Peters, 1997) in the tissues of *P. compressa* in both healthy and diseased samples (Fig. 2A). All putative bacterial aggregates were enumerated in a standardized 1712 × 1289 μm area of coral tissue approximately 1 cm below the branch tip.

3. Data analyses

The data for coral thickness, *Symbiodinium* cell densities and bacterial aggregate counts were checked for normality and equal variance. For coral thickness, these assumptions were met and a two-sample *t*-test was used to determine differences in tissue thickness between fragments affected by PBTL and control corals. Data for *Symbiodinium* cell and bacterial aggregate counts did not meet assumptions of normality and equal variance, so a non-parametric Mann–Whitney *U* test was used for comparisons.

4. Results

Branches affected by PBTL had significantly thinner tissue on the tip ($df = 51$, $t = 6.887$, $p < 0.001$) and sides ($df = 51$, $t = 2.322$, $p = 0.024$) than healthy controls (Table 1). Corals affected by PBTL also showed a significant decrease in *Symbiodinium* cell density (Mann–Whitney $U = 25$, $n = 53$, $p < 0.001$) and in the abundance of putative bacterial aggregates (Mann–Whitney $U = 148.5$, $n = 53$, $p < 0.001$) (Table 1). Of the healthy coral samples, 77% had putative bacterial aggregates in the examined tissue section versus 26% of PBTL-affected samples. Putative aggregates were round to oblong and ranged from approximately 170–1520 μm^2 in PBTL-affected samples and 175–1914 μm^2 in healthy samples.

Histological examination showed reductions in both *Symbiodinium* and melanin-containing granular cell densities that were more pronounced in the gastrodermis of the coenenchyme than

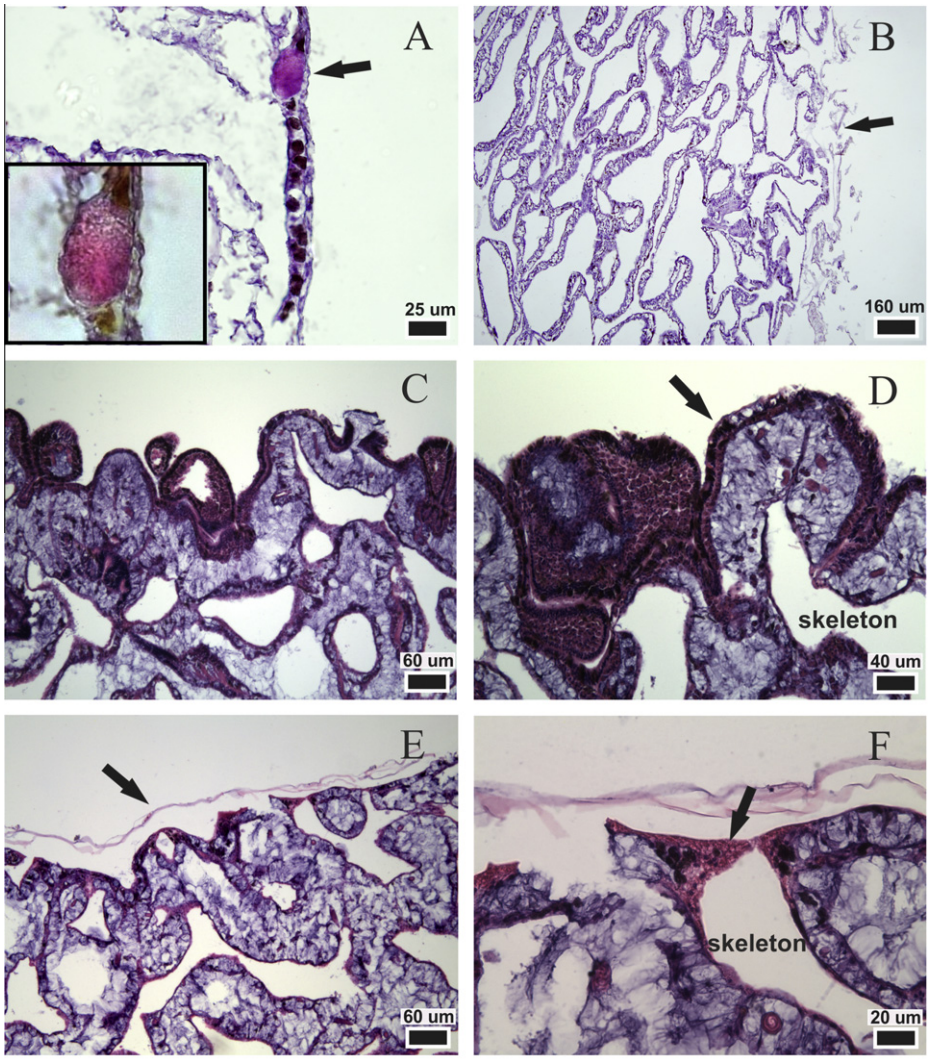

Fig. 2. (A) *P. compressa* with PBTL. Note putative bacterial aggregate in epidermis (arrowhead). Inset shows a close-up of the bacterial aggregate. (B) *P. compressa* with PBTL. Note ablation of the epidermis (arrowhead). (C and D) Normal *P. compressa*. Note regular columnar epidermis (arrowhead). (E) *P. compressa* with PBTL. Note attenuation and ablation of epidermis with overlaying hyaline membrane (arrowhead). (F) Close-up of E. Note hyaline membrane overlaying epidermis that manifests as cytoplasmic hypereosinophilia and karyorrhexis (arrowhead).

Table 1
Tissue thickness from the tip and side of the branches (mm), *Symbiodinium* cell densities (cells/cm^3) and putative bacterial aggregates (number/mm^2) in healthy control branches of *Porites compressa* and branches affected by PBTL. Values are mean ± SE and range. All pair-wise comparisons between healthy and PBTL-affected colonies were statistically significant ($p < 0.05$).

	Healthy	Range (min–max)	PBTL	Range (min–max)
Tissue thickness: tip (mm)	6.08 ± 0.18	4.69–8.21	4.38 ± 0.17	2.16–6.27
Tissue thickness: side (mm)	2.46 ± 0.07	1.84–3.19	2.21 ± 0.08	1.28–3.18
Symbiodinium density (cells/cm^3)	$1.4 \times 10^6 \pm 74533.1$	4.7×10^5–2.1×10^6	$4.8 \times 10^5 \pm 37027.8$	1.5×10^5–1.3×10^6
Putative bacterial aggregates (number/mm^2)	0.74 ± 0.14	0–2.3	0.19 ± 0.07	0–1.8

in the polyps. Ablation of the epidermis associated with algal overgrowth (Fig. 2B) was observed in 39% of samples affected by PBTL. Necrosis manifested as cytoplasmic hypereosinophilia and karyorrhexis overlaid by a hyaline membrane (Fig. 2E and F) and was observed in 19% of samples examined. Tissue fragmentation (Fig. 3A–C) was observed in 11% of PBTL-affected samples, and in two of these samples, helminths were observed in the degrading tissue (Fig. 3E). Clumps of diatoms were also found on the epidermis of one diseased sample (Fig. 3F). No other microbial or metazoan organisms were seen associated with diseased tissue for all remaining samples.

5. Discussion

Corals affected by PBTL showed a significant loss of their symbiotic dinoflagellates (*Symbiodinium* spp.) and melanin-containing granular cells, mainly from the gastrodermis of the coenenchyme.

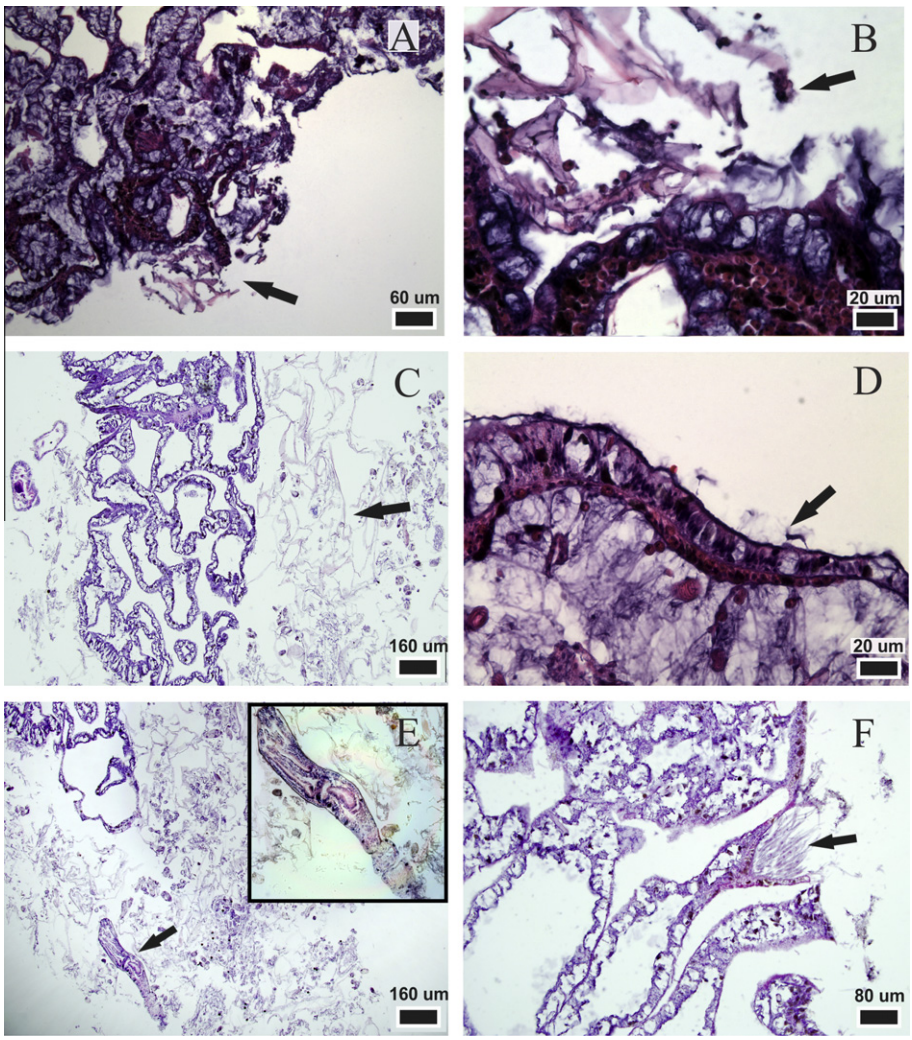

Fig. 3. (A) *P. compressa* with PBTL. Note tissue fragmentation and hyaline membranes effacing epidermis (arrowhead). (B) Close-up of A. Note cell debris (arrowhead) mixed with hyaline membrane. (C) *P. compressa* with PBTL. Note tissue fragmentation and cell debris (arrowhead). (D) Normal *P. compressa*. Note regular columnar epidermis with remnants of mucus (arrowhead). (E) *P. compressa* with PBTL. Note tissue fragmentation and helminths in the tissue debris (arrowhead). Inset shows a close-up of the helminths. (F) *P. compressa* with PBTL. Note clump of diatoms on epidermis (arrowhead).

This response was less pronounced in the polyps, thereby giving affected corals the typical "speckled" appearance (i.e. bleached coenenchyme and pigmented polyps). The extensive tissue loss of affected colonies observed in the field (Sudek et al., 2012) was found to result from tissue fragmentation and necrosis, leading to tissue mortality of affected areas.

PBTL-affected samples showed a 65% reduction in *Symbiodinium* cell density. This is similar to the loss seen in other corals that exhibit bleaching in response to disease. For example, bacterial bleaching in the reef coral *Pocillopora damicornis*, is characterized by a loss (>88%) and lysis of *Symbiodinium* cells due to an infection by *Vibrio* spp. (Ben-Haim et al., 2003). *Symbiodinium* cell loss (41–96.9%) is also seen in yellow-band disease (YBD), which affects *Montastraea* spp. from the Caribbean and starts as small blotches with reduced pigmentation, advancing over the colony and leaving dead skeleton behind (Cervino et al., 2001). PBTL, however, shows a very different and distinct bleaching pattern, with the coenenchyme bleaching first and the polyps remaining brown.

The loss of *Symbiodinium* cells may have contributed to the observed atrophy in affected samples (tissue thinning of 28% on the tip and 11% on the sides), which is indicative of a stressed coral colony. *Symbiodinium* cells can contribute over 90% of the coral's energy requirements through photosynthesis (Muscatine et al., 1984); a loss of *Symbiodinium* therefore leads to less energy being available for growth and other life processes such as reproduction and repair. Moreover, to counteract the prolonged loss of nutrition, corals may reabsorb their tissues (Szmant and Gassman, 1990). Atrophy can be observed in bleached corals (Glynn et al., 1985) and corals that are affected by sediment stress (Vargas-Angel et al., 2007).

Tissue fragmentation and necrosis were only observed in a few samples affected by PBTL, probably because the sampled coral colonies were at different stages of the disease. During sampling, branches in earlier stages of the disease (i.e. with intact tissue) were targeted and only a few could be collected with signs of tissue loss. Early stages of PBTL are mainly characterized by the loss of *Symbiodinium* cells, but it progresses to tissue fragmentation and necrosis in later stages. Cell death associated with tissue loss has been recorded in association with several coral diseases (McClanahan et al., 2003; Renegar et al., 2008; Williams et al., 2011; Work and Rameyer, 2005) and appears to be a common response to disease.

Ablation of the epidermis was associated with microalgal overgrowth, which may have contributed to tissue death. Other potentially opportunistic invaders, helminths and diatoms, were also observed in samples affected by PBTL. Helminths were found in

the tissue debris associated with tissue fragmentation that has also been reported in other coral diseases (Work and Aeby, 2011). Diatoms were observed on the epidermis of a diseased sample. Few studies have reported the occurrence of diatoms on the surface of corals (Johnston and Rohwer, 2007; Rublee et al., 1980) likely due to healthy coral's ability to protect themselves from settling organisms or sediment by mucus shedding (Brown and Bythell, 2005). Sorting out whether organisms such as helminths, diatoms or micro algae were primary invaders or sequelae to primary tissue loss will require longitudinal studies.

Using histology, bacterial aggregates have been observed in the tissues of corals (mostly *Acropora* spp.) affected by disease (Peters et al., 1983; Galloway et al., 2007), but no bacterial aggregates have been found in many other disease lesions (Ainsworth et al., 2007; Bythell et al., 2004). In this study, numerous clusters of putative bacterial aggregates were observed in both healthy and PBTL-affected corals but they were not associated with cell pathology, as seen in bacteria-induced diseases of vertebrates (Magi et al., 2009; Olsen et al., 2006), invertebrates (Johnson, 1976) and plants (Nelson and Dickey, 1970; Wallis and Truter, 1978). Indeed, a 74% reduction of putative bacterial aggregates was observed in corals affected by PBTL versus healthy colonies suggesting a disruption of the coral holobiont. If the symbiotic relationship between the coral and its associated microbial community is disrupted for any reason (for example changes in environmental factors), the whole balance of the holobiont could be compromised, ultimately contributing to a disease state (Vega-Thurber et al., 2009). Identifying how these bacteria interact with the coral host and their role in coral defense is a potentially fruitful avenue of investigation.

Given the lack of consistency between lesions and a particular etiological agent, we do not suspect that PBTL is caused by metazoans, bacteria or protozoans. However, smaller pathogens such as viruses, which are not easily detectable by light microscopy, cannot be ruled out and would necessitate ultra-structural examination of tissues. Other possible causes to consider are toxins and/or environmental triggers. Field studies have confirmed that PBTL can cause extensive tissue loss in affected areas of the colony (Sudek et al., 2012) so further research into the etiology of PBTL is underway to build a comprehensive case definition of this disease.

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