Identification of a primary pathogen involved in white patch syndrome, a newly-reported disease affecting the massive coral Porites lutea in the Western Indian Ocean

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IDENTIFICATION ET ETIOLOGIE DES MALADIES ASSOCIEES AUX CORAUX SCLERACTINIAIRES DANS LE SUD-OUEST DE L'OCEAN INDIEN

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Résumé

Abstract

During the past two decades, the emergence and spread of infectious diseases have caused substantial declines in the biodiversity and abundance of reef-building corals. Despite their increased global prevalence and virulence, little is known about coral diseases on Indian Ocean coral reefs. This study aims to fill this gap in knowledge by identifying the main coral diseases and quantify their prevalence at three localities Reunion, South Africa and Mayotte, determining their spatial distribution and seasonal variation. Additionally this research aimed to describe and characterise at ecological, histological, molecular and microbiological levels of the following two unreported coral diseases on the WIO reefs: *Porites* white patch syndrome (PWPS) and *Porites* black patch syndrome (PBPS).

Principal findings of this study demonstrated the presence of six main coral diseases including black band disease (BBD), white syndromes (WS), pink line syndrome (PLS), growth anomalies (GA), skeleton eroding band (SEB) and *Porites* white patch syndrome (PWPS). The overall disease prevalence was higher in Reunion (7.5 ± 2.2%; mean ± SE) compared to South Africa (3.9 ± 0.8%; mean ± SE) and Mayotte (2.7 ± 0.3%; mean ± SE). *Acropora* and *Porites* were the genera most vulnerable to disease. Spatial variability was detected in both Reunion and South Africa with BBD and WS more prevalent on shallow than deep reefs. There was also evidence of seasonality in two diseases: BBD and WS, their prevalence being higher in summer than winter.

Corals exhibiting signs of PWPS revealed extensive tissue fragmentation, generally associated with ovoid basophilic bodies resembling bacterial aggregates within the mesoglea of the body wall. Other organisms, including *Cyanobacteria*, Nematoda, Ciliata and endophytic algae, were also observed on diseased tissues and were generally associated with the dead epidermis and cell debris. Results of 16S rRNA sequence analysis revealed a high variability between bacterial communities associated with PWPS-infected and healthy tissues in *Porites lutea*. Several bacterial ribotypes affiliated to potential putative pathogens notably *Shimia marina* and *Vibrio hepatarius* were consistently found among the 16S rRNA sequences derived from the PWPS lesions, and absent and/or poorly represented in HT.

Primary pathogens involved in the PWPS were also investigated in this study using traditional culturing techniques and laboratory infection trials. Of the 14 isolates selected for the inoculation trials, only the bacterial strain P180R mostly phylogenetically closely related

to *Vibrio tubiashii* with its closest known sister taxon, *V. hepatarius*, was shown to cause signs resembling those of PWPS and satisfied the four Henle-Koch's postulates. P180R displayed focalised and progressive tissue paling 12 h after inoculation and visible lesions of PWPS were observed 12 h thereafter. Signs of PWPS appeared on 90% of the exposed coral fragments (27 of 30) under controlled environmental conditions. Moreover, the virulence of this marine pathogen was tested and seemed to be strongly dependent on seawater temperature, resulting in significantly higher tissue loss at 30°C than 28°C and 26°C.

Finally, a multidisciplinary approach involving field surveys, gross lesion monitoring, histopathology and 454-pyrosequencing was investigated to characterize an atypical form of BBD named PBPS. Surveys conducted within two geomorphological zones over two consecutive summers and winters showed that it manifested spatial and seasonal variability, with more infected colonies observed on the reef flat and during the summer season. Histology revealed cyanobacterial penetration of the compromised tissue as well as the presence of basophilic bodies resembling bacterial aggregates in the living tissue, adjacent to the bacterial mat. Bacterial 16S rRNA sequences yielded a broader diversity of bacterial taxa in PBPS-infected tissues than in healthy tissue, represented by the genus *Vibrio* (24.9%), followed by sulfate-reducers or sulfide-oxidizers such as *Desulfovibrio* (20%), *Clostridium* (12.9%) and *Arcobacter* (9.9%). PBPS appears to be a multi-stage disease triggered by cyanobacterial invasion and resulting in secondary infections by environmental bacteria that grow in mucus-like decomposing tissue.

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Chapter 1

General introduction

1. Coral reefs: ecological goods and services

Coral reefs are among the most productive, diverse and complex ecosystems in the world (Odum and Odum 1955) and support almost one third of the world's marine fish species (Newton et al. 2007). They provide many goods and services such as recreational activities and coastal protection (Moberg and Folke 1999). Ecosystem services that reefs provide are estimated to be worth 173 billion of $US (Martínez et al. 2007). They are also a valuable source of food for local communities (Bruckner 2000). More than 100 countries, representing approximately 500 millions of people, depend on coral reefs for part of their livelihood or for part of their protein intake (Salvat 1992; Bryant et al. 1998). Indeed, reef-related fisheries constitute approximately 2-5% of the total fish consumed by humans (Smith 1978; Pauly et al. 2002). In the Western Indian Ocean (WIO), approximately thirty million people depend directly or indirectly on the coastal environment for goods and services (McClanahan et al. 2008). Moreover, coral reefs provide many nautical activities and represent a recreational area for the local population (Piton and Taquet 1992). Finally, coral reefs are also important in coastal protection from currents, waves and storms, are essential in maintaining biological diversity and constitute important spawning, nursery and breeding areas (Moberg and Folke 1999).

2. The scleractinian coral, a complex and diverse micro-ecosystem

Coral reefs frameworks are formed mainly by scleractinian corals (Fig. 1A) that secrete a calcium carbonate (CaCO3) hard skeletal structure. Briefly, scleractinian corals are composed of numerous polyps interconnected by the tissue called the coenenchyme (Fig. 1B). The polyp tissue consists in three distinct layers (Veron 1985; Galloway et al. 2007); the epidermis, located either next to the external environment and the skeleton, contains several specific cells (Figs 1C, D) including the nematocytes (defence cells), calicoblastic cells (calcium carbonate

secretion) and the mucocytes (mucus secretion). The mesoglea generally located between the epidermis and the gastrodermis is a connective tissue layer with some isolated cells e.g. amoebocytes. The gastrodermis contains cells such as cnidocytes, amoebocytes, mucocytes but also the endosymbiotic algae called zooxanthellae (Galloway et al. 2007). The whole is covered by a viscous and transparent mucus layer and attached to the calcium carbonate skeleton.

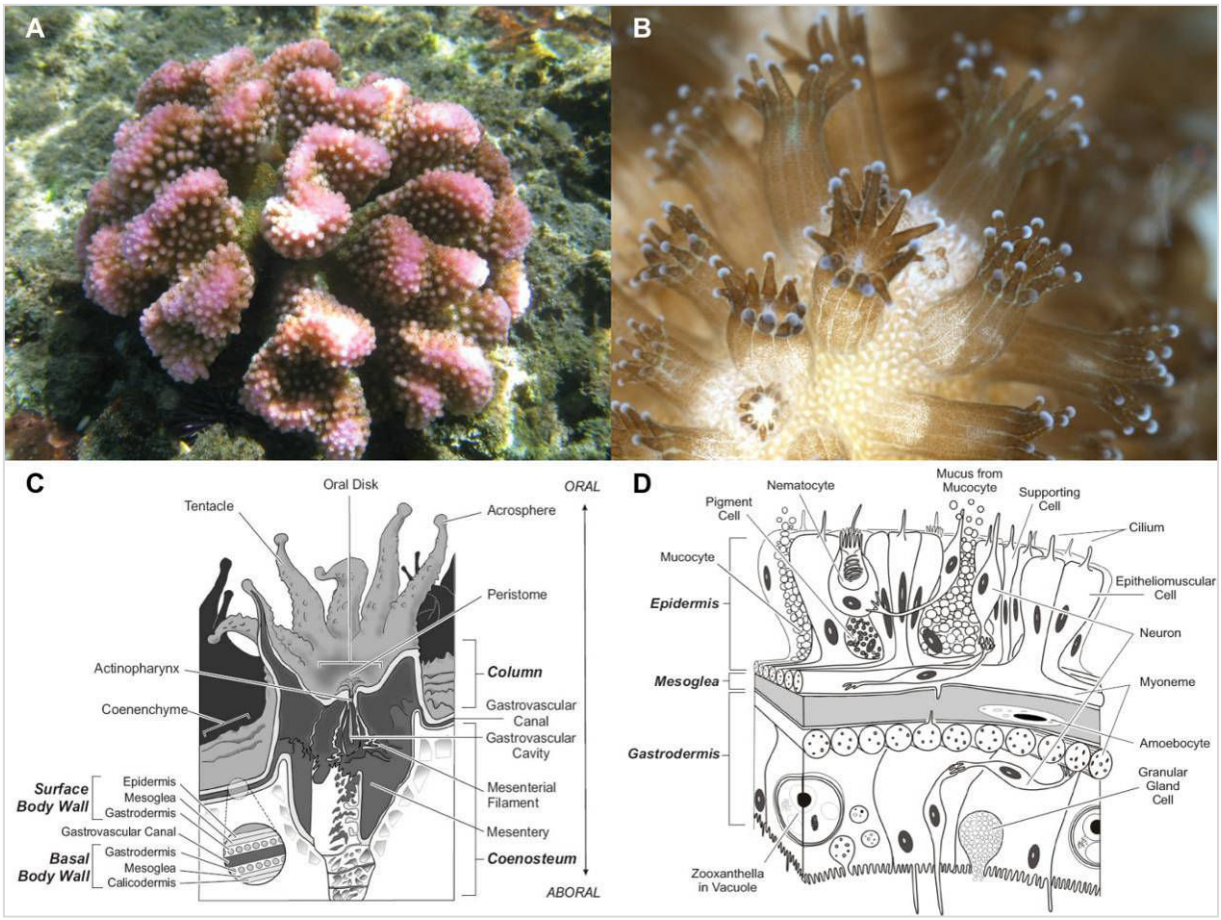

Figure 1: Simplified anatomy of scheratinian corals. A. Colony of Pocillopora sp. B. Colony of expended polyps of Pocillopora sp. C. Diagram of gross anatomy of a polyp. D. Three dimensional diagram representing the different tissue layers of the surface body wall of a scleractinian polyp (C and D taken from Galloway et al. (2007)).

Recent studies have shown that these coral structures/compartments (e.g. mucus layer, coral tissue and the skeleton) harbor a diverse, complex and abundant microbial population including endolithic algae (Fine and Loya 2002), protozoa (Toller et al. 2002; Sweet and Bythell 2012), archaea (Kellogg 2004; Wegley et al. 2004; Siboni et al. 2008), fungi (Bentis et al. 2000; Wegley et al. 2007a), bacteria (Rohwer et al. 2002; Knowlton and Rohwer 2003; Bourne and Munn 2005; Ritchie 2006; Wegley et al. 2007b; Bourne et al. 2009; Mouchka et al. 2010; Sweet et al. 2010; Barott et al. 2011) and viruses (Marhaver et al. 2008; Patten et al. 2008; Claverie et al. 2009; Van Oppen et al. 2009) providing diversified microhabitats (Sunagawa et al. 2010). This microbial-coral relationship termed "coral holobiont" is likely to play a key role in coral physiology and health (Rosenberg et al. 2007; Bourne et al. 2009; Ainsworth et al. 2010; Kvennefors et al. 2012). It has been suggested that microbial communities from the coral surface mucus layer (SML) may be used as a defense mechanism to protect the coral host against putative pathogens by displaying antimicrobial activity or though competition with non-resident bacteria for nutrition and space (Rohwer et al. 2002; Ritchie 2006; Shnit-Orland and Kushmaro 2009; Kvennefors et al. 2012). For instance, Shnit-Orland and Kushmaro (2009) found that 25-70% of cultivable bacteria isolated from the mucus of six different coral genera displayed antibacterial activity. Other studies has demonstrated that some bacterial strains also retrieved from the coral SML were able to inhibit growth of bacterial pathogens such as *Vibrio shiloi*, *V. coralliilyticus* and *Serratia marcescens* implicated in several coral pathology (Ritchie 2006; Nissimov et al. 2009; Rypien et al. 2010).

3. Infectious disease, an increasing threat to coral reef worldwide

Coral reefs are in serious decline worldwide with 20% of the world's coral reefs have already been degraded beyond the potential for recovery, 24% are under imminent risk of collapse and another 26% are under a longer term threat of collapse (Wilkinson 2004). These has been

attributed to increasing anthropogenic pressure (*e.g*. mining for lime production, destructive fisheries, poaching, pollution, SCUBA diving etc.) and natural factors (e.g. storms, solar radiations, and warm temperature anomalies (Bellwood et al. 2004)). These external factors acting individually or in synergy, could affect the resistance of corals by altering the complex microbial-coral associations and therefore stimulating the growth and severity of pathogenic organisms (Green and Bruckner 2000; Lesser et al. 2007).

Over the past 40 years coral diseases have substantially contributed to the decline in the biodiversity and abundance of reef-building corals (Peters 1984; Harvell et al. 1999; Hughes et al. 2003). Beside to be a major contributor to coral mortality, they are also known to generate progressive tissue loss (Bruckner et al. 1997), to affect the growth rate, reproductive capacity, recruitment and competitive ability of corals (Petes et al. 2003; Bruno et al. 2007; Weil and Cróquer 2008; Harvell et al. 2009; Borger and Colley 2010). The increase of frequency and intensity of disease outbreaks have contributed to massive mortalities that reduced population of several scleractinian species and generated shifts in coral communities at local and regional scales. For instance, repeated epizootic events have been the most significant source of mortality for Caribbean populations of the two most important framework-building species *Acropora palmata* and *A. cervicornis*, over the past 20-25 years (Aronson and Precht 2001; Patterson et al. 2002). In the Florida Keys more than 70% of *A. palmata* were decimated by white pox disease (WP) while 50% of sea fan tissue has been lost due to complete or partial disease-induced mortality (Kim and Harvell 2004). To date more than 30 distinct diseases of scleractinian corals, mainly identified according to their morphological aspect in the field (e.g. lesions or bands of tissue loss), have been reported with black band disease (BBD), white band disease (WBD), white plague disease (WPD), and growth anomalies found globally (Fig. 2) (Green and Bruckner 2000; Bruckner 2001; Frias-Lopez et al. 2002; Sutherland et al. 2004; Willis et al. 2004; Weil et al. 2006). Among these

diseases, only few putative agents including two ciliates (Cróquer et al. 2006; Bourne et al. 2008), one fungi (Sweet et al. 2013a) and six bacteria (Israely et al. 2001; Ben-Haim et al. 2003b; Sussman et al. 2006; Arboleda and Reichardt 2010; Sutherland et al. 2011; Ushijima et al. 2012) were characterised or/and identified experimentally by fulfilling Henle Koch's postulates (Koch 1891).

Figure 2: Coral diseases reported worldwide with (A) black band disease (BBD) on *Pavona* sp., (B) white band disease (WBD) on *Acropora* sp., white plague (WP) on *Montastraea annularis* (Bruckner 2002) and, growth anomaly (GA) on *Astreopora* sp.

Despite their increasing prevalence and virulence, little is known about the direct effects of biotic and abiotic factors on the infection, progression and transmission mechanisms of coral diseases. Nevertheless, several recent studies have found correlations mainly with temperature stress (Bruno and Selig 2007; Ward et al. 2007; Croquer and Weil 2009; Sokolow 2009;

Kuehl et al. 2011; Ruiz-Moreno et al. 2012) and nutrient loading (Bruno et al. 2003; Garren et al. 2009; Haapkylä et al. 2011; Kaczmarsky and Richardson 2011; Vega-Thurber et al. 2013). For instance in the Caribbean several coral diseases including BBD (Kuehl et al. 2011), WP (Patterson et al. 2002), and WPL (Harvell et al. 1999) were more prevalent or spread across colonies more rapidly during summertime. Similarly, on the Great Barrier Reef (GBR) white syndrome (WS) and atrementous necrosis (AN) were more prevalent in summer than winter (Jones et al. 2004; Willis et al. 2004). At microbial scale, anomalously high temperature could influence disease by altering basic biological and physiological properties of corals, particularly their resistance, impairing the balance between potential pathogen and host (Rosenberg and Ben-Haim 2002). The *Oculina patogonica-Vibrio shiloi* is an interesting model to illustrate the effects of thermal stress on host–pathogen interactions. At high summer seawater, this bacterium "*Vibrio shiloi*" expresses a cell surface adhesion required to fix the coral tissue and produces toxins that inhibiting photosynthesis and lysing the symbiotic zooxanthellae (Rosenberg and Ben-Haim 2002; Banin et al. 2003).

Pollutions associated with organic matter enrichment from run-off or sewage discharge have also been found to increase coral disease prevalence (Bruno et al. 2003; Kaczmarsky et al. 2005; Garren et al. 2009; Haapkylä et al. 2011; Vega-Thurber et al. 2013). For example, relationships between exposure to sewage and high prevalence were found for WPL, BBD, *Porites* ulcerative white spot disease (PUWSD), WP, Yellow blotch disease and AN (Patterson et al. 2002; Bruno et al. 2003; Kaczmarsky et al. 2005; Voss and Richardson 2006). In addition, experimental evidence showed that nutrient enrichment can increase the prevalence and/or severity of the dark spot syndrome (DSS) on *Siderastrea siderea* (Vega-Thurber et al. 2013) and yellow band disease (YBD) on *Montastrea annularis* (Haapkylä et al. 2011).

4. Status of coral diseases on the Western Indian Ocean reefs

The Western Indian Ocean (WIO) coral reefs range from, west to east, the coast of East Africa (Kenya to South Africa), Pemba, Zanzibar, Comoros islands (Anjouan, Moheli, Mayotte), Eparse Islands (Mozambique channel), Madagascar, Mascarenes (Reunion, Mauritius, Rodrigues), and Seychelles (Fig. 3). Coral reefs are divided into four reef systems (Obura 2005; Richmond 2011) including 1) the patch reefs mainly along the cost from South Africa to Mozambique, 2) fringing reefs which are the most common reef structure in the WIO (e.g. Reunion, Rodrigues, Mayotte, Zanzibar), 3) Barrier reefs in Mayotte and Mauritius, and 4) atolls such as Europa and Bassas de India.

Figure 3: Map of the Western Indian Ocean (Adapted from Mangubhai (2007)). The main coral reefs are in red on the map.

While coral bleaching have received intensive attention since the 1998 El Nino Southern Oscillation (ENSO) event (Bigot and Quod 2000; Cole et al. 2000; Goreau et al. 2000; McClanahan 2000; Spencer et al. 2000; Celliers and Schleyer 2002; Chabanet 2002; Conand et al. 2002; McClanahan et al. 2004a; Obura 2005; McClanahan et al. 2007), certainly the most destructive coral bleaching ever recorded in the region (50-60% mortality) (Obura 2005), very little research has focused on diseases of the WIO reefs (McClanahan et al. 2004a). To date, no report other than visual observations indicating their presence has been done in the region. In Zanzibar, bacteria-induced bleaching (Ben-Haim and Rosenberg 2002), black band, white band, and yellow band diseases were reported in isolated outbreaks (McClanahan et al. 2004b). In Kenya and Tanzania, a newly white syndrome associated with an infection of fungal hyphae, has been reported affecting populations of *Montipora*, *Astreopora* (McClanahan et al. 2004b). Finally Ernesto Weil (University of Puerto Rico) in 2005 has reported the presence of several diseases in Kenya and Tanzania including WS, GA, BBD, PUWS, compromised tissue responses and pigmentation response (Harvell et al. 2007).

5. Aims and objectives of this study

This PhD study aims at providing data on the prevalence and variability at local, regional and temporal scales, on three targets coral reef from the Western Indian Ocean. Moreover, this study focused on the characterization and identification of the agents of the pathogens combining with techniques from microbiology. The specific objectives are:

1. Identifying the main coral disease signs/ syndromes by systematically describing gross and microscopic lesions in scleractinian corals;
2. Investigating their impact on coral communities by quantifying their prevalence across three WIO reef ecosystems;

3. Determining the spatial distribution, and potential seasonal variations of coral disease outbreaks;
4. Characterizing microscopic morphology of lesions in corals;
5. Describing and comparing the bacterial communities associated with both healthy and disease coral colonies;
6. Identifying the putative pathogenic agents associated/responsible to the selected disease signs/syndromes.

6. PhD outlines

This thesis, comprising four main chapters (Chapter 2-5), is the first quantitative and qualitative study assessing the ecology and aetiology of infectious coral diseases on the Western Indian Ocean coral reefs. Chapter 2 identifies the main coral diseases and quantifies their prevalence at these localities, determining their spatial distribution and seasonal variation on three target coral reefs in Reunion, South African and Mayotte. Chapter 3 provides the first characterisation of bacterial communities associated with the recently reported disease, *Porites* white patch syndrome (PWPS, Séré et al. 2012) using both histopathology and culture-independent molecular techniques. The study in chapter 4 identifies the causal agent(s) of PWPS by combining traditional culturing methods and molecular techniques coupled with inoculation trials to test for Koch's postulates. Strains inducing PWPS in aquaria were further characterised using standard bacteriological tests. Effects of elevated seawater temperature, identified as an important abiotic factor enhancing the virulence several coral diseases were also tested in aquarium experiments. Chapter 5 provides a comprehensive characterization of an atypical black band disease by using a multidisciplinary approach involving field surveys, gross lesion monitoring, histopathology and 16S barcoding by 454-pyrosequencing to investigate bacterial community compositions.

Chapter 2

Identification and prevalence of coral diseases observed on three Western Indian Ocean (WIO) coral reefs

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1. Abstract

Coral diseases have caused a substantial decline in the biodiversity and abundance of reef-building corals. To date, more than 30 distinct diseases of scleractinian corals have been reported and are known to generate progressive tissue loss and affect coral growth rate, reproductive capacity, recruitment, species diversity and the abundance of reef-associated organisms. While coral disease investigations have increased over the last 40 years, very little is known about coral diseases in the Western Indian Ocean (WIO). Surveys conducted at multiple sites in Reunion, South Africa and Mayotte between August 2010 and June 2012 revealed the presence of six coral diseases: black band disease (BBD), white syndromes (WS), pink line syndrome (PLS), growth anomalies (GA), skeleton eroding band (SEB) and *Porites* white patch syndrome (PWPS). The overall disease prevalence was higher in Reunion (7.5 ± 2.2%; mean ± SE) compared to South Africa (3.9 ± 0.8%; mean ± SE) and Mayotte (2.7 ± 0.3%; mean ± SE). Compiling results from the three distant locations, *Acropora* and *Porites* were the genera most vulnerable to disease. Spatial variability was detected in both Reunion and South Africa with BBD and WS more prevalent on shallow than deep reefs. There was also evidence of seasonality in two diseases: BBD and WS, their prevalence being higher in summer than winter. This was the first study to investigate the ecology of coral diseases in the WIO reefs and surveys should be expanded to confirm these patterns.

Keywords: Coral diseases - Western Indian Ocean - Scleractinian corals - Seasonality - Spatial variability.

2. Introduction

The emergence and spread of infectious diseases have caused substantial declines in the biodiversity and abundance of reef-building corals during the last 40 years (Garzón-Ferreira et al. 2001; Weil et al. 2006). For example, white syndromes (WS) including white band disease (WBD), white plague (WP) and white pox disease (WPD), have been the most significant cause of mortality in Caribbean populations of the two most important framework-building species over the past 20-25 years, Acropora palmata and A. cervicornis (Patterson et al. 2002; Sutherland et al. 2004; Weil et al. 2006). To date, more than 30 distinct diseases, affecting at least 150 scleractinian corals, have been reported worldwide (Sutherland et al. 2004; Weil et al. 2006). Most of them are known to generate tissue loss and subsequently affect growth rate, reproductive capacity, recruitment and the competitive ability of corals (Petes et al. 2003; Sutherland et al. 2004; Bruno and Selig 2007; Weil and Cróquer 2008). For instance, several studies have shown that black band disease (BBD) can generate the loss of up to 2 cm of tissue per day by producing high concentrations of sulphide that kill the coral tissue (Boyett et al. 2007; Haapkylä et al. 2009; Sato et al. 2009). Despite their increased global prevalence and virulence, little is known about coral diseases on Indian Ocean coral reefs. Currently, surveys carried out on Maldivian reefs have reported the presence six scleractinian diseases including BBD, WBD, white spot, pink spot, WP and YBD, affecting eight coral genera (Montano et al. 2012). On Indian reefs, Thinesh and colleagues (2011) estimated that 21% of corals were affected by diseases (BBD, WBD, white spot, pink spot, WP and YBD). In contrast, very few coral diseases types have been reported on Western Indian Ocean (WIO) reefs. Bacteria-induced bleaching (Ben-Haim and Rosenberg 2002), BBD, WBD, and yellow band disease (YBD) have been observed in isolated outbreaks in Zanzibar (McClanahan et al. 2004b). In Kenya and Tanzania, a white syndrome associated with infection by fungal hyphae has been reported on *Montipora* and *Astreopora* (McClanahan et al. 2004b). However no in-

depth studies quantifying the current status of coral diseases have been performed on WIO reefs. Thus, the goals of this study were to investigate the prevalence and variability of coral diseases at temporal and spatial scales, focusing on three target coral reefs in Reunion, South African and Mayotte (fig. 1). The aims of the study were to identify the main coral diseases and quantify their prevalence at these localities, determining their spatial distribution and seasonal variation.

3. Methods

3.1. Study sites

Corals form fringing reefs at Reunion (21°07'S; 55°32'E; Fig. 4) and are 12 km² in area along 25 km of the coastline, mainly on the dry west coast. Three geomorphological zones are evident (Montaggioni and Faure 1980): 1) an outer reef slope (5-30 m) exposed to high turbulence and characterised by a basaltic substratum in alternating spurs and grooves, mostly covered by massive and encrusting corals, 2) a reef flat (0.5-2 m), generally composed of branching corals, and 3) an inner back-reef covered with sand and rubble (0.5-1 m). Due to their proximity to the coastline (± 500 m wide), these fringing reefs are subjected to high and increasing anthropogenic stressors such as water eutrophication from land-based pollution and overfishing and trampling.

South African reefs (1.9 km^2) are not typically accretive (Schleyer 2000); the corals grow on late-Pleistocene beach rock, originating from submerged, fossilised coastal sand dunes (Ramsay 1996). In topography, the reefs consist of shallow pinnacles (8-10 m), extensive deep subtidal reef flats (14-18 m) and a sloping fore-reef edge (24-27 m; Celliers and Schleyer (2008)). The warm Agulhas Current, the prevailing regional current (maximum speed 1.5 m s^{-1}), generates sub-tropical conditions in the area. All the reefs are located in marine protected areas and are in good condition.

Mayotte reefs consist of a large (15 km wide), deep (30-35 m) lagoon surrounded by a long barrier reef (150 km), which is 1.5 km wide in some areas and interrupted by 12 deep channels. Fringing reefs are also present along 210 km of the coastline of the island. A discontinuous, inner secondary barrier reef system (12 km long) is located on the south-west coast. Mayotte reefs are subjected to increasing pressures associated with human

development. Abnormally high sedimentation rates due to mangrove deforestation constitute the main stressors but the reefs are also damaged by waste-water discharge and overfishing.

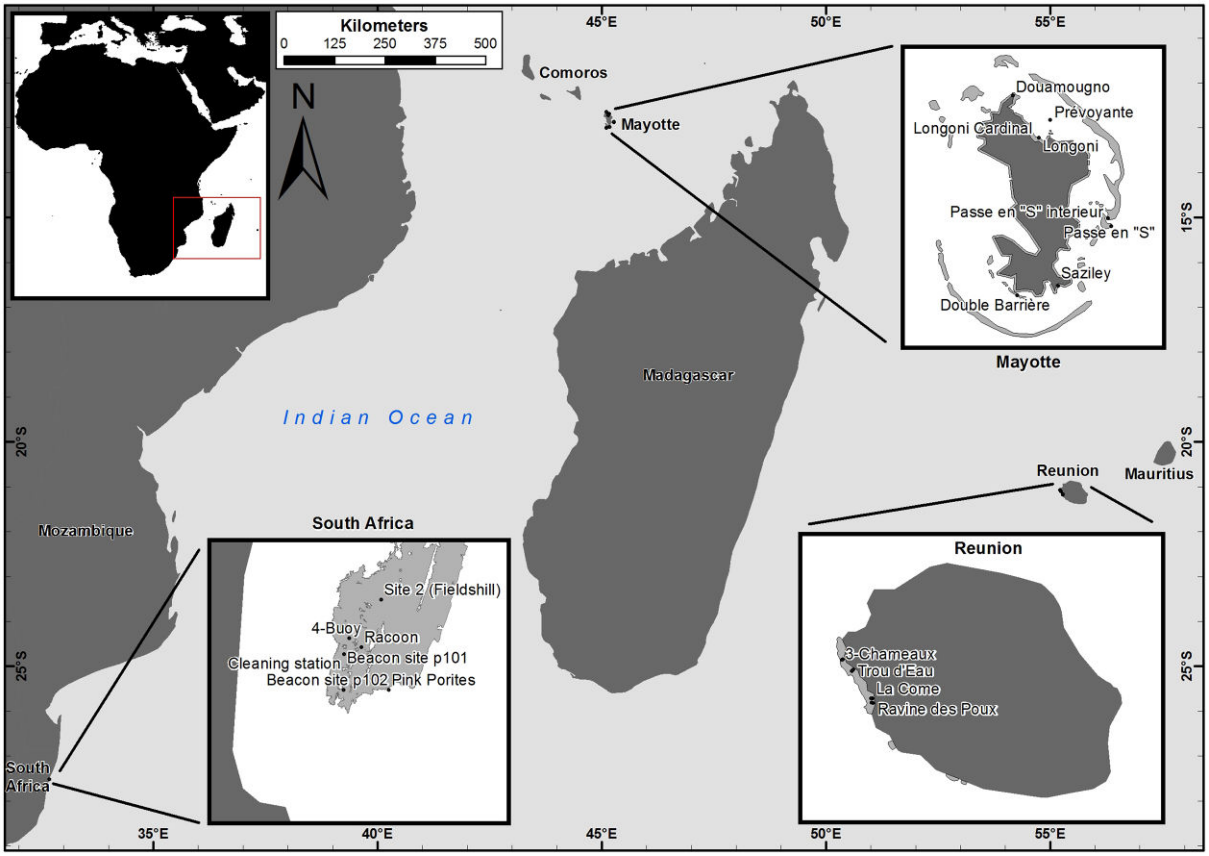


Figure 4: Map showing the study locations in the Western Indian Ocean

3.2. Survey methods

Surveys in South Africa were conducted on Two-mile Reef (TMR) in the central Maputaland reef complex at Sodwana-Bay in northern KwaZulu-Natal (27°24' S; 32°41'E). These were conducted at seven sites along a north-south gradient on TMR at two depth intervals: 8-10 m (shallow inshore region) and 12-16 m (deeper offshore region). On Mayotte, surveys were conducted at eight latitudinal sites on the barrier and fringing reef (Table 1 and Fig. 4). In Reunion, surveys were undertaken at four latitudinal sites (Table 1 and Fig. 4) on the outer reef slope and reef flat. Protocols were adapted to the different geomorphological zones. Five 10 × 2 m (1 m on each side of the transect line) transects were randomly laid parallel to depth

contours at each site at Sodwana Bay and Mayotte. A gap of 20 m was left between transects to ensure independence in the data for statistical analysis. At Reunion, the outer reef slope is characterized by a succession of spurs and grooves that represent different habitats. Spurs are covered mainly by hard corals, whereas grooves are often filled with sand and coral rubble. In order to stay within the coral community, five 10 m x 2 m belt-transects were laid along the spurs. Surveys on the inner reef flat were conducted along three 20 m x 2 m belt-transects positioned parallel to the coastline, again to avoid crossing different coral communities.

Table 1: Geographic coordinates and details of the survey sites.

Country	Reef	Sites	Profile	Depth (m)	Coordinates
South Africa	Two-mile Reef	Fields Hill	Deeper offshore region	14-15	-27.31010° ; 32.41419°
		4-Buoy	Shallow inshore region	9-11	-27.31276° ; 32.41206°
		Racoon Bommie	Deeper offshore region	15-16	-27.31325°; 32.41287°
		Cleaning station	Deeper offshore region	12-14	-27.31516° ; 32.41111°
		Pink Porites	Deeper offshore region	12-14	-27.31610° ; 32.41469°
		Beacon 101	Shallow inshore region	10-11	-27.31373° ; 32.41171°
		Beacon 102	Shallow inshore region	9-10	-27.31610° ; 32.41169°
Reunion	L'Ermitage	3-Chameaux	Reef flat	0.5-1	-21.080351° ; 55.219576°
			Reef slope	10-12	-21.081281° ; 55.217590°
	La Saline	Trou d'Eau	Reef flat	0.5-1	-21.103312° ; 55.242294°
			Reef slope	10-12	-21.106160° ; 55.239540°
	Saint-Leu	La Corne	Reef flat	0.5-1	-21.165960° ; 55.285080°
			Reef slope	10-12	-21.165940° ; 55.281930°
		Ravine des Poux	Reef flat	0.5-1	-21.176397° ; 55.285985°
			Reef slope	10-12	-21.175490° ; 55.283460°
Mayotte	North reefs	Douamougno	Fringing reef	3.5	-21.080351° ; 55.219576°
		Prévoyante	Barrier reef	7.0	-21.103312° ; 55.242294°
	North-east reefs	Longoni	Fringing reef	2.5-3.0	-21.165960° ; 55.285080°
		Longoni Cardinale	Fringing reef	2.0-3.0	-21.176397° ; 55.285985°
	East reefs	Passe en S extérieur	Barrier reef	10-12	-21.081281° ; 55.217590°
		Passe en S Intérieur	Barrier reef	5-10	-21.106160° ; 55.239540°
	South-reefs	Saziley	Fringing reef	3-6	-21.165940° ; 55.281930°
		Double Barrière	Barrier reef	6-10	-21.175490° ; 55.283460°

Scleractinian corals displaying evidence of disease were identified to the genus level and counted within each transect. Additionally, all coral genera exhibiting comparable gross lesions were considered to have the same disease (e.g. BBD, WS, etc.). Bleaching and necrosis were considered an impairment of normal function and were also recorded. The

prevalence of diseases was estimated as the number of diseased colonies divided by the total number of coral colonies >2 cm, identified to the genus level and counted within each transect in 1 × 1 m photoquadrats which covered the transect area (20-40 photo-quadrats per transect). Finally, surveys in both Reunion and South Africa were conducted over two consecutive summers and winters to gain a measure of seasonality in the prevalence of the diseases. In Mayotte, coral diseases could only be monitored during the summer and winter of 2012.

3.3. Disease identification

Gross lesions observed during the surveys were photographed and identified using the Underwater Cards for Assessing Coral Health on Caribbean and Indo-Pacific Reefs (Beeden R et al. 2008) and illustrations/descriptions available in the literature. It has been established that similar gross lesions can be manifested by multiple microscopic pathologies and/or different causal agents (Work and Rameyer 2005). Therefore, to avoid subjective interpretations and verify field observations, samples of each coral disease was described according to the systematic nomenclature developed by Work and Aeby (2006)

3.4. Statistical analysis

Disease prevalence, calculated per transect, was averaged for each site in each region. Data were tested prior to analysis for homoscedasticity (Levene's test) and normality of variance (Kolmogorov-Smirnov and Lilliefors tests) and were then log transformed [$\log_{10}$ (X)] for analysis of variance (ANOVA). Variations in the prevalence of coral disease between regions over the two survey years were tested in the consecutive summers and winters and across reef zones (shallow vs. deep) using two-way factorial ANOVA (STATISICA 8). Similarly, seasonality and spatial variations in the most prevalent diseases were examined using two-way factorial ANOVA. Finally, post hoc Fisher LSD tests were performed for multiple group comparisons.

4. Results

4.1. Description of the disease gross lesions in situ

A total of 76 coral disease surveys were conducted within the three regions at 22 sites between September 2010 and March 2012, covering an area of 7920 m^2 of reef. Photographs and samples taken from the reefs revealed the presence of six coral diseases manifesting discoloration, tissue loss and growth anomalies (Fig. 5). They included white syndromes (WS), black band disease (BBD), pink line syndrome (PLS), skeletal eroding band (SEB), growth anomalies (GA) and a newly identified coral disease, the *Porites* white patch syndrome (PWPS, Séré et al. 2012). These are characterised in Table 2.

4.2. Coral disease prevalence and susceptibility

While the most prevalent coral disease recorded in Reunion was PLS followed by PWPS, BBD and WS, the most common disease on both South African and Mayotte reefs was WS followed by a much lower prevalence of any other (Table 3). The prevalence of all the coral diseases encountered varied significantly between the three regions (ANOVA; F= 7.72, $p<0.01$; Table 4); the lowest total mean prevalence was recorded in Mayotte with 2.7 ± 0.3 % (mean ± SE) between 2011 and 2012 and the highest in Reunion with diseases affecting 7.0 ± 1.2 % and 8.1 ± 0.2 % (mean ± SE) of all coral colonies between 2010-2011 and 2011-2012 respectively. In South Africa, the proportion of infected coral colonies was low but quite variable between the two survey periods, with the average disease prevalence higher (ANOVA; F= 4.72, $p<0.01$) in 2011-2012 (4.9 ± 0.9%; mean ± SE) than 2010-2011 (2.9 ± 0.8%; mean ± SE).

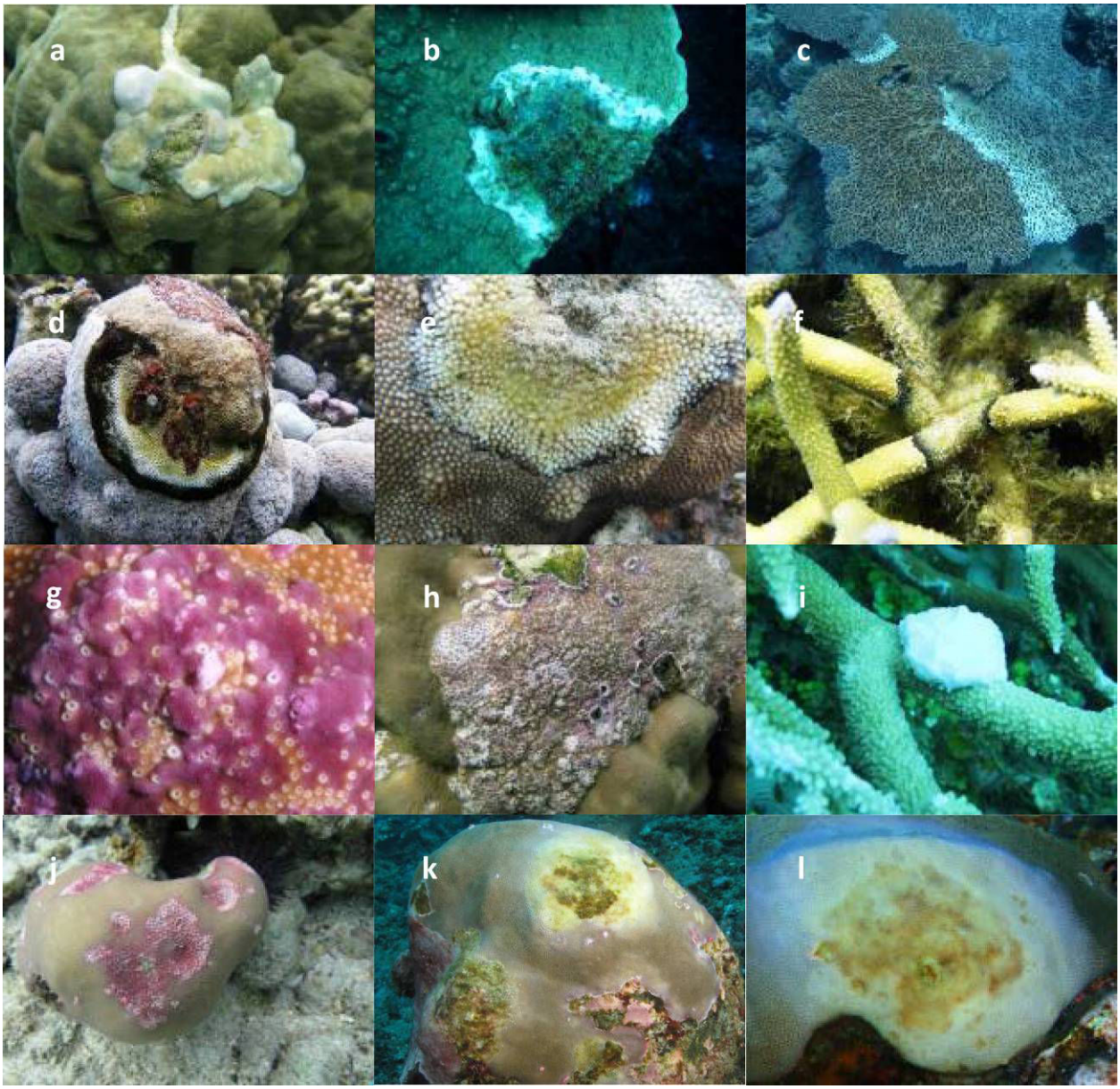


Figure 5: Principal coral diseases observed on the WIO reefs. White syndrome (WS) on (a) *Porites lutea* (b) *Montipora* sp. and (c) *Acropora* sp. Active black band disease (BBD) on (d) *Goniopora djiboutinensis* and (e) *Hydnophora* sp. Skeleton eroding band (SEB) on (f) *Acropora* sp. growth anomalies (GA) on (g) *Astreopora* sp., (h) *Porites lobata* and (i) *Acropora* sp. Pink line syndrome (PLS) on (j) *Porites lobata*. White patch syndrome (PWPS) on (k and l) *Porites lutea*.

Table 2: Description and characterisation of coral diseases observed on Reunion, Mayotte and South African reefs.

Coral diseases (code)	Description/Characteristics	Infected hosts
White syndrome (WS) (Fig. 5a-c)	Diffuse or distinct peripheral, basal or apical areas of bleached tissue, separating unaffected tissue from the intact but white skeleton or skeleton recently covered by green or brown algae.	*Acropora* spp., *Astreopora* spp., *Platygyra daedalea* and , *Pocillopora verrucosa*
Black band disease (BBD) (Fig. 5d, e)	Thin or wide undulating to serpiginous dark brown to black band, comprised predominantly of cyanobacteria. This band separates the unaffected tissue from a distinct area of tissue loss in which intact bare white skeleton is revealed (indicating acute to sub-acute tissue loss).	*Astreopora, Coscinarea, Echninopora, Favia, Favites, Hydnophora, Pavona* and, *Platygyra*
Pink line syndrome (PLS) (Fig. 5j)	Smooth to undulating band comprised of pink-coloured polyps, varying in width from a few millimetres to a few centimetres. This band separates the unaffected tissue from oblong to circular areas of tissue loss, the latter generally being diffused, centrally to peripherally situated and exposing skeleton covered by green or brown algae.	*Porites* spp.
Growth anomaly (GA) (Fig. 5g-i)	Focal, smooth to undulating surfaces, located principally at the surface of the colonies and containing partially-formed, disorganized, exert calices.	*Astreopora* and *Porites*
Porites white patch syndrome (PWPS) (Fig. 2k, l)	Diffuse, medium to large (50-300 mm diameter), circular to oblong tissue loss, surrounded by swollen white tissue. The older exposed skeleton is progressively colonized by endophytic algae and cyanobacteria.	*Porites lutea* and *P. lobata*
Skeletal eroding band (SEB) (Fig. 5f)	Thin, undulating to smooth black band, followed by a white band of bleached tissue, separating live tissue from a diffuse area of tissue loss. The exposed skeleton is generally colonised by endophytic algae.	*Acropora muricata*

Table 3: Overall occurrence (number of diseased coral colonies) and prevalence (the number of diseased coral colonies divided by the total number of colonies identified to the genus level within each transect; ± SE) of the main coral diseases in coral genera on Reunion (n= 23562 coral colonies), South African (n= 17140 coral colonies) and Mayotte (n= 19426 coral colonies) reefs between 2010 and 2012.

	Reunion		South Africa		Mayotte	
Disease condition	**Occurrence**	**Prevalence (%)**	**Occurrence**	**Prevalence (%)**	**Occurrence**	**Prevalence (%)**
Bleaching (Blea)	0.0	0.0	0.5 (1.4)	0.4 (0.6)	0.0	0.0
White syndrome (WS)	1.2 (0.5)	1.5 (0.5)	2.4 (1.1)	2.1 (0.7)	2.0 (1.1)	1.0 (1.4)
Pink line syndrome (PLS)	3.4 (1.2)	2.0 (0.9)	0.5 (0.6)	0.5 (0.6)	0.3 (1.2)	0.1 (1.5)
Porites white patch syndrome (PWPS)	2.7 (0.2)	2.3 (0.6)	0.2 (0.8)	0.2 (0.3)	1.0 (0.8)	1.0 (1.4)
Black band disease (BBD)	1.4 (1.2)	1.3 (0.5)	0.3 (0.7)	0.4 (0.2)	0.05 (0.4)	0.1 (0.6)
Necrose (Nec)	0.2 (0.3)	0.1 (0.5)	0.3 (0.5)	0.8 (0.7)	0.2 (0.8)	0.5 (1.8)
Growth anomaly (GA)	0.1 (0.6)	0.1 (0.1)	0.1 (0.8)	0.1 (0.3)	0.8 (2.0)	0.01 (0.8.7)
Skeletal eroding band (SEB)	0.2 (0.6)	0.2 (1.0)	0.0	0.0	0.0	0.0
All diseases	**9.3**	**7.5 (2.2)**	**3.8**	**3.9 (0.8)**	**4.2**	**2.7 (0.3)**

Table 4: Mean coral disease prevalence (± SE) in three Western Indian Ocean regions during successive winters and summers in 2010-2012.

Location	Period/Dates	Season	Prevalence
Reunion	September 2010	Winter 1	6.8 (2.2)
	December 2011	Summer 1	7.2 (2.4)
	2010-2011		**7.0 (2.4)**
	October 2011	Winter 2	8.3 (2.5)
	January 2012	Summer 2	7.8 (2.2)
	2011-2012		8.1 (0.2)
	Total		7.5 (2.2)
South Africa	February 2011	Summer 1	3.9 (1.8)
	July 2011	Winter 1	1.9 (0.9)
	2011		2.9 (0.8)
	February 2012	Summer 2	4.1 (0.3)
	June 2012	Winter 2	5.7 (0.7)
	2012		4.9 (0.9)
	Total		3.9 (0.8)
Mayotte	August 2011	Winter	2.3 (2.2)
	March 2012	Summer	3.1 (1.4)
	2011-2012		2.7 (0.3)
	Total		2.7 (0.3)

Acropora, *Goniopora*, *Hydnophora* and *Porites* seemed to be the most susceptible coral genera to disease on both the reef slope and reef flat in Reunion (Fig. 6). White syndromes (WS) were the most common disease affecting branching colonies of *Acropora* spp., only in the shallowest zone of the reef. Colonies of *Goniopora* sp., *Hydnophora* sp., and *Porites* spp. were most susceptible to BBD, especially those on the reef-flat. Massive colonies of *Porites* spp. appeared to be the most vulnerable to disease, exhibiting multiple infections including PLS, PWPS, BBD, WS, GA and Nec. In South Africa, 11 coral genera were observed with signs of disease (Fig. 7). The most susceptible coral genera were *Astreopora*, *Hydnophora*, *Pocillopora* and *Porites*. GA, WS and Nec were more prevalent on encrusting and massive *Astreopora* spp. in summer and winter during both survey years. Both BBD and WS were the most prevalent diseases on *Hydnophora* sp. on shallow reefs. Colonies of *Pocillopora* spp. exhibited high susceptibility to WS, whereas the massive corals *Porites lutea* and *P. lobata*, were more vulnerable to PWPS and PLS. Of the eight genera observed with diseases on both the barrier and fringing reefs of Mayotte (Fig. 8), *Acropora*, *Astreopora* and *Porites* seemed to be the most susceptible to disease. Colonies of *Astreopora* sp. were highly susceptible to GA and WS. *Acropora* spp. appeared to be particularly vulnerable to WS, whereas *Porites* spp, showed a particular susceptibility to GA, BB, PLS and Nec (Fig. 8).

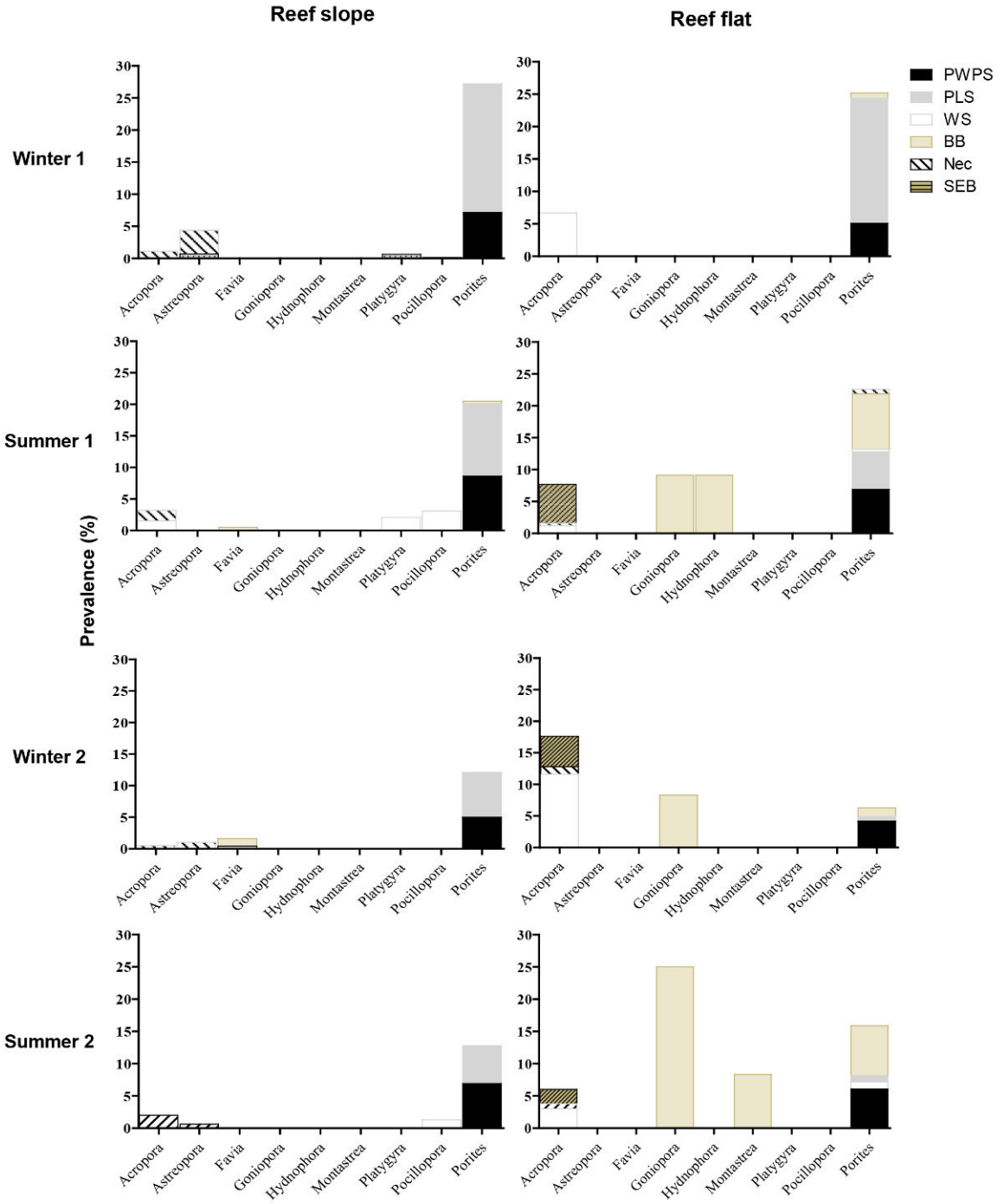


Figure 6: Prevalence (%) of the main coral diseases in nine scleractinian genera in Reunion: *Porites* white patch syndrome (PWPS), white syndromes (WS), pink line syndrome (PLS), black band disease, growth anomalies (GA) and skeleton eroding band (SEB). Prevalence is calculated relative to the total number of colonies in the respective taxa, per coral genus and per reef zone (reef flat and reef slope) for two consecutive summers and winters. Note that necrosis (Nec) is included in the analysis.

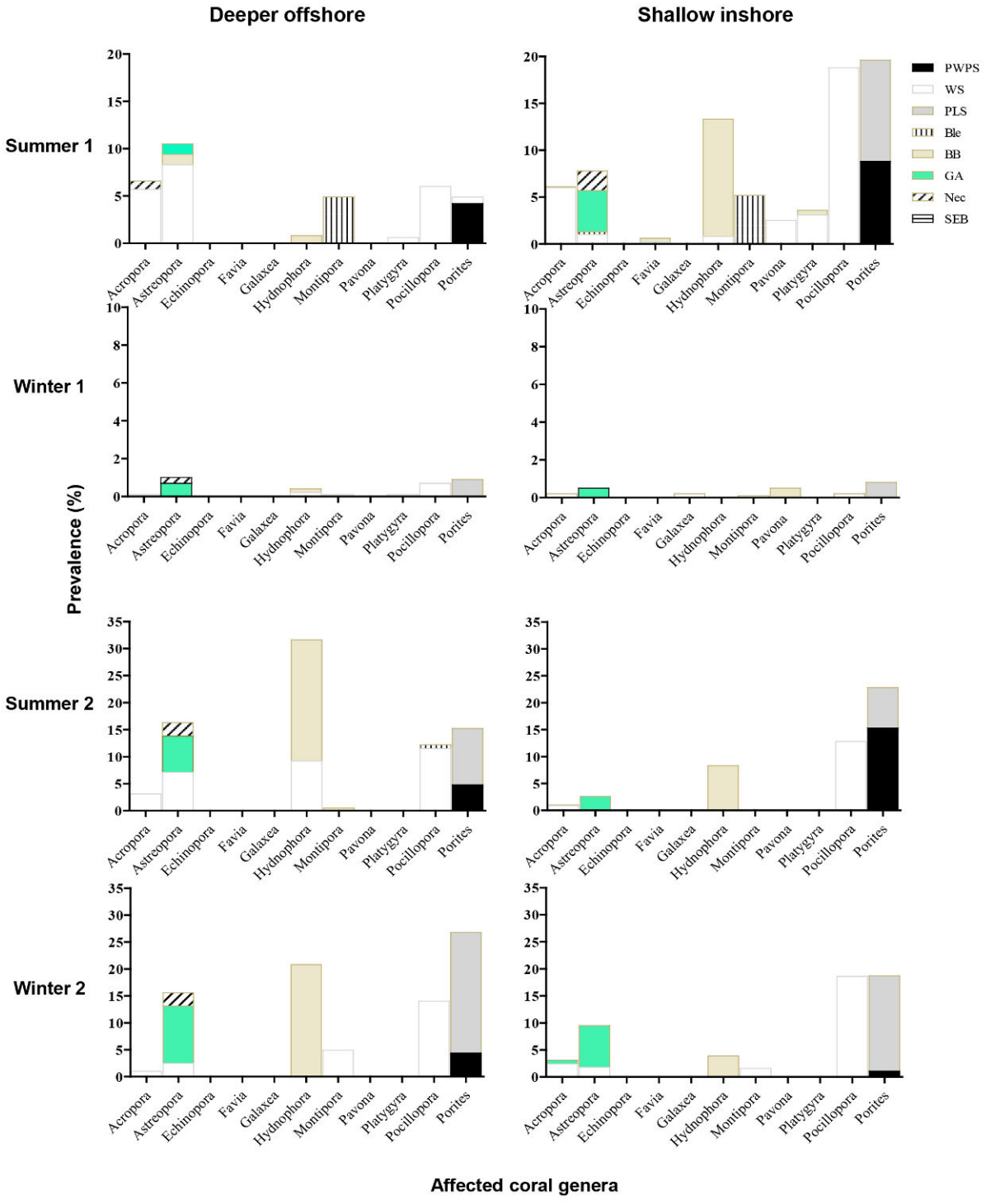


Figure 7: Prevalence (%) of the main coral diseases in 11 scleractinian genera at Sodwana-Bay, South Africa: *Porites* white patch syndrome (PWPS), white syndromes (WS), pink line syndrome (PLS), black band disease (BBD) and growth anomalies (GA). Prevalence is calculated relative to the total number of colonies in the respective taxa, per reef zone for two consecutive summers and winters. Note that bleaching (Ble) and necrosis (Nec) are included in the analysis.

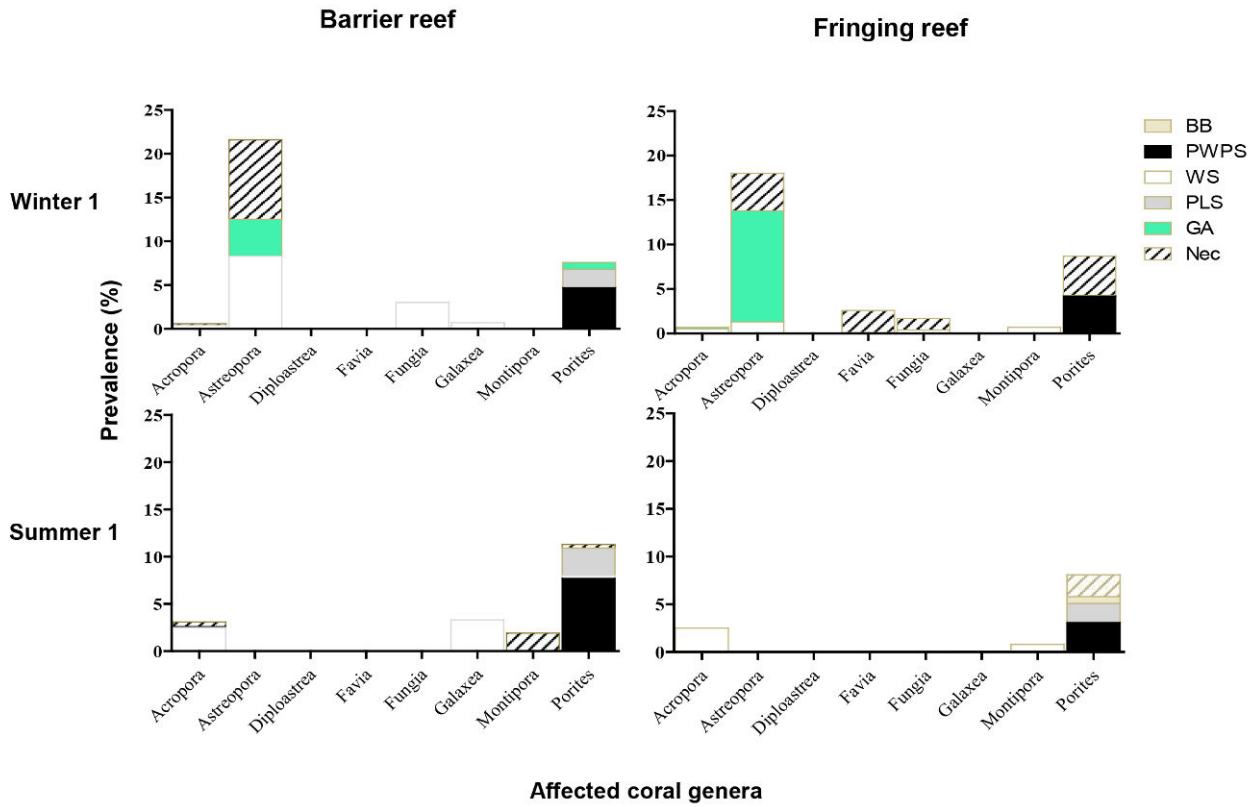


Figure 8: Prevalence of the main coral diseases in eight scleractinian genera in Mayotte: *Porites* white patch syndrome (PWPS), white syndromes (WS), pink line syndrome (PLS), black band disease (BBD) and growth anomalies (GA). Prevalence is calculated relative to the total number of colonies in the respective taxa per coral genus for one consecutive summer (March 2012) and winter (August 2011). Note that necrosis (Nec) are included in the analysis.

4.3. Seasonal and spatial variations in coral disease prevalence

There was no evidence of seasonality in the overall prevalence (all diseases pooled) of coral diseases between winter and summer at the studied localities (ANOVA, $p>0.05$; Table 5). However, coral disease prevalence differed significantly between reef zones in Reunion (ANOVA, $p<0.01$; Table 5) with a greater incidence on the reef flat than the reef slope (Fisher LSD, $p<0.001$). No difference was detected in South Africa and Mayotte between the shallower and deeper reefs (Fisher LSD, $p>0.05$). Of the seven coral disease states recorded in Reunion, the prevalence of only BBD seemed to vary between the reef zones and seasons

(Table 5). Its prevalence was significantly higher on the reef flat (ANOVA, $p<0.001$; Table 5) with the percentage of infected colonies being significantly higher in summer than winter (Fisher LSD, $p<0.001$). This spatial and seasonal pattern was observed particularly on *Porites* colonies which exhibited a higher mean BBD prevalence in summer (Fisher LSD, $p<0.001$; Table 5; Fig. 6) and at the shallowest sites (Fisher LSD, $p<0.05$). Finally, WS varied significantly between reef zones (ANOVA, $p>0.05$, Table 5) but no significant difference was found between seasons (ANOVA, $p>0.05$). Among diseases recorded on South African reefs, WS was seasonal on *Acropora* (ANOVA; $p<0.05$; Table 5; Fig. 7), and *Pocillopora* (ANOVA; $p<0.001$), with a higher percentage of infected colonies in summer than winter. A similar, seasonal trend was evident for PWPS but the difference between summer and winter was not significant (ANOVA, $p>0.05$; Table 3). Finally, a significantly higher prevalence of WS was recorded on *Pocillopora* (ANOVA; $p<0.01$; Table 5; Fig. 7) in the shallow South African reef zones (Fisher LSD, $p<0.001$). At Mayotte, WS on *Acropora* spp. varied significantly between seasons (ANOVA, $p<0.05$; Table 5; Fig. 8), with a higher prevalence in winter than in summer (Fisher LSD, $p<0.05$). However, no significant difference was recorded between the fringing and barrier reef (ANOVA, $p>0.05$).

Table 5: Summary of factorial ANOVA testing of seasonal and spatial variations in coral diseases on Reunion (REU), South African (RSA) and Mayotte (MAY) reefs across reef zones (Shallow=S; Deep=D) over two consecutive summers (S1-2) and winters (W1-2). Analyses were performed on the mean prevalence of coral disease between regions, the most prevalent diseases (BBD, PWPS and WS) and the most susceptible coral genera including *Acropora* (*Acr*), *Pocillopora* (*Poc*) and *Porites* (*Por*). *: Significant difference (p<0.05); **: Highly significant difference (p< 0.01); ns: no significant difference (p>0.05).

Factorial ANOVA					FISHER LSD			Interpretation
Location	**Disease**	**Factor tested**	***F***	**p**	**REU**	**RSA**	**MAY**	**Significant**
All locations	**All diseases**	Season	8.58	>0.05	ns	ns	ns	S = W
		Zone	5.71	*	**	ns	ns	REU: DE ≠ SH
		Season×Zone	0.97	>0.05	ns	ns	ns	S×DE.SH = W×DE.SH
	BBD	Season	2.04	0.1	ns	ns	ns	S = W
		Zone	16.62	**	**	ns	ns	REU: DE ≠ SH
		Season×Zone	5.35	*	**	ns	ns	REU: SH×S ≠ SH×W
	PWPS	Season	0.93	0.33	ns	ns	ns	Summer = winter
		Zone	0.70	0.49	ns	ns	ns	DE = SH
		Season×Zone	0.85	0.49	ns	ns	ns	S×DE.SH = W×DE.SH
	WS	Season	1.5	0.22	ns	ns	ns	S = W
		Zone	5.45	*	**	ns	ns	REU: DE ≠ SH
		Season×Zone	3.13	*	ns	ns	*	MAY: SH×S ≠ SH×W
Reunion	**PorBBD**	Season	5.07	*	**	-	-	S1 ≠ W1
		Zone	38.67	**	**	-	-	DE ≠ SH
		Season×Zone	2.73	*	*	-	-	S×SH≠W×SH
South Africa	**AcrWS**	Season	4.42	*	-	**	-	S1 ≠ W1
		Zone	1.12	0.30	-	ns	-	DE = SH
		Season×Zone	0.71	0.55	-	ns	-	S×SH=W×SH
	PocWS	Season	14.15	**	-	**	-	S1≠W1≠S2=W2
		Zone	17.87	**	-	**	-	DE ≠ SH
		Season×Zone	4.26	*	-	*	-	**Year 1**: S*DE.SH≠W×DE.SH **Year 2**: S×*DE≠W×DE
Mayotte	**AcrWS**	Season	4.85	*	-	-	*	S1 ≠ W1
		Zone	0.49	0.48	-	-	ns	DE = SH
		Season×Zone	0.10	0.74	-	-	ns	S×SH=W×SH

5. Discussion

This study constitutes the first assessment of coral diseases in the Western Indian Ocean (WIO). It provides baseline information with qualitative and quantitative data for three WIO countries, viz. Reunion, South Africa and Mayotte. Surveys revealed the presence of seven coral diseases: white syndromes (WS), black band disease (BBD), skeleton eroding band (SEB), pink line syndrome (PLS), growth anomaly (GA), necrosis (Nec) and a newly-identified disease, *Porites* white patch syndrome (PWPS). Except for PWPS, diseases recorded during this study have been previously reported within other regions across the Indian Ocean including the Chagos Archipelago (Sheppard et al. 2012), Republic of Maldives (Onton et al. 2011), South India (Thinesh et al. 2009; Thinesh et al. 2011) and the Indo-pacific region (Willis et al. 2004; Raymundo et al. 2005; Aeby et al. 2006).

The overall disease prevalence in Reunion (Table 6; 7.0-8.1%) was higher than that found in either South Africa (2.9-4.9%) or Mayotte (2.7%). Disease levels on Reunion reefs were also higher than those reported on other Indian Ocean reefs such as Ningaloo Reef in Australia (Onton et al. 2011), the Chagos Archipelago (Sheppard et al. 2012) or the Maldives (Montano et al. 2012). However, values obtained for Reunion were similar to those on Mandapam reefs in South India but were substantially lower than those recorded at Palk Bay, also in South India. This relatively high disease level may be attributable to the fact that fringing reefs in Reunion are young and adjacent to areas of high coastal development, and are thus subjected to stressors such as poor water quality caused by anthropogenic activities (urbanisation and agriculture in watersheds, waste-water discharge, sedimentation, over-exploitation and over-frequentation of reefs). This assumption was also proposed by Thinesh et al. (2011) to explain diseases patterns in Palk Bay and on Mandapam reefs. Nevertheless, more investigations are

needed to identify factors that may facilitate disease outbreaks or exacerbate their effects on coral reefs.

Table 6: Overall coral disease prevalence (± SE) and diseases recorded on Indian Ocean coral reefs. WS: white syndrome; BBD: black band disease; PWPS: *Porites* white patch syndrome; PLS: pink line syndrome; SED: skeleton eroding band; Nec: necrosis; GA: growth anomaly; BrB: brown band disease. WBD: white band disease; WP: white plague disease; YB: yellow band disease; PS: pink spot; UWS: ulcerative white spot; PDDr: *Porites* dark discoloration response.

Geographic distribution	Prevalence (%)	Coral diseases observed	Sources
Western Indian Ocean			
Reunion (2010-2011)	**7.0 (2.4)**	WS. BBD. PWPS. PLS. SEB. Nec. GA	**Present study**
Reunion (2011-2012)	**8.1 (0.2)**	WS. BBD. PWPS. PLS. SEB. Nec. GA	**Present study**
South Africa (2010-2011)	**2.9 (0.8)**	WS. BBD. PWPS. PLS. Nec. GA	**Present study**
South Africa (2011-2012)	**4.9 (0.9)**	WS. BBD. PWPS. PLS. Nec. GA	**Present study**
Mayotte (2011-2012)	**2.7 (0.3)**	WS. BBD. PWPS. PLS. Nec. GA	**Present study**
Eastern Indian Ocean			
Ningaloo Reef	2.3 (0.39)	WS. BBD. SEB. BrB	(Onton et al. 2011)
Central Indian Ocean			
Chagos Archipelago	5.2 (0.2)	WS. GA	(Sheppard et al. 2012)
Republic of Maldives	< 2	WS. BBD. SEB. PDDr. BrB. UWS	(Montano et al. 2012)
Northern Indian Ocean			
Mandapam (South India)	8.9 ± ND	WS. BBD.WBD. WP. YB. GA. PS	(Thinesh et al. 2009)
Palk Bay (South India)	21.0 ± ND	WS. BBD. WBD. PS. WP. YB	(Thinesh et al. 2011)

Among the coral communities at the three localities, the genera most vulnerable to disease were generally *Acropora* and *Porites*. These genera, commonly found on back, lagoon and fringing reefs, are important reef-building corals in Reunion, South Africa and Mayotte (Turner and Klaus 2005). *Acropora* was mainly represented by the species *Acropora muricata* in Reunion and Mayotte and exhibited signs of WS, SEB, GA and Nec. This high susceptibility is consistent with other research which has shown *Acropora* species to be particularly vulnerable to disease on Indian Ocean (McClanahan et al. 2004b; Thinesh et al. 2009; Thinesh et al. 2011) and indo-Pacific reefs (Willis et al. 2004; Aeby et al. 2006; Haapkylä et al. 2010; Aeby et al. 2011). Haapkylä et al. (2010) suggested that corals which

allocate greater energy to growth and reproduction (e.g. Acroporidae and Pocilloporidae) are more susceptible to disease than massive corals which seem to develop greater resistance through the allocation of more energy to colony maintenance. However, our results have shown that massive colonies of *Porites lutea* and *P. lobata*, generally considered robust and slow-growing genera (Raymundo et al. 2005), were affected by multiple infections, exhibiting signs of BBD, PLS and PWPS. The susceptibility of this genus to disease has also been reported worldwide (Table 7), notably on south-eastern Indian (Thinesh et al. 2009; Onton et al. 2011; Montano et al. 2012), Philippine (Santavy et al. 2001; Raymundo et al. 2005) and Indo-Pacific reefs (Sutherland et al. 2004; Haapkylä et al. 2009). Therefore, these results seem to contradict Haapkylä and colleague's (2009) assumption that disease vulnerability is related to life history traits, with the investment of energy into growth by fast-growing species being to the detriment of their pathogen resistance. Alternatively, the high susceptibility of massive *Porites* colonies to disease may be attributed to predation that compromises their health. During this study, fish bites were observed on almost every colony of *Porites lutea* and *P. lobata*. Corallivorous fishes are considered potential vectors of coral disease (Aeby and Santavy 2006; Raymundo et al. 2009; Chong-Seng et al. 2011) and Chong-Seng et al. (2011) found that fishes belonging to the families Blennidae, Chaetodontidae, and Pomacentridae feed preferentially on infected coral colonies and may spread coral diseases. Finally, *Hydnophora* spp. and *Goniopora* spp. exhibited particularly high susceptibility/sensitivity to BBD on both Reunion and South African reefs. For instance, all infected colonies of *Hydnophora* sp. recorded on the reef flat in Reunion died and, in subsequent surveys, were recorded as being colonised by opportunistic algae; no further *Hydnophora* colonies were encountered. This may suggest that BBD can re-structure reefs at the local scale, highlighting the importance of frequent monitoring to assess disease-related shifts in coral community structure.

Table 7: Disease conditions recorded worldwide on the genus *Porites*. WS: white syndrome; BBD: black band disease; PWPS: *Porites* white patch syndrome; PLS: pink line syndrome; SED: skeleton eroding band; Nec: necrosis; GA: growth anomaly; WBD: white band disease; WP: white plague disease; YBD: yellow band disease; PS: pink spot; UWS: ulcerative white spot; PDDr: *Porites* dark discoloration response; PR: pigmentation response; Trem: trematodiasis; TLS: tissue loss syndrome; BND: brown necrotizing disease; Por bl w/TL: *Porites* bleaching with tissue loss; PorDTTS: *Porites* discolored tissue thinning syndrome.

Species affected	Geographic distribution	Disease condition	Sources
Porites lutea	**Reunion**	WS, PWPS, PLS, BBD, SEB	**This study**
Porites lutea	**South Africa**	WS, PWPS, PLS, BBD, GA	**This study**
Porites lutea	**Mayotte**	WS, PWPS, PLS, BBD, GA	**This study**
Porites sp.	Indonesia	WS, PUWS, GA	(Haapkylä et al. 2009)
Porites sp.	Heron Island, Australia	PUWS	(Haapkylä et al. 2010)
Porites spp.	Florida keys	WS	(Porter et al. 2001)
Porites spp.	Philippines	GA, NS, PR, PUWS	(Raymundo et al. 2005)
Porites spp.	Caribbean	WS, BBD, GA, WP	(Sutherland et al. 2004)
Porites lutea	Indo-Pacific	BBD, WPL, GA, YBD, PLS	(Sutherland et al. 2004)
Porites lobata		GA	
Porites spp.		GA, YBD	
Porites lobata	Indo-Pacific	PUWS, PR	(Weil et al. 2012)
Porites lutea		PUWS, PR	
Porites spp.	Mexico	BBD, WP, YBD	(Ward et al. 2007)
Porites massive	Central Philippines	GA, PUWS	(Kaczmarsky 2006)
Porites asteroides	Eastern Brazil	BBD	(Francini-Filho et al. 2008)
Porites spp.	Hawaiian Archipelago	Trem, PorTLS, GA, PorBND, Por bl w/TL, PorDTTS	(Aeby et al. 2011)
Porites spp.	New-Caledonia	GA, MFBS	(Tribollet et al. 2011)
Porites spp.	South-western Caribbean	WP, YBD	(Garzón-Ferreira et al. 2001)
Porites sp.	Eilat, Gulf of Aqaba	WS, Nec	(Ainsworth et al. 2007)
Porites astreoides	Virgin Islands United states	WS, YBD	(Calnan et al. 2007)
Porites spp.	US Pacific Remote Island	WS, GA	(Vargas-Angel 2009)
Porites spp.	Red Sea	SEB	(Winkler et al. 2004)
Porites astreoides	Dominica, West Indie	WS	(Borger and Steiner 2005)
Porites lutea	Arabian Gulf Dubai	YBD	(Riegl 2002)
Porites lutea	Kavarati, Gulf of Kutch	BBD	(Ravindran et al. 1999)
Porites lutea	Papua New Guinea	BBD	(Frias-Lopez et al. 2004)
Porites lutea	Negros Island, Philippines	BBD	(Myers et al. 2007)
Porites spp.	Guam, Micronesia	BBD, SEB, WS, GA, PUWS	(Myers and Raymundo 2009)
Porites spp.	South-eastern India	PS, WS, WP, YBD, PR, PDDr	(Thinesh et al. 2011)
Poritidae	Ningaloo Reef (Australia)	Atramentous necrosis, SEB	(Harvell et al. 2007)

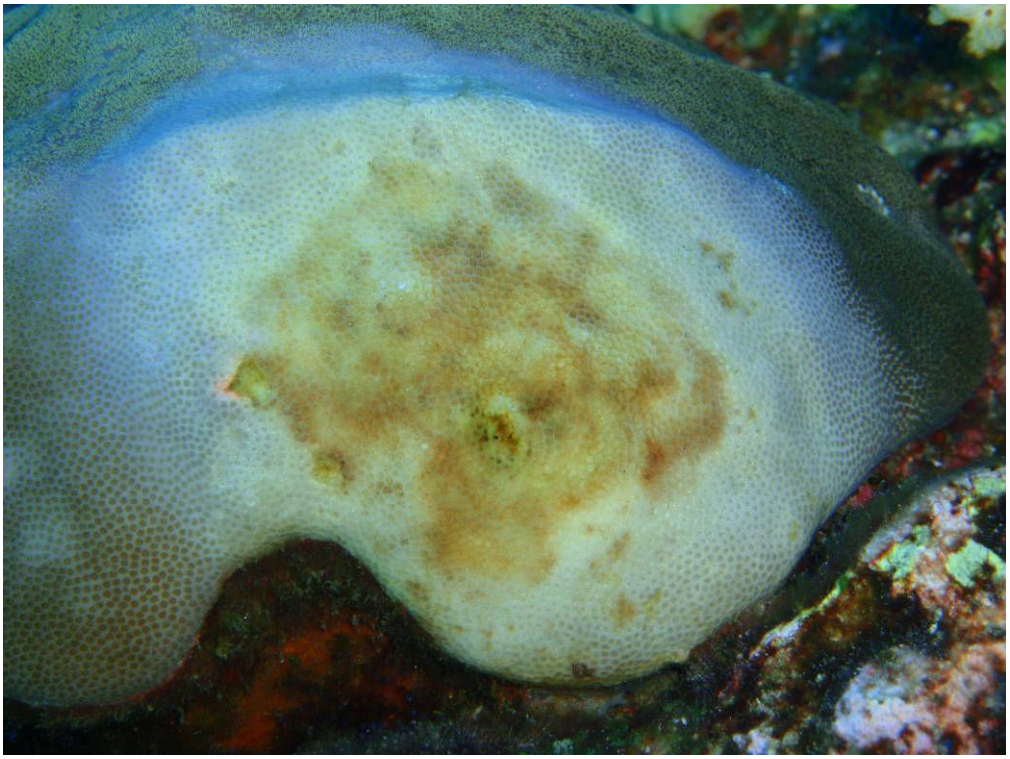
Spatial variability was detected in BBD and WS on both Reunion and South Africa reefs. For instance, BBD observed on *Porites* spp. in Reunion seemed to be depth-related, with more diseased cases observed in shallow than deep habitats. Similar trends were found in South Africa in colonies of *Pocillopora* sp. infected by WS. These results are consistent with patterns found in the Caribbean (Weil and Cróquer 2008), Republic of Maldives (Montano et al. 2012) and South India (Thinesh et al. 2009; Thinesh et al. 2011) where both WS and BBD are more abundant at shallow than deep sites. In Mayotte, no spatial variations were detected, despite fringing reefs being exposed to increasing and greater anthropogenic pressures than the barrier reef. As suggested previously by Weil et al. (2002), corals exposed frequently and continuously to the same stressors may develop resistance to them.

Of the diseases found on shallow reefs in Reunion, BBD seemed particularly to manifest seasonality. For instance, when recorded on massive *Porites* spp., it was first observed during the summer 2010. It decreased significantly during the winter of 2011 and then reappeared the next summer. In South Africa, warmer water temperatures seemed to be linked to a higher prevalence of disease in the first year during summer. This was the case for WS-infected *Acropora* spp. and *Pocillopora* spp. However, no variations were observed for the same syndromes between the summer and winter of 2012. PWPS was also more prevalent in summer on both the reef slope and reef flat but the results were not statistically significant. On Mayotte reefs, WS syndromes on *Acropora* spp. varied between seasons, with their prevalence being higher during the warmer months. Similar seasonal patterns have been reported for both BBD and WS in Australia (Willis et al. 2004; Boyett et al. 2007; Bruno and Selig 2007; Haapkylä et al. 2010) and the Caribbean (Bruckner et al. 1997). These patterns may be due to impairment of the host's disease resistance under summer conditions, generating a shift in the natural bacterial communities in the coral holobionts towards opportunistic pathogens. Previous work has shown that elevated seawater temperatures

increase disease progression (Willis et al. 2004; Bruno and Selig 2007) and tissue mortality (Boyett et al. 2007; Haapkylä et al. 2009; Haapkylä et al. 2010) by stimulating the growth of putative pathogens (Patterson et al. 2002; Ben-Haim et al. 2003a; Cervino et al. 2004; Rosenberg and Falkovitz 2004; Boyett et al. 2007) and altering the structure of coral-associated bacteria (Reshef et al. 2006; Mouchka et al. 2010) which may have an important role in disease-resistance (Ritchie 2006).

Chapter 3

Bacterial communities associated with *Porites* white patch syndrome (PWPS) on three Western Indian Ocean (WIO) coral reefs



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1. Abstract

The scleractinian coral *Porites lutea*, an important reef-building coral on western Indian Ocean reefs (WIO), is affected by a newly-reported white syndrome (WS) the *Porites* white patch syndrome (PWPS). Histopathology and culture-independent molecular techniques were used to characterise the microbial communities associated with this emerging disease. Microscopy showed extensive tissue fragmentation generally associated with ovoid basophilic bodies resembling bacterial aggregates. Results of 16S rRNA sequence analysis revealed a high variability between bacterial communities associated with PWPS-infected and healthy tissues in *P. lutea*, a pattern previously reported in other coral diseases such as black band disease (BBD), white band disease (WBD) and white plague diseases (WPD). Furthermore, substantial variations in bacterial communities were observed at the different sampling locations, suggesting that there is no strong bacterial association in *Porites lutea* on WIO reefs. Several sequences affiliated with potential pathogens belonging to the *Vibrionaceae* and *Rhodobacteraceae* were identified, mainly in PWPS-infected coral tissues. Among them, only two ribotypes affiliated to *Shimia marina* (NR043300.1) and *Vibrio hepatarius* (NR025575.1) were consistently found in diseased tissues from the three geographically distant sampling localities. The role of these bacterial species in PWPS needs to be tested experimentally.

Keywords: Coral disease - Western Indian Ocean - Scleractinian corals - *Porites* white patch syndrome - Bacterial communities - Causative agent.

2. Introduction

The scleractinian *Porites lutea*, commonly found on back reefs, lagoon and fringing reefs (Veron 2000), is an important reef-building coral in the western Indian Ocean (WIO) reefs. Despite its widespread distribution, this hermatypic coral has shown a particular susceptibility to natural pressures such as predation (Shafir et al. 2008; Cole et al. 2009) and infestation by parasites (Cole et al. 2008; Benzoni et al. 2010). Moreover, it seems to be more vulnerable to infectious disease than many other coral species (Wilkinson 2004).

Of the 30 coral diseases described to date (Sutherland et al. 2004; Weil et al. 2006), eight are known to affect *P. lutea* worldwide. On Indo-Pacific reefs, colonies of *P. lutea* have been recorded with signs of black band disease (BBD), white plaque syndrome (WPL), growth anomalies (GA), yellow band disease (YBD) and pink line syndrome (PLS) (Sutherland et al. 2004). Surveys conducted in the Gulf of Kutch (Ravindran et al. 1999), Papua New Guinea (Frias-Lopez et al. 2004) and Philippines have recorded BBD outbreaks in this scleractinian coral. In addition, a study performed on (Myers et al. 2007) coral health and diseases in the northern Egyptian Red Sea has revealed two other syndromes: *Porites* ulcerative white spot (PUWS) and a white syndrome (WS) so far unreported on *P. lutea* (Mohamed 2012). More recently, a white syndrome (WS) named *Porites* white patch syndrome (PWPS) was described on massive colonies of *P. lutea* on Western Indian Ocean (WIO) reefs (Séré et al. 2012). This syndrome was characterised by diffuse, medium to large (50-300 mm diameter), circular to oblong tissue loss, surrounded by swollen white tissue. The dead skeleton was progressively colonised by opportunistic algae and *Cyanobacteria* (Séré et al. 2012).

To date, nothing is known about the aetiology of PWPS. Previous studies on other white syndromes (white band disease (WBD), white plague disease (WP), progressive white syndromes (PWS), Australian subtropical white syndrome (ASWS), *Acropora* white

syndrome in American Samoa (AWS), and *Porites* bleaching with tissue loss (PBTL)) have characterized organisms (bacteria, ciliates, helminths, fungi, algae) associated with both healthy and diseased coral colonies. These investigations have allowed identification of a number of putative pathogens (Pantos et al. 2003; Pantos and Bythell 2006; Sunagawa et al. 2009; Cárdenas et al. 2011; Work and Aeby 2011; Godwin et al. 2012; Sudek et al. 2012; Sweet and Bythell 2012; Wilson et al. 2012). Evidence of the involvement of bacteria as causative agents has been suggested in some studies on several of the WS observed on scleractinian corals (Sussman et al. 2008; Arboleda and Reichardt 2010; Luna et al. 2010; Sutherland et al. 2011). For example *Serratia marcescens* has been reported to be linked with white pox disease (WPD) in the Elkhorn coral *Acropora palmata* (Sutherland et al. 2011) and *Vibrio owensii* to be the aetiological agent of *Montipora* white syndrome (MWS) in the Hawaiian coral *Montipora capilata* (Ushijima et al. 2012). However, some of these potential causative agents have i) not been fully characterised in terms of fulfilling all Koch's postulates (Bythell et al. 2004) or ii) have been biased by the execution of infection trials with a specific pathogen rather than testing for multiple potential pathogens. Finally, no clear link between the proposed pathogens and signs of disease has been demonstrated at the gross and cellular level in aquaria or the field.

This study aimed to provide the first characterisation of bacterial communities associated with healthy and PWPS-affected massive colonies of *P. lutea* in three WIO regions: Mayotte, South Africa and Reunion. The sampling sites included a continental African reef and two oceanic islands separated by over 1500 km of mostly oceanic water masses. These sites were selected to highlight general microbial patterns associated with this disease and the host. The investigation presented here used both histopathology and culture-independent molecular (clone libraries) techniques. The main objectives were to i) describe the microscopic

morphology of lesions in corals, and ii) identify the most represented bacteria associated with both healthy and infected coral tissues collected at the three localities.

3. Methods

The sampling of *Porites lutea* colonies for this study was authorised by the French Department of Ecology, Sustainable Development, Transportation and Housing (DEAL), the Isimangaliso Wetland Park (South Africa) and CITES (Permit no. FR1197400391-FR1197400394-1).

3.1. Sample collection

Individual samples of *Porites lutea* exhibiting signs of PWPS were collected in South Africa, Reunion and Mayotte (Fig. 9) using SCUBA. At each location, samples were collected from three healthy corals and colonies with signs of PWPS-infection (n_{total}=18 samples).

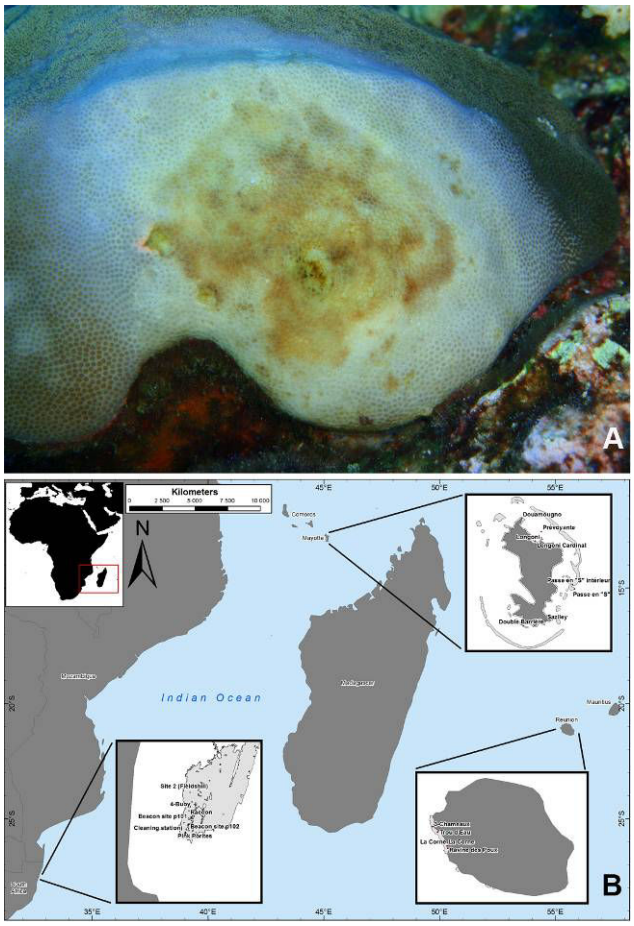

Figure 9: PWPS on *Porites lutea* (A) and map of the Western Indian Ocean showing the sampling locations (B).

Diseased tissues (DT) were sampled from the lesion boundary interface with visually healthy tissue (HT), and samples of HT were collected from visually healthy colonies with no signs of disease. Cores of both HT and DT (2.2 cm diameter to a depth of 0.5-1 cm) were collected using a sterile stainless steel core tube and placed individually in sterile disposable 50 ml polypropylene centrifuge tubes. Upon reaching the shore, the seawater within each tube was drained for 10 minutes, replaced with 100% ethanol and stored at -20˚C for subsequent DNA extraction and molecular analysis. Due to time and environmental constraints, corals exhibiting signs of PWPS could not be monitored for lesion progression.

Additional core samples were simultaneously collected at each location from five HT and five DT colonies using a hammer and chisel and fixed in 4% formalin for histopathology. Samples were protected in 1.5% (w/v) agarose to retain the spatial integrity of the tissues and microbial communities. They were then decalcified using 1% HCl and EDTA renewed every 12 h until the process was complete. Tissues from the decalcified fragments were dehydrated in a series of ethanol baths, cleared with xylene and embedded in paraffin wax. Sections of 6-8 μm were cut using a microtome, mounted on glass slides and stained with Harris haematoxylin and eosin with phloxine B to diagnose tissue fragmentation, necrosis and the presence of invasive organisms. Serial sections of the affected cells or tissues were examined under a light microscope at different magnifications (×200, ×400, ×1000) and photographed using NIS Element software (Nikon©). The number of colonies sampled per region for histology is presented in Table 8.

3.2. DNA extraction

Bacterial genomic DNA from both HT and DT were extracted using the NucleoSpin® Soil Kit (NucleoSpin Extract II, *Macherey-Nagel*, Düren, Germany). Refrigerated samples were dried at room temperature, after which approximately 150 mg of both tissue and skeleton

were scraped from their surface using a sterile scalpel blade, placed in a 1.5 ml centrifuge tube with 700 µl of lysis buffer and crushed using a fresh disposable plastic rod. Samples were then placed in lysing matrix tubes for DNA extraction. The DNA was eluted with 50 µl sterile elution buffer, verified by electrophoresis in agarose gels (1.5% wt/vol) stained with GelRed™ (Biotium Inc., Hayward, California, USA) and finally stored at -20˚C.

Table 8: Number of samples collected for histopathology, sections analysed and diagnosis partitioned by tissue category and region. HT = healthy tissue. DT = disease tissue.

	Reunion		**South Africa**		**Mayotte**	
Tissue categories	**HT**	**DT**	**HT**	**DT**	**HT**	**DT**
Samples	5	5	5	5	5	5
Cross-sections/sample	10	10	10	10	10	10
Samples with Ciliata	0	1	0	0	1	0
Samples with endophytic algae	0	5	0	5	0	5
Samples with *Cyanobacteria*	0	3	0	0	0	2
Samples with Nematoda	0	5	0	4	0	0
Samples with bacterial aggregates	0	4	0	2	1	3

3.3. PCR Amplification

16S rRNA genes were amplified from DNA extracts by PCR using universal primers 28F and 1492R (Frias-Lopez et al. 2002). PCR reactions were carried out in a final volume of 25 µl containing GoTaq®Hot Start Green Master Mix (Promega, Madison, WI), 0.5 mM of each primer and 10 ng of template DNA. Reaction mixtures were incubated in a GeneAmp®PCR System 2700 thermal cycler. Amplification conditions for the PCR included an initial denaturing step of 4 min at 95.5˚C, followed by 30 cycles at 94˚C for 30 sec, 55˚C for 60 sec and 72˚C for 90 sec followed by a final extension step of 15 min at 72˚C.

3.4. Cloning and Sequencing

PCR was carried out using DNA templates prepared from each of the three individual DT or HT samples. PCR products were verified for quality, size and quantity by electrophoresis and spectrophotometry. Equimolar quantities of PCR products were then pooled for the three DT

and HT samples at each locality (6 samples per locality) and each was subsequently separated by electrophoresis. Amplicons 1300-1500 bp long were excised from the gel under a UV transilluminator and the DNA was gel purified using NucleoSpin® Gel and a PCR Clean-up kit (*Macherey-Nagel*, Düren, Germany) for colony screening. The purified DNA was cloned into the pGEM-T vector system (Promega, Madison, WI) and ligation mixture was used to transform *Escherichia coli* JM109 competent cells. A total of 92-150 clones were randomly selected from each tissue category, spotted directly into 96-well plates and subjected to PCR with M13 forward and reverse primers (Inquaba Biotec™, Pretoria, South Africa). Amplification conditions for the PCR included an initial denaturing step at 94˚C for 5 min followed by 35 cycles of 94˚C for 1 min, 57˚C for 1 min and 72˚C for 1 min, with a final extension step at 72˚C for 7 min. PCR products were checked for quality, size and quantity by electrophoresis in agarose gels (1.5% wt/vol) as described above and sent to GENOSCREEN (Campus de l'Institut Pasteur, Lille, France) for Sanger sequencing.

3.5. Sequence analysis

The pair sequences obtained for each clone were edited and aligned using GENEIOUS™ Pro (V.5.6.3) sequencing software. All high quality consensus sequences (HQ> 65%) were submitted to BLAST at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) to determine their percentage similarity with known 16Sr DNA sequences. Sequences matching at a similarity level of 1) 97-100% were considered as belonging to the same species level, 2) 93-96% were considered as belonging to the same genus level and 3) <93% were considered to fall below the similarity of the genus level (Pantos and Bythell 2006). Bacterial rRNA sequences closely related to putative bacteria were aligned using Geneious alignment and rearranged manually. A phylogenetic tree was built using the Neighbor-Joining method of GENEIOUS™ Pro (V.5.6.3). All 16r RNA gene

sequences are accessible through the NCBI GeneBank database under accession numbers KF179641-KF180135.

3.6. Statistical analysis

Multidimentional Scale (MDS) analysis of bacterial communities associated with healthy and PWPS-affected tissues of Porites lutea collected at Reunion (R), South Africa (RSA) and Mayotte (M) was performed using PRIMER (V.6.1.14). Data were square root-transformed and MDS analysis was carried out using the Bray–Curtis similarity coefficient. Finally, the Shannon-Weaver index (H) was calculated for each tissue category to characterise pooled bacterial diversities in healthy and diseased coral samples

4. Results

4.1. Microscopic morphology and spatial structure

Histological cross-sections of PWPS revealed extensive tissue breakdown and necrosis within the lesion area between the exposed skeleton and living tissue (Fig. 10A). Ovoid basophilic bodies resembling bacterial aggregates were visible within the mesogloea of the body wall, mainly in DT (Table 8), especially in the area of tissue fragmentation (Figs 10B, D). These aggregates were seen in nine of the 15 samples collected from corals showing signs of PWPS at all three sampling locations. Among the 15 samples of HT, only one was found with such aggregates (Table 8). Other organisms, including *Cyanobacteria* (Fig. 2C, F), Nematoda (Fig. 10E), Ciliata (Fig. 10A), and algae (Fig. 10F) were also observed but only within dead tissue.

4.2. Bacterial communities associated with healthy tissues

A total of 91, 74 and 100 16S rRNA sequences (818-1627 bp), subdivided into seven, six and four classes (Table 2 and Fig. 3) were obtained from healthy tissues collected in Mayotte (HT-M), South Africa (HT-RSA) and Reunion (HT-R) respectively (Table 9). Sequences retrieved from HT-M (Table 9 and Fig. 11) were mainly identified as members of the *γ-proteobacteria* (42.0%), *α-proteobacteria* (19.0%), *Cyanobacteria* (11.0%), *Firmicutes* (6.0%), *Cytophagia* (6.0%) and *ε-proteobacteria* (3.0%).

Table 9: Number of samples collected, clones retrieved by tissue category and region and diversity index (Shannon-Weaver). HT = healthy tissue. DT = disease tissue.

	Reunion		South Africa		Mayotte	
	HT	**DT**	**HT**	**DT**	**HT**	**DT**
Samples collected	3	3	3	3	3	3
Pooled samples	1	1	1	1	1	1
Random clones from pooled samples	92	100	101	150	94	100
Consensus sequences	74	60	100	145	91	91
Sequence lengths (bp)	905-1498	904-1490	842-150	850-1498	1240-1483	855-1477
Sequence quality (%)	77.8-100	72.2-100	74.2-100	66.2-100	78.7-100	67.1-100
Class/subdivision	4	5	6	11	8	9
Species	12	26	21	53	36	39
Diversity (Shannon-Weaver index)	1.20	**2.83**	1.70	**3.37**	2.86	**3.29**

Of all sequences, 14.0% had no close relatives in the NCBI database and could only be classified as unknown bacterial clones. The bacterial diversity associated with HT-RSA (Table S1) was also dominated by sequences closely related to *γ-proteobacteria* (88.0%) followed by *Cyanobacteria* (5.0%), *α-proteobacteria* (2.0%), *Spirochaetes* (1.0%) and *Actinobacteria* (1.0%). HT-R (Appendix 1) seemed to contain less group diversity, mainly dominated by *γ-proteobacteria* (67.0%), *Cyanobacteria* (11.0%) and *Firmicutes* (6.0%). Unknown bacterial clones constituted 17.0% of all analysed sequences. The *γ-proteobacteria* retrieved from HT-M, HT-RSA and HT-R were dominated by bacterial species closely related to *Endozoicomonas elysicola* (accession no. NR041264), comprising 53.1%, 67.8 % and 81.8 % of the total *γ-proteobacteria* respectively. Sequences closely-related to *Vibrio fortis* (accession no. NR025575) were also common to bacterial communities associated with HT from all three sampling locations.

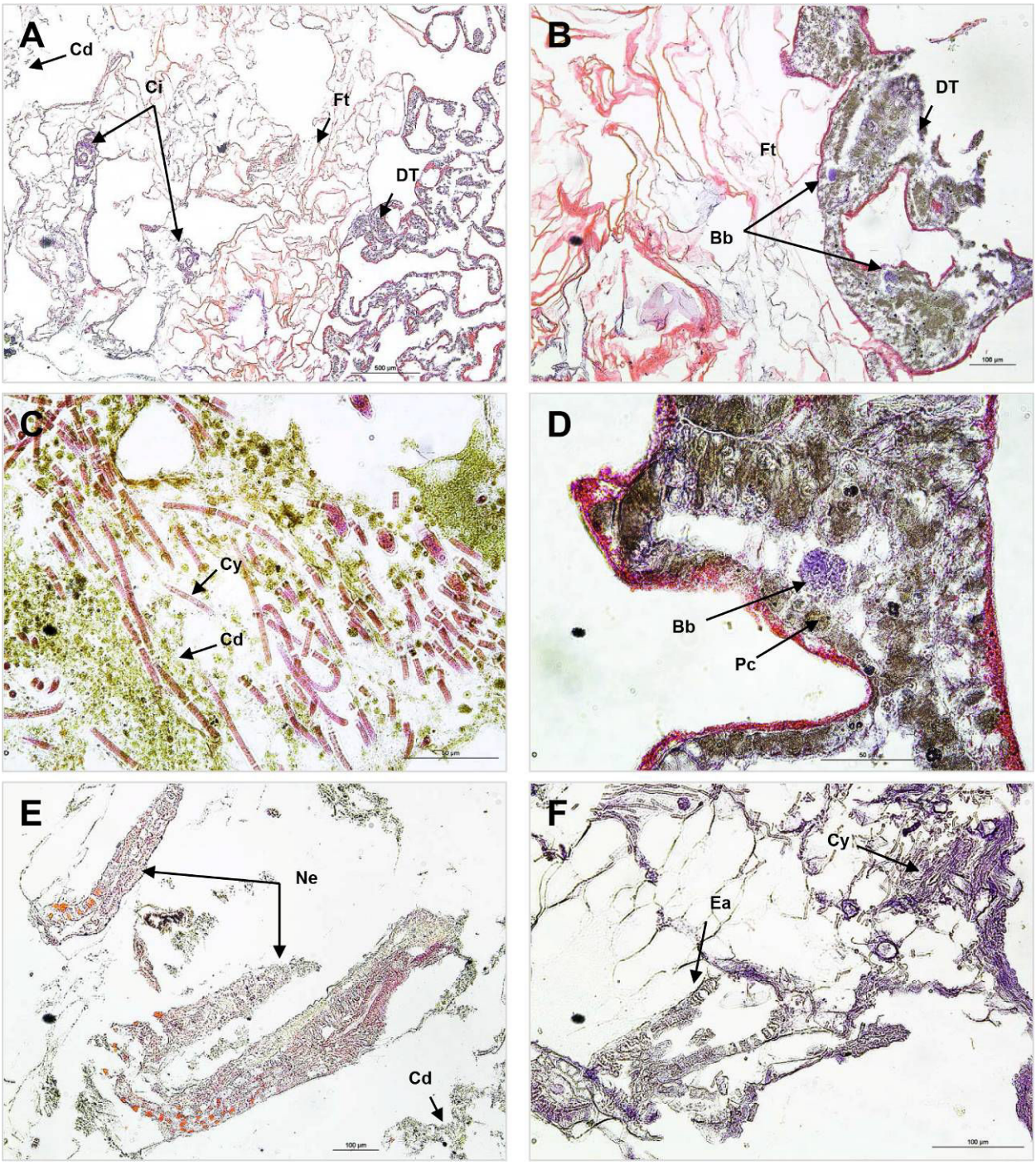

Figure 10: Photomicrographs of diseased Porites lutea coral tissues: Porites white patch syndrome (PWPS). A) Cross-section showing the well-defined boundary between fragmented (FT) and diseased tissue (DT); Cd = cell debris; Ci = Ciliata. B) P. lutea with PWPS. Note ovoid basophilic bodies (bb) like bacterial aggregates; Ft = fragmented tissue. C) Cross section of P.lutea affected by PWPS showing dead tissue full of cell debris (Cd) and Cyanobacteria (Cy). D) Close-up of an ovoid basophilic body (Bb). E) Nematoda (Ne) in the tissue debris. F) Endophytic algae (ea) in dead tissues.

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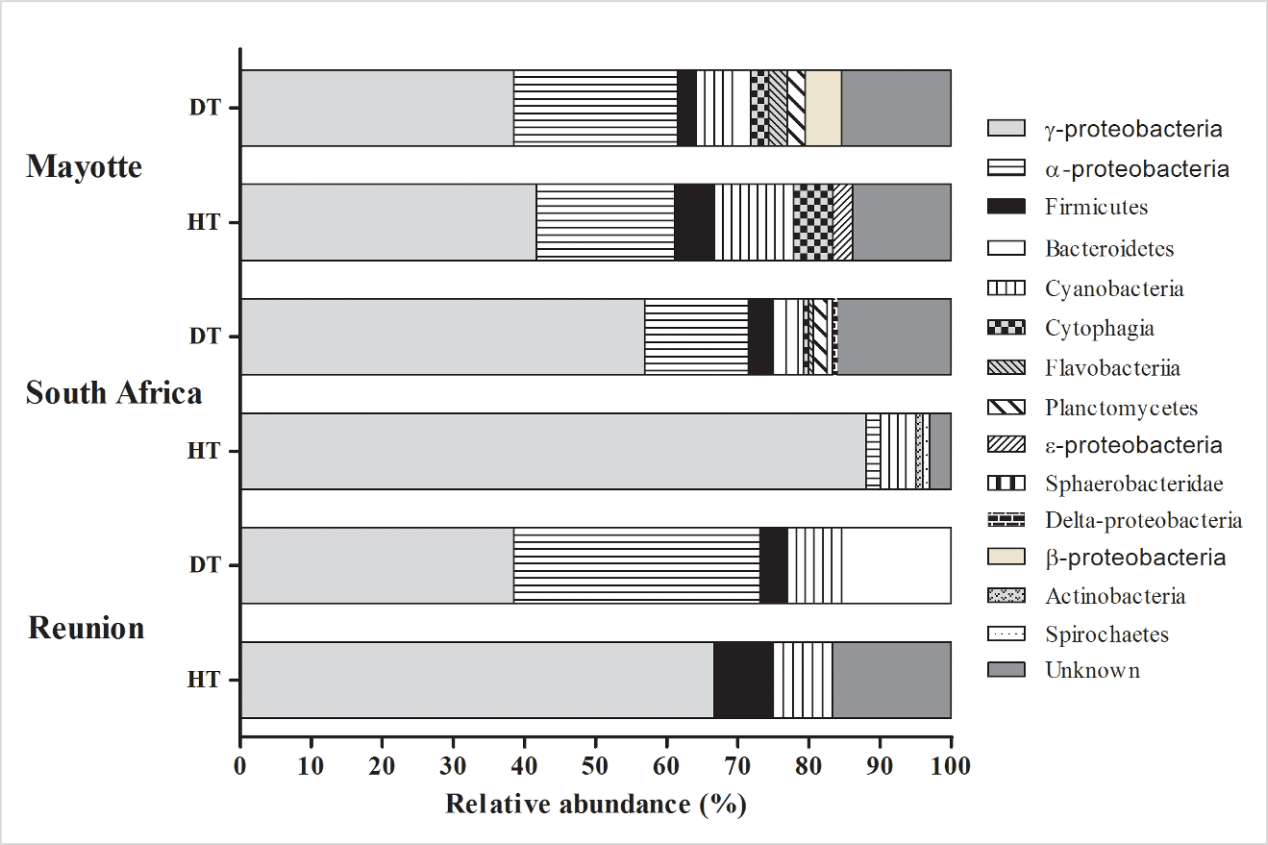


Figure 11: Relative abundance (%) of bacterial phyla retrieved from three diseased (DT) and three healthy (HT) samples of *Porites lutea* collected in Mayotte (MAY), South Africa (RSA) and Reunion (REU).

A total of 91, 145 and 60 16S rRNA gene clones (527-1602 bp), subdivided into 10, 12 and 5 classes, was obtained from diseased tissues collected in Mayotte (PWPS-M), South Africa (PWPS-RSA) and Reunion (PWPS-R) respectively (Appendix 1). PWPS-M samples exhibited high diversity (Fig. 11), dominated by members of the *γ-proteobacteria* (38.0%) and *α-proteobacteria* (23.0%), followed by *Cyanobacteria* (6.0%), *Cytophagia* (6.0%), *Firmicutes* (3.0%), *Bacteroidetes* (3.0%), *β-proteobacteria* (3.0%), *Chloroplasts* (3.0%), *Planctomycetes* (3.0%) and *Flavobacteriia* (3.0%). Of the analysed sequences, 17.0% had no close relatives in the NCBI database and could only be classified as unknown bacterial clones. Similar trends emerged for PWPS-RSA (Fig. 11), the bacterial classes being dominated by *γ-proteobacteria* (56.9%) and *α-proteobacteria* (14.6%), followed by *Cyanobacteria* (3.5%), *Firmicutes* (3.5%), *Planctomycetes* (2.1%), *Bacteroidetes* (0.7%), *Cytophagia* (0.7%), *Delta-proteobacteria* (0.7%), *Flavobacteriia* (3.0%) and *Sphaerobacteridae* (3.0%). Again, some

sequences (16.0%) had no close relatives in the NCBI database and could only be classified as unknown bacteria. PWPS-R (Appendix 1) samples were similarly dominated by members of the *γ-proteobacteria* (38.0%) and *α-proteobacteria* (23.0%), but contained only three other classes belonging to genera of *Bacteroidetes* (15.0%), *Cyanobacteria* (8.0%) and *Firmicutes* (4.0%).

Among the several bacterial classes found in this study, the *γ-proteobacteria Vibrio parahaemolyticus* (accession no. NR041838.1; n=15), *V. fortis* (accession no. NR025575.1; n=5) and *V. rotiferianus* (accession no. NR042081.1; n=4), as well as the *α-proteobacteria Paracoccus yeei* (accession no. NR029038.1; n=4), *Pseudoruegeria aquimaris* (accession no. NR043932; n=2) and *Shimia marina* (accession no. NR043300.1; n=3) were the best represented ribotypes in PWPS-M. The predominant bacterial ribotypes in PWPS-R were the *γ-proteobacteria E. elysicola* (accession no. NR041264; n=12), *Photobacterium damselae* (accession no. NR042975.1; n=4) and *Photobacterium* sp. (accession no. HQ697926; n=3). The next most abundant sequences were closely related to the *α-proteobacteria P. yeei* (accession no. NR029038.1; n=5), *Ruegeria pomeroyi* (accession no. NR028727; n=3), *S. marina* (accession no. NR043300.1; n=3) and *Silicibacter lacuscaerulensis* (accession no. NR029197; n=3). Among the bacterial strains retrieved from PWPS-RSA, the *γ-proteobacteria*, *E. elysicola* (accession no. NR041264; n=41) and *Oceanospirullum beijerinckii* (accession no. NR040784; n=5), the *α-proteobacterium S. marina* (accession no. NR043300.1; n=3) and the *Cyanobacterium Prochlorococcus marinus* (accession no. NR028762; n=5) were the most representative species.

4.3. Comparison of bacterial communities in healthy and diseased tissues

Distinctly partitioned ribotypes were detected among diseased and healthy tissues samples. In total, 31 (77.8%), 54 (90.0%) and 24 (92.3%) bacterial ribotypes were exclusively associated

with PWPS-M, PWPS-RSA and PWPS-R respectively, while 28 (77.8 %), 17 (73.9%) and 10 (83.3%) were found only in HT-M, HT-RSA and HT-R respectively (Fig. 12). Multidimentional scaling (MDS, Fig. 12B) analysis performed on the composition of bacterial 16S rRNA gene of each tissue categories revealed four distinct clusters representing four distinct bacterial communities. Similarities in bacterial composition in RDT and MDT were detected but SAHT, RHT, SADT and MHT samples exhibited more variability (Fig. 12).

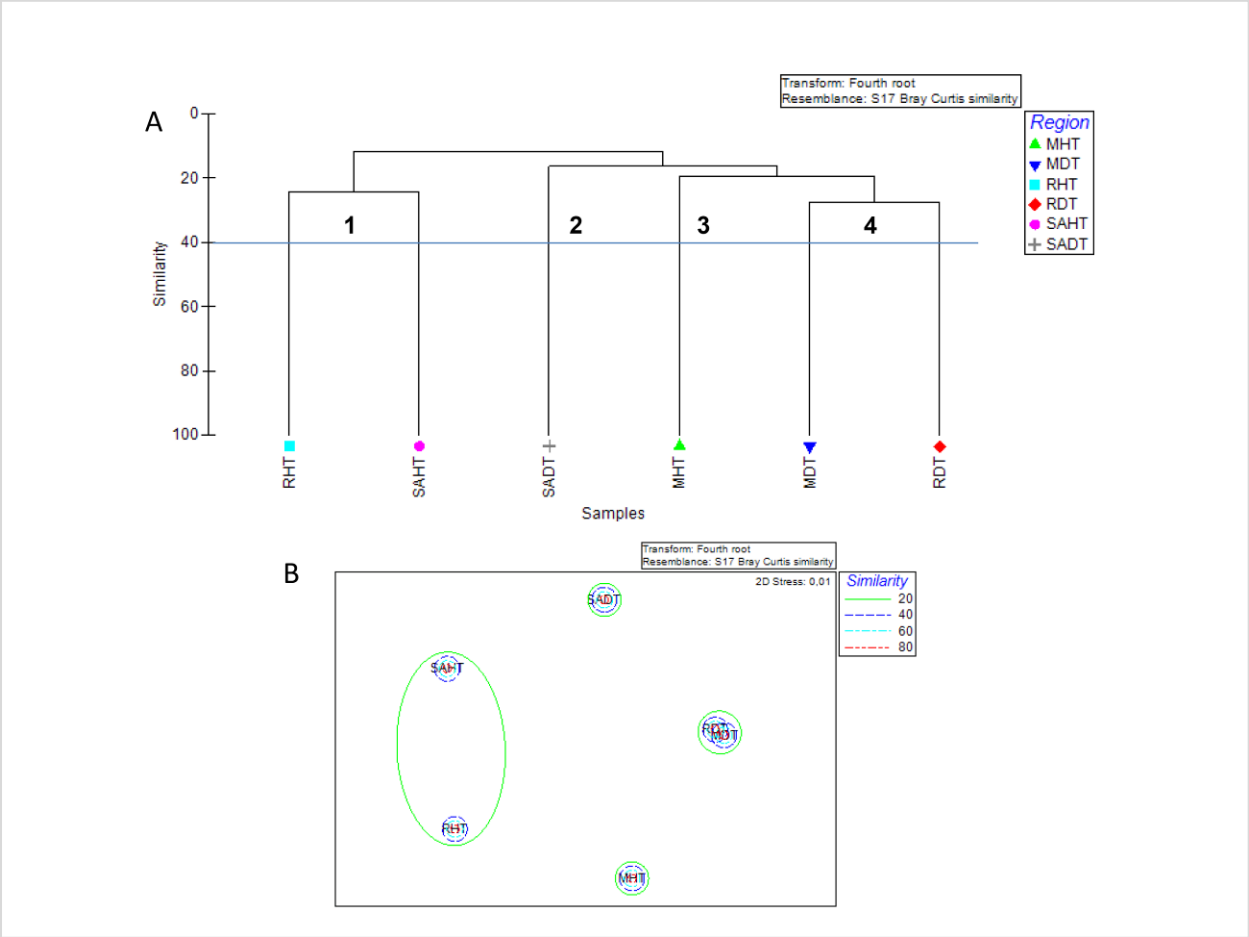


Figure 12: Multidimensional scaling (MDS) ordination of bacterial communities associated with healthy (HT) and PWPS-affected tissues (DT) of *Porites lutea* collected at Reunion (R), South Africa (RSA) and Mayotte (M).

In addition, the bacterial diversity identified in PWPS tissues collected on the three WIO coral reefs was higher than in HT (Table 9). For instance, 39, 60 and 26 16S rRNA gene sequences affiliated to bacterial genera/species were identified in PWPS-M, PWPS-RSA and PWPS-R respectively, whereas only 36, 23 and 12 were obtained in HT-M, HT-RSA and HT-R respectively (Table 9). Among these, only six ribotypes were commonly detected in PWPS samples from the three sampling localities and were closely related to *P. yeei* (accession no. NR025491), *P. aquimaris* (accession no. NR029038.1), *S. marina* (accession no. NR043300.1), *V. fortis* (accession no. NR025575.1), *V. hepatarius* (accession no. NR025575.1) and *V. parahaemolyticus* (accession no. NR041838.1). In HT, only 16S rRNA gene sequences affiliated to bacterial species *E. elysicola* (accession no. NR041264) and *V. fortis* (accession no. NR025575.1) were common in samples collected at the three sampling localities.

5. Discussion

5.1. Histological observations

Corals exhibiting signs of PWPS revealed extensive tissue fragmentation, generally associated with ovoid basophilic bodies resembling bacterial aggregates within the mesoglea of the body wall. These aggregates were seen in 60% of all samples collected from corals with signs of PWPS. However, these aggregates could not be directly linked with the pathology as there was no clear evidence of inflammatory response or tissue lysis associated with these ovoid bodies. In addition, some clusters of basophilic bodies were also observed in sections of one healthy sample of *P. lutea*, preventing definitive conclusion that they constituted a bacterial infectious agent in the PWPS lesions. Similar observations on bacterial aggregates have been previously reported in several histopathological studies, in both healthy and WS-infected colonies of *Acropora* spp., *Pocillopora meandrina* and *Porites compressa* (Galloway et al. 2007; Sudek et al. 2012). Direct identification from formalin-fixed, paraffin-embedded coral tissue combined with descriptions of cellular changes over time may be a viable option to identify the role of these aggregates in PWPS. Other organisms, including *Cyanobacteria*, Nematoda, Ciliata and endophytic algae, were also observed on diseased tissues and were generally associated with the dead epidermis and cell debris. No obvious evidence of the direct physical ingress of these organisms into the tissue in cross-sections was observed suggesting that these were potentially opportunistic invaders.

5.2. Variability in bacterial diversity in PWPS and HT

The bacterial communities in both healthy and PWPS-infected tissues of *P. lutea* were dominated at all three sampling locations by members of the *α-proteobacteria*, *γ-proteobacteria* and *Cyanobacteria* (Fig. 11). However, diseased corals exhibited higher bacterial diversity compared to healthy ones (Table 9). These results are consistent with

recent studies which have reported lower bacterial abundance and diversity in healthy corals than those displaying signs of WPD (Sunagawa et al. 2009; Cárdenas et al. 2011; Roder et al. 2013) and WBD (Pantos and Bythell 2006). Examination of 16S rRNA gene sequences using cloning as a culture-independent molecular technique, revealed high variability between bacteria associated with PWPS-infected and healthy tissues of *Porites lutea*, with only a few ribotypes commonly found in both diseased and healthy tissues (Appendix 1). Among them, ribotypes similar to *Pseudoalteromonas* spp., *Paracoccus yeei* and *Amphritea balenae* have been previously identified in seawater (Sweet et al. 2010), soil (Daneshvar et al. 2003) and sediment (Miyazaki et al. 2008) respectively, suggesting that these bacteria were present in the environment and opportunistically became resident in the coral mucus or associated with the healthy coral microbiota. Similar variations in the bacterial communities have been reported in several coral species affected by BBD (Voss et al. 2007), WPD (Pantos et al. 2003), WBD in the Caribbean (Pantos and Bythell 2006) and other WS in Australia and American Samoa (Kvennefors et al. 2010; Godwin et al. 2012; Wilson et al. 2012). The bacterial diversity found in PWPS was higher than in HT at all three localities. Our results were similar to those reported for white syndromes including *Acropora* white syndrome (AWS) on American Samoan reefs (Wilson et al. 2012) and white plague disease in the Caribbean coral *Montastrea annularis* (Pantos et al. 2003). This difference in the composition of bacterial communities may suggest that disease agents impair the structure of natural bacterial communities. It is likely that compromised or dead tissues represent a "micro-niche" that can be colonised by more competitive and opportunistic bacteria in the surrounding water and sediments or transmitted by other marine organisms (Pantos et al. 2003; Pantos and Bythell 2006; Gil-Agudelo et al. 2007; Cárdenas et al. 2011; Godwin et al. 2012).

Interestingly, comparisons of bacterial communities associated with both PWPS-infected and healthy tissues also revealed distinct populations at the three sampling locations (Appendix 1,

Fig. 12). This may suggest that no specific bacterial communities are associated with *P. lutea* on the WIO reefs. However some exceptions were recorded. For example, *E. elysicola* (accession no. NR041264) and *V. fortis* (accession no. NR025575.1) were found in both PWPS and HT collected at all the localities and seemed to be coral-specific. Another species, *V. rumoiensis* (accession no. NR024680) seemed to develop the same specific bacterial-coral association but was only found in HT sampled on South Africa and Mayotte reefs and not those at Reunion. These bacterial strains, apparently ubiquitous in HT, may play an important role in coral health and growth (Pantos and Bythell 2006). For instance the genus *Endozoicomonas*, found in several marine organisms (Kurahashi and Yokota 2007; Jensen et al. 2010; Bayer et al. 2013; Nishijima et al. 2013), seems to play an important role in corals, notably in the biogeochemical cycling of sulphur (Raina et al. 2009). *V. fortis* was initially described as a probiotic that out-competes pathogen strains (Thompson et al. 2003; Cunning et al. 2009) or is involved in the recycling of dimethyl-sulfoniopropionate (DMSP), which may be detrimental to coral health (Raina et al. 2009). However, further studies are needed to elucidate the ecological function of these genera in corals.

5.3. Potential pathogens associated with PWPS

In our study, several 16S rRNA gene sequences were closely related (97-100% similarity) to bacteria associated with coral diseases or known pathogens. Interestingly, one sequence was detected in PWPS from all three sampling localities but absent in healthy corals. Blast identification associated with phylogenetic analysis (Fig. 13) showed it to be closely related to the *γ-proteobacteria V. hepatarius* (accession no. NR025575.1), isolated for the first time from the healthy wild white shrimp *Litopenaeus vannamei* in Ecuador (Thompson et al. 2003). Other 16S rRNA gene sequences affiliated to the family *Vibrionaceae* were associated with PWPS-infected tissues (Fig. 13).

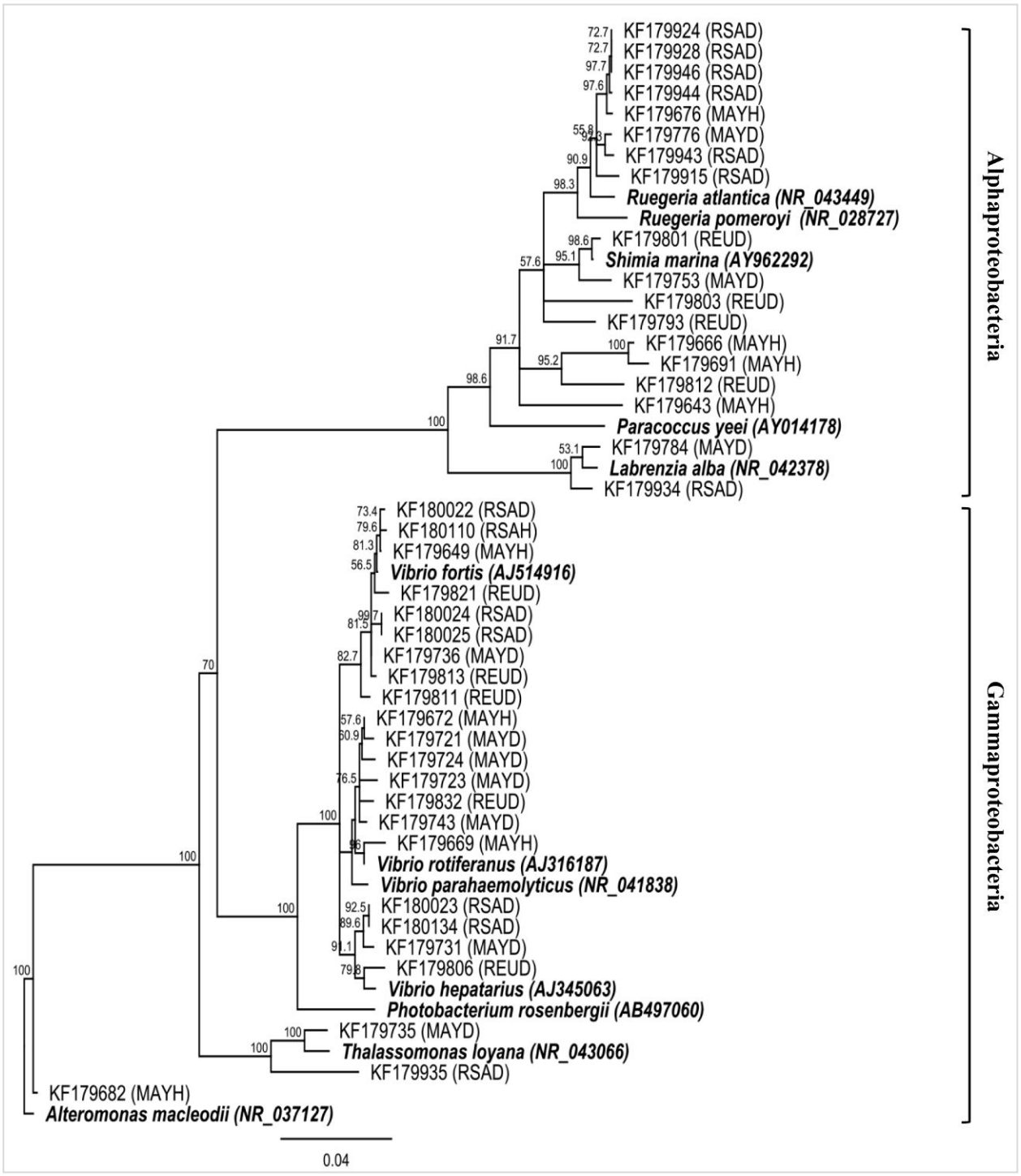


Figure 13: Neighbour-joining phylogenetic tree for the 16SrRNA gene sequences that were closely related to known and putative pathogens found in both healthy (HT) and *Porites* white patch syndrome (PWPS)-infected tissues (DT) of *Porites lutea* from Mayotte (MAY), South African (RSA) and Reunion (REU) corals. Numbers at each node are bootstraps values (%) obtained after 1000 iterations.

For instance, *V. fortis* was detected at all three sampling locations. This bacterium was first isolated from various marine organisms and has been reported to be pathogenic in corals (Boyett 2006), fish and crustacean (Austin et al. 2005), and is associated with several coral diseases including yellow band disease (YBD) in *Montastrea faveolata* (Cunning et al. 2009) and BBD in the Red sea (Barneah et al. 2007). However, sequences affiliated with this species were also found in HT from all three localities, making this a less likely candidate for PWPS pathogenesis. In addition, ribotypes similar to *V. parahaemolyticus*, known to induce disease in humans (Austin 2010) and many aquatic organisms (Cho et al. 2008), were identified as well as *V rotiferanus* associated with YBD in several Caribbean and Indo-Pacific scleractinian species (Cervino et al. 2008). However, similar sequences were found in healthy coral tissues or were not represented in diseased tissues at all three localities. Finally, sequences affiliated to *Shimia marina* (accession no. NR043300.1) were recorded only in PWPS-infected corals at all three sampling localities. This *roseobacter* was previously reported in the coral *Turbinaria mesenterina* infected by ASWS (Godwin et al. 2012) but no evidence of its pathogenicity has been established in previous studies. The potential pathogens related to the sequences obtained in this study thus need to be isolated, cultured and inoculated in laboratory corals to ascertain their ability to induce disease in corals.

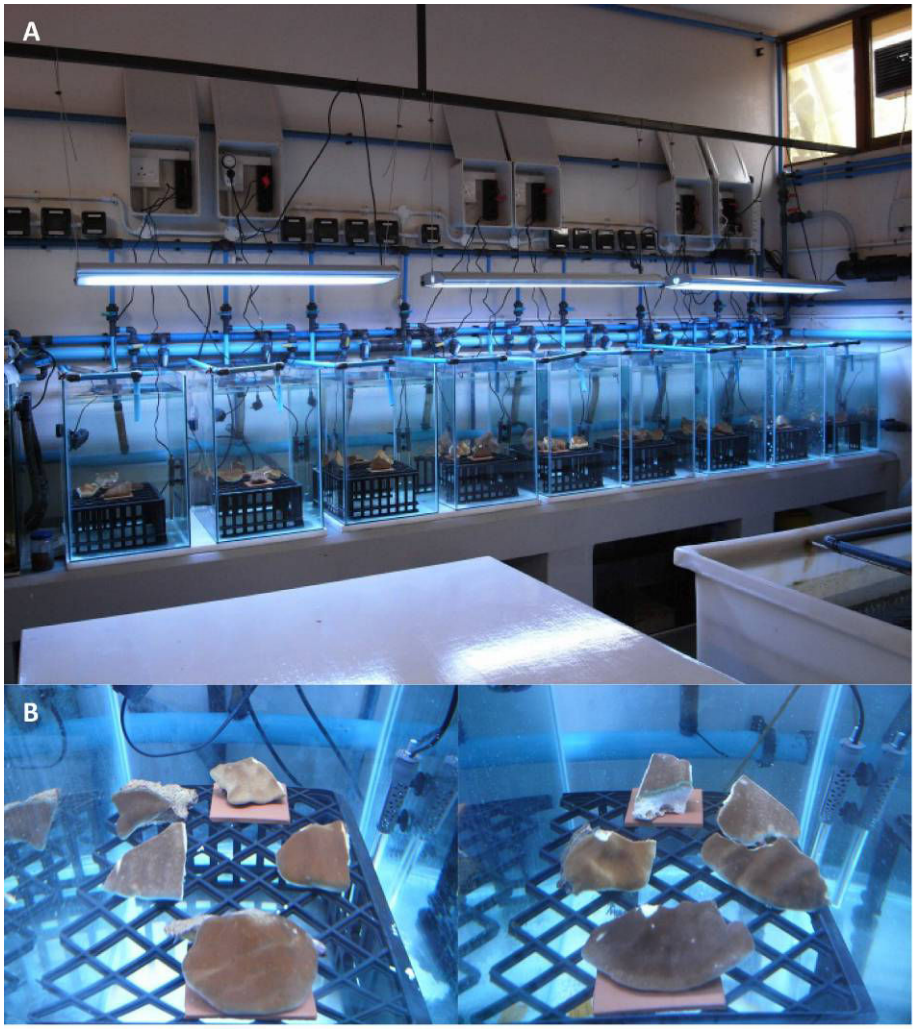
5.4. Conclusions

This is the first study characterising bacterial communities associated with healthy and PWPS-infected *Porites lutea* coral colonies on WIO coral reefs. Microscopy revealed the inclusion of basophilic bodies like bacterial aggregates in the coral epidermis within the lesion area. We established that the structure of the microbial communities is different between diseased and healthy coral tissues, and between localities, by cloning the 16S rRNA gene as a culture-independent molecular method. Furthermore, higher bacterial diversity was observed in PWPS-infected tissues. This shift may be explained by a perturbation of the

natural bacterial communities associated with coral holobionts which are progressively replaced by a succession of opportunistic bacteria including potential pathogens. Since the bacterial diversity at each of the three sites was assessed by analysing pooled samples, additional replicates including seasonal monitoring is needed to confirm the heterogeneity of bacterial species associated with PWPS in the areas studied. Several bacterial ribotypes affiliated to potential putative pathogens were consistently found among the 16S rRNA sequences derived from the PWPS lesions, and absent and/or poorly represented in HT. Isolation, culture and subsequent infection trials to satisfy Henle-Koch's postulates would be needed to prove their pathogenicity.

Chapter 4

Identification of a primary pathogen involved in white patch syndrome, a newly-reported disease affecting the massive coral *Porites lutea* in the Western Indian Ocean



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1. Abstract

A novel white syndrome, the *Porites* white patch syndrome (PWPS), was recently reported and described on Western Indian Ocean coral reefs. Bacterial communities associated with this coral disease were characterised using culture-independent molecular techniques (clone libraries), yielding a number of putative bacterial pathogens potentially involved as causative agents of this pathology. A total of 14 bacterial strains retrieved from PWPS-infected colonies of *Porites lutea* were subsequently used in experimental infection trials. Coral fragments exposed to a bacterial strain (P180R) closely related (99-100 % sequence identity) to *Vibrio tubiashii* elicited similar evidence of disease to that observed in the field. Following experimental infection, this pathogen was successfully re-isolated from the diseased tissues and re-inoculated in healthy corals colonies. Signs of PWPS appeared on 90% of the exposed coral fragments (27 of 30) under controlled environmental conditions. Moreover, virulence of this newly-identified marine pathogen seemed to be strongly dependent on seawater temperature, resulting in significantly higher tissue loss at 30°C than 28°C and 26°C. Nevertheless, further research is needed to determine if temperature is the primary factor that controls its virulence. Additional proof is needed to link P180R with PWPS at the gross and cellular level.

Keywords: Coral disease - Western Indian Ocean - Scleractinian corals - *Porites* white patch syndrome - Inoculation trials - Causative agent - *Vibrio tubiashii*

2. Introduction

Coral diseases are increasingly recognised as a significant threat to coral reefs (Bruno and Selig 2007; Harvell et al. 2007) with more than 30 syndromes reported to date (Weil et al. 2006). Among them, ten distinct white syndromes (WS), grossly characterized by advancing acute tissue loss (Willis et al. 2004; Sussman et al. 2008), have been described worldwide e.g. white plague (WP), white band disease (WBD), Australian subtropical white syndrome (AWS) (Godwin et al. 2012), progressive white syndrome (PWS) (Sweet and Bythell 2012), *Porites* ulcerative white spot disease (PUWS) (Arboleda and Reichardt 2010), white pox disease (WPD) (Sutherland et al. 2011)**,** *Montipora* white syndrome (MWS) (Ushijima et al. 2012), Indo-Pacific white syndrome (Sussman et al. 2008), acroporid white syndrome (AWS) (Aeby et al. 2011; Roff et al. 2011) and white plague-like disease (Barash et al. 2005).

Several studies have proposed bacteria as causative agents of these WS (Patterson et al. 2002; Sussman et al. 2008; Arboleda and Reichardt 2010; Luna et al. 2010; Sutherland et al. 2011; Godwin et al. 2012; Sweet and Bythell 2012; Ushijima et al. 2012). For example, *Thalassomonas loyana* has been suggested to cause white plague-like disease on Eilat coral reefs (Thompson et al. 2006) and *Aurentimona coralicida* to be the aetiological agent of WP type II (Denner et al. 2003). Several members of the genus *Vibrio*, *e.g*. *V. corallilyticus* (Ben-Haim and Rosenberg 2002), *V. harveyi* (Luna et al. 2010; Godwin et al. 2012) and *V. shilonii* (Israely et al. 2001; Kushmaro et al. 2001), have also been reported as putative pathogens in other WS. However, very few experimental studies involving inoculation trials have been undertaken on WS. To date, only six bacterial species have been identified as aetiological agents satisfying the following four Henle Koch's postulates (Israely et al. 2001; Ben-Haim et al. 2003b; Sussman et al. 2008; Arboleda and Reichardt 2010; Sutherland et al. 2011; Ushijima et al. 2012): a putative pathogen must be i) found only in diseased tissue, ii) isolated and grown in pure culture. The isolated and purified organisms must iii) cause disease when

inoculated into a healthy organism and iv) be re-isolated from the infected host (Koch 1982). For instance, the human pathogen *Serratia marcescens*, known to generate respiratory infections, pneumonia and meningitis (Grimont and Grimont 2006), has been found to generate WPD in the Elkhorn coral *Acropora palmata* (Patterson et al. 2002; Sutherland et al. 2011). Additionally, the *λ-proteobacterium Vibrio owensii*, has been recently described as a pathogen of MWS in *Montipora capitata* (Ushijima et al. 2012). However, despite having fulfilled all pathogenic criteria, no definitive proof linking some of these identified putative pathogens to the syndromes were demonstrated since inoculation trials were performed with one specific putative pathogen rather than multiple potential pathogens and/or strains of the same type.

Recently, a newly-identified WS, *Porites* white patch syndrome (PWPS), has been reported on massive *Porites lutea*, one of the most important reef-building coral on the Western Indian Ocean reefs (Séré et al. 2012). This WS is recognised by circular to oblong tissue loss (5.0–30.0 cm diameter) surrounded by a front of swollen and bleached tissues (1.0–20.0 cm width, Fig. 1). A recent molecular study on bacterial communities associated with PWPS-infected tissues in *P. lutea* conducted on three geographically distant sites of the western Indian Ocean (WIO), revealed the presence of several 16S rRNA gene sequences affiliated to potential causal agents (Séré et al. 2013). Of the several identified potential pathogens, bacterial 16S rRNA sequence genes phylogenetically closely related to *Shimia marina* and *Vibrio hepatarius* were found only in diseased coral tissues collected at three distant sites of the WIO.

This study aimed to isolate and identify the primary pathogen(s) of PWPS by combining traditional culturing methods and molecular genotyping coupled with inoculation trials to test for Koch's postulates. Strain(s) inducing signs resembling PWPS in aquaria were further characterised using standard bacteriological tests. Finally, the impact of elevated seawater

temperature, identified as an important abiotic factor enhancing the virulence several coral diseases, was also tested in aquarium experiments.

3. Methods

The sampling of *Porites lutea* colonies for this study was authorised by the French Department of Ecology, Sustainable Development, Transportation and Housing (DEAL), the Isimangaliso Wetland Park (KZN-Wildlife, South Africa) and CITES (Permit no. FR1197400391-I to FR1197400394-1).

3.1. Sampling, growth conditions and isolation of bacteria

Three core samples from three individual *P. lutea* colonies manifesting PWPS (Fig. 14) were collected at 10-15 m deep at Reunion (21 ° 07'S 55 ° 32'E) and Mayotte Islands (12 ° 41 'S, 45 ° 10' E).

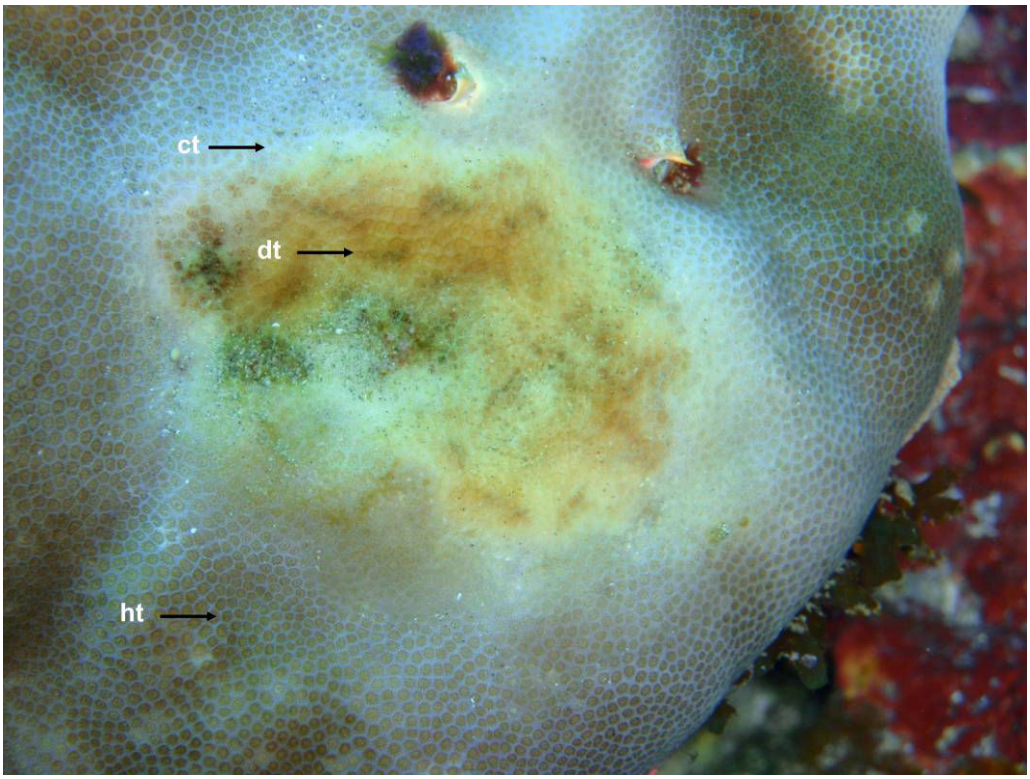


Figure 14: Colonies of *Porites lutea* exhibiting signs of PWPS characterised by oblong tissue loss surrounded by bleached and swollen tissue. ct = compromised tissue; dt = dead tissue; ht = healthy tissue.

One fragment of 5 g (wet weight) of each sample was crushed using a sterile mortar and pestle and diluted in 10 ml of 0.22 µm filtered seawater. Crushed samples were immediately streaked (100 µl) in triplicate serial dilutions (1:1, 1:10 and 1:100) on Marine Agar (1.8% Marine Broth, Difco-2216 USA, 0.9% NaCl, 1.8% Agar Bacto, Difco-214010, USA; MA) and thiosulfate citrate bile salts sucrose (TCBS) agar and incubated at 32°C for 24 h. Cultivable bacterial strains exhibiting a unique colony were routinely subcultured to purification under the same growth conditions for their subsequent use in inoculation experiments.

3.2. DNA extraction, PCR amplification and 16S rDNA sequencing

A total of 278 pure cultures were retrieved from diseased samples collected at Reunion and Mayotte. Genomic DNA was extracted from each bacterial colony by boil lysing in 100 µl of 5mM Tris/HCl at 100°C for 5 minutes. PCR amplification was carried out in a volume of 25 µl containing GoTaq®Hot Start Green Master Mix (Promega, Madison, WI), 0.5mM of 16S rRNA primers 27F (AGAGTTTGATCMTGGCTCAG)/1492R (GGTTACCTTGTTACGACTT) (Frias-Lopez et al. 2002), and 10 ng of template DNA. Amplification conditions for the PCR included an initial denaturing step of 4 min at 95.5˚C followed by 30 cycles at 94˚C for 30 sec, 55˚C for 60 sec, and 72˚C for 90 sec, with a final extension step of 15 min at 72˚C. Sequencing was carried out by Genoscreen (Lille, France). Partial sequences (700-1100 bp) obtained for each bacterial isolate were examined for error and edited using GENEIOUS™ Pro (V.5.6.6) sequencing software. All partial sequences were then submitted to BLAST at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) and compared with available sequences. The 16S rDNA consensus sequences of the bacterial isolates causing disease similar to PWPS were aligned with 15 reference rDNA gene sequences closely related to multiple strains of *Vibrio* species using the multiple sequence alignment method (93% identity). A phylogenetic tree based on the

alignment of a region of 1584 bp was built by the neighbour-joining method using the NKY model of GENEIOUS^{TM} Pro (V.6.1.5). Bootstrap values were based on 1000 replicates.

3.3. Coral collection, maintenance and experimental design

Healthy colonies of *Porites lutea* were collected in South Africa at Sodwana Bay at 15 m using SCUBA and placed in zip-lock plastic bags containing fresh seawater, filled with oxygen and placed in a cool box for transport to the ORI Research Aquarium. Here they were acclimated in a 1000 l plastic tank for 3 months under the following controlled conditions: T: 24˚C, with a photoperiod 12:12 h light: dark. A total of 45 small *P. lutea* fragments were then cut from each colony and monitored for two weeks until tissue had grown on the exposed skeleton. All bleached and lysed colonies were discarded. After the healing period, five coral fragments were randomly assigned to each of nine individual 100 l pre-sterilised aquaria (Fig. 15).

Figure 15: Experimental set-up of inoculation trials showing the nine 100 l tanks individually supplied with oligotrophic seawater and exposed to constant, artificial light.

Each aquarium was continuously supplied with oligotrophic seawater (70 l/h) pumped from the sea and filtered through a sand filter, a bio-filter (live rock) and a protein skimmer (RK2 Systems, USA) designed to remove floatables, grease and oils. To eliminate any external contamination by parasites, microalgae and diseases, the filtered seawater was purified by passage through a UV sterilizer (55 W, Ao Aqua Medic, Germany). Corals were exposed to a constant irradiance of 249.2 µmol photons m^{-2} s^{-1} using metal halide lamps (HAILEA®LFHO LAMP, 4 x 54 W/ Extra Reef tubes blue and white) and were slowly acclimated to 28˚C by increasing the initial temperature (24˚C) by 0.5˚C per day using heaters (SICCE, SCUBA 100, Italy). A flow pump (320 SEIO^{TM} PROP) was added to each aquarium to maintain aeration and water mixing. Experimental conditions, including pH, temperature, salinity, dissolved oxygen and light intensity, were kept constant and were assumed to be similar in each aquarium. The waste water was treated with another UV sterilizer (55 W, Ao Aqua Medic, Germany) before being discharged in a canal in order to avoid any risk of environmental contamination.

3.4. Phase 1: Screening of potential PWPS pathogens for infectivity

In total, 14 bacterial ribotypes retrieved from PWPS samples were selected for the inoculation experiments (Table 10). Only bacteria that were closely related (>95% identity) to identified pathogens found exclusively in PWPS-infected tissues at Reunion and Mayotte were selected. Candidate strains for the infection trials were also selected according to 16S rDNA sequences found in the molecular-based study conducted by Séré et al. (2013) using culture-independent molecular techniques. Each candidate strain was picked from pure cultures, incubated and grown at 28°C in sterile 15 ml PP Falcon tubes containing sterile Marine Broth (MB) until the end of the logarithmic phase (15-16h). The growth (CFU/ml) of each bacterial candidate was estimated by counting CFU on solid MA. Individual cultures were centrifuged at 3000 *g* for

10 min at 4°C and the pellets were transferred to sterile seawater (SSW) with a volume adjusted to contain a final concentration of ± 10^6 CFU ml^{-1}.

Table 10: List of strains retrieved from samples of PWPS collected on Mayotte and Reunion reefs and used during phase 1 inoculation trials.

Class/Subdivision	Closest species in GenBank database	Best match (%)	Similar 16S rRNA sequences gene		Strain Code	Sequence lengths (bp)	GenBank Acc. No
			MAY	**REU**			
Actinobacteria	*Micrococcus luteus*	99	*	*	**P230R**	812-947	NR_037113.1
Alphaproteobacteria	*Paracoccus yeei*	97-98	*	*	**P144M**	749-800	NR_029038
Firmicutes	*Staphylococcus aureus*	99	*	*	**P207R**	748-928	CP003033
	Shimia marina	99	*	*	**P57M**	769-775	NR_043300
Gammaproteobacteria	*Alteromonas marina*	99	*	*	**P74M**	847-853	NR_025260.1
	Photobacterium rosenbergii	99	*	*	**P3R**	857-891	NR_042343.1
	Vibrio coralliilyticus	98	*	*	**P74M**	852-739	NR_028014.1
	Vibrio fortis	98-100	*	*	**P6M**	857-980	NR_025575.1
	Vibrio harveyi	97-99	*	*	**P197R**	832-920	NR_043165.1
	Vibrio hepatarius	99	*	*	**P180R**	819-890	NR_025491.1
	Vibrio parahaemolyticus	99-100	*	*	**P160R**	635-893	NR_041838.1
	Vibrio rotiferianus	99	*	*	**P275R**	824-955	NR_042081
	Vibrio rumoiensis	99	*	*	**P17R**	885-898	NR_024680.1
	Vibrio sinaloensis	97-99	*	*	**P86R**	888-963	NR_043858.1

Coral fragments were carefully removed from each aquarium and placed into a plastic container containing 2 l of sterile seawater for inoculation. Prior to bacterial inoculation, each fragment was lightly scratched with a sterile pipette tip to break the mucus barrier and thereby create an entry point for the potential pathogens, simulating a natural trauma. Inoculation experiments were conducted by smearing 0.5 ml of isolate (± $1x10^6$ CFU ml^{-1}) onto the surface of each of the five healthy fragments of *P. lutea*. Control aquaria (N=2), also containing five healthy coral fragments, were treated using the same protocol but without bacterial inoculation. The coral fragments were then monitored and photographed on a daily basis. The number of individuals that displayed evidence of PWPS (morbidity) or that died as a result of infection (mortality) were recorded.

3.5. Phase 2: Infection trials:

To fulfil Henle-Koch postulates three samples of fragments infected in phase 1 were crushed, diluted 1:10 in sterile and filtered seawater and plated in triplicate on both MA and TCBS. Bacterial colonies that grew on both media overnight were subcultured to purification. Bacterial strains with 100% 16S rRNA gene similarity to the strain that initiated disease resembling PWPS during phase 1 of the inoculation trials were then re-inoculated on healthy colony fragments. Prior to the phase 2, the aquarium system was carefully cleaned and sterilised to eliminate residual bacteria from contaminating and biasing the results. Batches of five fresh coral fragments (N= 45 coral fragments) from apparently healthy colonies of *P. lutea* maintained in a 1000 l plastic tank were again re-distributed into each of the nine tanks. Infection experiments including preparation of the inoculums and inoculation were repeated as described above. Three of the nine aquaria containing five coral fragments (N=15 fragments) were not inoculated as controls. The coral fragments were again monitored and photographed for signs of PWPS.

3.6. Effects of temperature on virulence

Temperature experiments were conducted using the experimental procedure described above. Again, the aquarium system was carefully cleaned and sterilised prior to the experiment to prevent residual bacterial from contaminating and biasing the results. Fresh, apparently healthy coral fragments of *P. lutea* were divided into three treatments at three temperatures: **T1**: 26°C, **T2**: 28°C and **T3**: 30°C with two replicates and one control for each treatment. Each contained five coral fragments, randomly allocated to the nine 100 l tanks. Fragments were acclimatized for a week at their respective experimental temperatures and were monitored for apoptosis, bleaching and disease before commencing the temperature trials. The inoculum (± $1x10^6$ CFU ml^{-1}) was smeared onto the surface of each of healthy fragments as described in phases 1 and 2 and monitored and photographed. The mean proportions of

infected colonies and virulence (mean number of dead colonies per tank) were calculated. Finally, the rate of tissue loss (cm^2) was estimated for 170 h using image analysis software (AxioVison 4.8.2) to determine the progression rate of PWPS when exposed to different temperatures.

3.7. Multilocus sequence and phylogenetic analysis

A mutlilocus sequence typing (MLST) analysis using three additional housekeeping genes (*pyrH*, *recA* and *rpoA*) was performed to complete and confirm the 16S rRNA gene-based identification. The primers and PCR amplification conditions are presented in Table 11. Partial sequences of 16S rRNA (1422 bp), *pyrH* (590 bp), *recA* (766 bp) and *rpoA* (802bp) genes were examined and corrected manually and consensus sequences were determined using the two reads.

Table 11: Sequencing primers for 16S rRNA (Frias-Lopez et al. 2002), pyrH, rpoA, recA, (Thompson et al. 2005) genes and PCR amplification conditions used for multilocus sequence typing (MLST) analysis.

Gene	Primer	Sequence (5' -> 3')	PCR amplification conditions
16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	Initial denaturing step of 4 min at 95.5˚C followed by 30 cycles at 94˚C for 30 sec, 55˚C for 1 min, and 72˚C for 1 min 30 sec, with a final extension step of 15 min at 72˚C
	1492R	GGTTACCTTGTTACGACTT	
pyrH	pyrH-04-F	ATGASNACBAAYCCWAAACC	Initial denaturing step of 4 min at 95˚C followed by 30 cycles at 95˚C for 1 min, 52˚C for 1 min, and 72˚C for 1 min, with a final extension step of 10 min at 72˚C
	pyrH-02-R	GTRAABGCNGMYARRTCCA	
rpoA	rpoA-01-F	ATGCAGGGTTCTGTDACAG	Initial denaturing step of 5 min at 95˚C followed by 30 cycles at 95˚C for 35 sec, 55˚C for 1 min 15 sec, and 72˚C for 1 min 15 sec, with a final extension step of 7 min at 72˚C
	rpoA-03-R	GHGGCCARTTTTCHARRCGC	
recA	recA-01-F	TGARAARCARTTYGGTAAAGG	Initial denaturing step of 5 min at 95˚C followed by 30 cycles at 95˚C for 35 sec, 46˚C for 1 min 15 sec, and 72˚C for 1 min 15 sec, with a final extension step of 7 min at 72˚C
	recA-02-R	TCRCCNTTRTAGCTRTACC	

The consensus sequences obtained for each gene were then aligned with reference sequences related to multiple bacterial strains selected from NCBI using GENEIOUS alignment method. Additionally, sequences obtained for each gene were concatenated and aligned against concatenated sequences from 12 other species of *Vibrio* mainly identified as known pathogens (Thompson et al. 2005). Phylogenetic trees were built by the neighbour-joining method using

the NKY model of GENEIOUS^{TM} Pro (V.6.1.5) with bootstrap values based on 1000 replicates.

3.8. Metabolic characterisation of putative pathogen(s)

The metabolic characterisation of the selected bacterial isolates was performed with API 20E V4.1 strips following the manufacturer's instructions (Biomériux Durham, NC). Susceptibility to antibiotics was performed using antibiotic diffusion disc (Oxoid) incubated for 24h at 30°C (Thompson et al. 2003). The following antibiotic discs were tested: amikacin, amoxicillin, ampicillin, ceftiofur, cephalexin, chloramphenicol, ciprofloxacin, enrofloxacin baytril, metronidazole. The effect of temperature on growth was tested by incubating the bacterial cultures at 4°C, 20°C, 28°C and 37.5°C. Finally, the motility was assessed microscopically.

3.9. Statistical analysis

The proportion of infected coral fragments (virulence) and mortality in each tank were calculated for each aquarium. Means (± SE) of tissue loss rates for each infected fragments was calculated every 24 h at the three experimental temperatures. Data were log-transformed [$\log_{10}$ (X)] to satisfy assumptions of normality and homogeneity of variance. Repeated Measures ANOVA (STATISICA 8) was performed to test for differences in tissue loss between the temperature treatments and potential tank effects. When a significant difference was detected, a Fisher LSD post-hoc test was used to test for significance of the measured differences between the temperature treatments.

4. Results

4.1. Phase 1: Screening for potential PWPS pathogens (infectivity)

During phase 1 of the inoculation experiment, only one bacterial strain caused disease resembling PWPS, *i.e.* circular to oblong tissue loss surrounded by a bleached and swollen front of affected tissue (Fig. 16).This isolate (P180R) had close 16S rRNA gene genetic affiliation with the *Gamaprotebacteria*, *Vibrio hepatarius* (AJ345063, KF179862, and KF179731), and its closest known sister taxon, *V. tubiashii* (X74725, Fig.17). In total, 80% (N=4 over 5 coral fragments) of the coral fragments of *P. lutea* exposed to this strain became infected and exhibited signs of disease after 12 h incubation. Among the 13 remaining isolates used in this study, two other strains, P6M and P17R, genetically closely related (>95% identity) to *V. fortis* (AJ514916) and *V. rumoiensis* (AB013297) respectively (Table 10), were positive after 24 h incubation, displaying a "light focalised" bleaching reaction on 60% and 20% of the inoculated colonies respectively. All coral fragments placed in control tanks remained unaffected during the experiment. Scratches made to remove the mucus and facilitate entry of pathogens healed completely (100%) 20 days after the first inoculation trials in control and trial fragments.

4.2. Phase 2: Infection trials

The strain P180R was re-isolated (P180R-ri) from the experimentally diseased corals exhibiting signs of PWPS during phase 1 of the study. Genetic analysis of 16S rRNA gene sequences confirmed 100% identity (>1400bp) with the strain used in phase 1 of the inoculations trials. Results from the replicated inoculations trials showed that 90% (N=27) of the fragments were positive (infective), exhibiting signs of PWPS resembling those observed during phase 1 of the experiment and in the field. All *P. lutea* fragments exposed to the pathogen developed signs of disease after 12 h incubation. No fragment exposed to P180R-ri

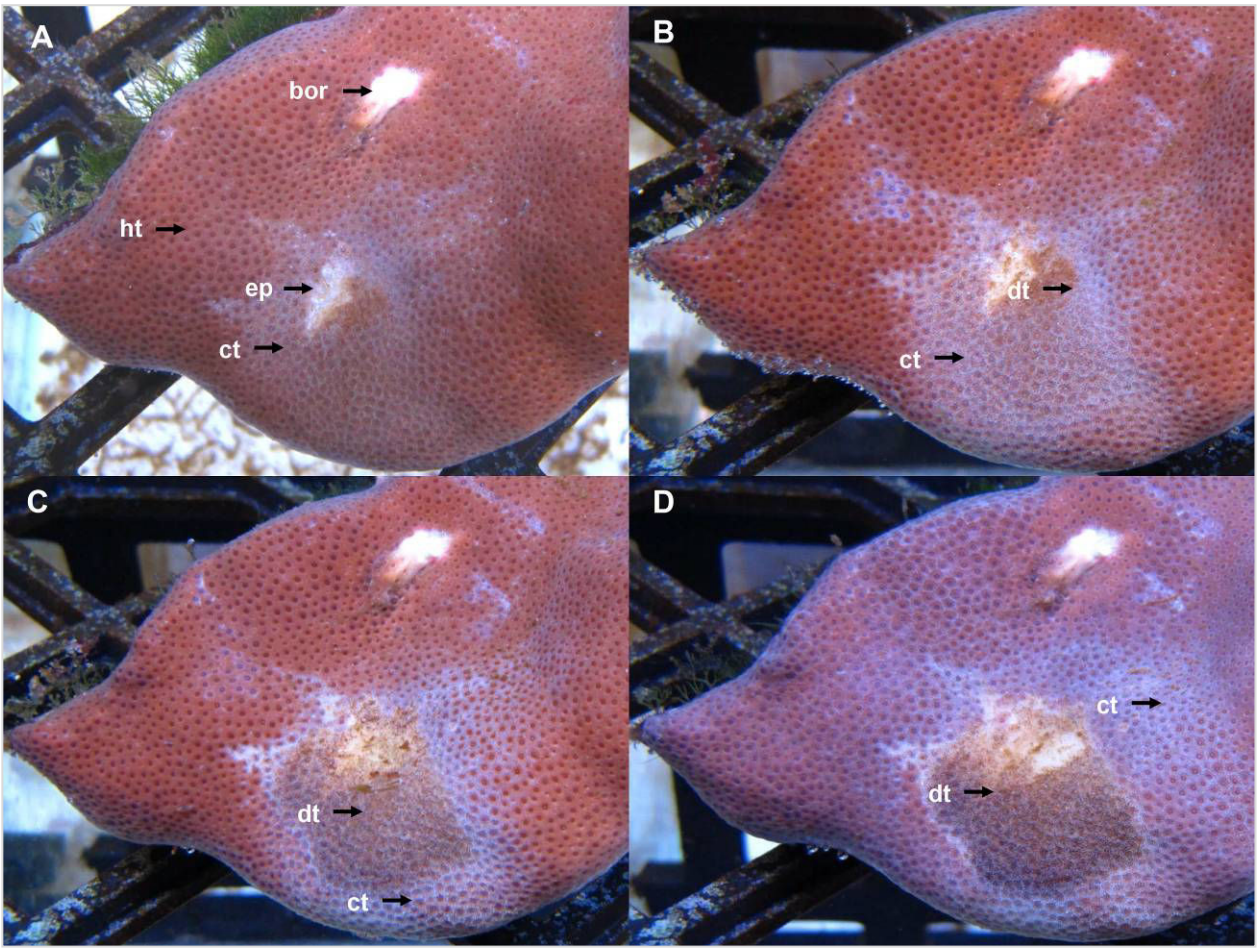


Figure 16: Time sequence photographs of *Porites lutea* fragments inoculated with a pure culture of the strain P180R manifesting disease A) 24 h, B) 48 h, C) 72 h and D) 96 h post-inoculation. Bor = borer; ht = healthy tissue; ep = entry point; ct = compromised tissue; dt = dead tissue.

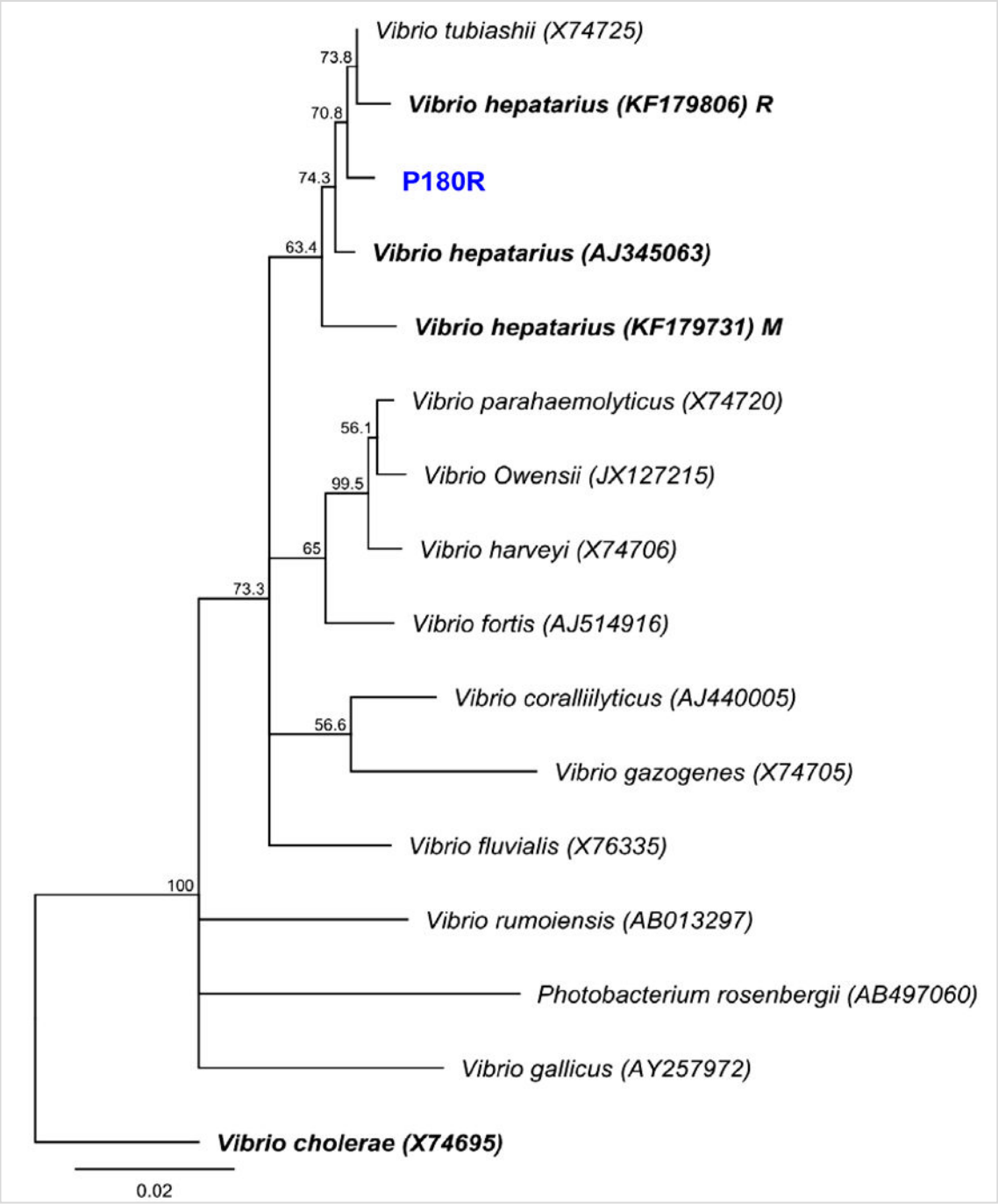


Figure 17: Neighbour-joining phylogenetic tree for the 16S rRNA gene showing the relatedness of the strain P180R with reference *Vibrio* strains. Numbers at each node are bootstraps values (%) obtained after 1000 iterations. The scale bar corresponds to the number of subdivisions per nucleotide position.

4.3. Temperature experiments

All colonies inoculated at the three temperature treatments had developed signs of PWPS 12h after inoculation (Fig. 5) but ANOVA analysis performed on means of average tissue loss revealed significant differences at the three temperature regimes (ANOVA: df= 2, F=39.77, P>0.01). The mean tissue loss at 30°C was significantly higher than at both 26°C (Fisher LSD post-hoc, p< 0.01) and 28°C (Fisher LSD post-hoc, p< 0.01). Fragments maintained in tanks at 28°C exhibited higher tissue loss than those at 26°C (Fisher LSD post-hoc, p< 0.01). Values of the disease progression rate in fragments exposed to 26°C, 28°C and 30°C were 0.51 ± 0.50 cm^2 day^{-1} (mean ± SE), 0.73 ± 0.80 cm^2 day^{-1} (mean ± SE) and 1.45 ± 0.85 cm^2 day^{-1} (mean ± SE) respectively. The PWPS in fragments exposed to both 26°C and 28°C had ceased to spread 96 h after infection, whereas the lesions on corals exposed at 30°C decreased slightly but had not halted in their spread after 170 hr (Fig. 5). Of the *P.lutea* fragments maintained at 30°C, 40% died three weeks after the experiment was terminated. However, those exposed to both 26°C and 28°C started to heal 126 h post-inoculation and recovered completely within one month of commencement of the infection trials.

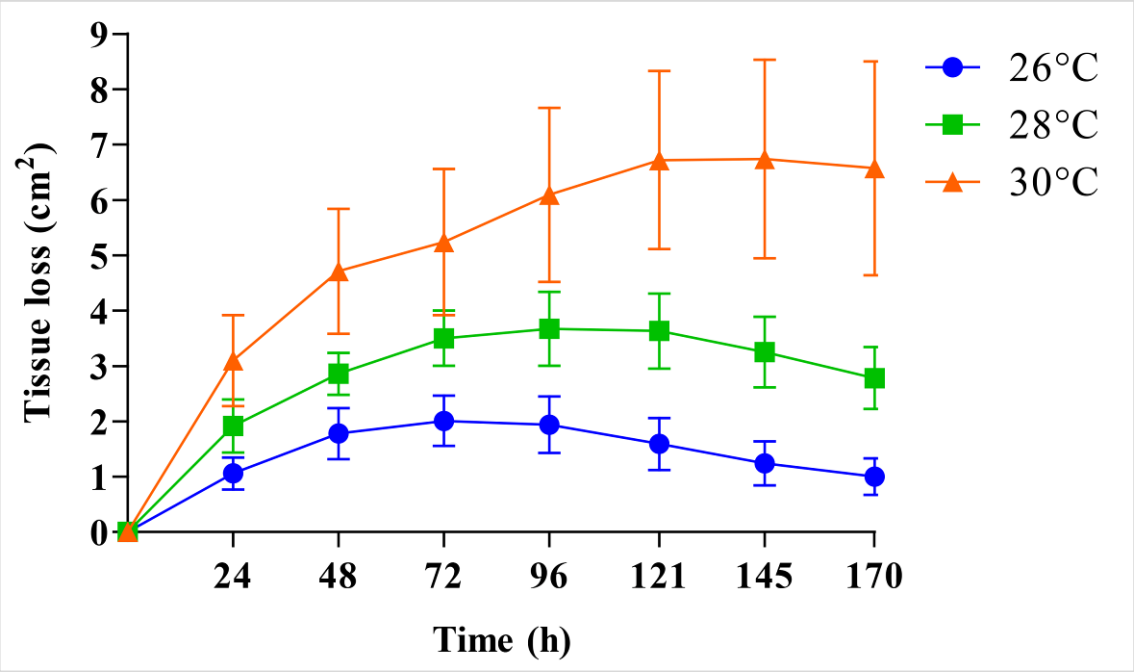


Figure 18: Mean tissue loss (cm^2 ± SE) of *Porites lutea* inoculated with P180R and held at 26°C, 28°C and 30°C.

4.4. Characterisation and identification of the primary pathogen P180R

P180R is a gram-negative bacterium forming beige/ cream colonies on MA, yellow on TCBS. Its culture yielded high growth at 37°C and emitted a strong sweet smell similar to sugar cane molasses. Tests on its phenotypic traits and susceptibility to antibiotics are presented in Table 12. While P180R appeared to be closely related to the *Gamaprotebacteria Vibrio hepatarius* (AJ345063, KF179862, and KF179731) using a region of 16S rRNA gene, additional analysis performed on *pyrH*, *recA* and *rpoA* genes revealed a greater affinity to *V. tubiashii* with 100% identity. Furthermore, phylogenetic analysis of the concatamerised genes (MLST) confirmed evidence that P180R is mostly phylogenetically related to the species *V. tubiashii* (Fig. 19).

Table 12: Biochemical and susceptibility tests on strains P180R and P180R-ri. - = non- susceptible, + = weakly susceptible, ++ = moderately susceptible, +++ = highly susceptible.

Tests	**P180R**
Colour on MA	Beige-cream
Colour on TCBS	Yellow
Growth at 2°C	Negative
Growth at 20°C	Positive
Growth at 28°C	Positive
Growth at 37.5°C	Positive
Motility	Positive
Biochemical tests	
B-galactosidase	Positive
Tryptophane Deaminase	Positive
Urease	Negative
Citrate utilisation	Positive
Indole production	Positive
Gelatinase	Positive
Fermentation/Oxidation	Positive
Susceptibility tests	
Amikacin	+
Amoxycillin/Clavulanic Acid	++
Ceftiofur	+++
Cephalexin	+
Chloramphenicol	+++
Ciprofloxacin	+
Enrofloxacin Baytril	-
Metronidazole	-

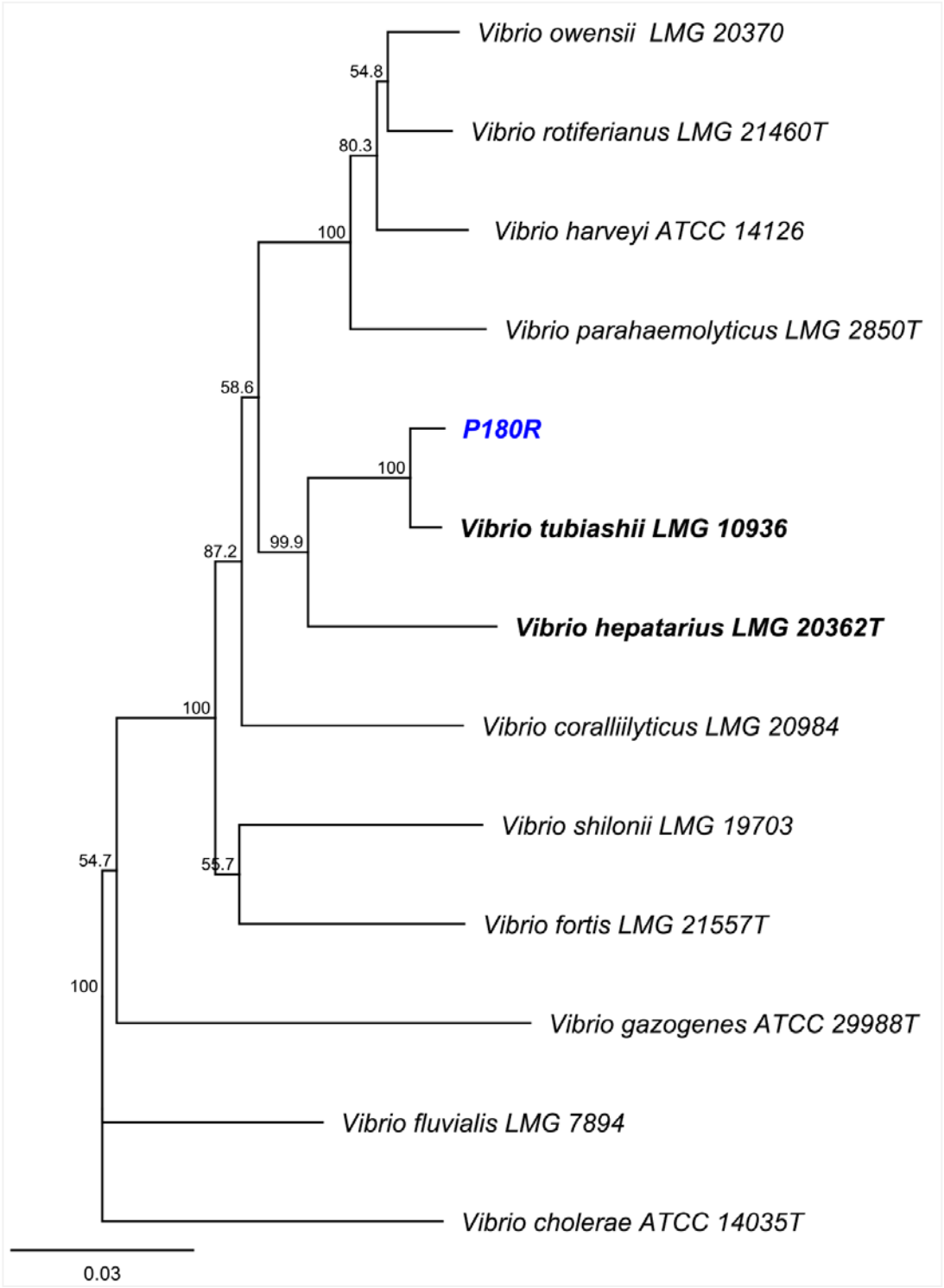


Figure 19: Neighbour-joining phylogenetic tree using the concatenated partial sequences (3580bp) of 16S rRNA, pyrH, recA and rpoA showing the relatedness of the strain P180R with reference concatenated *Vibrio* strains. Numbers at each node are bootstraps values (%) obtained after 1000 iterations. The scale bar corresponds to the number of subdivisions per nucleotide position.

5. Discussion

This is the first study to identify a primary causative agent of PWPS under controlled conditions. Bacterial strains cultured and used in laboratory based-infection trials were consistent with those identified recently in PWPS-infected *Porites* colonies using cultured-independent molecular techniques (Séré et al. 2013). Although marine bacterial culture is known to present challenges under laboratory conditions (Hill et al. 2002; Harvell et al. 2007; Bourne et al. 2009), 62.5 % of the 16S rRNA haplotypes identified using molecular methods and identified as putative pathogens (Séré et al. 2013) could be successfully cultured and subsequently inoculated on *P. lutea* in this study.

Of the 14 isolates selected for the inoculation trials, only the bacterial strain P180R mostly phylogenetically closely related to *V. tubiashii* was shown to cause signs resembling those of PWPS (Séré et al. 2012). This strain exhibited physiological traits similar to those reported for other pathogenic *Vibrio* spp. such as motility, oxidase and catalase activities and also a tolerance to temperature variations (Meron et al. 2009; Arboleda and Reichardt 2010; Ushijima et al. 2012). Interestingly, 16S rRNA gene sequences previously found in PWPS-infected tissues at three geographically distant WIO coral reefs (Séré et al. 2013) were closely related to P180R, suggesting its role as a primary pathogen of PWPS. *V. tubiashii* was initially recognised as a causative agent of a disease called vibriosis considered as a significant pathogen affecting early life stages of several species of bivalves (Hada et al. 1984; Elston et al. 2008; Hasegawa et al. 2008) and fish (Austin et al. 2005). This bacterial species is known to secrete and release toxins including haemolysin, cytolysin and a metalloprotease that are suspected to be pathogenicity factors inducing rapid onset of symptoms such as reduction in larval mobility and tissue necrosis (Kothary et al. 2001; Elston et al. 2008).

Time sequence photographs revealed a rapid response by fragments of *P. lutea* to the putative pathogen after inoculation, compared to other coral diseases. Indeed, coral tissues exposed to P180R displayed focalised and progressive tissue paling 12 h after inoculation and visible lesions of PWPS were observed 12 h thereafter. In contrast, previous experimental studies have reported that healthy *Acropora palmata* fragments infected by *Serratia marcescens* exhibited signs of WPD only 4-23 days after inoculation (Sutherland et al. 2011). Additionally, visual signs of PUWS in *Porites cylindrical* were visible only 15 days post-inoculation with a *Vibrio* sp., whereas *V. owensii* generated signs of WS within 30 days post-incubation under laboratory conditions (Ushijima et al. 2012). In this study, the infectivity rate of P180R was high (90%) compared to those reported for PUWS generated by a *Vibrio* sp. phylogenetically related to *V. natrieg*ens and *V. parahaemolyticus* (Arboleda and Reichardt 2010), and to WPD generated by *S. marcescens* (Patterson et al. 2002), but was relatively consistent with those reported for WS caused by the known pathogens *V. coralliilyticus* (Sussman et al. 2008) and *V. harveyi* (Luna et al. 2010). Nevertheless, no coral individuals died during the experiments and the infected tissues started to recover 20-29 days post-inoculation under laboratory conditions. This low pathogenicity may be related to i) the immune response in *P.lutea* which was not impaired under the experimental conditions (T°= 27.8 ± 0.05°C, pH= 8.27 ± 0.02, light = 249.2 µmol photons m^{-2} s^{-1}), and it was therefore able to fight infection; and/or ii) environmental conditions which did not allow full expression of the virulence of P180R (Brown et al. 2013).

Seawater temperature has been previously identified as an important abiotic factor impairing coral resistance to disease and enhancing bacterial virulence (Cervino et al. 2004; Barash et al. 2005; Bally and Garrabou 2007; Weil and Cróquer 2008). For example, tissue loss in *Pocillopora damicornis* (Ben-Haim et al. 2003b) and bleaching in *Oculina patagonica* (Kushmaro et al. 1997; Kushmaro et al. 2001) resulting from infections by *V. corralliilyticus*

and *V. shiloi* respectively have been reported to be temperature-dependant. Kushmaro et al. (Kushmaro et al. 2001) showed that *V. shiloi* caused bleaching at 25-26°C but not at 16°C. Furthermore, it has been demonstrated that *P. damicornis* inoculated with *V. coralliilyticus* generated no signs of infection at 22°C but bleached at temperatures of 24-26°C and developed rapid tissue lysis when exposed to temperatures of 27-29°C (Ben-Haim et al. 2003b). More recently, a study investigating the potential relationship between *Vibrio* infection and temperature in Yellow blotch disease (YBD) also identified temperature as an important driver of infection (Cervino et al. 2004). In this same study, coral colonies inoculated with a consortium of *Vibrio* species and exposed to 31°C resulted in 80% mortality, whereas those infected at 20°C still exhibited signs of YBD but without mortality (Cervino et al. 2004).

The potential effects of temperature on the virulence of PWPS were also tested in this study. The results yielded text-book model temperature dependencies with the virulence of P180R increasing progressively with increasing incubation temperatures. Additionally, all fragments of *P. lutea* that were exposed to 26-28 °C displayed signs of PWPS 24 h post-inoculation but the progression of infections diminished concomitantly 96 h after artificial infection. In contrast, PWPS infection of corals incubated at 30°C persisted for up to 170 h after commencement of the infection assays and resulted in 40% mortality several weeks after the end of the experiments. These responses to increased temperatures may be explained in different ways. First of all, high temperatures may compromise defence mechanisms in corals by impairing their immune system and thereby their ability to control pathogens (Harvell et al. 2007; Palmer et al. 2008; Brown et al. 2013). Healthy bacterial communities associated with corals are also known to provide important benefits (Rohwer et al. 2002; Lesser et al. 2004; Thurber et al. 2009; Littman et al. 2010; Rypien et al. 2010), notably antimicrobial compounds that protect the coral host against pathogens (Shnit-Orland and Kushmaro 2009).

In this regard, Alker et al. (Alker et al. 2001) have demonstrated that anomalously high temperatures reduced the efficiency of antifungal compounds produced by the sea fan *Gorgonian ventalina*. Thermal stress may also result in shifts in the natural bacterial communities associated with *P. lutea*, allowing the settlement and proliferation of opportunistic putative pathogens (Cervino et al. 2004). This might be investigated through deep sequencing, comparing complete bacterial communities between diseased and healthy corals over time when exposed to increasing temperatures. Finally, the virulence of pathogens may be enhanced at elevated temperatures, causing them to attain a toxic state and accelerating their growth rate (Lipp et al. 2002). For instance, Elston *et al*. (Elston et al. 2008) showed that virulence of *V. tubiashii* in shellfish hatcheries was strongly correlated with increasing sea surface temperature (SST).

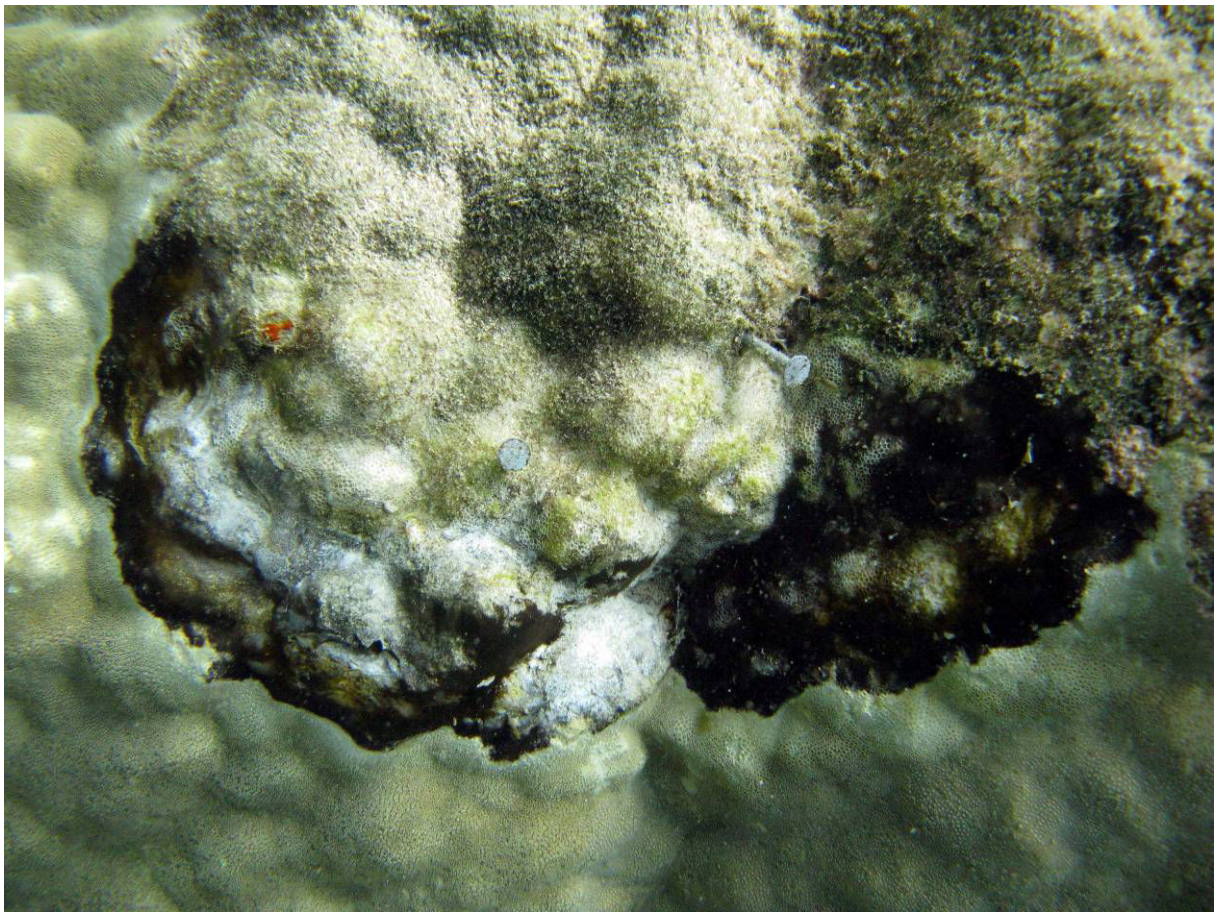
6. Conclusions

This study has shown the relevance of combining culture techniques and laboratory-based infection trials to identify putative coral pathogens. The role of P180R as a primary pathogen of PWPS has been demonstrated by satisfying Henle-Koch's postulates. Indeed, the strain used here induced the development of disease resembling that of PWPS during inoculation 1 and it was successfully re-isolated from inoculated corals that displayed the disease. This re-isolated strain was closely related to P180R (100% identity), again produced signs of disease in healthy *P. lutea* during phase 2 of the inoculation trials. Therefore, our results add another marine pathogen belonging to the Vibrionacea. Additionally, PWPS has been identified as a thermo-dependent coral disease as its severity increased at elevated seawater temperature. Nevertheless, additional proofs, using complementary approaches such as Transmission Electron Microscopy (TEM) or fluorescence in situ hybridization (FISH), are needed to link P180R with the disease signs at the gross and cellular level. Finally, further research should be conducted to establish whether thermal stress i) affects the coral immunity response, ii)

plays a primary role in the virulence mechanisms of *V. tubiashii* (toxin production) and compromises coral health, and/or iii) affects the coral-associated communities.

Chapter 5

Characterisation of *Porites* black patch syndrome, an atypical form of black band disease in the Western Indian Ocean



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1. Abstract

Recent surveys conducted on Reunion Island coral reefs revealed an atypical form of black band disease, the *Porites* black patch syndrome (PBPS), on two of the main framework building corals, *Porites lobata* and *P. lutea*. A multidisciplinary approach involving field surveys, gross lesion monitoring, histopathology and 454-pyrosequencing was employed to provide the first comprehensive characterization of this disease. Surveys conducted within two geomorphological zones over two consecutive summers and winters showed that it manifested spatial and seasonal variability, with more infected colonies observed on the reef flat and during the summer season. Histology revealed cyanobacterial penetration of the compromised tissue as well as the presence of basophilic bodies resembling bacterial aggregates in the living tissue, adjacent to the bacterial mat. Bacterial 16S rRNA sequences yielded a broader diversity of bacterial taxa in PBPS-infected tissues than in healthy tissue, represented by the genus *Vibrio* (24.9%), followed by sulfate-reducers or sulfide-oxidizers such as *Desulfovibrio* (20%), *Clostridium* (12.9%) and *Arcobacter* (9.9%). PBPS appears to be a multi-stage disease triggered by cyanobacterial invasion and resulting in secondary infections by environmental bacteria that grow in mucus-like decomposing tissue. While the reasons for the susceptibility of *Porites* spp. to cyanobacterial invasion are still unclear, the implications of the results are considered in terms of PBPS aetiology.

Keywords: Bacterial communities; coral disease; metabarcoding; *Porites* black patch syndrome; Reunion Island; scleractinian corals

2. Introduction

Black band disease (BBD) is one of the most widespread (Richardson 2004; Richardson et al. 2009), destructive (Richardson et al. 2009; Sato et al. 2009; Gantar et al. 2011) and intensively studied diseases on coral reefs worldwide (Rützler et al. 1983; Edmunds 1991; Al-Moghrabi 2001; Dinsdale 2002; Kuta and Richardson 2002; Boyett 2006; Voss and Richardson 2006; Boyett et al. 2007; Rodriguez and Croquer 2008; Sato et al. 2009; Zvuloni et al. 2009). Gross lesions of BBD are generally described (based on their presentation in the field) as a dark-coloured band (a few millimetres to centimetres wide, and up to 1 mm thick) separating living tissue from dead skeleton, and migrating across the coral colony (Antonius 1981; Rützler et al. 1983; Cooney et al. 2002; Myers and Richardson 2009; Gantar et al. 2011). As many as 70 coral species have been reported to be affected by BBD (Sutherland et al. 2004), particularly massive and slow-growing reef building corals (Richardson 2004; Gantar et al. 2011). Factors affecting susceptibility of corals to BBD or enhancing its progression and spread in corals are still not fully understood (Aeby and Santavy 2006; Voss and Richardson 2006; Boyett et al. 2007; Rodriguez and Croquer 2008; Sato et al. 2009; Zvuloni et al. 2009). However, a few experimental studies have linked nutrient enrichment, elevated temperature and light intensity to the pathogenesis of BBD in corals (Aeby and Santavy 2006; Voss and Richardson 2006; Boyett et al. 2007).

Historically, BBD pathology was first microscopically described as a microbial consortium dominated by filamentous cyanobacteria, associated with sulfate reducing (Garrett and Ducklow 1975) and sulphide oxidizing bacteria (Rützler et al. 1983). Later, studies using culture-independent molecular techniques revealed a dense and diverse microbial community classified into four functional groups, comprising photoautotrophs (cyanobacteria), sulphate reducers (*Desulfovibrio*), sulphide oxidizers (*Beggiatoa*) and organo-heterotrophs (*Vibrio*) (Cooney et al. 2002; Frias-Lopez et al. 2004; Richardson 2004; Sekar et al. 2006; Viehman et

al. 2006; Myers et al. 2007). Among these groups, a few bacteria have been suspected to be primary pathogens, including *Desulfovibrio* spp (Viehman et al. 2006) and *Vibrio coralliilyticus* (Arotsker et al. 2009); however, none of these species were tested experimentally and/or satisfied Henle Koch's postulates. In addition, variations have been detected in bacterial communities associated with BBD across geographic regions and between sympatric coral species (Voss et al. 2007). For instance, the presence of 16S rDNA sequences similar to *Trichodesmium* and *Oscillatoria* were reported in BBD-infected samples from Papua New-Guinea (Frias-Lopez et al. 2002), whereas members of the genera *Geitlerinema*, *Leptolyngbya*, *Lyngbya*, *Oscillatoria* and *Phormidium* were detected in BBD from the Caribbean and Philippines (Sekar et al. 2006; Myers et al. 2007). More recently, a transitional stage of BBD has been identified with different microbial communities and named "cyanobacterial patch (CP)". Molecular analysis revealed shifts in cynobacterial populations from sequences similar to *Bennothri*x sp. in CP to those related to *Oscillatoria* sp. in BBD (Sato et al. 2009; Sato et al. 2010). The high variability in BBD bacterial communities found between localities and infected host species may indicate that BBD actually derives from an earlier infectious stage which, after impairing the host's resistance, favours the infection and subsequent proliferation of opportunistic microorganisms such as cyanobacteria. However, beyond this highly speculative assumption and despite being intensively studied worldwide, the mechanisms of BBD development have remained unclear and no primary pathogens have been clearly identified.

Recent surveys, conducted on western Indian Ocean (WIO) coral reefs over two consecutive summers and winters between 2010 and 2012 (Séré et al. *submitted*), revealed an atypical form of black syndrome on two of the main framework building corals, *Porites lobata* and *P. lutea*, and termed here "*Porites* black patch syndrome" (PBPS). Following standardized terminology (Work and Aeby 2006), PBPS is characterized by a diffuse, centrally to

peripherally situated, medium to large black patch (1-10 cm in width), leaving behind it the dead skeleton generally covered by a whitish filamentous film (Fig. 20). The older exposed skeleton is progressively colonized by endophytic algae. Our study aimed at providing a comprehensive characterization of PBPS using a multidisciplinary approach involving field surveys, gross lesion monitoring, histopathology and molecular techniques. Here we have used 16S barcoding by 454-pyrosequencing to investigate bacterial community compositions in the i) diseased tissue, ii) apparently healthy tissue adjacent to the diseased region, and iii) healthy tissue taken from a separate healthy *Porites* colony.

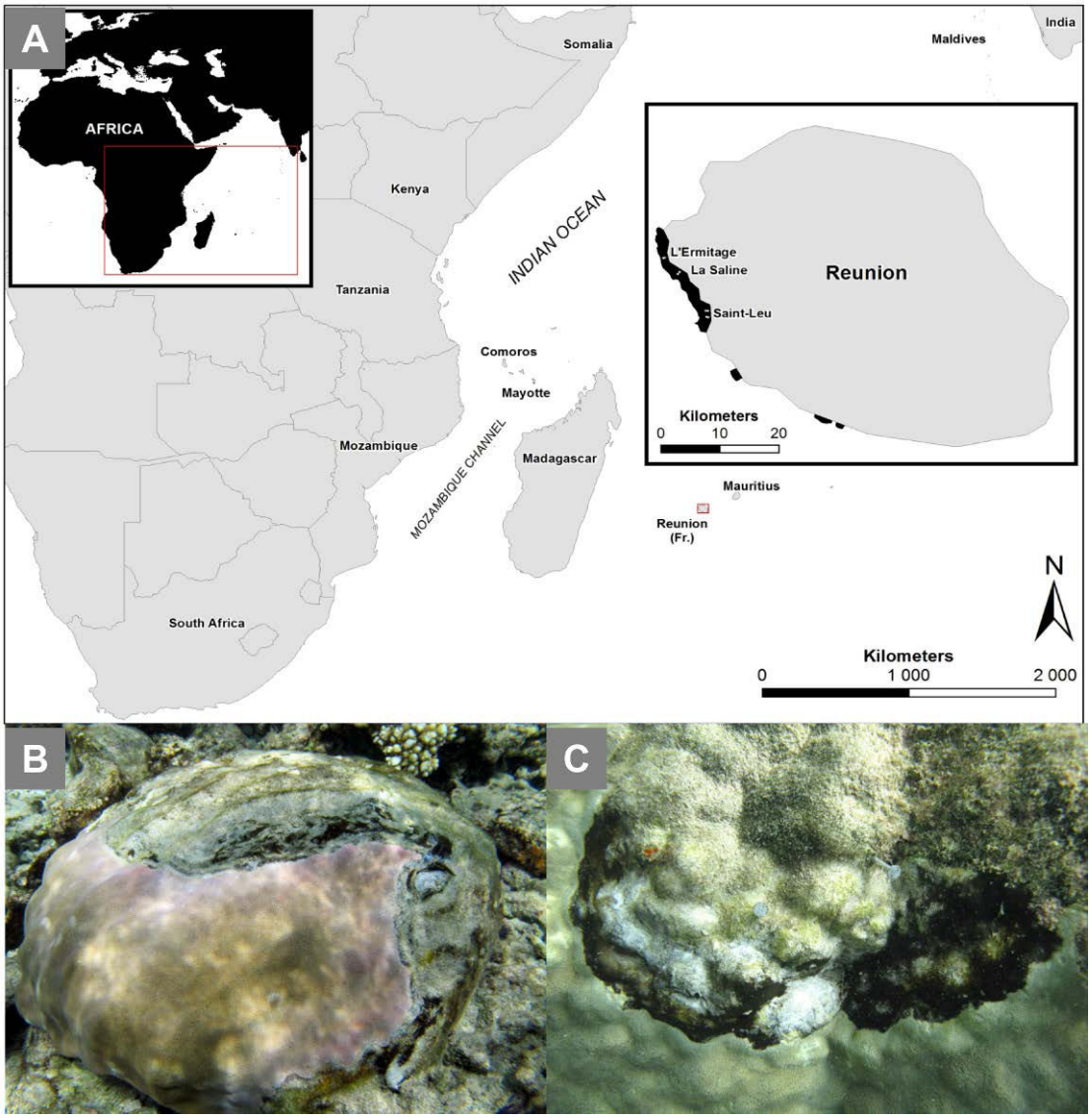


Figure 20: A) Map of the study sites, B) and C) Porites black patch disease on the massive coral Porites lutea. Note the whitish filamentous film that forms patches.

3. Material and methods

3.1. Field surveys and progression rate

Surveys were conducted in Reunion over two consecutive summers and winters, within four latitudinal sites and within two cross-shelf locations (Reef slope and Reef flat). Details of the sites are given in Table 13. The surveys were conducted in five 10 m x 2 m belt transects perpendicular or parallel to the depth contours on the reef slope, and three 20 m x 2 m belt transects parallel to the depth contours on the reef flat. All *Porites* displaying signs of PBPS were counted within each transect. The prevalence was estimated as the number of diseased colonies divided by the total number of *Porites* colonies (>2 cm) counted within each transect in 1 m × 1 m photo-quadrats. Disease fronts were monitored on a monthly basis from 30 November 2010 to 10 December 2011 to ascertain whether PBPS progressed. Nails were driven into the dead portions of five *Porites lutea* colonies behind disease front as reference markers for this purpose. The progression rate was recorded as the linear distance between the nails and the nearest live tissue by superimposing pictures taken in consecutive surveys.

Table 13: Location and depth of the reef sites and stations selected for this study.

Sites	Stations	Habitat	Reef depth (m)	Latitude/ longitude
L'Ermitage	3-Chameaux	Reef flat	0.5-1.0	-21.080351° ; 55.219576°
La Saline	Trou d'Eau		0.5-1.0	-21.103312° ; 55.242294°
Saint-Leu	La Corne		0.5-1.0	-21.165960° ; 55.285080°
Saint-Leu	Ravine des Poux		0.5-1.0	-21.176397° ; 55.285985°
L'Ermitage	3-Chameaux	Reef slope	10.0-12.0	-21.081281° ; 55.217590°
La Saline	Trou d'Eau		10.0-12.0	-21.106160° ; 55.239540°
Saint-Leu	La Corne		10.0-12.0	-21.165940° ; 55.281930°
Saint-Leu	Ravine des Poux		10.0-12.0	-21.175490° ; 55.283460°

3.2. Sample collection

Samples of *Porites lutea* exhibiting signs of PBPS were collected using SCUBA and snorkelling. Core samples of visually healthy (VHT) and diseased tissues (DT) from two diseased colonies and fixed in 4% formalin for histological examination of their tissue

structure. DT was sampled from the lesion boundary interface with visually healthy tissue (VHT) and healthy tissue (HT) was cored from an uninfected colony as a control. Fresh PBPS mat was collected from four infected *Porites lutea* colonies using needless and sterile syringe to identify the dominant cyanobacterial strains. Samples were placed in 10 ml centrifuge tubes with seawater and held in darkness at 20°C until return to the laboratory. Finally, two samples of DT, two samples of visually healthy tissue (VHT) and one sample of HT were collected using 10 mm core tubes to characterise the bacterial communities associated with PBPS. Samples were placed in 15 ml polypropylene centrifuge tubes and kept under low light conditions at 2°C in a cool box. Immediately upon the return to the laboratory, the seawater within each tube was decanted and the coral samples were immersed in absolute ethanol and stored at -80°C for molecular analysis.

3.3. Histopathology

Samples were protected in 1.5% (w/v) agarose to retain the spatial integrity of the tissues and microbial communities. They were then decalcified using 1% HCl and EDTA renewed every 12 h until the process was complete. Decalcified tissues were then dehydrated in a gradient of ethanol baths, cleared with xylene and embedded in paraffin wax. Cross sections of 6-8 µm thick were cut using a microtome, mounted on glass slides and stained with Harris haematoxylin and eosin (with phloxine B). These stains are used to diagnose tissue fragmentation, necrosis and identify invasive organisms (Work and Aeby 2011; Sudek et al. 2012). Serial sections were examined under a light microscope and photographed using NIS Element software (Nikon©).

3.4. Cyanobacterial culturing, isolation, and identification

Cyanobacterial filaments (one when possible) were isolated from the raw samples under a light microscope and transferred to agar plate containing Z8 medium (Kotai 1972) enriched with NaHCO3, $(NH_4)_2SO_4$ and Vitamin B12. Inoculated plates were incubated at 27°C with a 12h light:dark photoperiod and constant irradiance of 20 µmol photons m^{-2} s^{-1}. Bacterial strains were routinely passaged to purification until their use in molecular analysis. Genomic DNA was then extracted from each cyanobacterial isolate by boil lysing in 100 µL of 5mM Tris/HCl at 100°C for 5 minutes. PCR amplification was carried out in a volume of 25 µl GoTaq®Hot Start Green Master Mix (Promega, Madison, WI), 0.5mM of 16S rRNA gene cyanobacterial primers CYA781R/CYA106F (Nübel et al. 1997; Sussman et al. 2006; Rasoulouniriana et al. 2009), and 10 ng of template DNA. Amplification conditions for the PCR included an initial denaturing step of 5 min at 94˚C, followed by 35 cycles at 94˚C for 60 sec, 60˚C for 60 sec, 72˚C for 60 sec, and a final extension step of 5 min at 72˚C. Sequences obtained for each cyanobacterial strain were examined for error and edited using GENEIOUS™ Pro (V.5.6.6) sequencing software (Kearse et al. 2012). All consensus sequences were submitted to BLAST at the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) and compared with published sequences. The 16S rDNA sequences were aligned with reference rRNA gene sequences closely related to known cyanobacterial strains. A phylogenetic tree was built by the neighbour-joining method of GENEIOUS™ Pro (V.5.6.6). Bootstrap values were based on 1000 replicates.

3.5. Metagenomic profile of bacterial 16S rRNA Genes

3.5.1. DNA extraction

Bacterial genomic DNA was extracted from HT, VHT and DT using the NucleoSpin® Soil Kit (NucleoSpin Extract II, *Macherey-Nagel*, Düren, Germany). Approximately 150 mg of

both tissue and skeleton were scratched from the core surface using a sterile scalpel blade, placed in a 1.5 ml centrifuge tube with 700 µl of lysis buffer and crushed using a fresh disposable plastic rod. Samples were then placed in lysing matrix tubes for DNA extraction. The DNA was eluted with 50 µl sterile elution buffer, verified by electrophoresis in agarose gels (1.5% wt/vol) stained with GelRed™ (Biotium Inc., Hayward, California, USA) and finally stored at -20˚C.

3.5.2. PCR and 454 pyrosequencing

Bacterial 16S metabarcode sequencing of the bacterial communities associated with HT, VHT and DT samples was performed using 454-pyrosequencing technology (Roche, Nutley, NJ, USA) at GENOSCREEN (Campus de l'Institut Pasteur de Lille, France). In our design, 16S rRNA variant regions V3 and V4 were amplified using forward (TACGGRAGGCAGCAG) and reverse (GGACTACCAGGGTATCTAAT) primers. These primers were linked to 5' with MID tags, a GsFLX key and GsFLX adapters. Each sample was amplified independently twice with distinct MID tags, allowing the identification of each gene pool. Quality control was performed using the Agilent DNA 100 (Agilent Technologies). The quantity of each PCR product was measured with Picogreen and all products were mixed together in equimolar concentrations prior to 454 GsFLX sequencing.

3.6. Sequences analyses

All 454 GSFLX sequences (reads) were sorted by MID identification using GENEIOUS™ Pro (V.5.6.3). Raw sequences were first trimmed using the RDP Pyrosequencing Pipeline (available online at http://pyro.cme.msu.edu/index.jsp) using the following filter criteria: forward primer max edit distance, 2; reverse primer max edit distance, 1; max number of "Ns", 1 and min sequence length, 200. Trimmed reads were then compared against the NCBI reference 16S database (www.ncbi.nlm.nih.gov) using nucleotide BLAST, returning 50 hits

and descriptions for each hit. Blast hits were assigned to NCBI taxonomies with MEGAN software version 5.0.78 beta (Huson et al. 2007) using the lowest common ancestor (LCA) algorithm with all parameters kept at default values (min support, 5; min score, 35; top percent, 10.0; win score, 0.0). After the sequences were assigned to NCBI taxonomies, the output results were analysed using MEGAN v5.0.78 beta. Operational taxonomic units (OTUs) were identified for each major genus of the identified bacterial communities in healthy (HT), diseased (DT) and control tissues (CT) using MOTHUR (http://www.**mothur**.org) (Schloss et al. 2009). Consensus sequences were generated for each OTU at a genetic cut-off distance of 10% and a degeneracy cut-off of 50, which incorporated the fewest possible ambiguous bases into each consensus sequence. Phylogenetic trees were generated via maximum likelihood using the PhyML plugin for the Geneious Pro software package (V.5.6.6), incorporating related sequences of interest that were identified from literature searches and using the NCBI search tools. Trees were manipulated in Figtree (v.1.4). Cyanobacterial 16r RNA gene sequences are accessible through the NCBI GeneBank database under accession numbers KF957835- KF957838. Finally, raw 454-pyrosequencing reads were submitted in Study (BioProject PRJNA231011) to the NCBI Sequence Read Archive (SRA).

3.7. Statistical analysis

Disease prevalence was calculated per transect and for each site. Data were log-transformed [log10 (X)] to satisfy assumptions of homoscedasticity and normality of variance. Variations in the prevalence of PBPS over the two survey years in the consecutive summers and winters and across reef zones (shallow vs. deep) were tested using Factorial ANOVA (STATISTICA 8). Fisher tests were performed for post hoc multiple comparison. Rarefaction curves were performed on bacterial populations associated with each tissue category. Principal coordinate analysis (PCoA) and cluster analysis were performed using the Bray–Curtis similarity

coefficient to compare the bacterial community structures of the different tissue categories and the Simpson's and Shannon's diversity indices were calculated. Finally comparisons of average values of bacterial communities associated with different tissue categories were performed using the t-test in STATISTICA.

4. Results

4.1. PBPS Prevalence and virulence

A total of 3520 m^2 of reef and 5363 massive colonies of *Porites lutea* were surveyed between September 2010 and January 2012 (Table 13). PBPS varied seasonally and between the two geomorphologic reef zones. For instance, colonies of *Porites* exhibited significantly higher PBPS prevalence on the reef flat (ANOVA: $F = 1.18$, $p < 0.01$), affecting an average of 4.1 ± 2.0 % (mean ± SE) colonies compared to those observed at the deeper sites (0.2 ± 0.4; mean ± SE). The percentage of infected colonies recorded on both the reef flat and reef slope was significantly higher during summers than winters (ANOVA $_{\text{winter 1 vs. summer 1}}$: $F = 3.89$, $p < 0.01$; ANOVA $_{\text{winter 2 vs. summer 2}}$: $F = 0.6$, $p < 0.05$). The rate of tissue mortality measured on five massive colonies of *P. lutea* between 2011 and 2012 was 4.4 ± 0.12 (mean ± SE) mm day^{-1}. Among the monitored colonies, two died approximately 13 months after the beginning of the study.

4.2. Microscopic characterisation

Comparison of cross sections of PBPS-infected *P. lutea* colonies (Fig. 21A) revealed the presence of three distinct tissue regions; the first one being the oldest area of infection comprising dead and degraded tissue associated with cell debris, endophytic algae and other organisms such as cyanobacteria and ciliates (Fig. 21C). The second region was characterised by a dense mat of microorganisms visually dominated by filamentous cyanobacteria (Fig. 21C) which were mechanically perforating the compromised and dead tissue (Fig. 21E and F). Finally, the last portion of the lesion constituted viable (living) tissue with granular pigment cells in both the epidermis and gastrodermis (Fig. 21B). Basophilic bodies like bacterial aggregates were also observed in this tissue region and were regularly surrounded by the same granular cells.

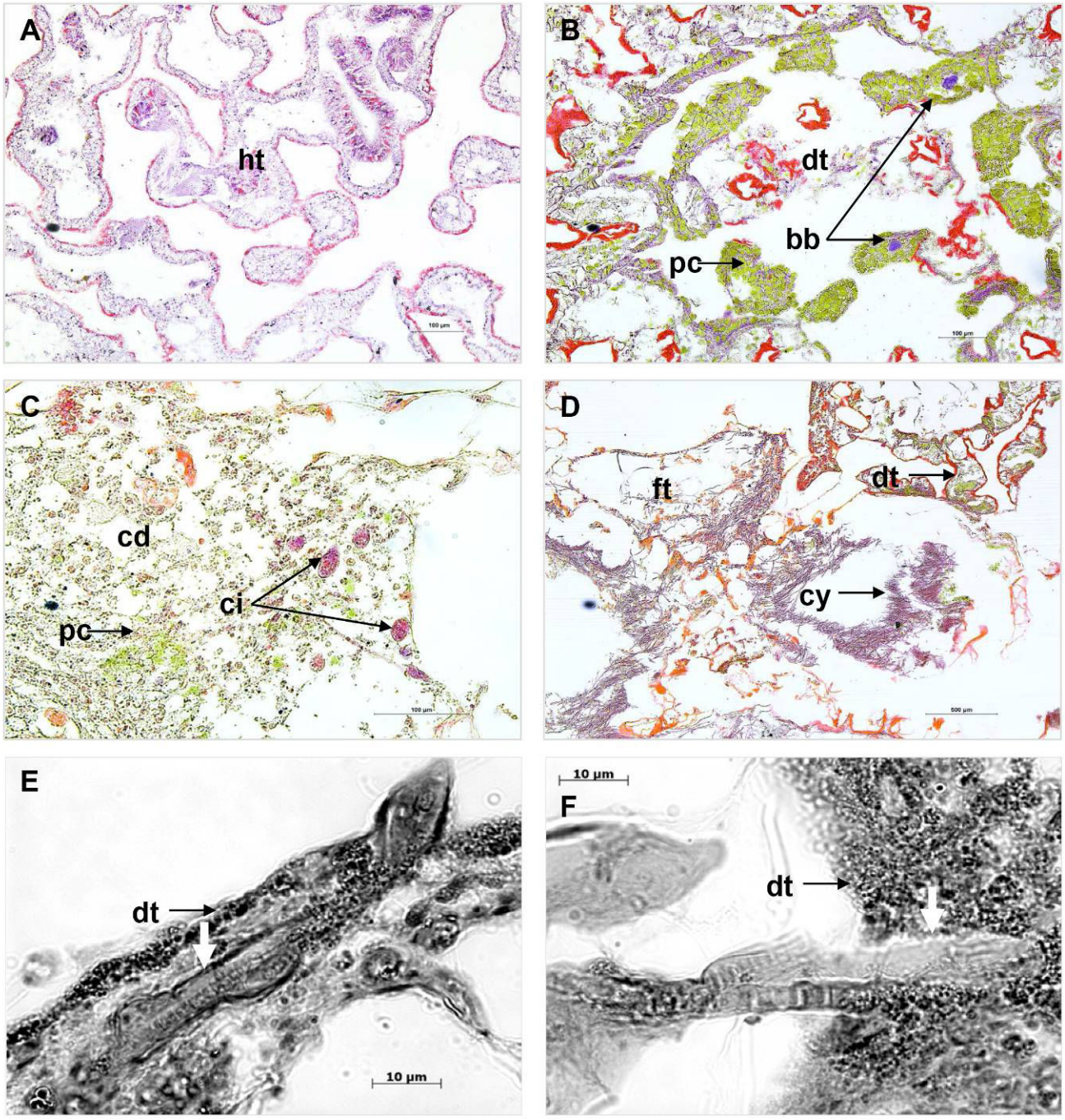


Figure 21: Histological sections of *Porites lutea*: A) Healthy tissue (ht). Note the integrity of the epidermis. B) Living polyp from PBPS-infected tissue (dt) showing high concentration of granular pigment cells (pc). Note the basophilic bodies (bb) surrounded by granular pigment cells. C) Degraded tissue with cell debris (cd) and residual of pigment cells (pc). Note the presence of ciliate-like organisms (ci). D) Disease (dt) band boundary characterised by a dense mat of filamentous cyanobacteria (cy) invading the fragmented tissue (ft). E) and (F) Penetration of filamentous cyanobacteria (white arrow) into the infected tissue (dt).

4.3. Identification of the dominant cyanobacterial strain

Cyanobacterial strains isolated from four individual PBPS-infected *Porites lutea* were characterised by dense clumps of brown filaments that were able to colonize the entire petri dish surface (Z8) in a single week (Fig. 22). The four strains were motile and appeared morphologically similar with pointed, arrow-like calyptra. The isolates were genetically close to each other (>98% identity) and phylogenetically affiliated to the cyanobacterium *Pseudoscillatoria coralii* (FJ210722) and *Roseofilum reptotaenium* (HM048872).

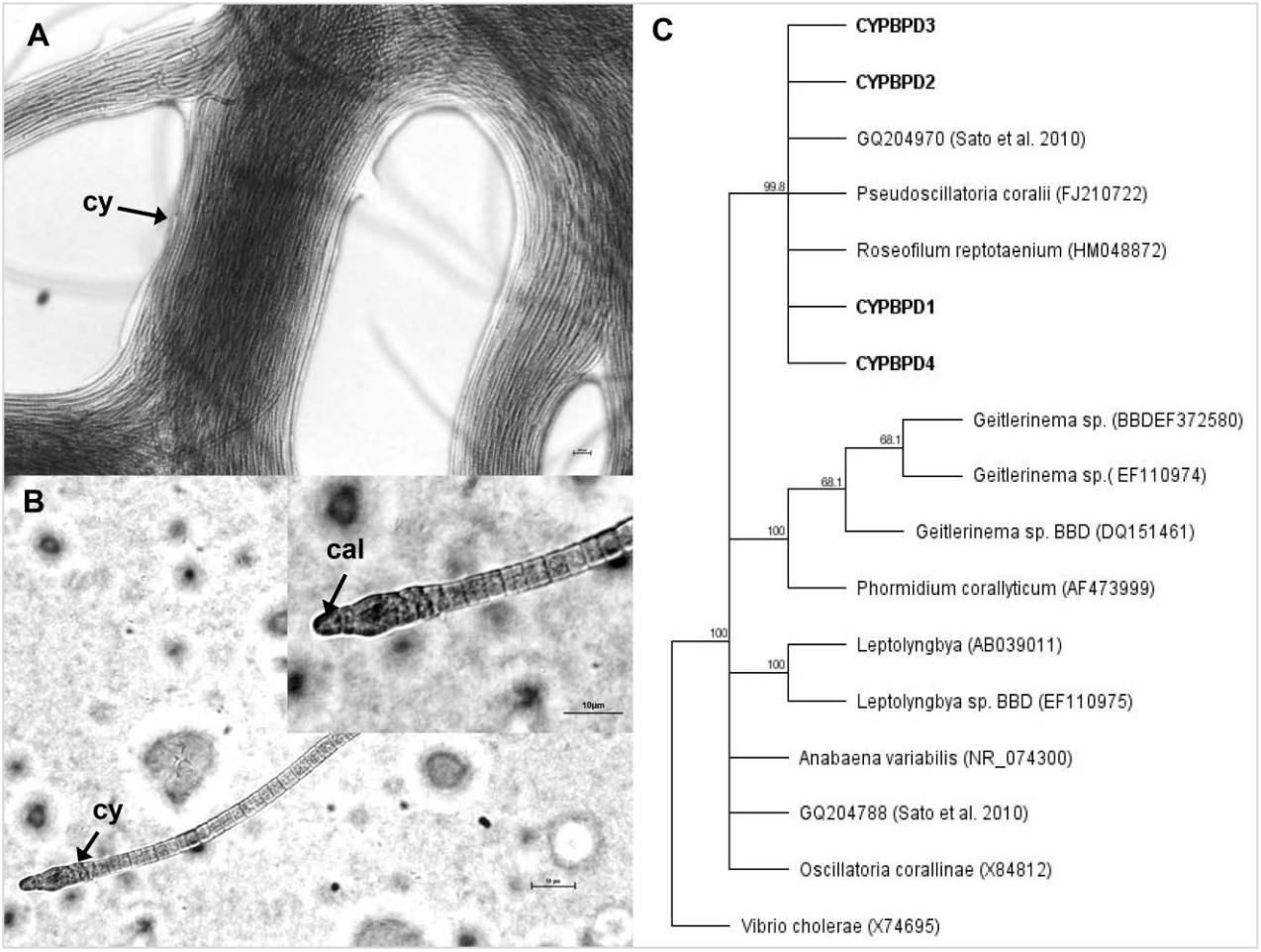


Figure 22: Cyanobacteria retrieved from *Porites* black patch syndrome (PBPS): A) Clumps of brown cyanobacterial filamments growing in a petri dish with Z8 medium. B) Photomicrograph of the cyanobacterial strain CYPBD1, closely related to the cyanobacterium *Pseudoscillatoria coralii* (FJ210722) and *Roseofilum reptotaenium* (HM048872), isolated from pure cultures. C) Neighbour-joining phylogenetic tree showing the relatedness of the strains CPPBPS1, CPPBPS2, CPPBPS3, and CPPBPS4 with reference *cyanobacterial* strains. Numbers at each node are bootstraps values (%) obtained after 1000 iterations.

4.4. Bacterial community structure

Following Roche 454-pyrosequencing, a total of 52 257, 38 778 and 21 309 sequence reads were obtained from samples of PBPS (DT1A-DT1B and DT2A-DT2B), apparently healthy tissue (HT1A-HT1B and HT2A-HT2B) and healthy tissue (HTA and HTB) respectively (Table 14). In the same order, 23 763 (45.47%), 30 602 (78.91%) and 13 806 (64.78%) sequences reads remained after trimmed analysis, with a minimum length of 250 bp.

Table 14: Total sequences read before and after sequence trimming, number of bacterial classes and genera and diversity indices for each sample and subsample of PBPS (DT1A-DT1B and DT2A-DT2B), apparently healthy tissue (HT1A-HT1B and HT2A-HT2B) and healthy tissue (HTA and HTB).

	DT1 A	DT1 B	DT2 A	DT2 B	VHT1 A	VHT1 B	VHT2 A	VHT2 B	HT A	HT B
Σ raw sequences	11 320	12 703	14 848	13 386	15 660	14 646	19 008	15 221	12 563	8746
Σ trimmed bacterial sequences	5785	5636	6505	5837	10 496	9419	10 687	8176	8259	5547
Σ bacterial Phylum	8	8	8	8	2	4	4	4	4	2
Σ genera	22	22	16	17	5	8	8	5	7	8
Shannon index	4.310	4.357	4.158	4.349	2.882	3.092	2.638	2.638	2.743	3.152
Simpson reciprocal index	11.947	12.162	10.756	13.063	6.730	7.745	6.059	6.061	6.064	8.131

The community structure in each subsample of the different tissue categories was compared with both Cluster and PCoA analysis in MEGAN (V5.0.77) based on Bray–Curtis similarity matrices (Fig. 23). Results revealed three clearly separated groups, including a Cluster 1 grouping all diseased samples, a Cluster 2 grouping only the HT1A and HTA subsamples and a Cluster 3 with the remaining healthy and visually healthy subsamples (Fig. 23A and B). Rarefaction curve analysis (Fig. 23 C) assessessing species richness showed almost all the samples nearly paralleled the x axis, indicating that the overall bacterial diversity was well represented. Only VHT2A and B did not level off, even with a respective sequence reads number of 10 687 and 8176 (Table 14). Bacterial diversity estimated with Simpson's and Shannon's diversity indices showed that DT had higher bacterial diversity than both VHT and HT (Table 14).

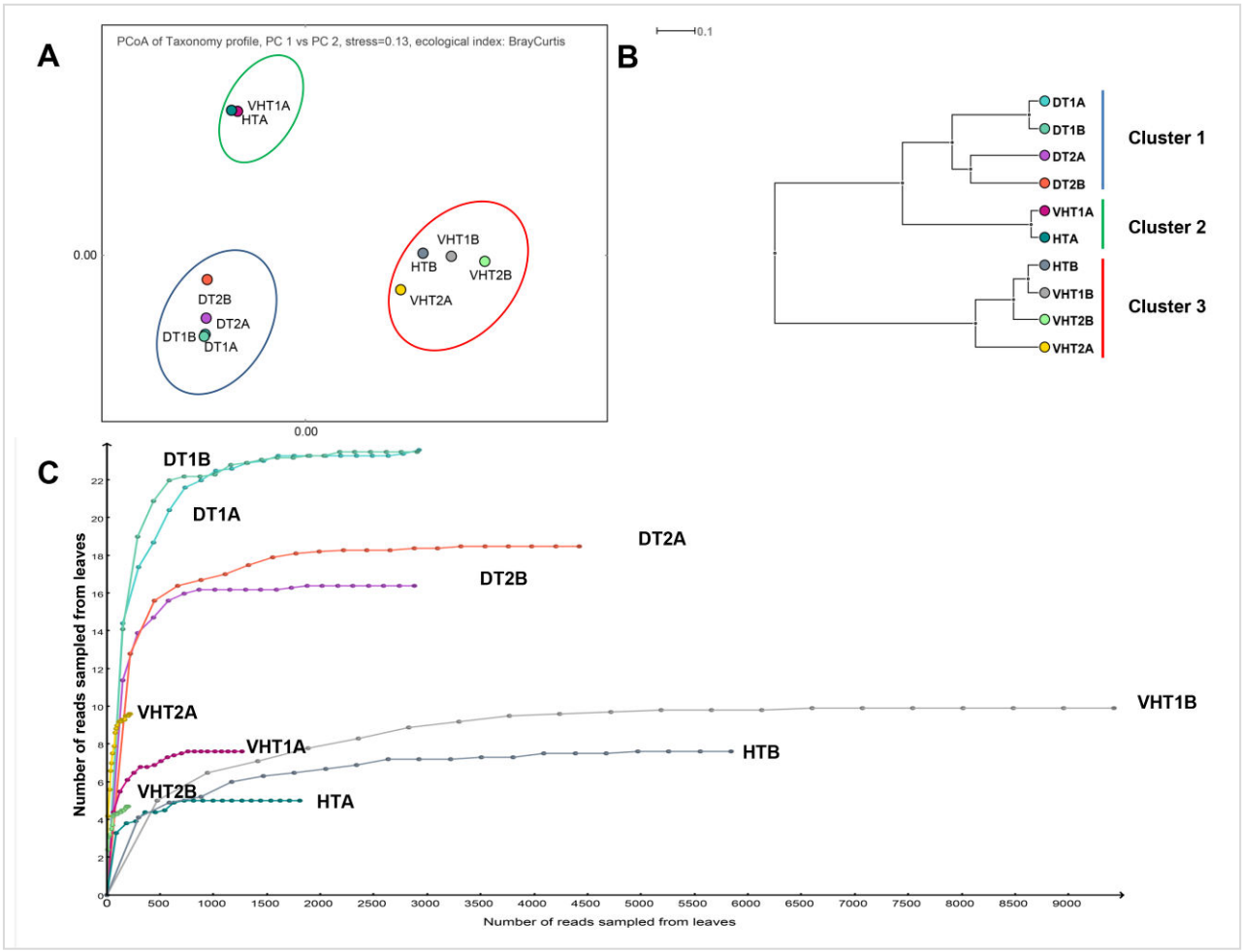


Figure 23: Comparative analysis of bacterial communities associated with three tissue categories of *Porites lutea*: A) Multidimensional scaling ordination, B) cluster diagram and C) rarefaction curves of bacterial communities associated with VHT1-2 A and B = apparently healthy tissue, HTA and B = healthy tissue and DT 1-2 A and B = diseased tissue of *Porites lutea*, created using MEGAN software version 5.0.78 beta.

4.5. Bacterial community composition in DT

Bacterial communities associated with PBPS tissues were represented by eight classes, including *Alphaproteobacteria*, *Clostridia*, *Cytophagia*, *Cyanobacteri*a, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria* and *Spirochaeta* (Fig. 24). In DT1, the most representative class was the *Alphaproteobacteria* (35.9%) followed by the *Gammaproteobacteria* (24.4%), *Deltaproteobacteria* (18.9%), *Cytophagia* (11.5%), *Firmicute* (5.1%), *Epsilonproteobacteria* (3.0%), *Spirochaeta* (1.0%) and Cyanobacteria

(0.2%). DT2 was dominated by *Gammaproteobacteria* (62.1%), followed by *Firmicute* (13.2%), *Epsilonproteobacteria* (10.2%), *Cytophagia* (5%), *Deltaproteobacteria* (4.0%), *Alphaproteobacteria* (3%), *Spirochaeta* (2.3%) and *Cyanobacteria* (0.2%).

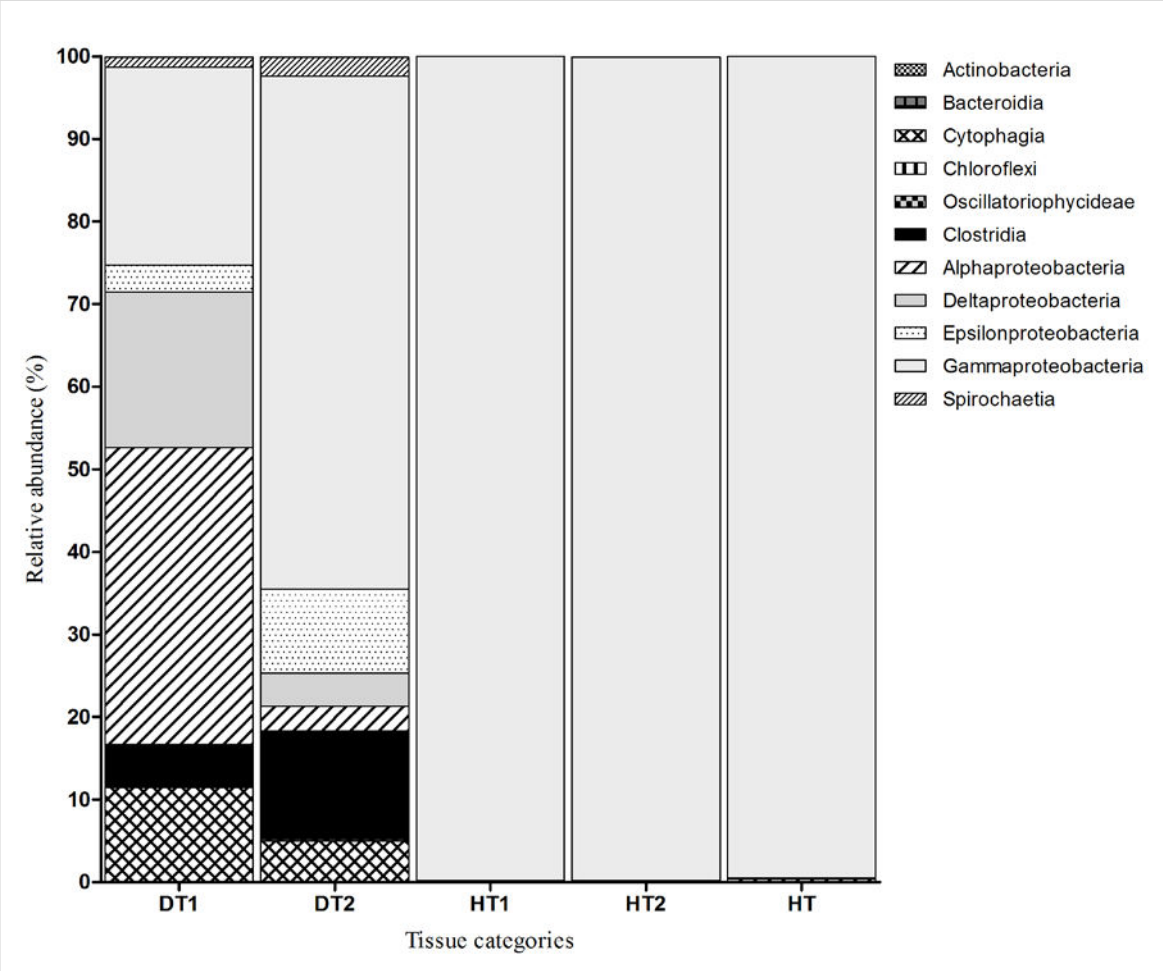


Figure 24: Relative abundance of bacterial classes associated with PBPS-infected tissues (DT1 and DT2), visually healthy tissues (VHT1 and VHT2) and healthy tissue (HT).

At a higher taxonomic level of MEGAN's cladogram, 16 to 22 genera were obtained from the four subsamples of DT (Table 14 and Fig. 25). Among them, nine genera were found in all disease samples/subsamples, including *Arcobacter*, *Clostridium*, *Cytophaga*, *Desulfovibrio*, *Flexibacter*, *Oceanospirillum*, *Oscillatoria*, *Spirochaeta* and *Vibrio* (Fig. 25).

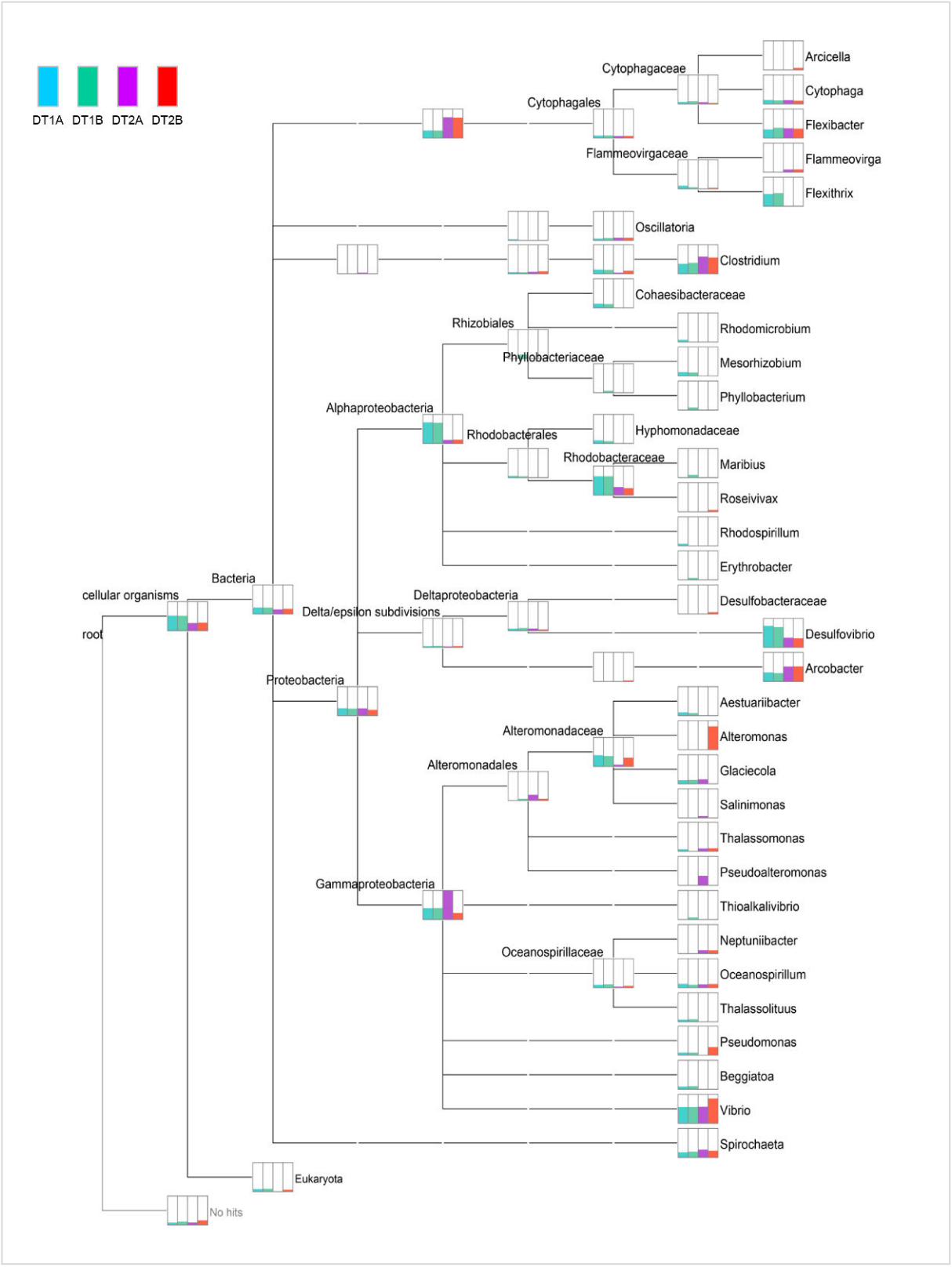


Figure 25: Taxonomic tree in MEGAN, profiling bacterial communities of two PBPS samples (DT1 and DT2) replicated in two subsamples (A and B). Each square and bar represents one subsample of diseased tissue. The scale of each bar was normalised and represents the number of sequence reads.

Considering the similarities between bacterial profiles and that no significant difference was found between the DT samples (t-test <0.05), the replicates were then pooled for detailed analysis. The most dominant genera observed in DTs were *Vibrio* (24.9%) followed by *Desulfovibrio* (20.1%), *Clostridium* (12.9%), *Arcobacte*r (9.9%), *Flexibacter* (6.2%) and *Spirochaeta* (2.7%).

4.6. Bacterial community composition in VHT and HT

Bacterial communities of VHT1, VHT2 and HT samples were almost exclusively dominated by the Class *Gammaproteobacteria* comprising 99.9%, 99.7% and 99.5% of the total sequences reads respectively (Fig. 26 and Table 14). A few additional sequences attributed to *Alphaproteobacteria* (0.06% in VHT1, 0.11 % in VHT2 and 0.38 % in HT) and *Deltaproteobacteria* (0.03% in VHT1, 0.03 % in VHT2 and 0.05 % in HT) were also found in common. In VHT1, additional sequence reads attributed to *Chloroflexi* (0.06%) were found while *Bacteroidia* (0.03%) and *Cytophagia* (0.05%) were obtained in VHT2.

Finally, the sequences obtained from HT, considered a negative control, were composed of a few extra reads affiliated to *Actinobacteria* (0.03%), *Photobacterium* (0.3%), *Acetobacter* (0.09%), *Wolbachia* (0.09%), *Rhizobium* (0.08%), and *Pseudomonas* (0.06%). In total, only three genera were common between VHT and HT samples and included *Spongiobacter* (80.1% in VHT1, 74.6% in VHT2 and 67.6% in HT), *Vibrio* (19.4% in VHT1, 7.0% in VHT2 and 67.6% in HT) and *Pseudomonas* (0.06% in VHT1, 2.6% in VHT2 and 0.06% in HT). A small proportion of sequence reads affiliated to *Photobacterium* (0.17%), *Chloroflexaceae* (0.1%), *Caulobacter* (0.06%), and *Bacteriovorax* (0.04%) were also found in VHT1, while better represented genera including *Porphyromonadaceae* (3.5%), *Arcobacter* (3.5%), sulfur-oxidizing symbionts (3.5%) and *Flexibacter* (2.6%) were obtained from VHT2.

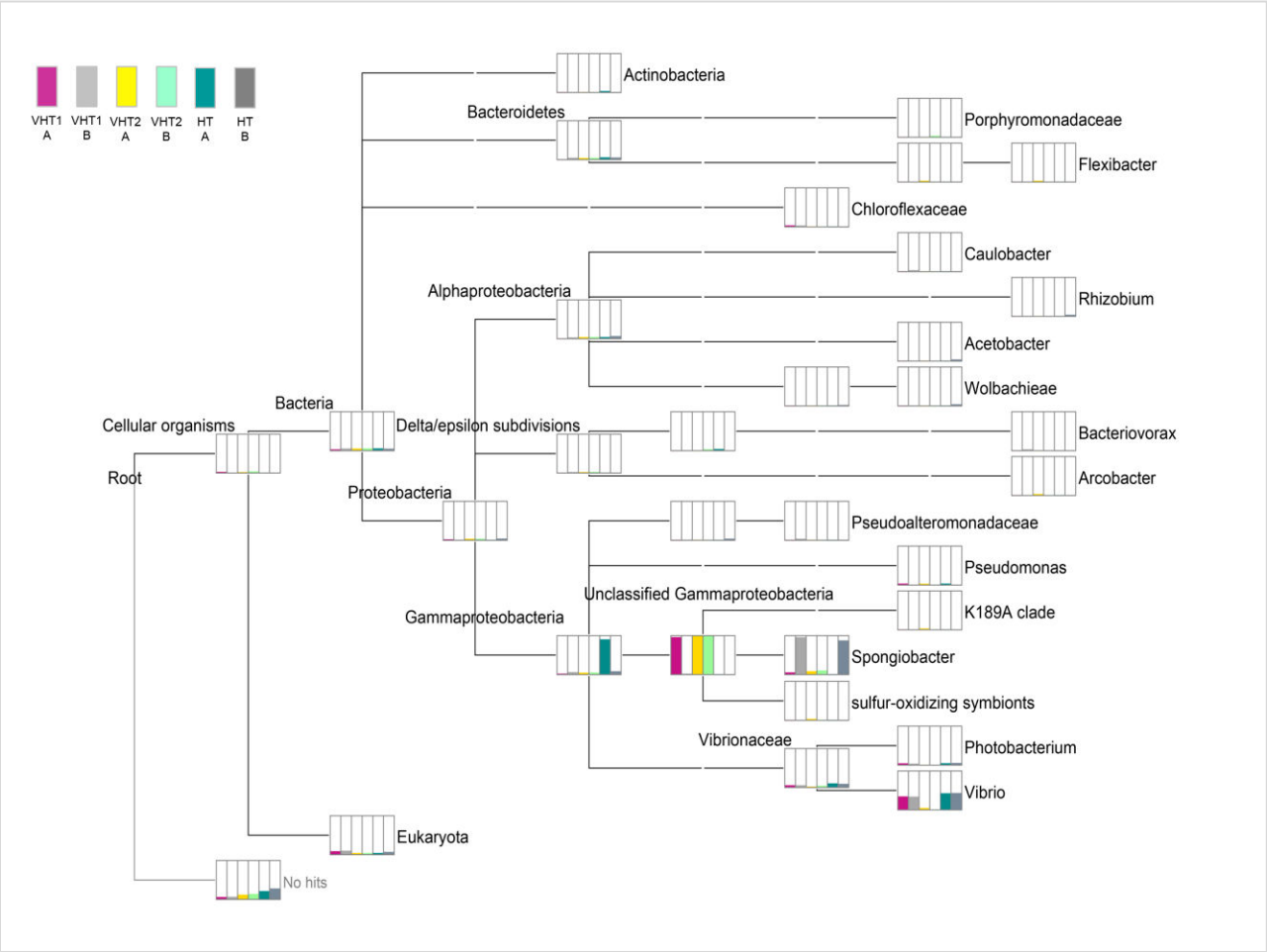


Figure 26: Taxonomic tree in MEGAN, profiling bacterial communities of VHT samples (DT1 and DT2) replicated in two subsamples (A and B). Each square and bar represents one subsample of diseased tissue. The scale of each bar was normalised and represents the number of sequence read

4.7. Comparative analysis of bacterial communities associated with DT, VHT and HT

Sequence reads from all samples and subsamples of each tissue category were combined (DT, VHT and HT) and compared statistically. Before analysis, all read counts were normalized to avoid bias due to datasets with different size (Mitra et al. 2009).The average number of taxonomic reads affiliated to bacterial genera in DT was significantly different from those in VHT (*t-test*: df=2 p<0.0001) and HT (*t-test*: df= 2 p= 0.0001). However no significant difference was obtained between VHT and HT (*t-test*: df= 2 p=0.001). Among the genera found in our study, only *Vibrio* and *Pseudomonas* were common between DT, VDT and HT (Figs 25 and 26).

5. Discussion

This study constitutes the first characterisation of *Porites* black patch disease (PBPS), an atypical form of BBD found on Reunion coral reefs. Surveys conducted on two geomorphological reef zones revealed spatial variability, with more infected colonies on the reef flat (0.5-1m) than the reef slope (10-20 m). Similar patterns were previously reported in Florida Keys (Kuta and Richardson 2002), the Republic of Maldives (Montano et al. 2012) and southern India (Thinesh et al. 2009; Thinesh et al. 2011) where BBD is more abundant at shallow than deep sites. This may be due to the proximity of the Reunion reef to the coastline (± 500 m wide), where it is constantly exposed to high and increasing anthropogenic stress from sewage discharges, land-based pollution and eutrophication with compounds such as nitrates, ammonium, and phosphate (Naim 1993; Chazottes et al. 2002).Several studies suggested that nutrient enrichment, sewage discharge and runoff may facilitate and increase disease outbreaks by enhancing the pathogen virulence and/or impairing host resistance (Voss and Richardson 2006; Rodriguez and Croquer 2008; Haapkylä et al. 2011).

Seasonal variations were also observed between 2010 and 2012: the average prevalence of PBPS recorded on the reef flat was at its highest in summer 2010 (8.7 ± 2.8%; mean ± SE), declined to low levels in winter 2011 (1.4 ± 1.0%; mean ± SE) and then increased the following summer (7.7 ± 2.5%; mean ± SE). Our results are consistent with those previously found during BBD surveys on the Great Barrier Reef in Australia (Boyett et al. 2007; Sato et al. 2009) and in Venezuela (Rodriguez and Croquer 2008), the Red Sea (Zvuloni et al. 2009) and on Caribbean reefs (Edmunds 1991). Seasonal fluctuations in BBD prevalence have been generally assumed to be driven by high summer sea temperatures, which may reduce host resistance or/and increased pathogen virulence (Rützler et al. 1983; Edmunds 1991; Kuta and Richardson 2002; Rodriguez and Croquer 2008; Sato et al. 2009; Sato et al. 2010). However, the hot season (December to March) in Reunion is also associated with heavy rainfall, leading

to high levels of ground water infiltration and surface water runoff, and an increased level of pollutants such as pesticide, fertiliser, sewage from septic systems and waste water in lagoon waters (Naim 1993; Chazottes et al. 2002).

Field monitoring performed on tagged colonies confirmed the virulence of PBPS with a mean tissue mortality rate reaching 4.4 ± 0.12 mm day^{-1} (mean ± SE). This value was higher than rates of BBD progression previously reported on other scleractininan corals in the Florida Keys (Kuta and Richardson 1997), in Australia (Boyett et al. 2007; Sato et al. 2010), Indonesia (Haapkylä et al. 2009) and India (Borger and Steiner 2005; Thinesh et al. 2009). However, regional comparisons may be confounded by differing innate immune responses between coral species (Mydlarz et al. 2008; Mydlarz et al. 2010). Nevertheless, our results still pinpoint the high destructive potential of this PBPS on the Reunion reefs, especially considering that 40% of the tagged colonies died after one year of monitoring.

Cross sections performed on PBPS-infected tissues revealed a dense microbial mat visually dominated by filamentous cyanobacteria adjacent to the living tissue. Similar cyanobacterial aggregates have been previously observed in several studies on typical BBD (Bythell et al. 2002; Ainsworth et al. 2007; Barneah et al. 2007; Sato et al. 2009). 16S rRNA gene sequences obtained from fresh and purified cyanobacteria strains isolated from the same infected colonies were closely related (99.8% identity) to two newly reported BBD cyanobacterial species. These were *Pseudoscillatoria coralii* (Rasoulouniriana et al. 2009) and *Roseofilum reptotaenium* (Casamatta et al. 2012), isolated on different coral hosts from the northern Red Sea and the Caribbean. The sequences were also 99.8% homologous to the BBD cyanobacterium found on infected colonies in the central Great Barrier Reef (Sato et al. 2010).

Morphologically, the isolated cyanobacterial strain possessed pointed terminal cells, or "calyptra", that may play a role in the tissue invasion observed in the histological cross-sections, and are probably involved in the secretion of toxins or other compounds (Whitton 2008). Previous studies have indeed suggested that cyanobacteria may facilitate their penetration by secreting cyanotoxins (Richardson et al. 2009; Stanić et al. 2011). The ability of cyanobacteria to penetrate coral tissue has been demonstrated in other studies of BBD (Barneah et al. 2007; Sato et al. 2010), highlighting their destructive potential in corals. Although these cyanobacteria clearly play a role in PBPS development, it remains unclear whether or not healthy corals would be infected by such bacteria in the absence of other aggravating factors including the potential role of other infectious bacteria (Sato et al. 2010). The precise mechanisms of coral invasion by cyanobacteria are thus unknown and require further investigation.

Granular and pigmented cells, resembling melanin deposits described by Palmer et al. (2008) in *Porites* sp., were found in high densities in PBPS-infected tissue of *P. lutea*. Such inflammations, found mainly in DT, are likely to be the result of an immune response, suggesting that healthy corals are capable of mounting a defence against invading organisms (Palmer et al. 2008; Mydlarz et al. 2010). However, basophilic bodies, resembling bacterial aggregates similar to those observed in PWPS (Séré et al. 2013), were found in the living tissue adjacent to the bacterial mat but were not observed in HT. Thus, it is possible that other bacteria may promote PBPS by initiating a primary infection that impairs the immune processes in corals. However, no evidence of direct physical destruction resulting from these basophilic bodies could be detected in the histological sections.

Bacterial community analysis via V3-V4, 16S metabarcoding suggested that the number of bacterial taxa identified in this study was higher than in other metagenomic analyses of bacterial communities associated with scleractinian corals (Wegley et al. 2007a; Littman et al.

2011). A higher diversity of bacterial taxa was found in PBPS-infected tissues than in healthy tissues. While VHT and HT mostly contained *Gammaproteobacteria*, DT samples yielded bacterial sequences affiliated to *Alphaproteobacteria*, *Clostridia*, *Cytophagia*, *Cyanobacteri*a, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria* and *Spirochaeta*. This result is consistent with patterns observed in other studies that have characterised microbial communities from other black syndromes (Cooney et al. 2002; Frias-Lopez et al. 2002; Sekar et al. 2006). However, the taxonomic complement of bacteria in both the HT and VHT was low compared to previous work that has focused on the bacterial community in the same *Porites* species using traditional methods (McKew et al. 2012; Li et al. 2013).

Although variations were detected between DT samples, the major bacterial classes found in this study were also observed in several previous works on typical BBD from distant locations and in different coral species (Cooney et al. 2002; Frias-Lopez et al. 2002; Sekar et al. 2006; Barneah et al. 2007; Sekar et al. 2008; Sato et al. 2010; Miller and Richardson 2011). Among them, *Vibrio* (*Gammaproteobacteria*) was the dominant genus in PBPS-infected tissues representing 24.9% of the overall sequence reads. Several members of this genus have been identified as pathogens of corals, their virulence being attributed to enzyme secretions that initiate tissue penetration and degradation (Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003b; Rosenberg and Falkovitz 2004). In the present study, phylogenetic analyses revealed the presence of sequences affiliated to *Vibrio* species that are known to be highly proteolytic (Appendix 2). These were found only in infected tissues and included the human pathogen *V. cholerae* (Halpern et al. 2006), the coral pathogen *V. coralliilyticus* (Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003b) and two other sequences groups related to *V. proteoliticus* and *V. rumoiensis*. The latter have previously been detected in BBD on Netherlands Antilles reefs (Frias-Lopez et al. 2002). Our results suggest that *Vibrio* spp. may play an important role in the pathogenicity of PBPS by creating an “entry point” that may

permit the settlement and the progression of the black patch microbial community. However, since vibrionic sequences were also abundant in non-infected tissues, their role in PBPS in *Porites lutea* needs to be individually assessed using multidisciplinary approaches combining bacterial culturing and inoculation/infection trials (Henle-Koch's postulates).

The second most represented bacteria in PBPS samples was *Desulfovibrio*, accounting for up to 20% of the overall 16S rRNA sequence genes. It has previously been found in BBD-infected corals from different locations and seems to be an important component in BBD aetiology (Viehman et al. 2006). However, none of sequences were related to known pathogens but the majority were closely related to *D. aespoeensis*, a sulfate-reducing bacterium from natural deep groundwater (Motamedi and Pedersen 1998). Additionally, we did not find any sequences related to sulfide-reducing bacteria in either VHT or HT, suggesting that they were opportunistic microorganisms that occupy an anoxic micro-environment (Cooney et al. 2002; Viehman et al. 2006; Sekar et al. 2008) rich in carbon compounds derived from cell debris and other organic nutrients produced during the lysis of coral tissue (Viehman et al. 2006). Other predominant genera found only in DT were *Clostridium*, followed by *Arcobacter*, which represented 12.9% and 9.9% of the total bacterial sequences respectively. Although their roles in BBD aetiology were not clearly identified, these genera have also been consistently found in BBD on several scleractinian corals at distant locations (Frias-Lopez et al. 2002; Sekar et al. 2006; Sato et al. 2013). *Arcobacter* is regularly found in sulfide rich environments similar to those found in BBD lesions (Glas et al. 2012). In this study, the majority of sequences affiliated to it were closely related to *A. sulfidicus*, an obligate marine microaerophile that oxidizes sulfides (Wirsen et al. 2002). Other sequences affiliated to *Flexibacter, Cytophaga*, *Oceanospirillum*, and *Spirochaeta* were also present in PBPS samples. As with *Desulfovibrio*, *Clostridium* and *Arcobacter*, these genera have been detected in the BBD microbial mat at different locations (Frias-Lopez et al. 2002;

Sato et al. 2013) and seem to be involved in the production of sulfated compounds suspected to exacerbate BBD virulence (Sato et al. 2009). However, their implications in PBPS aetiology warrant further investigations since none of them were phylogenetically closely related to known pathogens.

In summary, PBPS was found on two of the main framework building corals, *Porites lobata* and *P. lutea*. It manifested spatial and seasonal variability with more infected colonies observed on the reef flat and during the summer season. Histopathology performed on this patch-like form of BBD revealed a dense microbial mat visually dominated by a filamentous cyanobacterium genetically related to both *Pseudoscillatoria coralii* and *Roseofilum reptotaenium* which seemed to mechanically penetrate the coral tissue. Additionally, basophilic bodies were found only in the living tissue adjacent to the bacterial mat suggesting a potential implication of bacteria that initiate a primary infection. However, no direct evidence linking these basophilic bodies to the lesions could be highlighted using histopathology. This study also characterized the structural patterns of bacterial assemblage associated with PBPS using 16S barcoding by 454-pyrosequencing. We demonstrated that diseased tissue yielded a broader diversity of bacterial taxa than healthy tissue, dominated by the genus *Vibrio* followed by sulfate-reducers and sulfide-oxidizer found only in PBPS-infected tissue and which seem ubiquitous to BBD worldwide.

Chapter 6

General discussion

1. The Western Indian Ocean coral reefs (WIO) are also affected by coral diseases

This study represents the first quantitative and qualitative coral diseases assessment on the WIO coral reefs investigating the prevalence and variability of coral diseases at temporal and spatial scales (Chapter 2). While it was assumed that coral diseases were not as serious as they are on other reef ecosystems (McClanahan et al. 2004b), regional and seasonal field surveys conducted at three geographically distant WIO locations (Reunion, South Africa and Mayotte) during two consecutive years have demonstrated their potential threat on coral reefs. Principal findings from this study revealed the presence of six main coral diseases including white syndromes (WS), black band disease (BBD), skeleton eroding band (SEB), pink line syndrome (PLS), growth anomaly (GA), and *Porites* white patch syndrome (PWPS). Their prevalence was low on Mayotte and South African reefs (< 4%) compared with other coral reefs, but were relatively high in Reunion (7.5 %; Fig. 6.1). The genera *Acropora* and *Porites*, two important reef-building corals commonly found in Reunion, South Africa and Mayotte were the most vulnerable to disease. *Acropora* were more susceptible to WS, SEB, Nec and GA while *Porites* were regularly affected by signs of BBD, PLS and PWPS. Surveys conducted on different geomorphologic reef zones and between consecutive seasons provided important insights on potential factors driving coral diseases on WIO coral reefs. Indeed, spatial patterns in the prevalence of BBD and WS were found on both Reunion and South Africa reefs with more disease cases at shallow than deep sites. Additionally, BBD in Reunion and WS in Mayotte and South Africa seemed to manifest seasonality with higher prevalence during summer months. As warm temperature anomalies are expected to become more frequent or extreme during the next few decades, it is likely that coral diseases outbreaks will become more prevalent and severe. A recent study has predicted that WIO Sea Surface Temperature (SST) is increasing in most locations at about 0.01°C per year (Maina et

al. 2008). Furthermore, reefs located 10–15° South will be affected by lethal SST to corals every 5 years by 2010–2025 (Sheppard 2003).

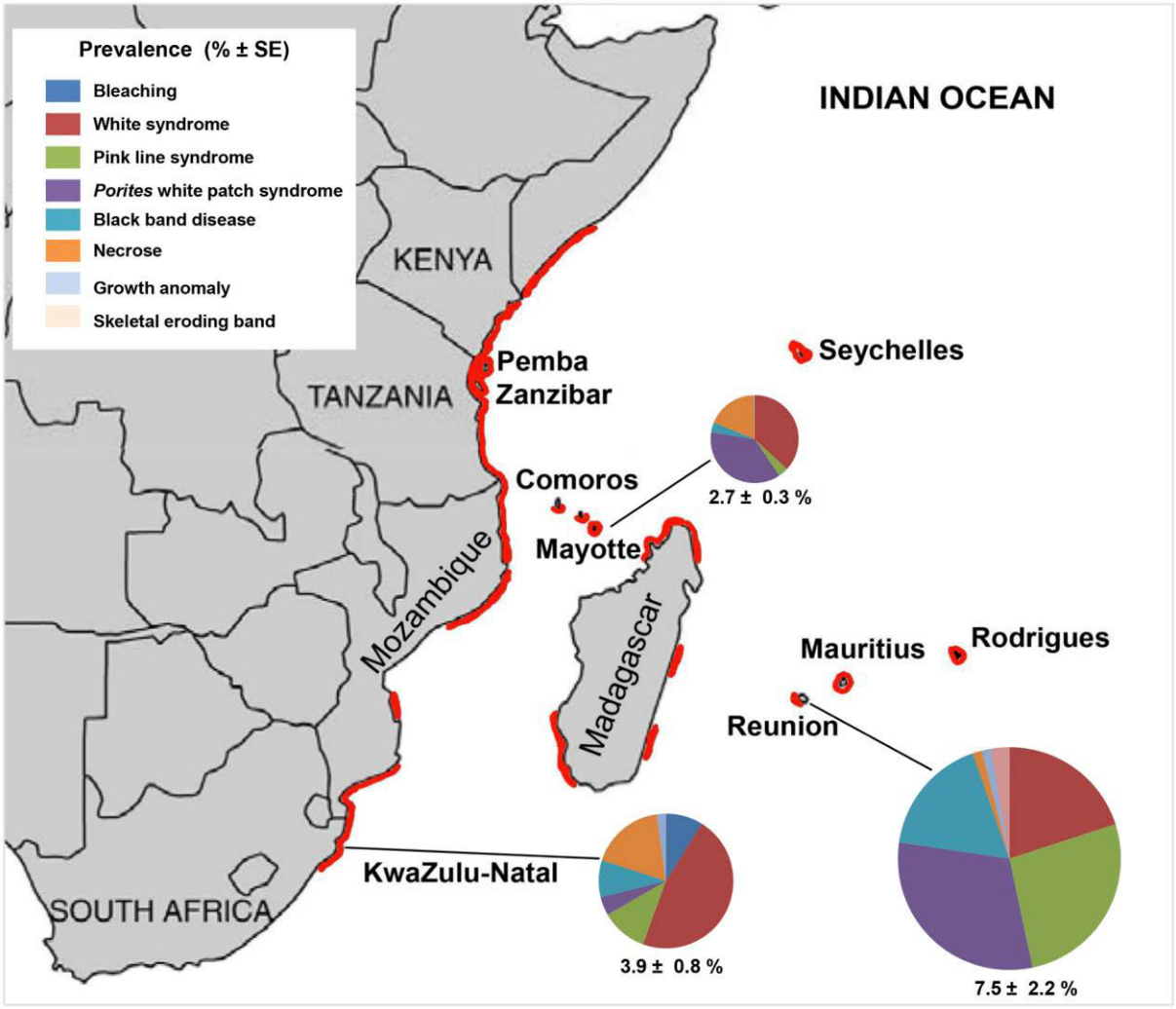


Figure 27: Map of the Western Indian Ocean showing results of the coral disease composition of each sampling and survey location and mean disease prevalence (% ± SE). Red bands represent the main coral reefs.

This is the first study to describe and characterise at ecological, histological, molecular and microbiological levels of two previously unreported coral diseases on the WIO reefs. The first one, *Porites* white patch syndrome (PWPS) was recorded on Reunion, South African and Mayotte reefs with prevalence (2010-2012) estimated around 2.3 ± 0.6%, 0.2 ± 0.3% and 1.0 ± 1.4% (mean ± SE) respectively (Chapter 2). PWPS affects massive *Porites lobata* and *P. lutea* by generating diffuse, medium to large (5.0–30.0 cm diameter), circular to oblong tissue loss, surrounded by a 1.0–20.0 cm width zone of swollen, paler tissues (Séré et al. 2012).

Histopathology of PWPS-affected tissues revealed extensive tissue fragmentations inhabited by ovoid basophilic bodies resembling bacterial aggregates. Other organisms e.g. Ciliata, Cyanobacteria and Nematoda were observed within the dead epidermis and cell debris. Principal findings from culture-independent analysis revealed substantial variability of bacterial communities not only between healthy and PWPS-infected tissues but also between the three geographically distant sampling localities. However, few bacterial ribotypes e.g. the γ-proteobacteria *Vibrio hepatarius* and the α-proteobacteria *Shimia marina* were consistently found from the lesions suggesting their potential role as primary pathogens of PWPS. In the same study, a deductively approach based on presence/absence of specific bacterial ribotypes (Chapter 3) associated with healthy and PWPS-infected tissues, traditional culturing techniques and laboratory infection trials was successfully used to identify potential primary causative agents (Chapter 4). Among the selected bacterial strains used in experimental infection trials, only the strain P180R closely related (100% sequence identity) to *Vibrio tubiashii* elicited similar evidence of disease to that observed in the field and satisfied the four Henle-Koch's postulates. Although no statistical difference between summer and winter was found in the field (chapter 2), seasonality was evident for PWPS. This "thermo-dependent" pattern of PWPS was demonstrated experimentally with its severity clearly increased with elevated seawater temperature (Chapter 4).

The second unreported coral disease was an atypical form of BBD termed here "*Porites* black patch syndrome" (PBPS) characterized by a diffuse, centrally to peripherally situated, medium to large black patch, leaving behind it at the rate of 4.4 ± 0.12 mm day^{-1} (mean ± SE), the dead skeleton generally covered by a whitish filamentous film (Chapter 5). Among the three distant studied locations, PBPS was only found at Reunion on two of the main framework building corals, *Porites lobata* and *P. lutea*. It manifested spatial and seasonal variability with more infected colonies observed on the reef flat and during the summer

season. Histopathology performed on this patch-like form of BBD revealed a dense microbial mat visually dominated by a filamentous cyanobacterium genetically related to both *Pseudoscillatoria coralii* and *Roseofilum reptotaenium* which seemed to mechanically penetrate the coral tissue. Additionally, basophilic bodies resembling those observed in PWPS (chapter 3) were found only in the living tissue adjacent to the bacterial mat suggesting a potential implication of bacteria that initiate a primary infection. However, no direct evidence linking these basophilic bodies to the lesions could be highlighted using histopathology. This study also characterized the structural patterns of bacterial assemblage associated with PBPS using 16S barcoding by 454-pyrosequencing. We demonstrated that diseased tissue yielded a broader diversity of bacterial taxa than healthy tissue, dominated by the genus *Vibrio* followed by sulfate-reducers and sulfide-oxidizer found only in PBPS-infected tissue and which seem ubiquitous to BBD worldwide.

3. PBPS: a primary or secondary infection?

During the course of this study two independent field observations revealed the development of PBPS from an original PWPS on *Porites* corals. From these observations we suggested that cyanobacteria likely form a secondary infection in *Porites* corals, already infected by other microbial organisms. It is possible that a succession of infections by different pathogens may increase, in a synergic way, the progression of coral diseases with black syndromes such as PBPS. Histological analysis revealed that PBPS shares some of the characteristics of PWPS. For instance basophilic bodies, resembling bacterial aggregates similar to those observed in PWPS (Séré et al. 2013), were found in the living tissue adjacent to the bacterial mat but were not observed in healthy tissues (HT). Thus, it is possible that other bacteria may promote PBPS by initiating a primarily infection impairing the immune processes in corals. This highly speculative assumption is consistent with results published by Sato et al. (2009) reporting morphological changes from a brown or green cyanobacterial-infected lesions

named cyanobacterial patches (CP) to typical BBD lesions associated with changes in microbial communities from CP dominated by sequences closely related to *Blennothrix* sp. and Alphaproteobacteria-affiliated to BBD dominated by sequences closey related to *Oscillatoria* sp. and Gammaproteobacteria.

4. Potential sources of ubiquitous bacteria associated with both PWPS and PBPS

When comparing all the 16S rRNA gene sequences taken from the diseased and healthy tissues (chapters 3 and 5) certain groups or species of bacteria were only present within infected one. For instance, 16S rRNA sequences closely related to *Vibrio hepatarius*, *Shimia marina* and *Pseudoruegeria aquimaris* were consistently found from PWPS while the bacterial genera *Desulfovibrio*, *Clostridium*, *Arcobacter, Flexibacter, Cytophaga*, *Oceanospirillum*, and *Spirochaeta* were found only in PBPS samples suggesting they might be introduced from external sources. It is possible that these bacteria which are potentially implicated in those two pathologies may have been transmitted by infected corallivorous organisms on healthy colonies. For instance during this study almost every PWPS-infected colony presented at the middle of the lesion a visible scar (Fig 6.2A, B) probably left by corallivorous fishes. Additionally, intense feeding or predation scars were regularly observed directly upon the PBPS-infected area. This suggests that coral-feeding organisms inflicting these scars may act as transmission vectors of disease. These observations are consistent with previous studies which have provided interesting insights into the likely contribution of marine corallivores in the initiation and dissemination of coral disease (Sussman et al. 2003; Raymundo et al. 2009; Chong-Seng et al. 2011; Nicolet et al. 2013). For example, on the Great Barrier Reef (GBR) six species of coral-feeding fishes were observed to feed intensively on BBD (Cole et al. 2009). More recently Chong-Seng et al. (2011) have shown that 25 species of fishes were observed to feed on coral infected with BBD and brown band disease (BrB) suggesting their potential role in disease transmission among adjacent healthy

coral colonies. Finally, a preliminary work conducted in Reunion during this study has shown the presence of several bacterial strains closely related to known pathogens associated with three species of damselfish, *Stegastes lividus*, *S. limbatus* and *S. nigricans* known to inflict intensive scars to corals (Appendix 3).

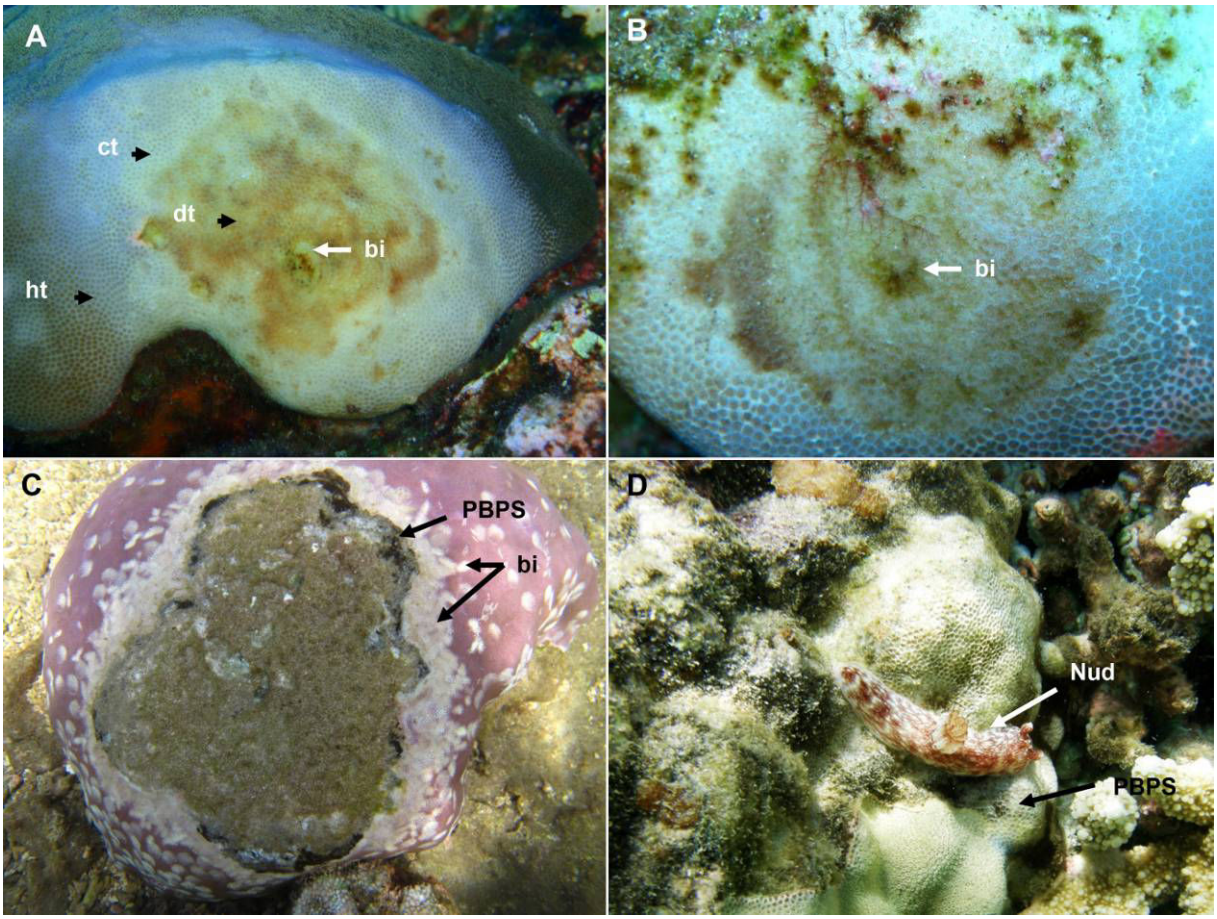


Figure 28: Massive colonies of *Porites lutea* exhibiting signs (A and B) of PWPS and (C and D) PBPS. Ct = compromised tissue; dt= diseased tissue; ht= healthy tissue; bi= bite; Nud= nudibranch

upon the PBPS-infected area (Fig. 28D). Previous researches have reported several cases of predation by corallivorous invertebrates and demonstrated their potential role in transmission of disease (Sussman et al. 2003; Dalton and Godwin 2006; Nugues and Bak 2009; Nicolet et al. 2013). For example, Sussman et al. (2003) showed that the fireworm *Hermodice carunculata* act as a winter reservoir and vector of *Vibrio shiloi* the causative agent of bleaching of the coral *Oculina patagonica*. Alternatively, benthic and endolytic algae

generally found adjacent or touching the corals (Fig. 28B-D), may also act as potential sources of pathogens. Indeed, a recent study has shown that algae contains bacterial pathogens and therefore may also act as vector of coral diseases (Sweet et al. 2013b).

5. Future directions

This study fills a gap in knowledge about coral disease prevalence worldwide and represents a baseline survey and a frame for future research programs in the area. It is also the first study to describe and characterise two unreported diseases PWPS and PBPS, using a multidisciplinary approach. However, this is a preliminary work based on surveys conducted only at three geographically distant localities and during two consecutive years. More investigations are needed notably on their drivers and transmission dynamics to improve our understanding of coral diseases and our ability to mitigate (prevent or reduce) their impacts at local and regional scales. Therefore, long-term disease surveys should be integrated and normalised to the existing monitoring programs regularly conducted on the WIO reefs such as the Global Coral Reef Monitoring Network (GCRMN) and Reef Check in order to expend surveys area and share valuable and consistent data. Finally, training should be provided to managers and non-expert marine scientist from WIO regions in order to optimize the quality and viability of the data collection and also enlarge the surveillance network.

Although temperature has been identified as the most likely environmental parameter influencing coral disease, our understanding on how other potential factors (acting individually or in synergy) could affect the resistance of corals and stimulate the growth and severity of pathogenic organisms remain unclear. Long term monitoring programs conducted on permanent transects set-up on different sentinel areas selected according to their state of disturbance could be an efficient tool to measure potential effects of biotic and abiotic factors on coral disease incidence and virulence. Thus, regular measures of physical and chemical

parameters (temperature, pH, salinity, nutrient load, and metal concentrations), organic matter and microbial communities in the water column should be recorded regularly at the same selected site simultaneously with coral disease monitoring. Therefore combined results may guide managers to better identified, mitigate or even control the main factors leading to disease incidence.

Among the 30 coral diseases identified to date only six bacterial species have been identified as aetiological agents (Israely et al. 2001; Ben-Haim et al. 2003b; Sussman et al. 2008; Arboleda and Reichardt 2010; Sutherland et al. 2011; Ushijima et al. 2012). Therefore, it is important to develop standardized methodologies from the coral disease sampling to the characterisation and identification of primary pathogens. Thus, a multidisciplinary approach based to that used in this study could be proposed for future researches. It includes the following steps:

i. Characterisation of lesions using histopathology to describe lesions at the cellular level and detect presence (or absence) of potential pathogens.
ii. Discrimination of potential pathogens using molecular techniques (Cloning, 454 pyrosequencing) based on presence/absence of bacterial ribotypes in healthy/diseased colonies.
iii. Culturing and isolation of potential pathogens using general and specific growth media and candidates selection based on step (ii) for infections trials.
iv. Inoculation trials to test the infectivity of the selected bacteria and determine disease causality by fulfilling Koch's Postulates.
v. Metabolic characterisation and identification using MLST and phylogenetic analysis.

Nevertheless, not all bacteria are culturable with exciting commercial media. Thus, more efforts need to be allocated to the development of new specific and generic marine media since bacterial culturing is the crucial step in coral disease identification.

Another important challenging area of research is the control of coral diseases. The best approach would always be the complete rehabilitation of the coral reef ecosystem notably by applying efficient control of reef fisheries and improving the watersheds and coastal management (main sources of water pollution). However this would be only possible with an intensive and interoperable effort among governments, municipalities and researcher. Alternatively, the best line of defence seems to be the "biological control" of coral disease. While actions plans such as aspirating or covering the affected area of diseased corals, vaccinations and antibiotic treatments seem unrealistic in term of logistic, expenses and ethic, the "biological control" is likely the more natural and realistic approach to mitigate coral diseases. Therefore, more investigations are needed to:

a. Understand interactions among coral associated bacteria and their links with the host.
b. Identify the role and functions of associated microorganisms in coral health.
c. Indentify, isolate and test on diseased corals potential "probiotic" candidates exhibiting specific properties such as biocides or antibiotics production that inhibit virulence of the pathogens and super competitors.

Finally, more research is needed to assess the potential role of corallivorous organisms as reservoirs and vectors of coral diseases. Investigations on the identification of local vertebrate and invertebrate corallivores and their feeding behaviour coupled with descriptions of their foraging modes is required to ascertain their potential implications in spread and progression of diseases. Additionally, screening of microorganisms associated with corallivores may provide interesting insight into potential reservoirs which can serve as vector for the transmission of putative pathogens.

Appendices

Appendix 1 : Bacterial 16S rRNA gene sequences from samples of apparently healthy (HT) and PWPS-diseased (DT) *Porites lutea* tissues collected at Mayotte (M), Reunion (R) and South Africa (SA).

Group/Subdivision affiliation	Closest genus/species in GenBank database	Best match (%)	GenBank Acc. No	M-HT	M-DT	R-HT	R-DT	SA-HT	SA-DT
Actinobacteria	*Kineosporia rhamnosa*	97	NR028600					+	
α-proteobacteria	α-proteobacterium	99	AB571945		+				
	Henriciella marina	96	NR044345						+
	Erythrobacter aquimaris	99	NR025789						+
	Erythrobacter vulgaris	99	NR043136.1	+					
	Hyphomonas adhaerens	95	NR024937	+					
	Kiloniella laminariae	94	NR042646.1				+		
	Labrenzia alba	98	NR043040		+				+
	Labrenzia marina	99	NR043040						+
	Leisingera aquimarina	98	NR042670				+		
	Loktanella koreensis	97	NR043741						+
	Loktanella maricola	95	NR044163						+
	Oceanicaulis alexandrii	96	NR025456.1				+		
	Mesorhizobium albiziae	99	NR043549						+
	Methylobacterium salsuginis	94-96	NR044038					+	
	Paracoccus yeei	97-99	NR029038.1	+	+		+		+
	Parvularcula lutaonensis	99	NR044474.1	+					
	Pseudoruegeria aquimaris	97-98	NR043932		+		+		+
	Pseudovibrio denitrificans	98	NR041040.1	+					
	Roseovarius aestuarii	97	NR044424		+				
	Roseovarius crassostreae	96	NR041731						+
	Ruegeria pomeroyi	98	NR028727				+		+
	Ruegeria atlantica	97	NR043449						+
	Shimia marina	98	NR043300.1		+		+		+
	Silicibacter lacuscaerulensis	98	NR029197				+		+
	Sphingomonas echinoides	99	NR024700.1				+		
	Sphingopyxis flavimaris	100	NR025814		+				
	α-proteobacterium	94	JQ579969.1	+					
	Rhodospirillales sp.	95	HM798908.1	+					
	Roseobacter sp.	99	EF092256		+				
	Thalassobius aestuarii	98	NR042903						+
	Thalassobius gelatinovorusc	98	NR043447						+
	Thalassobius sp.	99	FJ403051		+				
Bacteroidetes	*Bacteroidetes* sp.	96	HM593523						+
	Flavobacteria sp.	95	AM279213		+				
	*Fabibacte*r sp.	98	HQ270264.1				+		
	Flexibacter elegans	100	NR040908.1				+		
	Lewinella nigricans	98	NR028695				+		
	Marinoscillum furvescens	100	NR040920.1				+		
β-proteobacteria	*Delftia tsuruhatensis*	99	NR024786.1		+				
Chloroplast	Uncultured organism	97	GU119563.1		+				
Cyanobacteria	*Halospirulina tapeticola*	92	NR026510				+		+
	Halospirulina tapeticola	92	NR026510.1			+			
	Limnothrix sp.	96	DQ889938.1	+					
	Planktothricoides raciborskii	92	NR040858						+
	Prochlorococcus marinus	97	NR028762	+	+				+
	Pseudophormidium sp.	100	AB512143.1	+					
	Uncultured cyanobacterium	99	HM474900.1	+					
	Uncultured cyanobacterium	99	HQ242399.1		+				
	Uncultured cyanobacterium	94	FJ516952.1				+		
Cytophagia	*Flammeovirga* sp.	98	NR041394.1	+					
	Flammeovirga sp.	98	AB681285.1		+				
	Flexibacteraceae sp.	91	FJ425608.1	+					

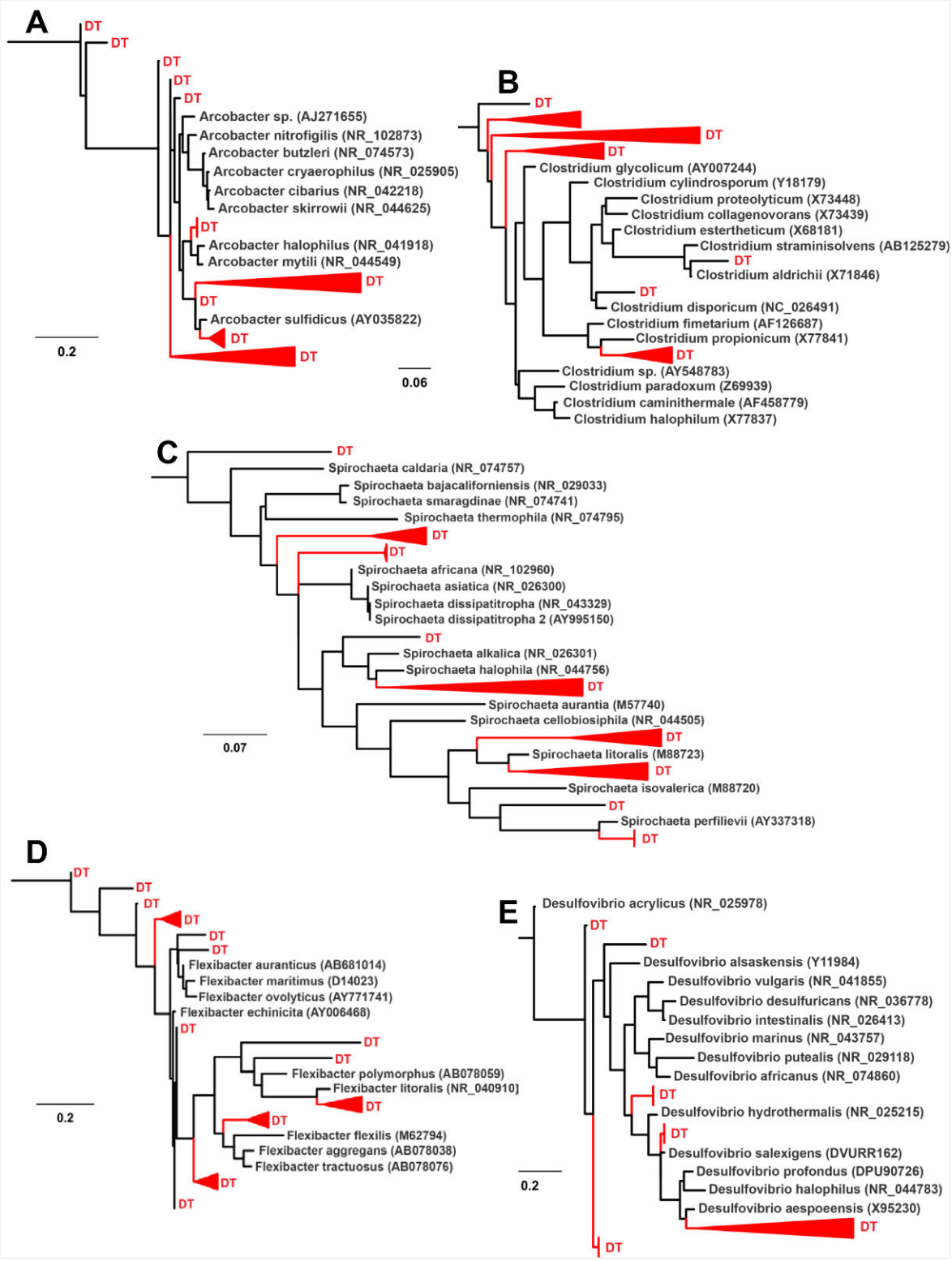
Marinoscillum furvescens	94	NR040920	+

Appendix 1: Continued

Group/Subdivision affiliation	Closest genus/species in GenBank database	Best match (%)	GenBank Acc. No	MHT	MDT	RHT	RDT	SAHT	SADT
εproteobacteria	*Arcobacter* sp.	97	DQ917897.1	+					
λ-proteobacteria	λ-proteobacterium clone	99	HM593548						+
Firmicutes	*Alkaliphilus crotonatoxidans*	100	NR041892				+		
	Bacteroides capillosus	100	NR025670.1	+					
	Clostridium clariflavum	100	NR041235						+
	Cryptanaerobacter phenolicus	94	NR025757.1			+			
	Epulopiscium sp.	95-96	DQ917864		+				
	Gemella haemolysans	100	NR025903						+
	Proteiniborus ethanoligenes	97	HM585026.1	+					
	Sporomusa malonica	100	NR025416						+
Flavobacteriia	*Flavobacterium* sp.	95	FJ745113		+				
	Gaetbulibacter marinus	97	NR044090						+
γ-proteobacteria	*Aeromonas bivalvium*	94	NR043885					+	
	Aeromonas hydrophila	92	NR042155					+	
	Alteromonadales sp.	99	FJ952789.1		+				
	Alteromonas genovensis	94	NR042667.1	+					
	Alteromonas macleodii	99	NR037127	+	+		+		
	Alteromonas sp.	99	FJ952780.1		+				
	Amphritea atlantica	93-94	NR042455					+	+
	Amphritea balenae	98	NR041617.1				+		
	Azorhizophilus paspali	93	NR042070					+	+
	Azotobacter beijerinckii	92	NR042071					+	+
	Dasania marina	93-94	NR043175						+
	Endozoicomonas elysicola	92-99	NR041264	+		+	+	+	+
	Enterovibrio coralii	98	NR042342.1				+		
	Ferrimonas balearica	94	NR027602						+
	Halomonas aquamarina	100	NR042063					+	
	Marinobacter lutaoensis	93	NR025116					+	
	Neptuniibacter caesariensis	97	NR042749		+		+		
	Oceanospirillum beijerinckii	95-100	NR040784						+
	Photobacterium damselae	96-98	NR042975.1			+		+	+
	Photobacterium frigidiphilum	97	NR042964					+	
	Photobacterium halotolerans	99	NR042975					+	
	Photobacterium lutimaris	98-99	NR043902						+
	Photobacterium rosenbergii	99	NR042343.1	+					+
	Photobacterium sp.	97-98	HQ697926			+			
	Pseudoalteromonas agarivorans	93	NR025509						+
	Pseudoalteromona haloplanktis	95	NR044837						+
	Pseudoalteromonas mariniglutinosa	98	NR028992	+	+	+	+		
	Pseudoalteromonas phenolica	98-100	NR028809	+	+				
	Pseudoalteromonas sp.	99	AF343949.1		+				
	Pseudoalteromonas sp.	99	FJ457155	+					
	Pseudoalteromonas sp.	99	FJ170037.1	+					
	Pseudoalteromonas sp.	99	HQ342691	+					
	Pseudoalteromonas sp.	99	AB457045	+					
	Pseudomonas alcaliphila	93	NR024734					+	
	Pseudomonas fragi	94	NR024946.1			+			
	Pseudomonas indica	98	NR028801						+
	Pseudomonas lutea	92	NR029103						+
	Pseudomonas mosselii	91	NR024924.1	+					
	Pseudomonas sp.	100	AJ551160.1				+		
	Rhodanobacter lindaniclasticus	96	NR024878						+
	Thalassomonas loyana	98	NR043066.1		+				+
	Alteromonadales sp.	99	FJ403097.1		+				
	Vibrio crassostreae	98	NR044078			+			

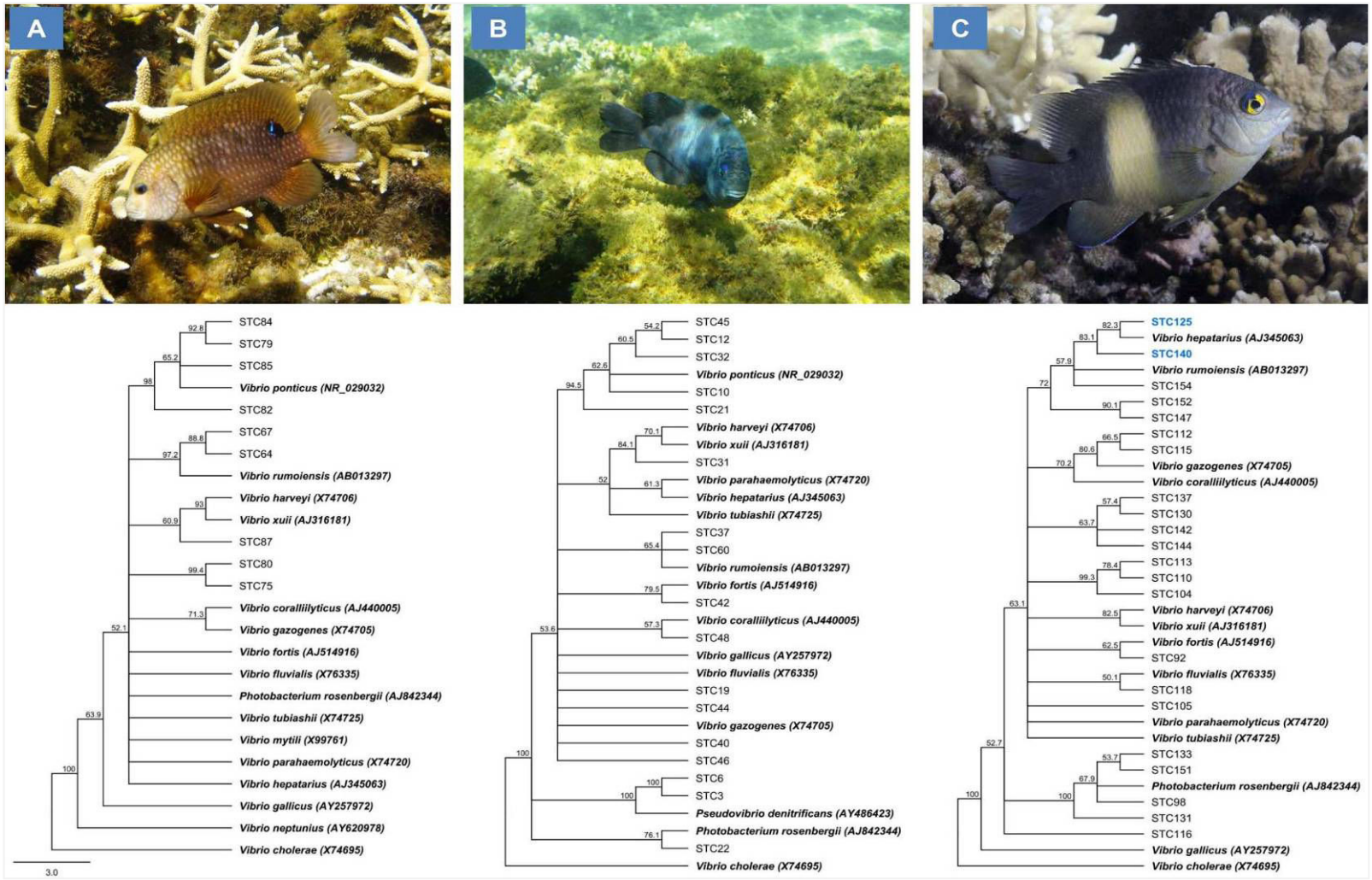
Appendix 1. Continued

Group/Subdivision affiliation	Closest genus/species in GenBank database	Best match (%)	GenBank Acc. No	MHT	MDT	RHT	RDT	SAHT	SADT
	Vibrio fortis	98-99	NR025575.1	+	+	+	+	+	+
	Vibrio furnissii	99	NR036790						+
	Vibrio gallicus	100	NR025740						+
	Vibrio gazogenes	98	NR029256					+	
	Vibrio harveyi	99	NR043165.1	+	+				
	Vibrio hepatarius	99	NR025491.1		+		+		+
	Vibrio natriegens	97	NR026124.1			+			
	Vibrio parahaemolyticus	98-99	NR041838.1	+	+		+		
	Vibrio rotiferianus	99	NR042081.1		+		+		
	Vibrio rumoiensis	98	NR024680	+				+	
	Vibrio sp.	98	AB470934		+				
	Zooshikella ganghwensis	93	NR025668					+	+
Planctomycetes	Planctomycetes	95-97	JF443763		+				
	Rhodopirellula baltica	100	NR043384						+
	Singulisphaera acidiphila	97	NR042662						+
Sphaerobacteridae	*Sphaerobacter thermophilus*	100	NR042118						+
Spirochaetes	*Leptospira borgpetersenii*	99	JQ988862					+	
Unknown	Uncultured bacterium	98-99	EU636648	+					
	Uncultured bacterium	95	GU119164.1	+					
	Uncultured bacterium	99	GU118981.1	+					
	Uncultured bacterium	96	FJ202586.1	+					
	Uncultured bacterium	96	GU119041.1	+					
	Uncultured bacterium	99	JQ347309.1		+				
	Uncultured bacterium	97	HM768687		+				
	Uncultured bacterium	99	FJ203318		+				
	Uncultured bacterium	99	FJ202906.1		+				
	Uncultured bacterium	99	FJ202762		+				
	Uncultured bacterium	98	JF514283.1		+				
	Uncultured bacterium	97-98	GU220747.1			+			
	Uncultured bacterium	92	JF261520.1			+			
	Uncultured bacterium	98-99	HM445412	+					
	Uncultured bacterium	98	DQ200473						+
	Uncultured bacterium	98	JF272035						+
	Uncultured bacterium	99	GU293218						+
	Uncultured bacterium	99	GU472290						+
	Uncultured bacterium	97-98	FJ202885						+
	Uncultured bacterium	98	JF915116						+
	Uncultured bacterium	97	FJ952694						+
	Uncultured bacterium	97-99	FJ202885						+
	Uncultured bacterium	96	FJ203501						+
	Uncultured bacterium	99	FJ203506						+
	Uncultured bacterium	99	FJ202970					+	



Appendix 2: Unrooted phylogenetic trees of bacterial 16S rRNA sequences obtained from samples of *Porites* black patch syndrome (PBPS), generated via maximum likelihood using the PhyML plugin, incorporating related sequences of interest. (A) *Arcobacter*, (B) *Clostridium*, (C) *Spirocheta*, (D) *Flexibacter* and (E) *Desulfovibrio*

Appendix 2 (continued): Unrooted phylogenetic trees of bacterial 16S rRNA sequences obtained from samples of Porites black patch syndrome (PBPS), generated via maximum likelihood using the PhyML plugin, incorporating related sequences of interest. (F) *Vibrio*, (G) *Oceanospirilum*, and (H) *Cytophagia*



Appendix 3 : Neighbour-joining phylogenetic tree for the 16SrRNA gene sequences that were closely related to known and putative pathogens found in *Stegastes lividus* (A), *S. limbatus* (B) et *S. nigricans* (C). Numbers at each node are bootstraps values (%) obtained after 1000 iterations.

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