1.1 Overview

Scleractinian corals form the structural basis of coral reefs, which with their very high species diversity are arguably the most diverse and spectacular marine ecosystems on earth (Bellwood & Hughes 2001). Coral dominated ecosystems have traditionally been vital to the lives of millions of people in tropical and subtropical regions around the world as sources of food, cultural items, and income (Serageldin 1998). In modern times they also form the basis of thriving multimillion-dollar tourism industries in many countries. The potential for discovery of a wide range of useful pharmaceuticals and other products from coral reefs has also recently been appreciated (Bernan et al. 1997, Adey 2000). However, despite the immense value of coral reefs to the welfare of human beings around the world and their intrinsic value as beautiful and unique biological systems, these fragile natural metropolises are facing destruction on a global scale due to increasing incidences of lethal disease outbreaks.

The threat of emerging diseases of reef organisms has led to an increased effort by researchers to understand the causes and mechanisms of coral mortality. In particular, the roles played by the microbial communities associated with corals have recently begun to receive a great deal of attention. It has long been appreciated that the key to the tremendous diversity of coral reefs is the ability of reef-building corals to thrive in oligotrophic waters where limited nutrients would be expected to limit productivity. Historically, the success of scleractinian corals in nutrient-limited environments was attributed exclusively to the partnership formed between the cnidarian hosts and the photosynthetic symbionts (zooxanthellae) that reside within them (Ruppert & Barnes 1994). This symbiosis allows corals to harvest light energy and fix carbon in the absence of sufficient supplies of particulate organic matter. Recent research however,

suggests that that scleractinian corals should not be considered as consisting of an autonomous cnidarian host coupled with one or two species of algal symbionts. It is now clear that corals harbour dense and diverse microbial communities, whose members perform vital functions for the coral host. As such, it has become clear that it is more appropriate to consider the biology of the coral 'holobiont' encompassing the cnidarian host, along with zooxanthellae and a suite of prokaryotes, algae and fungi (Rohwer & Kelley 2004).

This chapter will focus on: (1) A summary of the current state of knowledge regarding the structure and function of the microbial communities associated with scleractinian corals, with particular emphasis on bacteria. The roles of various microbial groups in the function of the coral holobiont and the consequences of perturbation of these communities will also be discussed. (2) A summary of the current state of knowledge regarding the best characterised syndromes and diseases of scleractinian corals worldwide. (3) An overview of the ecology of scleractinian corals in subtropical eastern Australia and the history of coral disease research in the region.

1.2 The coral host as a habitat

Scleractinian corals provide a unique environment for the growth of microbes. In one of the earliest investigations of the microbial communities found on coral surfaces, Ducklow and Mitchell (1979) revealed that the mucus layer of living corals harbours a bacterial community that is distinct from the surrounding water and substrate. The first studies to apply culture independent techniques to survey the bacteria associated with corals made it clear that corals harbour unique and diverse bacterial communities (Rohwer et al. 2001, Rohwer et al. 2002). More recently, it has been demonstrated

that the coral tissue offers further microbial niches, which are occupied by communities of bacteria which are distinct from those inhabiting the mucus layer (Bourne & Munn 2005, Koren & Rosenberg 2006, Koren & Rosenberg 2007). Investigations of the porous interior of the calcium carbonate skeleton of corals has also revealed a distinct microbial community dominated by filamentous algae and fungi (Jeffery 1968, Lukas 1974, Le Campion-Alsumard et al. 1995, Ferrer & Szmant 1998).

The coral host presents a particular range of challenges that can be met by specialised organisms. The microbial communities associated with corals are forced to exist in relatively extreme oxidative conditions. During the day, the photosynthetic activity of the zooxanthellae results in the supersaturation of the coral mucus and overlying boundary layer of water with oxygen. At night however, respiration by the coral tissue creates microaerophilic or even anoxic conditions (Shashar et al. 1993). The microbes associated with the coral tissue and surface layers have adapted to these extreme diel fluctuations by expressing protective enzymes such as superoxide dismutases, catalases and peroxidases which allow them to survive in the presence of free radicals and other oxidative species (Lesser et al. 1990, Banin et al. 2003).

Those microbes that have adapted to survive in the extreme oxidative conditions of the coral surface are able to exploit a rich source of organic carbon in the coral mucus. Mucus secreted by corals and other reef organisms supply a substantial percentage of the total organic carbon found in many reef systems (Wild et al. 2004). Whereas planktonic bacteria in the water column are generally extremely small (Paul et al. 1986) and rely mostly on photoautrophy to meet metabolic requirements, the bacterial

cells found in coral mucus are large (Paul et al. 1986), diverse (Koren & Rosenberg 2006) and highly metabolically active (Paul et al. 1986, Ritchie & Smith 1995a, Ritchie & Smith 1995b).

1.3 Corals form specific associations with bacteria

Appreciation of the important role of bacteria in the coral microbial community has begun only recently with the application of culture-independent techniques. Rohwer et al. (2002) established that different coral species harbour distinct bacterial communities. Interestingly, they also found that in corals of the same species, the composition of these communities is maintained across colonies separated by large spatial and temporal scales. These findings suggest that coral species have adapted to harbour specific bacterial communities. Further support for this idea has been provided by Ritchie and Smith (2004), who demonstrated that metabolic profiles of coral associated bacteria could be clustered in patterns resembling the phylogenetic relationships between the host coral species. This simple model of specific host-bacterial associations has been complicated by Koren and Rosenberg's (2006, 2007) findings that coral colonies of the same species that are exposed to differing environmental conditions harbour different bacterial communities. They showed that in the coral *Oculina patagonica,* healthy, bleached and naturally azooxanthellate colonies found in caves each harboured a distinct bacterial community. They propose that the coral holobiont may adapt to changing environmental conditions through changes in its bacterial communities (Reshef et al. 2006, Rosenberg et al. 2007).

There is mounting evidence to show that corals actively regulate the bacterial communities associated with their mucus and tissue layers. It has been shown that

other anthozoan species, including anemones (Herndl & Velimirov 1985) and octocorals (Kelman et al. 1998, Harder et al. 2003) regulate the composition and densities of bacterial populations on their surfaces by secreting various antibacterial compounds. Although little antimicrobial activity has been detected in scleractinian corals using traditional assays (Kelman et al. 2006), it has been suggested that these corals may regulate the bacterial populations on their surfaces through other means, such as sloughing of the mucus layer (Ducklow & Mitchell 1979, Rublee et al. 1980), or production of specific metabolites that target bacterial phenotypes, rather than killing the bacteria outright (Kelman et al. 2006). Scleractinian corals are also capable of rapidly producing broad spectrum antibacterials in response to mechanical stress (Geffen & Rosenberg 2005). This ability may act as a defence against infection following injury caused by predation. If hosts have developed specialised mechanisms of regulating the bacterial communities occurring on their surfaces, it follows that these communities must play roles with adaptive significance in the functioning of the holobiont.

1.4 The function of bacterial communities in the coral holobiont

The precise metabolic roles of coral associated bacteria are still unclear, but it is clear that they perform a range of nutrient processing functions (Ritchie & Smith 2004) (Figure 1.1). One important functional role that would appear to be vital to the survival of the cnidarian host and its algal symbionts is nitrogen fixation. Accessible nitrogen is a limiting factor in the growth of many corals (Shashar et al. 1994). Although corals are able to take up both inorganic and organic dissolved nitrogen from the water column, and supplement this uptake with heterotrophic uptake of particulate matter (Mills et al. 2004), it is thought that nitrogen fixation carried out by

microbial members of the coral community may be responsible for 55-65% of the coral's nitrogen requirements (Ferrer & Szmant 1998). Shashar et al. (1994) found that colonies of the massive coral *Favia favus* were capable of nitrogen fixation only when supplied either with light or glucose. They deduced that bacteria present in the coral skeleton were able to fix nitrogen if they had an available supply of organic carbon, which under normal conditions is presumably generated by the photosynthetic activity of the coral's zooxanthellae. Recently, Lesser et al. (2004) discovered a novel species of nitrogen-fixing cyanobacteria living inside the cells of the massive coral *Montastrea cavernosa*. This discovery suggests that endosymbiotic cyanobacteria may play a vital role in making limiting nitrogen available to the coral host and zooxanthellae as well as to other microbial components of the coral holobiont. This newly discovered endosymbiosis may even represent an early step towards the evolution of a new type of organelle. This notion seems entirely plausible when it is considered that both mitochondria and chloroplasts are generally accepted to have evolved from similar symbioses with prokaryotes (Margulis 1981), and that intracellular nitrogen-fixing bodies of cyanobacterial origin have been found in some diatoms (Prechtl et al. 2004).

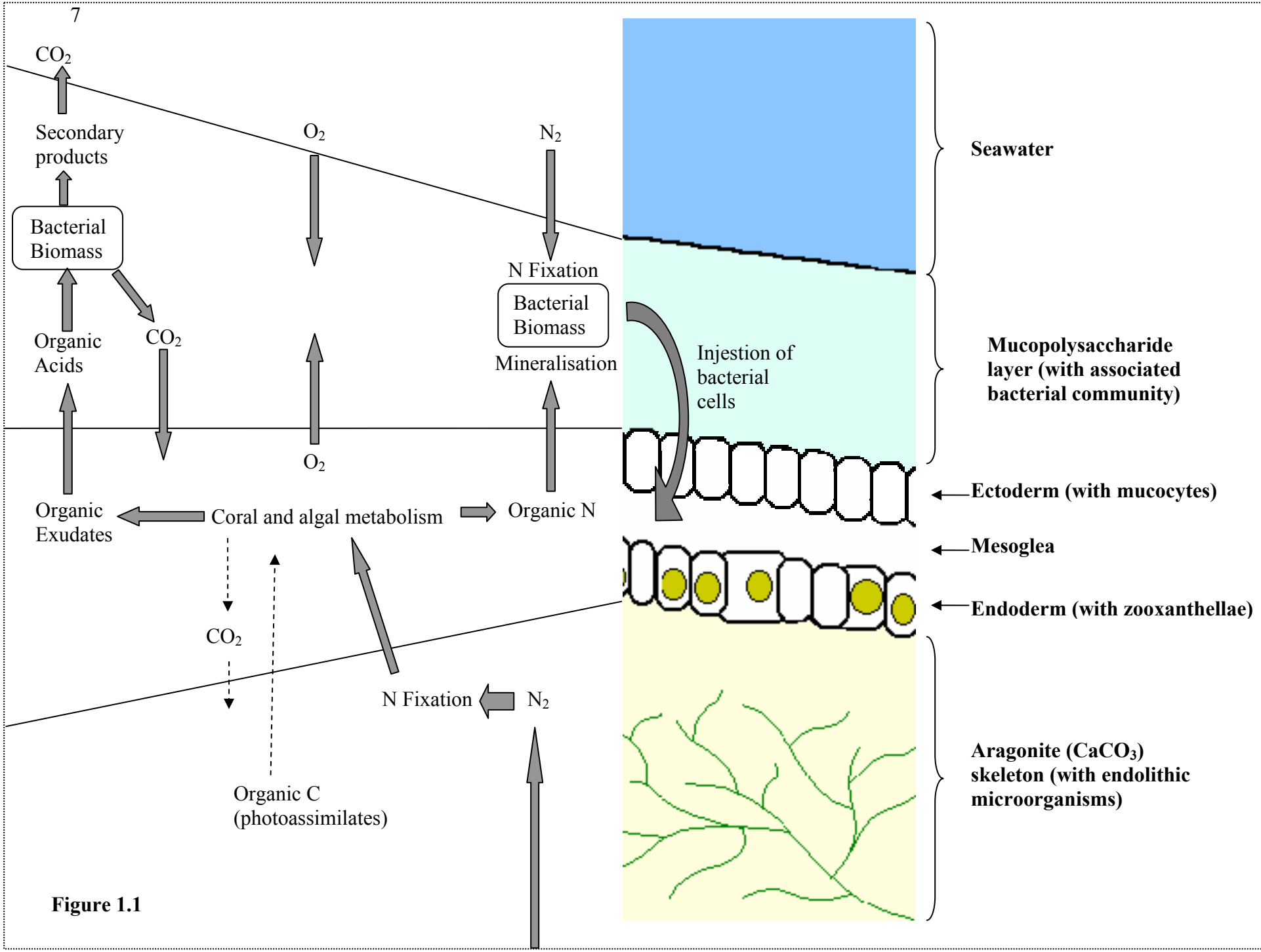
CO_2
Secondary products
Bacterial Biomass
Organic Acids
CO_2
O_2
N_2
N Fixation
Bacterial Biomass
Mineralisation
O_2
Organic Exudates
Coral and algal metabolism
Organic N
CO_2
N Fixation
N_2
Organic C (photoassimilates)
Injestion of bacterial cells
Seawater
Mucopolysaccharide layer (with associated bacterial community)
Ectoderm (with mucocytes)
Mesoglea
Endoderm (with zooxanthellae)
Aragonite ($CaCO_3$) skeleton (with endolithic microorganisms)

Figure 1.1

Figure 1.1 A model of probable flux of carbon, nitrogen and oxygen between the water mass, coral tissue, mucopolysaccharide layer, and skeleton during daylight hours. Adapted from Ritchie and Smith (2004) and (Rohwer & Kelley 2004). The mucus layer is likely to be stratified with respect to oxygen concentration, with microaerophilic or anaerobic conditions towards the centre due to activity of the associated bacteria (Ritchie & Smith 2004). In this model, organic carbon and nitrogen in the form of complex carbohydrates and proteins is secreted by the coral host as mucus and broken down by the bacterial community (Ritchie & Smith 2004). N_2 fixation by the coral-associated bacteria followed by ingestion of bacterial cells by the coral polyps supplies the coral tissues and algal symbionts with nitrogen (Kushmaro & Kramarsky-Winter 2004). Fixed nitrogen and photoassimilates such as carbohydrates are also supplied by the endolithic microbial community (indicated by dotted lines) (Le Campion-Alsumard et al. 1995, Ferrer & Szmant 1998, Fine & Loya 2002)

Many bacterial species almost certainly play a more direct role in the nutrition of the coral host by serving as a source of food. Bacteria, by virtue of their high surface area to volume ratio and high affinity transport systems, are much more efficient than relatively large organisms like corals at harvesting micronutrients such as phosphorous and iron from the water column (Ducklow 1990). In comparison to eukaryotic cells, bacteria are also particularly rich in nitrogen and phosphorous due to their high protein and nucleic acid content, and relative lack of carbohydrate–rich structural material (Simon & Azam 1989). These characteristics make bacteria a potentially valuable source of nutrients for heterotrophic corals. Ferrier-Pages et al. (1998) used radiolabelled marine bacteria and ciliates to measure the rates of heterotrophy in the coral *Stylophora pistillata*. They showed that the corals, even when maintained in sufficient light to allow the photosynthetic activity of their zooxanthellae to satisfy 100% of their energy requirements, ingested both bacterial and ciliate cells. They suggest that this feeding behaviour may provide the coral with nutrients not supplied by the zooxanthellae, such as nitrogen, phosphorus and vitamins (Ferrier-Pages et al. 1998). Further evidence for the active consumption of bacteria by corals is provided by Kushmaro & Kramarsky-Winter's (2004)

observations of dense bacterial-like aggregates on the surfaces of some coral species. The density of these aggregates is highest near the mouths of polyps, leading Kushmaro and Kramarsky-Winter (2004) to suggest that they may be actively transported towards them by the coral.

A third likely role of the bacterial communities normally found in association with healthy corals is prevention of infection by pathogenic microorganisms. As corals lack an adaptive immune system equivalent to that of vertebrates, it is likely that mutualistic bacteria help to prevent infection either by expressing antimicrobials or other metabolites to prevent growth of pathogens or by competing with pathogens for resources and physical space on the coral surface. Reshef et al. (2006) proposed that environmental conditions such as temperature and nutrient availability exert selective pressure on the entire holobiont, resulting in shifts in microbial community structure that allow the coral to rapidly adapt to changing conditions. Several studies provide support for this hypothesis. Koren and Rosenberg (2006) found differences between the bacteria communities associated with the coral *Oculina patagonica* in summer and winter. Ritchie and Smith (1995a, 1995b) also showed that the composition of the culturable bacterial communities associated with the mucus layer of corals changed during periods of stress, and other culture independent assessments of the bacterial communities associated with healthy and diseased corals have shown that the community structure associated with apparently healthy tissues of diseased corals differs from that associated with tissues from completely unaffected colonies (Pantos & Bythell 2006). These observations suggest that either the coral holobiont is able to shift its community of bacterial symbionts in order to resist infection, or that a shift in

community composition where probiotic species are excluded may make the colony more susceptible to disease.

1.5 Departure from normal microbial function and the definition of disease

Although much research is still required, it is now clear that scleractinian corals harbour unique and diverse microbial communities. Since the various members of these communities carry out a range of functions that are important to the health of the coral holobiont, it is logical to assume that a disturbance that disrupts any of these important functions is likely to impact on the health of the community. In other words, if the balance of processes in the coral microbial community is shifted away from normal, disease will result. A definition of the term 'disease' is required to clarify this statement. Disease may be broadly defined as 'A pathological condition of a part, organ, or system of an organism resulting from various causes, such as infection, genetic defect, or environmental stress, and characterised by an identifiable group of signs or symptoms.' (Stedman's Medical Dictionary 2002). For the purposes of this discussion, disease will be defined as any pathological, or unhealthy, condition of corals that is characterised by identifiable physiological or microscopic signs or symptoms. In corals, these symptoms chiefly include loss of colour (bleaching) and tissue degradation.

Pantos et al. (2003) propose that disease may arise as a result of perturbation of the normal bacterial community on the coral surface, leading to a loss of bacterial diversity and dominance of the system by a few species, which may become oppurtunistic pathogens. This hypothesis is supported by experimental work performed by Kuntz et al. (2005) and Kline et al. (2006). They found evidence of non-

specific pathologies in Caribbean corals arising from enrichment of the surrounding water with nutrients. They propose that increased nutrient loads over-stimulate growth of some bacterial species, leading to disruption of the normal coral-microbial interactions and development of disease. Further support for this hypothesis was provided by Smith et al. (2006), who demonstrated that exposure to the labile carbon produced by algae results in mortality of corals in aquaria.

Other modes of perturbation of the normal bacterial community that may lead to disease may include a change in temperature that favours the growth of pathogens over beneficial bacteria (Koren & Rosenberg 2006), or a change in light regime that affects photosynthesis and mucus production (Koren & Rosenberg 2007).

1.6 Bacterial diseases of scleractinian corals

1.6.1 The approach to studying coral pathology

There are presently at least 20 distinct described diseases affecting corals worldwide (Sutherland et al. 2004, Rosenberg et al. 2007). Many of these diseases present similar symptoms, as they all involve progressive loss of live coral tissue, resulting in exposure of the white aragonite skeleton. This means that it may be exceedingly difficult to distinguish between different conditions by simple visual observations in the field. This difficulty, combined with the fact that so far the aetiologies of only 6 of these 20 diseases have been determined, has led to great deal of confusion among coral biologists. This confusion has made it virtually impossible to formulate a strategy for management of any coral disease. Informed management decisions regarding coral disease outbreaks will require an understanding of the processes involved in infection, disease transmission and host resistance (Rosenberg & Ben-

Haim 2002). A critical step towards gaining this understanding is to understand the structure of the microbial communities associated with the coral. Microbiological and molecular analyses should therefore be a priority in any coral disease study.

According to standard practice, organisms responsible for an infectious disease must be verified by fulfilment of Koch's postulates as follows: (1) The organism must be present in all cases of the disease. (2) It must be isolated in pure culture. (3) Inoculation of healthy corals with this pure culture must produce a disease with the same symptoms. (4) The organism must be recovered from individuals in which disease was induced by inoculation. Once infectious agents have been verified in this manner, steps can be taken towards identifying their modes of transmission and reservoirs, thus opening possibilities for disease management through prevention of spread. The difficulty with verifying a pathogenic agent by fulfilment of Koch's postulates is that many marine microorganisms may be very difficult to isolate in pure culture (Amann et al. 1995, Eguchi 1999, Fuhrman et al. 2002).

The following section outlines the current state of knowledge for each of the relatively well characterised bacterial coral diseases and syndromes, and gives a brief summary of the research involved in attaining this understanding.

1.6.2 Bacterial bleaching of *Oculina patagonica*

The bleaching of the Mediterranean coral *Oculina patagonica* by the bacterium *Vibrio shiloi* is currently the best characterised microbial coral disease. Kushmaro et al. (1996) first observed that bleached colonies of this coral were associated with dense aggregates of rod-shaped bacteria, which were not present in healthy colonies. They

proceeded to isolate a bacterium from bleached colonies and demonstrated that healthy colonies became bleached when inoculated with this organism. The bacterium was reisolated from newly infected colonies, thus fulfilling Koch's postulates (Kushmaro et al. 1996, Kushmaro et al. 1997). The bacterium responsible for bleaching of *O. patagonica* was classified as a new species in the genus *Vibrio*, and has since been named *Vibrio shiloi* (Kushmaro et al. 2001).

From the perspective of the coral pathologist, the *V. shiloi*/*O. patagonica* system makes an excellent model for understanding the process by which corals may be infected by bacteria. The elucidation of the virulence mechanisms of *V. shiloi* has revealed a number of critical steps that have implications for the study of other coral diseases. These steps are: (1) The chemotactic attraction of *V. shiloi* cells to the coral mucus (Banin et al. 2001a). (2) Adhesion of the bacterial cells to a β-D-galactoside-containing receptor in the mucus (Toren et al. 1998). (2) Penetration of coral epidermal cells by the bacteria and intracellular multiplication (Banin et al. 2000b). (3) Differentiation of the pathogen into a viable but not culturable (VBNC) state (Banin et al. 2000b, Israely et al. 2001) (4) Expression of virulence factors that bleach and lyse zooxanthellae (Ben-Haim et al. 1999, Banin et al. 2001b), and allow the pathogen to survive in the hostile oxidative conditions of the host tissue (Banin et al. 2003).

Importantly, it has also been shown that the expression of virulence factors that enable the pathogen to adhere to the coral mucus, survive inside the cells of the host, and destroy zooxanthellae are all temperature dependant (Kushmaro et al. 1998, Banin et al. 2000a, Israely et al. 2001). These findings explain the observed seasonal pattern of

bleaching of *Oculina patagonica*, in which corals become bleached in summer when water temperatures reach a maximum of 31 ºC, and recover during winter (Kushmaro et al. 1998). The finding that *V. shiloi* is unable to survive in coral tissues at low temperatures implied that a reservoir must exist that allowed the bacteria to persist in the environment during the winter months and reinfect coral colonies in summer. Sussman et al. (2003) showed that this was indeed the case. They identified the marine fire worm *Hermodice carunculata* as the winter reservoir and vector of *V. shiloi*, and demonstrated that the pathogen infects the worms and enters a VBNC state as it does in coral tissues (Sussman et al. 2003).

These findings have important implications for the study of other coral pathologies. The discovery that *V. shiloi* is able to enter a VBNC state suggests that if other coral pathogens also differentiate into a state in which they are no longer culturable, it may prove difficult or impossible to isolate them in pure culture and fulfil Koch's postulates by using them to reinfect healthy corals.

1.6.3 Bleaching and tissue lysis of *Pocillopora damicornis* by *Vibrio coralliilyticus*

The discovery that bleaching in *Oculina patagonica* is caused by bacterial infection led to the hypothesis that bacteria may be involved in other cases of coral bleaching. In order to address the question of the generality of the bacterial bleaching hypothesis, it was necessary to find a more typical model than the temperate *O. patagonica*/*V. shiloi* system (Ben-Haim Rozenblat & Rosenberg 2004). Ben-Haim et al. (2003a) conducted a search for coral pathogens in the tropical waters off Zanzibar which led to the discovery of the novel pathogen *Vibrio coralliilyticus* in bleached colonies of the common branching coral *Pocillopora damicornis*. Infection experiments

conducted using cultured *V. coralliilyticus* and aquarium-maintained *P. damicornis* fragments revealed that the effect of the pathogen is temperature dependant. At moderate temperatures (24 – 25 ºC) *V. coralliilyticus* causes bleaching, but the coral tissue itself remains intact (Ben-Haim et al. 2003b). At high temperatures (≥26 ºC) the tissue of coral fragments inoculated with as few as 30 bacteria per mL is completely destroyed, leaving only bare skeleton (Ben-Haim & Rosenberg 2002). This tissue lysis is probably caused by an extracellular protease produced by *V. coralliilyticus* at high temperatures (Ben-Haim et al. 2003b).

It is widely recognised that most cases of coral bleaching are closely related to higher than normal seawater temperatures (Jokiel 2004), but this bleaching may not necessarily be due to direct stress effects on the coral or zooxanthellae, as has been previously assumed. Rosenberg (2004) proposes that coral bleaching triggered by high temperatures may be due to the induction of virulence factor expression in coral bleaching pathogens. Temperature is an important regulating condition in the expression of virulence factors in many other pathogenic microorganisms, including bacteria (Garner et al. 2004, Grundling et al. 2004), fungi (Kraus et al. 2004) and yeast (Singleton & Hazen 2004). The findings that both *P. damicornis* and *O. patagonica* are susceptible to bacterial induced bleaching, and the fact that in both cases the effect of the pathogen is highly temperature dependant, provides support for the bacterial disease hypothesis of coral bleaching (Ben-Haim Rozenblat & Rosenberg 2004, Rosenberg 2004). However the question of how general bacterial bleaching of corals may be is yet to be fully addressed. It has been found that many coral species can survive for months in aquaria maintained at 30 ºC, and that some individuals which bleach at higher temperatures contain higher numbers of colony

forming *Vibrio* spp. than controls (Ben-Haim Rozenblat & Rosenberg 2004). It has also been shown that in the massive coral *Montastrea annularis*, bleaching is associated with a shift in bacterial community structure from *Pseudomonas* spp. to *Vibrio* spp. (Ritchie et al. 1994). Further research is required to determine the significance of these observations.

1.6.4 Black Band Disease

Black band disease (BBD) was first reported as affecting corals on Caribbean reefs in 1973 (Antonius 1976). The disease affects a wide range of species, including scleractinian corals and gorgonians (sea fans) and has been discovered in the Indo-Pacific, including on the Great Barrier Reef (GBR), since its first appearance in the Caribbean (Antonius 1984, Miller 1996, Dinsdale 2000). BBD has presented marine microbiologists with a particularly interesting challenge, as the black band that characterises the disease consists of a diverse microbial community, which is completely different from the normal coral community and the bacterioplankton assemblages in the surrounding seawater (Frias-Lopez et al. 2002). The community is dominated by filamentous cyanobacteria (Rützler et al. 1983), and forms a mat which migrates horizontally across the coral skeleton, consuming live coral tissue as it goes and leaving white coral skeleton in its wake.

In functional terms, the black band mat is similar to sulphur-cycling microbial mats which occur in other marine habitats (Carlton & Richardson 1995, Richardson 1996, Richardson et al. 1997), such as near deep sea methane hydrate lakes (Boetius et al. 2000) and in illuminated environments where microbial processes are driven by photosynthesis (Jørgensen et al. 1979). Sulphate reducing species including

Delsulfovibrio (Viehman et al. 2006), and sulphide oxidisers morphologically characterised as *Beggiatoa* spp (Garret & Ducklow 1975) have been identified in BBD mats. Much research effort has been dedicated to studying the community comprising the black band mat and trying to identify which of its components are directly responsible for disease causation in corals.

The fact that the intact mat had been shown to be infectious whereas individual organisms that were isolated from the mat seemed unable to cause infection (Rützler et al. 1983), led Carlton and Richardson (1995) to propose that the disease was caused not by a single pathogen, but by a pathogenic consortium of microorganisms found within the mat. They showed that the activity of the sulphur cycle bacteria results in the formation of distinct chemical microgradients between the upper and lower levels of the mat. The anoxic conditions and high H_2S concentrations in the lower portion of the mat are extremely hostile for living coral tissue, which dies as the mat progresses across it (Carlton & Richardson 1995).

Recently, several studies have applied molecular microbial ecology techniques to examine the composition and function of the BBD microbial community. These studies have forced previous models of the disease to be reconsidered. In similar studies, Frias-Lopez et al. (2002) and Cooney et al. (2002) conducted surveys of the bacterial community associated with BBD by amplifying 16S rRNA genes from the band. They showed that a single cyanobacterial ribotype was present in all corals affected by the disease in the Caribbean, but found that this ribotype differed from the cyanobacterial species detected in BBD mats in the Indo-Pacific (Frias-Lopez et al. 2003). These studies have consistently failed to detect nucleotide sequences related to

organisms of the genus *Beggiatoa*. The true identity of the filamentous sulphide-oxidising bacterium previously identified by microscopic observation as a *Beggiatoa* species remains a mystery.

These findings illustrate the importance of taking an integrated approach to the study of new coral pathologies, as the information supplied by microscopic observation, culture-based analysis of microbial communities or molecular characterisation alone may not be sufficiently useful to allow the identification of causative agents.

1.6.5 'White syndromes' - White band disease, white plague and white pox

Most coral diseases that are associated with tissue destruction result in similar macroscopic symptoms. Typical cases involve the exposure of the white coral skeleton as disease progresses from one side of the colony to the other. The exposed skeleton is generally colonised by various species of algae, resulting in most cases in a narrow white band of recently exposed skeleton, occurring between the healthy coral tissue and the older algae-covered skeleton, which moves gradually across the colony. Unless there is some distinguishing characteristic, such as the obvious dark coloured microbial mat typical of black band disease, many coral diseases may be impossible to distinguish by field observations alone (Bythell et al. 2004). This problem has resulted in a proliferation of arbitrarily assigned names for coral diseases that result in the exposure of the white coral skeleton. For the purposes of field-based ecological studies, it has become acceptable practice in Australia to pool all of these diseases under the single heading 'white syndrome' (Willis et al. 2004), at least until such time as aetiology for each can be determined. When these diseases are examined at the microscopic or molecular level, it becomes apparent that white syndromes that

are indistinguishable in the field may be caused by completely different pathogens. This section will summarise the current state of knowledge regarding the aetiologies of each of the 'white syndromes' that have been determined thus far.

1.6.5.1 White Plague

The term 'white plague' was originally used to describe a necrotic condition resulting in skeletal exposure in massive and tabulate Caribbean corals (Dustan 1977). The name is now applied to at least three distinct coral diseases, designated white plague types I, II and III, which are defined on the basis of phenotype and the range of coral species that they affect (Bythell et al. 2004). Only one of these three diseases, white plague type II (WPII) has a known aetiology. WPII is caused by infection with *Aurantimonas coralicidia* (Richardson et al. 1998a, 1998b), an alphaproteobacterium belonging to the order *Rhizobales* (Denner et al. 2003). *A. coralicidia* has been detected on fronds of the macroalgae *Halimeda opuntia*, and it has been proposed that this algae acts as a reservoir and vector of the pathogen (Nugues et al. 2004).

A recent molecular investigation of the bacterial ecology of a plague-like disease, which may be identical to WPIII, affecting *Montastrea anularis*, has identified several potential pathogens (Pantos et al. 2003). Samples of diseased tissues consistently yielded an α-protebacterium closely related to the bacterium responsible for juvenile oyster disease. The same group also identified this species in many BBD microbial mats (Cooney et al. 2002). The causative agent of WPII was not identified in any samples of this disease. In addition it was shown that there are substantial differences between the compositions of the bacterial communities of healthy tissues and diseased tissue. Interestingly, it was found that in *M. annularis* colonies affected by the

disease, the apparently healthy living tissue distant from the actively progressing disease front harboured a less diverse bacterial community than the tissue of completely healthy colonies (Pantos et al. 2003).

1.6.5.2 White band disease

White band disease (WBD) was first observed on Caribbean acroporid coral in the 1970's (Gladfelter et al. 1977, Gladfelter 1982). WBD is currently distinguished from plague only by the species that it affects. Whereas the name plague is generally used to refer to diseases of massive and encrusting species, WBD is used to refer to similar diseases of branching acroporid corals, in particular the ecologically important Caribbean species *A. palmata* and *A. cervicornis*. The first recorded epizootic of WBD in Caribbean during the 1980's is believed to responsible for the large-scale phase shift of some reefs from coral-dominated to algae dominated (Aronson & Precht 2001).

WBD typically progresses in a similar manner to WP. An expanding area of dead tissue results in a band of recently exposed white skeleton sandwiched between the healthy tissue, and older dead skeleton colonised by algae. As the species affected have branching growth forms, the disease spreads as white bands along each branch. Casas et al. (2004) used 16S rRNA gene analyses to examine the bacterial communities associated with type I white band disease in acroporids. They found no obvious differences in the bacterial communities associated with corals from reefs with a high occurrence of WBDI and reefs with low WBDI occurrence. They suggest that a non-bacterial pathogen may be involved in WBD.

A second variant of WBD was identified in 1995 by Ritchie and Smith (1998). In the field, type II white band disease (WBDII) is distinguishable from WBDI in that a different range of coral species are affected and the disease lesion is separated from the healthy coral tissue by a thin margin of bleached tissue. Microbiological analysis of the lesions caused by WBDII have revealed increased numbers of a *Vibrio* spp. in the bleached area adjacent to the exposed skeleton (Ritchie & Smith 1995a, Ritchie & Smith 1998). Gil-Agudelo et al. (2006b) provided further evidence for the involvement of a *Vibrio* species as the causative agent of WBDII when they demonstrated that healthy corals in the field developed identical symptoms when it was inoculated *Vibrio harveyi* cultured from naturally occurring WBDII lesions. The observation of a *Vibrio* species associated with the bleached tissue of WBDII affected corals agrees with Rosenberg's (2004) observations of increased numbers of *Vibrio* spp. in samples of other bleached coral species, and the general hypothesis that coral bleaching may be related to bacterial infection.

1.6.5.3 White Pox Disease (Acroporid serratiosis)

First reported in 1996 (Bruckner & Bruckner 1997), white pox disease affects *Acropora palmata*, the Caribbean's most common reef building species. Since its emergence, white pox disease has decimated *A. palmata* populations, with an average loss of live coral cover of 88% (Patterson Sutherland & Ritchie 2004). White pox can be distinguished from other white syndromes by the characteristic pattern of tissue loss that it causes. Rather than progressing as a band or front across the coral colony, it causes small localised patches of tissue death. This results in round white spots of exposed skeleton surrounded by large areas of apparently healthy tissue, hence the

names white pox (Patterson Sutherland & Ritchie 2004, Sutherland et al. 2004), and patchy necrosis (Bruckner & Bruckner 1997).

White pox disease has been shown to be caused by the ubiquitous bacterium *Serratia marcescens* Patterson et al. (2002). This gram-negative, coliform bacteria is found in association with wide variety of habitats including freshwater, soil, plants and the gut tracts of many animals including humans and insects (Grimont & Grimont 1994) cited in (Patterson Sutherland & Ritchie 2004). (Patterson et al. 2002) first isolated the bacterium from syringe samples of the mucopolysaccharide layer of white pox affected *A. palmata* colonies. Inoculations of healthy corals were performed in the field by adsorbing the cultured bacterium onto sterilised calcium carbonate sediment and placing this sediment directly on the coral surface. The bacterium was then reisolated from the coral fragments after development of typical white pox lesions. Although the mechanisms of infection and virulence are yet to be determined, white pox disease appears to be highly transmissible and outbreaks are correlated with higher seawater temperatures (Patterson Sutherland & Ritchie 2004). Patterson et al. (2002) suggest that the fact that *S. marcescens* is a common human enteric bacterium may indicate a link between human faecal effluent and coral diseases.

1.6.6 Yellow Band/Blotch Disease

Yellow band disease (YBD) was first observed in 1994 on corals in the Florida Keys. (Santavy & Peters 1997). It has since been observed on reefs throughout the Caribbean, and a similar condition was reported to affect corals in the southern Arabian Gulf (Korrûbel & Riegl 1998). The disease presents as an expanding area of yellow or pale brown tissue, which may form a ring or band as it spreads across a

colony (Santavy et al. 1999). The condition is best described as a form of bleaching, as it appears to primarily affect the algal symbionts rather than the host tissue itself (Santavy & Peters 1997, Cervino et al. 2001). In cases where the affected corals are further stressed by high water temperatures the tissue in the centre of a discoloured patch will die, exposing the skeleton. This may result in a lesion that expands as the disease spreads across the coral colony (Cervino et al. 2004). The condition affects a wide range of coral species including massive *Diploria*, *Favia, Montastrea* and *Porites* species in the Caribbean (Gil-Agudelo et al. 2004), and several *Acropora* and *Porites* species in the Arabian Gulf (Korrûbel & Riegl 1998).

The aetiology of YBD has only recently been investigated. Cervino et al. (2004) first observed high numbers of *Vibrio* species in association with affected *Montastrea spp.* specimens. These bacteria were not present in healthy samples. When these bacteria were isolated and used to inoculate healthy specimens a particular consortium of four species was found to cause symptoms that matched the disease phenotype. The extent and rate of spread of the symptoms caused by infection with these four *Vibrio* spp. bacteria is greatest at high temperatures. As is the case in the field, it was found that YBD affected corals could survive at moderate temperatures (20 ºC) but suffered 80% mortality at higher temperatures (31 ºC). It seems likely that YBD provides another example of bacterial induced coral bleaching, but further research is required to determine the exact mechanisms of pathogenesis (Cervino et al. 2004).

1.7 Study Areas in Subtropical Eastern Australia

The bulk of coral disease research reported in the literature focuses on diseases that affect coral dominated ecosystems in tropical regions (Sutherland et al. 2004). Shallow water marine communities dominated by scleractinian corals also occur in subtropical regions, and since the corals in these regions often exist at the limits of their ranges of tolerance for environmental conditions such as temperature, light intensity and nutrient levels, these communities are particularly vulnerable to damage by outbreaks of coral disease.

The subtropical waters of eastern Australia comprise a unique biogeographical region with a high level of species diversity. A key factor in the biodiversity of the region is its position with respect to the warm Eastern Australian Current (EAC). This current carries warm water from the GBR south to the NSW coast, where it mixes with cool temperate waters (Figure 1.2). The EAC also carries a variety of larval fishes, corals and other invertebrates from the Great Barrier Reef (Dight et al. 1990, Wilson & Harrison 1998, O'Hara & Poore 2000, James et al. 2002), many of which are able to settle and contribute to the biodiversity of the area. The interaction of cool and warm currents in the region results in a broad range of both tropical and temperate species.

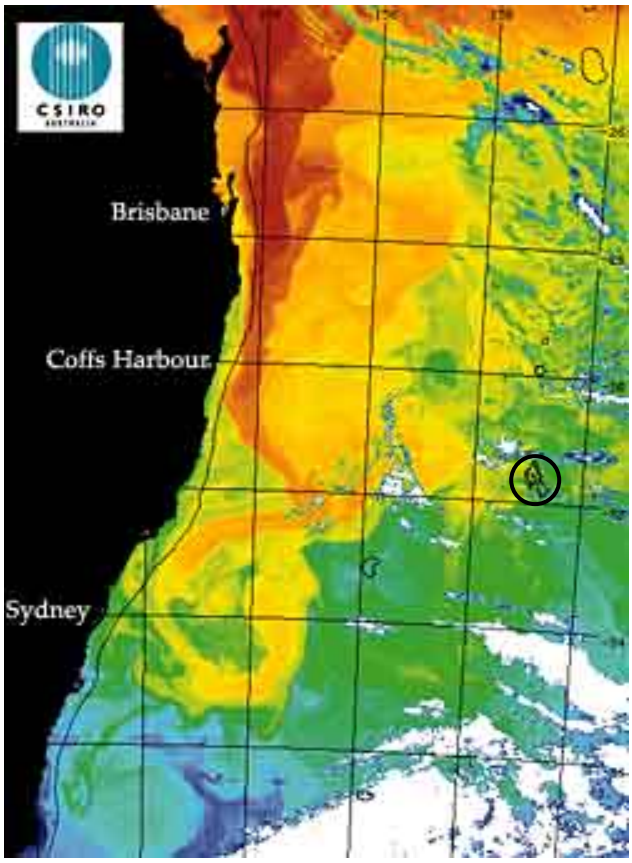

Figure 1.2 Thermal satellite image mapping sea surface temperatures adjacent to the east coast of Australia. Regions of highest temperature are coloured red, while regions of lowest temperature are coloured blue. This image graphically illustrates the position of the warm East Australian Current, and the mixing of warm (red/orange) and cool (green) water that occurs along the northern coast of New South Wales, and at Lord Howe Island (circled) (CSIRO).

1.7.1 The Solitary Islands Marine Park

The 75,000 ha Solitary Islands Marine Park (SIMP) extends for 75 km along the mid north coast of NSW, from Muttonbird Island (30.3 ºS), near Coffs Harbour, to Plover Island (29.6 ºS), adjacent to the mouth of the Sandon River. The park is named after the five small rocky islands that it encompasses. The shallow waters surrounding each of these islands, and several other rocky outcrops, islets and shoals, support unique and diverse coral dominated communities (Veron et al. 1974, Harriot et al. 1994), while the islands themselves provide important nesting grounds for migratory seabirds (Mitchell 1997). The park also contains important coastal, estuarine, open ocean and

soft sediment habitats and forms part of the route for the annual migration of Australia's east coast humpback whale population (Marine Parks Authority). The SIMP's natural beauty, abundant marine life, and location close to the major tourism centre of Coffs Harbour make it a popular destination for many groups of users including researchers, surfers, sport divers, recreational fishers, whale watchers and boaters.

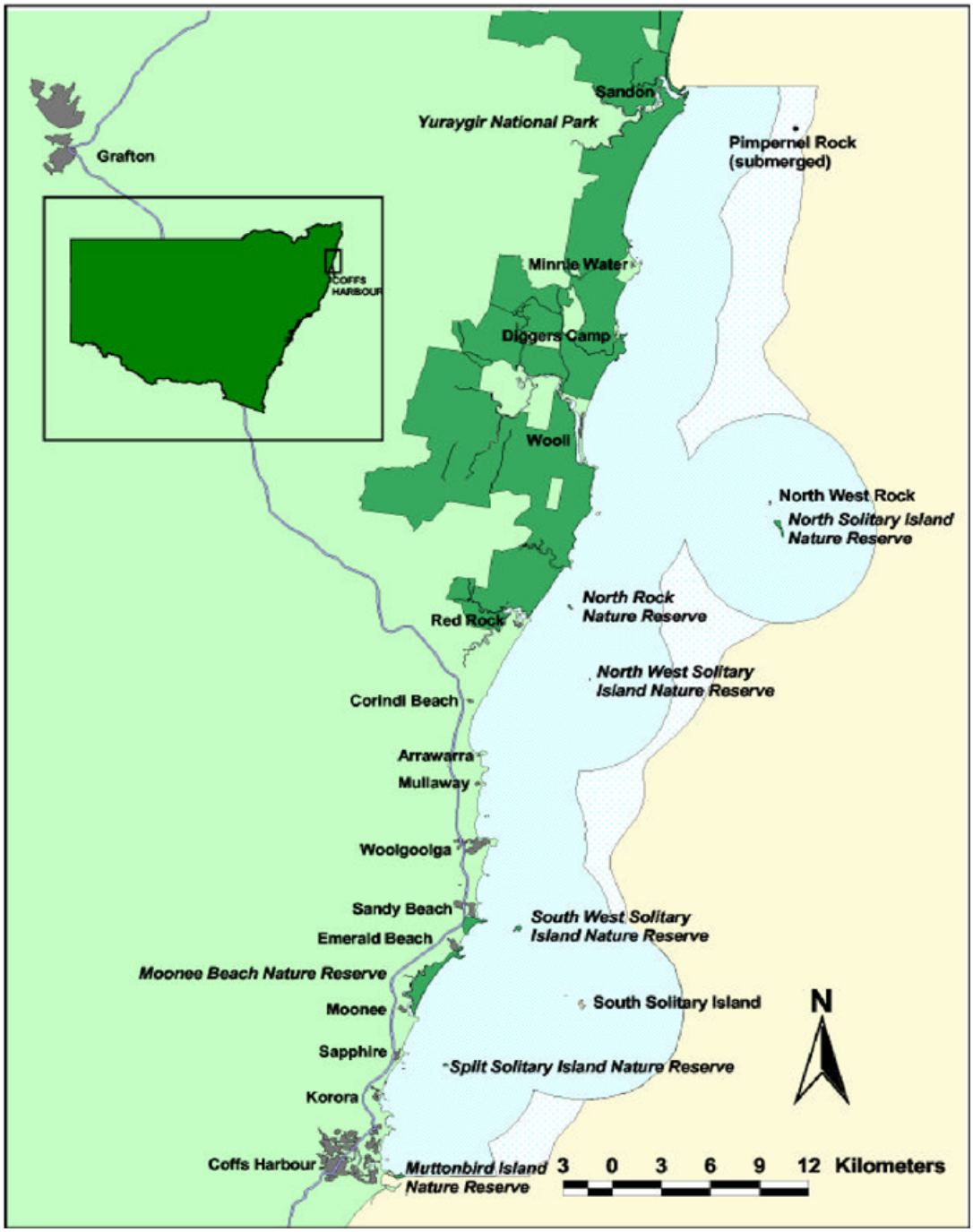

Figure 1.3 The Solitary Islands Marine Park. The blue areas indicates the boundaries of the park (NSW Marine Park Authority).

The coral communities within the SIMP are diverse and unique. A total of 90 scleractinian coral species have been recorded, including 77 tropical, 11 subtropical and one temperate species (Veron et al. 1974, Harriot et al. 1994). These corals must contend with environmental parameters that differ greatly from those experienced by

most tropical scleractinian coral communities. Low water temperatures, turbidity, and regular episodes of heavy wave action in the SIMP restrict the growth rates of hermatypic corals (Harriott 1999). In addition low rates of sexual reproduction and larval recruitment (Harrison & Wallace 1990, Harriot 1992, Harriot & Banks 1995) affect the re-establishment of corals after patches of substratum are cleared by storms. As in other reef systems, corals in the SIMP must also compete for substrate with a large range of other sessile organisms including sponges, macroalgae, anemones, soft coral and corallimorphs. The synergistic effect of these factors prevents the accretion of skeletons and the formation of true coral reefs in the SIMP, even though hermatypic corals comprise up to 50.9% of the benthic cover at some locations in the park (Harriot et al. 1994). Combined with the natural pressures on corals in SIMP, coral disease has the potential to radically alter the composition and structure of coral dominated communities in this unique subtropical area.

1.7.1.1 Coral Bleaching and Disease in the SIMP

The severe El Nino weather pattern that caused higher than normal sea surface temperatures during the southern hemisphere summer of 1997/98 resulted in a global mass coral bleaching event (Douglas 2003). Following this event, members of the voluntary Solitary Island Underwater Research Group (SURG) noted increased incidences of coral bleaching in the SIMP. A monitoring program was established to follow the occurrence of bleaching in the park, and continued for a period of 2.5 years from 2000 to 2003. Low levels of patchy bleaching were recorded throughout this period (Edgar et al. 2003). In 2002 a second mass bleaching event was recorded in the GBR when seawater temperatures rose in late summer (Wilkinson 2002). Temperature logger data from the SIMP indicated that water temperatures were

higher in 2002 than in the previous year, but no mass bleaching was reported by the SURG monitoring program (Edgar et al. 2003). Coral disease, defined as mortality spreading gradually across coral colonies, was also recorded (Edgar et al. 2003). Coral disease in the SIMP usually presents as the classic 'white syndrome' with gradual loss of living tissue beginning at one point, then spreading outwards across the coral colony, exposing the underlying white skeleton (Dalton & Smith 2005). As the range of coral species affected by disease is different to that previously reported for other regions (Sutherland et al. 2004, Willis et al. 2004), the name 'subtropical white syndrome' (SWS) has been adopted to describe diseases with these symptoms in subtropical eastern Australia (Steven Dalton, personal communication).

Dalton and Smith (2005) recorded further progression of SWS in the SIMP during 2002-2004, correlated with a decrease in overall coral cover. SWS appears to affect at least ten coral species in the park, although rates of progression vary between species (Dalton 2003). Of particular concern are the high levels of mortality that occur in the dominant structure forming species, which create vital habitats for many other reef species. The tabulate corals *Acropora solitaryensis* and *Turbinaria mesenterina*, which are responsible for forming much of the benthic structure in the SIMP, are potentially highly vulnerable, as their growth rates are very slow (Harriott 1999), reducing their ability to recover from disease outbreaks. Disease was recorded in 32% of tagged *Acropora* colonies and 35% of tagged *Turbinaria* colonies surveyed between 2000 and 2003, and was presumed responsible for the total mortality of 16% and 21% of these colonies respectively (Edgar et al. 2003).

Unlike white plague in the Carribean, which does not appear to be related to temperature (Borger 2003, Voss & Richardson 2006), SWS is more virulent at higher temperatures. Dalton et al. (submitted, 2008) showed that SWS lesions on *Turbinaria mesenterina* progressed faster at 26 ºC than at 21 ºC.

1.7.2 Lord Howe Island

Lord Howe Island (LHI) is the eroded remnant of a large volcano, the summit of a seamount that rises at least 1800 m from the ocean floor (McDougall et al. 1981). The island lies approximately 600 km east of the coast of New South Wales, and is surrounded by the most southerly coral reefs in the world (Allen & Paxton 1974). In 1982 the island and the surrounding waters were added to the UNESCO world heritage list in recognition of the unique ecology and large number of endemic species in the region. The coral communities surrounding the island include both tropical species that are living at the southern limit of their distribution, and subtropical species that are rare or absent on the GBR (Harriot et al. 1995). Extensive coral reefs enclose a large lagoon on the western side of the island (Figure 1.4, 1.5), with significant coral communities also occurring on the rocky reefs surrounding the island (Harriot et al. 1995). The diversity of the coral communities at LHI is high, with a total of 83 scleractinian coral species having been recorded (Harriot et al. 1995).

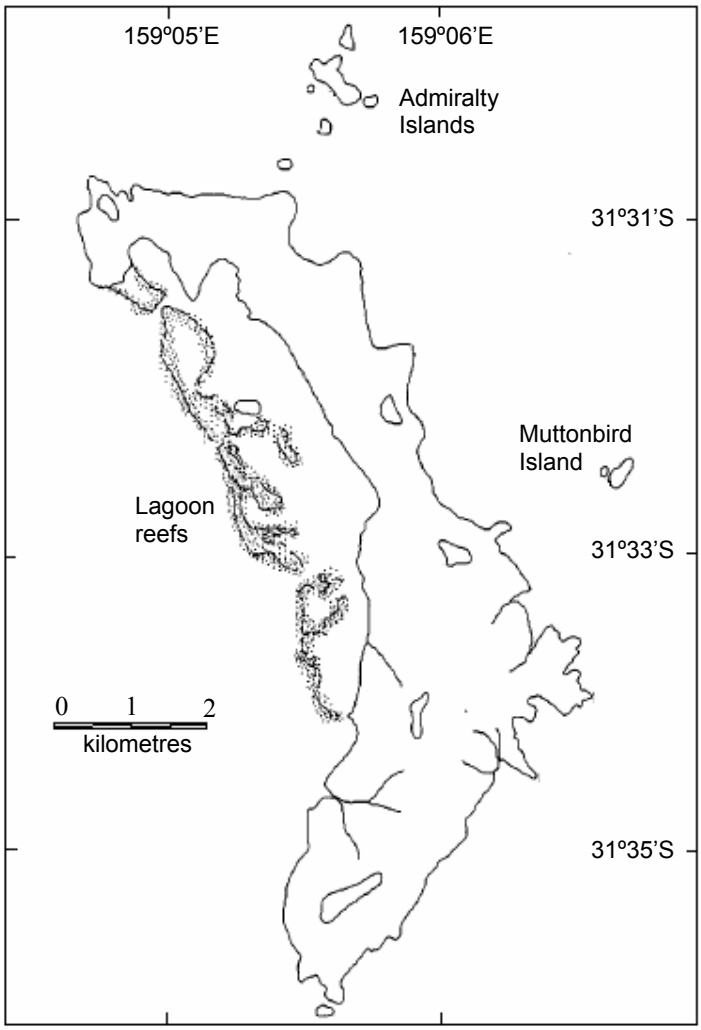

Figure 1.4 Map of Lord Howe Island, showing major geographical features and coral reefs. Adapted from Harriot et al. (1995).

Figure 1.5 A section of reef at Stephen's hole, in the outer lagoon reef at LHI, showing the diverse community of scleractinian corals and other reef organisms.

1.7.2.1 Coral disease at LHI

Previously reported surveys of the coral communities at LHI make no specific mention of disease (Veron & Done 1979, Harriot et al. 1995, Harriott 1999), although some mortality of *Acropora* spp. was noted in the northern part of the lagoon between 1984 and 1995 (Harriot et al. 1995). A survey conducted in 2005 detected disease with symptoms consistent with SWS in several coral taxa including *Acopora, Porites* and *Pocillopora*, but overall disease prevalence at the sites surveyed was lower than in the SIMP (Dalton & Godwin 2005).

1.8 Coral species examined in this study

Following preliminary trials using *Acropora*, *Pocillopora*, *Goniastrea* and *Turbinaria* species to determine which could be most practically maintained in aquaria, the two similar coral species *T. frondens* and *T. mesenterina* were chosen as models for this study. These species are abundant at the sites surveyed, and form a large proportion of the benthos, particularly in the SIMP (Harriot et al. 1994). In addition, these species yielded the most consistent results in experiments involving the extraction of DNA and PCR amplification of bacterial rRNA genes. As the two species are morphologically very similar, occupy the same ecological niche and are difficult to distinguish in the field, it was decided to pool all data related to them, both for the work reported in this thesis, and for ecological field studies (Steven Dalton, personal communication). As *T. mesenterina* appears to be the dominant of the two species in the SIMP, the *T. mesenterina*/*T. frondens complex* will be collectively referred to as *Turbinaria mesenterina* for the remainder of this thesis.

1.9 Study Objectives

The aim of the work presented in this thesis is to provide a basic understanding of the pathology and bacterial ecology of SWS in *T. mesenterina*. Specifically, it aimed to address the following questions: (1) Is SWS caused by an infectious microbial agent, and if so, is this agent likely to be bacterial, fungal, protozoan or viral in nature? (2) What is the structure of the bacterial communities associated with SWS affected *T. mesenterina*, and which members of those communities are likely to be involved in the disease process?

Chapter 2 of this thesis provides the first basic description of the microscopic characteristics of SWS in *T. mesenterina*. This chapter also demonstrates that SWS is caused by an infectious biotic agent that is transmissible by direct contact between diseased and healthy *T. mesenterina* fragments, but not via the water column. Evidence is also porvided that this infectious agent is likely to be a bacterium. Aspects of this chapter have been submitted to the journal Diseases of Aquatic Organisms (Dalton et al. submitted, 2008).

In Chapter 3 a survey of the culturable bacteria associated with *T. mesenterina* is described. Comparisons made between healthy and SWS affected *T. mesenterina* within the SIMP indicate clear differences between apparently healthy, diseased and dead sections of SWS affected colonies, and between completely healthy colonies and the apparently healthy tissues of affected colonies. A strain of the bacterium *Vibrio harveyi* was identified as a potential pathogen, as it was detected in all samples of SWS affected *T. mesenterina*, but not in samples from healthy colonies. This chapter also compares the bacterial communities associated with healthy *T. mesenterina* in two different subtropical locations. It is demonstrated that the bacterial communities associated with *T. mesenterina* in a near shore environment with extensive anthropogenic influences (SIMP) differ from those in remote offshore location where anthropogenic impact is much more limited (LHI). The extensive culture collection that was developed will be available for use in any future experiments.

Chapter 4 describes a culture-independent survey of the bacterial communities associated with healthy and SWS affected *T. mesenterina* in the SIMP performed using bacterial gene profiling technique (Oligonucleotide fingerprinting of bacterial

ribosomal genes). There were significant differences between the bacterial communities associated with apparently healthy, diseased and dead sections of SWS affected colonies. Significant differences were also observed between the apparently healthy tissues of SWS affected colonies and completely healthy colonies, indicating that the development of SWS symptoms is associated with changes in the entire colony. The *Vibrio* species identified as a potential pathogen in the culture based surveys was not detected, but bacteria belonging to the *Roseobacter* clade were significantly more abundant in samples from SWS lesions than they were in healthy tissue, suggesting a possible aetiological role for these bacteria in SWS. This chapter will be submitted to Applied and Environmental Microbiology.

Chapter 2 – Pathology of Subtropical white syndrome of *Turbinaria mesenterina*: The role of bacteria as biotic factors in disease progression and transmission

2.1 Introduction

Subtropical white syndrome (SWS) was first observed affecting *Turbinaria mesenterina* in the waters of subtropical eastern Australia in 2002 (Edgar et al. 2003). SWS results in an expanding area of exposed white calcium carbonate skeleton on the surface of affected coral colonies (Figure 2.1), and appears similar to diseases such as white plague (WP) and white band disease (WBD) described in other coral species worldwide (Bythell et al. 2004). Although the appearance of the disease is superficially similar, the range of coral species affected by SWS is different to the range affected by WBD or WP. 'White' type diseases have not been reported in *Turbinaria* spp. in other areas, although Willis et al. (2004) have observed black band disease in *Turbinaria* spp. on the Great Barrier Reef. Considering that the occurrence of disease in *Turbinaria* is apparently unique to subtropical eastern Australia it is likely that SWS in this genus has a unique aetiology, distinct from the aetiologies of previously reported 'white' diseases.

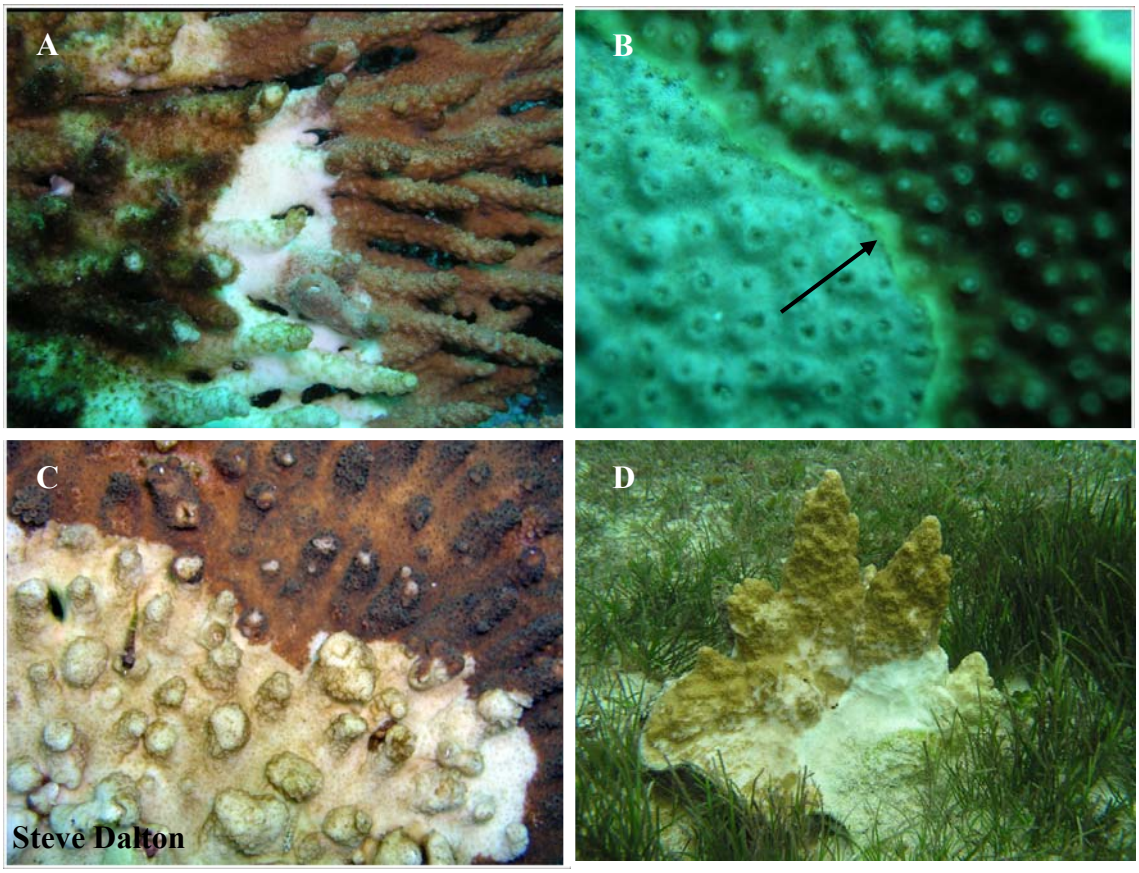

Figure 2.1. Examples of coral colonies affected by SWS, demonstrating the demarcation between live tissue (dark coloured) and recently exposed skeleton (white), with the colonisation of older dead skeleton by algae. **A**. *Acropora* sp., North Solitary Island. **B**. *Turbinaria mesenterina*, Split Solitary Island, showing signs of recovery along the disease margin (arrow). **C**. *Acropora glauca*, South West Solitary Island. **D**. *Acropora palifera*, North Bay, Lord Howe Island lagoon.

There has previously been no investigation of the causes of SWS of *T. mesenterina*. Basic questions that need be addressed are; (1) Is the disease caused by a biotic infectious agent (pathogen), abiotic stressors in the environment, or a combination of these factors? (2) If the disease is caused by an infectious microbial agent, is this agent bacterial, fungal, protozoan or viral in nature? (3) If a pathogen is involved, is it an organism normally found in association with healthy corals, or is it a novel pathogen that has been introduced from elsewhere?

Field observations of the distribution of SWS in the SIMP suggest that it is most likely caused by an infectious microbial agent (Dalton 2003, Dalton & Smith 2005). The disease causes the formation of distinct lesions on affected colonies. These lesions usually originate at one point and expand across the colony at a rate of >1 mm per day (Dalton & Smith 2005). The disease does not cause death of the entire colony at once. If the disease was a result of a simple response by the coral host to an abiotic environmental stressor, and did not involve the coral-associated microbial community, it would be expected that the effects of the disease would be displayed uniformly across the whole colonies, and across all colonies of the same species located in the same area. This is not the case however. Dalton and Smith (2005) reported that only up to 27.2% of *T. mesenterina* colonies at South West Solitary Island were affected by SWS between 2002 and 2004.

Currently all of the infectious diseases of scleractinian corals for which the aetiologies are understood have bacterial pathogens (Ritchie & Smith 1995a, Smith et al. 1996, Kushmaro et al. 1997, Patterson et al. 2002, Ben-Haim et al. 2003b, Denner et al. 2003, Cervino et al. 2004, Richardson 2004, Thompson et al. 2006). Although SWS presents symptoms comparable to other bacterial diseases of scleractinian corals, close investigation is necessary before it can be classified as a bacterial disease. Conclusive evidence that any disease is caused by a particular pathogen or pathogens can only be provided by the fulfilment of Koch's postulates, however, this may not always be possible due to difficulties associated with isolating the putative pathogen in culture, or with maintaining live host organisms in the laboratory for manipulative experiments.

A number of alternative approaches have been used by other researchers to identify microbes associated with diseases of marine organisms as potential pathogens. Kushmaro et al. (1996) observed aggregates of bacterial cells at the boundary between bleached and unbleached tissues in *Oculina patagonica* using scanning electron micrographs of diseased coral samples. They also demonstrated that bleaching of *O. patagonica* could be blocked with antibiotics, providing further evidence that the disease was caused by a bacterial pathogen. Similarly, Boettcher et al. (1999) interpreted the delayed onset of juvenile oyster disease in oysters treated with antibiotics as evidence that this disease has a bacterial aetiology. Microscopy evidence is also important in providing basic descriptions of new pathologies and in the identification of potential pathogens if Koch's postulates have not been fulfilled. Rützler et al. (1983) used microscopic examinations of the microbial mat associated with black band disease to propose that a novel cyanobacterium was involved in its aetiology.

The data presented here comprise the first detailed description of the microscopic characteristics of SWS of *T. mesenterina* in the SIMP. These results also provide strong evidence which demonstrates that the disease is caused by a biotic infectious agent (or agents), which is transmissible by direct contact between corals, and that this infectious agent is mostly likely a bacterium.

2.2 Materials and Methods

2.2.1 Direct observations of disease affected corals

SWS affected corals were observed and photographed *in situ* by scuba diving at depths of 10-20 m at locations within the SIMP including Split Solitary Island, South West Solitary Island and North Solitary Island. Fragments of healthy and SWS affected *T. meseneterina* colonies were also collected for microscopic investigation. Fragments were removed using a hammer and chisel and carried to the surface in clean plastic Ziploc bags. Upon surfacing these bags were immediately placed in 60L containers of seawater for transport back to the laboratory. The coral samples collected were examined under a stereo dissecting microscope to search for signs of microbial growth associated with the disease lesions such as mats or fungal hyphae. Scrapings of the coral surfaces on each side of the margin of disease lesions were further examined using compound light microscopy.

2.2.2 Transmissibility of the disease

In order to establish whether or not SWS is caused by an infectious biotic agent, a transmission experiment was conducted to investigate the ability of the disease to be transmitted from one coral fragment to another. This experiment will be reported in Diseases of Aquatic Organisms (Dalton et al. submitted, 2008). Healthy and SWS affected *T. mesenterina* fragments were collected from Split Solitary Island and returned to the National Marine Science Centre (NMSC) in Coffs Harbour, where they were fragmented into ca. 25 cm^2 pieces. These fragments were then placed in aerated outdoor aquaria shaded with 70 % shade cloth and supplied with constant flow-through seawater. The fragments were monitored for one week. At the end of

this period fragments from healthy colonies were only used if they displayed signs of tissue recovery along broken edges and showed no sign of loss of tissue or colour.

The fragments were placed in 12 separate 7 L aquaria, each containing 6 L of filtered and aerated seawater maintained at 21 ºC. The coral fragments were arranged such that each aquarium contained two healthy coral fragments situated approximately 5 cm apart. In six of these aquaria a third coral fragment, taken from a SWS affected colony, was placed on top of one of the two healthy fragments and oriented so that the margin of the disease lesion was in direct contact with the tissue of the healthy fragment. The remaining six aquaria served as controls in which a third healthy coral fragment was placed in contact with a healthy fragment. The coral fragments were maintained at 21 ºC and monitored for signs of disease such as tissue loss or bleaching. The water in aquaria was replaced daily with filtered natural seawater that had first been equilibrated to 21 °C. Samples of necrotic tissue that sloughed off the skeleton of diseased fragments were examined by light microscopy in order to observe any changes that occurred in the microbial community associated with the coral tissue during the disease process.

2.2.3 Inoculation of healthy corals with cultured bacteria

To investigate the potential of the most abundant culturable bacteria associated with the infected corals to cause disease in healthy corals, an attempt was made to fulfil Koch's postulates. Samples of the mucus and necrotic tissue of one of the coral fragments infected in the disease transmission experiment were taken using a sterile syringe and needle and spread on SLB agar plates as described in section 3.2.1. The plates were incubated for 16 hours at 30 ºC, then examined for the formation of

bacterial colonies. The two most abundant colony types were designated the names T1 and T2. These strains were isolated and subcultured on SLB agar. Each of these bacteria was then grown to stationary phase in liquid culture (see section 3.2.2). The bacterial cells were washed by centrifuging 5 ml aliquots of the liquid cultures, then resuspending the cells in 5 ml filtered and autoclaved seawater (FSW). This washing step was performed twice for each strain. The concentration of bacterial cells in the inoculum was determined by spreading serial dilutions on SLB agar plates and enumerating the colonies that formed after 16 hours incubation at 30 ºC.

Healthy *T. mesenterina* fragments (collected and maintained at 21 ºC in separate 7L aerated aquaria in the same manner as described in section 2.2.2) were inoculated with approximately 10^6 bacterial cells by lowering the water level until the coral surface was exposed, then gently pipetting 1 ml of bacterial suspension directly onto the coral surface. Six fragments were inoculated with strain T1, and six were inoculated with strain T2. As a procedural control another six fragments were inoculated with sterile FSW. After sixty seconds the aquaria were then refilled and the fragments were monitored for signs of tissue loss.

2.2.4 Effects of antibacterials on disease progression

To verify that coral-associated bacteria play a role in the progression of disease lesions on *T. mesenterina*, diseased coral fragments were exposed to antibiotics in controlled aquarium experiments. Sections of disease affected coral colonies were collected from South West Solitary Island (SWSI, 30º 10’ S, 153º 14’ E, approximately 10 km NNE of SSI) using a hammer and chisel as described above (section 2.2.2). These sections were divided into fragments of approximately 15 - 30

cm^2. The position of the disease margin (M) at the interface between apparently healthy coral tissue (H) and dead coral skeleton (D) was marked by drilling a small hole (3 mm diameter). To ensure that only fragments with actively advancing disease lesions were used in the experiment, the fragments were placed in aquaria containing natural seawater maintained at 26 ºC for 48 hours prior to the addition of antibiotics. (The fragments were maintained at 26 ºC because earlier experiments by Dalton et al. (submitted, 2008) had demonstrated that the disease progresses faster at this temperature. 26 °C was also the maximum seawater temperature recorded during sampling dives when high incidences of diseased corals were observed) During this period the fragments were monitored for disease progression. Fragments on which the disease margin did not progress at least 2 mm in 48 hours were not used for the experiment.

Twenty four fragments with actively progressing disease lesions were placed in separate 2.5 L plastic containers, each containing 2.0 L of filtered natural seawater at 26ºC. The temperature experienced by the coral fragments was maintained at 25 – 26ºC throughout the experiment and each container was separately aerated. The coral fragments were lit by four 40W 10,000K and four 40W actinic fluorescent globes for 12 hours per day.

Twelve of the 24 replicate coral fragments were randomly selected for treatment with antibiotics. Antibiotic stock solutions consisted of 100 mg ml^{-1} ampicillin in distilled water and 10 mg ml^{-1} tetracycline hydrochloride in 70% ethanol. The antibiotics were added to the water to give final concentrations of 25 ug ml^{-1} ampicillin and 10 ug ml^{-1} tetracycline. The remaining 12 fragments served as controls and were maintained in

filtered seawater without any additions. Every one to two days, the coral fragments were removed from the containers and the progression of the disease lesion was determined by measuring the distance from the reference hole to the disease margin. The fragments were then transferred to clean containers containing fresh filtered seawater at 26 ºC, and fresh antibiotics were added to the treated fragments. The experiment was continued in this manner for 12 days.

2.2.4.1 Statistical analysis

Prior to statistical analysis, disease progression measurements were converted to overall rates of progression in mm day^{-1} by dividing the total distance the lesion had progressed by the time since the addition of antibacterials. Data were tested for homogeneity of variance and normality using Levene's test. The data was cube root transformed to correct for non-normal distribution. Separate two-sample T-tests were used to test for differences between rates of disease progression between the treatment and control replicates at each time point. All analyses were performed using MINITAB version 13.1.

2.2.4.2 Enumeration of antibacterial resistant bacteria

Samples of coral fragments maintained in the presence and absence of antibacterials were taken to examine the effect of the antibacterials on the composition of the associated bacterial communities. Two treated replicates and two control replicates were each ground into a smooth paste with sterile mortars and pestles. One gram of this paste was suspended in 9 ml of sterile FSW. 100 µl serial dilutions of this suspension (up to 10^{-4} in FSW) were spread on four sets of SLB agar plates which contained; (1) 100 µg/ml ampicillin, (2) 25 µg/ml tetracycline, (3) both 100 µg/ml

ampicillin and 25 μg/ml tetracycline, and (4) no antibacterials. These plates were incubated for 16 hours at 30 ºC, then the numbers of viable culturable bacteria per gram of wet coral sample were determined by enumeration of the colony forming units (cfu) on each plate.

2.3 Results

2.3.1 Macroscopic and microscopic characteristics of SWS

Coral colonies affected by SWS typically exhibit common signs of a progressive loss of tissue which originates from one point and spreads gradually across the colony. As the tissue is destroyed, the white aragonite skeleton underneath is exposed. Then, as the disease lesion progresses across the colony, the denuded skeleton is colonised by algae. Typical cases of disease are therefore characterised by a clean white band of recently exposed skeleton, with a clear boundary between the living tissue on one side and an area with varying degrees of colonisation by algae and other encrusting organisms on the other side (Figure 2.1). *Turbinaria* spp. colonies were sometimes observed that showed signs of recovery along the disease margin. This was displayed as a thin band of yellow coloured tissue, with a distinct lip where new aragonite skeleton was being laid down over the old skeletal material exposed by disease (Figure 2.1B).

Direct microscopic examination of the surface of fragments of disease affected corals revealed no observable evidence of microbial mats, fungal hyphae or significant numbers of protists. There was a clear demarcation between the live, pigmented tissue and the exposed skeleton (Figure 2.2). Small fragments of remnant tissue and zooxanthellae could be observed in dead areas very close to the margin of the disease

lesion, but skeleton more than 5mm away from the margin was completely devoid of tissue (Figure 2.2). The surface of the exposed skeleton appeared intact, with no evidence of mechanical damage. The coral surface on the other side of the disease margin appeared normal. It was darkly pigmented and visually indistinguishable from tissue of completely healthy colonies unaffected by disease. There was no band of bleached tissue between the apparently healthy tissue and the exposed skeleton. Scrapings taken from the apparently healthy tissue close to the disease margin contained large numbers of zooxanthellae which appeared intact and identical to zooxanthellae from unaffected coral fragments (Figure 2.2b). In areas where the tissue appeared normal, corallites contained live polyps, which were responsive to tactile stimulus with a probe.

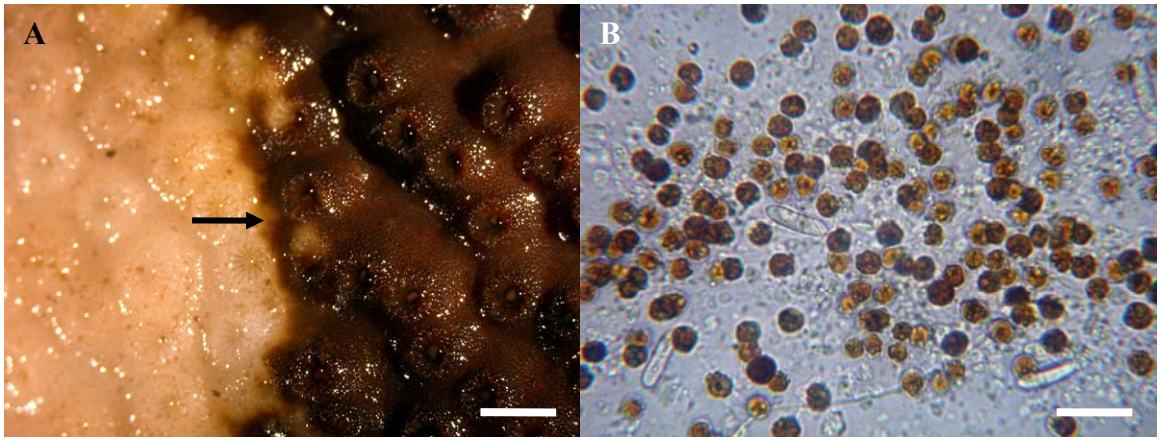

Figure 2.2 A. Surface of a SWS affected fragment of *Turbinaria mesenterina* collected from the field (bar is 5 mm). Live tissue (dark coloured) was normal in appearance. The disease margin is indicated by the arrow. **B**. Zooxanthellae from a scraping of apparently healthy tissue of a SWS affected *T. mesenterina* fragment (bar is 25 μm)

2.3.2 Transmissibility of the disease

Transmission of disease from SWS affected to healthy *T. mesenterina* fragments was observed only between fragments in direct contact. Healthy coral fragments that were not in direct contact with diseased fragments (but still in the same aquarium as a diseased fragment), and healthy fragments in contact with other healthy fragments showed no signs of tissue loss or stress. However, partial bleaching due to shading was observed in all of the healthy control fragments in the region covered by another healthy fragment.

Within two days of the start of the experiment two of the six fragments placed in direct contact with SWS affected coral displayed symptoms of tissue necrosis. These symptoms were typical of those seen in SWS affected corals in the field, with an expanding area of exposed coral skeleton radiating outward from the initial point of infection (Figure 2.3). Within six days, four of the six fragments (66%) in direct contact with SWS affected coral displayed signs of tissue loss. By the tenth day tissue loss had stopped expanding in two of the infected fragments, however the other two fragments appeared to develop a secondary infection by a microorganism that formed white, filamentous growths on the coral surface. These growths originated on the exposed skeleton and rapidly expanded over the surviving tissue, completely covering the fragments, resulting in death of 100% of the coral tissue (Figure 2.4C).

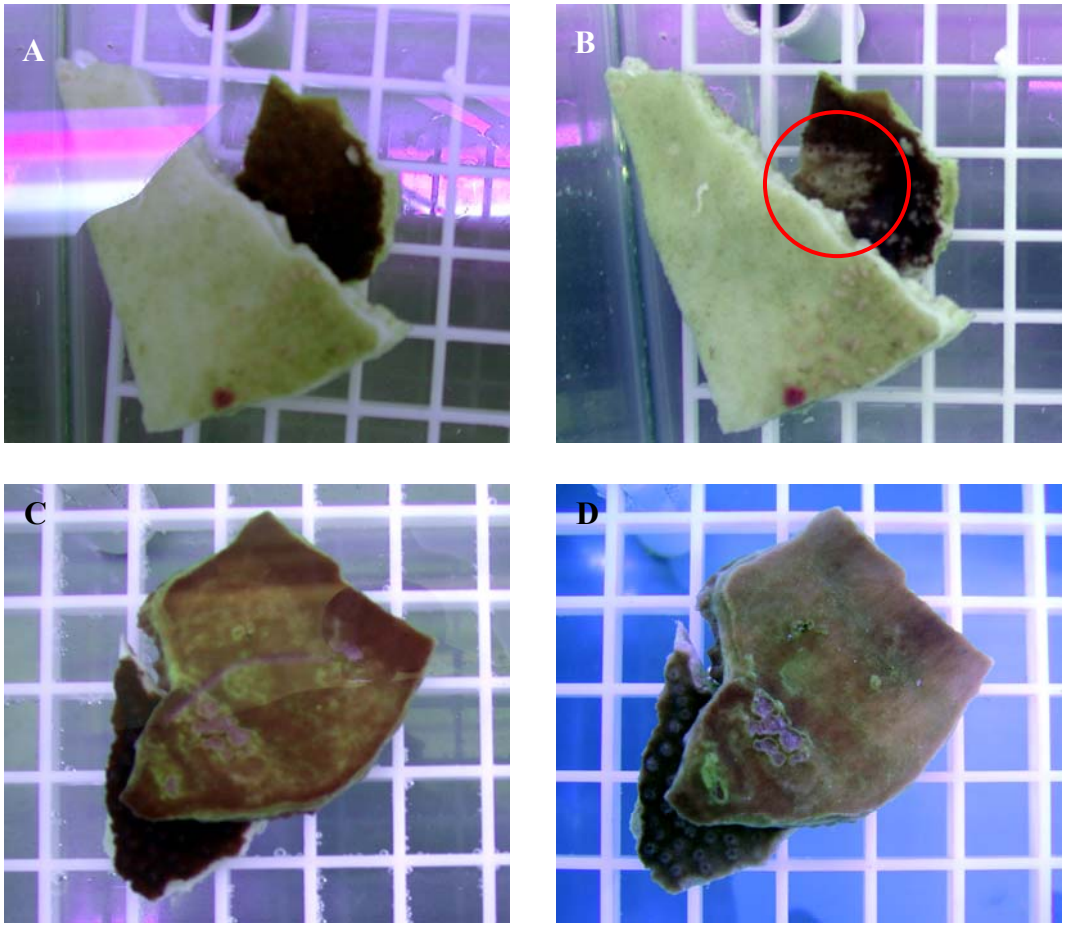

Figure 2.3 A. A healthy *T. mesenterina* fragment in direct contact with a SWS affected fragment (inverted) on the first day of the aquarium transmission experiment. **B**. The same fragment two days later. The circled area indicates exposed coral skeleton where tissue loss has occurred. **C**. A healthy *T. mesenterina* fragment in direct contact with a healthy fragment (inverted). **D.** The same fragments 13 days later. Slight bleaching (loss of colour) of the polyps is visible in the bottom fragment, but the tissue is otherwise healthy and intact.

Microscopic assessment of the infected coral fragments indicated a distinct succession of microbial colonisation. Scrapings taken from the margin of the disease lesion immediately following initial tissue loss revealed large numbers of motile rod and spiral-shaped bacteria (Figure 2.4F,H). During subsequent days the tissue of the infected fragments sloughed off the skeleton in sheets. Samples of this necrotic tissue contained large numbers of ciliates which were observed engulfing free zooxanthellae, bacteria, and small particles of coral tissue (Figure 2.4G). The two infected fragments that appeared to develop a secondary infection were covered in a

mat of white filaments which produced the odour of hydrogen sulphide. Microscopic examination of this growth revealed filaments up to 2 μm in diameter which exhibited gliding motility and contained reflective sulphur granules (Figure 2.4D,E). This morphology is consistent with bacteria of the genus *Beggiatoa*, which have been previously reported in association with dying corals (Richardson 2004). These mats harboured a diverse community of other microorganisms, including large numbers of motile bacteria, ciliates, flagellates and other protozoans (Figure 2.4F).

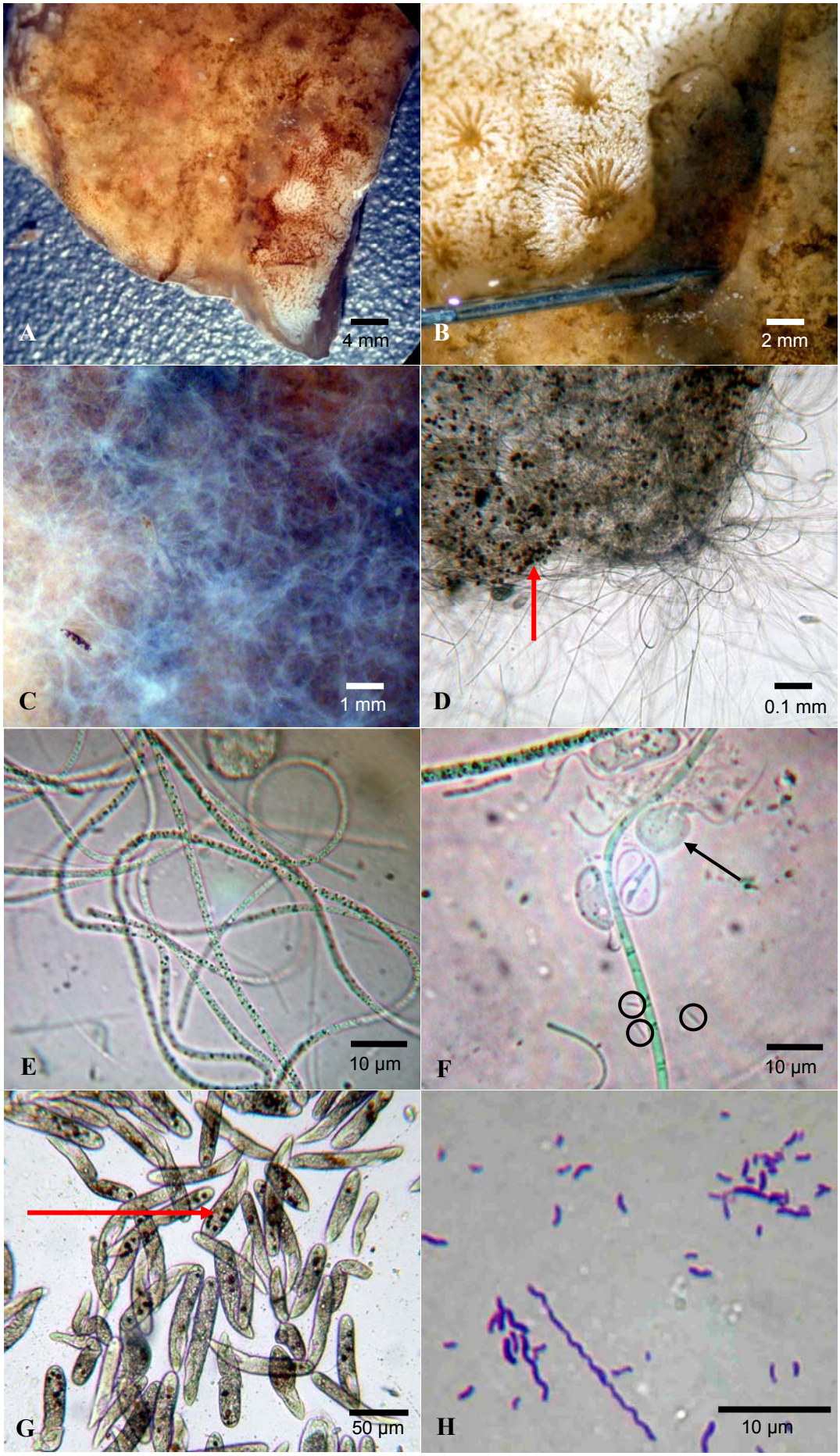
A
4 mm
B
2 mm
C
1 mm
D
0.1 mm
E
10 µm
F
10 µm
G
50 µm
H
10 µm

Figure 2.4 Diseased tissues and associated micro-organisms of *T. mesenterina* fragments infected by direct contact with SWS affected fragments in aquaria. **A**. The surface of a diseased coral fragment approximately 24 hours post infection, showing necrotic tissue. **B.** Sloughing of necrotic tissue away from the underlying coral skeleton. **C**. Surface of a dead coral fragment covered in a mat of white filamentous bacteria approximately 36 hours post infection. **D**. A fragment of necrotic tissue, showing bacterial filaments and zooxanthellae remaining in coral tissue (arrow). **E**. Bacterial filaments at 1000x magnification, showing the distinctive reflective sulphur granules characteristic of the genus *Beggiatoa*. **F**. Flagellates (arrow) and motile rod shaped bacteria (circled) in a scraping taken from necrotic tissue. **G**. Ciliates in a sample of mucus taken from a diseased coral fragment. The arrow indicated zooxanthellae visible inside the cells. **H**. Crystal violet stain of bacterial cells from a scraping of necrotic coral tissue.

2.3.3 Re-infection trials using bacteria isolated from diseased coral

The coral fragments inoculated with bacterial isolates T1 and T2 failed to develop any of the characteristic signs of disease, such as loss of colour or tissue death. An observable increase in mucus production occurred during the first two to three hours post inoculation. However, it is likely that this was a response to exposure to air during inoculation, as a similar increase in mucus production was also observed in the control fragments inoculated with sterile FSW. The fragments were monitored for ten days before the experiment was terminated when both the treatments and controls began to display signs of stress.

Similar experiments were also conducted using bacterial strains isolated from diseased corals in the field. Various experiments were conducted in which bacteria were inoculated onto coral fragments in aquaria as washed cells, unwashed culture (using the bacterial growth medium as a control inoculum) axenic cultures and mixed inocula containing up to ten bacterial strains. None of these experiments resulted in the development of significantly more stress or disease symptoms in the inoculated coral fragments than in the controls.

2.3.4 Effect of antibacterials on disease progression

Of the 24 coral fragments initially used in the experiment, 8 developed characteristics atypical of SWS or died suddenly without a gradual progression of the disease lesion. These fragments were discarded from the analysis, leaving a total of 7 fragments treated with antibacterials and 9 control fragments. Initially, the addition of antibacterials to disease affected *Turbinaria mesenterina* fragments resulted in a reduction of the mean rate of disease progression from 1.3 mm day^{-1} to 0.7 mm day^{-1} within 48 hours of the commencement of the experiment (Figure 2.5). This reduction in rate of spread continued for the first seven days following the addition of antibacterials. In contrast, the disease lesions on the untreated control fragments progressed at an accelerating mean rate for the first three days of the experiment and remained high before slowing to a rate similar to that observed in the treated fragments (Figure 2.5). By the end of the experiment, disease progression had halted in 57% of the fragments treated with antibiotics, and 44% of the control fragments. Two sample T-tests performed on the data at each time interval indicated that the effect of the antibacterials was most significant between 2 and 7 days after the beginning of the experiment. The strength of the effect lessened after 7 days (Table 2.1).

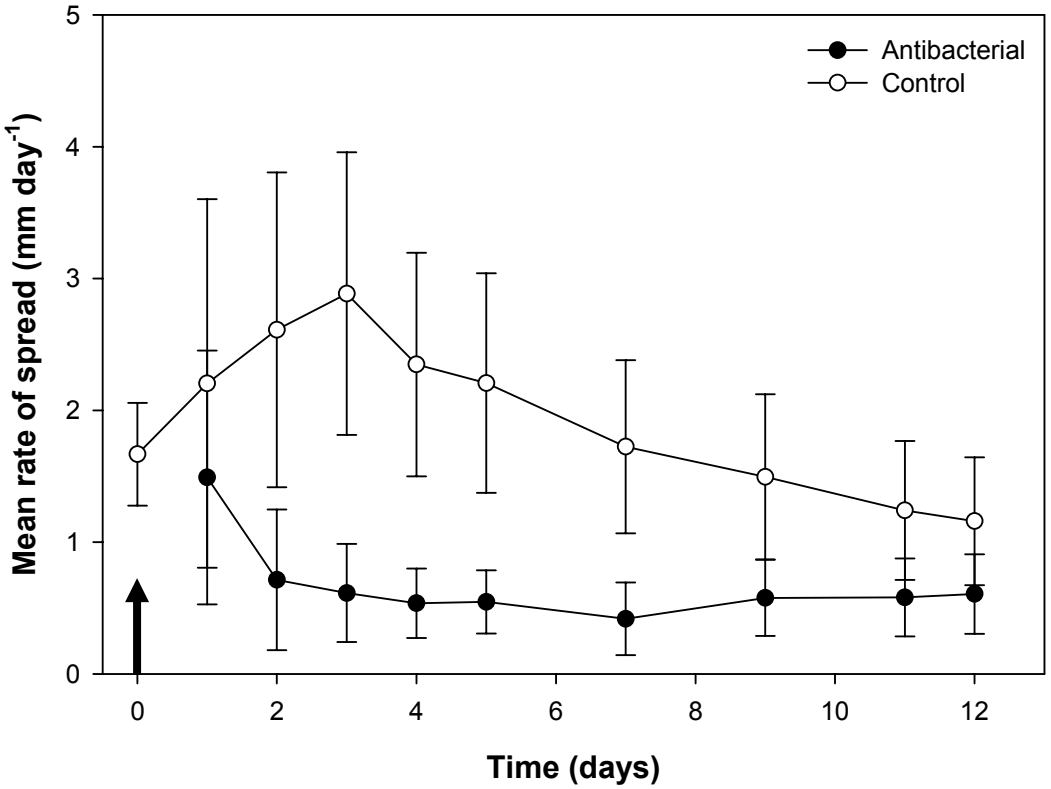

Figure 2.5 Mean rate of spread of disease lesions (± standard errors) on *T. mesenterina* fragments maintained at 26 ºC in the presence and absence of antibacterials. Both control and treatment fragments were maintained for two days before the addition of antibacterials (indicated by arrow).

Table 2.1. Two-sample T-tests for rate of spread of disease lesions in the presence and absence of antibacterials. (DF – Degrees of freedom)

Time (days)	DF	T	p
1	14	-0.43	0.675
2	14	-1.59	0.135
3	14	-1.61	0.131
4	14	-1.27	0.226
5	14	-1.18	0.258
7	14	-0.78	0.450
9	14	-0.56	0.581
11	14	-0.26	0.796
12	14	-0.16	0.873

2.3.5 Effect of antibacterials on coral associated bacterial communities

Exposure of diseased *T. mesenterina* fragments to antibacterials resulted in increased numbers of antibacterial resistant organisms in the coral-associated community. Corals maintained in the presence of ampicillin and tetracycline contained 107.5 times more culturable bacteria that were resistant to ampicillin, and 4.5 times more culturable bacteria that were resistant to tetracycline in comparison to untreated controls. Corals treated with antibacterials also contained 16.9 times more culturable bacteria that were resistant to both ampicillin and tetracycline than controls (Figure 2.6).

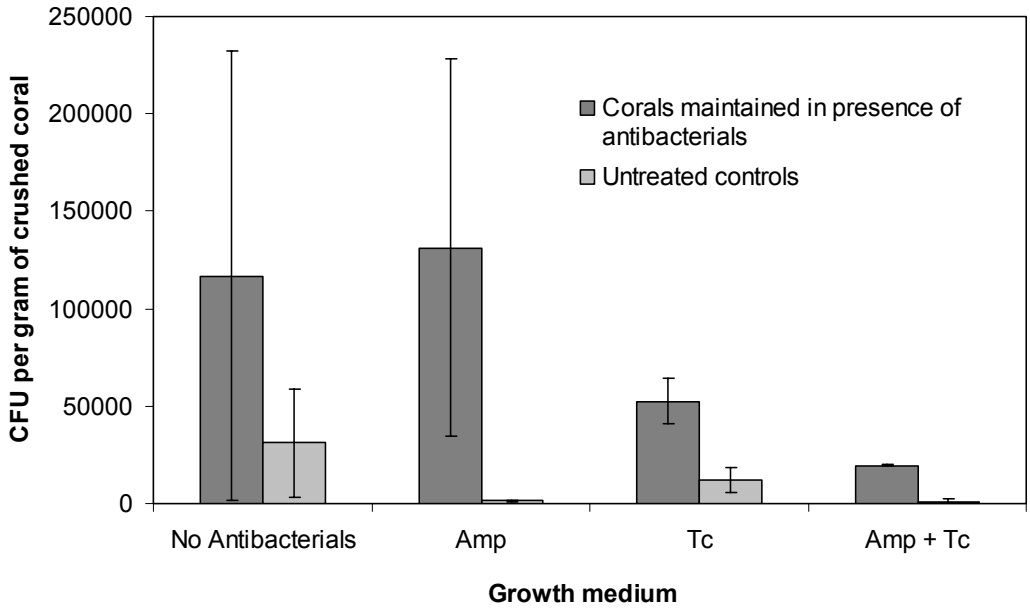

Figure 2.6 Mean numbers (± standard error) of antibacterial resistant viable bacteria recovered from coral fragments maintained in the presence and absence of antibacterials. A. Bacteria grown on SLB agar containing 100 mg/ml ampicillin. T. Bacteria grown on SLB agar containing 25 mg/ml tetracycline. A+T. Bacteria grown on SLB containing both 100 mg/ml ampicillin and 25 mg/ml tetracycline.

2.4 Discussion

The results of these experiments demonstrate that SWS of *T. mesenterina* is an infectious disease, and provide substantial evidence that it is caused by a bacterial pathogen. As a coral disease that causes progressive loss of tissue, SWS exhibits superficial characteristics common to many coral diseases that have been described worldwide (see Bythell et al. 2004 for review). The typical band of clean white skeleton, which is exposed as the margin of the disease lesion progresses across the colony, is similar in appearance to the patterns of spreading mortality observed in other 'white' coral diseases, such as white band disease and white plague (Bythell et al. 2004). At the present time all the diseases of scleractinian corals that cause progressively expanding lesions of tissue lysis that have known pathogens are caused by bacteria (Ritchie & Smith 1995a, Smith et al. 1996, Kushmaro et al. 1997, Patterson et al. 2002, Ben-Haim et al. 2003b, Denner et al. 2003, Cervino et al. 2004, Richardson 2004, Gil-Agudelo et al. 2006b, Thompson et al. 2006). Evidence from preliminary microscopic investigations of SWS is also consistent with a bacterial, rather than fungal or protozoan, aetiology, although the potential involvement of viral pathogens can not be excluded purely on the basis of this evidence.

The results of the disease transmission experiment clearly indicate that SWS is caused by an infectious biotic agent which is transmissible by direct contact between colonies, but not through the water column. This pattern of infectivity has also been verified in the field. Dalton et al. (submitted, 2008) showed that SWS was transmitted to healthy *T. mesenterina* colonies that had been placed in direct contact with fragments that included portions of the disease margin taken from SWS affected colonies. The currently wide distribution of SWS in the SIMP (Dalton & Smith 2005),

and the fact that SWS can be transmitted only by direct contact and not through the water column, implies that a vector must be involved in the transfer of the pathogen between coral colonies. Sussman et al. (2003) demonstrated that the marine fireworm *Hermodice carunculata* is the vector responsible for transfer of the pathogenic bacterium *Vibrio shiloi* between *Oculina patagonica* colonies in the Mediterranean. Similarly, Nugues et al. (2004) have proposed that the macroalga *Halimeda optunita* acts as a reservoir and vector of *Aurantimonas coralicida*, the causative agent of white plague type II. A candidate vector for the causative agent of SWS has been identified. Dalton and Godwin (2006) noted that healthy *T. mesenterina* colonies that came into contact with a corallivorous nudibranch (*Phestilla* sp.) developed the characteristic symptoms of SWS. Only two specimens of this nudibranch have been found, each at different times, so further experiments to investigate its possible role as a vector of bacteria associated with SWS have not yet been conducted.

The results of the antibiotic trial provide further evidence that SWS in the SIMP is caused by a bacterial pathogen or pathogens. Although the effect of antibacterials was not highly significant, it was certainly notable, and the reduction in the rate-of-spread indicates that bacteria are most likely responsible for the tissue lysis. The variability in the effect of exposure to antibacterials was too high to conclusively say whether a large change in the overall numbers of viable culturable bacteria associated with coral fragments occurred. It did cause a measurable shift in the composition of the bacterial communities however. This shift was as expected, with antibacterial resistant strains becoming more numerous in corals maintained in the presence of antibacterials. Large numbers of viable bacteria were able to survive the extended exposure to the antibiotics, so it is possible that at the antibiotic concentrations used (25 $\mu g\ ml^{-1}$

ampicillin and 25 μg ml^{-1} tetracycline), the pathogen/s were partially inhibited, but not eliminated. It is possible that a stronger effect would be observed at higher antibiotic concentrations. The inhibitory effects of the antibacterials on any pathogenic bacteria may have also been mitigated by their effects on any probiotic bacteria that form part of the coral's natural defence against infection (Reshef et al. 2006). It is also possible that exposure to antibacterials affected the coral hosts directly, stimulating an immune response or otherwise altering their resistance to disease progression.

Secondary colonisation of dead coral surfaces by microbial mats dominated by gliding filamentous bacteria appeared to enhance the rate of spread of coral tissue mortality, as once these mats were established, tissue death progressed very rapidly. These bacteria appear morphologically to be members of the genus *Beggiatoa*, although this is not a certain assumption as no other evidence is available to identify them. *Beggiatoa* spp. are sulphide oxidising bacteria normally associated with gradient environments in which sulphide is supplied from below while oxygen is supplied from above (Schmidt et al. 1987). The structure of the mat formed by the *Beggiatoa* filaments aids the growth of the bacterium by trapping sulphide on its underside and excluding oxygen (Moller et al. 1985). These conditions are hostile to coral tissues (Richardson et al. 1997). It is not likely that *Beggiatoa* dominated mats are the primary biotic cause of SWS, as they have not been observed on SWS affected corals in the field, and they only developed on two out of four coral fragments that were infected in the disease transmission experiment. It is possible however, that the nutrient enriched conditions created by the initial death of coral tissue due to infection by a different pathogen allowed the development of a microbial community in which

sulphur reducing bacteria produced sufficient amounts of sulphide to promote the growth of *Beggiatoa*, which forms the structural basis of a complex microbial mat. Once established the mat could in turn, because of the chemical microenvironment that it creates, lead to further tissue death as it expanded into areas of previously healthy coral tissue. This mechanism of coral tissue destruction is similar to the one proposed by Richardson (2004) for black band disease. In this case however, the structural basis of the mat is formed by *Beggiatoa* filaments, rather than a filamentous cyanobacterium as proposed by Richardson (2004).

There were differences between the characteristics of specimens of SWS affected *T. mesenterina* collected from the field and fragments of the same species infected in the laboratory. The sloughing of sheets of necrotic tissue and the presence of *Beggiatoa* dominated microbial mats have not been observed in the field. This may be explained by the lack of sufficiently vigorous water movement in the aquaria used in the infection experiments. Although the aquaria were aerated, the water movement provided was relatively gentle in comparison to the constant effects of currents, surge and wave action experienced in the field. The SIMP is frequently exposed to heavy wave action, and this is one of the primary factors preventing the accretion of true coral reefs in the area (Harriot et al. 1994, Harriot et al. 1999). It was noted that sloughing tissue and microbial mats were easily dislodged from the surface of corals in aquaria when they were picked up to be examined. It is likely that that the clean appearance of the disease margins observed in the field results from the continual removal of sloughing tissue by surge and wave action. The same water movement would most likely prevent the formation of loosely attached microbial mats like the *Beggiatoa* mats observed in the aquarium experiments.

It is possible that the water movement observed in the field benefits corals by preventing infection. There is ample anecdotal evidence that water movement is important for the health of corals maintained in aquaria (Borneman 2001). It has also been shown by Nakamura & van Woesik (2001) that exposure to high water flow enhanced the survival of corals on Japanese reefs during the 1998 coral bleaching event. Nakamura and van Woesik (2001) proposed that water movement enhances coral survival by flushing away toxins which may otherwise build up to lethal concentrations in the boundary layers close to coral surfaces. It is possible that water movement also benefits corals by preventing the build-up of mucus and associated bacteria on the coral surface. *Porites* spp. corals have been observed shedding layers of mucus with large numbers of associated bacteria (Forest Rohwer, personal communication). Water movement may also wash away pathogens before they can become established. The first two necessary steps in the infection of the coral *Oculina patagonica* by the pathogenic bacterium *Vibrio shiloi* are the chemotactic attraction of the motile bacteria to the coral mucus (Banin et al. 2001a), followed by adhesion of the bacteria to a receptor, which is also located in the mucus (Toren et al. 1998). As chemotactic movement of bacterial cells is only effective over short distances (Macnab & Koshland 1972), it is conceivable that vigorous water movement helps to prevent pathogens from moving from the water column to the coral surface.

Other abiotic factors related to the maintenance of corals in aquaria may have also contributed to the differences observed between SWS in the field and in the laboratory. Transfer of healthy corals from the field to aquaria causes stress which is manifested in a number of ways. *T. mesenterina* fragments placed in aquaria

illuminated with artificial fluorescent lighting invariably undergo changes in colour over a period of several weeks, usually becoming darker overall and losing green and fluorescent pigments. This change is presumably due to changes in pigment expression by the resident zooxanthellae or even a shift in the composition of the suite of algal symbionts associated with the coral as it adjusts to the new light regime (Coles & Brown 2003, Buddemeier et al. 2004). Excessive production of mucus is a generally recognised sign of coral stress (Brown & Bythell 2005) and is often observed when *T. mesenterina* fragments are first transferred to aquaria. It is expected that stresses that result in changes in the host physiology will also affect the composition of the associated bacterial community. A number of studies have shown that coral associated bacterial communities can change as a function of external factors such as depth, incident light, water quality and coral health (Ritchie & Smith 1995a, Ritchie & Smith 1995b, Klaus et al. 2004, Klaus et al. 2005, Koren & Rosenberg 2006, Koren & Rosenberg 2007). It has also been recently demonstrated that coral associated bacterial communities undergo significant changes when corals are moved from the field to aquaria (Kooperman et al. 2007). Changes in the dynamics of the interactions between the coral host, pathogenic bacteria and other coral associated bacteria due to the stresses of the aquarium environment may explain some of the differences between the appearances of SWS in the field and in aquaria.

Chapter 3 – The culturable bacterial diversity associated with *Turbinaria mesenterina*

3.1 Introduction

Until recently, traditional approaches to identifying the causative agents of disease, such as microscopy, culture of micro-organisms and fulfilment of Koch's postulates have been applied to the study of various coral pathologies (Kushmaro et al. 1996, Kushmaro et al. 1997, Richardson 1997, Richardson et al. 1997, Richardson 1998, Richardson et al. 1998b, Kushmaro et al. 2001, Ben-Haim & Rosenberg 2002, Ben-Haim et al. 2003b). It is becoming increasingly clear however, that this simplified approach is limited in its ability to elucidate the aetiologies of coral diseases that may be caused by complex interactions between the coral host, environmental factors, and a number of microbial species. Corals harbour distinct bacterial communities that differ from the communities found in the surrounding water column (Ducklow & Mitchell 1979). Rohwer et al.(2002) reported that the composition of these bacterial communities are maintained amongst different colonies of the same species, even when the colonies are separated by large geographical distances. It is now recognised that bacterial communities play important roles in the health of the coral by carrying out various metabolic functions and by protecting it from infection (Rosenberg et al. 2007) (see chapter 1).

Recently, the focus of coral disease research has shifted from searching for a single pathogenic agent to examining the structure and function of the entire coral-associated bacterial community (Cooney et al. 2002, Frias-Lopez et al. 2002, Rohwer et al. 2002, Pantos et al. 2003, Frias-Lopez et al. 2004, Pantos & Bythell 2004, Bourne & Munn

2005, Gil-Agudelo et al. 2006a, Koren & Rosenberg 2006, Lampert et al. 2006, Pantos & Bythell 2006, Kooperman et al. 2007, Koren & Rosenberg 2007). Studies of this nature aim to improve the understanding of the roles of the various members of the bacterial community in the context of the coral holobiont, and may eventually lead to the ability to predict the consequences of specific changes to the community.

The broad aim of this study was to examine the culturable bacterial communities associated with the common subtropical scleractinian coral species *T. mesenterina* in order to identify bacteria that may play important roles as pathogens or as beneficial components of the coral holobiont. The study was conducted in two parts. Firstly, the culturable bacterial communities associated with healthy *T. mesenterina* colonies were compared between two different subtropical locations; the SIMP, where SWS of *T. mesenterina* affects up to 58% of colonies at some locations (Dalton & Smith 2005), and LHI, where disease was not recorded in *Turbinaria* species (Dalton & Godwin 2005). Secondly, the culturable bacterial communities associated with healthy and diseased *T. mesenterina* colonies within the SIMP were compared in order to identify bacteria with potentially important roles in the disease process.

3.2 Materials and Methods

3.2.1 Field site descriptions and sample collections

Coral samples were collected from rocky reefs at depths of 10-20 m by scuba diving at Split Solitary Island, Solitary Islands Marine Park, NSW (30º14.5' S, 153º11.0' E) and Lord Howe Island (31º30.5' S, 159º3.5' E, Figure 3.1). Diseased coral colonies were identified by visual inspection. When selecting diseased coral samples, only colonies that displayed an actively advancing disease lesion with a clean area of recently exposed white aragonite skeleton were selected for sampling (Figure 3.2). Healthy coral colonies were defined as those with no visible signs of disease lesions, bleaching (loss of colour), predation scars or sediment damage. All samples described in this study were collected in autumn between the months of March and May in 2004 and 2005.

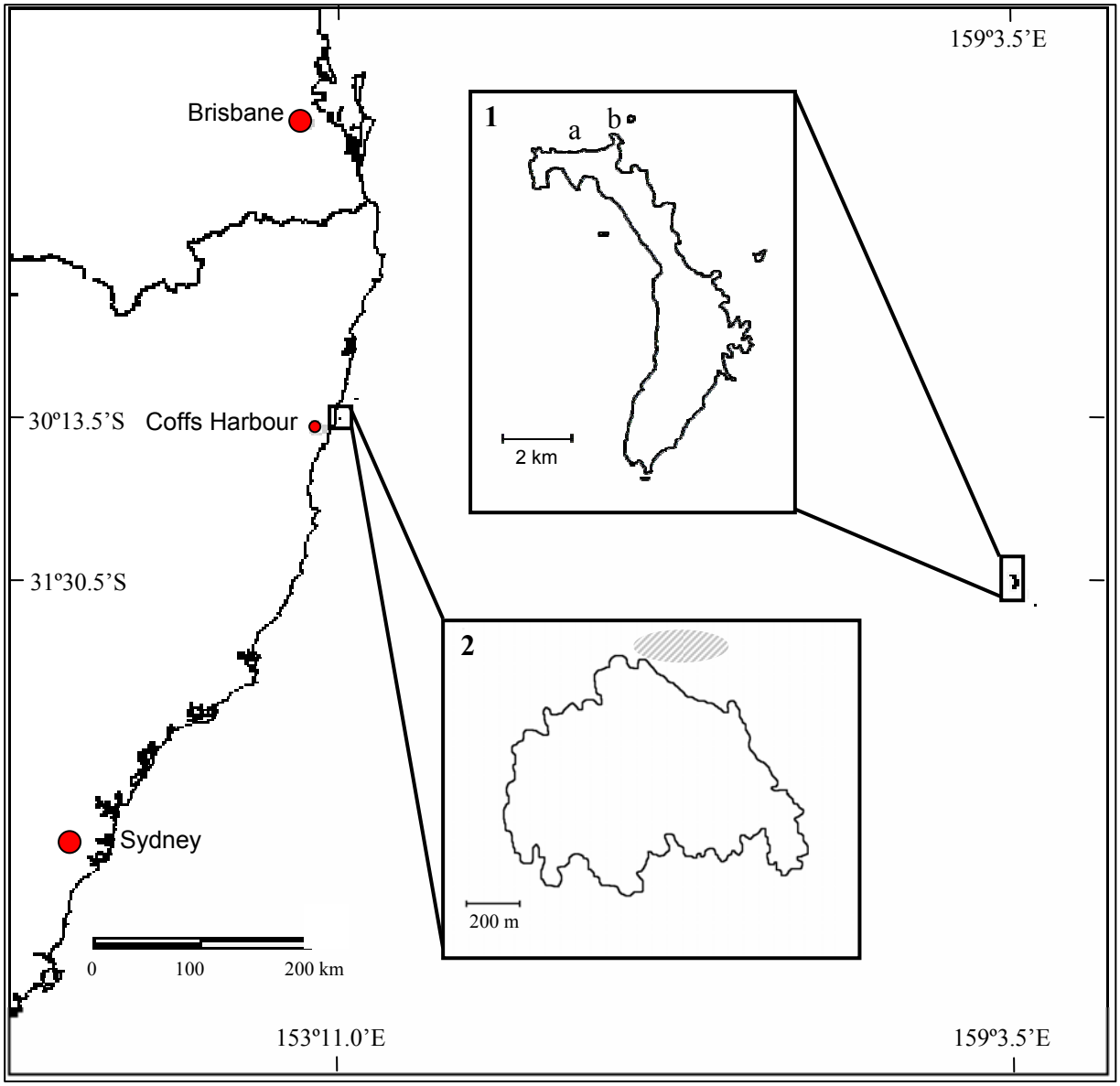

Figure 3.1. Map of subtropical sampling locations in Eastern Australia. 1. Lord Howe Island. Samples were collected from 10-20 m depth at the sites indicated: a. Malabar Shoal, b. Noddy's Island. 2. Split Solitary Island, Solitary Islands Marine Park. Samples were collected at depths of 10-15 m from reefs on the northern side of the island (shaded area).

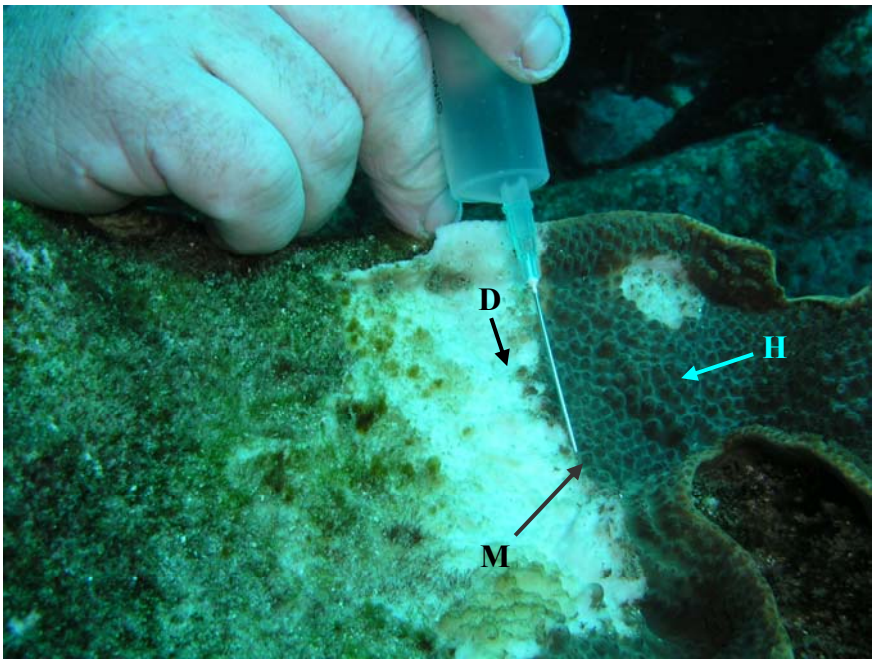

Figure 3.2. Sampling of a diseased *Turbinaria mesenterina* colony. Arrows indicate the sections of the colony where each of the disease samples were collected from. H - Apparently healthy tissue, M - Margin of disease lesion, D - Dead skeleton devoid of coral tissue. The band of clean white skeleton without algal growth indicates recent progression of the disease lesion. Control samples (C) were collected from nearby colonies with no visible signs of disease. (Photograph by Steven Smith)

3.2.1.1 Sampling methods

Samples of the coral surface mucus layer were taken by applying suction with a 20 ml syringe, with a 23 gauge needle attached, while drawing it across the coral surface (Figure 3.2). Coral fragments were also collected by removing small sections (approx 15 cm^2) from the coral colony using a hammer and chisel. Disease samples were taken from the active margin of the disease lesion (M). Samples were also taken from the apparently healthy (identified by the presence of normally pigmented tissue) (H) and dead (D) regions of disease affected colonies approximately 2 cm on either side of the disease margin (Figure 3.2). Control samples were collected from completely healthy colonies (C) nearby. These fragments were placed immediately in clean separate Ziploc bags underwater. Upon surfacing, the bags containing fragments and

the syringes were placed in a 60 L container of seawater in order to maintain a constant temperature during transport back to the laboratory.

3.2.2 Microbiological media

Nutrient rich media were chosen for the isolation of bacteria from coral samples so that the widest possible range of heterotrophic strains could be detected. Bacterial strains were isolated and maintained on Salt Luria Broth (SLB) agar (Sar & Rosenberg 1987). Liquid cultures were prepared by inoculating 5 ml Marine Yeast Tryptone (MYT) medium (0.8% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract prepared in 75% v/v natural filtered seawater) with single colonies picked from pure subcultures grown overnight on SLB agar. Cultures were shaken at 180 rpm at 30 ºC for 16 to 24 hours, (depending on the bacterial strain). Glycerol stocks of the bacterial isolates were prepared by mixing 500 µl of fresh liquid culture with 500 µl sterile glycerol and were stored at -70 ºC.

3.2.3 Isolation of bacteria from coral samples

Coral fragments were placed in a sterile mortar to which approximately 20 ml of autoclaved filtered seawater (FSW) was added. The fragments were ground into a smooth slurry with a sterile pestle. In order to break up sticky aggregates of mucus and coral tissue, this slurry was vigorously passed several times in and out of a sterile 20 ml syringe first without a needle, and then with a 21 gauge needle attached. Four serial dilutions of 10^{-1} to 10^{-4} of the resulting slurry were prepared in sterile FSW. 100 µl aliquots of each dilution were spread on SLB agar plates, which were incubated at 30 ºC for up to five days. Mucus samples were treated in the same manner, except that the crushing step was not required.

Inoculated plates were checked every twelve hours for the formation of bacterial colonies, as some colonies grew to observable size much faster than others. Estimates of the total number of colony forming units (cfu) on each plate were recorded after 48 hours incubation. Colonies were differentiated and enumerated based on their morphology (shape, size, texture and colour) and representatives of the most abundant colony types were subcultured on SLB agar plates. It is important to note that when attempting to enumerate bacteria on agar plates it was found that it was difficult to obtain accurate counts of each colony type, as many bacteria formed colonies with very similar morphologies, and colonies of the same bacteria often adopted different morphologies depending on the degree of crowding and the neighbouring species on the agar plate. For this reason, the numbers of cfu reported here for each isolate should be regarded as approximate estimates of abundance, not absolute quantitative measures.

All isolates were subcultured at least twice on agar plates to ensure that cultures were axenic. Broth cultures were prepared from each isolate and used for the preparation of glycerol stocks as described above (Section 3.2.2). Bacterial isolates were designated names in the format: 2.3.05 2MS3, where the date is the date the original sample was collected, followed by the serial number of the coral colony sampled on that date (1,2,3 etc), the section of the coral colony the sample was taken from (category H,M,D or C. see Figure 3.2), the sampling method used (S for syringe or C for crushed fragment) and finally a number indicating the order in which the strain was isolated from the original agar plates.

3.2.4 Identification of bacterial isolates by ribosomal DNA sequence analysis

Bacterial isolates were identified by 16S rDNA sequence analysis. Isolates were revived from cryopreserved glycerol stocks by streaking onto SLB agar plates and incubating overnight at 30 ºC. Liquid cultures for bacterial DNA extraction were then prepared using single colonies picked from the fresh plates. The cultures were grown as described above (Section 2.2.2). The bacterial cells were recovered by centrifugation of 250 µl aliquots of the fresh culture (12,000 rpm for 5 minutes). Genomic DNA was extracted from these cells with the Qiagen DNeasy blood and tissue kit (Qiagen-Hilden, Germany), using the protocol recommended for Gram-negative bacteria. This protocol was successful in isolating DNA from almost all bacterial isolates. The resulting genomic DNA extracts were stored at -20 ºC.

16S rDNA was amplified from the genomic DNA extracts in multiple 25 µl reactions containing 1.5 mM $MgCl_2$, 250 µM of each deoxynucleoside, approximately 5U of *Taq* polymerase, 1 µl of bacterial genomic DNA extract, and the forward primer (27F 5'gagctccagagtttgatcmtggctcag) and reverse primer (1492R 5'cacgytaccttgttacgactt) (Lane 1991) at a concentration of 400 nm each. PCR cycling parameters were 94 ºC for 3 minutes, followed by 35 cycles of 94 ºC for 30 seconds, 48 ºC for 30 seconds and 72 ºC for 2 minutes, with a final extension step of 72 ºC for 10 minutes. PCR products were cleaned up using the Qiaquick spin PCR cleanup kit (Qiagen-Hilden, Germany), eluted in 10 mM Tris/HCl (pH 8.0) and quantified on agarose gels against 1kb standard DNA ladders (New England Biolabs).

3.2.4.2 16S rDNA sequence analysis

DNA sequencing reactions consisted of 20 to 80 ng of cleaned 16S rDNA and 10 pmol of the forward primer 27F (5'gagctccagagtttgatcmtggctcag) in a final volume of 16 µl. DNA sequencing was performed by the Sydney University Prince Alfred Molecular Analysis Centre (Sydney, NSW). The nucleotide sequences obtained were examined in Chromas 1.45 (McCarthy 1996). Sequences containing unread bases ('Ns') were trimmed from the 5' and 3' ends and misread bases were corrected where possible. The sequences were then subjected to nucleotide-nucleotide BLAST searches to identify their nearest relatives in the GenBank database (http://www.ncbi.nlm.nih.gov/blast). Classification of isolates to genus level was achieved by submitting their 16S rDNA sequences to the Ribosomal Database Project (RDP) Classifier (Wang et al. 2007), which assigns a classification with at least 80% confidence based on known sequences contained in the RDP (Cole et al. 2007). Sequences were then aligned against the complete 16S rDNA sequences of *E. coli* and representative type strains belonging to the classes Gammaprotebacteria, Alphaproteobacteria and Bacteroidetes taken from the RDP (Cole et al. 2007). Alignment was performed in MEGA 4.0 (Tamura et al. 2007) using the default ClustalW algorithm (Chenna et al. 2003). The aligned sequences were then cropped to produce an alignment of the 570 bp region corresponding to positions 130-700 of the *E. coli* 16S RNA gene. This was done to ensure that only high quality completely overlapping regions of the sequences were used. This region spans all of the third hypervariable region (V3) of the bacterial 16S RNA gene, and most of the second (V2) and fourth (V4) variable regions. Phylogenetic trees were inferred in MEGA 4.0 (Tamura et al. 2007) using the neighbour-joining and minimum evolution algorithms.

All sequences were submitted to the GenBank database and will appear with the accession numbers EU267607-EU267670.

3.3 Results

The data presented here include the results of two comparative studies of the culturable bacterial communities associated with *T. mesenterina*. The first compares bacteria isolated from healthy *T. mesenterina* colonies at two subtropical locations; The Solitary Islands Marine Park (SIMP), a near-shore environment influenced by terrestrial runoff and nutrient laden currents where SWS is known to occur in *T. mesenterina*, versus Lord Howe Island (LHI), an offshore oceanic environment where SWS has not been recorded in any *Turbinaria* species. The second study compares the culturable bacterial communities associated with healthy and diseased *T. mesenterina* within the SIMP.

A total of 52 bacterial strains were isolated from healthy corals at LHI, and a total of 95 strains were isolated from all sample categories (H, M, D and C) in the SIMP. 16S rDNA sequences were obtained from 93 of these 147 isolates (37 from LHI and 56 from the SIMP). Of the 93 isolates identified by 16S rDNA sequencing, 76 (82%) belonged to the class *Gammaproteobacteria*. All of the remaining isolates belonged to the class *Alphaproteobacteria* (17%), with exception of a single isolate (2.3.053CC3), that belonged to the family *Flavobacteriaceae*, in the class *Bacteroidetes*. Many isolates formed clusters of very closely related (>99% sequence identity) or identical sequences. It is likely that closely related isolates within each of these clusters represent the same species (Stackebrandt & Goebel 1994, Stackebrandt & Rainey

1995). It is evident from these clusters that the same species were isolated multiple times from different samples, and sometimes more than once from the same sample.

Because of the difficulties associated with differentiating bacteria purely on the basis of their colony morphology on agar plates, the cfu counts per 100 μL of undiluted sample recorded for each bacterial isolate were approximate, as indicated above (Section 2.2.3). Cfu counts per plate for highly abundant bacteria were recorded as simply '>100', so in later comparative analyses these isolates were given cfu values of 100. It should be noted therefore that the actual contributions of the most abundant bacterial groups to each sample are underestimated in the results presented here. It should also be noted that strictly anaerobic bacteria were not isolated in this work, as all the cultures used were prepared and incubated in air. Despite the semi-quantitative nature of the data, there were clear differences between the structures of culturable bacterial communities associated with *T. mesenterina* colonies at Lord Howe Island and the Solitary Islands (Section 3.3.1). Within the Solitary Islands samples there were also clear differences between each of the four sample categories (H, M, D and C, section 3.3.2).

Full details of the colony morphology, cfu counts, and closest relatives of bacterial isolates identified by 16S rDNA sequence analysis, including the accession numbers for the 16S rDNA gene sequences generated in this work are presented in Appendix 1. Micrographs of the gram stained bacterial isolates are presented in Appendix 2.

3.3.1 Culturable bacterial communities associated with *T. mesenterina* at LHI versus the SIMP

3.3.1.1 Coral disease occurrence at the sites surveyed

T. mesenterina is not as abundant at LHI as it is in the SIMP, and is only found in a limited number of sites (Harriot et al. 1995). Due to time constraints and sampling difficulties related to unfavourable weather conditions, only two of these sites were sampled (1a and 1b, Figure 3.1). There were no incidences of SWS observed in *T. mesenterina* at LHI or the adjacent reefs that were surveyed, although incidences of disease were observed in other coral species. Most notably affected by disease were *Acropora* and *Porites* spp., within the shallow sheltered reefs inside the lagoon on the western side of the island (Dalton & Godwin 2005). All *T. mesenterina* colonies sampled at LHI were therefore completely healthy colonies, which had not been affected by sediment damage or predation. The *T. mesenterina* colonies sampled in the SIMP included both entirely healthy and SWS affected specimens.

3.3.1.2 Differences between the culturable bacterial communities associated with healthy *T. mesenterina* colonies at LHI and in the SIMP

A total of 53 separate bacterial isolates were retrieved from the mucus and tissue of healthy coral samples at LHI and a total of 22 strains were isolated from healthy corals in the SIMP. These isolates comprised a total of approximately 443 cfu in LHI samples and 49 cfu in SIMP samples. A large proportion of the culturable bacteria associated with healthy *T. mesenterina* did not survive in axenic culture. It is possible that they represent fastidious organisms that comprised large proportions of the communities associated with living coral tissue. Because they could not be grown in

pure culture, it was not possible to extract genomic DNA from these bacteria for 16S rDNA analysis. Approximately 29% (131) of the total number of bacterial cfu from LHI samples and 57% (28) of bacterial cfu from healthy SIMP samples did not survive in axenic culture and will be referred to as 'unidentified' isolates. In LHI samples this proportion of unidentified bacteria consisted primarily of a single isolate (10.5.051CC2) that was very abundant in a sample collected from Malabar shoal, whereas in the SIMP small numbers of unidentified bacteria were encountered in all samples.

Samples from LHI contained several taxa that were not detected in samples from the SIMP (Figure 3.3). Most notable are the three isolates belonging to the family *Sphingomonadaceae*. The RDP classifier placed these isolates in the genus *Erythrobacter*. Two of these isolates (11.5.051CS3 and 10.5.051CC3) are likely the same strain as they had the same appearance on SLB agar (Appendix 1) and differed by only one base pair in their 16S rDNA across the 735 bases that were sequenced. This strain was abundant in the *T. mesenterina* colony sampled at Malabar shoal, and shared 99% 16S rDNA sequence similarity with an uncultured *Erythrobacter* isolated from a deep sea octocoral.

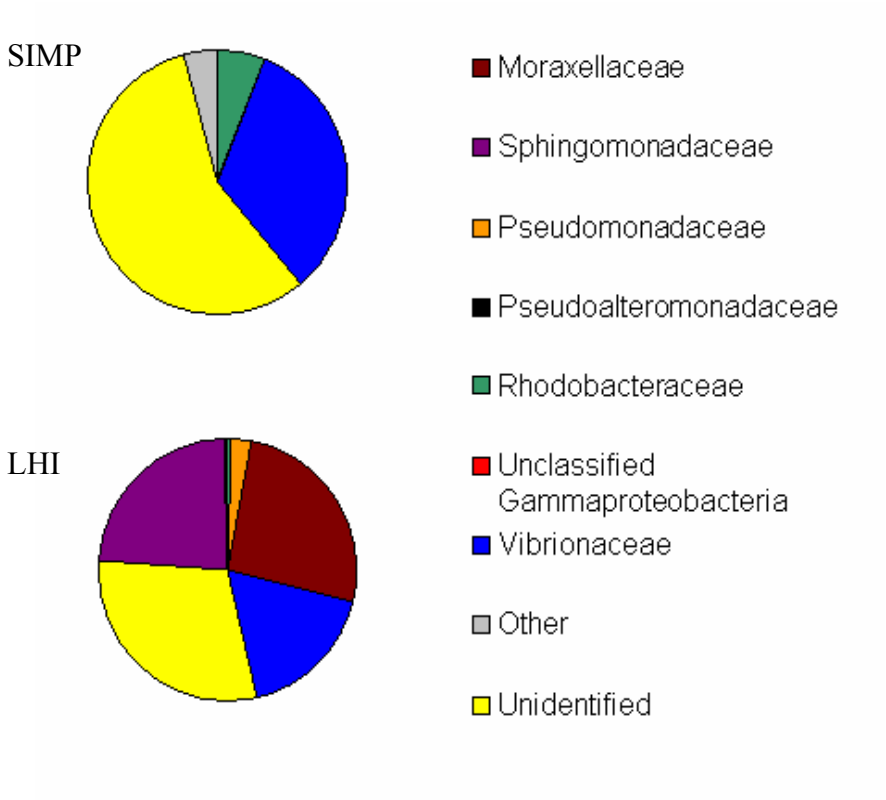

Figure 3.3 Differences between the culturable bacterial communities associated with the mucus and tissues of healthy *T. mesenterina* colonies at LHI and in the SIMP. The sections of each pie represent the approximate proportion each bacterial family contributed to the total number of colony forming units counted. The total numbers of cfu recorded were 49 in the SIMP, and 443 at LHI.

Members of the family *Moraxellaceae* were also present only in samples from LHI. Representatives of this family were detected in all samples from LHI. The most abundant isolate in this family (10.5.051CC1) shared an average of 96% 16S rDNA sequence similarity with *Psychrobacter celer*. A single isolate with the same 16S rDNA sequence and colony morphology (2.3.053HC2) was detected in apparently healthy tissue of SWS affected *T. mesenterina* in the SIMP, but not in completely healthy colonies.

Vibrio spp. comprised 18% of the bacterial cfu in LHI samples, and 33% of cfu in SIMP samples. Phylogenetic analysis indicated that the majority of the *Vibrio* strains isolated occurred in both locations (Figure 3.5). An exception was isolate 11.5.052CC11 which was obtained from a sample of crushed coral collected at LHI, and was not detected in samples from the SIMP. This isolate shared 97% 16S rDNA sequence similarity to *V. shiloi,* the causative agent of bacterial bleaching of *Oculina patagonica* (Kushmaro et al. 1997). The most abundant *Vibrio* isolate in LHI samples was 11.5.051CC2, which shared >99.5% 16S sequence similarity with isolates 11.5.051CC1, 11.5.052CC5, 11.5.053CC1 and 11.5.052CC7. Phylogenetic analysis placed these isolates amongst the non-pathogenic strains of the *V. harveyi* group (Figure 3.5).

3.3.2 Differences amongst culturable bacterial communities associated with healthy and diseased *T. mesenterina* in the SIMP.

Culturable bacteria were approximately 3-9 times more abundant in samples of dead coral (D) and disease margin (M) than in samples of living tissue (H and C). Mean numbers of 59, 201, 219 and 25 cfu per 100 µL of sample (crushed coral or mucus) were counted for H, M, D and C categories respectively. As was the case with bacteria associated with completely healthy *T. mesenterina*, a large proportion of the colony forming bacteria associated with the apparently healthy living tissue of SWS did not survive in axenic culture. Strains comprising 57% of total cfu(28 cfu, 13 of the 53 isolates) of colony forming bacteria from C samples and 46% (108 cfu) of colony forming bacteria from the H samples did not survive in axenic culture and could not be identified by 16S rDNA sequence analysis. In contrast, only 12% (73 cfu) and 2%

(20 cfu) of colony forming bacteria from the M and D sample categories consisted of strains that failed to grow in axenic culture.

The culturable bacterial communities associated with dead (D) and disease margin (M) sections of SWS affected *T. mesenterina* colonies in the SIMP were dominated by members of the *Rhodobactereacae* and *Vibrionaceae* families (Figure 3.4). *Vibrio* isolates comprised approximately 51% (443 cfu) and 38% (230 cfu) of cfu's isolated from D and M samples respectively. The most abundant *Vibrios* in samples of dead skeleton (D) and lesion margins (M) of SWS affected corals were members of the *harveyi* group (Section 3.3.3, Figure 3.5). *Vibrio* spp. also comprised considerable proportions of the bacteria isolated from living coral tissue (12% (28) of cfu's isolated from H samples and 33% (16) of cfu's isolated from C samples). However, with the exception of a single isolate (2.3.052HS2) that occurred only once in samples of the surface of apparently healthy tissue of SWS affected colonies (H), these bacteria were taxonomically different from the *Vibrio* spp. that dominated dead and diseased sections of *T. mesenterina* colonies. While BLAST analysis of the *Vibrio* spp. associated with dead coral (D) and disease margin (M) samples indicated that they were related to species associated with disease and mortality of marine organisms, *Vibrio* spp. associated with living coral tissue were most closely related to previously reported strains from seawater, and were not associated with disease (Section 3.3.3).

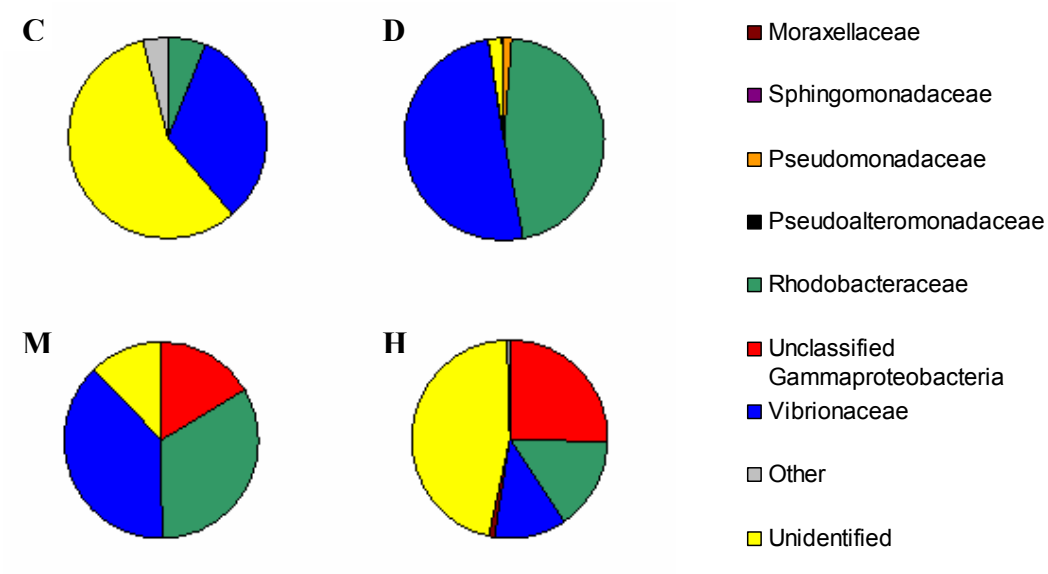

Figure 3.4 Differences amongst culturable bacterial communities associated with the mucus and tissues of healthy and diseased corals in the SIMP. (D) Dead skeleton adjacent to disease lesions on SWS affected colonies. (M) Samples taken along the margin of active disease lesions. (H) Sections of colonies with apparently healthy coral tissue adjacent to disease lesions. (C) Completely healthy colonies with no signs of SWS located within 1-2 m of SWS affected colonies. The sections of each pie represent the approximate proportion each bacterial family contributed to the total number of colony forming units counted in each sample category. The total numbers of cfu recorded were; C - 49, D - 877, M - 603 and H - 234.

Rhodobactereaceae species were the second most numerically dominant group of bacteria isolated from SWS affected colonies, comprising 46% (401 cfu), 33% (200 cfu) and 15% (35 cfu) of the dead (D), margin (M) and healthy (H) sample categories respectively (Figure 3.4). With the exception of the isolate 2.3.053DC8, all of the *Rhodobactereaceae* spp. isolated from these categories were closely related to *Shimia marina* (Figure 3.5), a bacterium isolated from fish farm biofilms. Members of this family comprised only 6% (two isolates – 4.5.0412CS12 and 2.3.053CC4) of bacteria isolated from completely healthy coral colonies (C). These isolates were different from members of the same family isolated from diseased colonies.

One strain was particularly abundant in samples taken from only one of the disease affected *T. mesenterina* colonies. This organism was isolated twice, once from the disease margin (M) and once from the apparently healthy tissue (H) of this colony, but was not found on any other coral colony, either in the SIMP or at LHI. These two isolates (2.3.052MS2 and 2.3.052HS1) were designated 'unclassified gammaproteobacteria' by the RDP classifier. These isolates are most likely the same species as they shared 100% 16S rDNA identity over the region sequenced. Taken together with data from the other replicate samples they comprised approximately 16% (100 cfu) of bacteria isolated from all disease margin samples and 25% (60 cfu) of all bacteria isolated from apparently healthy tissue adjacent to disease lesions. The two closest relatives to this species identified by BLAST analysis were unclassified marine bacteria from a microbial fuel cell (94% sequences similarity), and from sea urchin intestine (also 94% similarity) (accession numbers AY785252.1 and AY770722.1). Microscopic examination revealed gram-negative, spiral shaped cells which did not resemble any of the other bacteria isolated (Appendix 2). Given the lack of family level classification and the low level of sequence similarity to previously reported bacteria this organism is likely to be a new species belonging to an undescribed genus and family.

3.3.3 Phylogenetic relationships of bacterial isolates

Data from all the samples taken both in the SIMP and at LHI were pooled to infer their phylogenetic relationships with each other and with bacterial 16 rDNA sequences downloaded from public databases. Figure 3.5 illustrates the inferred phylogenetic relationships of all of the sequenced isolates compared to related bacterial sequences from the RDP database (Cole et al. 2007).

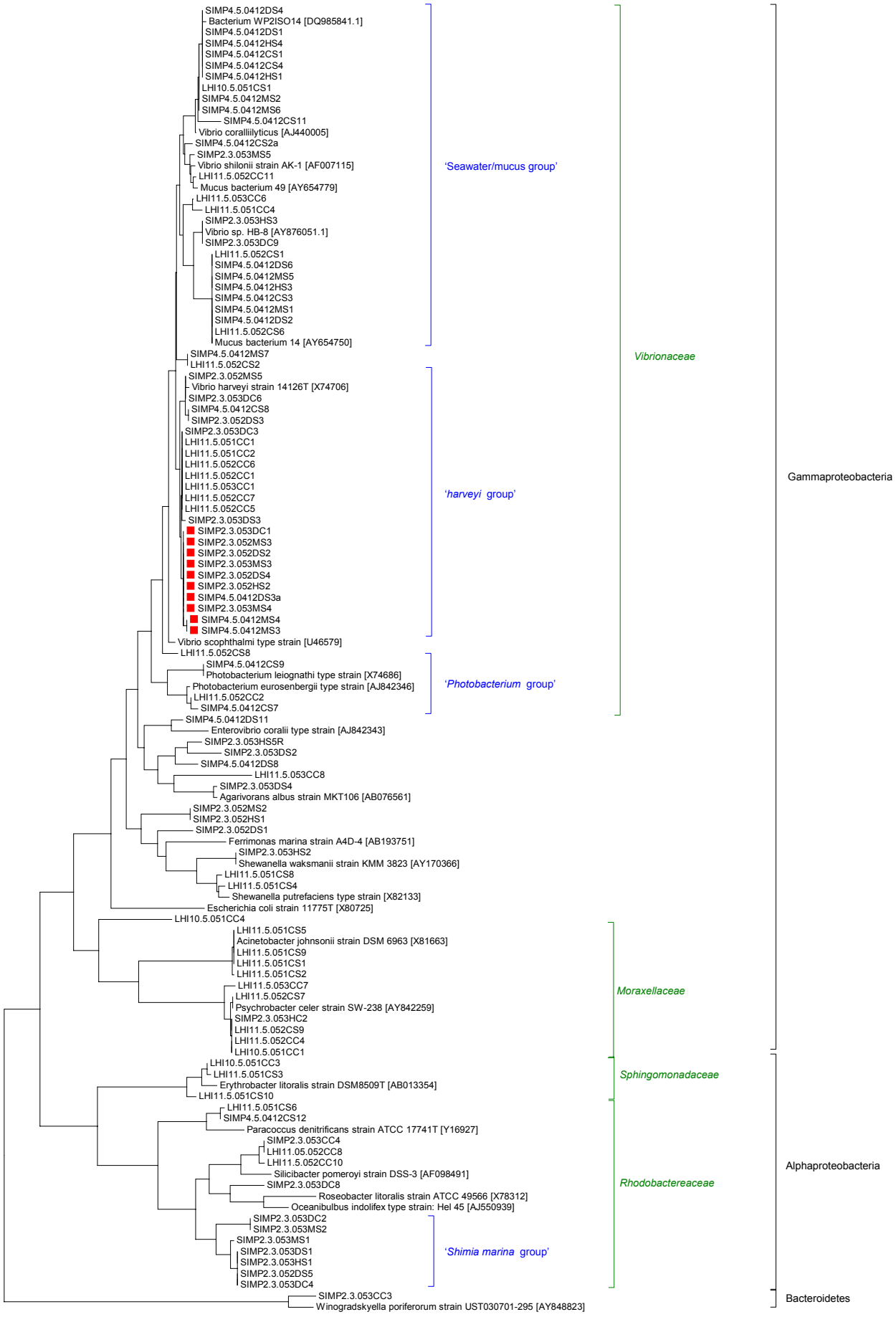
SIMP4.5.0412DS4
Bacterium WP2ISO14 [DQ985841.1]
SIMP4.5.0412DS1
SIMP4.5.0412HS4
SIMP4.5.0412CS1
SIMP4.5.0412CS4
SIMP4.5.0412HS1
LHI10.5.051CS1
SIMP4.5.0412MS2
SIMP4.5.0412MS6
SIMP4.5.0412CS11
Vibrio coralliilyticus [AJ440005]
SIMP4.5.0412CS2a
SIMP2.3.053MS5
Vibrio shilonii strain AK-1 [AF007115]
LHI11.5.052CC11
Mucus bacterium 49 [AY654779]
LHI11.5.053CC6
LHI11.5.051CC4
SIMP2.3.053HS3
Vibrio sp. HB-8 [AY876051.1]
SIMP2.3.053DC9
LHI11.5.052CS1
SIMP4.5.0412DS6
SIMP4.5.0412MS5
SIMP4.5.0412HS3
SIMP4.5.0412CS3
SIMP4.5.0412MS1
SIMP4.5.0412DS2
LHI11.5.052CS6
Mucus bacterium 14 [AY654750]
'Seawater/mucus group'
SIMP4.5.0412MS7
LHI11.5.052CS2
SIMP2.3.052MS5
Vibrio harveyi strain 14126T [X74706]
SIMP2.3.053DC6
SIMP4.5.0412CS8
SIMP2.3.052DS3
SIMP2.3.053DC3
LHI11.5.051CC1
LHI11.5.051CC2
LHI11.5.052CC6
LHI11.5.052CC1
LHI11.5.053CC1
LHI11.5.052CC7
LHI11.5.052CC5
SIMP2.3.053DS3
SIMP2.3.053DC1
SIMP2.3.052MS3
SIMP2.3.052DS2
SIMP2.3.053MS3
SIMP2.3.052DS4
SIMP2.3.052HS2
SIMP4.5.0412DS3a
SIMP2.3.053MS4
SIMP4.5.0412MS4
SIMP4.5.0412MS3
'harveyi group'
Vibrio scophthalmi type strain [U46579]
LHI11.5.052CS8
SIMP4.5.0412CS9
Photobacterium leiognathi type strain [X74686]
Photobacterium eurosenbergii type strain [AJ842346]
LHI11.5.052CC2
SIMP4.5.0412CS7
'Photobacterium group'
Vibrionaceae
SIMP4.5.0412DS11
Enterovibrio coralii type strain [AJ842343]
SIMP2.3.053HS5R
SIMP2.3.053DS2
SIMP4.5.0412DS8
LHI11.5.053CC8
SIMP2.3.053DS4
Agarivorans albus strain MKT106 [AB076561]
SIMP2.3.052MS2
SIMP2.3.052HS1
SIMP2.3.052DS1
Ferrimonas marina strain A4D-4 [AB193751]
SIMP2.3.053HS2
Shewanella waksmanii strain KMM 3823 [AY170366]
LHI11.5.051CS8
LHI11.5.051CS4
Shewanella putrefaciens type strain [X82133]
Escherichia coli strain 11775T [X80725]
LHI10.5.051CC4
LHI11.5.051CS5
Acinetobacter johnsonii strain DSM 6963 [X81663]
LHI11.5.051CS9
LHI11.5.051CS1
LHI11.5.051CS2
LHI11.5.053CC7
LHI11.5.052CS7
Psychrobacter celer strain SW-238 [AY842259]
SIMP2.3.053HC2
LHI11.5.052CS9
LHI11.5.052CC4
LHI10.5.051CC1
Moraxellaceae
LHI10.5.051CC3
LHI11.5.051CS3
Erythrobacter litoralis strain DSM8509T [AB013354]
LHI11.5.051CS10
Sphingomonadaceae
LHI11.5.051CS6
SIMP4.5.0412CS12
Paracoccus denitrificans strain ATCC 17741T [Y16927]
SIMP2.3.053CC4
LHI11.05.052CC8
LHI11.5.052CC10
Silicibacter pomeroyi strain DSS-3 [AF098491]
SIMP2.3.053DC8
Roseobacter litoralis strain ATCC 49566 [X78312]
Oceanibulbus indolifex type strain: Hel 45 [AJ550939]
Rhodobactereaceae
SIMP2.3.053DC2
SIMP2.3.053MS2
SIMP2.3.053MS1
SIMP2.3.053DS1
SIMP2.3.053HS1
SIMP2.3.052DS5
SIMP2.3.053DC4
'Shimia marina group'
Alphaproteobacteria
SIMP2.3.053CC3
Winogradskyella poriferorum strain UST030701-295 [AY848823]
Bacteroidetes
Gammaproteobacteria
0.05

Figure 3.5 Phylogenetic tree showing the taxonomic relationships of the 95 bacterial isolates characterised by 16S rDNA sequencing to related sequences from public databases. Black brackets indicate taxonomic categories at division level. Green brackets indicate bacterial families that were particularly abundant. Blue brackets indicate arbitrary clusters of closely related bacteria (see text for details). Isolates closely related to pathogenic strains of *Vibrio harveyi* are indicated with red markers. The phylogeny was inferred using the Neighbour-Joining method (Saitou & Nei 1987). The optimal tree with the sum of branch length = 1.66260874 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 466 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

3.3.3.1 *Vibrionaceae* Isolates

Of the isolates within the gammaproteobacteria, 53 (60% of all strains isolated) were members of the family *Vibrionaceae*. These isolates may be broadly divided into three groups on the basis of their phylogeny: *Photobacterium* spp., the '*Vibrio harveyi* group', and the seawater and mucus bacteria group.

The *Photobacterium* group contained two isolates originating from the same healthy coral colony in the SIMP (4.5.0412CS9 and 4.5.0412CS7) and one isolate originating from a healthy coral colony at LHI (11.5.052CC2). All of these isolates were closely related (97-99% sequence identity) to *Photobacterium* species, and produced luminescent colonies on SLB agar. No *Photobacterium* strains were isolated from *T. mesenterina* colonies affected by disease.

The *Vibrio harveyi* group contained 21 bacterial isolates which all shared >99% sequence similarity. All members of this group exhibited similar morphological characteristics. They were gram-negative rods (Appendix 2) which formed round, pale coloured colonies on SLB agar. Blast analysis indicated that these isolates were

all closely related to *Vibrio harveyi* strains. This group of isolates is of particular interest because it contained several strains (isolates 4.5.0412MS4, 4.5.0412MS3, 2.3.052DS2, 2.3.052DS4, 2.3.05HS2, 2.3.052MS3, 2.3.053DC1, 2.3.053DC6, 2.3.053MS3 and 2.3.053MS4, indicated with red markers in Figure 3.5) which originated from SWS affected *T. mesenterina* colonies in the SIMP. These strains were closely related to pathogenic *V. harveyi* strains from fish and strains associated with mortality of lobsters grown in aquaculture (accession numbers DQ314530.1, DQ314529.1, DQ831087.1, DQ831116.1 and DQ831086.1).

With exception of two isolates (2.3.053DC3 and 2.3.053DS3), which both originated from a dead section of the same disease affected colony in the SIMP, all of the remaining members of the group were isolated from healthy *T. mesenterina* colonies with no signs of disease, both in the SIMP and at LHI. BLAST analysis indicated that these remaining isolates were more closely related to *V. harveyi* strains from seawater and marine algae samples, and were not associated with disease in any organism.

The third group included the remaining *Vibrio* isolates, which were more diverse than the *harvey*i group. Phylogenetic analysis grouped them into a cluster with other *Vibrio* species found in seawater or associated with coral and other marine organisms (Figure 3.5). Noteworthy isolates in this group included 4.5.0412CS1, which was isolated from a healthy coral colony in the SIMP. Although the nearest relative of this isolate identified by BLAST analysis was an uncultured bacterium from seawater, the next closest relative, with 96% sequence similarity, was an unclassified *Vibrio* strain known to inhibit pathogens in molluscs (accession number AY034144.1). Other isolates of interest included 2.3.053HS3 and 2.3.053DC9 which were respectively

isolated from apparently healthy (H) and dead (D) areas of the same SWS affected coral colony. These isolates both exhibited >97% sequence similarity to *Vibrio* sp. HB-8 (accession number AY876051.1), which is associated with brown band disease in *Acropora muricata* on the Great Barrier Reef. Three strains (4.5.0412MS7, 11.5.052CC11, 11.5.052CS2) were also identified that shared >97% sequence similarity with *Vibrio shiloi*, the causative agent of bacterial bleaching of the coral *Oculina patagonica* (Kushmaro et al. 2001).

3.3.3.2 Other Gammaproteobacteria

The remaining isolates classified within the gammaproteobacteria were diverse and included representatives of the families *Moraxellaceae*, *Shewanellaceae*, *Alteromonadaceae*, *Ferrimonidaceae*, *Pseudoalteromonadaceae*, and *Pseudomonadaceae.* Two isolates with 100% sequence identity (2.3.052MS2 and 2.3.052HS1) represent a single organism that was unrelated to described families within the gammaproteobacteria and was designated 'unclassified' by the RDP (Wang et al. 2007). Isolate 2.3.053DS4 was classified as belonging to the genus *Agarivorans*, which falls amongst genera of uncertain status in incertae sedis 7 of the *Alteromonadales*.

Members of the family Moraxellaceae represented approximately 10% of the sequenced bacterial isolates. However, with the exception of a single isolate (10.5.051CC1), which comprised >30% of the total cfu from samples collected at Malabar Shoal, LHI, these bacteria were not abundant in any sample. BLAST analysis of sequences obtained from these isolates indicated close relationships to members of the genera *Psychrobacter* and *Acinetobacter*.

3.3.3.3 Alphaproteobacteria isolates

Of the 16 sequenced isolates classified by the RDP as Alphaproteobacteria, 13 (81%) belonged to the *Rhodobacteraceae* family. Seven of these isolates (2.3.053DC2, 2.3.053MS2, 2.3.053MS1, 2.3.053DS1, 2.3.052DS5, 2.3.053DC4 and 2.3.053HS1) formed a cluster with >97% shared sequence identity. BLAST analysis indicated that these isolates were most closely related (with an average of 97% sequence identity) to *Shimia marina*, a novel bacterium isolated from fish farm biofilms (accession number AY962292.1). Members of this cluster were abundant in samples taken from a diseased colony in the SIMP, but were not identified in samples from healthy corals. The remaining 19% of Alphaproteobacteria isolates belonged to the family *Sphingomonadaceae*.

3.4 Discussion

3.4.1 General observations

Clear differences were observed between sample categories in both the size and composition of the culturable bacterial communities. There were less culturable bacteria in samples of live tissue (H and C categories) than in samples from dead surfaces and the disease margin (D and M categories) of SWS affected corals. Comparable disparity in the abundance of culturable bacteria associated with healthy and diseased corals has been reported by Beleneva et al. (2005), who found higher numbers of culturable organisms in samples of bleached coral than in samples of healthy colonies of the same species. There are several possible explanations for this observation. Although extracts of scleractinian coral tissues have not exhibited strong antibacterial properties (Koh 1997), the living tissue of many coral species is able to produce broad spectrum antibacterials in response to mechanical stress (Geffen &

Rosenberg 2005). The sampling techniques used in this study unavoidably subjected the live coral tissue to mechanical damage, so it is likely that the antibacterial response (if it exists in *T. mesenterina*) would have been elicited, resulting in lower numbers of viable bacterial cells in the samples. The coral-associated bacteria themselves may also play a role in regulating their own community. Ritchie (2006) demonstrated that the mucus and associated bacteria of healthy *Acropora palmata* colonies had antimicrobial properties. It is possible that antimicrobial compounds are produced by the symbiotic bacterial community associated with the living tissue of *T. mesenterina* which regulate bacterial densities and inhibit the growth of invasive bacteria, thereby reducing the total number of culturable bacteria.

It is also possible that the bacterial community associated with living coral tissue contains a higher proportion of fastidious organisms than the populations associated with disease margins or dead skeleton. Bacteria growing on living coral tissue may require specific substrates present only in the coral mucus or on the coral skeleton. As members of a complex microbial community they may also rely on the metabolites of other bacterial species for growth. This idea is supported by the observation that a higher proportion of the bacteria isolated from live coral tissue failed to grow in axenic culture (37% of cfu counted from H and C samples compared to 6% from D and M samples). The metabolic roles of coral associated bacteria are poorly studied, but previously reported surveys using culture independent methods indicate that a wide variety of bacteria with a potentially diverse range of metabolic capabilities can be found on living corals (Rohwer et al. 2002, Bourne & Munn 2005). A model of the coral holobiont in which the bacterial community is responsible for converting complex organic coral exudates to simpler substrates has been proposed by Ritchie

and Smith (2004). Such a community would contain a number of species with complex or specific nutritional requirements. It is likely that heterotrophic bacteria associated with dead and dying coral are less specialised and are able to utilise a wider range of substrates. This interdependency in the bacterial community of living coral tissue may account for the failure of such a large proportion of culturable bacteria from H and C samples to grow in axenic culture. Qualitative observations of colonies of some of the bacterial isolates in this study suggest that their metabolic processes were influenced by neighbouring colonies. Colonies of isolate 2.3.052DS3 produced an intense purple pigment when first observed on SLB agar plates in mixed culture (Appendix 1), but were white in colour in axenic culture. Evidence of antimicrobial activity has also been observed in bacteria isolated from other coral species in the SIMP, which produced zones of inhibition around their colonies in mixed cultures on SLB agar (data not shown).

3.4.2 Geographic variation in coral-associated bacterial communities

The structure of bacterial communities observed on healthy *T. mesenterina* colonies from the SIMP differed considerably from those observed on colonies from LHI. These results appear to contradict Rohwer et al.'s (2002) findings that corals form species-specific associations with bacteria and that the structure of the bacterial communities is maintained over geographic distances. It is possible that the differences observed here are related to factors such as increased freshwater runoff and nutrient levels in the nearshore environment of the SIMP. The SIMP is heavily influenced by freshwater runoff from the Clarence River, which drains the second largest catchment area in NSW. Bleaching of corals in the SIMP has been correlated with periods of heavy influxes of freshwater from the Clarence (Edgar et al. 2003). In

comparison, LHI has much smaller catchment area, and the effects of ocean currents are likely to be much more significant than terrestrial runoff. Kline et al. (2006) and Kuntz et al. (2005) have demonstrated that nutrient loading, particularly with elevated levels of dissolved organic carbon (DOC), can cause coral mortality through perturbation of the normal coral-associated bacterial community. If elevated DOC levels result in shifts in coral-associated bacterial communities they may help to explain the differences in culturable bacterial community structure observed in this study. There are several potential sources for DOC in the SIMP which are not present at LHI. These include high volumes of terrestrial runoff from the adjacent coastline, anthropogenic inputs such as treated sewerage water and possibly the higher occurrence of macroalgal assemblages (Harriot et al. 1994, Harriot et al. 1995). Smith et al. (2006) demonstrated that fixed carbon released into the water column by macroalgae can induce changes in the coral-associated bacterial community that lead to coral mortality.

The *Vibrio* strains isolated from healthy *T. mesenterina* samples were detected at both LHI and the SIMP. There were striking differences however between the other dominant bacterial groups. Members of the genera *Erythrobacter* (family *Sphingomonadaceae*), *Psychrobacter* and *Acinetobacter* (family *Moraxellaceae*) were at least 100 times more abundant in samples from LHI (Figure 3.3). Members of these families have been reported in association with healthy corals by other investigators. Studies by Santavy et al. (1995) and Beleneva et al. (2005) both identified *Moraxella* spp. in association with healthy *Porites* spp corals. Bourne and Munn (2005) identified *Acinetobacter* spp. in clone libraries from healthy *Pocillopora damicornis* on the Great Barrier Reef, and members of this genus have been isolated from

samples of *Oculina patagonica* from coastal waters of Israel (Koren & Rosenberg 2007). The role of these bacteria in the context of the coral holobiont is not clear, but their apparent association with healthy corals of several taxa, and absence from samples of diseased corals, suggests that they may play important roles as probiotics (Reshef et al. 2006). This role could be carried out by the production of compounds that inhibit the growth of pathogens. Isolates belonging to the genus *Erythrobacter* may also have potential as probiotics. Although antimicrobial activity has not been reported from this genus, members of the related genus *Sphingomonas* are able to inhibit the growth of a wide range of bacteria (Romanenko et al. 2007). If that is the case, the lower abundance of these bacteria on *T. mesenterina* in the SIMP may even contribute to the higher incidence of coral disease in that area relative to LHI.

3.4.3 Partitioning of bacterial communities between diseased and healthy corals in the SIMP

The disease margin, dead skeleton and apparently healthy tissues of SWS affected *T. mesenterina* colonies each harboured a distinct culturable bacterial community, all of which differed from the community associated with completely healthy colonies unaffected by SWS. The clear succession of bacterial communities from the living tissue of infected colonies, to the advancing disease margin, and exposed dead skeleton is consistent with previously reported observations of the bacterial communities associated with other coral diseases such as black band disease, white band disease and white plague (Frias-Lopez et al. 2002, Pantos et al. 2003, Pantos & Bythell 2006). It is expected that the bacterial community will change as the disease progresses. Death of the coral tissue will drastically alter the microenvironment available for bacterial colonisation, and it is likely that oppurtunistic heterotrophs that

can take advantage of the nutrients available in the decaying coral tissue will become dominant.

The differences between the culturable bacterial communities associated with apparently healthy tissue of SWS affected colonies (H) and the tissue of completely healthy colonies (C), is also consistent with observations of other coral species affected by diseases such as WP and WBD (Pantos et al. 2003, Pantos & Bythell 2006). These differences indicate that a shift in the bacterial community associated with the living coral tissue occurs before the onset of disease symptoms. This shift may be the result of an integrated response to infection by the entire coral holobiont, or the shift may precede infection and occur in response to an abiotic stressor. In either case the observation lends support to Reshef et al's (2006) hypothesis that the bacterial communities of corals are able to modify the structure in response to external stimuli in order to rapidly adapt to changing conditions.

3.4.4 Potential causative agents of SWS

The ten isolates that were closely related to pathogenic *Vibrio harveyi* strains (Figure 3.5) represent a single bacterial strain which was detected in the 'D' and 'M' samples of all three of the SWS affected colonies sampled. It was also detected in the apparently healthy tissues (H) of two of the same three affected colonies, but was not observed in any healthy colonies (C). The strong association of this strain with SWS affected corals suggest that it may a role as a pathogen. *V. harveyi* is well known as a pathogen of wide range of marine organisms, including bony fish, sharks, prawns, holothurians, lobsters and abalone, and has been reported as causing infections in shark-bite wounds in humans (Austin & Zhang 2006). *V. harveyi* strains have also

been reported in association with diseased corals. Ritchie and Smith (1998) observed a strong association of *V. charchariae* (a junior synonym of *V. harveyi*) with WBDII in *Acropora cervicornis* and proposed that it may be the causative agent responsible for the disease. Recently, Gil-Agudelo et al. (2006b) reported preliminary evidence from field infection trials to suggest that this may indeed be the case. *V. harveyi* has also been recently reported in association with rapid tissue necrosis of *Pocillopora damicornis* (Luna et al. 2007). Considering its wide host range it is likely that *V. harveyi* is simply an oppurtunistic pathogen of stressed or immunocompromised marine organisms. The potential role of this bacterium in SWS pathogenesis should be investigated further.

An alphaproteobacterium closely related to *Shimia marina* was also observed in high numbers only in SWS affected *T. mesenterina* colonies and was not detected in healthy controls. Other members of the family *Rhodobacteraceae* are known to cause disease in oysters (Boettcher et al. 2005). The role of this bacterium in SWS warrants further investigation, however as this strain was only isolated from two of the three diseased colonies sampled it does not seem to be as strong a candidate as the *Vibrio* isolate, which was identified in all disease samples.

Preliminary infection trials using cultures of these two strains inoculated into the water column of aquaria containing healthy *T. mesenterina* did not result in disease (data not shown). However it is now understood that direct contact with diseased coral tissue, or contact with a vector carrying the causative agent is required for transmission of SWS from one colony to another (Section 2.3.2). It is possible that infection trials using a different method of inoculation would produce different

results. Gil Agudelo et al. (2006b) were able to reproduce symptoms of white band disease type II in the Caribbean coral *Acropora cervicornis* by inoculating pieces of sterile gauze with putative bacterial pathogens and tying these gauze pieces to healthy coral colonies. In similar experiments, Patterson et al. (2002) inoculated healthy *Acropora palmata* colonies with previously sterilised sediment onto which bacterial cells had been adsorbed to show that white pox disease in this coral is caused by *Serratia marcescens*. In both of these cases inoculation using bacteria adsorbed onto solid material was done because the coral species in question can not be maintained in laboratory conditions, and infection experiments had to be conducted in the field. Much of the difficulty encountered in performing infection trials on *T. mesenterina* was related to problems with maintaining large numbers of replicate coral fragments in aquaria for sufficiently long periods of time. Performing infection experiments in the field may allow these difficulties to be avoided, but introduces other problems related to the variable conditions in the field. The ethical consideration of introducing potential pathogens to healthy corals in the field must also be taken into account. If improved methods could be developed to maintain *T. mesenterina* in controlled laboratory conditions, it would be well worthwhile pursuing further infection experiments using different inoculation techniques. At this point none of the bacterial strains isolated in this study can be definitively ruled out as potential pathogens.

Chapter 4 - Analysis of bacterial communities associated with subtropical white syndrome of *Turbinaria mesenterina* by oligonucleotide fingerprinting of ribosomal genes

4.1 Introduction

Attempts to isolate pathogenic bacterial agents for many coral diseases using traditional microbiological techniques have often proved unsuccessful (Bythell et al. 2004, Gil-Agudelo et al. 2007). This is not surprising given that estimates of total bacterial numbers by DNA staining procedures have shown that less than 1% of the bacteria associated with scleractinian corals are culturable (Koren & Rosenberg 2006). The strong biases inherent in culture techniques also mean that only a very small subset of the total community can be studied if these techniques are used to survey bacterial community structure. The emergence of culture-independent 16S rDNA gene profiling techniques for bacterial community analysis has allowed a much more comprehensive understanding of the true extent of bacterial diversity in a variety of different habitats, including on corals.

The present study is the first report of a culture-independent survey of the bacterial communities associated with the scleractinian coral *Turbinaria mesenterina.* The culturable bacterial communities associated with both healthy and SWS affected *T. mesenterina* have been extensively sampled (Chapter 2). However, attempts to identify the causative agent of SWS by inoculating potentially pathogenic bacteria onto healthy corals have not been successful.

This study was conducted to provide data complementary to the culture-based study described in chapter 3 by generating more comprehensive profiles of the bacterial communities associated with healthy and diseased *T. mesenterina*, In order to cost-effectively sample as large and representative a section of the communities as possible, the oligonucleotide fingerprinting of ribosomal genes method (OFRG) (Valinsky et al. 2004) was employed. This approach allowed a total of 8448 16S rDNA clones to be analysed without extensive nucleotide sequencing.

4.2 Materials and Methods

4.2.1 Fieldwork and sample collection

Coral samples were collected from approximately 10 metres depth by scuba diving off Split Solitary Island (30º 14'S, 153º 11'E, Figure 3.2). Diseased coral colonies were located by visual inspection. Only colonies that displayed an actively advancing disease lesion, with a clean area of recently exposed white aragonite skeleton, were selected for sampling. Samples were taken by removing small sections (approximately 15 cm^2) from the coral colony using a hammer and chisel. Samples were collected from the active disease margin, apparently healthy and dead regions of diseased *T. mesenterina* colonies (Figure 3.1), and from completely healthy colonies nearby. These fragments were placed immediately into separate clean Ziploc bags underwater. Upon surfacing, the bags were placed on ice for transport back to the NMSC laboratory in Coffs Harbour, then frozen at -20 ºC. The next day the frozen samples were transported to UNE, Armidale, on dry ice and prepared for DNA extraction by freezing in liquid nitrogen and crushing into a fine powder in a mortar and pestle. This powder was stored at -70 ºC. The 11 samples in the final set consisted of three replicate samples of apparently healthy sections of diseased corals (**H**), two replicate

samples of disease margins (**M**), four replicate samples of dead sections of diseased colonies (**D**) and two replicate samples from completely healthy colonies (**C**).

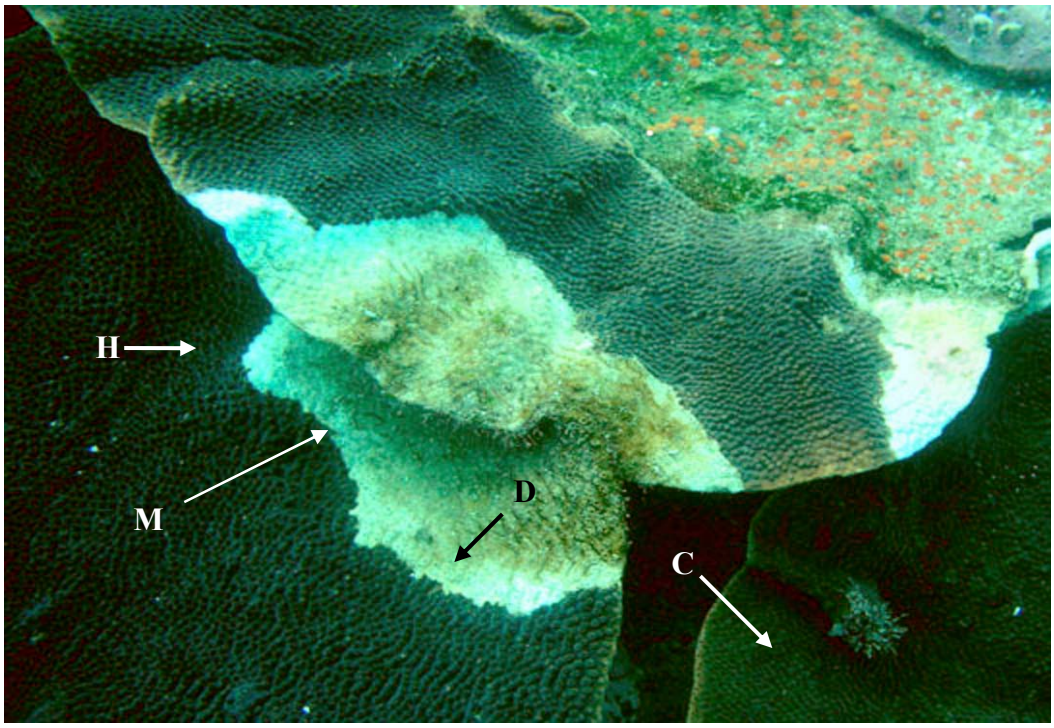

Figure 4.1 Diseased *Turbinaria mesenterina* colonies indicating the regions sampled for bacterial community analysis. Dark areas of the coral surface are covered in living tissue, white areas are recently exposed calcium carbonate skeleton. (H) – Apparently healthy tissue of disease colony, (M) – Margin of disease lesion, (D) – Dead coral skeleton (C) – Healthy tissue from a nearby colony unaffected by disease.

4.2.2 Extraction of total DNA from coral samples

Two alternative methods were initially trialled for extraction of DNA from the coral samples in order to minimise skewing of the resulting bacterial community profile caused by bias in the extraction protocol. In the first method (UREA), adapted from Asahida et al. (1996) and Bourne (2005), 500 µL urea extraction buffer (10 M urea, 0.5 M NaCl, 160 mM Tris-HCl, 0.03 M EDTA and 0.5 M sarcosine) was added to 500 mg ground coral sample, which was then shaken for 5 minutes at 37 ºC. Standard phenol/chloroform extraction was performed by adding an equal volume of

phenol/chloroform/isoamylalcohol (25:24:1) and shaking for 5 minutes at 37 ºC. The aqueous phase was then extracted with chloroform/isoamylalcohol (24:1). The DNA was precipitated by addition of 50 µL sodium acetate (3M) and 500 µL isopropanol, and pelleted by centrifugation (30,000 g for 10 min). The pellet was washed with 70% ethanol, then dried and resuspended in 200 µL Tris/HCl (pH 8.0). The resulting crude extract was purified by agarose gel electrophoresis to remove humic acids and other PCR inhibitors. 60 µL of crude extract was loaded on 1% agarose gels, and high molecular weight (>1.5 kb) DNA was excised after overnight electrophoresis at 20 V. DNA was purified from the agarose using the Qiagen gel extraction kit (Qiagen, Hilden, Germany).

The second DNA extraction method (KIT) utilised a commercial kit (Powersoil DNA isolation kit, MoBio, Carlsbad, CA), which employs a combination of chemical and mechanical (beadbeat) treatments to lyse bacterial cells and release DNA. 250 mg of each ground coral sample was processed according to the manufacturer's instructions. The resulting DNA preparation was used for PCR without any further processing.

4.2.3 Terminal restriction fragment length polymorphism (TRFLP) analysis

In order to estimate the efficacy of each extraction protocol, TRFLP assays were performed to compare the diversity (number of unique ribotypes) of bacteria detected in DNA samples prepared using the UREA and KIT methods. DNA was separately prepared from one disease margin (**M**) and one dead coral (**D**) fragment using each method.

TRFLP was performed by the method of Liu et al. (1997). Universal bacterial primers 9F (5'-GAGTTTGATYMTGGCTC) with a 5' carboxyfluorescine (FAM) label and 1509R (5'-GYTACCTTGTTACGACTT) were obtained from Geneworks Pty. Ltd. (Thebarton, SA, Australia). The 25 μL polymerase chain reaction mixtures contained Taq polymerase buffer (Qiagen) giving a final reaction concentration of 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 μM, the FAM-labelled forward primer 9F and unlabelled reverse primer 1509R each at a concentration of 400 nM, 0.5 U of *Taq* polymerase (Qiagen) and 1 μL of total coral DNA prepared by either the KIT or UREA method. The thermocycling parameters were; an initial denaturation of 5 minutes at 94 ºC, then 35 cycles of 94 ºC for 1 min, 55 ºC for 1 min, and 72 ºC for 2 min, followed by a final extension step of 72 ºC for 5 minutes.

Replicate PCR products were combined to a total volume of 300 μL, and purified using a PCR clean up kit (Qiagen). Purified DNA was eluted with 30 μL water. 10 μL aliquots of purified DNA were separately digested with RsaI, HhaI and MspI in foil covered tubes. Digested products were sent to the Australian Genome Research Facility (AGRF, St Lucia, Qld, Australia) for fragment analysis. TRFLP profiles were created by plotting fragment size against peak height. These profiles were used to compare the diversity of ribotypes amplified from DNA extracts prepared by the two extraction methods.

4.2.4 PCR for 16S rDNA library construction

Final DNA extracts were prepared from coral samples using the KIT method. 16S rDNA was amplified from the extracts in multiple 25 μL reactions containing 1X PCR

buffer (Qiagen), 1μL Q solution (a propriety reagent designed to aid in the amplification of difficult samples (Qiagen), 25mM $MgCl_2$, 250μM of each deoxynucleoside, 5U of *taq* polymerase, 1μL of coral DNA extract, and the forward primer (27F 5'**GGAGACAU**GAGCTCAGAGTTTGATCMTGGCTCAG) and reverse primer (1492R 5'**GGGAAAGU**CACGYTACCTTGTTACGACTT) at a concentration of 400 nM each. These primers included eight nucleotide modifications (indicated in bold) to allow cloning with the USER friendly cloning kit (New England Biolabs). PCR cycling parameters were 94ºC for 2 minutes, followed by 35 cycles of 94ºC for 30 s, 48ºC for 30 s and 72ºC for 2 min, with a final extension step of 72ºC for 3 min. PCR products were quantified on agarose gels, and 4-5 replicate reactions were combined for each sample. The pooled product was evaporated to approximately 10μL on a Savant Speed Vac (Savant). The 1.5 kb product corresponding to16S rDNA was excised from 1.5% agarose gels and purified with a Qiagen Minelute kit (Qiagen).

4.2.5 Clone library construction

Construction of the clone libraries, and the printing and hybridisation of macroarrays for this experiment was performed during a visit to the University of California, Riverside (UCR). Purified PCR products were assembled into the pNEB205A vector (New England Biolabs) and transformed into *Escherichia coli* DH5α competent cells (Invitrogen, Carlsbad, California) by heat shock. Transformed cells were grown overnight at 37 ºC on Luria-Bertani (LB) agar containing 100 μg/mL ampicillin, 200 μg/mL isopropyl-β-D-1-thioglactopyranoside (IPTG) and 70 mg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). For each of the 11 replicate coral samples (see Section 4.2.1 for sample categories), 768 white colonies were randomly

selected using a QPix robotic colony picker (Genetix, Hampshire, UK) and transferred to 384 well culture plates containing 30μL LB medium supplemented with 8% glycerol and 100 μg/mL ampicillin per well. Clones were grown in a shaking incubator (300 rpm) for 16 hours at 37ºC. Clone libraries were stored at -70ºC.

4.2.6 PCR for macroarray contruction

9600 clone arrays were constructed by spotting PCR amplified rDNA genes onto nylon membranes. To amplify the cloned genes, 1 μL portions of freshly grown overnight clone library cultures were transferred to 384 well PCR plates using a 384-pin split pin replicator (V&P Scientific, San Diego, California). Each well contained a 15μL reaction mixture of 50mM Tris (pH 8.3), 500 μg/mL bovine serum albumin, 2.5 mM $MgCl_2$, 250 μM of each deoxynucleoside triphosphate 400 nm each of the forward primer (UserOFRGFor2- 5'TCGAGCTCAGGCGCGCCTTATTAAGCTGA) and reverse primer (UserOFRGRev2- 5'GCCAAGCTTCCTGCAGGGTTT AAACGCTGA) and 0.75U *taq* polymerase. Plates were sealed with Thermo-seal foil (Marsh Bio Products) using a preheated Thermo-sealer (Abgene, Epsom, United Kingdom). PCR was performed by alternately submerging the sealed plates in waterbaths held at 72 ºC and 94 ºC. The cycling parameters were 94 ºC for 10 min followed by 35 cycles of 94 ºC for 1 min and 72 ºC for 2 min. A final extension step was performed at 72 ºC for 5 min. An additional library of 96 control clones with defined nucleotide sequences was required for analysis of the OFRG data (see section 4.2.9). These clones were processed alongside the sample library in three replicate 384 well plates, each containing 4 replicates of the 96 control clones.

4.2.7 Array printing

The PCR products were printed onto nylon membranes using a QPix robot (Genetix) as described by Bent et al. (2006). In addition to the 768 clones from each of the coral samples, 3 replicates of the 384 control clone library were included in the arrays, giving a total of 9600 clones per array. A total of 45 macroarrays (11 cm × 7 cm each) were printed. These replicate arrays were used in the hybridisation experiments.

4.2.8 Array hybridisation

38 separate hybridisation experiments were performed as described by Valinsky et al. (2004). The tenmer bacterial probes (Table 4.1) were designed using a previously described simulated annealing algorithm (Borneman et al. 2001).

Following hybridisation each membrane was washed twice in 1×SSC buffer for 15 minutes. The wash conditions were identical for all probes. After washing the arrays were allowed to dry completely, then taped to stiff paper to hold them flat and exposed directly to a phosphoimaging screen (Bio-Rad). The screens were scanned using a Personal Molecular Imager (Bio-Rad). Probes B39 and B74 were not included in the final analysis, as they did not produce useful signals due to an error made during the hybridisation process.

Table 4.1 Tenmer DNA probes used in hybridisation experiments. The reference probe is designed to hybridise to all clones (see section 4.2.9)

Probe Name	**Sequence**	**Probe Name**	**Sequence**
Reference	GCTGCTGGCA	B64	GCTAACGCAT
B2	GGGCGAAAGC	B66	CATTCAGTTG
B5	GAGACAGGTG	B73	GTCTCAGTTC
B21	CCAGACTCCT	B74	AGGCTAGAGT
B25	CGTGGGGAGC	B79	CAACCCTTGT
B30	ACGTAATGGT	B80	GCGTGAGTGA
B31	TCCAGAGATG	B87	GGTGCAAGCG
B38	CCTTCGGGAG	B89	AGCTAGTTGG
B39	CCTACCAAGG	B92	AATACCGGAT
B42	GATGAACGCT	B94	TGTGGGAGGG
B44	GTGGGGTAAA	B98	CCCGCACAAG
B46	GGTAATGGCC	B99	CGGTACAGAG
B51	CCGTGAGGTG	B100	ACCAAGGCAA
B52	AGTCGAACGG	B101	TTGCCAGCGG
B56	TTGGTGAGGT	B102	ACCGCGAGGT
B57	GCCGTAAACG	B106	GCCTTCGGGT
B58	GTAACGGCTC	B107	TTGCCAGCAT
B59	CCGCAAGGAG	B108	GCTAACGCGT
B63	GAACGCTGGC	B109	ACGGTACCTG

4.2.9 Construction of hybridisation fingerprints from array data

Analysis of the array information was performed as described by Bent et al. (2006). OFRG produces a hybridisation fingerprint for each clone by assigning a score of 0, 1 or N to each probe/clone combination according to whether the probe hybridised or did not hybridise to the clone. An example of a fingerprint obtained for a single clone using a combination of 35 probes is 00100001000011001101000101l0N001001. These scores are generated by quantifying the intensity of spots on the arrays using suitable array analysis software. The quantified data are then transformed by defining threshold intensities for the assignment of positive (1), negative (0) and uncertain (N) scores according to the hybridisation patterns of the control clones, which have defined nucleotide sequences and theoretically calculated expected fingerprints.

In this work, hybridisation data were quantified using Imagene v5.6 software (BioDiscovery Inc., Segundo, CA). The program's output consists of signal intensity values for the spots on the arrays, and the background area surrounding each spot. Background values were subtracted from signal intensity values using a background subtraction macro in excel (Bent et al. 2006). The background-subtracted signal intensities were then normalised by dividing the values obtained from the differential probes by the values obtained from the reference probe, which is designed to hybridise to all clones. The normalised intensity values were transformed into 1, 0 or N scores according to the threshold values determined using the 96 control clones as described by (Bent et al. 2006). This process resulted in a fingerprint for each clone.

4.2.10 Fingerprint analysis

To place the clones into closely related taxonomic groups, fingerprints were clustered using the Greed Clique Partition Package Tool (GCPAT) (Figueroa et al. 2004), which first resolves uncertain 'N' values and clusters identical fingerprints using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm (Swofford 2002). Since clusters consist of groups of clones with the same fingerprint, each cluster can be defined as an operational taxonomic unit (OTU). Clusters consisting of six or more clones were subjected to correlation analysis using SAS v8.2 (SAS Institute, Inc., Cary, NC) in order to identify OTUs that contained significantly higher numbers of clones in each coral tissue category (H, M, D or C). Because the number of replicate samples was not the same in each category, and because the cloning and array printing process unavoidably results in the loss of some clones from the final array, the numbers of clones in each OTU had to be adjusted to account for the total sample size (number of clones) in each sample category. This was done by dividing the number of clones in each OTU by the total number of clones in each array. An arbitrary variable X of either 1 or 0 was then assigned to each sample category, and correlations were run between the number of clones per category and X for each OTU. OTUs that were positively correlated with categories M (margin of disease lesion) or C (control colonies unaffected by disease) with $p<0.3$ were selected for identification by nucleotide sequence analysis. Note that a large p-value was used because the aim was to identify interesting OTUs rather than perform a rigorous statistical analysis of the data.

To quantify the overall levels of similarity between each clone library, the nonparametric maximum likelihood estimator of similarity θ (Yue & Clayton 2005)

was calculated for every possible pairwise comparison between libraries. Two replicate libraries were randomly selected from each sample category and pooled. Calculations were performed using SONS (Schloss & Handelsman 2006), which allows comparisons to be made between sets of community data based on user-defined operational taxonomic units, rather than discrete species.

4.2.11 Comparison of OFRG survey with culture-dependent survey

Because the culture based survey (Chapter 2) had suggested that some groups of bacteria, particularly *Vibrio* species, are associated with SWS affected *T. mesenterina*, an experiment was conducted to find out if these culturable bacteria could be detected in the clone libraries used for the OFRG experiment. All the steps of this experiment were performed at UNE. Hybridisation fingerprints of 109 of the cultured bacterial isolates described in chapter 3 (Table 4.2) were constructed so that the clone libraries could be screened for their presence. Revival of the isolates from glycerol stocks, growth in broth, and extraction of genomic DNA was performed as described previously (Section 3.2). The 16S rRNA gene was then amplified from each genomic DNA extract using the modified universal PCR primers and cycling conditions described above (Section 4.2.4). The PCR products were also assembled into the pNEB206a vector and transformed into *E. coli* DH5α as described above (Section 4.2.5). White colonies were manually picked from LB/ampicillin/X-gal agar plates using sterile toothpicks and transferred to separate wells in 384 well culture plates held on ice. Each well contained 60μL LB media supplemented with 8% glycerol and 100 μg/mL ampicillin. The culture plates were kept on ice and a higher volume of culture medium per well was used to prevent excessive evaporation from the wells. This was necessary because manual picking of colonies was much slower than

automatic picking using a robot. The culture plates were grown in a shaking incubator (300 rpm) for 16 hours at 37 ºC. Once grown, the clone library was stored at -70 ºC.

Re-amplification of the 16S rDNA inserts from the clone library was as described above (section 4.2.6), except that rather than performing the PCRs in waterbaths, they were performed in batches of 50 reactions in 25 µl volumes in separate tubes in a thermocycler. This was done because of difficulties associated with maintaining a waterbath at 94 ºC in the laboratory at UNE. A control clone library consisting of a single 384 well plate with four replicates of the same 96 clone library used in the culture independent experiment was amplified alongside the marine bacterial clones so that each array consisted of the 384 control clones plus the 109 clones derived from cultured isolates.

The PCR products were transferred to 384 well plates, then arrays were constructed manually by transferring 1 µl from each well to nylon membranes using a 384-pin split pin replicator (V&P Scientific, San Diego, California). The 25 µl PCR volume used for each clone allowed a total of 10-15 replicate arrays to be printed before printing became inconsistent (spots missing). The DNA was cross-linked to the membranes by two minutes exposure to ultraviolet light on a previously calibrated UV transilluminator.

Table 4.2 Cultured bacterial isolates from which 16S rRNA gene clones were produced for fingerprint construction

Array Position	Isolate Name	RDP Classifier Taxon
A1	2.3.05 3DC5	Unclassified Gammaproteobacteria
A2	2.3.05 3DS5	Unknown
A3	2.3.05 3DC2	Rhodobacteraceae (genus Silicibacter)
A4	2.3.04 3DS4	Incertae sedis 7 (genus Agarivorans)
A5	2.3.05 3DC1	Vibrionaceae (genus Vibrio)
A6	2.3.05 2DS3	Vibrionaceae (genus Vibrio)
A7	2.3.05 3HC2	Moraxellaceae (genus Psychrobacter)
A8	2.3.05 3CC4	Rhodobacteraceae (genus Silicibacter)
A9	2.3.05 2MS3	Vibrionaceae (genus Vibrio)
A10	2.3.05 3MS1	Unclassified Rhodobacteraceae
A11	2.3.05 3HS5G	Pseudoalteromonadaceae (genus Pseudoalteromonas)
A12	2.3.05 3MS4	Vibrionaceae (genus Vibrio)
A13	2.3.05 3DC4	Unclassified Rhodobacteraceae
A14	2.3.05 3CC3	Flavobacteriaceae (genus Winogradskyella)
A15	2.3.05 3HS1	Unclassified Rhodobacteraceae
A16	2.3.05 2DS4	Vibrionaceae (genus Vibrio)
A17	2.3.05 2MS5	Vibrionaceae (genus Vibrio)
A18	2.3.05 3DS2	Pseudoalteromonadaceae (genus Pseudoalteromonas)
A19	2.3.05 2HS2	Vibrionaceae (genus Vibrio)
A20	2.3.05 3MS3	Vibrionaceae (genus Vibrio)
A21	2.3.05 3DS3	Vibrionaceae (genus Vibrio)
A22	11.5.05 2CC11	Vibrionaceae (genus Vibrio)
A23	11.5.05 1CC1	*Unclassified Vibrionaceae*
A24	11.5.05 2CS7	Moraxellaceae (genus psychrobacter)
B1	11.5.05 3CC1	Vibrionaceae (genus Vibrio)
B2	11.5.05 2CS6	Vibrionaceae (genus Vibrio)
B3	11.5.05 2CC8	Rhodobacteraceae (genus Silicibacter)
B4	10.5.05 1CC1	Moraxellaceae (genus psychrobacter)
B5	11.5.05 2CS1	Vibrionaceae (genus Vibrio)
B6	11.5.05 2CS9	Vibrionaceae (genus Vibrio)
B7	4.5.04 12HS3	Vibrionaceae (genus Vibrio)
B8	4.5.04 12DS11	Vibrionaceae (genus EnteroVibrio)
B9	4.5.04 12MS5	Vibrionaceae (genus Vibrio)
B10	4.5.04 12MS1	Vibrionaceae (genus Vibrio)
B11	4.5.04 12CS13	Unknown
B12	4.5.04 12CS11	Vibrionaceae (genus Vibrio)
B13	4.5.04 12DS6	Vibrionaceae (genus Vibrio)
B14	4.5.04 12MS2	Vibrionaceae (genus Vibrio)
B15	4.5.04 12CS9	Vibrionaceae (genus Photobacterium)
B16	4.5.04 12MS4	Vibrionaceae (genus Vibrio)
B17	4.5.04 12CS12	Rhodobacteraceae (genus Paracoccus)
B18	11.5.05 3CC5	Unknown
B19	4.5.04 12CS8	Vibrionaceae (genus Vibrio)
B20	4.5.04 12CS1	Vibrionaceae (genus Vibrio)
B21	4.5.04 12MS3	Vibrionaceae (genus Vibrio)
B22	4.5.04 12CS7	Vibrionaceae (genus Photobacterium)
B23	4.5.04 12DS3A	Vibrionaceae (genus Vibrio)
B24	4.5.04 12HS4	Vibrionaceae (genus Vibrio)
C1		Unknown
C2	11.5.05 2CS2	Vibrionaceae (genus Vibrio)
C3	11.5.05 1CS8	Shewanellaceae (genus Shewanella)
C4	4.5.04 12DS8	Pseudoalteromonadaceae (genus Pseudoalteromonas)
C5	10.5.05 1CS1	Vibrionaceae (genus Vibrio)

Array Position	Isolate Name	RDP Classifier Taxon
C6	10.5.05 1CC4	Pseudomonadaceae (genus Pseudomonas)
C7	11.5.05 3CC7	Moraxellaceae (genus psychrobacter)
C8	11.5.05 1CS10	Sphingomonadaceae (genus Erythrobacter)
C9	4.5.04 12DS1	Vibrionaceae (genus Vibrio)
C10	11.5.05 1CS4	Shewanellaceae (genus Shewanella)
C11	11.5.05 1CS2	Moraxellaceae (genus Acinetobacter)
C12	11.5.05 2CC2	Vibrionaceae (genus Photobacterium)
C13	4.5.04 12MS7	Vibrionaceae (genus Vibrio)
C14	11.5.05 3CC6	Vibrionaceae (genus Vibrio)
C15	4.5.04 12CS2a	Vibrionaceae (genus Vibrio)
C16	11.5.05 1CS3	Sphingomonadaceae (genus Erythrobacter)
C17	4.5.04 12CS4	Vibrionaceae (genus Vibrio)
C18	10.5.05 1CC3	Sphingomonadaceae (genus Erythrobacter)
C19	11.5.05 2CC7	Vibrionaceae (genus Vibrio)
C20	10.5.05 1CS6	Unknown
C21	4.5.04 12CS3	Vibrionaceae (genus Vibrio)
C22	11.5.05 3CC8	Alteromonadaceae (genus Alteromonas)
C23	4.5.04 12MS6	Vibrionaceae (genus Vibrio)
C24	4.5.04 12DS4	Vibrionaceae (genus Vibrio)
D1	11.5.05 2CC6	Vibrionaceae (genus Vibrio)
D2	11.5.05 3CC6	Vibrionaceae (genus Vibrio)
D3	4.5.04 12DS2	Vibrionaceae (genus Vibrio)
D4	4.5.04 12CS9	Vibrionaceae (genus Photobacterium)
D5	11.5.05 1CS9	Moraxellaceae (genus Acinetobacter)
D6	4.5.04 12HS1	Vibrionaceae (genus Vibrio)
D7	4.5.04 12MS4	Vibrionaceae (genus Vibrio)
D8	11.5.05 1CS6	Rhodobacteraceae (genus Paracoccus)
D9	11.5.05 2CS8	Vibrionaceae (genus Vibrio)
D10	11.5.05 1CS4	Shewanellaceae (genus Shewanella)
D11	11.5.05 1CS1	Moraxellaceae (genus Acinetobacter)
D12	4.5.04 12MS6	Vibrionaceae (genus Vibrio)
D13	2.3.05 2DS4	Vibrionaceae (genus Vibrio)
D14	2.3.05 2MS3	Vibrionaceae (genus Vibrio)
D15	2.3.05 3HS2	Shewanellaceae (genus Shewanella)
D16	2.3.05 3MS5	Vibrionaceae (genus Vibrio)
D17	2.3.05 3DS3	Vibrionaceae (genus Vibrio)
D18	11.5.05 2CC10	Rhodobacteraceae (genus Silicibacter)
D19	4.5.04 12MS2	Vibrionaceae (genus Vibrio)
D20	10.5.05 1CS4	Shewanellaceae (genus Shewanella)
D21	11.5.05 3CC2	Unknown
D22	11.5.05 1CS2	Moraxellaceae (genus Acinetobacter)
D23	11.5.05 3CC1	Vibrionaceae (genus Vibrio)
D24	11.5.05 1CC4	Vibrionaceae (genus Vibrio)
E1	11.5.05 2CC4	Moraxellaceae (genus psychrobacter)
E2	11..5.05 1CC2	Vibrionaceae (genus Vibrio)
E3	11.5.05 2CC5	Vibrionaceae (genus Vibrio)
E4	2.3.05 2MS2	unclassified Gammaproteobacteria
E5	2.3.05 2DS5	Unclassified Rhodobacteraceae
E6	11.5.05 2CC1	Vibrionaceae (genus Vibrio)
E7	11.5.05 1CS5	Moraxellaceae (genus Acinetobacter)
E8	2.3.05 3MS2	Rhodobacteraceae (genus Silicibacter)
E9	2.3.05 2HS1	Unclassified Gammaproteobacteria
E10	2.3.05 3DC9	Vibrionaceae (genus Vibrio)
E11	2.3.05 3DC3	Vibrionaceae (genus Vibrio)
E12	2.3.05 2DS2	Vibrionaceae (genus Vibrio)
E13	2.3.05 3DS1	Unclassified Rhodobacteraceae
E14	10.5.05 1CS4	Unknown

Hybridisation of the membranes with the oligonucleotide probes was conducted as described above (section 4.2.8). Following hybridisation the membranes were washed twice in 1 × SSC buffer for 15 minutes. The wet membranes were then covered with plastic wrap and exposed to autoradiography film (Kodak BioMax MR) for 12 – 24 hours at room temperature. The films were developed manually. Hybridised probes were stripped from the membranes by the addition of boiling stripping buffer (1 × SSC, 0.1 % SDS, 200 mM Tris, pH 7.5). The stripping step was performed twice for each membrane. After stripping, the membranes were reused for further hybridisations. No membrane was reused more than three times. Analysis of the hybridisation signals was performed as described above (section 4.2.9).

4.3 Results

4.3.1 Evaluation of DNA extraction procedures.

In all cases the TRFLP profiles obtained from DNA extracts prepared using the KIT protocol were more diverse, with higher signal intensities than the profiles obtained using extracts prepared by the UREA protocol (eg. Figure 4.2, see Appendix 3 for all TRFLP profiles constructed). The KIT protocol was therefore used to prepare all DNA extracts used in the OFRG analyses.

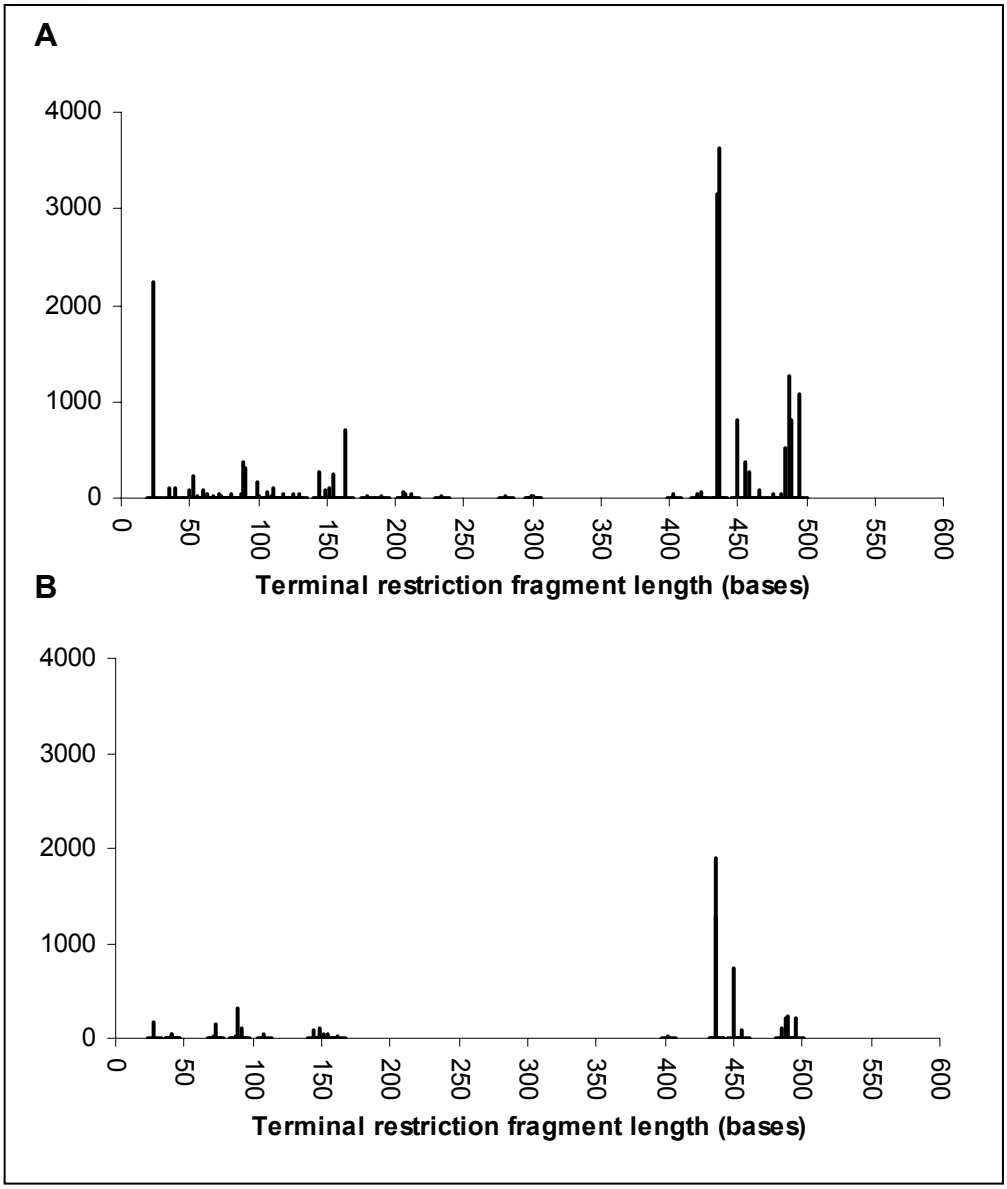

Fig. 4.2 TRFLP profiles of 16S rDNA amplified from total DNA extracted from coral sample 1M and digested with *Msp*I The extract produced using KIT method (a) contained a higher diversity of ribotypes and gave a stronger signal than the extract produced using UREA method (b).

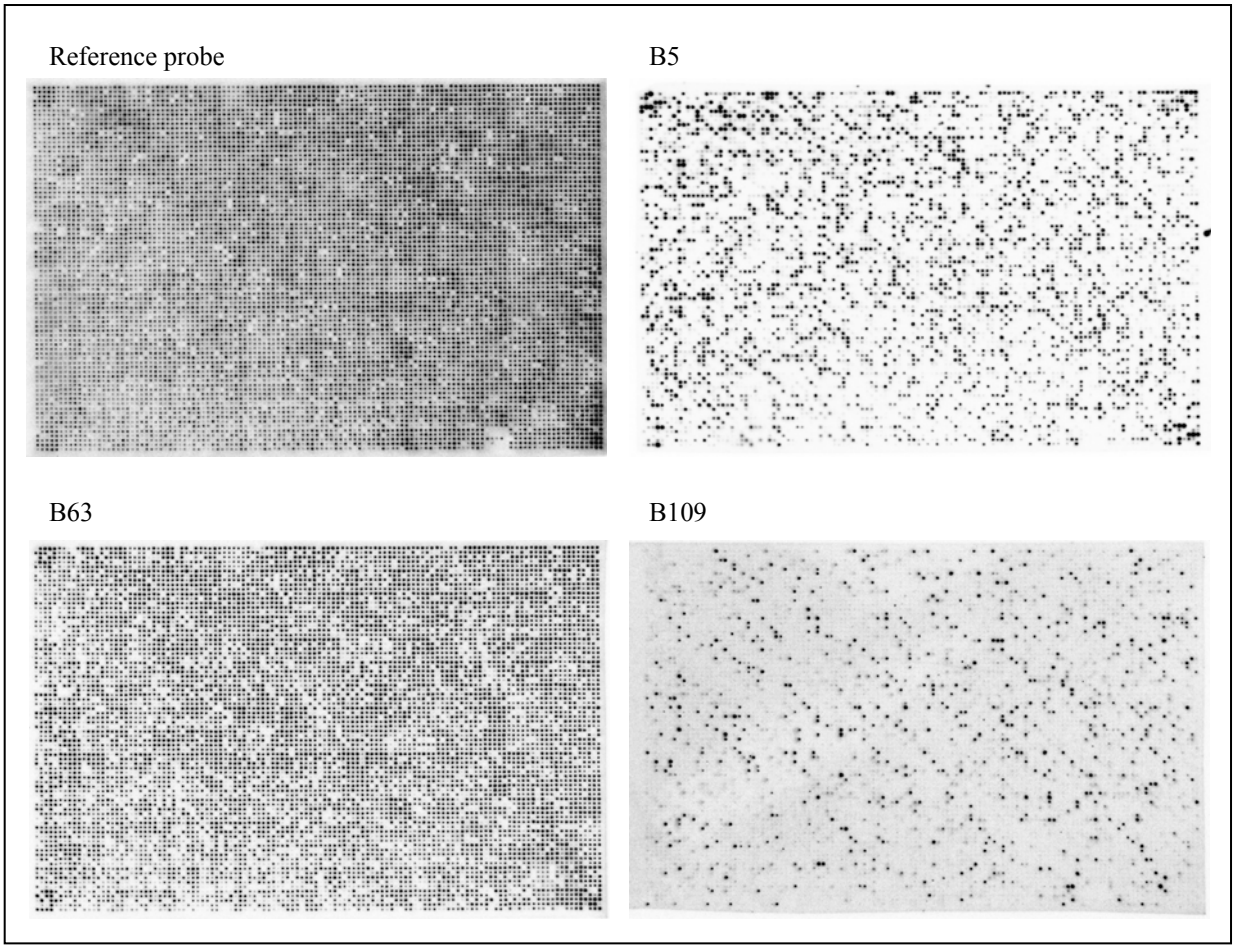

Figure 4.3 Examples of images produced by hybridisation of labelled oligonucleotide probes with 9600 clone macroarrays. The probe used in each of these arrays is indicated. The reference probe is designed to hybridise to all clones. The blank spaces in the reference probe array correspond to positions where either the reamplification or printing of 16S rRNA genes was unsuccessful. Probes B5, B63 and B109 are differential probes.

4.3.2 Hybridisation fingerprints and clustering of clones

Figure 4.3 illustrates examples of the hybridisation patterns produced with the reference probe and differential probes. The 9600 clones analysed in the OFRG experiment produced a total of 8094 fingerprints. GCPAT analysis of these fingerprints grouped them into 3208 clusters of clones with identical fingerprints. Nucleotide sequence identity within clusters ranged from 90.0% to 100.0%, with a mean identity level of 97.9% (data not shown). It has been previously demonstrated that the OFRG method is able to discriminate between 16S rDNAs at near-species

level (Valinsky et al. 2002). The clusters generated can be considered operational taxonomic units (OTUs). Of these 3208 OTUs, 201 contained six or more clones, and 43 of these (comprising a total of 1527 clones) were selected for identification by sequence analysis. (Table 4.3). Of the 43 OTUs identified by sequence analysis, 33 were significantly more abundant in the active margin of disease lesions (M), and approximately half (16) of these OTUs were present in all M samples and absent from all unaffected control (C) samples.

4.3.3 Identities of selected OTUs.

BLAST analyses indicate that the majority of the bacterial clones were of marine origin (Table 4.3). Of the 43 OTUs identified by sequence analysis only three (OTUs 248, 2921 and 2937) were identified as closely related to bacteria of terrestrial origin. Six of the 43 OTUs selected for 16S sequencing and BLAST analysis were identified as members of the α-proteobacteria division. These OTUs represented 77.5% of the subset sample of 1527 clones. Other bacterial taxa represented included γ-proteobacteria (5.5% of clones), Cyanobacteria (3% of clones), Cytophaga-Flexibacter-Bacteriodes (6%), Firmicutes (7.5% of clones) and Verrucomicrobia (0.5% of clones). These proportions agree with those recently reported for the bacterial communities associated with Caribbean acroporid corals affected by white band disease (Pantos & Bythell 2006).

Of particular interest are OTUs 530 and 534 (Table 4.3, highlighted) which exhibited high levels of sequence similarity (≥97%) to *Rhodobactereaceae* species associated with juvenile oyster disease (Boettcher et al. 2005) and black band disease in corals (Cooney et al. 2002), respectively. Both of these OTUs were present in all samples of

the active margin of disease lesions (M) and absent from samples of unaffected control colonies (C). OTU 530 was closely related (97% sequence identity) to *Roseovarius crassostreae*, the apparent aetiological of juvenile oyster disease (Boettcher et al. 2000, Boettcher et al. 2005). Bacteria related to this pathogen have also been detected in association with corals affected by a number of other disease conditions (Cooney et al. 2002, Pantos et al. 2003, Pantos & Bythell 2006).

Table 4.3 OTUs identified by sequencing representative clones. The nearest relatives of only one of each of the two clones sequenced per OTU is reported. Values in bold indicate the sample categories in which each OTU was significantly more abundant.

OTU	Nearest relative [accession number]	% Identity to nearest relative	RDP classifier taxon (80% confidence threshold)	Mean number of clones per sample category			
				H	M	D	C
OTUs significantly abundant in margin of disease lesion							
175	Uncultured alpha proteobacterium from squid mucus [AJ633968]	99	*Rhodobactereaceae* (Genus *Silicibacter*)	56	**129**	34	21
204	Marine bacterium ATAM173a_16 [AF359531] isolated from a culture of Alexandrium tamarense (dinoflagellate)	96	*Rhodobactereaceae* (unclassified)	2	**7**	4	6
210	*Thalassobius mediterraneus* [AJ878874]	99	*Rhodobactereaceae* (unclassified)	3	**12**	5	1
265	Uncultured bacterium (from sponge) [AY845242]	98	*Rhodobactereaceae* (Genus *Roseobacter*)	10	**26**	19	4
329	Rhodobacteraceae bacterium [AY177714]	98	*Rhodobactereaceae* (Genus *Roseovarius*)	2	**17**	4	3
307	Rhodobacteraceae bacterium [AY177714]	*98*	*Rhodobactereaceae* (Genus *Roseovarius*)	2	**3**	1	1
384	Rhodobacteraceae bacterium [AY177714]	98	*Rhodobactereaceae* (Genus *Roseovarius*)	1	**2**	0	0
388	Rhodobacteraceae bacterium [AJ810844]	*96*	*Rhodobactereaceae* (unclassified)	1	**4**	2	0

OTU	Nearest relative [accession number]	% Identity to nearest relative	RDP classifier taxon (80% confidence threshold)	Mean number of clones per sample category			
				H	M	D	C
449	Uncultured alpha proteobacterium (from squid) [AJ633989]	96	*Rhodobactereaceae* (unclassified)	0	**7**	3	0
452	Uncultured bacterium (from seafloor sediment) [AY35417]	90	Unclassified Gammaproteobacteria	0	**5**	1	0
459	Uncultured epsilon proteobacterium (deep sea isolate) [AB113178]	90	Unclassified Bacteria	0	**5**	1	0
469	Marine alpha proteobacterium [AJ391182]	93	*Rhodobactereaceae* (unclassified)	2	**5**	2	2
480	*Thalassobius mediterraneus* [AJ878874]	99	*Rhodobactereaceae* (unclassified)	1	**10**	4	2
491	Uncultured bacterium (sea sediment) [AY373402]	93	Unclassified Gammaproteobacteria	1	**10**	6	0
497	Rhodobacteraceae bacterium (from fish farm biofilm) [AY962292]	96	*Rhodobactereaceae* (unclassified)	1	**7**	4	3
521	Uncultured bacterium (from sponge) [AY845242]	98	*Rhodobactereaceae* (Genus *Roseovarius*)	1	**5**	2	0
525	Uncultured *Thiothrix* (from marine amphipod) [AY426613]	92	*Thiotrichaceae* (Genus *Thiotrix*)	0	**2**	1	0
530	*Roseovarius crassostereae* [AF114484] tied with CVSP bacterium [AF114485] (both associated with juvenile oyster disease)	97	*Rhodobactereaceae* (Genus *Jannaschia)*	<1	**11**	2	0
534	Uncultured alpha proteobacterium (associated with black band disease in corals) [AF473915]	97	*Rhodobactereaceae* (unclassified)	0	**3**	<1	0
539	Uncultured Bacteroidetes (from hypersaline microbial mat) [DQ330308]	89	*Saprospiraceae* (Unclassified)	0	**4**	0	0
562	Flavobacterium (artic marine sediment) [DQ514308]	93	*Flavobacteriaceae (Genus Algibacter)*	0	**4**	1	1

OTU	Nearest relative [accession number]	% Identity to nearest relative	RDP classifier taxon (80% confidence threshold)	Mean number of clones per sample category			
				H	M	D	C
609	Marine Flavobacterium [AY028207]	95	*Flavobacteriaceae (Unclassified)*	0	**2**	1	0
616	*Thiothrix* sp. (marine isolate) [DQ067608]	96	*Thiotrichaceae* (Genus *Thiotrix*)	0	**6**	0	0
851	*Plectonema* sp. (cyanobacterium) [AF091110]	94	Unclassified cyanobacteria	0	**4**	2	0
1017	Uncultured marine bacterium [DQ071069]	96	*Verrucomicrobiaceae* (Genus *Verrucomicrobium*)	1	**2**	0	1
1310	Three-way tie; Pleurocapsa sp. [X78681], Myxosarcina sp. [AJ344561], Pleurocapsa sp. [AJ344561	95	Unclassified subsection 2 Cyanobacteria	0	**3**	0	0
1574	*Thalassobius mediterraneus* [AJ878874]	97	*Rhodobactereaceae* (unclassified)	0	**3**	0	1
2011	Marine bacterium Flavobacterium [AY028207]	95	*Flavobacteriaceae* (Unclassified)	0	**3**	0	0
2049	*Roseovarius crassostereae* [AF114484] tied with CVSP bacterium [AF114485] (both associated with juvenile oyster disease)	96	*Rhodobactereaceae* (unclassified)	0	**2**	1	0
2051	Marine bacterium Flavobacterium [AY028207]	95	*Flavobacteriaceae* (Unclassified)	0	**10**	1	0
2094	*Thiothrix* sp. [DQ067608]	96	*Thiotrichaceae* (Genus *Thiotrix*)	0	**3**	0	0
OTUs significantly abundant in apparently healthy tissue of disease affected colonies							
195	Uncultured alpha proteobacteria (associated with whale carcass) [AY922246]	97	*Sphingomonadaceae* (Genus *Sphingopyxis*)	**3**	0	<1	<1

OTU	Nearest relative [accession number]	% Identity to nearest relative	RDP classifier taxon (80% confidence threshold)	Mean number of clones per sample category			
				H	M	D	C
270	Escherichia coli 536 [CP000247]	99	*Enterobacteriaceae* Genus *Escherichia*	**2**	1	0	1
285	Uncultured *Bactereroidetes* bacterium (asscociated with sea urchin) [AY222319]	96	Unclassified *Bacteroidetes*	**2**	0	<1	1
OTUs significantly abundant in unaffected control colonies							
1094	*Muricauda* sp. (Marine isolate) [AY576776]	93	*Flavobacteriaceae* (Genus *Muricauda*)	1	0	0	**11**
2802	Uncultured *Bactereroidetes* bacterium (marine bacterioplankton) [AY580710]	94	Unclassified *Bacteroidetes*	0	0	<1	**5**
2921	Uncultured bacterium (isolated from rainbow trout gut) [AM179918.1]	99	*Clostridiaceae (Genus Sarcina)*	0	1	0	**54**
2937	Uncultured bacterium (from pig gut) [AF371938.1]	95	Unclassified *Clostridales*	0	0	0	**5**
269	Uncultured gamma proteobacteria (marine picoplankton) [AY726915.1]	89	Unclassified Gammaproteobacteria	3	2	0	**7**

4.3.4 Community structure comparisons

All treatment categories were very diverse (Simpson's index (1-D) > 0.9). Comparative analysis of bacterial community structure was performed by calculating the Yue and Clayton estimator of similarity (θ) (Yue & Clayton 2005) for pairwise comparisons of randomly pooled pairs of replicates from each sample. The results indicate strong partitioning of bacterial communities between dead, apparently healthy and disease affected coral sections (Table 4.4). Samples from the active disease lesion (M) exhibited the highest degree of community similarity with samples from the dead (D) and apparently healthy (H) sections of disease affected corals. This

is expected, as the M samples unavoidably contained some of the dead and apparently healthy coral which occurred on either side of the very narrow margin of the lesion. Much lower degrees of similarity were observed between the dead and apparently healthy sections. Samples from completely unaffected control colonies (C) exhibited low degrees of similarity with all samples from disease affected colonies, including the apparently healthy tissue, indicating a whole-organism response to the disease.

Figure 4.4 illustrates the differences between sample categories. It is particularly notable that a number of OTUs appeared in the healthy corals unaffected by SWS (C) that did not appear in the apparently healthy tissues of SWS affected colonies (H).

Table 4.4 Values of the Yue and Clayton nonparametric maximum likelihood estimator of similarity θ for pairwise comparisons of each sample category. The numbers in the table are the means of the statistic calculated for every possible pairwise comparison between the sample categories. The θ statistic is an estimator of similarity scaled between 0 and 1, where 0 indicates no similarity between populations, and 1 indicates identical community composition.

Sample Category	Margin	Dead	Control
Healthy	0.462600	0.229664	0.277400
Margin	-	0.420750	0.209600
Dead		-	0.115557

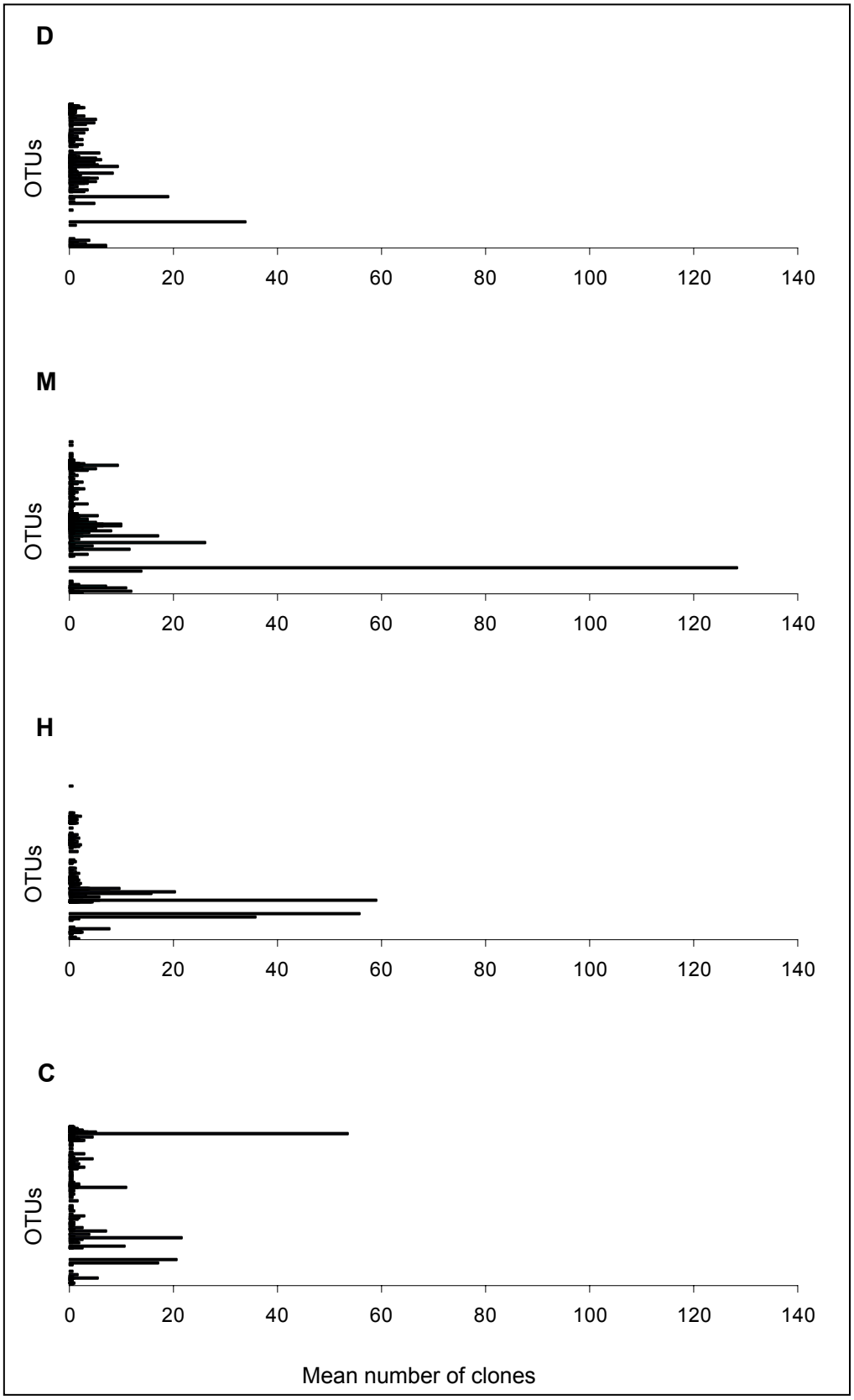

Figure 4.4 Comparative profiles displaying mean numbers of clones for all OTUs in each sample category (D) Dead skeleton of SWS affected colonies, (M) Active disease margin, (H) Apparently healthy tissue of SWS affected colonies, (C) Healthy control colonies unaffected by disease.

4.3.5 Comparison of OFRG survey with culture-dependent survey

A number of technical problems were encountered when attempting to construct hybridisation fingerprints for 16S rRNA genes of the cultured bacteria. The hybridisation experiments produced unexpected results in the control clone library, with many clone/probe combinations that were not expected to hybridise giving positive signals. Several strategies were used to try to rectify this problem, including reducing the probe concentration, and increasing the temperature of the wash steps from 11 ºC to room temperature (data not shown). The non-specific probe binding could not be sufficiently reduced however, and the fingerprint data obtained from this experiment was not useable. Three examples of the 37 array images produced in this experiment, demonstrating the high levels of non-specific probe binding are presented in Appendix 4.

4.4 Discussion

This is the first reported culture-independent assessment of the bacterial communities associated with the subtropical coral *Turbinaria mesenterina*. The efficacy of the OFRG method for analysing an array of 9600 16S rDNA clones has been demonstrated previously (Bent et al. 2006). It has been previously argued that the libraries used to represent diverse bacterial communities from many environments are often not large enough (Kemp & Aller 2004). The advantage of this high-throughput technique over other bacterial community profiling methods is that it allows very large clone libraries to be analysed, without the expense of extensive nucleotide sequence analysis. The shortcoming of the OFRG method is its inability to resolve closely related bacterial ribotypes to acceptable taxonomic levels. As the probe set

used in this study was originally developed for profiling bacterial communities in soil, it was not known how well it would be able to differentiate between closely related marine bacteria. For this reason, the resolution generated by the probe set was checked by sequencing two clones from each OTU identified. Some OTUs belonging to the *Rhodobactereacae* family exhibited some degree of dissimilarity between sequenced clones, with 16S rDNA sequence identities as low as 90%. The 16S rDNA sequences of clones from these OTUs will be included in future datasets used to generate probe sequences in order to improve resolution in this family (Elizabeth Bent, personal communication). The resolution of other taxonomic groups in this study was high. In most cases 16S sequences of clones belonging to the same OTU were >97% identical (data not shown), indicating near-species level resolution.

Recent development in OFRG fingerprint analysis techniques has revealed that it is possible to infer higher order taxonomic relationships from dendograms produced by UPGMA clustering of fingerprints (Elizabeth Bent, personal communication). It should therefore be possible to define larger OTUs at the level of bacterial family, order or sub-division and make meaningful comparisons between the abundances of these OTUs in different sample categories. This technique will be applied to the data generated in this experiment before it is submitted for publication.

4.4.1 Bacterial community structure shifts

There were clear shifts in bacterial community structure as the coral moved through healthy (H), diseased (M) and dead (D) states. Moreover, the bacterial community associated with the apparently healthy tissue of colonies affected by SWS (H) differs from the community associated with completely unaffected control colonies (C). This

difference is apparent in the low level of overlap between the two communities, and in the presence of specific ribotypes which occurred in the (C) samples, but not in the (H) samples, and vice versa. This observation suggests that a shift in the bacterial community composition precedes the development of disease characteristics, and indicates a whole-organism response to the disease lesion. Such a response would be consistent with the whole-colony response to mechanical lesions observed by Oren et al. (2001). This pattern of bacterial community change also follows those described for other coral disease states such as plague like disease of *Montastrea annularis* (Pantos & Bythell 2006), and type I white band disease of *Acropora palmata* (Pantos et al. 2003). Pantos et al. (2003) proposed a number of explanations for the whole-colony effect.

The important question to address is whether the observed community shift occurs before disease develops, or in response to the presence of the disease lesion. If the shift precedes lesion development, the changes may occur as a result of an environmental stimulus that affects the bacterial community directly by favouring the growth of certain species over others, or indirectly by altering the physiology of the host. In this case, it is possible that exposure to environmental stressors such as elevated water temperature may result in a shift in the composition of the normal bacterial community associated with the corals. Shifts in coral bacterial communities in response to seasonal variation in temperature has been reported (Koren & Rosenberg 2006). A second possibility is that exposure to sediments and dissolved organic nutrients carried into the SIMP by terrestrial runoff results in perturbations of the normal bacterial communities associated with the corals. Increased concentrations of dissolved organic carbon have also been shown to cause coral disease, presumably

by creating favourable conditions for the growth of some microbes, which may then become oppurtunistic pathogens (Kuntz et al. 2005). It has also been proposed recently that some members of the bacterial community associated with corals serve a probiotic role (Reshef et al. 2006). If perturbation of the normal bacterial community resulted in the loss of species which have important roles in maintaining the health of the coral holobiont, the coral, which lacks an adaptive immune system, may become more likely to succumb to infection. The fact that many OTUs were conspicuously present in C samples and absent or present at much lower levels in H samples provides support for this idea.

An alternate possibility is that the shift in community structure is caused by infection with a pathogen, which can be present without causing visible symptoms of disease. Many bacterial pathogens of other organisms, including humans, are known to establish as systemic infections before causing any symptoms of infection (Bennett et al. 1998, Bricknell et al. 1999).

4.4.2 OTUs identified by sequence analysis

Sequence analysis of selected OTUs suggests that the bacterial communities associated with both healthy and diseased *Turbinaria mesenterina* are dominated by members of the division alphaproteobacteria. This finding contrasts with the results of the culture based survey (Chapter 2), which suggested that the bacterial communities of both healthy and SWS affected *T. mesenterina* were dominated by *Vibrio* spp. The OFRG analysis indicated that members of the γ-proteobacteria represented only a small proportion of OTUs that correlated with the disease affected corals, and the *Vibrionaceae* were not represented at all amongst the OTUs identified by sequence

analysis. It is unlikely that the failure to detect *Vibrio* species was due to bias in the PCR amplification of 16S genes from the coral samples, as the same PCR primers used in this study are effective in amplifying 16S rDNA from cultured *Vibrio* isolates. This result highlights the importance of using both culture-based and culture-independent studies in efforts to identify unknown pathogens from environmental samples.

The presence of ribotypes with high degrees of similarity to *Roseovarius crassostereae*, the bacterium suspected as the causative agent of juvenile oyster disease, is consistent with the findings reported by authors investigating the bacterial communities associated with corals affected by white band disease (Pantos & Bythell 2006), black band disease (Cooney et al. 2002) and a plague-like disease (Pantos et al. 2003). These bacteria, which belong to the diverse and cosmopolitan *Roseobacter* clade, were present in all samples from the margin of the disease lesion (M), and were not detected in samples from unaffected control colonies (C). The fact that these bacteria are associated with a range of different types of disease lesions, on a range of different coral species suggests that they may be present simply as opportunists, and are not directly responsible for causing the disease. The possible role of these bacteria as pathogens can not be ruled out however, and although attempts to isolate them in culture have thus far been unsuccessful (data not shown), future work should focus on enriching for *Roseobacter* so that they may used in infection experiments to attempt to fulfil Koch's postulates.

Chapter 5 – Discussion

5.1 Differences between culture-based and culture-independent approaches

It is well established that the vast majority of bacteria in the marine environment are not culturable using standard microbiological techniques (Amann et al. 1995, Eguchi 1999, Fuhrman et al. 2002). Culture-independent enumeration of coral-associated bacteria using DNA staining techniques has also shown that 99.8% of bacteria associated with coral mucus and tissues do not form colonies on agar media (Koren & Rosenberg 2006). These limitations mean that the bacteria isolated from *T. mesenterina* in this study represent a very small subset of the total community. It is not unexpected therefore, that the bacterial community profiles constructed in the culture dependent study differed considerably from those constructed using the OFRG method.

It was surprising however to note that although *Vibrio* species formed a substantial proportion of the culturable bacteria associated with all the corals sampled, they were not identified in the culture independent survey. An experiment designed to search the fingerprint database generated in the OFRG work for the bacteria isolated in culture did not yield useful results (Section 4.3.5, Appendix 4). It is possible that the molecular survey methods used were biased against this group of bacteria (discussed below), but *Vibrio* species have been detected in other culture independent surveys which used the same PCR primers and similar techniques (Bourne & Munn 2005). The efficacy of the PCR primers used in his work for amplifying 16S rRNA sequences of *Vibrio* spp. was also demonstrated with cultured *Vibrio* isolates (Chapter 3). As only a small subset of OTU's were identified from each clone library by

sequence analysis it is also possible that any *Vibrio* species that were present clustered in smaller OTU's and were simply missed during the selection of OTU's for identification. Since the culture based work suggested an important role for *Vibrio* spp. in the development of SWS, it is hoped that further analysis of the OFRG dataset will yield more information related to this group of bacteria (Elizabeth Bent, personal communication).

5.2 Limitations of 16S rRNA gene surveys

Although culture independent approaches to bacterial community surveys can provide a great deal of useful information, they have some serious limitations which should be considered when drawing conclusions from the data generated in these experiments. These limitations are often overlooked in the coral bacterial ecology studies reported in the literature. Each step in the molecular characterisation of bacterial communities that relies on the direct extraction of genomic DNA from environmental samples and the amplification of bacterial 16S rRNA genes has the potential to introduce biases and error (von Wintzingerode et al. 1997). During sample collection and handling care must be taken to prevent enrichment of specific bacterial groups. This can be achieved by freezing the samples as soon as possible after collection to stop microbial activity (Rochelle et al. 1994). Further biases may be introduced in the initial extraction of DNA from the sample. Differences in the efficiency of bacterial cell lysis can have serious effects. The comparative assessment of the two DNA extraction techniques used in the present study revealed substantial differences not only in the relative abundances of bacterial groups, but also in the number of unique ribotypes identified (Appendix 3). Rohwer et al. (2001) also recorded discrepancies between community profiles of coral associated bacteria that were constructed using different

DNA extraction techniques. The method of DNA extraction used should be an important consideration when comparing profiles of bacterial community structure reported by different authors.

An additional problem with most of the available techniques for extracting DNA from environmental samples is the co-purification of humic substances, which strongly inhibit DNA modifying enzymes such as *Taq* polymerase (Tebbe & Vahjen 1993). This problem was frequently encountered when attempting to amplify 16S rRNA genes from DNA extracts prepared from coral samples. Less that 50% of the samples processed yielded acceptable quantities of PCR product, even when high concentrations of high molecular weight DNA were present (data not shown). It was interesting to note that it was generally more difficult to obtain PCR product from samples of healthy living coral tissue than from dead skeleton or bleached tissues of the same species (data not shown). It is possible that derivatives of the photosynthetic pigments in living coral tissue co-purify with DNA and inhibit *Taq* polymerase.

Biases are also likely to be introduced during the amplification of 16S rRNA genes from the total DNA extract. Although 'universal' 16S primers are designed to anneal to the highly conserved regions of the gene, variations can still occur between taxa which may prevent the amplification of some rRNA genes. The primers used in this study were modified versions of the standard universal primers 27F and 1492R (Lane 1991) which included degenerate bases to allow a greater variety of rRNA genes to be amplified. Newer versions of these primers have also been developed for the construction of 16S rRNA gene libraries which contain additional degeneracies, and would be expected to allow the detection of a wider range of bacterial taxa (Valinsky

et al. 2004). Even when all possible precautions have been taken to ensure that the primers used will anneal to as wide a range of 16S rRNA genes as possible, it is likely that differences in amplification efficiencies will introduce artefacts that affect the estimation of the relative abundances of species in a sample. Sipos et al (2007) demonstrated that mismatches between primer and template can result in serious bias in estimates of relative abundances of bacterial species in mixed samples, particularly at high annealing temperatures. Other sources of bias include inconsistent copy numbers of ribosomal genes in different bacterial species (Farrelly et al. 1995), the presence of DNA-associated macromolecules that inhibit polymerase binding (Waterhouse & Glover 1993) and variable GC content in rRNA genes, which can affect template denaturation efficiency (Baskaran et al. 1996, Reysenbach et al. 1996) For these reasons it is important not to place too much emphasis on the relative proportions of bacterial species in coral samples as determined by culture independent techniques. The goal of PCR dependent molecular surveys of coral-associated bacteria should be to determine the presence or absence of certain species with potentially interesting functional roles, not to make strong statements about their relative abundances.

A further limitation of 16S based surveys is that they often provide very little useful information about the functions of the various species that make up bacterial communities. The rapid development of methodologies for molecular characterisation of microbial communities has led to the compilation of large databases of ribosomal gene sequences of uncultured microorganisms, which give meaningful information about phylogenetic relationships, but the functional aspects of these organisms have to be inferred by comparison to their closest cultured relatives. In this work, bacteria

were frequently encountered with no known close relatives. Examples included OTUs 452, 491, 601, 248 and 269 in the OFRG experiment which were classified only as 'unclassified gammaproteobacteria' by the RDP. It is important that molecular surveys of bacterial communities are complemented by culture based work designed to enrich for and cultivate interesting bacteria so that their functions may be examined.

5.3 Bacterial community shifts and disease processes

There were clear patterns in the bacterial communities associated with *T. mesenterina* that were consistently identified in both studies. An ecological succession in bacterial communities can be observed as the coral moves through healthy, SWS affected and dead states.

It is clear that the development of disease in scleractinian corals is linked with shifts in the structure of the bacterial community associated with the coral. Ritchie and Smith (1995a) first demonstrated bacterial community shifts related to white band disease in acroporids. More recently Pantos et al (2003) and Pantos and Bythell (2006) have shown that the bacterial communities found on the apparently healthy, living tissue of disease affected coral colonies differ from the communities associated with completely healthy colonies that are not affected by disease. A similar pattern has been reported for the bacterial communities associated with gorgonian corals affected by aspergillosis. Gil-Agudelo et al. (2006a) showed that surviving tissue of infected gorgonians harboured a bacterial community that was distinct from that found on unaffected colonies. The observations of bacterial community shifts associated with SWS in *T. mesenterina* are consistent with these other reports.

Substantial differences were found between the bacterial community associated with the healthy tissue of unaffected colonies and the apparently healthy tissue of SWS affected colonies in both the culture-based and culture independent surveys.

The current literature presents two alternative models of bacterial coral disease development that consider the role of the coral-associated bacterial community. The first, proposed by Rosenberg et al (2007) suggests that corals harbour specific bacterial communities which benefit the coral host by protecting it from infections by foreign bacteria (Reshef et al. 2006, Rosenberg et al. 2007). They propose that shifts in coral-associated bacterial communities allow corals to adapt to changing environmental conditions such as light and temperature (Koren & Rosenberg 2006, 2007). According to this model, infectious diseases of corals occur when an invasive pathogen is able to evade the coral holobiont's innate and bacterial defences (Reshef et al. 2006). Coral pathologies that have been shown to have simple and specific aetiologies (i.e. those caused by a single pathogenic species or a defined pathogenic consortium) may be explained by this model.

The second model, proposed by Kline et al. (2006), suggests that coral disease occurs as a consequence of perturbation of the coral-associated bacterial community. They suggest that shifts in the microbial community may result in an imbalance where overgrowth of some native bacterial species leads to a breakdown of the holobiont which manifests as disease (Kline 2004, Kuntz et al. 2005, Kline et al. 2006). They show that perturbation of the bacterial community can be triggered by nutrient loading, particularly with excess organic carbon, and suggest that the elevated levels of fixed carbon produced by macroalgal communities in close proximity to corals may

be responsible for some disease outbreaks (Smith et al. 2006). This model of coral disease draws close parallels to Marsh's (2003) description of human dental diseases as 'ecological catastrophes' that occur when the bacterial plaque communities shift away from their normal composition because of changes in environmental conditions in the mouth (specifically low pH), thereby allowing pathogenic bacteria to become dominant over probiotics (Marsh 2003, Lovegrove 2004, Kumar et al. 2006). A similar type of bacterial community shift, where the relative balance of bacterial species already present is changed by environmental cues is accepted as the cause of bacterial vaginosis in humans (Spiegel 1991, Kafarskaia et al. 2002). Kline et al.'s (2006) model of coral pathogenesis may help to explain cases of coral disease where there does not appear to be any specific aetiology.

A third possible model has also been proposed by Ainsworth et al. (2007). They showed that tissue loss in Acroporid corals affected by white syndrome on the Great Barrier Reef occurs due to programmed cell death (PCD), rather than necrosis. They also demonstrated a lack of bacterial activity at the actively advancing margins of disease lesions. On the basis of these observations, Ainsworth et al. (2007) propose that coral tissue death may occur by mechanisms that do not require the involvement of microbial pathogens. PCD can not be ruled out as at least part of the mechanism of tissue death in SWS, however, the observation that the disease is infectious and can be transmitted from diseased to healthy colonies indicates that microbial pathogens are most likely involved at some critical step in the formation of disease lesions.

Although a bacterial community shift is associated with SWS, it does not appear to be strictly analogous to the 'ecological catastrophe' that occurs in dental disease,

vaginosis and carbon loading in corals. If SWS was the result of certain bacterial groups that were already present on the coral becoming dominant due to environmental changes it would be expected that disease lesions would develop spontaneously on healthy fragments maintained under the same conditions as fragments with actively progressing lesions. This was not the case however. The results of the disease transmission experiment show that a foreign pathogen must be introduced for disease to develop, as healthy coral fragments that were maintained under the same conditions as fragments with actively progressing SWS lesions did not develop disease unless they were placed in direct contact. The pattern of SWS lesion progressions on coral colonies in the field also indicates that a pathogen must be involved. SWS lesions usually appear at one point on a colony and expand outwards. If SWS was the result of a general bacterial community shift that directly caused disease it would be expected that coral tissue mortality would occur more or less uniformly across entire affected colonies.

It is unclear whether the observed changes in the bacterial communities associated with apparently healthy tissues of corals affected by SWS and other diseases occur before or after infection. If the shift in surviving parts of the holobiont occurs after the development of disease symptoms it may mean that changes in the host physiology in response to the infection are affecting the bacterial community. It is known that entire coral colonies can make an integrated response to damage at a single site (Oren et al. 2001). When the close interdependence of the bacterial community and the host is considered in the context of the coral holobiont, it is expected that an integrated response to injury or infection by a coral colony will affect its associated bacteria. Shifts in the bacterial community are especially likely to occur if physiological

changes in the host result in altered bacterial growth conditions through, for example, changes in the amount or composition of mucus produced, or photosynthetic changes that result in different oxidative conditions in the coral tissues. Alternatively, a shift that precedes lesion development may indicate that the bacterial community has been perturbed by abiotic stresses such as increased temperature (Koren & Rosenberg 2006) or nutrient loading (Kline et al. 2006). This may occur due to direct selective pressure on the community by the abiotic stressor, or as a flow-on effect of changes in the host physiology.

In addition to the work presented in this thesis, our group has made several important observations that help to elucidate the SWS disease process, and allow informed guesses to be made regarding the order in which the processes of infection, bacterial community shift and lesion development occur. Dalton et al. (submitted, 2008) showed that SWS is more virulent at higher temperatures, with lesions on infected fragments progressing faster at 26 ºC than at 21 ºC. In addition, aquarium experiments have shown that healthy *T. mesenterina* fragments can be maintained at 26 ºC for extended periods without developing SWS symptoms (unpublished data). These observations suggest that both high temperatures and the presence of a pathogen are required for the development of SWS lesions. It is likely that the pathogen is transferred between colonies by a predatory vector. The corallivorous *Phestilla* sp. nudibranch is a good candidate vector, as aquarium observations indicate that the lesions inflicted by its grazing on *T. mesenterina* continue to expand in manner characteristic of SWS, even after the nudibranch is removed from the aquarium (Figure 5.1) (Dalton & Godwin 2006).

Figure 5.1 The corallivorous nudibranch *Phestilla* sp. grazing on *T. mesenterina*. The areas of denuded coral skeleton visible in this photograph continued to expand even after the nudibranch was removed from the coral.

Dalton et al. (submitted) also performed field experiments which showed that the apparently healthy tissues of *T. mesenterina* colonies that are already affected by SWS are more likely to succumb to a second infection by direct contact with a diseased coral fragment than the healthy tissues of colonies that were not previously affected by SWS. This observation indicates that changes in some aspect of the holobiont make it more susceptible to SWS. 100% of SWS affected colonies developed a second infection when exposed to infected fragments whilst only 60% of previously unaffected colonies did (Dalton et al. submitted, 2008). This suggests that the change in the holobiont that reduces resistance to the pathogen may be a necessary condition for infection. It follows that this change would have to occur before the pathogen comes into contact with the coral, rather than as a later response to infection. The difference in SWS susceptibility between the healthy tissues of SWS affected and unaffected colonies is consistent with the observed differences between the bacterial

communities associated with the healthy tissue of SWS affected colonies and the tissues of completely healthy colonies.

What is not clear is whether or not the reduced resistance of the coral to infection is directly related to the altered bacterial community structure. If coral-associated bacteria perform essential defensive roles as proposed by Reshef et al (2006), it is possible that the bacterial shifts observed in SWS are involved in the reduced resistance of affected colonies to further infection. There is some evidence to suggest that bacteria associated with healthy *T. mesenterina* play such roles. One of the culturable bacteria isolated from a healthy *T. mesenterina* colony in the SIMP (4.5.0412CS1) was most closely related to a *Vibrio* species known to inhibit the growth of pathogenic bacteria in molluscs (accession number AY034144.1) (Riquelme et al. Unpublished). Members of the *Shingomonadaceae* were also isolated only from healthy colonies. Although antimicrobial activity has not been recorded in the strains that were isolated (Ivanova et al. 2005), other members of the family are known to produce broad spectrum antibacterials (Romanenko et al. 2007). It is also worth noting that these isolates were most closely related to *Erythrobacter vulgaris,* which exhibits resistance to a wide range of antibacterials, suggesting that they may be adapted to live in the presence of antibacterial compounds (Ivanova et al. 2005). The OFRG survey also identified evidence of antibacterial producing species in association with healthy *T. mesenterina*. Members of the *Clostridales* were detected only in samples of living tissue, and were strongly associated with completely healthy colonies unaffected by SWS. Although these bacteria are commonly associated with pathogenesis in humans and other mammals, it has been shown that marine isolates in

this family are strongly antagonistic against other marine bacteria (Grossart et al. 2004).

Evidence of bacterial defences in the coral holobiont has also been observed in other coral species. Ritchie (2006) recorded antimicrobial activity in 20% of cultured bacteria from the mucus of *Acropora palmata*, and proposed that bacterial activity is responsible for antimicrobial properties of the mucus itself. Ritchie (2006) also noted evidence of bacterial community shifts in the apparently healthy tissue of stressed corals that potentially reduce their ability to resist infection. She reports that mucus from the apparently healthy tissues of *Acropora palmata* colonies sampled during a mass bleaching event when seawater temperatures were high did not contain antibiotic-producing bacteria and was dominated by *Vibrio* spp (Ritchie 2006). This observation provides strong support for the idea that stress-induced shifts in bacterial community composition can compromise the defences of the coral holobiont and leave it vulnerable to infection and disease.

5.4 A tentative model of the SWS disease process

When all of the information above is considered, it is possible to construct a tentative model of the processes leading to SWS lesion development in *T. mesenterina* (Figure 5.2)

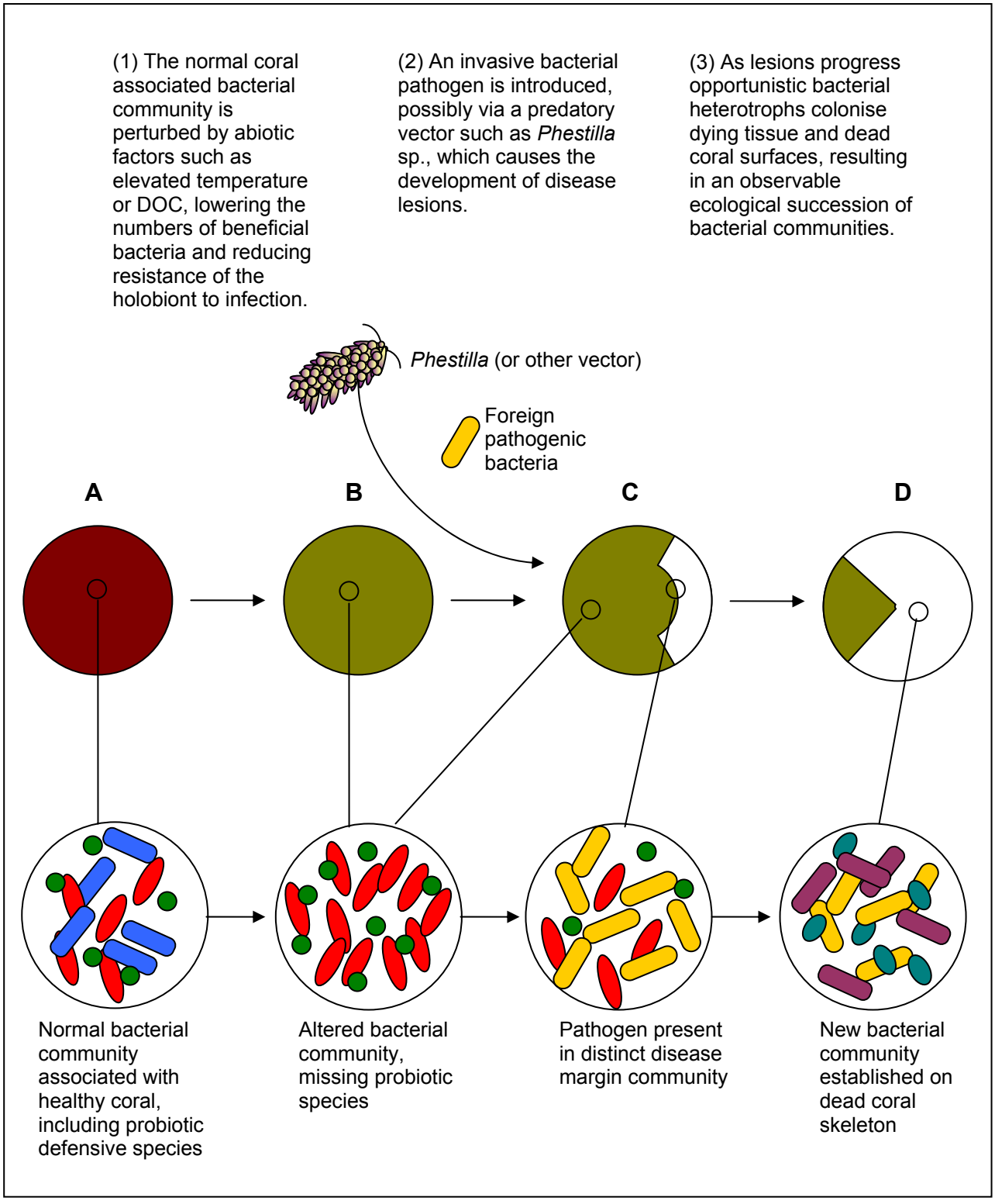

Figure 5.2 A model of the possible processes leading to SWS in *T. mesenterina*. A healthy colony (A) is exposed to a change in environmental conditions, such as elevated temperature, which results in a shift in the composition of native bacteria associated with the healthy coral tissues, possibly accompanied by changes in the physiology of the host and algal symbionts. The modified holobiont (B) remains

healthy, but is compromised in its ability to resist infection by the SWS pathogen. When the pathogen is introduced, either via a vector or when the expanding disease lesion of an adjacent, touching colony reaches the coral, it causes death of the coral tissue (C). As the disease lesion expands it leaves exposed coral skeleton in its wake (D). A succession of bacterial communities can be observed as the coral tissue progresses from healthy, to immunocompromised (stressed), diseased and dead.

5.5 Unanswered questions and avenues for future research

Although the currently available information on SWS fits the model proposed in figure 5.2, several questions will need to be addressed before it can be accepted with confidence:

(1) Can changes in the bacterial communities associated with healthy *T. mesenterina* be observed in response to abiotic stresses such as elevated temperature or nutrient levels? If so, do these changes resemble the observed differences between completely healthy colonies and the apparently healthy tissue of SWS affected colonies? The differences observed between healthy colonies at the SIMP and LHI already suggest that such changes may occur, although it is not known if these differences are due to selection pressure by abiotic factors, or are simply a result of natural variations between corals. Koren and Rosenberg (2006, 2007) have demonstrated that corals of the same species can harbour different bacterial communities under different conditions. It is likely that certain mutualistic bacterial species which form an essential part of the holobiont will be maintained across colonies of the same species under a given set of environmental conditions. However, it has been suggested that a proportion of the bacterial community associated with corals consists of transient 'visitors' which originate from the water column or other sources such as animal vectors and become trapped in the mucus layer (Ritchie 2006). Since each individual coral colony will have a unique history of interaction with 'visiting' bacteria it is

likely that some variation in bacterial community structure will naturally occur between colonies. This potential source of variation should be considered when examining differences between the bacterial communities associated with healthy corals.

(2) Is there a correlation between bacterial community structure changes and disease resistance? Although Dalton et al. (submitted, 2008) showed that apparently healthy sections of SWS affected colonies were more susceptible to second infections, experiments have not been performed to measure the susceptibility of previously unaffected colonies under stress conditions. To satisfactorily answer this question it would be necessary to assess both disease susceptibility and the associated bacterial community structure of healthy *T. mesenterina* before and after an abiotic stressor such as elevated temperature was applied.

(3) What is the identity of the bacterial pathogen involved in SWS? Although there is evidence to suggest that *Vibrio harveyi* may play an important role in SWS (Section 3.4.4) it will be necessary to verify the ability of this bacterium to infect healthy *T. mesenterina* experimentally before it can be identified as a pathogen. Future work should also focus on enriching for *Roseovarius* spp, as the results of the OFRG survey and other reported surveys of diseased corals (Cooney et al. 2002, Pantos et al. 2003) suggest that these bacteria may play a role as pathogens. Once a pathogen is verified by fulfilment of Koch's postulates it may be possible to investigate the role of putative vectors such as *Phestilla* using molecular detection techniques. Sussman et al. (2003) were able to identify the marine fireworm *Hermodice carunculata* as the vector of the coral bleaching pathogen *Vibrio shiloi*, and Pinzon et al. (2006) used

molecular probes to investigate potential reservoirs of the WPII pathogen *Aurantimonas coralicida*. A more detailed study of *Phestilla* itself woud also provide useful information about its associated bacterial communities and may lead to the discovery of more potential causative agents of SWS.

(4) Can bacteria with antagonistic activity against putative SWS pathogens be isolated from healthy or diseased *T. mesenterina*? If the causative agent of SWS is isolated in culture and verified, it will be possible to screen other culturable bacteria associated with healthy and SWS affected *T. mesenterina* for antagonistic activity against it. Radjasa et al. (2005) showed that a coral-associated *Pseudoalteromonas* species is able to inhibit the growth of *V. harveyi*, and Ritchie (2006) demonstrated antibacterial activity of bacteria associated with healthy *Acopora palmata* against the white pox pathogen *Serratia marcescens.* If bacterial antagonists of a SWS pathogen could be isolated from healthy *T. mesenterina* it would provide support for the idea that loss of probiotic species reduces the coral's resistance to infection.

5.6 Summary and conclusions

The results presented in this thesis demonstrate that SWS in *T. mesenterina* is an infectious disease which most likely has a bacterial aetiology. This is supported by observations of the bacterial communities associated with healthy and SWS affected *T. mesenterina* colonies, which indicate a succession of communities as the coral moves through apparently healthy, diseased and dead states. Furthermore, the observed differences between the bacterial communities associated with the apparently healthy tissue of surviving regions of SWS affected colonies and the tissue of completely healthy unaffected colonies indicates that SWS infection involves a shift in the bacterial community associated with the entire coral colony. Based on the

currently available information on SWS and the coral disease literature, a tentative model of the SWS disease process is proposed in which an abiotic stressor such as elevated temperature causes shifts in the native bacterial community associated with healthy corals. This shift reduces the coral's resistance to infection by lowering the numbers of probiotic bacteria which play defensive roles in the holobiont. The immunocompromised coral is infected by a pathogenic bacterium, carried by an animal vector, and SWS lesions develop. Further research is warranted to test this model.

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Appendix 1A - Cultured bacteria isolated from Split Solitary Island Samples. Isolates of interest are highlighted.

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.052DS1	EU267664	1	Ferrimonidaceae (genus Ferrimonas)	**Best hit**		
Intense purple				Ferrimonas kyonanensis [AB245514.1]	97%	Selenate-reducing bacteria belonging to the Gammaproteobacteria isolated from Tokyo Bay
				Next best hit		
				Ferrimonas futtsuensis [AB245515.1]	96%	Selenate-reducing bacteria belonging to the Gammaproteobacteria isolated from Tokyo Bay
2.3.052DS2	EU267642	>100	Vibrionaceae (genus vibrio)	**Best hit four-way tie**		
Small round, translucent				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	96%	Unknown
				Vibrio sp. YASM15 [DQ314530.1]	96%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. YASM14 [DQ314529.1]	96%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. Lu1 [AF094701.1]	96%	
				Next best hit 14-way tie		
				Vibrio sp. BA2 [EF187016.1]	96%	Coastal water sample, China
				Vibrio campbellii strain BL9 [DQ980029.1]	96%	Arsenic resistance in marine bioluminescent *Vibrio campbelli* BL9
				Uncultured gamma proteobacterium clone UA07 [DQ269050.1]	96%	Surface of marine macro-alga *Ulva australis*
				Vibrio sp. OSH4 [DQ317691.1]	96%	Luminous bacteria from fish guts
				Vibrio sp. OSUP1 [DQ317690.1]	96%	Luminous bacteria from fish guts
				Bacterium OLO3 [DQ317686.1]	96%	Luminous bacteria from fish guts
				Vibrio sp. OYSC1 [DQ317682.1]	96%	Luminous bacteria from fish guts
				Vibrio sp. OIMK4 [DQ317681.1]	96%	Luminous bacteria from fish guts
				Vibrio campbellii strain 90-69B3 [AY738129.1]	96%	Unknown
				Vibrio campbellii [AY544985.1]	96%	Unknown
				Vibrio harveyi strain UQM 2849 [AY264924.1]	96%	Unknown
				Vibrio harveyi strain LB15 [DQ146938.1]	96%	Marine luminous bacteria from shallow coastal waters of Taiwan

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Vibrio harveyi strain LB13 [DQ146937.1]	96%	Marine luminous bacteria from shallow coastal waters of Taiwan
				Vibrio harveyi strain LB4 [DQ146935.1]	96%	Marine luminous bacteria from shallow coastal waters of Taiwan
				Bacterium CWISO3 [DQ334343.1]	96%	Water column of a *Panulirus ornatus* larval rearing system
2.3.052DS3	EU267648	20	Vibrionaceae (genus vibrio)	**Best Hit**		
Round, white, textured surface				Vibrio sp. V639 [DQ146989.1]	98%	Unknown marine sample
				Next best hit tie between		
				Vibrio harveyi strain LMG4404T [AY750576.1]	98%	Unknown
				Vibrio harveyi strain NCIMB1280T [AY750575.1]	98%	Unknown

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.052DS4		>100	Vibrionaceae (genus vibrio)	**Best hit**		
	EU267630					
Probably same as 2DS2				Vibrio harveyi strain LB4 [DQ146935.1]	99%	Shallow coastal waters of Taiwan
				Next best hit five way tie		
				Uncultured bacterium isolate LCPISO4 [DQ831087.1]	99%	Stage 1 cultured, live *Panulirus ornatus* at time of a larval mass mortality event
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	99%	Unknown
				Vibrio sp. YASM15 [DQ314530.1]	99%	Pathogen in *Scopthalmus maximus* (turbot)
				Vibrio sp. YASM14 [DQ314529.1]	99%	Pathogen in *Scopthalmus maximus* (turbot)
				Vibrio sp. Lu1 [AF094701.1]	99%	Tyrrhenian Sea coastal waters off northeastern Sicily
2.3.052DS5		1	Unclassified Rhodobacteraceae	**Best hit**		
	EU267649					
Very small, round, white				Rhodobacteraceae bacterium CL-TA03 [AY962292.1]	96%	Biofilm in a coastal fish-farm
				Next best hit		
				Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	96%	East China Sea
2.3.052DS6 Medium, round, yellow/brown, translucent ~10		10		No sequence data		
2.3.052HS1		60	Unclassified Gammaproteobacteria	**Best hit**		
	EU267665					
Small, round, pale brown, slightly translucent				Uncultured Vibrio sp. clone 12 [AY785252.1]	93%	Microbial fuel cell anode

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Nest best hit Uncultured gamma proteobacterium from sea urchin Paracentrotus lividus clone PIS51 [AY770722.1]	93%	Sea urchin *Paracentrotus lividus* (intestinal tract)
2.3.052HS2 Medium, round, translucent yellowish	EU267640	1	Vibrionaceae (genus Vibrio)	**Best hit**		
				Vibrio sp. YASM14 [DQ314529.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Next best hit four-way tie		
				Vibrio sp. YASM14 [DQ314529.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	97%	
				Vibrio sp. YASM15 [DQ314530.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. Lu1 [AF094701.1]	97%	Unknown
2.3.052HS3 Very small, round, greyish		1		No sequence data		
2.3.052MS1 Small, round, yellow, translucent		50		No sequence data		

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.052MS2		>100	unclassified Gammaproteobacteria	**Best hit**		
	EU267650					
Small, round, pale, slightly translucent				Uncultured Vibrio sp. clone 12 [AY785252.1]	94%	Microbial fuel cell anode
				Next best hit		
				Uncultured gamma proteobacterium from sea urchin Paracentrotus lividus clone PIS51 [AY770722.1]	94%	Sea urchin *Paracentrotus lividus*
2.3.052MS3		20	Vibrionaceae (genus Vibrio)	**Best hit**		
	EU267662					
Medium, round, creamy off-white, opaque				Vibrio harveyi strain LB4 [DQ146935.1]	99%	Marine luminous bacteria from shallow coastal waters of Taiwan
				Next best hit six-way tie		
				Bacterium WP2ISO1 [DQ985828.1]	99%	Great Barrier Reef seawater
				Uncultured bacterium isolate LCPISO4 [DQ831087.1]	99%	*Stage 1 cultured, live Panulirus ornatus* at time of a larval mass mortality event
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	99%	
				Vibrio sp. YASM15 [DQ314530.1]	99%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. YASM14 [DQ314529.1]	99%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. Lu1 [AF094701.1]	99%	Marine luminous bacteria

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.052MS4 Similar to 2MS2, but samller and more translucent		20		No sequence data		
2.3.052MS5 Medium-large, opaque, similar to 2MS3	EU267643	1	Vibrionaceae (genus Vibrio)	**Best hit 9-way tie**		
				Bacterium WP2ISO12 [DQ985839.1]	99%	Great Barrier Reef seawater
				Uncultured bacterium isolate DCPISO1 [DQ831116.1]	99%	stage 1-2 cultured, dead *Panulirus ornatus* at time of a larval mass mortality event
				Uncultured bacterium isolate LCPISO1 [DQ831086.1]	99%	stage 1 cultured, live *Panulirus ornatus* at time of a larval mass mortality event
				Vibrio harveyi strain S35 [AY750578.1]	99%	Unknown
				Vibrio harveyi strain S20 [AY750577.1]	99%	Unknown
				Vibrio fischeri isolate EH701 [AY292941.1]	99%	Unknown
				Vibrio harveyi strain EcGS020802 [AY332565.1]	99%	Pathogenetic *Vibrio harveyi* from estuary cod, *Epinephelus coioides*
				Vibrio harveyi strain EcGY020401 [AY332564.1]	99%	Pathogenetic *Vibrio harveyi* from estuary cod, *Epinephelus coioides*
				V.carchiariae (ATCC 35084T) [X74693.1]	99%	Unknown

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit 3-way tie		
				Vibrio harveyi strain LMG4404T [AY750576.1]	99%	Unknown
				Vibrio harveyi strain NCIMB1280T [AY750575.1]	99%	Unknown
				Vibrio carchariae [AF134581.1]	99%	Causes infectious necrotizing enteritis and mortality in summer flounder *(Paralichthys dentatus*) during intensive culture
2.3.053CC1 Yellow, round, probably same as 3HC4. very thin translucent margin		1		No sequence data		
2.3.053CC2 Small, round, similar to 3CC1, but smaller, slightly paler colour		6		No sequence data		
2.3.053CC3 Small round, orange	EU267644	2	Flavobacteriaceae (genus Winogradskyella)	**Best hit**		
				Winogradskyella poriferorum strain UST030701-295 [AY848823.1]	97%	Winogradskyella poriferorum sp. nov., a novel member of the family Flavobacteriaceae isolated from a sponge in the Bahamas
				Next best hit		
				Coccinimonas marina strain IMCC1846 [EF108213.1]	96%	Coccinimonas marina gen. nov. sp. nov. a novel marine bacterium in the family flavobacteriaceae
2.3.053CC4 Medium, round white	EU267623	2	Rhodobacteraceae (genus Silicibacter)	**Best hit five-way tie**		
				Ruegeria atlantica [AB255399.1]	98%	Unknown
				Ruegeria atlantica strain 1111 [DQ888840.1]	98%	Abundance, bioactivity and ecological function of cultured sponge-associated bacteria from the Mediterranean Sea
				Marine bacterium NJ1-1-1	98%	Marine alga *Lomentaria catenata*

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Alpha proteobacterium SOMOC20, isolate SOMOC20 [AJ936960.1]	98%	Cuttlelfish (*Sepia officinalis*)egg case
				Alpha proteobacterium SOGA14 [AJ244791.1]	98%	Bacterial symbiont isolated from the cuttlefish *Sepia officinalis* accessory nidamental glands
				Next best hit Alpha proteobacterium SOMBO57, isolate SOMBO57 [AJ936937.1]	98%	Bacteria associated with the egg cases of Sepia officinalis (Cephalopod:Decapod)
2.3.053CC5 Medium small, round brownish		2		No sequence data		
2.3.053DC1 Small, round, semi-translucent pale brown/cream colonies. Flat, glossy surface	EU267631	1	Vibrionaceae (genus vibrio)	**Best hit** Vibrio harveyi strain LB4 [DQ146935.1]	97%	Marine luminous bacteria from shallow coastal waters of Taiwan

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit seven-way tie		
				Vibrio sp. BA2 [EF187016.1]	97%	China: Qingdao offing, Shandong Province
				Uncultured bacterium isolate LCPISO4 [DQ831087.1]	97%	Stage 1 cultured, live *Panulirus ornatus* at time of a larval mass mortality event
				Vibrio harveyi strain LB13 [DQ146937.1]	97%	Marine luminous bacteria from shallow coastal waters of Taiwan
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	97%	Unknown
				Vibrio sp. YASM15 [DQ314530.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. YASM14 [DQ314529.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. Lu1 [AF094701.1]	97%	Tyrrhenian Sea coastal waters off northeastern Sicily
2.3.053DC2 Very small, round, semi-translucent pale brown	EU267632	>100	Rhodobacteraceae (genus Silicibacter)	**Best hit** Rhodobacteraceae bacterium CL-TA03 [AY962292.1]	96%	Biofilm in a coastal fish-farm
				Next best hit Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	95%	Coastal waters, East China Sea

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.053DC3	EU267666	1	Vibrionaceae (genus vibrio)	**Best hit**		
Small-medium, round, semitranslucent, pale creamy colour, maybe same as 3DC1				Uncultured gamma proteobacterium clone UA07 [DQ269050.1]	97%	Surface of marine macro-alga (*Ulva australis*)
				Next best hit tie between		
				Vibrio harveyi strain LB4 [DQ146935.1]	97%	Shallow coastal waters of Taiwan
				Vibrio sp. 98CJ11027 [AF246980.1]	97%	Unknown
2.3.053DC4	EU267651	>100	Unclassified Rhodobacteraceae	**Best hit**		
Very tiny, round, white				Rhodobacteraceae bacterium CL-TA03 [AY962292.1]	98%	Shimia marina gen. nov., sp. nov., a novel bacterium of the Roseobacter clade isolated from biofilm in a coastal fish farm
				Next best hit		
				Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	98%	Coastal waters - East China Sea
2.3.053DC5			Unclassified Gammaproteobacteria	**Best hit**		
Small, round, pale brown, possibly same as 3DC1, 3DC3		1		Vibrio sp. V068 [DQ146975.1]	97%	*Latris lineata* (Striped trumpeter)
				Next best hit		
				Vibrio sp. O2 [DQ235159.1]	96%	Alginate lyase producing bacterium from seawater in Mihonoseki Harbor, Japan

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.053DC6	EU267633	>100	Vibrionaceae (genus vibrio)	**Best hit 12-way tie**		
Pale, slightly irregular, flat, translucent, many				Bacterium WP2ISO12 [DQ985839.1]	94%	Great Barrier Reef seawater
				Uncultured bacterium isolate DCPISO1 [DQ831116.1]	94%	stage 1-2 cultured, dead *Panulirus ornatus* at time of a larval mass mortality event
				Vibrio harveyi strain S35 [AY750578.1]	94%	Unknown
				Vibrio harveyi strain S20 [AY750577.1]	94%	Unknown
				Vibrio fischeri isolate EH701 [AY292941.1]	94%	Unknown
				Vibrio harveyi strain EcGS020802 [AY332565.1]	94%	Pathogenetic *Vibrio harveyi* from estuary cod, Epinephelus coioides
				Vibrio harveyi strain EcGY020401 [AY332564.1]	94%	Pathogenetic *Vibrio harveyi* from estuary cod, Epinephelus coioides
				Vibrio harveyi [DQ068936.1]	94%	Unknown
				Vibrio sp. BB4 [AF319768.1]	94%	Enrichment culture from a deep-sea hydrothermal vent
				V.harveyi (ATCC 14126T) [X74706.1]	94%	Unknown
				V.carchiariae (ATCC 35084T) [X74693.1]	94%	Unknown
				Vibrio carchariae [AF134581.1]	94%	Causes infectious necrotizing enteritis and mortality in summer flounder *Paralichthys dentatus* during intensive culture
				Next best hit tie between		
				Vibrio harveyi strain LMG4404T [Y750576.1]	94%	Unknown
				Vibrio harveyi strain NCIMB1280T [AY750575.1]	94%	Unknown
2.3.053DC7 Small, round, convex, yellow, opaque. Possible contaminat				No sequence data		

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.053DC8	EU267637	>100	Unclassified Rhodobacteraceae	**Best hit**		
Small, perfectly round, white, slightly translucent, convex				Unidentified bacterium clone WP2OTU8 [DQ985908.1]	96%	Great Barrier Reef seawater
				Next best hit		
				Unidentified bacterium clone WP3OTU9 [DQ985926.1]	99%	Great Barrier Reef seawater
2.3.053DC9	EU267645	1		**Best hit**		
Medium, brownish, slightly irregular, probably same as 3DC1				Vibrio sp. HB-8 [AY876051.1]	98%	Acorpora muricata coral showing symptoms of Brown Band (BrB) syndrome
				Next best hit		
				Vibrio fortis, type strain LMG 21557T [AJ514916.1]	98%	Marine samples
2.3.053DS1	EU267646	>100	Unclassified Rhodobacteraceae	**Best hit**		
Very small, round, white opaque.				Rhodobacteraceae bacterium CL-TA03 [AY962292.1]	98%	Novel bacterium affiliated with family Rhodobacteraceae, isolated from a biofilm in coastal fish-farm

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit		
				Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	98%	Coastal waters - East China Sea
2.3.053DS2		10	Pseudoalteromonadaceae (genus Pseudoalteromonas)	**Best hit three-way tie**		
	EU267641					
Small, round 'fried eggs' orange/brown centre with white margin				Unidentified bacterium clone WP2OTU33 [DQ985917.1]	96%	Great Barrier Reef seawater
				Uncultured gamma proteobacterium , clone:IB300-1 [AB197184.1]	96%	Bacteria that colonised a control colonization device deployed in Deep sea hydrothermal field (Iheya North field in the Mid-Okinawa Trough)
				Uncultured Pseudoalteromonas sp. , clone:IBRC-1 [AB175553.1]	96%	Bacteria that colonised a control colonization device deployed in Deep sea hydrothermal field (Iheya North field in the Mid-Okinawa Trough)
				Next best hit		
				Pseudoalteromonas sp. 03/034, strain 03/034 [AJ874351.1]	96%	*Crassostrea gigas* (oyster) blood
2.3.053DS3		10	Vibrionaceae (genus Vibrio)	**Best Hit**		
	EU267667					
Small, round, creamy yellowish white, bioluminescent				Vibrio sp. LC1-199 [AB239472.1]	99%	Sagami Bay, Japan
				Next best hit		
				Vibrio harveyi isolate 1 [DQ842240.1]	99%	Marine plankton
2.3.053DS4		1	Incertae sedis 7 (genus Agarivorans)	**Best hit**		
	EU267652					

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
White. Degrades agar.				Agarivorans albus, strain:MKT112 [AB076562.1]	96%	Agar liquefiable bacteria isolated from marine creatures
				Next best hit		
				Agarivorans albus, strain:MKT89 [AB076560.1]	96%	Agar liquefiable bacteria isolated from marine creatures
2.3.053DS5		>100		Bad sequence		
Small, round, yellowish white, partially translucent						
2.3.053HC1		3		No sequence data		
Medium, yellowish brown, Translucent, irregular, flat						
2.3.053HC2		2	Moraxellaceae (genus Psychrobacter)	**Best Hit**		
	EU267668					
Small white, slightly irregular, textured, dry surface, opaque				Psychrobacter celer strain KOPRI24932 [EF101550.1]	99%	*Undaria pinnatifida* (korean brown algae)
				Next best hit tie between		
				Psychrobacter celer [AY842259.1]	99%	Seawater, South Sea, Korea
				Psychrobacter sp. B-5151 [DQ399761.1]	99%	Unknown marine samples

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.053HC3 Small round, very pale brown/creamy, opaque, slightly raised		12		Bad sequence		
2.3.053HC4 Yellow, round, opaque, convex		12		No sequence data		
2.3.053HC5 Round, translucent brown, slightly raised, smooth		5		No sequence data		
2.3.053HS1 Very small, round, white opaque	EU267669	35	Unclassified Rhodobacteraceae	**Best Hit** Rhodobacteraceae bacterium CL-TA03 [AY962292.1]	97%	Biofilm in a coastal fish-farm
				Next best hit Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	97%	East China Sea
2.3.053HS2 Small-medium, round, brown, semitranslucent	EU267629	1		**Best hit** Shewanella sp. C321 [DQ005902.1]	99%	*Corallina officinalis* (coralline algae)
				Next best hit Shewanella waksmanii [AY170366.1]	99%	Isolated from a sipuncula (*Phascolosoma japonicum*)

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.053HS3	EU267634	15		**Best hit**		
Medium, round, creamy white, opaque				Vibrio sp. HB-8 [AY876051.1]	97%	Acorpora muricata coral showing symptoms of Brown Band (BrB) syndrome (great barrier reef)
				Next best hit		
				Vibrio fortis, type strain LMG 21557T [AJ514916.1]	97%	Unknown marine samples
2.3.053HS4		10		Bad sequence		
Similar to 3HS3, but with a brown tinge						
2.3.053HS5		1	Pseudoalteromonadaceae (genus Pseudoalteromonas)	**Best hit**		
	EU267635					
Small, round, blood red 1 (also grey/green mutant idenfied as same by 16S sequence)				Marine bacterium LMG1 [AY082666.1]	98%	Isolated from Gulf of Mexico
				Next best hit 12-way tie		
				Uncultured bacterium isolate CWISO5 [EF195258.1]	97%	Water column of a *Panulirus ornatus* larval rearing system
				Pseudoalteromonas sp. SMB16 [DQ868681.1]	97%	Marine biofilm
				Pseudoalteromonas sp. JL-96 [AY745871.1]	97%	Marine carotenoid-containing heterotrophic bacteria. Isolated from Eastern Chinese Sea
				Bacterium K2-89o [AY345481.1]	97%	Lake Kauhako, water, Hawaii
				Pseudoalteromonas sp. X153, isolate X153 [AJ581533.1]	97%	Isolated from a roller associated with macro-algae (Rhodophyceae and chlorophyceae).Conatins novel antimicrobial protein
				Marine bacterium PWF3 [AY082667.1]	97%	Isolated from Gulf of Mexico
				Pseudoalteromonas viridis , strain:G-364 (=MBIC03136) [AB231329.1]	97%	Pseudoalteromonas viridis sp. nov., a green and red pigmented bacterium isolated from marine algae

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Pseudoalteromonas flavipulchra	97%	Laboratory culture collection
				Pseudoalteromonas piscicida, strain:O-7 [AF297958.1]	97%	Chitinolytic marine bacterium. Source unknown
				Pseudoalteromonas piscicida, strain:IAM 12932 [AB090232.1]	97%	Chitinolytic marine bacterium. Source unknown
				Pseudoalteromonas sp. AS-43 [AJ391204.1]	97%	Isolated from the Adriatic Sea
				Pseudoalteromonas sp. AS-29 [AJ391190.1]	97%	Isolated from the Adriatic Sea
2.3.053MS1	EU267636	>100	Unclassified Rhodobacteraceae	**Best hit**		
Very small, round, white				Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	96%	East China Sea
				Next best hit tie between		
				Marine arctic deep-sea bacterium HD9 [AJ557871.1]	95%	50 m above the sediment of the Hakon Mosby Mud Vulcano (arctic sea)
				Marine arctic deep-sea bacterium HH8 [AJ557864.1]	95%	50 m above the sediment of the Hakon Mosby Mud Vulcano (arctic sea)
2.3.053MS2	EU267638	>100	Rhodobacteraceae (genus Silicibacter)	**Best Hit**		
Similar to 3MS1, but slightly larger				Rhodobacteraceae bacterium CL-TA03 [AY962292.1]	94%	Biofilm in a coastal fish-farm
				Next best hit		
				Uncultured alpha proteobacterium clone JL-ECS-X8 [AY663968.1]	94%	East China Sea
2.3.053MS3	EU267639	>100	Vibrionaceae (genus Vibrio)	**Best hit**		
Medium, round, creamy, off-white, slightly translucent. Thin transparent margin				Vibrio harveyi strain LB4 [DQ146935.1]	98%	Shallow coastal waters of Taiwan
				Next best hit four way-tie		

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	98%	Unknown
				Vibrio sp. YASM15 [DQ314530.1]	98%	Pathogen in *Scophthalmus maximus* (turbot)
				Vibrio sp. YASM14 [DQ314529.1]	98%	Pathogen in *Scophthalmus maximus* (turbot)
				Vibrio sp. Lu1 [AF094701.1]	98%	Unknown
2.3.053MS4	EU267647	1	Vibrionaceae (genus Vibrio)	**Best hit**		
Similar to 3MS3, but darker with yellow/brown tinge				Vibrio harveyi strain LB4 [DQ146935.1]	99%	Shallow coastal waters of Taiwan
				Next best hit six way tie		
				Vibrio sp. BA2 [EF187016.1]	99%	Coastal water - Qingdao offing, Shandong Province, China
				Vibrio harveyi strain LB13 [DQ146937.1]	99%	Shallow coastal waters of Taiwan
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	99%	Unknown
				Vibrio sp. YASM15 [DQ314530.1]	99%	Pathogen in *Scophthalmus maximus* (turbot)
				Vibrio sp. YASM14 [DQ314529.1]	99%	Pathogen in *Scophthalmus maximus* (turbot)
				Vibrio sp. Lu1 [AF094701.1]	99%	Unknown

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
2.3.053MS5		>100	Vibrionaceae (genus Vibrio)	**Best hit**		
	EU267670					
Possibly same as 3MS3				Bacterium WP2ISO6 [DQ985833.1]	98%	Great Barrier Reef seawater
				Next best hit		
				Vibrio sp. SOMBO38, isolate SOMBO38 [AM162586.1]	98%	*Sepia officinalis* (cuttlefish) egg capsule
4.5.0412CS1		4	Vibrionaceae (genus vibrio)	**Best hit**		
	EU276994					
Medium, off white, very thin transparent margin, very similar to 12hs1				Bacterium WP2ISO14 [DQ985841.1]	97%	Great Barrier Reef seawater
				Next best hit		
				Vibrio sp. C33 [AY034144.1]	96%	Inhibits mollusk pathogen
4.5.0412CS2		6		No sequence data		
Large, creamy off white, slightly translucent						
4.5.0412CS2a		3	Vibrionaceae (genus vibrio)	**Best hit**		
	EU267654					
Very simlar to 2, but smoother with irregular margin				Bacterium WP2ISO6 [DQ985833.1]	98%	Great Barrier Reef seawater
				Next best hit		
				Bacterium WP2ISO8 [DQ985835.1]	98%	Great Barrier Reef seawater
4.5.0412CS3		3	Vibrionaceae (genus vibrio)	**Best hit three way tie**		
	EU267612					
Similar to 2, but more translucent and brownish				Bacterium WP3ISO9 [DQ985870.1]	99%	Great Barrier Reef seawater
				Vibrio sp. D1234 [DQ480141.1]	99%	Unknown
				Vibrio sp. D4051 [DQ480140.1]	99%	Unknown

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit		
				Vibrio splendidus biovar II [AB038030.1]	99%	North-western Pacific Ocean
4.5.0412CS4	EU267616	2	Vibrionaceae (genus vibrio)	**Best hit**		
Medium, opaque, white				Bacterium WP2ISO14 [DQ985841.1]	99%	Great Barrier Reef seawater
				Next best hit		
				Bacterium WP3ISO10 [DQ985871.1]	99%	Great Barrier Reef seawater
4.5.0412CS5 Flat, irregular, translucent, orange		1		No sequence data		
4.5.0412CS6 Medium, amorphic, translucent brown/yellow		2		No sequence data		
4.5.0412CS7	EU276996	1	Vibrionaceae (genus Photobacterium)	**Best hit**		
Medium, opaque, white, glossy				Photobacterium lutimaris strain DF-42 [DQ534014.1]	97%	Tidal flat sediment in Korea
				Next best hit		
				Photobacterium sp. 2220 [DQ219367.1]	97%	Seawater
4.5.0412CS8		1	Vibrionaceae (genus vibrio)	**Best hit three way tie**		
Medium, opaque yellow/brown, convex				Vibrio sp. V639 [DQ146989.1]	98%	Abalone
				Vibrio harveyi strain LMG4404T [AY750576.1]	98%	Unknown
				Vibrio harveyi strain NCIMB1280T [AY750575.1]	98%	Unknown
4.5.0412CS9	EU267624	1	Vibrionaceae (genus Photobacterium)	**Best hit**		
Small, round, translucent				Photobacterium sp. YL2 [EF187015.1]	99%	Coastal water,China

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit		
				Photobacterium leiognathi, strain:LC1-283 [AB243249.1]	99%	Light organ symbiont of fish
4.5.0412CS10 Small, round, translucent		1		No sequence data		
4.5.0412CS11	EU277000	1	Vibrionaceae (genus vibrio)	**Best hit**		
Small, round, translucent orange/brown				Vibrio sp. V051 [DQ146974.1]		*Latris lineata* (fish)
				Next best hit		
				Uncultured bacterium isolate LCPISO3 [DQ831088.1]		The Indo-Pacific lobster *Panulirus ornatus*
4.5.0412CS12	EU276992	1	Rhodobacteraceae (genus Paracoccus)	**Best hit twelve-way tie**		
Small, opaque, orange brown				Paracoccus sp. JL1105 [DQ985064.1]	97%	Unknown marine samples
				Paracoccus sp. MSCB-2 [EF103199.1]	97%	Snow from mountain in western china
				Paracoccus sp. NPO-JL-65 [AY745834.1]	97%	Sea water
				Bacillus subtilis, isolate B-1082 [AM110930.1]	97%	Unknown marine samples
				Paracoccus marcusii isolate SCH0403 [AY881236.1]	97%	Marine water samples - Korea
				Paracoccus marcusii [Y12703.1]	97%	Contaminant on a nutrient agar plate
				Paracoccus sp. MBIC1143 [AB008114.1]	97%	Unknown marine samples
				Paracoccus marcusii strain T1947D [DQ298023.1]	97%	Chironomid egg mass
				Paracoccus sp. B-1018 [DQ270725.1]	97%	Unknown marine samples
				Paracoccus sp. B-1017 [DQ270724.1]	97%	Unknown marine samples
				Paracoccus carotinifaciens [AB006899.1]	97%	Soil
				Paracoccus sp. B-1082 [DQ248331.1]	97%	Unknown marine samples

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Paracoccus sp. JL1080 [DQ985061.1]	97%	Unknown marine samples
4.5.0412CS13 Very small, round, bright yellow		6		No sequence data		
4.5.0412CS14 Medium, pale yellow, smooth, glossy		1		No sequence data		
4.5.0412CS15 Very small, opaque whie margin, brown centre, convex but sunken in centre		1		No sequence data		
4.5.0412CS16 Small, round, translucent brown.yellow, simialr to 3		1		No sequence data		
4.5.0412DS1 Medium, round smooth, translucent white	EU267608	3	Vibrionaceae (genus vibrio)	**Best hit**		
				Bacterium WP2ISO14 [DQ985841.1]	97%	Great Barrier Reef seawater
				Next best hit		
				Vibrio sp. CAIM 695 [DQ451210.1]	96%	*Lutjanus guttatus* (spotted rose snapper) - spleen sample

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
4.5.0412DS2	EU267617	1	Vibrionaceae (genus vibrio)	**Best hit three-way tie**		
Large, round, lfat, smooth, translucent yellow/brown, green colonies on TCBS, smells of ammonia				Bacterium WP3ISO9 [DQ985870.1]	97%	Great Barrier Reef seawater
				Vibrio sp. D1234 [DQ480141.1]	97%	Marine samples, QingDao Coast, China
				Vibrio splendidus biovar II [AB038030.1]	97%	North-western Pacific Ocean
				Next best hit		
				Vibrio chagasii, strain LMG 13237 [AJ490157.1]	97%	Unknown marine samples
4.5.0412DS3 Medium/small, smooth, translucent white, more opaque in centre		2		No sequence data		
4.5.0412DS3a	EU276997	1	Vibrionaceae (genus vibrio)	**Best hit five-way tie**		
Small, transparent, round, smooth, flat				Uncultured bacterium isolate LCPISO4 [DQ831087.1]	95%	Stage 1 cultured, live *Panulirus ornatus* (lobster) at time of a larval mass mortality event
				Vibrio sp. R-14939 [AJ316187.1]	95%	Aquaculture samples
				Vibrio sp. YASM15 [DQ314530.1]	95%	Pathogenic bacteria isolated from *Scophthalmus maximus* (turbot)
				Vibrio sp. YASM14 [DQ314529.1]	95%	Pathogenic bacteria isolated from *Scophthalmus maximus* (turbot)
				Vibrio sp. Lu1 [AF094701.1]	95%	coastal waters off northeastern Sicily
				Next best hit		
				Bacterium WP2ISO1 [DQ985828.1]	95%	Great Barrier Reef seawater

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
4.5.0412DS4		2	Vibrionaceae (genus vibrio)	**Best hit**		
Medium/small, round, opaque white, thin transparent margin	EU267655			Bacterium WP2ISO14 [DQ985841.1]	98%	Great Barrier Reef seawater
				Next best hit		
				Vibrio sp. CAIM 695 [DQ451210.1]	97%	Lutjanus guttatus (spotted rose snapper)
4.5.0412DS5		1		No sequence data		
Irregular, medium, opaque, white						
4.5.0412DS6		1	Vibrionaceae (genus vibrio)	**Best hit two way tie**		
Amorphic film, translucent yellow/brown, green, textured colonies on TCBS	EU276991			Bacterium WP3ISO9 [DQ985870.1]	96%	Great Barrier Reef seawater
				Vibrio sp. D1234 [DQ480141.1]	96%	Marine heterotrophic bacteria, QingDao coast, China
				Next best hit		
				Vibrio sp. D4051[DQ480140.1]	96%	Marine heterotrophic bacteria, QingDao coast, China
4.5.0412DS7		1		No sequence data		
Similar to 6, but lighter in colour						
4.5.0412DS8	EU267609			**Best hit**		
Small, round, transparent margin		2	Pseudoalteromonadaceae (genus Pseudoalteromonas)	Bacterium WP3ISO11 [DQ985872.1]	96%	Great Barrier Reef seawater
				Next best hit		
				Pseudoalteromonas sp. JL1003 [DQ985032.1]	96%	Unknown marine samples

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
4.5.0412DS9 Small, round, white, opaque		1		No sequence data		
4.5.0412DS10 Small, translucent white, sunken in centre		1		No sequence data		
4.5.0412DS11 Medium/small, round, translucent yellowish white	EU277001	2	Vibrionaceae (genus Enterovibrio)	**Best hit** Enterovibrio norvegicus strain LMG	98%	Isolated from the gut of turbot (Scophthalmus maximus) larvae
				Next best hit Vibrio sp. LMG 19839, strain LMG 1	98%	Pectolytic Pectobacterium causing soft rotting of potatoes grown in New Zealand
4.5.0412DS12 Medium, round, smooth, white, opaque		1		No sequence data		
4.5.0412HS1 medium/small, round, opaque creamy off-white, thin transparent margin, smooth, flat	EU267656	7	Vibrionaceae (genus vibrio)	**Best hit** Bacterium WP2ISO14 [DQ985841.1]	99%	Great Barrier Reef seawater
				Next best hit Bacterium WP3ISO10 [DQ985871.1]	99%	Great Barrier Reef seawater
4.5.0412HS2 Small, round, white, opaque		2		No sequence data		

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
4.5.0412HS3	EU277002	1	Vibrionaceae (genus vibrio)	**Best hit nine-way tie**		
Medium/large, yellowish white, concentric rings				Vibrio sp. A2 [EF467288.1	98%	Sea dragon
				Vibrio sp. gq [EF187006.1]	98%	Coastal water
				Vibrio gigantis strain CAIM 25 [EF094888.1]	98%	Unknown
				Vibrio crassostreae strain CAIM 1405 [EF094887.1]	98%	Unknown
				Vibrio sp. Do-194 [AB257566.1]	98%	Fish farm sediment
				Mucus bacterium 6 [AB257566.1]	98%	Mucus of Oculina patagonica
				Mucus bacterium 14 [AY654750.1]	98%	Mucus of Oculina patagonica
				Vibrio splendidus strain 630 [AY620974.1]	98%	Oyster
				Vibrio splendidus strain 636 [AY620972.1]	98%	Oyster
4.5.0412HS4	EU276998	4	Vibrionaceae (genus vibrio)	**Best hit**		
Small-very small, convex, yellow				Bacterium WP2ISO14 [DQ985841.1]	97%	Microbial diversity within mid-stage Palinurid and Scyllarid phyllosoma obtained from Great Barrier Reef waters
				Next best hit		
				Uncultured bacterium isolate LCPISO3 [DQ831088.1]	97%	The Indo-Pacific lobster *Panulirus ornatus*
4.5.0412HS5 Very small, translucent, clustered closely on plate (maybe badly spread?)		60		No sequence data		
4.5.0412HS6 Very small, orange		1		No sequence data		

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
4.5.0412HS7 Small, translucent, pale brown		1		No sequence data		
4.5.0412HS8 Medium, translucent brown		1		No sequence data		
4.5.0412MS1	EU276989	2	Vibrionaceae (genus vibrio)	**Best hit four-way tie**		
Amorphic film, appears identical to 12ds6, large patches, green, textured colonies on TCBS				Bacterium WP3ISO9 [DQ985870.1]	98%	Microbial diversity within mid-stage Palinurid and Scyllarid phyllosoma obtained from Great Barrier Reef waters
				Vibrio sp. D12349 [DQ480141.1]	98%	Marine Heterotrophic Bacteria Identification and Distribution in QingDao Coast
				Vibrio chagasii [AJ490157.1]	97%	Unknown marine samples
				Vibrio splendidus biovar II [AB038030.1]	98%	Seawater - north-western Pacific Ocean and Otsuchi Bay, Japan
				Next best hit		
				Vibrio sp. D4051 [DQ480140.1]	97%	Coastal water - QingDao Coast, China
4.5.0412MS2	EU276993	1	Vibrionaceae (genus vibrio)	**Best hit**		
Amorphic, small, white similar to 12DS7, no growth on TCBS				Vibrio sp. PH1 [AF513461.1]	97%	Hawaii marine sediments
				Next best hit		
				Uncultured bacterium clone pt50m [DQ890425.1]	97%	Empty intestine of *Pestarella tyrrhena* (burrowing decapod crustacean)

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
4.5.0412MS3	EU276999	1	Vibrionaceae (genus vibrio)	**Best hit**		
Amorphic, very simialr to 1 and 2, smooth round dark green colonies on TCBS				Vibrio harveyi strain LB4 [DQ146935.1]	97%	Shallow coastal waters of Taiwan
				Next best hit seven way tie		
				Vibrio sp. BA2 [EF187016.1]	97%	Coastal water
				Vibrio harveyi strain OVL 0-53535-F [AY264925.1]	97%	Laboratory culture collection
				Vibrio harveyi strain LB13 [DQ146937.1]	97%	Shallow coastal waters of Taiwan
				Uncultured bacterium clone PDC-OTU2 [AY700621.1]	97%	Bacteria associated with the coral *Pocillopora damicornis* from the Great Barrier Reef
				Vibrio sp. R-14939, strain R-14939 [AJ316187.1]	97%	Unknown
				Vibrio sp. YASM15 [DQ314530.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. YASM14 [DQ314529.1]	97%	Pathogenic bacteria isolated from *Scophthalmus maximus*
				Vibrio sp. Lu1 [AF094701.1]	97%	Unknown
4.5.0412MS4	EU267657/EU 276995	1	Vibrionaceae (genus vibrio)	**Best hit three way tie**		
Appears identical to 12ms3, but regions of contact between colonies of 3 and 4 remain separate, smooth blue/green colonies on TCBS				Vibrio sp. BA2 [EF187016.1]	97%	Coastal water - Qingdao offing, Shandong Province, China
				Vibrio harveyi strain LB13 [DQ146937.1]	97%	Shallow coastal waters of Taiwan
				Vibrio harveyi strain LB4 [DQ146935.1]	97%	Shallow coastal waters of Taiwan

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit then Uncultured bacterium isolate LCPISO4 [DQ831087.1]	97%	The Indo-Pacific lobster *Panulirus ornatus*
4.5.0412MS5	EU276990	1	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Large round colony, smooth, translucent, appears identical to 12ds2				Bacterium WP3ISO9 [DQ985870.1]	98%	Great Barrier Reef seawater
				Vibrio sp. D1234 [DQ480141.1]	98%	Coastal water - QingDao Coast, China
				Next best hit Vibrio sp. D4051 [DQ480140.1]	97%	Coastal water - QingDao Coast, China
4.5.0412MS6	EU267653/ EU259885	1	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Medium small, round, opaque white centre with transparent margin				Bacterium WP3ISO10 [DQ985871.1]	99%	Great Barrier Reef seawater
				Bacterium WP3ISO1 [DQ985862.1]	99%	Great Barrier Reef seawater
				Next best hit Vibrio sp. DS40M5 [AF199438.1]	99%	Seawater isolate
4.5.0412MS7	EU267659	1	Vibrionaceae (genus vibrio)	**Best hit tie between**		
medium, round, translucent whitish				Bacterium WP2ISO9 [DQ985836.1]	98%	Great Barrier Reef seawater
				Vibrio shiloi strain SW-2 [AY911395.1]	98%	Swire ocean research station, Hong Kong

Isolate Name and colony morphology on SLB agar	Accession Number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit four-way tie		
				Mucus bacterium 49 [AY654779.1]	98%	Culturable bacteria from the mucus of *Oculina patagonica*
				V.mediterranei (CIP 103203T) [X74710.1]	98%	Unknown
				Vibrio sp. SOMBO52, isolate SOMBO52 [AJ936957.1]	98%	*Sepia officinalis* (cuttlelfish) egg case
				Vibrio shiloi [AF007115.1]	98%	Laboratory culture collection
4.5.0412MS8 small round, intense violet centre with transparent margin		1		No sequence data		
4.5.0412MS9 Irregular, transparent, flat, textured appearance		2		No sequence data		

Appendix 1B - Cultured bacteria isolated from Lord Howe Island samples. Isolates of interest are highlighted.

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
10.5.051CC1		>100	Moraxellaceae (genus psychrobacter)	**Best hit two-way tie**		
Medium/small, round, white, opaque, glossy	EU276984			Psychrobacter celer [AY842259.1]	98%	Sea water of the South Sea in Korea
				Psychrobacter sp. B-5151 [DQ399761.1]	98%	Unknown
				Next best hit		
				Psychrobacter celer strain KOPRI24932 [EF101550.1]	97%	Associated with the Korean brown alga, *Undaria pinnatifida*
10.5.051CC2 Small, round, yellowish		>100		No sequence data		
10.5.051CC3		>100	Sphingomonadaceae (genus Erythrobacter)	**Best hit 3-way tie**		
Small, round, orange/brown	EU267618			Erythrobacter sp. JL274 [EF512736.1]	97%	Unknown marine samples, China
				Erythrobacter vulgaris strain JL271 [EF512708.1]	97%	Unknown marine samples, China
				Uncultured organism clone ctg_NISA074 [DQ395997.1]	97%	Deep-sea octocoral
				Next best hit tie between		
				Uncultured organism clone ctg_CGOF118 [DQ395626.1]	96%	Deep-sea octocoral
				Alpha proteobacterium MBIC2351 [AB012061.1]	96%	Unknown

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
10.5.051CC4		10	Pseudomonadaceae (genus Pseudomonas)	**Best hit 23-way tie**		
	EU267615					
Irregular, slightly orange, raised				Pseudomonas stutzeri A1501, complete genome [CP000304.1]	98%	Metabolically versatile and root-associated nitrogen-fixing bacterium
				Pseudomonas stutzeri strain GIST-BDan2 [EF429003.1]	98%	Anaerobic water from BuDeul-Construct Wetland, South Korea
				Uncultured alpha proteobacterium clone TK-NH8 [DQ463739.2]	98%	Lake Tanganyika anoxic hypolimnion
				Pseudomonas stutzeri ATTC 17594 [AY905607.1]	98%	Unknown
				Pseudomonas sp. IBUN MAR3 [DQ813309.1]	98%	Sea-sludge - beaches of the Caribbean Sea, Colombia
				Pseudomonas sp. E4-1 [DQ227349.1]	98%	Denitrifier isolated from deep sea
				Mucus bacterium 10 [AY654828.1]	98%	Culturable bacteria from the mucus of *Oculina patagonica*
				Pseudomonas sp. JPL-1 [AY030314.1]	98%	Unknown
				Pseudomonas sp. R-25343, strain R-25343 [AM084028.1]	98%	Unknown
				Pseudomonas stutzeri [AF094748.1]	98%	Denitrifying bacteria from activated sludge from a municipal wastewater treatment plant
				Pseudomonas sp. 17-c2 [AY561567.1]	98%	Vadose-zone sediments contaminated with high level nuclear waste
				Pseudomonas sp. 17-b2 [AY561565.1]	98%	Vadose-zone sediments contaminated with high level nuclear waste
				Pseudomonas sp. 17a-4 [AY561562.1]	98%	Vadose-zone sediments contaminated with high level nuclear waste

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Pseudomonas sp. 17a-3 [AY561561.1]	98%	Vadose-zone sediments contaminated with high level nuclear waste
				Pseudomonas sp. 12b-1 [AY561550.1]	98%	Vadose-zone sediments contaminated with high level nuclear waste
				Pseudomonas sp. 12a-1 [AY561549.1]	98%	Vadose-zone sediments contaminated with high level nuclear waste
				Pseudomonas stutzeri, strain DSM 5190T [AJ288151.1]	98%	Unknown
				Pseudomonas sp. BRW3 [AF025351.1]	98%	Unknown
				Pseudomonas sp. BRW1 [AF025349.1]	98%	Unknown
				Pseudomonas putida isolate SR21 [DQ288952.1]	98%	Deep oil-bearing drilling cuttings
				Pseudomonas stutzeri isolate FM22 [DQ289075.1]	98%	Deep oil-bearing drilling cuttings
				Pseudomonas stutzeri CCUG 11256 [U26262.1]	98%	Laboratory culture collection
				Pseudomonas stutzeri [U25432.1]	98%	Laboratory culture collection
				Next best hit 12-way tie		
				Pseudomonas stutzeri strain KSC_Ak10C [DQ870705.1]	97%	National Aeronautics and Space Administration clean room
				Uncultured bacterium clone RL311_aam24d02 [DQ803347.1]	97%	Human faeces
				Uncultured bacterium clone JSC2-C6 [DQ532187.1]	97%	Johnson Space Center
				Uncultured bacterium clone QHO-B41 [DQ675028.1]	97%	Production water of a high-temperature petroleum reservoir
				Pseudomonas sp. Zp2 [DQ659594.1]	97%	Soil in oil refinery fields, Shanghai, China

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Pseudomonas sp. FD41 [DQ462181.1]	97%	Oil degrading bacteria
				Pseudomonas sp. E1-4 [DQ227347.1]	97%	Denitrifier isolated from deep sea
				Pseudomonas sp. A7-1 [DQ227346.1]	97%	Denitrifier isolated from deep sea
				Pseudomonas stutzeri [DQ224384.1]	97%	Unknown
				Uncultured Pseudomonas sp. clone 349_11 [AF467304.1]	97%	Associated with ectoparasitic chewing lice of pocket gophers
				Pseudomonas stutzeri isolate Fe31 [DQ289057.1]	97%	Sandstone core found 3386m deep in oil well
				Pseudomonas stutzeri strain DNSP21 [U26414.1]	97%	Unknown
10.5.051CS1	EU267625	2	Vibrionaceae (genus vibrio)	**Best hit**		
Medium, round, white, opaque, glossy, transparent margin				Bacterium CWISO20 [DQ334358.1]	97%	Water column of a *Panulirus ornatus* (lobster) larval rearing system
				Next best hit		
				Vibrio sp. DS40M5 [AF199438.1]	97%	Seawater
10.5.051CS2 Small round yellow/brown		1		No sequence data		

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
10.5.051CS3 Small round, very pale brown		1		No sequence data		
10.5.051CS4 Large, convex, yellowish, soft, smooth		1		Bad sequence		
10.5.051CS5 Small, round, pale brown		1		No sequence data		
10.5.051CS6 Very small, round, very plae brown/white.		10		Bad Sequence		
11.5.051CC1	EU276985		Unclassified Vibrionace	**Best hit**		
				Vibrio sp. Y4tang [EF187013.1]	99%	Seawater, Qingdao offing, Shandong Province, China
				Next best hit		
				Vibrio sp. GAS8 [EF584050.1]	99%	Seawater, Camp Cove, Sydney Harbour, Australia
11.5.051CC2	EU276986	50	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Medium, round, opaque, creamy yellowish white				Vibrio sp. Y4tang [EF187013.1]	98%	Seawater, Qingdao offing, Shandong Province, China
				Vibrio harveyi strain LB4 [DQ146935.1]	98%	Shallow coastal waters of Taiwan
				Next best hit		

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Uncultured gamma proteobacterium clone UA07 [DQ269050.1]	98%	Surface of marine macro-alga

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.051CC3 Very large, irregular, slimy translucent film		1		No sequence data		
11.5.051CC4 Medium/small, round, translucent	EU276979	10	Vibrionaceae (genus vibrio)	**Best hit five way tie**		
				Vibrio sp. F 10, strain F 10 [AM159567.1]	97%	*Sparus aurata* (cultured Gilthead sea bream)
				Vibrio ponticus, strain 380 [AJ630203.1]	97%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: sea
				Vibrio ponticus, strain 292 [AJ630202.1]	97%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: cultured mussel
				Vibrio ponticus, type strain CECT 5869T [AJ630103.1]	97%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: cultured mussel
				Vibrio ponticus, strain CECT 5870 [AJ630102.1]	97%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: *Sparus aurata* (Gilthead sea bream)
				Next best hit		
				Vibrio sp. V859 [DQ146996.1]	97%	*Lates calcarifer* (barramundi)

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.051CC5 Small, round, yellow, concave		3		No sequence data		
11.5.051CC6 Small, round, white		1		No sequence data		
11.5.051CS1		8	Moraxellaceae (genus Acinetobacter)	**Best hit**		
	EU267610					
Medium/small, round, white, thin trasparent margin				Uncultured bacterium clone BANW441(98%) [DQ264438.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Next best hit four way tie		
				Acinetobacter johnsonii isolate F26 [EF204266.1]	98%	Raw milk
				Acinetobacter johnsonii isolate F25 [EF204265.1]	98%	Raw milk
				Kartchner Caverns bacterium HI-O4 [DQ205305.1]	98%	Cave sediment (Kartchner Caverns, Arizona, USA)
				Uncultured bacterium clone BANW589 [DQ264550.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
11.5.051CS2		1	Moraxellaceae (genus Acinetobacter)	**Best hit three way tie**		
	EU267626					
Medium, round, creamy				Acinetobacter sp. EN69 [DQ842492.1]	98%	Oil degrading bacteria
				Uncultured bacterium clone BY9 [DQ394301.1]	98%	Broilers intestine
				Uncultured Acinetobacter sp. clone DE3.4 [AY588958.1]	98%	Pacific Ocean deep sea sediment

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit eight way tie		
				Uncultured Acinetobacter sp. clone DS103 [DQ234186.2]	98%	Component of mangrove bacterioplankton community
				Uncultured bacterium clone BANW676 [DQ264618.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Uncultured bacterium clone BANW663 [DQ264609.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Uncultured bacterium clone BANW582 [DQ264544.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Uncultured bacterium clone BANW565 [DQ264533.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Uncultured bacterium clone BANW519 [DQ264497.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Uncultured bacterium clone BANW443 [DQ264440.1]	98%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Uncultured bacterium clone EV818BHEB5102702SAS66 [DQ256363.1]	98%	Subsurface water of the Kalahari Shield, South Africa
11.5.051CS3	EU267660	2	Sphingomonadaceae (genus Erythrobacter)	**Best hit five-way tie**		
Small, round, orange				Erythrobacter sp. JL274 [EF512736.1]	99%	Unknown marine samples
				Erythrobacter vulgaris strain JL271 [EF512708.1]	99%	Unknown marine samples
				Uncultured organism clone ctg_NISA074 [DQ395997.1]	99%	Deep-sea octocoral
				Erythrobacter sp. MB-16 [AF325446.1]	99%	Manganese oxidising bacterium isolated surface sediments of San Diego Bay, San Diego, Calif

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Erythrobacter vulgaris strain 022 2-10 [AY706935.1]	99%	Starfish *Stellaster equestris*
				Next best hit tie between		
				Erythrobacter vulgaris strain 022 4-7 [AY706938.1]	99%	Soft coral
				Erythrobacter vulgaris strain 022 2-12 [AY706937.1]	99%	Starfish *Stellaster equestris*
11.5.051CS4	EU267663	1	Shewanellaceae (genus Shewanella)	**Best hit tie between**		
Medium, round, creamy				Shewanella sp. ANA-3, complete genome [CP000469.1]	98%	Arsenate reducing bacterium
				Shewanella taiwanensis [AB263191.1]	98%	Facultative Microorganism Shewanella sp. which Can Decolorize Azo Dyes - Isolated from heat exchange cooling system of petroleum refinery
				Next best hit		
				Shewanella sp. ANA-3 [AF136392.1]	97%	Arsenate reducing bacterium
11.5.051CS5	EU276980	1	Moraxellaceae (genus Acinetobacter)	**Best hit**		
Medium, round, similar to 1CS2, 1CS4				Uncultured bacterium clone BANW441 [DQ264438.1]	97%	Subsurface groundwater during polylacetate stimulated chromate bioremediation
				Next best hit		
				Acinetobacter johnsonii isolate F26 [EF204266.1]	96%	Raw milk

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.051CS6	EU267611	1	Rhodobacteraceae (genus Paracoccus)	**Best hit is eleven way tie**		
Small irregular, red, textured surface				Paracoccus sp. JL1105 [DQ985064.1]	97%	Unknown marine samples
				Paracoccus sp. MSCB-2 [EF103199.1]	98%	Mountain snow in western china
				Paracoccus sp. NPO-JL-65 [AY745834.1]	98%	Marine samples - North Pacific Ocean
				Bacillus subtilis isolate B-1082 [AM110930.1]	98%	Unknown
				Paracoccus marcusii isolate SCH0403 [AY881236.1]	98%	Coastal marine samples - South Korea
				Paracoccus marcusii	98%	Unknown
				Paracoccus sp. MBIC1143 [AB008114.1]	97%	Unknown marine samples
				Paracoccus marcusii strain T1947D [DQ298023.1]	98%	Chironomid egg mass
				Paracoccus sp. B-1018 [DQ270725.1]	97%	Unknown
				Paracoccus carotinifaciens [AB006899.1]	98%	Soil - Kanagawa Prefecture, Japan
				Paracoccus sp. B-1082 [DQ248331.1]	98%	Unknown marine samples
				Next best hit		
				Paracoccus sp. JL1080 [DQ985061.1]	97%	Unknown marine samples

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.051CS8	EU267614	1	Shewanellaceae (genus Shewanella)	**Best hit three-way tie**		
Medium, large creamy				Shewanella sp. ANA-3, complete genome [CP000469.1]	98%	Arsenate reducing bacterium
				Shewanella sp. MR-7, complete genome [CP000444.1]	98%	Nitrogen reducing bacterium
				Shewanella sp. CL256/73 [AF387346.1]	98%	Isolated from human cerebrospinal fluid
				Next best hit		
				Uncultured bacterium clone aab65d10 [DQ814211.1]	97%	Conventionally-raised adult zebrafish (*Danio rerio*) digestive tract
11.5.051CS9	EU267627	1	Moraxellaceae (genus Acinetobacter)	**Best hit tie between**		
Medium, round, slightly translucent similar to 1CS7				Acinetobacter johnsonii isolate F26 [EF204266.1]	96%	Raw milk
				Acinetobacter johnsonii isolate F25 [EF204265.1]	96%	Raw milk
				Next best hit		
				Uncultured Acinetobacter sp. clone 88_6 [AF467299.1]	96%	Associated with ectoparasitic chewing lice of pocket gophers

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.051CS10		1	Sphingomonadaceae (genus Erythrobacter)	**Best hit 9-way tie**		
Medium, round, white, slightly translucent. Similar to 1CS8, 1CS7, 1CS2	EU267628			Erythrobacter flavus strain JL95 [EF512715.1]	96%	Unknown marine samples
				Unidentified bacterium clone WP2OTU3 [DQ985903.1]	96%	Great Barrier Reef seawater
				Erythrobacter sp. JL-378 [DQ285076.1]	96%	Unknown
				Uncultured organism clone ctg_NISA106 [DQ396272.1]	96%	Deep-sea octocoral
				Uncultured organism clone ctg_NISA275 [DQ396019.1]	96%	Deep-sea octocoral
				Bacterium K2-13 [AY345436.1]	96%	Water samples, Lake Kauhako, Hawaii
				Erythrobacter flavus strain SW-52 [AF500005.1]	96%	East Sea, Korea
				Erythrobacter flavus strain SW-46 [AF500004.1]	96%	East Sea, Korea
				Uncultured bacterium clone PDC-OTU4 [AY700623.1]	96%	Associated with the coral *Pocillopora damicornis*
				Next best hit tie between		
				Uncultured organism clone ctg_NISA352 [DQ396179.1]	96%	Deep-sea octocoral
				Uncultured organism clone ctg_NISA303 [DQ396111.1]	96%	Deep-sea octocoral

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.052CC1 Large, round, creamy off-white, appears brown when held up to light	EU276981	1	Vibrionaceae (genus Vibrio)	**Best Hit** Uncultured gamma proteobacterium clone UA07 [DQ269050.1]	95%	Surface of marine macro-alga Ulva australis
				Next best hit Vibrio sp. BA2 [EF187016.1]	95%	Coastal water - China
11.5.052CC2 Large, round, opaque white	EU267620	1	Vibrionaceae (genus Photobacterium)	**Best hit** Photobacterium eurosenbergii, strain LMG 22223T [AJ842344.1]	95%	Photobacterium eurosenbergii sp. nov. and Enterovibrio coralii sp. nov., vibrios associated with coral bleaching
				Next best hit Photobacterium eurosenbergii, strain LMG 22225 [AJ842345.1]	95%	Photobacterium eurosenbergii sp. nov. and Enterovibrio coralii sp. nov., vibrios associated with coral bleaching
11.5.052CC3 Large, round, slightly translucent with transparent margin		1		No sequence data		

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.052CC4		1	Moraxellaceae (genus psychrobacter)	**Best hit tie between**		Sea water - South Sea, Korea
Medium/small, round, very pale brown	EU276982			Psychrobacter celer [AY842259.1]	95%	
				Psychrobacter sp. B-5151 [DQ399761.1]	96%	Unknown marine samples
				Next best hit Psychrobacter sp. R-23191 [AJ969173.1]	95%	Adventitious microflora of a smear-ripened cheese
11.5.052CC5		1	Vibrionaceae (genus vibrio)	**Best hit**		
Medium/large, round, very similar to 2CC1	EU276983			Uncultured bacterium clone BB3S16S-17 [EF089472.1]	96%	Associated with Black Band disease (BBD) in stony corals from Eilat, Red Sea, Israel
				Next best hit Vibrio sp. FLPN2 [DQ317677.1]	96%	Fish gut
11.5.052CC6		1	Vibrionaceae (genus vibrio)	**Best hit**		
Very similar to 2CC5	EU267607			Uncultured gamma proteobacterium clone UA07 [DQ269050.1]	97%	Surface of marine macro-alga *Ulva australis*
				Next best hit Vibrio sp. 98CJ11027 [AF246980.1]	96%	Unknown marine samples

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.052CC7	EU267619	1	Vibrionaceae (genus vibrio)	**Best hit**		
Very similar to 2CC6, 2CC5 but lighter in colour				Uncultured gamma proteobacterium clone UA07 [DQ269050.1]	95%	Surface of marine macro-alga *Ulva australis*
				Next best hit		
				Vibrio sp. 98CJ11027 [AF246980.1]	95%	Unknown marine samples
11.5.052CC8	EU276972	1	Rhodobacteraceae (genus Silicibacter)	**Best hit four-way tie**		
Small, round, slightly translucent, white				Ruegeria atlantica strain 1111 [DQ888840.1]	98%	Bacterial symbionts in the accessory nidamental glands of the sepioid *Sepia officinalis* (Cephalopoda, Sepiida, Sepiidae)
				Marine bacterium NJ1-1-1 [AY626827.1]	98%	Marine alga *Lomentaria catenata*
				Alpha proteobacterium SOMOC20	98%	Associated with the egg cases of *Sepia officinalis*
				Alpha proteobacterium SOGA14 [AJ936960.1]	98%	Bacterial symbionts in the accessory nidamental glands of the sepioid *Sepia officinalis* (Cephalopoda, Sepiida, Sepiidae)
11.5.052CC9 Fungus, probably contaminant				No sequence data		
11.5.052CC10	EU276977	1	Rhodobacteraceae (genus Silicibacter)	**Best hit three way tie**		
Small, round white				Alpha proteobacterium MBIC1876 [AB026194.1]	96%	Marine sponge
				Alpha proteobacterium D29 [DQ227659.1]	96%	Marine sponge *Pseudoceratina clavata*

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Mucus bacterium 103	96%	Mucus of *Oculina patagonica* (coral)

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.052CC11	EU276973	1	Vibrionaceae (genus vibrio)	**Best hit**		
Large, round, pale brown, transluent margin				Vibrio shiloi strain SW-2 [AY911395.1]	97%	Sea water- Hong Kong
				Next best hit		
				Bacterium WP2ISO9 [DQ985836.1]	97%	Great Barrier Reef seawater
11.5.052CS1	EU276974	1	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Large, creamy, slightly irregular				Vibrio chagasii strain LMG 13237 [AJ490157.1]	96%	Marine samples
				Vibrio splendidus biovar II [AB038030.1]	97%	North-western Pacific Ocean and Otsuchi Bay, Japan
				Next best hit		
				Bacterium WP3ISO9 [DQ985870.1]	96%	Great Barrier Reef seawater
11.5.052CS2	EU267661	1	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Small, round, slightly irregular, flat, opaque centre and very transparent margin				Bacterium WP2ISO9 [DQ985836.1]	98%	Great Barrier Reef seawater
				Vibrio shiloi strain SW-2 [AY911395.1]	98%	Hong Kong: Swire ocean research station
				Next best hit		
				V.mediterranei (CIP 103203T) [X74710.1]	98%	Laboratory reference strain

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.052CS3		1	Vibrionaceae (genus vibrio)	**Best hit three-way tie**		
Small, round, red				Bacterium WP3ISO9 [DQ985870.1]	98%	Great Barrier Reef seawater
				Vibrio sp. D1234 [DQ480141.1]	98%	Unknown marine samples, QingDao Coast, China
				Vibrio sp. D4051 [DQ480140.1]	98%	Unknown marine samples, QingDao Coast, China
				Next best hit		
				Vibrio splendidus biovar II [AB038030.1]	98%	North-western Pacific Ocean
11.5.052CS4		2	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Small, round, slightly transparent margin, creamy opaque centre				Vibrio sp. CAIM 695 [DQ451210.1]	97%	Spotted rose snapper (*Lutjanus guttatus*) spleen
				Bacterium WP2ISO14 [DQ985841.1]	97%	Great Barrier Reef seawater
				Next best hit		
				Vibrio sp. CAIM 797 [DQ451211.1]	96%	Spotted rose snapper (*Lutjanus guttatus*) spleen
11.5.052CS5		1		No sequence data		
Medium/small,						

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.052CS6	EU276975	1	Vibrionaceae (genus vibrio)	**Best hit tie between**		
Large, round, concentric rings, possibly same as 2CS1				Bacterium WP3ISO9C [DQ985870.1]	98%	Great Barrier Reef seawater
				Vibrio sp. D1234 [DQ480141.1]	98%	QingDao Coast
				Next best hit		
				Vibrio sp. D4051 [DQ480140.1]	98%	QingDao Coast
11.5.052CS7	EU276976	1	Moraxellaceae (genus psychrobacter)	**Best hit two way tie**		
Medium, round, white, opaque				Psychrobacter celer [AY842259.1]	97%	Sea water of the South sea in Korea
				Psychrobacter sp. B-5151 [DQ399761.1]	97%	Unknown
				Next best hit		
				Psychrobacter celer strain KOPRI24932 [EF101550.1]	96%	Associated with the Korean brown alga, Undaria pinnatifida
11.5.052CS8	EU267613	1	Vibrionaceae (genus vibrio)	**Best hit**		
Small, round, opaque creamy off-white				Vibrio sp. V639 [DQ146989.1]	95%	Abalone

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit three way tie		
				Vibrio harveyi strain LMG4404T [AY750576.1]	95%	Unknown
				Vibrio harveyi strain NCIMB1280T [AY750575.1]	95%	Unknown
				Vibrio harveyi strain LB4 [DQ146935.1]	95%	Shallow coastal waters of Taiwan
11.5.052CS9		1	Moraxellaceae (genus psychrobacter)	Psychrobacter sp. B-5151 [DQ399761.1]	96%	Unknown
	EU276978					
Very similar to 2CS8 but more opaque, convex				Psychrobacter celer [AY842259.1]	96%	Sea water of the South sea in Korea
11.5.053CC1		2	Vibrionaceae (genus vibrio)	**Best hit**		
	EU276987					
Large, round, white				Vibrio harveyi strain LB5 [DQ146936.1]	100%	Marine Iminous bacteria from shallow coastal waters of Taiwan
				Next best hit tie between		
				Vibrio sp. FLLU3 [DQ317675.1]	99%	Luminous bacteria from fish guts
				Vibrio sp. LMG 20370, strain LMG 20370 [AJ345066.1]	99%	Unknown
11.5.053CC2		1		No sequence data		
Medium, round, white, opaque, convex, glossy						

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
11.5.053CC3 Small, round, yellow		6		No sequence data		
11.5.053CC4 Small, round creamy pastel pinkish		1		No sequence data		
11.5.053CC5 Medium, round, white, similar to 3CC2		1		sequence contaminated		
11.5.053CC6 Medium, translucent, flat	EU267621	1	Vibrionaceae (genus vibrio)	**Best hit five-way tie**		
				Vibrio sp. F 10, strain F 10 [AM159567.1]	95%	*Sparus aurata* (cultured Gilthead sea bream)
				Vibrio ponticus, strain 380 [AJ630203.1]	95%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: sea water
				Vibrio ponticus, strain 292 [AJ630202.1]	95%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: cultured mussel
				Vibrio ponticus, type strain CECT 5869T [AJ630103.1]	95%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: cultured mussel

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Vibrio ponticus, strain CECT 5870 [AJ630102.1]	95%	Vibrio ponticus sp. nov., a neighbour of V fluvialis-V. furnissii clade, isolated from gilthead sea bream, mussels and seawater. Source: *Sparus aurata* (Gilthead sea bream)
				Next best hit Vibrio sp. V859 [DQ146996.1]	94%	*Lates calcarifer* (barramundi)
11.5.053CC7	EU267622/EU 276988	1	Moraxellaceae (genus psychrobacter)	**Best hit**		
Medium, round, white similar to 3CC5				Psychrobacter sp. MJYP.15.12 [AB094456.1]	94%	Coastal subseafloor sediments from the sea of Okhotsk
				Next best hit Unidentified bacterium clone WP1OTU6 [DQ985888.1]	94%	Great Barrier Reef seawater
11.5.053CC8	EU267658	1	Alteromonadaceae (genus Alteromonas)	**Best hit 41-way tie**		
Medium/large, round, creamy off-white				Alteromonas sp. NJSX54 [EF061436.1]	99%	China Sea
				Alteromonas sp. NJSX51 [EF061435.1]	99%	China Sea
				Alteromonas sp. NJSX38 [EF061430.1]	99%	China Sea
				Alteromonas sp. NJSX35 [EF061428.1]	99%	China Sea
				Alteromonas sp. NJSX31 [EF061425.1]	99%	China Sea
				Alteromonas sp. NJSX25 [EF061424.1]	99%	China Sea

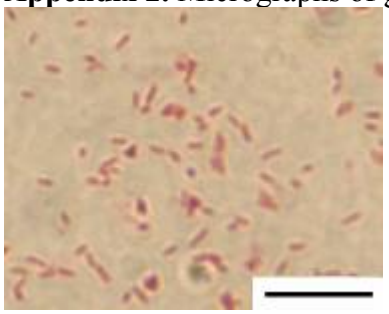
Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Alteromonas sp. NJSX18 [EF061421.1]	99%	China Sea
				Alteromonas sp. NJSS36 [EF061417.1]	99%	China Sea
				Alteromonas sp. NJSS35 [EF061416.1]	99%	China Sea
				Alteromonas sp. NJSS16 [EF061414.1]	99%	China Sea
				Alteromonas sp. NJSS12 [EF061412.1]	99%	China Sea
				Alteromonas sp. NJSS3 [EF061409.1]	99%	China Sea
				Alteromonas sp. NJSS2 [EF061408.1]	99%	China Sea
				Bacterium WP3ISO14 [DQ985875.1]	99%	Great Barrier Reef seawater
				Bacterium WP1ISO12 [DQ985822.1]	99%	Great Barrier Reef seawater
				Alteromonas sp. PH1228, isolate PH1228 [AJ876737.1]	99%	PHA producing bacteria isolated from a deep sea hydrothermal vent
				Alteromonas sp. SPB-8 [DQ412077.1]	99%	Mn(II)-oxidizing bacteria isolated from submarine basalts at Loihi Seamount
				Alteromonas sp. SPB-5 [DQ412075.1]	99%	Mn(II)-oxidizing bacteria isolated from submarine basalts at Loihi Seamount
				Proteobacterium SW1 [AY780442.1]	99%	Seawater isolate
				Uncultured Pseudoalteromonas sp. clone JL-ETNP-F14 [AY727023.1]	99%	2850m-depth layer of the eastern tropical North Pacific Ocean

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Uncultured gamma proteobacterium clone JL-ETNP-Z61 [AY726969.1]	99%	1000m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured gamma proteobacterium clone JL-ETNP-Z46 [AY726955.1]	99%	1000m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured gamma proteobacterium clone JL-ETNP-Y46 [AY726910.1]	99%	1000m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured Alteromonas sp. clone JL-ETNP-Y28 [AY726894.1]	99%	1000m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured gamma proteobacterium clone JL-ETNP-S55 [AY726866.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured Alteromonas sp. clone JL-ETNP-S53 [AY726864.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured gamma proteobacterium clone JL-ETNP-S47 [AY726858.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured Alteromonas sp. clone JL-ETNP-S26 [AY726839.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured gamma proteobacterium clone JL-ETNP-S12 [AY726830.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured Alteromonas sp. clone JL-ETNP-R52 [AY726811.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Uncultured gamma proteobacterium clone JL-ETNP-R16 [AY726788.1]	99%	100m-depth layer of the eastern tropical North Pacific Ocean
				Uncultured gamma proteobacterium clone JL-ESNP-I18 [AY664203.1]	99%	Eastern subtropical North Pacific
				Uncultured Alteromonas sp. clone JL-WNPG-U55 [AY664094.1]	99%	West Pacific Gyre
				Uncultured Alteromonas sp. clone JL-SCS-L60 [AY664007.1]	99%	South China Sea
				Uncultured Alteromonas sp. clone JL-SCS-L45 [AY663996.1]	99%	South China Sea
				Alteromonas sp. SSN-6 [AY626838.1]	99%	Unidentified sea-snail
				Uncultured bacterium clone HF500_A8_P1 [DQ300676.1]	99%	PCR amplicon from a single fosmid library pool from 500m depth, HOT station ALOHA 22.45 deg. N 158 deg. W
				Marine bacterium July88 small subunit ribosomal RNA gene, partial sequence [AY082664.1]	99%	Unknown
				Uncultured marine bacterium clone Surf1.44 [DQ071164.1]	99%	Gulf Stream Ring surface at 2 m
				Bacterium CWISO17 [DQ334355.1]	99%	water column of a *Panulirus ornatus* larval rearing system
				Pseudoalteromonas sp., isolate PRLIST2 [Y15323.1]	99%	Unknown
				Alteromonas macleodii, strain DSM 6062 [Y18228.1]	99%	Mediterranean seawater mesocosm

Isolate Name and colony morphology on SLB agar	Accession number	CFU	RDP Classifier Taxon (80% confidence threshold)	Nearest Relative [accession number] (two best hits are reported)	% Identity to nearest relative	Source/ Comments
				Next best hit Alteromonas sp. NJSS10 [EF061411.1]	99%	China Sea
11.5.053CC9 Small, round, white		1		No sequence data		

Appendix 2. Micrographs of gram stained bacterial isolates (scale bar is 10 µm).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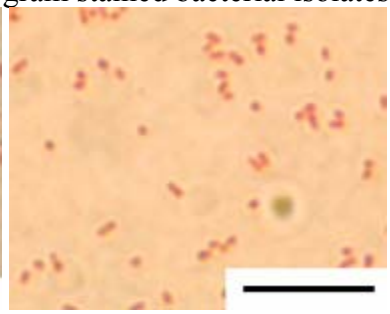
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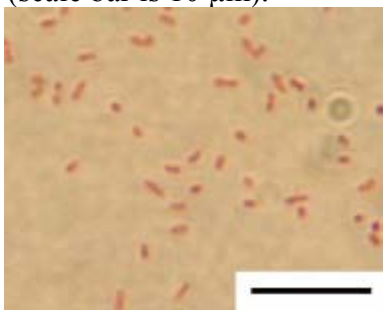
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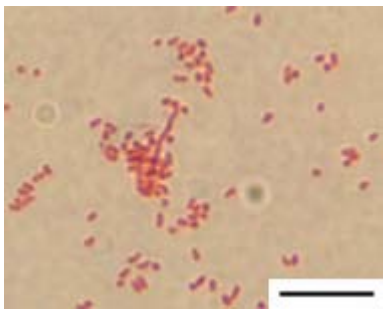
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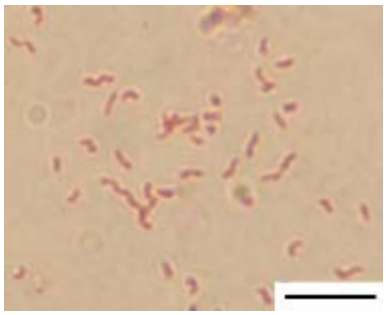
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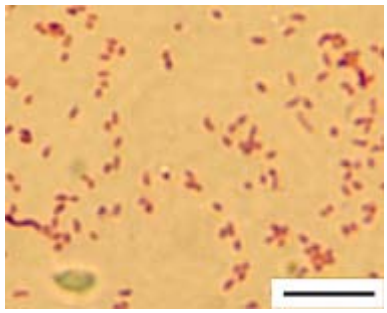
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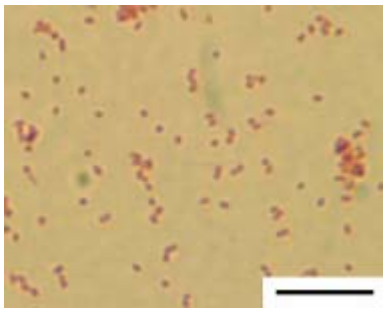
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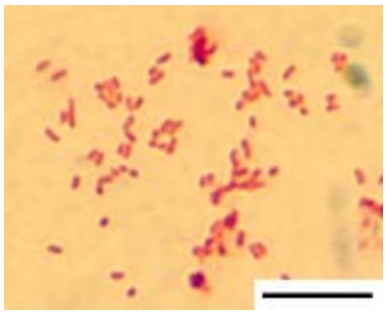
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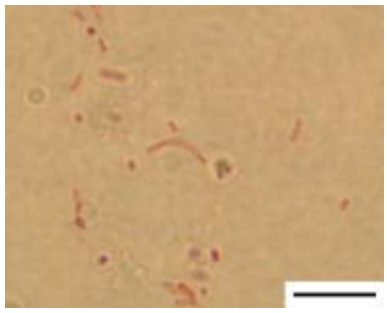
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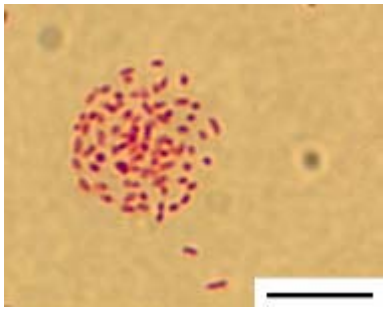
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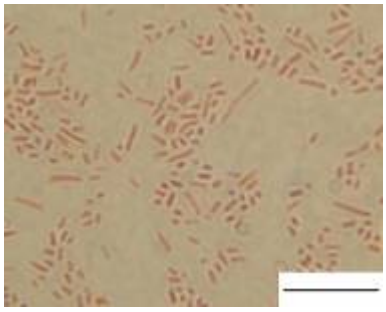
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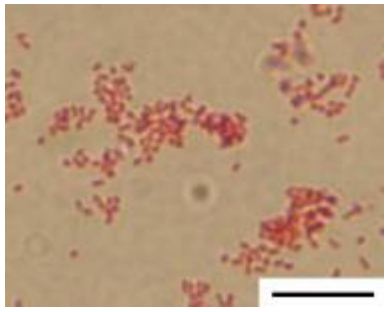
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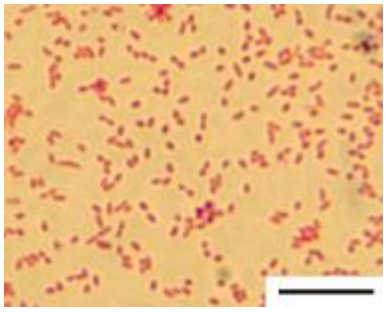
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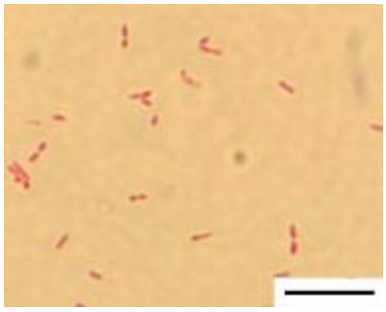
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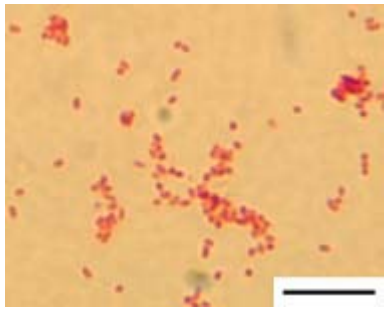
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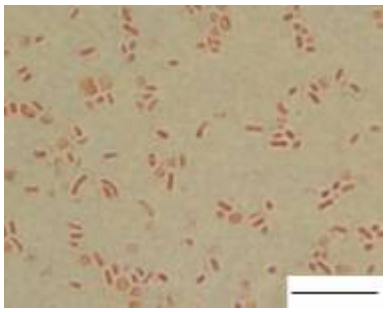

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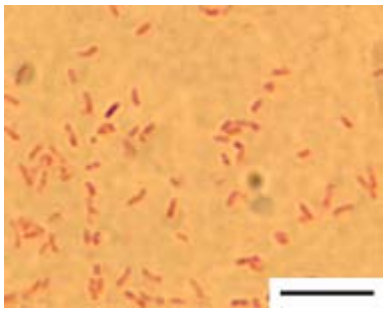

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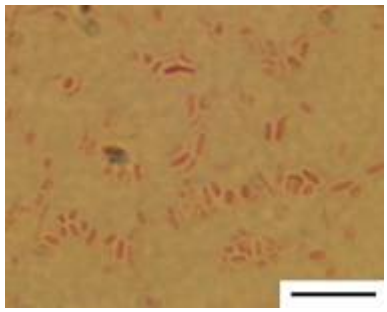

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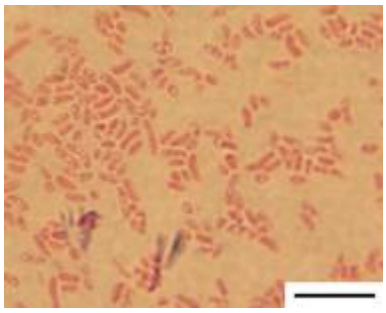

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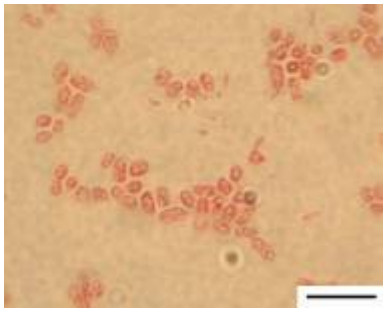

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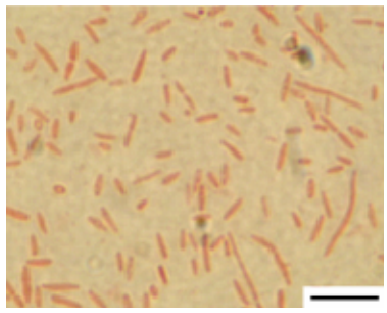

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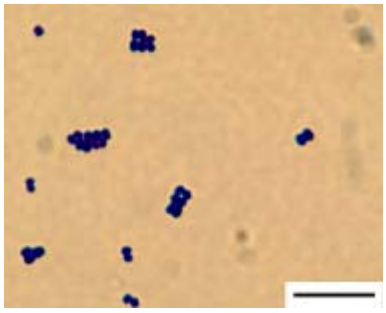

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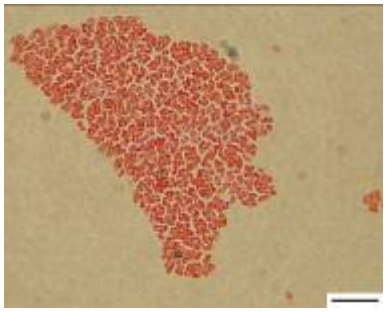

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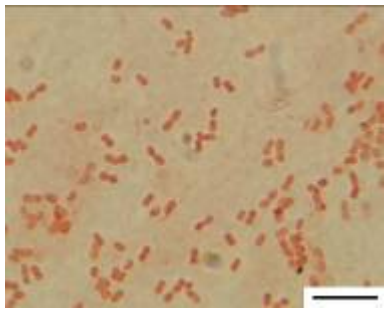

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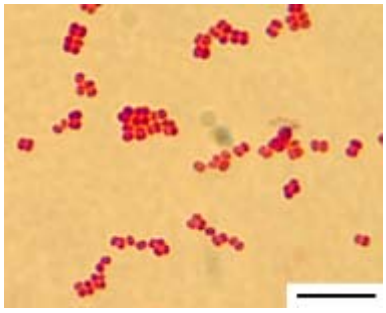

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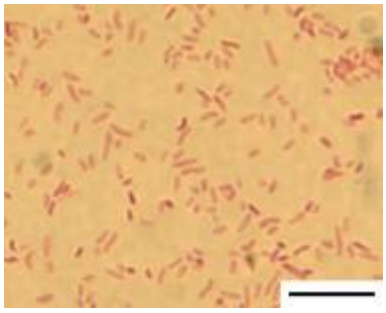

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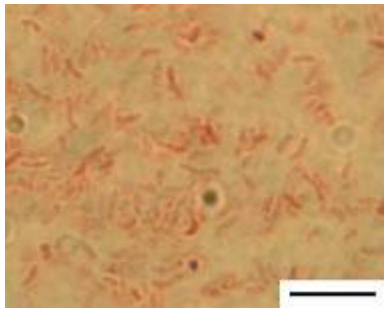

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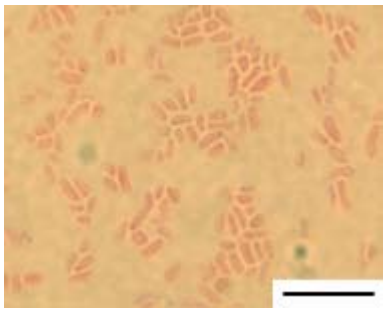

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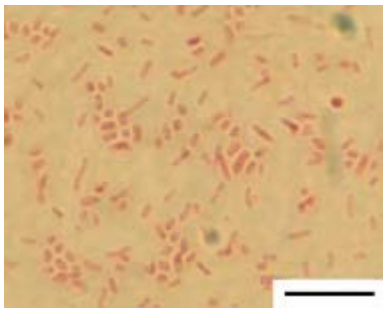

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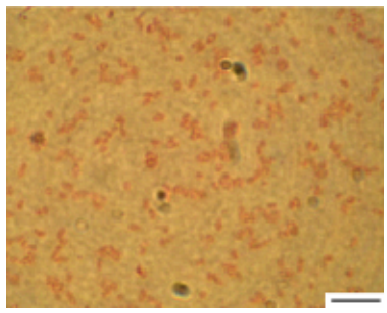

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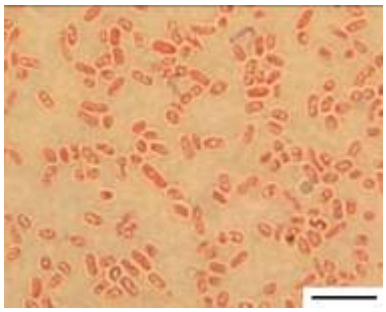

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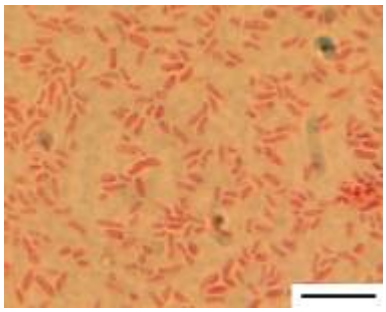

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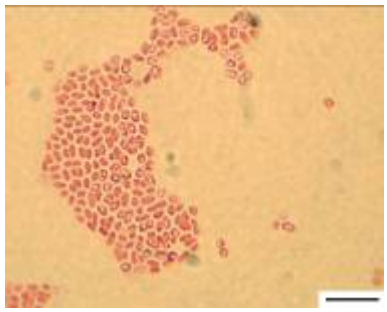

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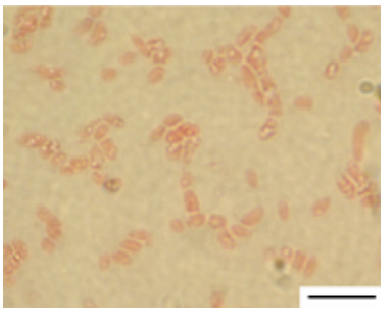

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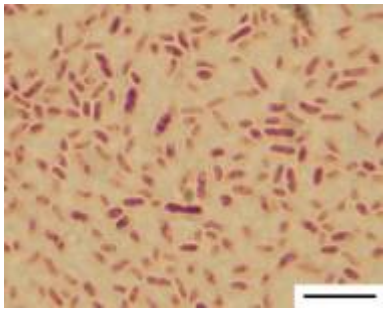

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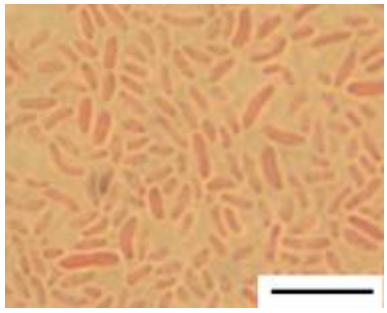

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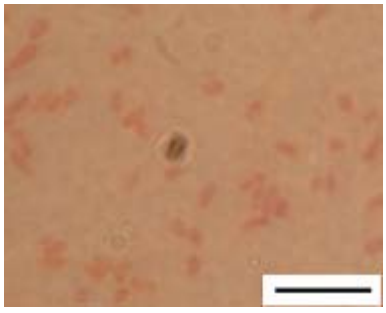

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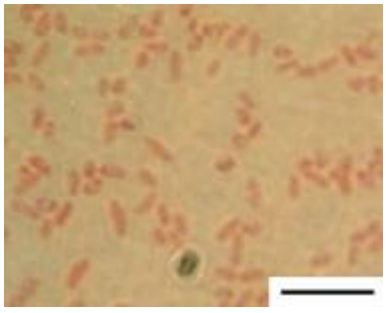

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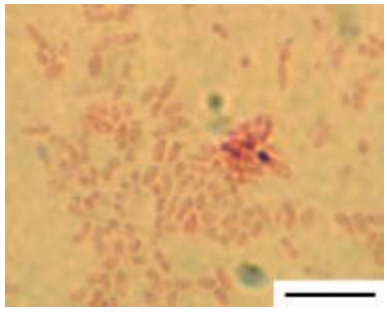

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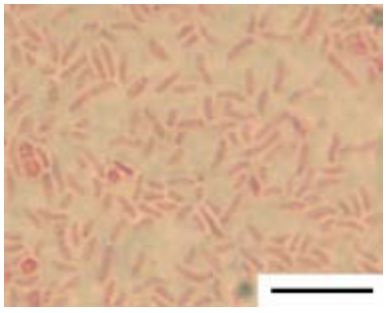
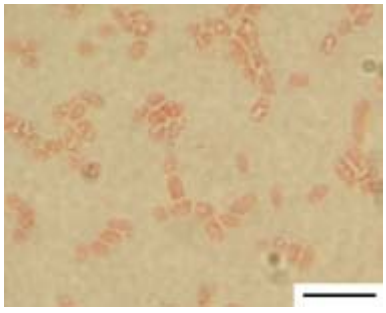
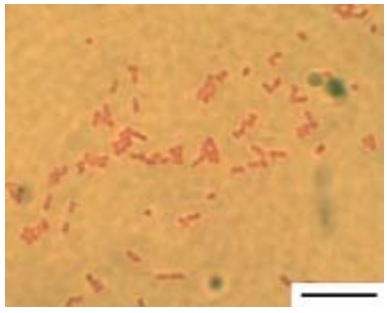


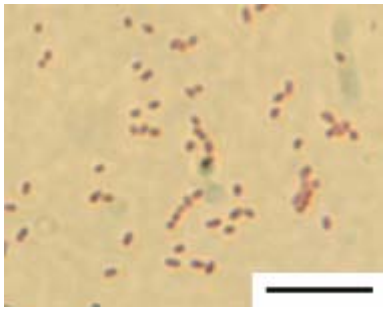
4.5.05 12MS6



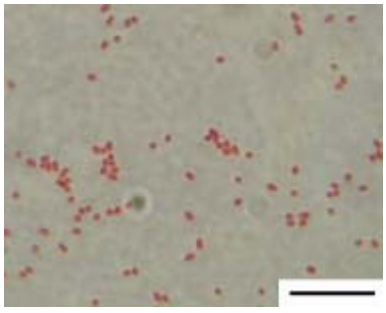
4.5.05 12MS7



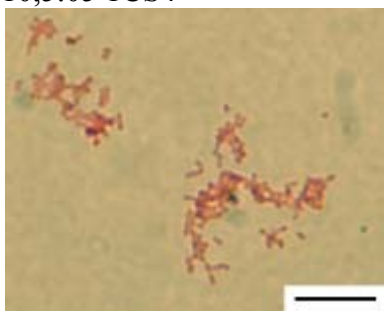
10,5.05 1CS4



11.5.05 1CC1



11.5.05 1CC2



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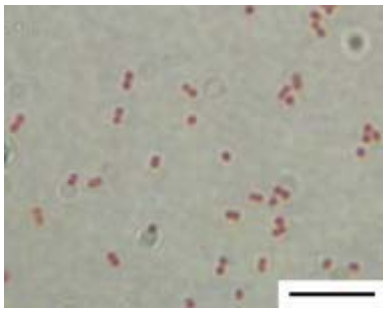

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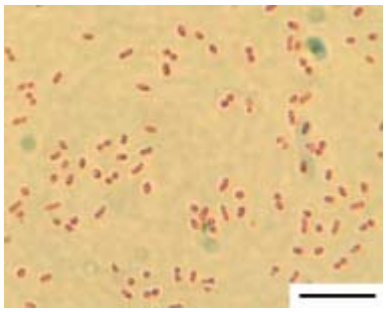
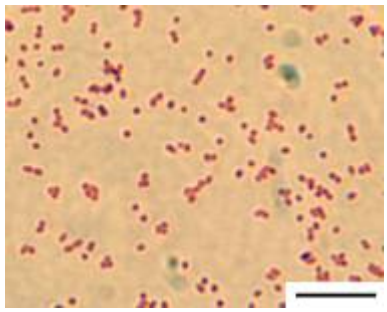


11.5.05 2CC2



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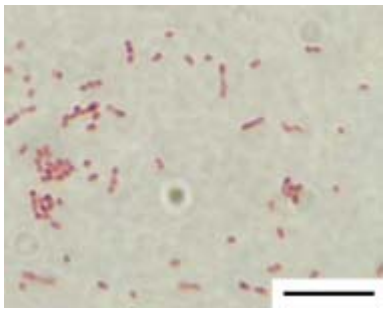

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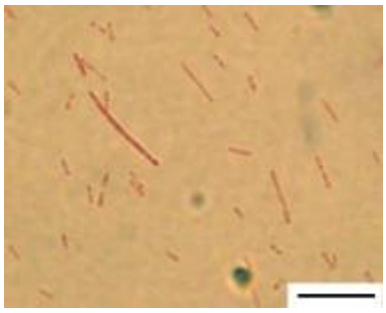
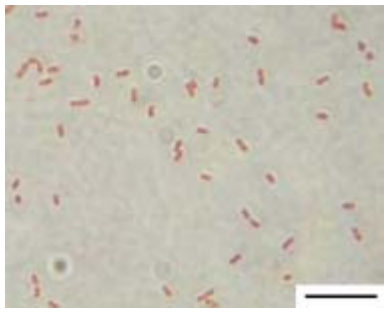


11.5.05 2CC10



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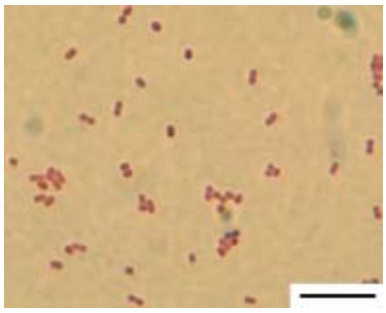

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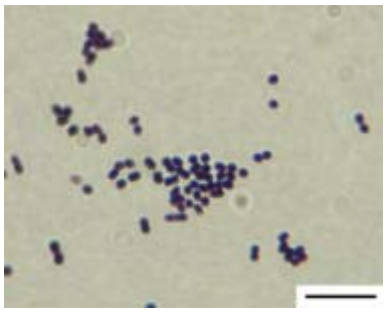

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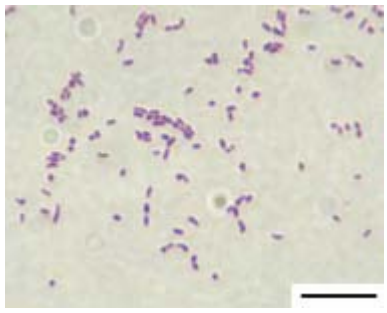
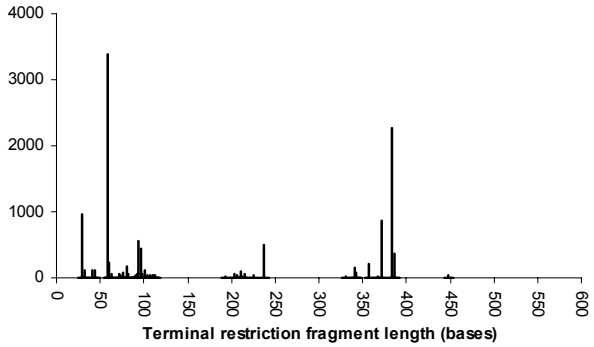
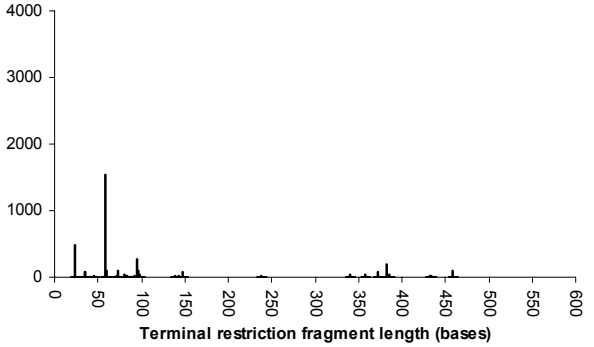


11.5,05 3CC2

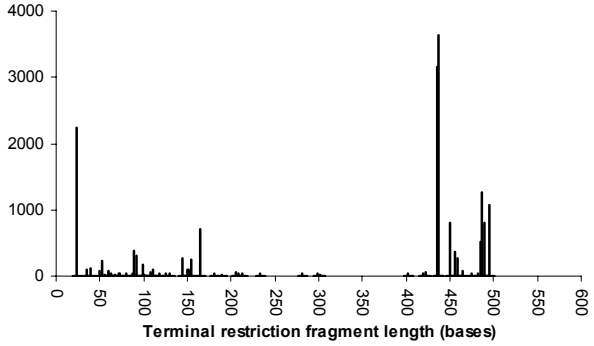
Appendix 3. Terminal restriction fragment length profiles of 16S rDNA amplified from total DNA extracted from coral samples 1M and 1D by the KIT and UREA methods.



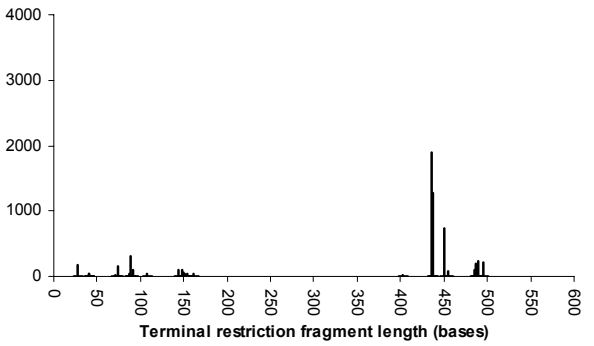
1. IM (Kit extract) digested with HhaI



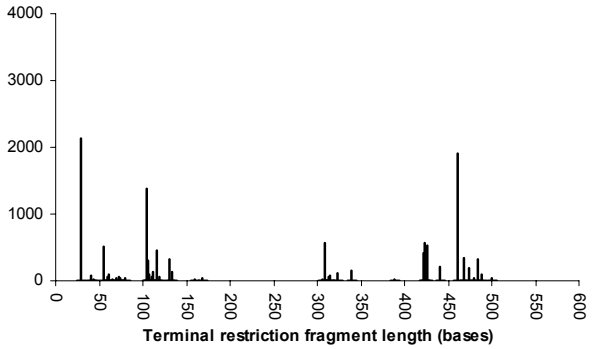
2. 1M (Urea extract) digested with HhaI



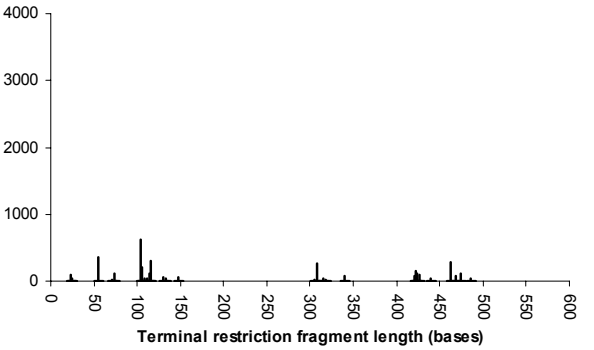
3. 1M (Kit extract) digested with MspI



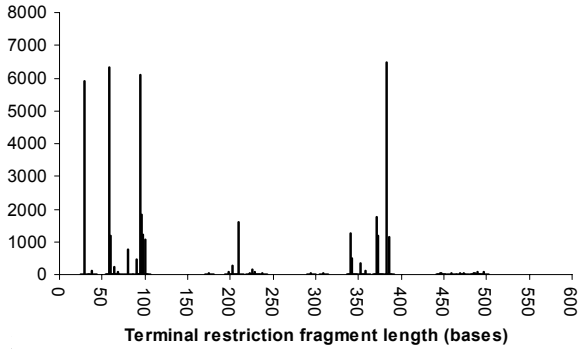
4. 1M (Urea extract) digested with MspI



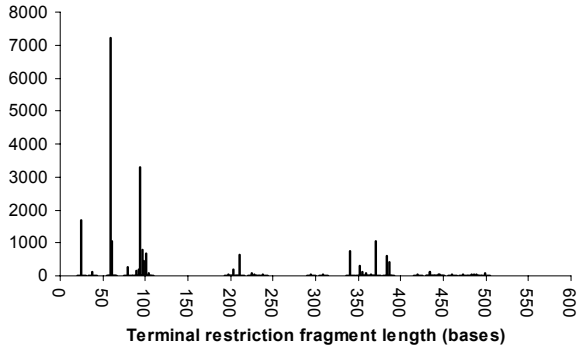
5. 1M (Kit extract) digested with RsaI



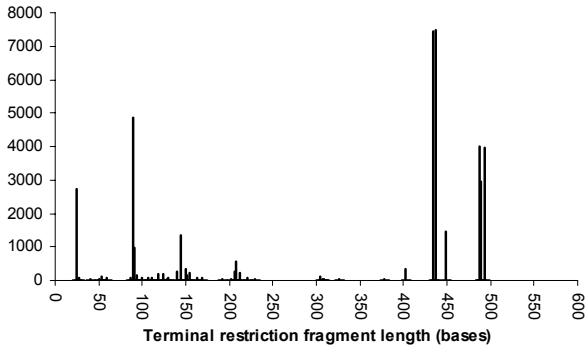
6. 1M (Urea extract) digested with RsaI



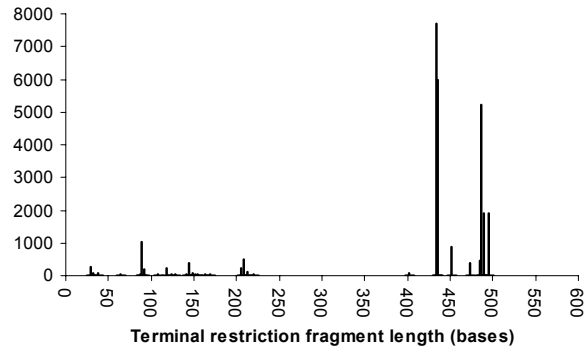
7. ID (Kit extract) digested with HhaI



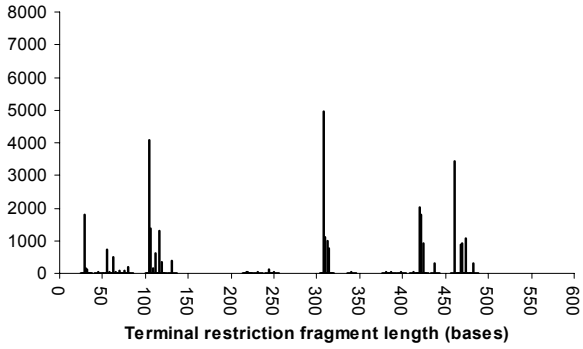
8. 1D (Urea extract) digested with HhaI



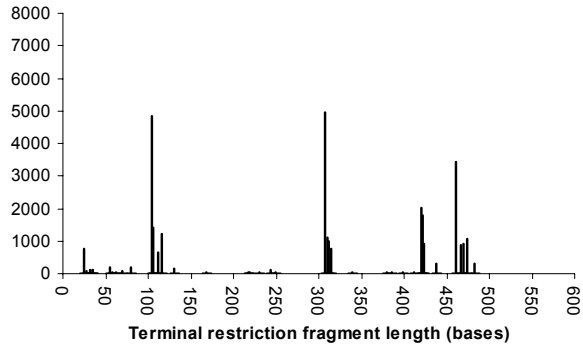
9. 1D (Kit extract) digested with MspI



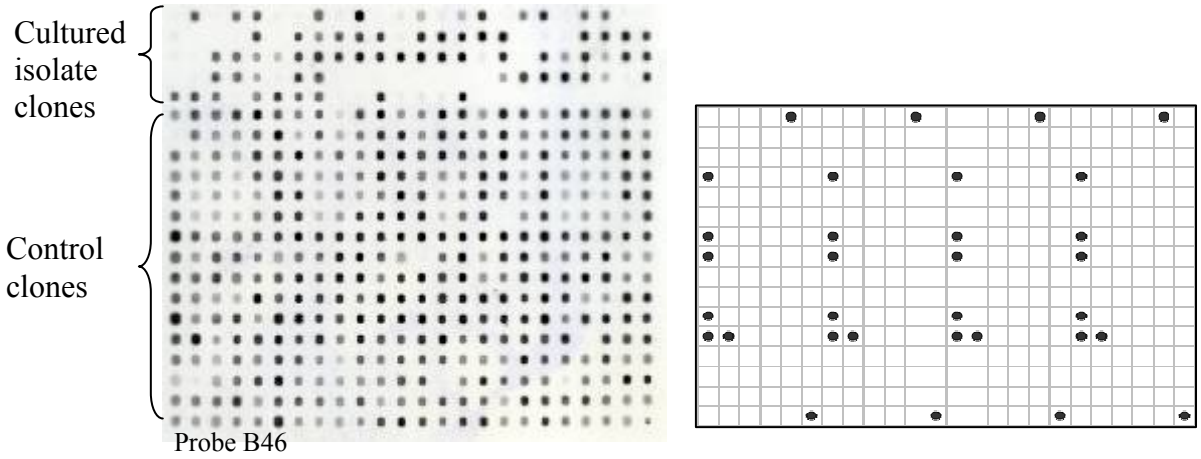
10. 1D (Urea extract) digested with MspI

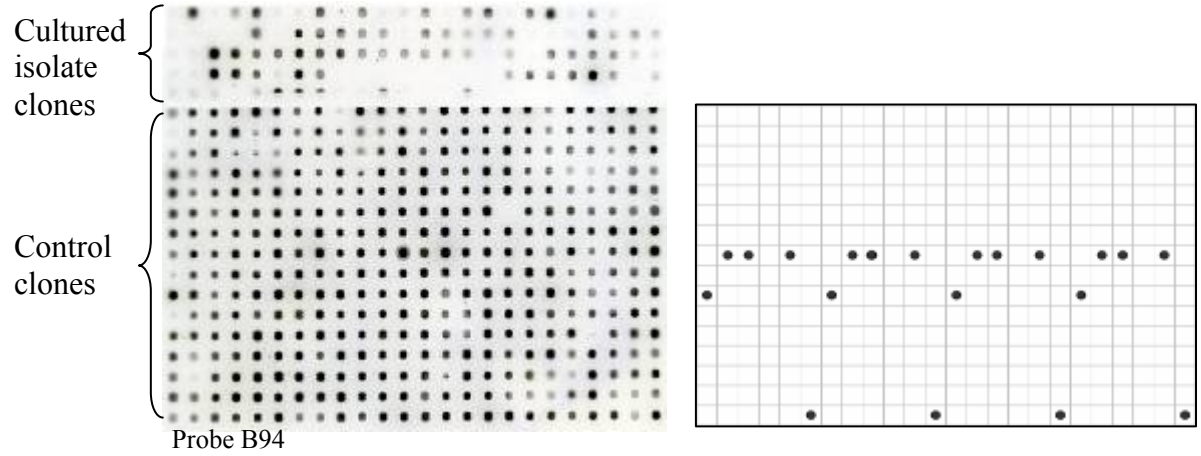


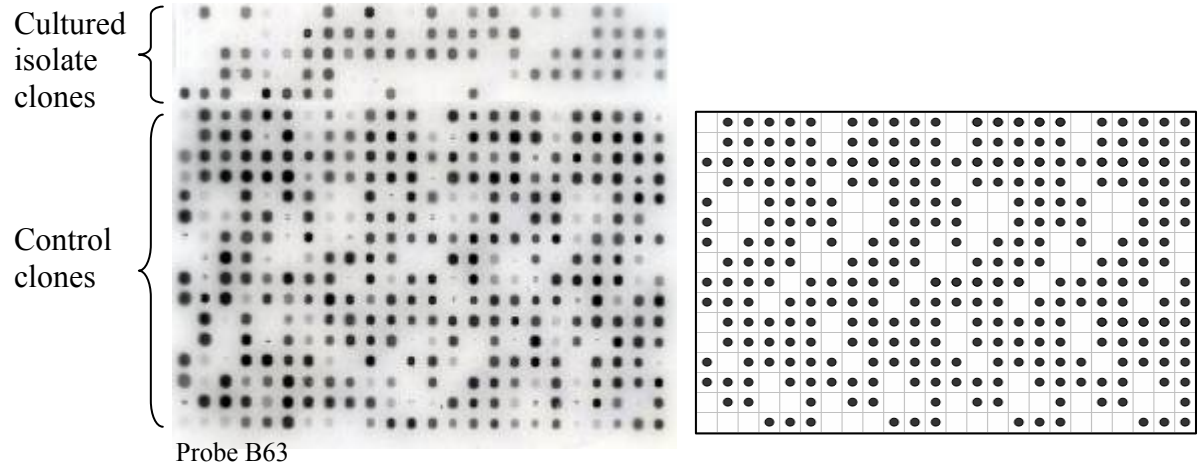
11. 1D (Kit extract) digested with RsaI



12. 1D (Urea extract) digested with RsaI







Appendix 4. Examples of macroarray images produced by hybridisation of labelled oligonucleotide probes with 16 rRNA genes arrayed on nylon membranes. The images on the left show the actual hybridisation signals produced in the experiment. The images on the right indicate the theoretical hybridisation pattern expected for the control clone library. The high number of false positive signals made the hybridisation data unusable. The name of the oligonucleotide probe used in each hybridisation experiment is indicated below the images.