Contrasting microbial communities across anthropogenic pollution gradients: MV *Rena shipwreck* versus urban pressures

A thesis submitted in partial fulfilment
of the requirements for the degree
of
Master of Science
in Biological Sciences
at
The University of Waikato
by

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The University of Waikato
2016

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Abstract

With the catastrophic environmental event that was the MV *Rena* ship wreck and oil spill in the Bay of Plenty area in 2011, a serendipitous opportunity to laterally explore a variety of impacts in the marine biosphere emerged. One of those opportunities is studied here; how microbial communities respond to stress in context of existing ambient local anthropogenic influences. The locational backdrop to this study is also relevant and provides an additional element of complexity as the Bay of Plenty region is located in the middle of the Taupo Volcanic Zone, hence microbial communities are likely to reflect the regional geology. Therefore a research project was possible where a pollutant gradient was selected based on a range of locations reflecting different elements of natural versus human long term and acute impact stresses. The concept was to establish a platform of preliminary study from which more detailed could follow. In addition this study aimed at providing a link between microbial environmental responses to stress in a context that could permit uptake of scientific evidence for cultural impact assessments given the strong cultural focus on MV *Rena* impacts by government and the public.

A significant element of the exploratory process was to identify methodologies which may be of use in ascribing microbial community composition with the microenvironment in a manner that allows comparison with research reported in the literature.

Review of all results showed that sites from widely varying geologies and environmental/stress condition were more similar than was initially predicted. This is in sharp contrast to expectations generated from the literature.

At the sites of Astrolabe Reef (Otaiti) (impacted by the ship wreck, major oil spill and heavy metal pollution event) and Maketu (impacted by a eutrophic estuary), similar consortia transpired, despite strongly different sources of stress, substantiating the microbial biogeography theory: '*Everything is everywhere*' and '*the environment selects*'. However at Tauranga Harbour long term human activity appears to have had a significant 'additional' effect on microbial communities. Our challenge has hence become one to separate out natural versus a range of anthropogenic stressors.

Underlying the general overall trends, subtle community effects such as at Astrolabe Reef, which supported significantly different dominant communities to the other two sites. It was expected that Tauranga Harbour and Maketu Estuary (adjacent coastally), would be more similar. Of all the sites one at the impacted Maketu, site 1 (M1) and the impacted Tauranga site 2 (T2), were highly similar at 96% from statistical analysis suggesting, the original assumption of visually similar site characteristics (impacted sites with low residence time and thick algal matts) do support similar microbial communities. Substantiating the theory that 'everything is everywhere' at these two sites, even though the variables and elemental composition were dissimilar.

Rare and abundant species were separated out and analysed to elucidate patterns or trends in the communities. Overall the abundant species clustered, into impacted versus non impacted sites, for the rare species Tauranga and Maketu clustered

clearly following the same trend; while the Astrolabe reef sites clustered together. The clear differentiation in the rare species from the Astrolabe reef sites, suggests that those species may make up the endemic bacterial communities at Astrolabe Reef with additional effects of *Rena* pollution suggested at some of the sites close to the wreck.

A general discussion of the cultural and economic impacts of the *Rena* oil spill, gave context to the holistic human impacts sustained by the local Māori, specifically the disconnect of their precious livelihoods and unique relationship with the environment. These holistic concepts could compromise a thesis in themselves so in this report their definitions are by no means finite.

This study attempted to interrelate both cultural, ecological biology and microbiology science components for the purpose of a more comprehensive investigation of microbial responses to the *Rena* oil spill and contrast these to local anthropogenic pressures. However being the first attempt to catalogue the previously unknown microbial communities in the Bay of Plenty area, meant that sample regimes, techniques, processing and analysis were all experimental. Despite this, effects attributable to the Rena were identified that lay beyond the large scale variability identified for Bay of Plenty microbial community assemblages.

These research results provide an important primary contribution to microbial understandings in the New Zealand context, and as a baseline mapping exercise for the Bay of Plenty region.

Acknowledgements

First and foremost I thank the Lord - that I have finally completed this thesis, all glory to God.

The biggest gratitude and thanks go out to my patient, persistent and ever encouraging supervisors. My primary supervisor Chris Battershill who against all odds supported me and encouraged me to finish, in between all his own international and national commitments, and all the curve balls after curve ball I threw at him, we finally got there, I am forever in your debt and can't thank you enough!! Your patience, support and generous giving of your time and experience has not gone unnoticed and words could never truly express how grateful I have been over this journey.

My secondary supervisor Craig Cary, opened my eyes to a microbial world I would have never been exposed to in any other place, the learning curves and journey has been spectacular and I will never forget all the effort your poured in to guide me, I am truly grateful.

My second secondary supervisor, Charles Lee, an absolute genius, I am in total awe and am always humbled by your presence. The skills you guided me to discover and develop will set me above and apart in the workforce. What challenged me the most was how you constantly challenged me to raise my thinking to the next level, then push past that, to the next level, that was so inspirational, being under your guidance was an utter privilege I will never forget. And like Chris and Craig, you made time for me in between your international and national research projects and commitments, I am indebted to your generous giving of time and teaching.

To the technicians at Sulphur Point, Dave and Rex, and Phil from ERI thank you for the hard work sampling, especially at White Island I know that trip was a total bore and such a drag, thank you so much for diving and hanging out with me there, it was such a privilege. To the other technicians and consultants who gave their precious time and knowledge especially Roanna Richards – Babbage (lab queen), Dudley Bell, Barry Obrien (all things microscopy king) and Steve Cameron (chemistry guru). To the other professors who influenced or advised along the way especially Alistair Wilkins (retired genius) and Michelle Prinsep (organic chemistry wizard),

I need to thank Kim Pritchard who edited and fired feedback to me in record time, to keep me on track and aid in any way she could, thank you, thank you so much!!

A big thank you to my peers at the Coastal Marine Research Station at Sulphur Point especially Te Puea Dempsey (mother hen), Vanessa and Caleb the writing retreat was such a great experience and you guys inspired me to keep going even when I wanted to throw it in, thank you so much, and to Te Puea for sharing her data with me, you made this research happen.

And a big thank you to my peers at the international research centre in Hamilton, especially Stephen Archer whose advice and directions lead me through, and his willingness to help in any way, so grateful, thank you so much.

To anyone else I may have forgotten at this time, it is not that you weren't appreciated just time has eroded my fickle memory, thank you!!

Last but by no means least, my ever suffering family, to my children who were uprooted and dragged across the motu, to live in the sunny Bay of Plenty and enjoy a life of sun, surf and beach, I know this was hard and you have suffered much, but thank you for your support and love, it is appreciated. To my parents who have been

unconditionally supportive I love you, thank you for everything. And Paul what a journey, I'm there, all my love and thank you.

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Chapter 1

General Introduction

The Global environment has seen an escalation in aquatic pollution since the industrial age in estuaries and coastal environments; micro-algal blooms have been considered a natural response to excess nutrients entering the ecosystem. However, such blooms have increasingly become associated with toxic or polluting events where a range of factors may influence within bloom species composition, their extent and duration.

The research presented here constitutes the first study to map the broad scale bacterial community consortia in the Bay of Plenty area, with an emphasis on examining 'contamination or pollutant related responses' by microbial community assemblages. As the Bay of Plenty region is renowned for its volcanic origins and continuing activity, an added factor is examined; that of responses that may be associated with natural environmental extremes and those associated with anthropogenic activity. Specifically, influences of land sourced enrichment or pollution are contrasted against the effects of a major maritime event – the MV Rena ship grounding and pollution, against a backdrop of volcanic activity exemplified by the active system at Whakaari, White Island. The research is additionally set into the context of impact on Mauri[1] and hence, the cultural impact on human health and wellbeing, with reference to the indigenous New Zealand Māori also discussed.

[1] Part of the *Rena* recovery plan as sanctioned by the Ministry of Environment is to consider Māuri, and the impact from the grounding, as pertaining to the local iwi (tribe), they have published an impact report on Māuri, specifying this in detail, see Bennett, (2015).

1.1 Our Oceans Microbes and their communities

The principal component of life support on planet Earth is the vast ocean gyre. Aquatic habitats occupy 70% of the Earth, of which >97% is held in the ocean's hydrosphere (Pommier et al 2006; Thakur *et al.* 2008) and within that biome billions of microorganisms are sustaining the biogeochemical functioning of the marine ecosystem (Zhang *et al.* 2014). How these microbial communities function, interact, disperse and the extent of their diversity is providing insight into the planet's beginnings and contemporary evolution (Pommier *et al.*, 2006; Fuhrman, 2009).

The role of microbial communities in coastal and oceanic processes is significant. Biogeochemical processes such as the marine carbon, nitrogen and sulphur cycles are being understood in much greater detail than ever before. For example, the use of dissolved organic material, as one of the largest carbon sinks in the earth's biosphere and can be metabolised into a single carbon energy source by the heterotrophic bacteria cluster Cytophaga-Flavobacteria-Bacteroides (CFB) (Kirchman, 2002). An estimated 50% of global carbon primary production is created from phytoplankton metabolising CO_2 into organic carbon (Thakur *et al.*, 2008).

Microbes' most beneficial role is arguably the ability to recycle primary elements, especially carbon, oxygen and nitrogen, into useable forms for uptake by other organisms (Todar, 2008). Modern molecular techniques are enabling fresh insight into this functional role, importantly providing information on their response mechanisms and resilience to external pressures.

The importance of bacteria and their role in biogeochemical processing is significant to sustaining all life forms. One important aspect of bacterial functioning in the earth's ecosystem, is the role they play in bioremediation of anthropogenic

pressures such as increased nutrient loading and oil spills (Kostka *et al.*, 2011; Paerl *et al.*, 2014). Observing the bacterial consortia which inaugurate bioremediation processes is critical to understanding recovery and resilience of bacterioplankton populations and the resultant effects on wider ecosystems. The development of molecular techniques has advanced understanding of how these processes occur and what genera are involved. This growing field has direct relevance to biotechnological advances and opportunity in bioremediation, as the enzymatic functionalities can be utilised. In addition, such marine microbiological research can be used in other industry sectors such as food, chemical and pharmaceutical (Thakur *et al.*, 2008; Todar, 2008).

While microbial function in our oceans is only just being fully appreciated, we are dealing with a marine ecosystem, under increasing threat. Environmental stress comes from anthropogenic sources, a multitude of which culminate in a changing marine climate through warming seas and ocean acidification. Future climate change predictions are increased temperatures, enhanced vertical stratification of aquatic ecosystems, alterations of seasonal and inter-annual weather patterns – including droughts, tsunamis, flooding, etc. (Paerl *et al.*, 2014). On a more regional scale sources of pollution are of increasing additional concern, yet the microbial response is not well understood.

One environmental stressor of significant concern globally is spilt oil; the substantial quantity of crude oil spilled in one incident can cause catastrophic ecological devastation, for example, the Nakhoda tanker accident, Japan (Head *et al.*, 2006); Deepwater Horizon, Gulf of Mexico (Head *et al.*, 2006; Kostka *et al.*, 2011; Redmond & Valentine, 2012); Exxon Valdez, Prince William Sound, Alaska (Leahy & Colwell, 1990); Texas, Rhode Island, Delaware (Leahy & Colwell, 1990);

Terra Nova Bay and Ross Sea, Antarctica (Yakimov *et al.*, 2004); Scott Base and McMurdo Station, Antarctica (Negri *et al.*, 2006), Browns Bay, O'Brien Bay and Casey Wharf, Antarctica (Powell *et al.*, 2003), to name but a few.

Therefore research comparing various marine habitat types and the ecological drivers imposed by each at the microbial level can aid in establishing marine molecular biology as an independent and relevant discipline (Thakur *et al.*, 2008). How microbial interactions through space and time reflect anthropogenic influence which affects population sizes, extinction, recruitment, resilience and recovery strategies is of the utmost importance. These kind of data are valuable to understand bioremediation as a mechanism for natural biodegradation and waste management in the environment.

1.2 Marine Microbial communities under stress

A common activator of bacterial biodegradation or bioremediation is spilt oil. Although there is a natural occurrence of oil seepage in the ocean estimated at 200 million tonnes per year (National Research Council (U.S.), 2003), large inputs from anthropogenic oil spills activate populations of microbial communities, which swell in abundance as mediation of the hydrocarbons are initiated. A pollution/diversity gradient is observed in several studies such as Webster *et al.*, (2001) and Negri *et al.*, (2002, 2006) with correlations being made to various species of macro- and micro-organisms which activate under these remediation conditions. Kostka *et al.* (2011) reported a consortia of Gammaproteobacteria - Alcanivorax, Marinobacter, and Alphaproteobacteria – Rhodobacteraceae were key players in oil degradation of the Deepwater Horizon spill in the Gulf of Mexico. Another key consortia reported in oil bioremediation is the CFB cluster and gram negative microbes. MacNaughton *et al.*, (1999) found that community structure shifted substantially in

laboratory studies of oil additions, and that CFB and gram negative consortia responded the most positively.

Leahy & Colwell (1990) found that microbial composition of biodegraders was dependent on oil quality and concentration. For example, toluene mineralisation was associated to its low viscosity and highly solubility (Leahy & Colwell, 1990), while higher molecular weighted (HMW) aromatic hydrocarbons such as naphthalene and phenanthrene correlated solely with solubility rather than concentration (Leahy & Colwell, 1990). Other biodegraders include fungi, viruses and archaea, that are commonly found in terrestrial and aqueous biomes (Head *et al.*, 2006; Thakur *et al.*, 2008). The community composition of biodegraders exposured to hydrocarbons is a result of the enzymatic capacity of the microbes to utilize that substrate (Leahy & Colwell, 1990).

1.2.1 Biogeography

To understand how microbial assemblages respond under stress, the concept of patchiness associated with naturally occurring communities is reviewed here. Molecular techniques have increased our understanding of bacterial community assemblage, functioning and interactions but still we pursue the paradox of how did they get there? Biogeography as a theory has been debated since Baas-Becking succinctly proposed the concept in 1934 that "Everything is everywhere and the environments selects" (Martiny *et al.*, 2006; Nemergut *et al.*, 2011). The essence of the debate is that dispersal mechanisms and barriers to microbial distribution are not limited as in macro-ecology. Bacteria or microbes were considered to be outside these principles as they could be found in any environment and were therefore deemed 'cosmopolitan', existing anywhere conditions were favourable (Fenchel, 2003, Martiny *et al.*, 2006; Pommier *et al.*, 2006). Based on their huge population

densities it is assumed that this evokes high diffusion and very low extinction rates (Fuhrman, 2009). However, Papke *et al.*, (2003) refuted this idea by asserting that phylogeny and distribution patterns in four geological hot spring locations were geographically isolated and the genotypes found were not identical, therefore the premise could not be true, and historical legacies had an effect on bacterial community structure. Fuhrman (2009) deliberates that this may in fact be true, but proving the contrary is impossible as you cannot prove complete absence of an organism, but he does assert that within discernible biotopes biogeographical patterns do exist.

Other studies have centred on the 'environment selects' notion, citing microbial assemblages are determined by contemporary environmental factors, which influence the spatial variation of these communities (Martiny *et al.,* 2006; Pommier *et al.,* 2006; Green, 2008). The essence of this concept is founded in macro-ecology with fundamental patterns revealed as species-area gradients (Green *et al.,* 2008), and isolation as a geographical barrier to ubiquitous dispersal (Papke *et al.,* 2003), and therefore it is inferred that functional traits contribute to community patterns. This may in fact be the case in macro-organisms, but in micro-organisms the complexity of functional variation, fitness strategies and horizontal gene transfer mechanisms suggest bacterial uniqueness (Martiny *et al.,* 2006; Green *et al.*, 2008; Barton & Northrup, 2011). In the case of micro-organisms limitations such as historical contingencies and current evolutionary traits are based on contemporary environmental factors which, Martiny *et al.* asserts, empirically defines the opposing characteristics of micro and macro organisms (Martiny *et al.,* 2006).

Zhang *et al.* (2104) demarcated a difference in community presence and activity within structures, and although correlation of environmental factors were revealed,

geographic distance was inconsequential. Active members of the bacterial communities were more sensitive to external influences and a depth gradient was observed in microbial communities. Findings from this study verified that not only dispersal mechanisms but recruitment success were supported by the distance-decay relationship (Zhang *et al.,* 2104). The active subset of the microbial community positively exhibited a distance-decay relationship from comparisons between the 16S rRNA and 16S rDNA (Zhang *et al.,* 2104).

Earlier observations by Zhang *et al.* (2007) on bacterioplankton community characteristics were defined as 'particle attached' and free living assemblages, in the polluted and non-polluted waters of Victoria Harbour, Hong Kong. This study surmised that the 'particle attached' communities are more vigorous, dominant and productive than their free living counterparts. It is increasingly evident from the research that community, co-occurrence and symbiotic relationships drive productivity in the microbial world. Symbiotic relationships within and between microorganisms are well documented for example sponges and bacterial symbionts (Webster *et al.*, 2001, 2004), microbial co-occurrence (Kirchman, 2002; Fuhrman, 2009), bioremediation consortia (Leahy & Colwell, 1990; MacNaughton *et al.*, 1999; Kostka *et al.,* 2011). So as an area of research for both biotechnology and bioremediation there are clear opportunities focusing directly on community substructure and their elements.

For planktonic organisms other discussions involve endemic, rare or abundant species concepts as critical aspects determining bacterioplankton community structure. In the marine gyre, endemic species are thought to be the rarity, therefore diversity over a spatial scale is thought to be very low (Pommier *et al.*, 2006; Green *et al.,* 2008; Zhang *et al.,* 2014,). Evidence supports the long-tailed species

abundance curve indicating the large presence of 'rare' species occupying most ecological systems (Fenchel & Finlay, 2004; Fuhrman, 2009; Zhang *et al.*, 2014). As Fuhrman (2009) emphasises 'rare' and abundant species are in the range of 0.1 to 1% of communities, and that the rare percentage contribute the least in biogeochemical processes.

Hubbell (2005) examines a competitive niche paradigm which was developed based on the idea that resource availability determines consumer species abundance. In this resource based theory, trade-offs in functional goods and services occur as a form of coexistence and competition for a limited resource, therefore the 'strongest survives' in the niche biotope (Hubbell, 2005). Hubbell (2005) defined this notion as *functional equivalence* or *symmetry* where heterogeneity is diverse but there is a ceiling on *per capita* rates, which is a constant, relative to resource availability.

In contrast to the niche theory in macro-ecology, the neutral theory, as explained by Hubbell (2005) is a simplistic community assembly model which explicitly ignores functional traits and variations. Accordingly its success is in its rudimentary conceptualisation, however, it requires empirical patterning to test its hypotheses so underlying processes may be obscured (Green *et al.*, 2008). In plant ecology (Hubbell, 2005) defends the role of dispersal limitations and stochastic processes as essential in the assembly of natural communities de Wit & Bouvier (2006) agree. At its core it suggests that stochastic births, deaths and immigration are the key functional aspects in community assemblages. Therefore, communities are finite consumer populations and only through death can replacement opportunities arise, via births or recruitment: thus a neutral dynamic model (Fenchel & Finlay, 2004; Sloan *et al.*, 2006).

However, these models assume stable constants such as competition being equal and the absence of predation, so although predictions made on patterns of diversity and abundance are reflective of actual communities, they are not necessarily accurate. In Sloan *et al.* (2006) a parameterisation matrix is used to define the detection threshold of relative abundance so microbial field data can be incorporated into the model to quantify its results. Evidence from that study shows that it is in fact chance and immigration that model patterns in prokaryote community assemblages at the scale that they are typically observed. Although the model may not be acutely correct, it can still be useful for gathering data.

In summary, biogeographic analysis lays a platform to explore bacterial community assemblages. What is evident is that bacteria are ubiquitous but how, why and where they disperse can be influenced by an array of factors. It is obvious that co-occurring consortia are most productive and may even increase in productivity with symbiotic relationships with other micro and macro-organisms. For a methodological theory, biogeography is a means to measure and understand community assemblages and what consortia may be expected in the environments that have been selected for this study.

1.3 Bacterial community assemblages

Looking closer at the patchiness of ambient assemblages, bacterial communities at the microcosm scale also show distinct patterns and orders.

Previous research indicates a hierarchy of dominance in bacterial community assemblages (Zhang *et al.*, 2007; Bottos *et al.*, 2008; Niederberger *et al.*, 2015). In Zhang *et al.* (2007) the community structure was revealed from particle-attached and free living organisms in Victoria Harbour, Hong Kong. In the particle-attached

communities, *Cyanobacteria* were the third dominant phyla, but in free living organisms *Actinobacteria* were the third dominant (Zhang *et al.,* 2007). Similar patterns were revealed in Antarctica soils from Niederberger *et al.* (2015) where wet soils and one dry soil samples revealed *Cyanobacteria* as the third dominant phyla, but in dry soils *Actinobacteria* were third most dominant. From the American culture collection *Firmicutes* are the second most dominant phyla then *Actinobacteria* is third (Floyd *et al.,* 2005). A study from the Japan coast by Suzuki *et al.* (2001) found *Cyanobacteria* as the second most dominant phyla followed by *Firmicutes.* Then there are studies which reveal the dominance of rare species specific to those biomes, such as *Deinococcus thermus* and sulphate reducing or green sulphur bacterium from Antarctica in Webster & Negri (2006).

Indications of bacterial community assemblages suggest that a hierarchy of structure can be expected in the order of *Proteobacteria*, *Bacteroidetes*, and then *Cyanobacteria,* but this hierarchy shifts depending on factors or variables specific to the sites. In Barton & Northrup (2011) findings show that aquatic habitats have different phylogenic groups and these differences are even more evident at lower taxonomic units such as genus. Zhang *et al.* (2007) determined algal blooms to be particle attached communities, therefore it is expected that the findings from this study should reflect similar community assemblages, dictated by current environmental influences (*Rena* oil spill) paired to environmental factors. So the community assemblages sampled here should therefore be snap shots of the primary production consortia in the selected biomes.

1.3.1 Genera and their ecological characteristics

Specific genera have preferences for energy sources and these can reflect their ecological characteristics. Microorganism energy use is metabolised from reduced

organic compounds or inorganic materials in the anaerobic and aerobic environments (Fernández-Gómez *et al.,* 2013; Zhang *et al.,* 2014). The energy supplied from the cell metabolism is a mechanism for motility and nutrient transportation (Kostka *et al.,* 2011; Fernández-Gómez *et al.,* 2013). What defines microorganisms from other organisms is that the single cell is the whole life form whereas most other life forms are multiple cells constituting the whole form. Cell propagation is asexual in bacteria therefore biomass fluxes are connected to nutrient increases. Environmental changes stimulate selection criteria within cells which favour the genetic content most likely to thrive in the new environmental conditions. These forms of adaptation can reside within populations as induction or gene repression and although the geochemical shift may be short term it may occur frequently such as in algal blooms (Barton & Northrup, 2011; Kostka *et al.,* 2011).

Communities which have previously been exposed to increased loading or nutrients show higher resilience, recovery rates and positive response mechanisms than assemblages from pristine habitats (Atlas, 1991; Yakimov *et al.,* 2004). Shifts in community structure changes are signalled by chemical inductions known as auto-inducers. When population densities are swelling auto-inducers signal stressor responses, and these signals accumulate until they cross a threshold which induces a shift in gene expression. The change in target gene expression influences community behaviour and is called quorum-sensing (Barton & Northrup, 2011). The quorum-sensing phenomenon is a protection mechanism for bacterial communities against predation and is considered a stressor response. Additional to the stress response mechanisms in the quorum-sensing phenomenon are mortality and predation as an influence of community behaviour shifts. Protists and viruses are the main predators of bacteria (Barton & Northrup, 2011). Although viruses are

ubiquitous across all habitats, their role in regeneration of organic matter in aquatic habitats lies in transforming carbon and other nutrients by lysing their energy requirements out of their prey.

The current phylogenetic classification system used here, (*Proteobacteria*, *Bacteroides* and *Cyanobacteria)*, is evolving at such a pace due to the explosion in discovery of molecular techniques which can identify more in-depth functionality, with new classification categories are being revised from geochemical discoveries that in two years' time the current classification used here, may be redundant (Cummings, 2006; Kirchman, 2002).

1.3.2 Proteobacteria

Proteobacteria are one of the largest and most diverse eubacterial groups and are gram negative. At present while there is ongoing debate about how many classes of Proteobacterial categories there are, for the purposes of this study I have chosen to use the following five classes as per Cummings, (2006) and Barton & Northrup (2011), Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria. Each class level has distinct characteristics which describe their physiological differences (Cummings, 2006, Barton & Northrup, 2011).

In general, Alphaproteobacteria include most oligotrophic and purple non-sulphur bacteria. They are flexible in their energy sourcing, not sulphur or sulphate oxidising, and prefer habitats with high organic matter and low sulphide levels. They also include nitrogen fixing, nitrifying, ammonia and ammonium reducing bacterial genera, as well as pathogenic genera. One genera which indicates nutrient

poor marine environments and can also be a contaminant in sample processing is *Caulobacter* (Prescott, 2001; Cummings 2006).

The class Betaproteobacteria overlaps with Alphaproteobacteria but their preference is for nutrient starved habitats. They use this biosphere to metabolise dissolved organic matter. They are considered to be a diverse group with a wide range of metabolic capacity; some abilities include reducing nitrate to nitrite, oxidising hydrogen sulphide and reducing sulphur to sulphate. Pathogens associated with this class include human diseases such as whooping cough and meningitis (Prescott, 2001; Cummings, 2006).

The Gammaproteobacteria are the largest and most diverse group of all the Proteobacteria*:* in the Bergey's Manual (Boone *et al.*, 2001) of systematic bacteriology, it is divided into 13 orders, 19 families and around 130 genera (Prescott, 2001). This group includes methane oxidisers, facultative anaerobic genera who metabolise carbohydrates, oxidisers of hydrogen sulphide and intracellular pathogens like *Legionella pneumophila*.

The Deltaproteobacteria, one of the smaller known classes, are a group of unusual genera which are chemo-organotrophs, organisms which obtain their energy from oxidising electron donors from their environments. One order is the Desulfovibrio, which are strict anaerobes using elemental sulphur or sulphur compounds as electron acceptors during anaerobic respiration. This particular order plays a key role in sulphur cycling and thrives in polluted sediments as they digest methane. This class also has one of the only prey bacterial organisms, the Bdellovibrio which alternates between a predator and an intracellular reproductive phase (Prescott, 2001; Cummings, 2006).

The smallest order (with two families and two significant genera) is the Epsilonproteobacteria. The two families *Campylobacter* and *Helicobacter* have only recently been reclassified in this order and are associated with human infections and vibrio–like organisms which cause gastric infections, ulcers and cancers and nervous system disorders (Todar, 2008).

1.3.3 Bacteroidetes

Consistently recognised as one of the three most abundant bacteria in the ocean, *Bacteroidetes* account for between 10-30% of total bacterial counts in coastal areas (Fernández-Gómez *et al.*, 2013). Some genera are better known for their specialist processing of polymeric organic matter in mammalian guts (Fernández-Gómez *et al.*, 2013). In aquatic habitats they are synonymous with post-algal bloom occurrence suggesting that they have a penchant for polymers. They are commonly correlated to specialist degrading of HMW compounds and particle matter in general.

The preferred growth mechanisms of *Bacteroidetes* are in attaching themselves to particles, surfaces and algal cells. Of the many adaptive characteristics they possess, their polymer degrading abilities include peptidases, glycoside hydrolases, glycosyl transferases as well as adhesion proteins and genes for gliding motility. Proteorhodopsin which is associated with the *Bacteroidetes* order has two specific characteristics of note - their small genome size and a higher number of genes involved in CO_2 metabolism per Mb (Fernández-Gómez *et al.*, 2013). Findings from their study found that co-occurrence between the proteorhodopsin gene and anaplerotic CO_2 fixation gene in the genomes of Bacteroidetes do not sense or utilise light. These characteristics are thought to be significant as survival mechanisms, as they freely float in the nutrient poor surfaces of the ocean.

Bacteroidetes are known to be the largest polymer degrading enzyme community, confirming their role as primary particulate matter degraders which indicate a preference for proteins. The functionality of the *Bacteroidetes* suggests their complimentary role in the carbon cycling of the oceans to *Cyanobacteria* and *Alphaproteobacteria*.

1.3.4 Cyanobacteria

Cyanobacteria are an important but understudied group of marine micro-organism. At the New Zealand regional scale, Cyanobacteria and their toxins, are recorded from the late 1990's (Hamill, 2001), with marine varieties not being well understood or investigated. Therefore the research presented here provides a much needed preliminary examination on marine Cyanobacterial varieties which influence our kaimoana (sea food). Of relevance are anecdotal links to paralytic shellfish poisoning (PSP) and suggestions that such events can co-occur with major pollution incidents (R Reichelt, GBRMPA; D Tang SCIO pers com)

Research to date on cyanobacteria in New Zealand has tended to concentrate on freshwater cyanobacteria due to their direct association with drinking water supplies. Several reviews and risk assessment reports have been commissioned by the New Zealand Government and local regional bodies in order to establish monitoring programmes and protocols to protect the public from exposure to nonlethal toxins (Wood *et al.*, 2006, 2009). Similar research is occuring globally to address local issues eg: Baltic Sea (Mazur-Marzec & Plińsk i, 2009); India (Rashid *et al.*, 2013); Lake Victoria, Africa; Lake Erie and Michgan, US–Canada; Lake Okeechobee and Lake Ponchartrain, USA; Lake Taihu, China; Lake Biwa, Japan; and Caspian Sea, west Asia (Paerl & Huisman, 2009).

In New Zealand, the first toxin-associated poisonings were dog deaths in the late 1990's, and observations of a growing presence of blue-green mat-like occurrences in streams and lakes (Hamill, 2001). From mouse bioassays, toxins were identified but could not isolate a singular toxin. Studies found that the presence of one or more similar *Oscillatoria* species of cyanobacteria were responsible for the dog poisonings. However, the toxins were not specific to a species type but it was discovered that the favourable conditions allowed the toxins to produce, however, it is not known what the exact favourable conditions are e.g. temperature, co-occurrence etc (Hamill, 2001).

A recently released study revealed evidence of global warming, watershed degradation and increased nutrient loading as contributing factors to the frequency, distribution, extent and increasing severity of harmful algae blooms: emphasises the lack of knowledge pertaining to health risks to animals and humans from accumulative nonlethal toxin exposure (Backer *et al.*, 2013). A national survey of New Zealand water bodies in 2001 to 2004, ascertained the extent of cyanotoxins and saxitoxins.

While saxitoxins were considered to be much lower in concentration than cyanotoxins, an increasing presence notably from the mat-like formations is causing alarm (Wood *et al.*, 2006). That study aimed to explore the likely producers of these cyanotoxins the extent and distribution of the species responsible. What was evident was cyanotoxins were more widely spread than first thought in New Zealand waterways and that algal mats were found to be the primary source of the cyanobacterial communities and cyanotoxins. The study advocated for more research to be done on species occurrence, distribution and extent (Wood *et al.*, 2006).

Cyanobacterial physiology is that of an oxygenic photosynthetic aerobe which uses water as its electron donor and expels O_2. Cyanobacteria have phycobilins or light receptors in addition to chlorophyll which allows them to harvest light from a broader spectrum range than can be acquired by chlorophyll. The advantage of such receptors in the aquatic environs is a considerable benefit, as each wave length of light is absorbed at varying frequencies from the water column (Barton & Northrup, 2011).

The need for further research on marine varieties of cyanobacterial blooms and their associated bacterial consortium is highlighted in the studies mentioned above. Specifically, information is needed on abundance, distribution and their extent to characterise bacterial populations. Findings reveal that a plethora of variables impact bacterial assemblages based on environmental factors which stimulate community production; therefore variables which will aid in answering the hypothesis such as DO, Conductivity, pH, temperature, elemental composition of surrounding habitat have been targeted.

1.3.4.1 Cyanobacterial background

Considerable research indicates that cyanobacteria are some of the oldest organisms to occupy the earth. They are believed to be the seed population of earth's modern vegetation (Sharma *et al.*, 2011) and their only requirement for genesis is sunlight, utilising a wide spectral range. Cyanobacteria are resilient to pH and oxygen extremes, and can exist in water depleted of nitrates or ammonia (Mazur-Marzec & Pliński, 2009; Sharma *et al.*, 2011). The total lack of growth requirements reveals their highly developed structures and mechanisms, which allows them to proliferate in every habitat on earth including extreme climatic conditions such as desert,

volcanic and arctic Gaia (Mazur-Marzec & Pliński, 2009; Sharma *et al.,* 2011; Schirrmeister *et al.,* 2011, 2013; Marshall, 2012). Although cyanobacteria are one of the oldest and most resilient organisms found on earth, to date very little is known about the origins, evolution and extent of their vast diversity.

Although fossil data dates cyanobacteria back to deep in the pre-Cambrian era, they are considered to be one of three major branches of life forms on the earth. Before the 1970s these organisms were classified by morphological characteristics, however, with the introduction of genomics and sequencing it was found that morphological features were not consistent in categorising relatedness. The debate on identification and classification has been raging for decades (Ojvind, 2014), as microalgal species present as cryptic and pseudo cryptic genera; based on similarities and dissimilarities.

Contemporary classification of cyanobacteria consigns them as the only prokaryotes with oxygenating photosynthetic abilities (Sharma *et al.*, 2011; Schirrmeister *et al.,* 2011, 2013; Marshall, 2012). Although diverse in morphology, the three distinct morphological groupings for cyanobacteria are unicellular, multicellular and filamentous forms (Percival & Williams, 2014). Multicellular has been further defined as heterocysts or filamentous forms with or without heterocysts (Kumar *et al.,* 2010). Current research has found that heterocysts are the originators for cell differentiation tasks within the cyanobacteria, colonies and clusters (Sharma *et al.,* 2011).

Results from Schirrmeister *et al.* (2011, 2013) studies suggests that cyanobacteria multicellularity existed before the Great Oxidation Event (GOE) and that their evolutional rate increased in abundance and distribution after this event. It is believed that the increase in oxygen offered niche biotopes which increased

speciation and created the availability of diverse habitats for cyanobacteria colonisation. Cyanobacteria are noted as a key element in the GOE, which capitalised on a niche event, and is believed to have occurred 2.45 billion years ago. However, the role they played is still uncertain, although fossils dating 2.2 billion years back support the explosion in cyanobacterial diversity. Fossil data is limited by availability and evolutional processes which can identify divergent events to an extent, states Schirrmeister in an interview by Marshall (2012).

Although referred to as bacteria, cyanobacteria have traits of both bacteria and algae, hence the name. Similarity to bacteria is found in the cellular structure but cyanobacteria have multicellular adhesion with a protective sheath membrane that grows in a singular direction (Kumar *et al.,* 2010). The chloroplast organelles are the subunits within the cells that harvest light as the main source of energy; they then expel their byproduct of oxygen. As some of the most successful organisms on earth they are gram-negative bacteria, to date this has allowed their successful identification (Sharma *et al.,* 2011). With these exploitative characteristics cyanobacteria are able to out compete most other phytoplankton organisms on the earth (Mazur-Marzec & Pliński, 2009).

As primary producers cyanobacteria generate 30% of oxygen production on the earth (Sharma *et al.,* 2011). They are known to be key players in the nitrogen and carbon cycles and in other biogeochemical processes. Some genera are able to fix nitrogen directly from the atmosphere, and store nitrates and phosphates in their cells. Recent research has discovered that some genera have gas vacuoles that allow them vertical mobility so that they can optimize light and nutrient conditions (Mazur-Marzec *et al.,* 2006; Hamilton, 2013).

Cyanobacteria, or blue-green algae as they are commonly known (named from their blue-green hue as they float on the water), are extremely fast-growing and opportunistic when nutrient levels are in excess of the natural carrying capacity in their habitat. In waterways they have become synonymous with anthropogenic pollution and act as a bio-indicators of increased nutrients (Soltani *et al.*, 2012).

Harmful algal blooms are usually associated with *Lyngbya* in the marine context; they have far reaching effects such as skin irritations, rashes and swelling. Anecdotal evidence has seen them connected to seafood poisonings and mortalities, their impact is being extended to commercial economic growth in marine food industries and recreational activities (McLean & Sinclair, 2013).

1.3.4.2 Cyanobacteria in the Bay of Plenty

The *Lyngbya* genus (**Plate 1**) is one of the consortia of cyanobacteria that are commonly found in the aquatic environment. *Lyngbya* is a filamentous strand like weed with a tough sheath exterior. The visual appearance can be in a coagulated mat form of hair like strands to slime-like slick covering the surface of a water body. Its easily identifiable appearance makes it an excellent sampling choice, as it is readily available.

Plate 1: *Lyngbya sp*. smothering *Gracilaria* strands at Maketu site 1.

Lyngbya is ubiquitous in its distribution in waterways and is thought to be a causal agent of algal blooms. However, to the author's knowledge from literature searches, there has been no research in the Bay of Plenty area specifically on *Lyngbya*, its toxin producing capabilities, its abundance, distribution or extent to characterise populations for comparison.

The genus *Lyngbya* has become synonymous with land use and marine pollution over the last few decades (National Geographic, 2013). Freshwater cyanobacterial species have received a plethora of research regarding their biological and physiological attributes. However, research on the *Lyngbya* genus and the marine varieties has been limited here in New Zealand.

In summary, ecological characteristics of specific bacterial genera are of significant interest as they indicate environmental adaption mechanisms, based on changes in their specific environments. While empirically identifying signatures of *Rena* crude oil in each environment is beyond the scope of this research, inferences of influences can be determined, from geochemical and molecular analysis techniques.

1.4 MV *Rena* grounding - microbial community considerations of a major maritime pollution event

With the grounding of the MV *Rena*, a unique opportunity arose to test research possibilities which had previously been unavailable in New Zealand. Although it could be seen as a serendipitous opportunity, at each scale from macro- to micro-cosm, studies with exploratory concepts for monitoring right through to communities being mapped have presented themselves. A platform to test previous assumptions has presented itself and is reported here.

The following research focuses on bacterial communities with associations to the MV *Rena* oil spill and ship wreck, which occurred following the ships grounding in October 2011, and its ecological impact in the Bay of Plenty area. As a means to explore marine microbial responses, algal biofilm structures were used as environmental bio-indicators in developing a hypothesis relating to bacterial community reaction and possible bioremediation activities. In addition, there was concern expressed by the public that the oil spill may trigger phytoplankton blooms possibly containing harmful species.

Algal blooms have had an increasing visual presence in the marine environment of the Bay of Plenty region over the last few decades (BOPDHB, 2011; BOPRC, 2015a). Algal blooms are believed to be a co-occurrence consortia consisting of heterosigma (a phytoflagellate), cyanobacteria (*Synechococcus sp*.) and diatoms, (Bronicheski, 2014). Therefore, based on anecdotal evidence that crude oil additions from the spill are contributing to the increase in algal blooms in the region, algal biofilms were selected as a mechanism to investigate bacterial assemblages associated with anthropogenic stressors (MacNaughton *et al*., 1999; Kostka *et al.*, 2011; Paerl *et al*., 2014), using the *Rena* oil spill as a baseline reference.

Marine environmental biofilms are the primary colonising communities which consist of macromolecules, attached bacterial assemblages enmeshed with extracellular polymers (Webster & Negri, 2006). Understood to be key players in signaling invertebrate larval settlement and other biogeochemical processes which establish primary marine microbial communities they are essential in fundamental marine functioning and re-colonising habitats after stressor events such as an oil spill (Webster & Negri, 2006; Thakur *et al.* 2008; Kostka *et al.* 2011). Therefore new biofilm assemblages created as a successional response to the *Rena* oil spill

could create new space or altered bacterial consortia as a precursor to future altered communities.

The MV *Rena* grounding on Otaiti (Astrolabe Reef) had immediate impact on the surrounding ecology. The initial grounding resulted in a first stage release of heavy fuel oil and the debris from containers which washed off the cargo ship and were strewn along the coast and on the seabed floor around the reef. Some of the containers lost their contents which included an assortment of contaminants, food stuffs, metals and silicon beads (-including large quantities of metals including over 50 tonnes of finely milled copper clove and 500 tonnes of cryolite, McSweeney, 2015). A significant amount of TBT was released from the damaged hull of the ship (Ross & Battershill, 2014). While the initial clean up and continued monitoring is still being overseen by the Bay of Plenty Regional Council, the residual impacts now are less visible but are being observed as contamination in the trophic cascade of reef dwelling species around the wreck (Ross & Battershill, 2014). A series of studies as part of the *Rena* Long Term Environmental Recovery Program report (Battershill & Schiel, 2013) on various aspects of ecology have revealed the extent of the impact over the years since the spill, for example, the effects on juvenile paua (McSweeney, 2015); intertidal beach fauna (Culliford & Fairweather, 2013); and toxicity in marine algae (Reihana, 2013), to name a few.

A great deal of scour of surfaces on the reef and indeed on the ship's hull occurred and continues to occur in a highly energetic marine environment (Ross & Battershill, 2014) . This provides novel surfaces for the establishment of biofilms as a precursor to later phase algal and invertebrate succession. Indeed the 'greenish fuzz' on surfaces and the ship's hull was obvious from the outset on the ship's wreckage on the reef (*see* Plate 2 and Plate 3). This green 'fuzz' was reported in the press as

being indicative of fast recovery of the reef and successful colonisation of what was assumed to be healthy life over the ship with the thought that anything green was good. However, without understanding exactly what these biofilms are comprised of, this view was misleading and significantly affected the tenor of the consent case.

Plate 2: Monitoring diver Phil Ross on the Hull of the MV *Rena* shipwreck, clear colonization of the novel surface for biofilm establishment. Image courtesy of Phil Ross.

Plate 3: *Rena* hull with successional colonization. Image courtesy of Ministry for the Environment (2012).

As there were no comprehensive baseline ecological characterisation data prior to the wreck of *Rena*, assessment of impacts on Astrolabe Reef were based on anecdotal information on the reefs' biodiversity backed up with comparative reviews of adjacent reefs immediately after the *Rena* grounded (and before she broke up). No information on 'natural' microbial biofilms on any of the reef systems (pre Rena) was available. Ecological loss and how it is measured has been the impetus of research on how to gauge the full extent of the oil spill.

For the resource consent application to leave the wreck on the reef, the report by Brodie et al (2014), (see also Consent Hearing evidence (BOPRC, (2015b)), defines the ecological character and its impact with 2% of the total Astrolabe Reef area affected.

In the Brodie *et al* (2014) report, a summary of the pre-*Rena* ecology is assessed from vicinity locations and assumptions, based on available records and they assume the state of the reef to be of a high natural value and in almost pristine condition; abundance of benthic biodiversity and having well managed maintained fisheries. Although they state that the risk is considered low and estimated the impact area to be only 2% of the total site, they also comment that within the impact zone the ecology is 'completely wiped out', and contaminated by toxic chemicals. The chemical of most concern is copper and its toxicity to marine biota. Other toxicants are the plastic pellets, a serious pollutant and danger in themselves to animals through consumption; ferrosilicon, and cryolite as sporadic toxicity release agents and are not considered to be a high risk (Brodie *et al.*, 2014).

In summary, the importance of this research in investigating post anthropogenic stressors, *Rena* oil spill and the successional novel biofilms bacterial assemblages

and their response mechanisms is critical in informing metagenomics, specifically bioremediation and biofouling as functional bio-indicators.

1.5 Economic and cultural impacts of the *Rena* event as measured by microbial community perturbations.

An environmental catastrophe such as the *Rena* oil spill must include the effects on humans as part of any analysis as well as consideration for the economic and cultural values of the places impacted. Seldom are these considerations made with reference to shifts in the microbial community composition or dynamic, yet such changes can inform review of fundamental issues in environmental impacts given these communities are the base line to most other ecological pathways. In terms of successional events that lead to recolonisation of reef surfaces for instance, examination of biofilm development and maturation is viewed as being essential. Given that a precedent has been created in the *Rena* case, where the then Minister for the Environment required that a review of long term effects and recovery in response to the *Rena* wreck, needed to be taken into consideration practically the impact on Mauri (life force) (Puia, 1990; Roberts *et al.*, 1995; Durie, 2003) of the environment (MfE, 2012), then examination of the microbial realm would be a fundamental first step. This is a prime aim for the work described in this thesis. The work embodied herein is the first of its kind to link marine microbial science to providing input into cultural health assessments and to underpin assessment of the effects of a maritime disaster on the Mauri of the ecosystem.

1.5.1 Cultural impact

The cultural significance is centred on the indigenous people of New Zealand, the Māori. The role in this research is based on the integral relationship Māori have

with the environment. Under the Resource Management Act 1991, specifically sections 1, 5 and 8, the Crown has an obligation to actively protect the relationship Māori have with their culture, traditions, ancestral lands, water, sites, wāhi tapu (sacred sites) and other tāonga (a highly regarded object or natural resource) (NZ Government, 1991). Incorporating this outlook is integral in research, as it pertains to the environment or Te Tāiao, which is inherently linked to the physical and mental well-being of Māori.

A portion of the 6-part recovery plan established by the MfE, is to address cultural impacts (MfE, 2012). A measurement on the cultural impact in the Maketu coastal area was done by Bennette, (2015) using the Mauri model. The Mauri model is a multidimensional paradigm considering the physical, spiritual and economic state and impact on wellbeing of a Māori community. Definitive pre- and post-*Rena* measures are stipulated in the report and propositions for remediation thresholds are identified. However, this model does not take into consideration the ecological connectivity especially that generated from the microbial realm.

As with any perspective, how a concept, idea, even an impact is value-weighted is a subjective judgement based on an individual's (among other things) knowledge base, experiences and cultural world view *(pers. comm*. Hamill, *2015)*. As we make value judgement calls based on our subjective observations it is through our personal lens we value weight an effect or impact.

The Mauri model utilised to make an environmental assessment based on the value weight of the Māori culture is a subjective tool, which represents the interest of the individuals involved, in this particular case Te Arawa ki Tai Trust. Their value-weighting of impact or value, however, may not be consistent with Māori in general

but be a reflection of the iwi (tribe) or hapū (sub-tribe) who were involved, based on their specific Mātauranga (body of knowledge), tikanga (customs) and kawa (protocol).

With the ability to look at all aspects of the environment in this case microbial, previously inaccessible data can now be added to the contemporary knowledge base that is Mātauranga. These new tools and information can support the higher level taxa impacts on Mauri such as, fisheries and kaimoana to aid in restorative measure programmes. Microbial community trends can predict recovery of higher level taxa, and shifts in the habitat from natural and anthropogenic stressors.

1.5.2 Economic impact

In line with cultural impact, economic impact is value-based on the subjective judgement of the vested interests of groups or individuals. With economic impact, interest groups can be defined as commercial or private, recreational or personal harvesting (e.g.; wild food gathering, medicinal, naturopathy, etc.). One aspect of loss associated with economic value is monetary which can be applied to gathering of a resource, time and costs expended, wholesale and retail resale value. While economic impacts are cited in several newspaper articles (Linnell, 2011, Massey University, 2011) actual dollar figures are not mentioned and so costs can only be inferred.

The economic impact is also a major contributing factor in research for the Long term recovery plan (MfE, 2012). As microbial processes underpin much of the wider ecology including that which generates food for cultural take, fisheries both recreational and commercial; microbial mechanisms of function within the marine

ecosystem can predict trends on the complex energy flow, bioremediation and economic impact to the aquaculture industry .

In summary, the cultural and economic impact from the *Rena* oil spill is significant from the perspective of the local Māori, culturally and economically it has had negative connotations based on their value judgement weighting and cultural assessment tool, the Mauri model (Bennette, 2015). This study represents a first of its kind in that it addresses some of these issues from the viewpoint of examining the microbial response to a pollution event, highlighting successional assemblage functioning from novel biofilm colonisation. It builds on the fact that microbial processes underpin a wider ecological reaction and recovery to environmental stress. Major goals are to characterise these populations and identify their niche functioning so as to indicate the environmental variables which influence community structure. The contrasting various marine habitats will elucidate a range of data to increase marine ecology and metagenomics development.

1.6 MĀORI EPISTEMOLGY

1.6.1 Māori worldview

'Ko te kai hoki I waiaua'
(To be the food bowl that feeds the world)
Whakatohea whakatauki (proverb from Te Whakatohea iwi)

Māori cosmology is the culmination of traditional knowledge which seeks to answer the origins of the universe. Fundamental to Māoritanga (Māori culture) are the key concepts of whakapapa (genealogy) and natural phenomenon (within the human race and their interactions with their environment) (Te Rake & Rout, 1926, Roberts, 2005). These intergenerational concepts consider the tinana (body or

physical) (Te Rake & Rout, 1926), hinengaro (mental or mind) and wairua (spiritual or soul) well-being of the culture as core beliefs for sustainability of resource and all people. These intrinsic concepts determine the health and well-being of Māori people and are described by James (1990), as the notion of being part of the whole ecological system of the earth. Unlike the common European notion of living apart as a separate entity from Earth's natural cycles (James, 1990), these concepts embody the concept that all things are interrelated and interconnected as part of the Earth's bionetwork. It is the delicate balancing of these three metaphysical aspects which guide Māori in a life of unity with one's surrounding biosphere. These notions are consistently recorded and upheld by Māori today (James 1990; Puia 1990; Roberts *et al.*, 1995; Durie, 2003; Morgan, 2004; Harmsworth & Awatere, 2013).

As part of these metaphysical notions, caretaking of resources which sustain Mauri (life force) is given the highest regard (James 1990, Harmsworth & Awatere 2013; Roberts *et al.*, 1995). The understanding of action and reaction with the regard to environment translates to an understanding of consequence from human activities, so any such action was thoroughly thought through, especially regarding future sustainability of a resource. These ideals lay the foundation of Mātauranga and Māori lore, of which traditions such as tapu (sacred) and rahui (ban) were implemented to ensure future generational use. Rahui on resources included seasonal harvesting on species, timing of the harvesting year and size limits in some areas. While tāpu was designed to restrict practices which were detrimental to the future use of resources, for example, human waste being deposited in the moāna (sea), it included prevention of damaging fishing and shellfish beds by techniques such as dredging (Hutching & Walrond, 2012).

Understanding of these metaphysical relationships were well developed and entrenched in Māoritanga, which are still an ethos held by Māori today (Te Rake & Rout, 1926; Williams, 2004; MfE, 2010; Wehi *et al.*, 2013).

1.6.2 Traditional Ecological knowledge (TEK)

From these understandings of the world we participate in, come the basis of Māori TEK or IK (Indigenous knowledge) principles. Whākapapa is the substrate of TEK and from these aspects come the formalities in action such as kaitiaki (resource guardian) and Mauri (Te Rake & Rout, 1926; Roberts *et al.*, 1995; Durie, 2003). Features of these principles are the products of a dynamic system as an integral part of the physical and social environment of communities and how these work in the best interest of the collective good. These principles protect the resources and its future use by understanding its role in the ecological cycle, its reproduction times, appropriate times for harvesting and population; recruitment and recovery (Johannes, 1993; Durie, 2003; Chinn, 2007; Harmsworth & Awatere, 2013). With colonization came dislocation from the land and environment, which fractured these integral resource management systems.

However, TEK has seen a resurgence over the last few decades initiated by the indigenous people of the world at the permanent forum for indigenous issues established at the United Nations in the late 1980's (Durie, 2003). Essentially being defined as the drawing out of the observational and quantitative environmental health indicators then following up with action orientated management practices (Chinn, 2007). In the transdisciplinary fields of science and education, discourse on these concepts were being described as 'environmental literacy' as coined by Disinger and Roth (1992), in a concerted effort to produce environmentally literate people.

An example of TEK in action in New Zealand is from Ngati Kanohi and Ngati Rere in the South Island in conjunction with Department of Conservation (DOC) and Ministry for the Environment (MfE), where a toolbox of environmental indicators for marine health and species biology is maintained to assess the state of environment. This toolbox was commissioned in order to inform and define how Māori kāitiaki (watch over) over their marine reserves and resources (MfE, 2010).

These ideals are embodied throughout indigenous people around the world as an example by Chinn (2006) who uses the Hawaiian state science content standard to convey this understanding from a Hawaiian saying; *"Malama I kA 'iAna, sustainability'* translated further and defined by Kanahele as *"we are but stewards of the 'aina (land) and kai (sea), trusted to take care of these islands on behalf of the gods, our ancestors, ourselves and our children"*.

1.6.3 Māori and the sea

The sea for Māori is not only a food source but a place of physical interaction which feeds their spiritual sustenance or ones mental state of mind (Durie, 2003; Harmsworth & Awatere ,2013). The moāna is considered a living organism which shares her bounty of fish, shellfish, sea vegetation and birds (Te Rake & Rout, 1926; Hutching & Walrond, 2012). Food resources were also an exchangeable trade between inland and coastal tribes. Algae had its place in Māoritanga as a primary food source: for Māori the traditional harvested seaweed was Karengo, rimu rapa, rehia and tupahiki as part of the staple diet (Colenso, 1880; Rake & Rout 1926; Johnston, 1965, 1970; Schonfeld-Leber 1979; Puia 1990).

With the acute inter-relational understanding Māori had of ecological life cycles, while cyanobacteria is not specifically mentioned or more correctly recognised as a significant primary food source on record, the understanding that main food

sources would require sustenance of its own can be assumed. Cyanobacteria as a primary producer is the food source for many main dietary protein sources, such as the many varieties of fish and shellfish that are eaten by Māori both traditionally and currently, for example crabs, mussels, pips, cockles, paua, kina etc. (Doyle, 2011, Dunlop et al 2013).

1.7 Thesis motivation

Pollution oriented microbial research to date in New Zealand has tended to concentrate on freshwater cyanobacteria due to their direct association with drinking water supplies. In the marine context, algae are the basis of the food web and accumulation up trophic levels can lead to consumption of poisoned shellfish. Cyanobacteria are known to cause irritations to human skin and orifices, stinging rashes and swelling, and have also been known to incite respiratory conditions.

It is also relevant to note that in the Bay of Plenty region there has been prolonged paralytic shellfish poisoning (PSP) contamination since the late 1980s, with a current ban on kaimoana (seafood) sanctioned since December 3rd 2014.

An intrinsic part of the Bay of Plenty region are local Māori, their customary traditions, worldview and how pollutants have impacted them, and their ability to kaitiaki "the circumstance of watching over something" (Williams, 2004) of their resources within the region. The most tangible concept Māori have as a measure of degradation in the environment, in western scientific measures is the Mauri (life force which binds the physical and spiritual aspects of any living beings; Roberts *et al.*, 1995) model by Morgan (2004, 2006). However, Traditional Ecological Knowledge (TEK) is fast becoming part of the future co management tenet on ecological resource management globally.

To Māori TEK is known as Mātauranga – generational learned accumulated natural knowledge, which is past down and experimented with from generation to generation (Roberts *et al.*, 1995; Williams, 2004). A major guiding principle of Mātauranga is Mauri, the living life force within every living organism (Morgan, 2006). The Mauri of an organism indicates its health and wellbeing in its optimal living manifestation, any adverse alteration which impedes this optimal wellbeing is a deterioration of its living ability, in essence, a negative effect. Such effects can ripple up through the trophic systems to inevitably have impacts on humans, their health and well-being, the core concern of any culture.

1.8 Thesis objectives

Selected locations from the Bay of Plenty region were identified to permit sampling across a range of contaminants sources for comparison. Cyanobacteria being the core consortia associated with algal blooms have been selected as a common component and a target phylum for analysis: where possible *Lyngbya sp.* has been the focus of sampling, due to its visual presence.

The latest molecular and geochemical techniques were utilised in order to create a comparative basis to characterise and document bacterial consortium from the selected Bay of Plenty locations.

When the MV *Rena* grounded on Astrolabe Reef in 2011, amongst the devastation and cataclysmic ecological fall out, an array of opportunities arose in regards to marine ecology and biological research. One such opportunity is this study of microbial community structures and how they shift in response to a stressor event, including the role local environmental variables play in affecting the biodiversity.

How the microbial communities have responded against their local anthropogenic influences will be contrasted to elucidate influence from the *Rena* oil spill. Investigating microbial assemblages from phylum down to genera level will allow statistical probabilities of the severity of any impacts found. The 'instinct' of natural microbial communities to remediate oil from a spill is well documented (Leahy & Colwell, 1990; Head *et al.*, 2006, Kostka *et al.*, 2011, Redmond & Valentine, 2012).

As the marine biotope is the source of an estimated 50% of the world's biodiversity and a huge natural source of untapped pharmacology products, the paucity of knowledge around marine microbes offers endless potential. Therefore this research will be of value to marine microbiology, marine ecology, material sciences, marine natural products, fisheries, aquaculture, bio-security industries and many more.

1.9 Research aims

The aim of this research is to investigate bacterial community responses to a variety of anthropogenic disturbances on coastal habitats in as much as bacterial assemblages underpin the wider ecology of the ecosystem. Specifically the effects of the MV *Rena* Oil spill and ship wreck event will be contrasted with the bacterial community structure of the wider Bay of Plenty with preliminary examination of the natural variations in gross community assemblage in an ecosystem that additionally has influence from urban presence (in Tauranga Harbour) and where the presence of active and remnant volcanic systems (e.g. Whakaari or White Island volcano) are also likely to contribute to diversity.

Site selection was based on putative estimates of high, medium and low anthropogenic impacts. Astrolabe Reef as the initial impact zone is the high impact site; Maketu Estuary, one of the first points of contact with the mainland coast for

debris and oil and a residual transition zone with rural influences is the medium impact site; and Tauranga Harbour, a large port with urban influences is the relatively low impact site.

At each mainland location (Maketu Estuary and Tauranga Harbour), three sites were selected for sampling bacterial communities: two 'impacted' sites (based on visual assessment of algal blooms and with low to no residence water movements) and a 'control' site (bearing algal vegetation with high water residence movement in the middle of channels).

For Astrolabe Reef (Otaiti), three sample sites in the debris field and on the wreck itself were selected solely by visual assessment by the recovery divers, and a control site outside these obviously impact areas. As algal blooms in this study are consigned as particle attached communities, this study focused on these communities being the most abundant and productive.

Compounding factors from each site of individual local anthropogenic pressures should be captured in the sampling regime and will be identified and considered in analysis.

The objectives of this research are:

- To construct a broad preliminary genera level library of microbial community composition within the Bay of Plenty coastal region reflecting various levels of anthropogenic disturbance (urban environments versus a major ship wreck).
- Characterise bacterial communities on natural and artificial surfaces within the selected habitats across a pollution profile.

- Based on reported correlations between taxonomic grouping and micro environmental conditions, raise hypotheses as to community assemblage functionality and response to stress.
- Examine the relevance of microbiological research to understanding environmental impact assessment and human impacts, specifically cultural health and economic assessments as are pertinent to Māori.

Contrasting habitats will reveal different geological and physiochemical composition of each location and allow examination of the null hypothesis: 'that there are no significant differences between bacterial community assemblages in locations affected by varying degrees of human induced pollution'. Based on this premise the research offers a unique opportunity to test these assumptions.

This research will utilise the robust and universal DNA-based polymerase chain reaction (PCR) assay, together with targeting the V4 region of the 16S rRNA gene data will be extrapolated to observe diversity with automated rRNA intergenic space analysis (ARISA). Distribution and abundance observations from Ion Torrent sequencing (ITs) will reveal community structure and functional faculties which can then be used to separate out the various local anthropogenic pressures and influences from the *Rena* oil spill.

Local geochemistry (ICP-MS) and environmental variables will be examined in conjunction with bacterial community structure DNA analysis. This will emphasise parameters governing biodiversity of these coastal habitats, indicating responsive functional linkages within the microbial assemblages.

1.10 Thesis format

This thesis is based on a published article format which entailed a chapter written specifically for publishing in a peer reviewed scientific journal, PLOS ONE.

The thesis format contextualises the physical and metaphysical (Mauri) aspects of the study then proceeds into microbial range and spatial patterning before finishing off with and an overall synthesis of the published article.

Chapter 1 is an extensive introduction of the many threads of interest which were woven into this experimental spatial mapping exercise. From the outset the work constituted the first broad scale exploration of bacterial consortia present in the Bay of Plenty maritime estate, with a subsequent examination of bacterial community assemblages associated with algal biofilms in locations of environmental stress. It was serendipitously possible for the first time in New Zealand, to examine the marine microbial response to a major maritime disaster the MV Rena ship wreck and pollution event. A summary of the thesis objectives, is then followed by the literature review on microbial history, context and contemporary analytical techniques.

A current overview is provided of microbial communities, their ecophysiology, functional characteristics, known heterogeneity and habitat preferences. The environmental event which activated this research is introduced, the *Rena* oil spill, the selected locations from the Bay of Plenty region which were identified to permit sampling across a range of contaminants sources for comparisons. Following this is the consideration of economic and cultural impacts from the *Rena* grounding.

Then expanding on the cultural aspects from the grounding is the indigenous Māori perspective, summarised here are the effects on both the physical and metaphysical.

A review of the concept of Mauri and the unique cultural significance of this work is discussed.

Chapter 2 introduces the analytical methodologies and contemporary sampling techniques which were identified to be most appropriate. The geological setting of each site, the historical anthropogenic alterations and discharge inputs. In essence this chapter describes the regional environmental backdrop to the study, and includes the environmental zonation of natural seascape and landscape features. With the material methodologies clearly in context, the practical methods of sampling, processing and analysing the collected data is described here in detail.

Chapter 3 reviews observational bacterial community data and associated statistical interpretation, with the intent to elucidate spatial patterns, ranges of relative abundance and points of interest which have been emphasised from the data analysis.

Chapter 4 is a manuscript readied for submission to PLOS ONE and represents the combined science of the work contained in this thesis. (There is of necessity some repetition from the previous Chapters 2 & 3) The chapter contains further site and location analysis, functional traits observations with an overall summary of the research.

Chapter 5 provides a summary and conclusions of the research. This includes discussion of observed correlates of bacterial species assemblages with pollution stress, then widens the synthesis to examine the importance of microbial research to understanding ecosystem response to pollution events and the relevance to Māori, Mauri and assessments of environmental impact, and the change of status (eg an

area of ‘Significant conservation value’ to ‘Regionally significant natural feature and landscape’) and what legal inferences these may have.

Chapter 2

Materials and Methods

2.1 Analytical Techniques

2.1.1 Metagenomics

The acceleration of molecular techniques offers unique opportunities for bridging traditionally segregated disciplines in the science realm. Metagenomics or ecogenomics is one of these emerging fields in biological sciences (Thakur *et al.*, 2008; Gobler *et al.*, 2011) which unites geneticists, physicists and structural chemists in a common goal to better understand the structure and function of microbial genes. These new insights offer solutions for global warming, ocean acidification, harmful algal blooms, environmental quality, restoration programs and monitoring of shifts in ecosystems. Marine molecular biology is increasingly observing biological events from the perspective of the physiochemical properties of the molecules (Thakur *et al.*, 2008).

Two hypothesis developed by plant ecologists explored trait based effects on ecosystem processes (Laughlin, 2014; Fierer *et al.*, 2014). One hypothesis proposes that a 'mass ratio' exerts influence in community structure, where community drivers are in direct proportion to primary production. Here the assumption is that the most dominant species', which are driving primary production in an ecosystem, are unaffected by the richness of rarer or transient species interacting with the community (Grime, 1998).

Plant ecologists have defined communities taxa in three distinct categories, dominant species, subordinates (rare) and transients (seed pool or recruiters) so

while the theory may have valid propositions, taxa definitions' may not hold true in the microbial application (Grime, 1998).

The second proposition is 'the diversity' theory where the range of functional traits in a community drive ecosystem functioning through complementary resource use (Laughlin, 2014; Fierer *et al.*, 2014). Laughlin (2014) articulates that the effects of these two theories may work in complementary fashion to each other. He also suggests that what may be most beneficial is a framework which can incorporate trait diversity not just the means of statistical data, into a species abundance distributions.

Fierer *et al.* (2014) suggests that from a lack of conceptual strategies for exploring the 'mass ration' and 'the diversity' theories, community-aggregated traits (CATs) may benefit microbiologists in understanding and predicting the functional attributes of microbial communities.

The advantage of using such an approach is to gain an overview of the functional capabilities as a community is affected or responds to changes in biotic and abiotic conditions, while still considering the range of natural variability. The benefit of using this approach in the marine microbial context is the ability to predict the functional capabilities of the community as a whole rather than individuals in isolation, inferring a more accurate depiction of an ecosystem at a certain time and space.

From the advancement of shotgun DNA sequencing, characterisation of the complex interactive network in microbial communities can capture a truer representation of the communities *in vitro*. The advantage of *in-vitro* data is that

documentation of *in situ* laboratory resistant microbes can now be included in the whole community picture. While dominant microbial genera may be crucial to primary production and community functioning, rare species may hold specialist roles in biogeochemical processes from the marine environment. One such bacteria is the *Roseobacter* group which mediates competing pathways of dimethylsulfoniopropionate (DMSP), rapidly cycling dissolved organic matter into sources of marine carbon and sulphur (Howard *et al.*, 2008). Therefore the role of dominant and rare microbes in theories such as neutral and niche must be further elucidated.

2.1.2 Molecular techniques

Understanding microbial ecology, has been accelerated by the development of molecular techniques such as denaturing gradient gels electrophoresis (DGGE), Ion Torrent sequencing (ITS), Pyrosequencing and many other DNA sequencing and analysis techniques (MacNaughton *et al.*, 1999, Webster & Negri 2006, Archer *et al.*, 2014). Libraries of clones and sequenced environmental samples that have been acumulating since the discovery of such informative techniques are catapulting our understanding not just of microbes but of our entire world including humans, the atmosphere and our environment. What has opened up from these techniques is the unique opportunity to explore the spatio-temporal community concepts of function, structure, fitness mechanisms, diversity and interactions at the molecular level (Paerl *et al.*, 2002, Pommier *et al.*, 2006, Webster & Negri 2006, Fuhrman 2009).

2.1.3 ARISA

A high throughput fingerprinting method used to elucidate community characterisation and relative diversity, which is inexpensive and widespread is the automated rRNA intergenic spacer analysis (ARISA) (Cary 2013; Wood *et al.*,

2008). From each bacterial sample the intergenic transcriber spacer region of the ribosomal gene between 16S and 23S, is amplified by Polymerase chain reaction (PCR). The assayed product of the PCR are then quantified with Qubit quant and Nanodrop, before sequence analysis (Jami *et al.*, 2014; Cary, 2013; Wood *et al.*, 2008).

2.1.4 ITs techniques

Ion Torrent sequencing (ITs) is used to study microbial community abundance and assemblage. It has traditionally been used for clinical studies in medical research but advances have now enabled it to be a powerful tool in environmental microbiology (Fujimoto *et al.*, 2014). Ion Torrent sequencing outputs data as operational taxonomic units OTUs which can then be analysed in the appropriate statistical software. Primer 7 is the latest update version, and universally used to analyse complex sequence sets (Archer *et al.*, 2014). Sequence matching and DNA registration, are typically logged in the National Centre for Biotechnology Information database, USA (NCBI 1993).

Considerations which need to be included in any study are the limitations and biases of ARISA and ITs techniques. In brief, limitations can be: primers and their target specific sequences amplify most, but not all, abundant or rare organisms (Nübel *et al.*, 1997, Sloan *et al.*, 2006); sampling sizes are too small and not a true representation of the community (Nemergut *et al.*, 2011); under represented or over representation from analysis techniques (Webster *et al.*, 2001); and the traditional method of cultured sample media being unable to support the entire suite of environmental bacteria, bacterial adaption, growth rates, etc, (Ashby *et al.*, 2007, Brettar *et al.*, 2007).

2.1.5 Geochemical techniques

Typical geochemical analysis can identify environmental elements which can correlate drivers or influences in habitats. Bacterial population dominance and specific genera associated with increased inputs can indicate functional linkages. The analysis techniques used in Webster *et al.* (2001) and Negri *et al.* (2006) to elucidate element concentrations was inductively coupled plasma-atomic emission spectrometer (ICP-AES), and gave clear indications of environmental impact and effects of elements of concern such as Cu, Pb, Cd, Zn, As and Cd to name a few.

An advanced development of this technique is inductively coupled plasma – mass spectrometry (ICP-MS), as utilised by Archer *et al.* (2014): in their study it depicts geochemistry between their sites for comparison. Geochemical analysis of environmental elements using ICP-MS can indicate anthropogenic stressors and elucidate understanding of function, distribution, presence and concentrations of trace elements (Jenner *et al.*, 1990, Rosen & Hieftje 2004). The technique is also able to define the speciation of a compound, by identifying its chemical characteristics and its molecular weighting. Its ability to analyse small sample sizes, with good limits of detection, excellent accuracy and precision, make it a valuable analysis technique (Archer *et al.*, 2014, Jenner *et al.*, 1990). The main limitations of this particular technique are matrix, drift and interferences, to minimise against these limitations in processing, standards and calibrations are run at regular intervals through sample processing to counter matrix and drift issues. Dilution factors are used to limit interferences from the saline waters as the technique was developed for freshwater analysis (Archer *et al.*, 2014).

2.2 Study Sites

The Bay of Plenty region was named so by Captain James Cook, due to its gentle climate, stunning coastline and luxurious forests, which are still draw cards of the region today. With an abundance of outstanding natural features from mountains, estuaries and volcanic islands this meandering coastline is held in sacred regard by the local iwi (tribes) of the region (McKinnon, 2014).

Located on the eastern side of the North Island in New Zealand, Figure 1, the Bay of Plenty is a natural coastline sweep which encapsulates the coastal lifestyle of sun, surf and recreation. Tauranga is the only city in the region and has one of New Zealand's largest ports. The majority of the land in the area is used in rural industries such as dairying, kiwifruit and avocado's (McKinnon, 2014).

The rolling terrain is peppered with volcanoes and volcanic islands which sit out in the gulf, these features are a result of the area being located in the Taupo volcanic zone, created by the Pacific and Australian tectonic plates (University of Otago 2014).

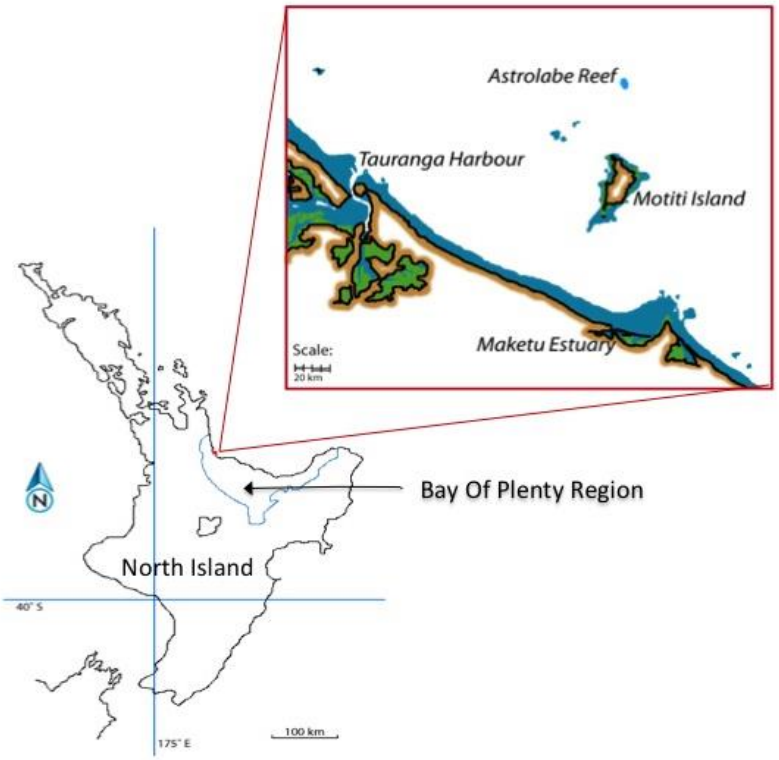

Figure 1: Regional map of Bay of Plenty, NZ

The MV *Rena* was enroute with its cargo to the Tauranga port, when it grounded itself on Astrolabe Reef (Otaiti), significantly impacting the coastline of the Bay of Plenty region. The sites selected for this research had the following criteria

- Location and vicinity to grounding
- Considered impact from the grounding, such as oil and debris
- Coastal versus open ocean
- Access to comparable habitats

Considered impact was identified as high, medium and low. High being Astrolabe Reef (Otaiti), Medium being Maketu Estuary and Low being Tauranga Harbour Figure 2.

2.2.1 Astrolabe Reef (Otaiti)

Here the context, for Astrolabe reef or Otaiti, with regards to zonation, perceived value and how authorities have viewed the area over time before and after the *Rena*

grounding. The MV *Rena* grounding occurred at Astrolabe Reef, a sub-tidal reef located approximately 35 km from Tauranga Harbour, Figure 1 and Figure 2. It is identified by Robertson (2014), from the Operative Regional Coastal Environment Plan, 2003, as an 'Area of significant conservation value', however, in the 2006 review the 'Outstanding natural features and landscapes' (ONF/L) status changed, and Astrolabe Reef became included under the Motiti Island, Motuhaku and Motunau Island ONF/L status. Regionally significant natural features and landscapes status (RSNF/L) calls for a national profile for categorisation, and reference is made in this report to Astrolabe Reef being a regionally significant site for its fur seal habitat and as a nationally recognised dive site. Under the 2012 Bay of Plenty coastal environmental assessment, for Policy 13 of the New Zealand Coastal Policy Statement (NZCPS), the above Islands and Astrolabe Reef were re-categorised as Coastal Sector 23, identified as 'outstanding natural character' with Astrolabe Reef categorisation deferred due to the grounding, but noted that it most likely would have been described as such prior to the event.

The most significant impact was sustained by the reef and the neighbouring Motiti Island, then the coastline of the Bay of Plenty region. The coastline absorbed varying degrees of debris and crude oil washed up from the spill. For months following the grounding, containers and debris washed ashore with the extent of monitoring stretching from Ohiwa Harbour, Ohope in the south to Waihi Beach in the north.

Due to the significance of such a catastrophic environmental event in such a significantly and nationally outstanding natural seascape, a range of impacts sites was selected to compare colonising biofilm communities as an observed reaction to anthropogenic stressors on the ecosystem. Sites identified as previously mentioned

are Astrolabe Reef, Tauranga Harbour, and Maketu Estuary. The preliminary scope of this study included Whale Island as a low impact site and White Island as a control, however, geological diversity deemed these sites to be adverse in making relative comparisons, so they were removed from formal analysis in this thesis. The work (Appendix 1) will be written up separately at later date.

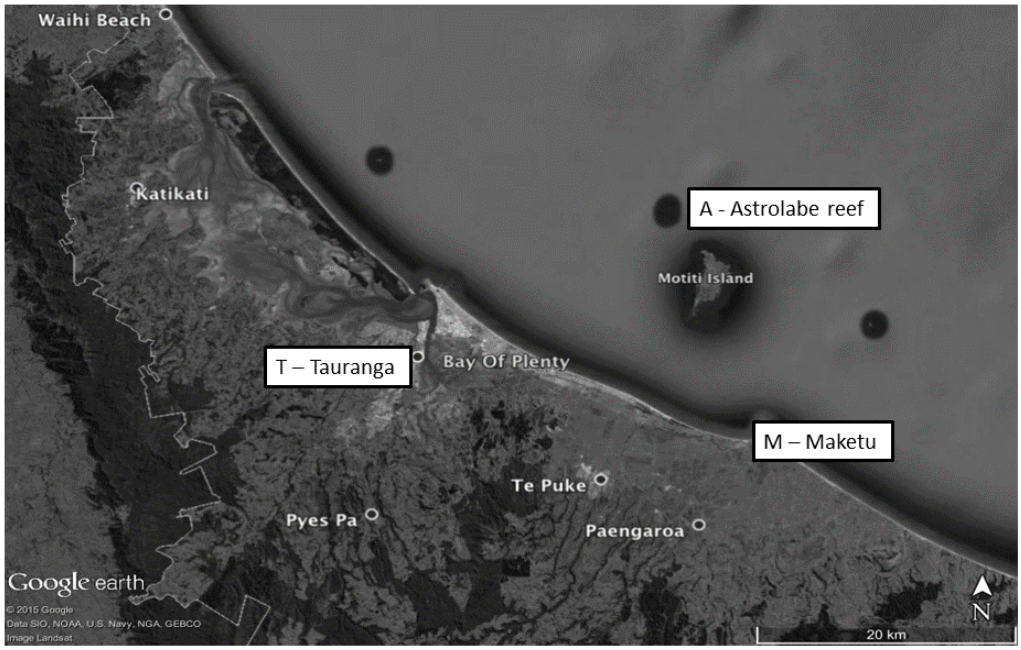

Figure 2: Regional location map of selected sampling sites

2.2.2 Astrolabe Reef context and characteristics

Natural character is defined by the New Zealand Institutes Landscape Architects as " … the expression of natural elements, patterns and processes in a landscape." In addition there are several widely recognised referenced sources which establish the definition under the RMA (Robertson, 2014). In the report by Robertson (2014) the natural character is further delineated as physical and perceptual attributes.

In brief, the physical attributes are a highly energetic environment with currents up to 0.66m/s and a significant wave height of up to 7.8 m as shown in the location map, Figure 3. Over the general area, the currents are NW-SE, dominated by E-SE currents, with faster currents assumed at near-surface levels. The complete reef

structure comprises of an isolated pinnacle structure consisting of Motunau, Tokerau Shoal, Motuhaka (Schooner Rocks), Okaparu Reef, Penguin Shoal and Pudney Rock in an inshore soft sandy mud sediment shelf (Robertson, 2014).

The dynamic physical structure of Astrolabe Reef reflects in the heterogeneity of the pelagic community including but not limited to sponges, algae, molluscs and urchins etc. From the pelagic to the mammalian habitat for the resident fur seal community, to the diverse marine invertebrate and fisheries of native and exotic species such as snapper, terakihi, gurnard, marlin, swordfish etc., the biological characteristics of Astrolabe Reef - are a valued resource protected under the current conservation status, as an outstanding natural national seascape feature (Robertson, 2014).

Perceptual attributes are defined by two contexts: the local, and wider or national awareness. Since the *Rena* grounding, knowledge of the reef and its unique character as a significant natural feature has escalated. As a submerged seascape feature, its beauty or significance cannot be fully appreciated unless under the water therefore, for the general public, mental associations as a feature are not highly valued. Its location hidden behind Motiti Island also plays a part in its physical visual presence as it is not easily recognised as islands above the sea level are. Although it is considered a significant and outstanding feature, due to its lack of physical above the water, the perceived value as a natural seascape feature is not really experienced by the wider population.

In the local context, its attributes are well harvested through commercial and private or wild food gathering traditions, customs and livelihoods to not only the Motiti Island residents but other frequent recreational divers (McSweeney, 2015). From

the recreational and commercial diving perspective, prior to the wreck this was a well-known spot for an underwater experience, due to its natural dynamic physical structural (caves, shelves, vertical drops, depths etc.) and ecological diversity (Robertson, 2014).

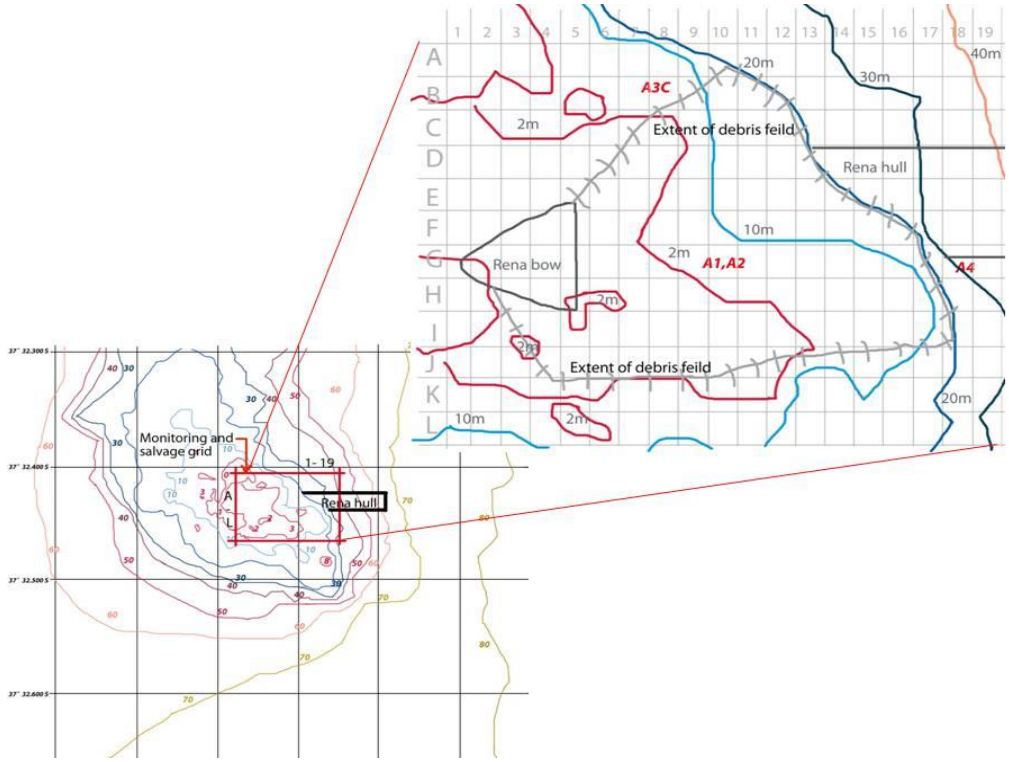

Figure 3: Astrolabe Reef co-ordinates and monitoring grid.
KEY: A =A1, A2, A4 are the 'impact' site locations. A3C = control site.

2.2.3 Tauranga Harbour context and characteristics

The physical features of Tauranga Harbour offer a complex overlaying of essentially two hydrological basins which interact within a natural lagoon. From post-glacial rises and tectonic subsidence a lagoon has formed enclosed by spit formations and a barrier island (Cromarty & Scott, 1996).

From this geological formation two tidal phases occur which discharge through the two entrances to the harbour area, the Mt Maunganui entrance and the Katikati entrance Figure 5. While the water quality is considered of 'good quality'

(Cromarty & Scott, 1996), localised eutrophication and other pollution sources are known (*pers. comm*. *Battershill,* 2012; Huteau, 2015: Taikato, 2015*)*. Direct and indirect human activity has contributed to the anthropogenic inputs in the Tauranga Harbour, this has reflected in the increase of mangroves forests over the last few decades, and loss of large areas of saltmarsh from siltation (Van Meeuwen-Dijkgraaf *et al.,* 2010).

Tauranga is a major port in New Zealand (the largest commercial port in the country), which includes wharves and servicing areas located in the southern part of the harbour at Mt Maunganui and Sulphur Point. Extensive land reclamation has been done in response to the exponential growth of this region from ports operations, various marine industries - from recreational and commercial, tourism and transport requirements (a four way expressway along the Waikareao Estuary claimed 9 ha of tidal land) (Cromarty & Scott, 1996).

The majority of the surrounding catchment is farmed or in horticultural use and the intensive horticultural development continues to modify landuse and hydrological pattern which discharges into Tauranga Harbour. Water quality of the harbour is in decline as impacts from human development and land use have been sustained for many decades (Cromarty & Scott, 1996). The direct impact from the catchment land use reflects in a change in the harbour biota from pollutant discharges such as herbicides, fertilisation, septic tanks, effluent, industrial waste spillages and discharges (Cromarty & Scott, 1996). The fluctuations and slow decline in species richness can be seen in benthic monitoring data from Park (2000), Figure 4.

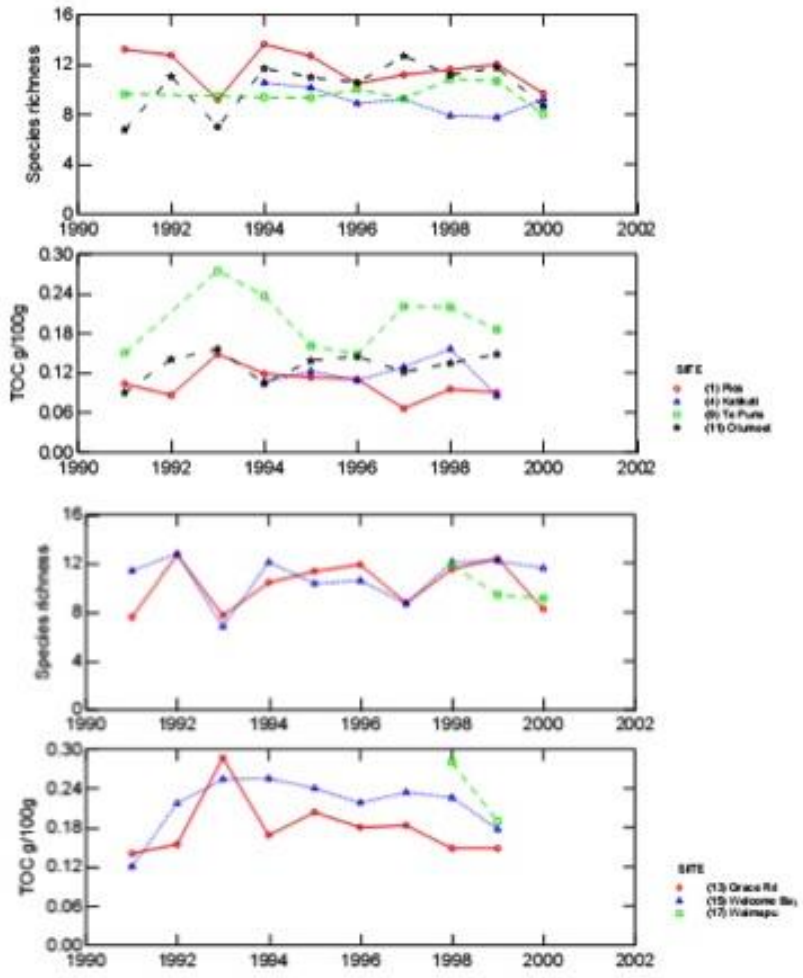

Figure 4: Tauranga monitoring sites. Sourced from Park (2000: Figures 3.2 & 3.3).

Tauranga city's sewerage outfall also discharges into the harbour near Sulphur Point; this has seen an increase in nuisance *Ulva* sp. stands. Other sources of leachates and discharges direct to the harbour are local authority rubbish tips along the coastline, there are just the known sources and do not account for the unknown one-off or diffused continual pollutant sources draining down the freshwater streams and into the harbourscape basins (Cromarty & Scott, 1996).

Tauranga has a long history of Māori occupation which is supported by the extensive archaeological history in the area, so Tauranga Harbour has rich historical and cultural value, this is recorded in the Western Bay of Plenty District's Planning Scheme, Tauranga Districts Planning Scheme which has 5 planning zones covering

this area, the RMA 1991, and DOC: all have vested conservation and cultural heritage value over this area. The harbour is ranked by the Ministry of Fisheries as an outstanding "wetland of national importance" and is noted to have important spawning and nursery areas of native marine fish species, freshwater fish and shellfish etc. (Cromarty & Scott, 1996). The Department of Conservation has also recognised this area as an 'outstanding site of special wildlife interest' reflecting habitats which remain unmodified in the area (Cromarty & Scott, 1996).

The cultural importance of this harbour is summarised by Tauranga County Council (Scheme change 9 to District Council Scheme second review 1986). Māori have occupied this area for over 1000 years and, as such, have a long occupational history entwined with the environment for sustenance and traditional associations with landmarks, harbour features, wāhi tapu (sacred sites), urupa (burial sites), pa (fortified villages), kainga (unfortified villages) food storage pits, middens and terraces etc. (Cromarty & Scott, 1996).

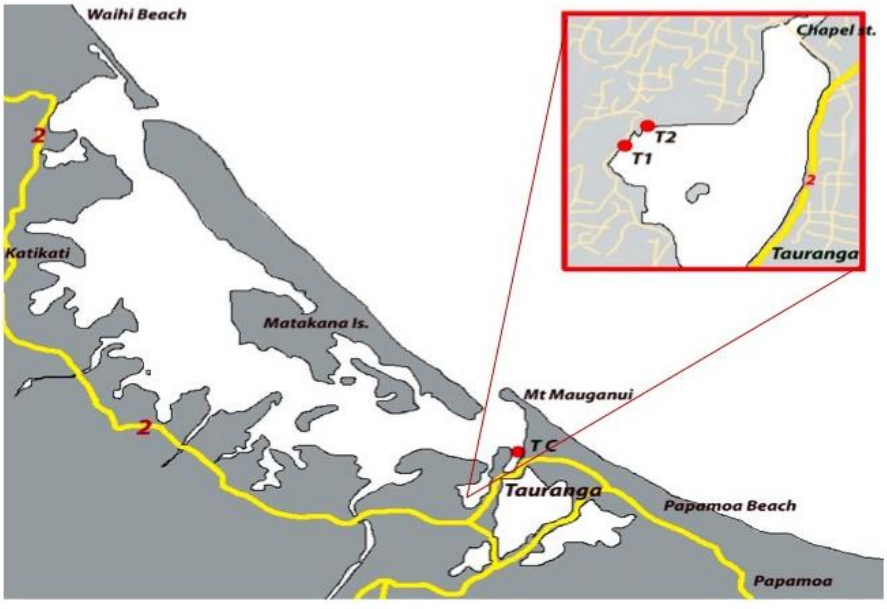

Figure 5: Tauranga Harbour sampling sites.

2.2.4 Maketu Estuary characteristics

The origin of the Kaituna River comes from the Rotoiti lakes through a series of carved out gorges to the flood plains on the coast. The original river channel discharged into the Maketu Estuary and was constrained from the open ocean by a sand dune barrier known as the Maketu Spit, Figure 6. The Kaituna River flood plain was swampy and prone to frequent flooding therefore human modifications began as far back as the 1890s (KRTA, 1986).

The river consistently breached the coastal shoreline at Te Tumu so Fords Cut was implemented by the Kaituna River Board in 1922 in an attempt to redirect the river. Because flooding still persisted a detailed report was commissioned in 1951 and a temporary solution allowing the river to connect directly to the coast from Te Tumu was approved in 1954 as an immediate relief measure and an opportunity to buy some time while long term solutions were investigated. The completion of these measures in 1957 saw the Te Tumu Cut with the additions of river stop banking, drainage pumps and flood gates as part of the mitigation measures. Further modifications were implemented well into the 1960s (KRTA, 1986, Hamill, 2014).

These modifications came with substantial adverse alterations to the estuary and ecology of the area (KRTA, 1986). The issues of significant ecological concern as identified by Hamill (2014) in brief are: accelerated infilling, loss of tidal prism (smaller estuary volume), loss of mussel beds, decline in size and abundance of other kaimoana species, substantial loss of wetlands (approximately 160 ha), and a decline in Mauri.

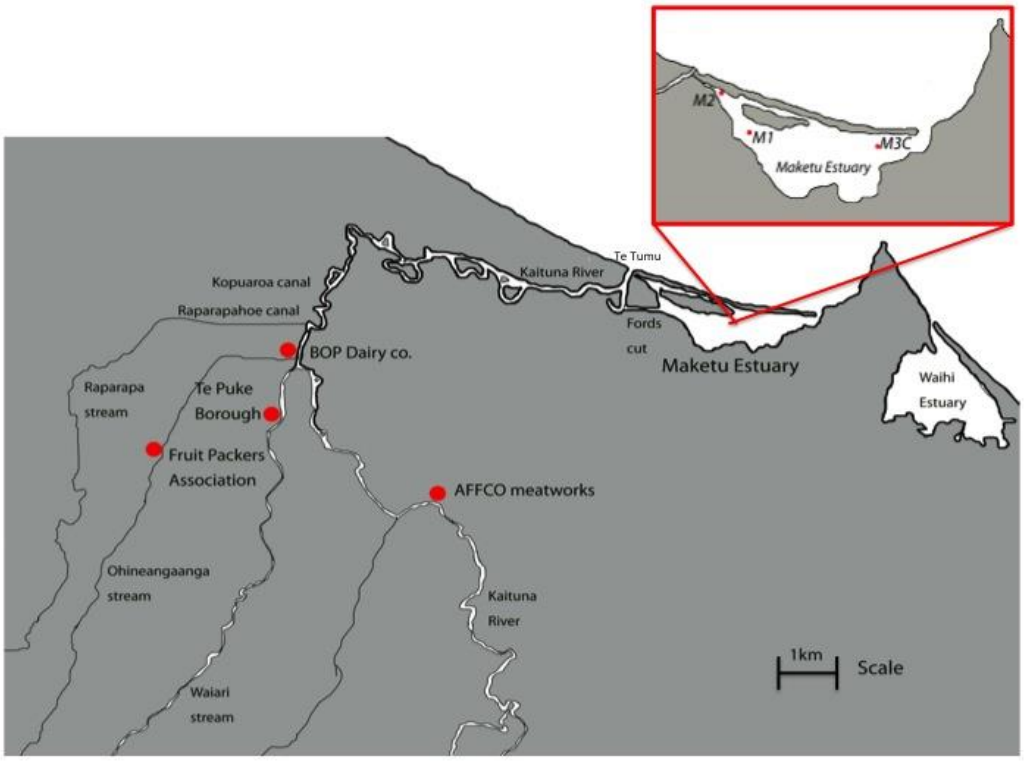

Figure 6: Maketu Estuary map

The estuary has a highly dynamic nature with massive sand movements over large areas, and the majority of the sand entering from the estuary mouth. The sand movements influence dune migration along the estuarine barrier, shallowing of the estuary and channel realignment within the estuary. This motile environment is reflected in the low average species richness recorded in the monitoring update released by Park (2000) (Figure 7).

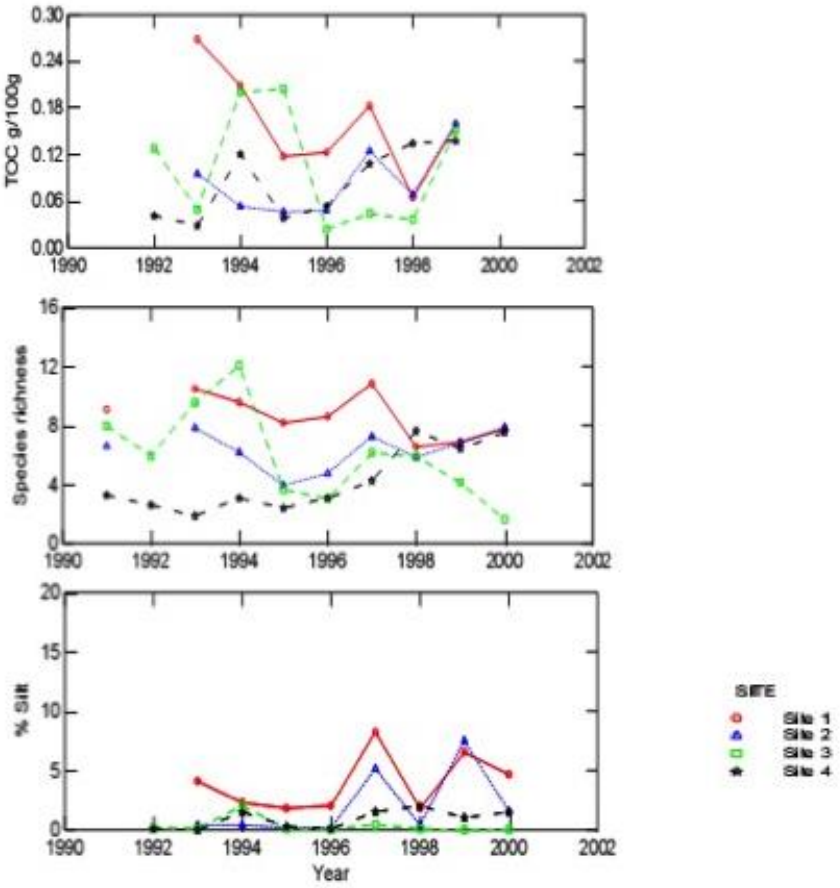

Figure 7: Species richness, Sediment TOC levels, percentage silt content graph. Sourced from Park (2000: Figure 3.5).

Known pollution sources which feed directly into the Kaituna River from the commercial discharge consents are the BOP Diary Co, Te Puke Borough sewerage treatment plant, Fruit Pickers' Association and AFFCO meat works, Figure 6. These companies have rights to discharge into the river from 1966, were renewed in 1987 and 1991, and will expire in 2016. Bacterial concentrations were recorded as high from the point of output and remained high right through to the sea dispersal (KRTA, 1986). So while assessments in the KRTA (1986) report claim that the water quality of the Kaituna River complies with the classifications, a noticeable presence of dense algal growth is visible and areas are considered sensitive to eutrophication.

Algal coverage within the estuary was reported in Hamill (2014) and an estimated 30% (71 ha) had greater than 50% algae coverage. Algal species within the estuary are predominately: *Ulva pertusa* in the lower end or closer to the mouth; the margins and mid upper estuary are dominated by *Gracilaria* sp.; and *Ulva intestinalis* adjacent to Maketu Road area or the southern lower end of the estuary. In the upper estuary zone where there is low water residence and thick algal mats, the cyanobacteria *Oscillatoria* sp. grew as an epiphyte and *Lyngbya* was also identified (Hamill, 2014). Algal coverage overall in the estuary is believed to have increased in the last 10 to 25 years within the estuary, with particular intensification of *Gracilaria* and *Ulva clathrata* species in the last 5 years. The majority of the estuary substrate is firm sand with anoxic mud overlays where algal accumulations occurred (Hamill, 2014).

Māori in Maketu are documented from the arrival of the Te Arawa canoe some 600 years ago where the traditions and cultural heritage are intrinsically intertwined to the area. Māori still own a significant amount of land surrounding the estuary, including Papahikahawai Island in the estuary, while the rest of the land is in Crown and private ownership. A set of wildlife reserves is proposed: 17 ha in the Ford Road lagoon area, and the spit is a recreation reserve set aside as a breeding zone for the local shorebirds and gulls, all to be managed by the Western Bay of Plenty District Council. This year, 2015, consent has been granted to redirect a portion of the Kaituna River to flow back into the Maketu Estuary as well as three significant wetlands restoration projects on the surviving wetlands areas.

2.3 Sampling sites and locations

Samples were collected from three locations Tauranga Harbour (Figure 4), Maketu Estuary (Figure 5) and Astrolabe Reef (Figure 2). Tauranga (latitude 37º 71’63”S)

and (longitude 176 º 15’99” E) and Maketu (latitude 37º 75’43”S) and (longitude 176 º 43’60” E) are coastal habitats with samples for this study taken in shallow embayment’s, no deeper than 50 cm as described by Hamill (2014). Astrolabe Reef, (latitude 37º 33’37”S) and (longitude 176 º 23’47” E) samples were collected from the wreck and debris sites on and around the grounded *MV Rena*, by the monitoring divers. Due to access restrictions on the area, authorised salvage and monitoring divers were the only people with access to the *Rena* grounding site, which is why the samples were collected by those dive teams.

Samples were collected based specifically on visual assessment, indicated by algal blooms and in low residence locations. Stagnant sites and debris field were classed as ‘impacted’ sites and algal vegetation in high residence locations such as the centre of channels and outside debris field were classed as ‘non-impacted’. A hierarchical sampling regime was designed based on Kirchman (2002) and Rashid *et al.* (2013) protocols. Physical characteristics of each location are varied, with several hundred kilometers separating each location. However, similar sampling sites within locations were chosen based on observational characteristic similarities.

The Astrolabe Reef sites were located in the sub tidal reef habitat characterised by an array of rock platforms, shelves, and sand and sediment boulder medleys. Samples were collected at two depths: ≤ 10 m and 30 m. Sites were selected at diver’s discretion and assessed at the time, which allowed more sites at this location. Sites A1 and A2 were selected on the first dive and several months’ later the second set of sites, A3 control and site A4 were selected.

Tauranga Harbour is a natural lagoon enclosed with spits and an island barrier. Tauranga city sits at one end flanked by a major commercial cargo port and

surrounded by a catchment dominated by dairy farms and horticulture. Impacted samples (T1 & T2) were collected in one of the embayment estuaries, Figure 5, with the control (T3C) retrieved from marine fish tanks located at the Sulphur Point coastal research centre. The source water of these tanks was collected from the Tauranga main channel. Over a period of three months algae grew in the tank and was used in this study as the harbor non-impacted control.

Maketu sites were selected based on the surveying undertaken in the Hamill (2014) report, Site M1 and M2 were known as impacted locations with cyanobacterial growth. A3c was selected as a clear non impacted site, located in the central channel where no know cyanobacterial blooms had previously been identified Figure 6.

2.4 Sampling protocol

Following the protocols of Garcia-Pichel *et al.* (1996), natural samples of algal matts were collected at each site, algal vegetation and water samples were collected in 50 cc falcon syringes, and one 15 ml amber bottle of organic algal matter preserved in 10% formalin for taxonomic identification of algal genera.

Water samples were filtered with 0.2 µm and 0.45 µm Sartorius membrane filters into 50ml falcon tubes, and frozen at -20º within two hours of collection as per the protocols of Powell *et al.* (2003) and Archer *et al.* (2014) .

Organic algae matter was collected and placed in 50ml falcon tubes and frozen at -20˚ within two hours of field collection as described by Garcia-Pichel *et al.* (1996) and Powell *et al.* (2003)

At each site, environmental parameters (pH, dissolved oxygen (DO), specific (SPC) conductivity and temperature) were recorded using a hand held Aquaread AP 2000

meter (Aquaread Ltd, UK) as described by Pommier *et al.* (2006), Bottos *et al.* (2008) and Larsen *et al.* (2012).

2.5 Trace element analysis

Trace metal analysis was performed on each water sample, which was firstly filtered with 0.2 µm and 0.45 µm Sartorius membrane filters into 15ml falcon tubes as per the protocols of Archer *et al.* (2014). Inductively coupled plasma mass spectrometry (ICP-MS), was then performed using a Mass Spectrometer ELAN DRC II (Perkin Elmer Inc., Münster, Germany). Samples prepared for ICP-MS analysis were diluted with Type 1 water at 20%, then 9.8µl of the sample was measured out and 0.2µl of nitric acid to achieve a 0.02% concentration in samples as a preservative, following the protocols described in Jenner *et al.* (1990) and Archer *et al.* (2014).

2.6 DNA extraction

Samples from all sites were extracted with a standard CTAB lab extraction protocol as described by Archer *et al.* (2014) and then compared to a commercial isolation MoBio Power soil (MoBio, Carlsbad, CA, USA) (Cary, 2013).

Weak amplifications of DNA as shown in SI App. 1, lead to amended protocols to eliminate inhibitors and extract higher quality DNA amplicons.

CTAB protocols as described by Bottos *et al.* (2014) in brief is as follows; the frozen raw algal matter was thawed on ice then, 270µL phosphate buffer (100mM NaH_2PO_4) was added to the 1.5mL eppendorf tube and vortexed for 10s. The sample was then transferred to a new 1.5mL screw capped conical bottomed polypropylene tube containing 0.5gm each of 0.1 mm and 2.5 mm silica-zirconia beads. 270µL SDS lysis buffer (100mM NaCI 500mM Tris ph 8.0 10% SDS) was

added to clean out remaining sample in tube then beat beaten for 15s then horizontally shaken on a vortex genie for 10mins. This process was repeated twice to lyse open algal sheath. Samples were then centrifuged at 13 000rpm for 3mins to compact sample. A CTAB buffer solution made up of 50% BME: 1 mL. of CTAB buffer, 180µL of working solution was added to sample then vortexed for 10s and incubated at 300rpm at 60º for 30 mins. Centrifuged again for 30s, and then transferred to a new eppendorf tube with 350µL chloroform: isoamyl alcohol to lysate DNA and then vortexed for 15s then placed on a hula mixer for 20mins. Centrifuged again for 5 mins, then the upper aqueous layer removed to a new eppendorf tube. To complete the lysate stage 10M ammonium acetate was added to a final concentrate of 2.5M vortexed for 10s then centrifuged at 13 000rpm for 5mins. The aqueous layer again transferred to a new eppendorf tube, then was washed with 0.54 times the volume of sample and manually inverted 20 times before incubation at -20ºC for 48 hours. Pellet was then washed with 1mL cold 70% AR grade ethanol, then centrifuged for 1min, this step was repeated 2 more times. Once pellet was dried it was resuspended in 50µm of sterile LO-TE, vortexed for 10s then this process was repeated another 10 times.

MoBio clean up extractions were used to process samples which were considered to have high carbon content (Astrolabe Reef and some of the other weak amplifications) as per manufacturer's instructions with the exception of an increased supernatant volume of 700µL and 640µL for the lysate stages then centrifuged for 1mins, with this step repeated three times.

For the MoBio ULTRA CLEAN 15 kit the manufacturers' protocols were used as per the manuals instructions, with an increase in the ULTRABIND ratio of 12µL: 2µL to the DNA template for amplification.

Extraction efficiency was determined by diagnostic gels and PCR, trials gel images are shown in SI App. 1. PCR reactions were performed in triplicate and combined to overcome later biases during DNA sequencing. To determine and compare relative diversity between all sites, automated ribosomal intergenic spacer analysis (ARISA) was used. Amplification of the intergenic spacer of the rRNA operon was carried out in 25µl reactions containing 10mM of forward primer FAM labelled primer PET-CY-ARISA-F (50 - PET/TG GYC AYR CCC GAA GTC RTT A - 30) and 10mM of reverse primer 23S30R (50 - CHT CGC CTC TGT GTG CCW AGG T - 30) as described by Barrett *et al.* (2006), Sokol *et al.* (2013) and Wood *et al.* (2008).

2.7 Ion Torrent sequencing

To prepare samples for Ion Torrent sequencing (ITs), the V4 region of the 16S rRNA gene was amplified using the forward primer linked with Ion adapter "F" sequence,

(5'CCATCTCATCCCTGCGTGTCTCCGACGATGTGCCAGCMGCCGCGGT AA '3, and Ion Xpress barcode sequences were inserted between adapter "F" and the primer. The reverse primer was linked to Ion adapter "p1" sequence 5'CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTA CHVGGGTWTCTAAT '3 as described by Fujimoto et al (2014). The Ion express bar codes table are shown in SI App. 2. PCR amplicons were performed in 25µL reactions, using 2µL of DNA template, BSA 1.0µL, 1.0µL of primers, 3.0µL of 10X buffer, dNTPS, $MgCL_2$ and 0.12µL of U Platinum Taq, with the remaining made up of MilliQ H_2O. PCR thermocycler conditions were 94ºC for 3mins, a cycle of 94ºC for 45s, 50ºC for 1min, then 30 cycles of 72ºC for 1.5min, a final extension of 72ºC for 10mins before holding at 4ºC until refrigerated overnight. Pooled DNA

amplifications were viewed on a 2% TAE agarose gel, which had been run for 35mins at 75V and stained with 'SYBR safe' (Invitrogen ltd).

PCR products were then cleaned again with the SPRIselect process to remove primer dimers. SPRI was added to DNA template at a ratio of 0.8 to 1µL, mixing together by manual pipetting 10x. Incubated at room temperature for 1min, and then placed on a magnetic tube rack to allow settling for 2mins. The supernatant was discarded and the pellet washed with 180µL AR grade 85% EtOH, returned to the magnetic rack and allowed to incubate at room temperature for 30s. The EtOH was discarded and pellet allowed to dry at room temperature. Once dry elute pellet with 20µL 1 X TE and pipette to mix solution, then incubate at room temperature. Place tube on magnet again then transfer eluent to a new tube, repeat final step twice to remove any residue contaminate. All DNA products were quantified using Nanodrop ND-1000 at 260nm DNA product was checked on a Qubit Fluorometer (Invitrogen Ltd).

2.8 Data processing

Ion Torrent sequence data was processed into their unique name files and cluster sequences of operational taxonomic units (OTUs) with Mothur 1.17.0 (Archer et al 2014). Phylogenic assignments were analysed using Ribosomal Database Project (RDP) release 10, update 15 and converted into fasta files for processing as defined by Fujimoto *et al.* (2014).

Chimera purging was completed with RDP levels and a threshold criteria. First level criteria was Family confidence of <38%, Genus confidence at <30%, Class confidence level at < 90%, Phylum confidence level at <96%, then Domain at <99%. After each RDP level was verified, cyanobacteria were selected out due to uncertainty and misclassification with macroalgae. Cyanobacteria had a threshold

criteria of >97% (Zhang *et al.* 2014), all cyanobacteria at <97% were assigned as macroalgae (Fujimoto *et al.* 2014). Each OTU identified below the filtered threshold was run through the Basic Logic Alignment Search tool (BLAST) (Webster & Negri, 2006), firstly in a nucleotide BLASTN check, then a second check through a MEGA BLASTN as verification.

Abundant selection criteria had a cut off threshold of 1% to capture the centric core species and for the rare species threshold criteria was 0.05%. Eleven site samples generated 14 666 reads, with 250+bp. ITs data verified OTUs used for rare species were 201 in total, and abundant species were 148 OTUs.

Sequence analysis was accomplished with software package Primer 7 (Primer-E: Lutton, Ivy Bridge, United Kingdom) to investigate community patterns (Kostka *et al.*, 2011). Similarity matrices were generated using the Bray-Curtis measure on bacterial assemblages at each site. To compare the sites and locations further, between the rare and abundant communities, CLUSTER analysis was used, and then SIMPROF to identify the resemblance relationships. ANOSIM (one way) was used to identify R statistics and pairwise significance levels for similarities between locations, with a principle coordinate ordination (PCO) cluster to assess the distance matrix between impacted and non-impacted sites.

The ARISA data was used to generate a Non Metric multidimensional (nMDS) diversity profile to represent distance matrices between samples. The stress levels of the nMDS plots were 0.06 for 2D, and 0.02 for 3D as a means of considering the true representation of the dimensionality distance of the data. Then a SIMPER analysis was done on both rare and abundant species to quantify the similarity and dissimilarity between samples.

2.8.1 Environmental variables

ICP-MS trace elemental data was analysed with BEST to identify elements of influence correlated with the Spearman rank method, measured by Euclidean distance. An element threshold criterion was selected at (0.9) for correlations of significance. All environmental variables above the threshold were then compared in a Principle component analysis (PCA) against all sites to elucidate elemental influences. The trace metal suite identified in Negri *et al.* (2006) showed sediment contamination in Antarctica was utilised as a baseline comparative analysis. In this study the same suite of metals was analysed with PCA to correlate ecological influences (Negri *et al.*, 2006; Zhang *et al.*, 2007; Kostka *et al.*, 2011; Zhang *et al.*, 2014). RELATE analysis revealed *rho* statistics which gave linear correlation coefficients between the sites and factors of influence (Powell *et al.* 2003).

2.8.2 Community assemblages

Community assemblages were then compiled from the OTUs, these were converted into a representation percentage of the community at RDP level, phylum and genera. Overall comparisons were made between sites, and then within locations to establish average dominate bacterial community structures, similar to comparisons made in Pommier *et al.* (2006),Webster & Negri (2006) and Aguiló-Ferretjans *et al.* (2008). ANOSIM One way pairwise analysis elucidated similarity of the relative diversity from the abundant and rare species, then BOOTSTRAP alignments measured the data's dependability (Baldauf, 2003)

Chapter 3

Broad Community Analysis

This chapter reports a broad overview of the dominant phylogenic patterns which were observed from the statistical interpretation of data and observational analyses. The aim here is to document possibly undescribed bacterial communities, where the *Rena* grounding offered a unique opportunity to examine a severe organic and inorganic pollution event. To date very little microbial community composition data has been collected and catalogued in New Zealand generally and in association with major pollution events specifically. Internationally several studies have explored microbial communities with various associations with pollution gradients (Webster *et al.*, 2001; Negri *et al.*, 2002; Zhang *et al.*, 2007). Fewer studies have had the opportunity to collect data across pollution gradients in order to compare and explore possible microbial community drivers in response to major pollution events. As no pre-existing literature of bacterial assemblages have been recorded for the Bay of Plenty, this study has now generated a baseline catalogue as a point of reference.

3.1 Methods

The methods and materials used for this chapter have been described in Chapter 2. The ICP-MS analysis used a pseudo 'best' replacement for the proposed industry standard High Performance Liquid Chromatography (HPLC) and Liquid chromatography – Mass spectrum (Parent ions) – Mass spectrum (Daughter Ions) LC-MS-MS. Both of these sensitive techniques are used to identify ions and molecular biology in cyanobacterial studies (Lawton *et al.*, 1994; Ciminiello *et al.*, 2005; Bogialli *et al.*, 2006). Toxicology of cyanobacterial communities and novel

organic chemical compounds which had previously not been explored was one possible avenue of investigation in this study. However, due to logistic constraints the proposed HPLC, LC-MS-MS analysis to be accomplished for this study was not carried out and will be developed in future research.

The original study design included White Island (Appendix 1) as locations of novel cyanobacterial biofilm substrates (given these were active volcanic systems) to represent background geological environment without Rena oil spill influence. However, the distinct microbial diversity recorded was so dissimilar to other regional locations that it was deemed unrealistic to attempt to use these locations as natural stress controls, hence this data will be worked up separately at a later date.

The original research concept was to survey microbial assemblages from algal biofilm substrates as particle attached communities which to date had not been mapped. Hence the unique opportunity that the *Rena* grounding offered was a baseline survey which could be contrasted against the context of each site's local anthropogenic stressors.

3.2 Results

Observations of community patterns and consortium compositions are generally discussed and each site consortium is shown in Table 1**.** The statistical analysis was summarised in a table to identify general patterns, results are shown in Table 2, with the ARSIA Diversity statistical data presented in Table 3.

3.2.1 Statistical analysis

3.2.1.1 Geochemical

First a BEST fit analysis of the statistical probability that the environmental factors could influence community structure, with a significance level of 1%, therefore

highly probable that the environmental factors were asserting influence over the community structure. To assess how significant the relationship between variables and influence as drivers of community structure RELATE analysis gave a significance level of 0.1%. Principle component analysis (PCA) plots revealed the various proportions of influence each factor and element asserts SI App. 3

Table 1: Site break down of dominate bacterial components

	M1	M2	MC	T1	T2	TC	A1	A2	A3C	A4.0	A4.1
Alpha	Hyphomonas	Methylobacterium	Anderseniella	Erythrobacter	Hyphomonas	Hyphomonas	Jannaschia	Hyphomonas	Hyphomonas	Jannaschia	Hyphomonas
	Anderseniella	Methylobacterium	Hyphomonas	Ruegeria	Sphingomonas	Roseovarius	Hyphomonas	Roseovarius	Roseovarius	Hyphomonas	Roseovarius
	Sphingomonas	Hyphomonas		Hyphomonas		Anderseniella	Roseovarius	Anderseniella	Anderseniella	Roseovarius	Anderseniella
		Anderseniella					Anderseniella			Anderseniella	
		Sphingomonas									
Beta	Methylophilus	Ralstonia	Methylophilus		Methylophilus		Methylophilus				
		Ralstonia									
		Ralstonia									
		Methylophilus									
Gamma		Marinomonas	Alteromonas				Marinomonas		Haliea	Marinomonas	Haliea
		Haliea	Haliea				Alteromonas		Shewanella	Haliea	
			Alteromonas				Haliea			Alteromonas	
							Alteromonas			Shewanella	
							Shewanella				
Delta	Desulfopila	Desulfopila	Desulfopila								
Bacteroidetes											
Flavobacteriia/Cytophagia	Flavobacterium	Tenacibaculum	Bizionia	Flavobacterium	Flavobacterium	Bizionia	Bizionia	Bizionia	Bizionia	Bizionia	Bizionia
		Tenacibaculum	Aquimarina				Aquimarina	Aquimarina	Aquimarina	Aquimarina	Aquimarina
			Dokdonia				Dokdonia	Dokdonia	Tenacibaculum	Tenacibaculum	Tenacibaculum
			Tenacibaculum			Tenacibaculum	Tenacibaculum	Tenacibaculum	Tenacibaculum	Tenacibaculum	Tenacibaculum
			Tenacibaculum			Maritimimonas	Tenacibaculum	Tenacibaculum	Maritimimonas	Actibacter	Maritimimonas
			Actibacter				Actibacter	Actibacter	Aquimarina	Maritimimonas	Aquimarina
			Aquimarina				Maritimimonas	Maritimimonas		Aquimarina	
							Aquimarina	Aquimarina			
Epsilonproteobacteria	Sulfurovum	Sulfurovum	Sulfurovum				Sulfurovum	Sulfurovum	Sulfurovum	Sulfurovum	Sulfurovum
	Sulfurovum	Sulfurovum	Sulfurovum				Sulfurovum	Sulfurovum			
Cyanobacteria											
		Prochlorococcus	Prochlorococcus	Chroococcales,	Chroococcales,	Chroococcales,	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta
				Synechococcus	synechococcus	synechococcu					
				Oscillatoriales	Oscillatoriales	Oscillatoriales	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta
		Rhodophyta	Rhodophyta	cryptic	cryptic	cryptic	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta
				Oscillatoriales	Oscillatoriales	Oscillatoriales	Lyngbya	Rhodophyta	Prochlorococcus	Prochlorococcus	Rhodophyta
		Prochlorococcus	Prochlorococcus	cryptic	cryptic	cryptic					
				Oscillatoriales	Oscillatoriales	Oscillatoriales	Prochlorococcus	Lyngbya	Rhodophyta	Rhodophyta	Prochlorococcus
				cryptic	cryptic	cryptic					
						Oscillatoriales	Rhodophyta	Prochlorococcus	Chroococcales,	Chroococcales,	Rhodophyta
							Chroococcales,	Rhodophyta	Prochlorococcus	Prochlorococcus	Chroococcales,
							synechococcus	Chroococcales,			synechococcus
							Prochlorococcus	synechococcus			
								Prochlorococcus			

3.2.1.2 Site and consortium comparisons

CLUSTER analysis indicated similarities between sites and locations at phylum level, from abundant and rare species. Astrolabe reef is significantly different from Tauranga and Maketu locations. Impacted sites versus non-impacted sites are significantly different at each location with the exception of Astrolabe reef. At this location the rare species clustered altogether including the control, therefore statistically significant rare species at Astrolabe reef are highly similar and dissimilar to any other site or location. The other exception is T2 and M1, in both dominant and rare species these two sites cluster separate to all other sites. With a similarity index of 78% in dominant and 60% in the rare species the ANOSIM analysis indicated significant dissimilarity between Tauranga and Maketu, then the comparison between Astrolabe reef, Tauranga and Maketu sites also highlighted the significant dissimilarity between all locations. The distance matrix analysed by PCO graph plotting emphasised the impacted and non-impacted distinctions again and revealed statistically how distinct the microbial community consortium at each site are, see Table 2.

An ANOSIM analysis on class level OTU's revealed similarities between all Astrolabe reef sites at 66.7% with all other site comparisons yielding a similarity index of 33.3%. The BOOTSTRAP average analysis between all sites showed the bootstrap regions to be at 95%, with a *rho* value of 0.992, indicating the dependability of the data overall, the 2D stress is 0.06, with the 3D stress at 0.02, the min stress is at .01 with a Kruskal formula of 1, visualisation of the analysis are plotted in Figure 8.

Table 2: ***Analysis and data significance summary table at Phylum level***

Analysis type	Significance
Similarity between sites and locations at Phylum level	
CLUSTER analysis – between sites Bray Curtis similarity Figure 5: chapter 4	**Abundant species** **Similarity of:** **T2 & M1 = Significance at 78%** **A1 & A2 = Significance at 60%** **A4.0 & A4.1 = Significance at 40%** **SIMPROF test : Resemblance** **Bray Curtis similarity** **Significance level 5%** **M1 & T2 – significance of 99.6%** **A1 & A2 - significance of 71.2%** **A4.0 & A4.1 – significance of 70.9%** **Rare species** **Significance level 5%** **SIMProf test : Resemblance** **Similarity of:** **T2 & M1 = significance at 60%** **SIMPROF test : Resemblance** **Bray Curtis similarity** **Significance level 5%** **M1 & T2 – significance of 98.6%** **A4.0 & A4.1 - significance of 3.9%**
ITS: Abundant species between locations ANOSIM – ONE way site similarities	• Pairwise test R value for A & M .549 at 1.8% • Pairwise test R value for A & T .579 at 3.6% • Pairwise test R value for T & M -0.074 at 30% • Overall level of significance = 2.1% • Sample stat 0.442
ITS: Rare species between locations ANOSIM – ONE way site similarities	• Pairwise test R value for A & M .846 at 1.8% • Pairwise test R value for A & T .744 at 1.8% • Pairwise test R value for T & M -0.111 at 30% • Overall level of significance = 0.5% • Sample stat 0.603
PCO cluster analysis Figure 6:Chapter 4	• Abundant – all control sites cluster , all Astrolabe reef sites cluster, then M1 & T2 cluster, then singles - T1, M2 • Rare – Maketu and Tauranga control site cluster, all astrolabe reef site cluster, same M1& T2 cluster, and same single clusters T1, M2.

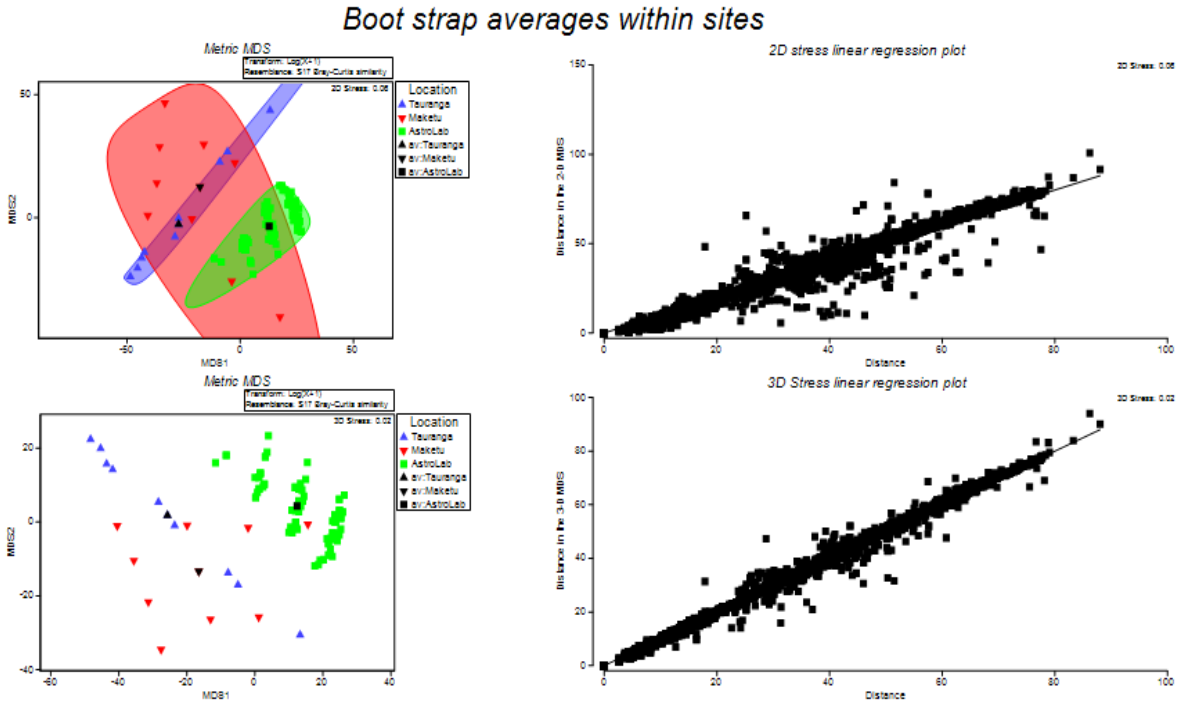

Figure 8: Bootstrap plots, non-metric MDS bray Curtis, plots showing class level relatedness. Linear regression 3D and 2D plots indicating dimensional stress gradients.

In the ARISA MDS analysis on the cyanobacterial component of the microbial communities clustering of assemblages revealed very different consortium patterns as shown in Table 3 . The first groupings were Astrolabe reef control and site 4 were highly similar to Maketu site 2. Then a second group combination of Tauranga site 1 and 2 with Astrolabe reef Site 2, the remaining sites were not similar or associated with any others and are quite distinct in their microbial consortium as well. The Kruskal formula was 1, with a min stress of 0.01, a 2D stress of 0.06 and a 3D stress of 0.02.

Table 3: Diversity profile for ARISA analysis

Analysis type	Significance
Diversity profile	
ARISA – CLUSTER similarity between sites Cophenetic correlation 0.95%	• A1 & A2 = 86.25% • T2 & M1 = 84.68% • A4.0 & A1, A2 (12) = 78.36% • T1 & A3 = 76.33% • T2, M1 & T1, A3 = 66.75% • A4.1 & A4.0, A1, A2 = 66.69% • TC & A4.0, A4.1, A1, A2 = 54.92% • M2 & TC, A4.0, A4.1, A1, A2 = 53.05% MC & T2, M1, T1, A3 = 34.61%
ARISA – MDS similarity between sites Figure 7: Chapter 4 • To reveal the monotonic relationship between dissimilarities of the sites, and the Euclidean distance between them, it locates the low dimensional space between them. 2D and 3D stress gradients. Stress being a dimensionless quality, of distances which are relative not absolute. 3D ordination is a satisfactory representation to consider the true dimensionality of the data.	• Clusters C1 = A4, A3 and M2 outside the 20% highly similar • C2 = A2 at 20% with T1, T2 then at T1, T2 at 40% to each other highly similar • M1, A1 and TC not similar to any other, distinctively not similar to any other site. • Kruskal formula : 1, min stress 0.01 • Dim stress 2D = 0.06 and 3D = 0.02

3.2.2 Bacterial consortium

Further class level investigations revealed the Cyanobacteria's phylum fragmentation into macroalgae and assigned genuine Cyanobacteria, with (≤ 2%) presence at Maketu and Tauranga, and a (22.2%) presence at Astrolabe Reef. The macro algae substrate appears to be most dominant at Astrolabe Reef A3C site (44.43%), Maketu M1 (23.87%) and Tauranga T1 (63.83%) and overall most dominate at the Tauranga location, Figure 9.

The Proteobacteria phylum is dominated by Alphaproteobacteria, a consistent (15-18%) range across all sites, Betaproteobacteria dominate at Maketu with a (27.67%) presence, and Gammaproteobacteria with a low range of (4.8 – 7.18%) presence across all sites, Figure 9.

There was a notable presence of Epsilonproteobacteria at all Astrolabe and Maketu sites of (≤4%), Flavobacteria are most dominant with (22.92%) at Astrolabe, (14.13%) at Maketu and only (5.64%) at Tauranga. There is a small representation of Sphingobacteria $\leq 2\%$ at all sites with a Cytophagia presence at Tauranga 2 and Maketu 1 sites only. Actinobacteria and associated classes are constant with a range of ($0 \leq 5\%$) across all sites.

The relative abundance at class level from each site emphasised the distinct patterns between impact versus non impacted sites, Figure 9. Bacterial structural assemblages are very different at control sites compared to impacted sites; gradients can be seen in figure 8.

At the Tauranga control site the Alphaproteobacteria were dominant with (18.91%), then Flavobacteriia (8.95%), then Actinobacteria (7.61%). At the Tauranga 1 and 2 site, Eukaryota dominated with (63.83%) and (32.33%) then at T1 Alphaproteobacteria (4.56%) then Betaproteobacteria (3.95%). At the T2 site the second most dominant class were Betaproteobacteria (7.11%) then Alphaproteobacteria (5.22%). At the Maketu control site Betaproteobacteria were most dominant with (36.32%), then Alphaproteobacteria (11.25%), then Eukaryota (10.14%). At Maketu site 1 Eukaryota was most dominant with (23.87%), then Alphaproteobacteria (6.99%) then Betaproteobacteria (5.73%). At Maketu site 2 the most dominant group was Flavobacteriia (21.50%), then Alphaproteobacteria

(4.91%) then Gammaproteobacteria (4.62%), with Epsilonproteobacteria closely behind with (4.49%).

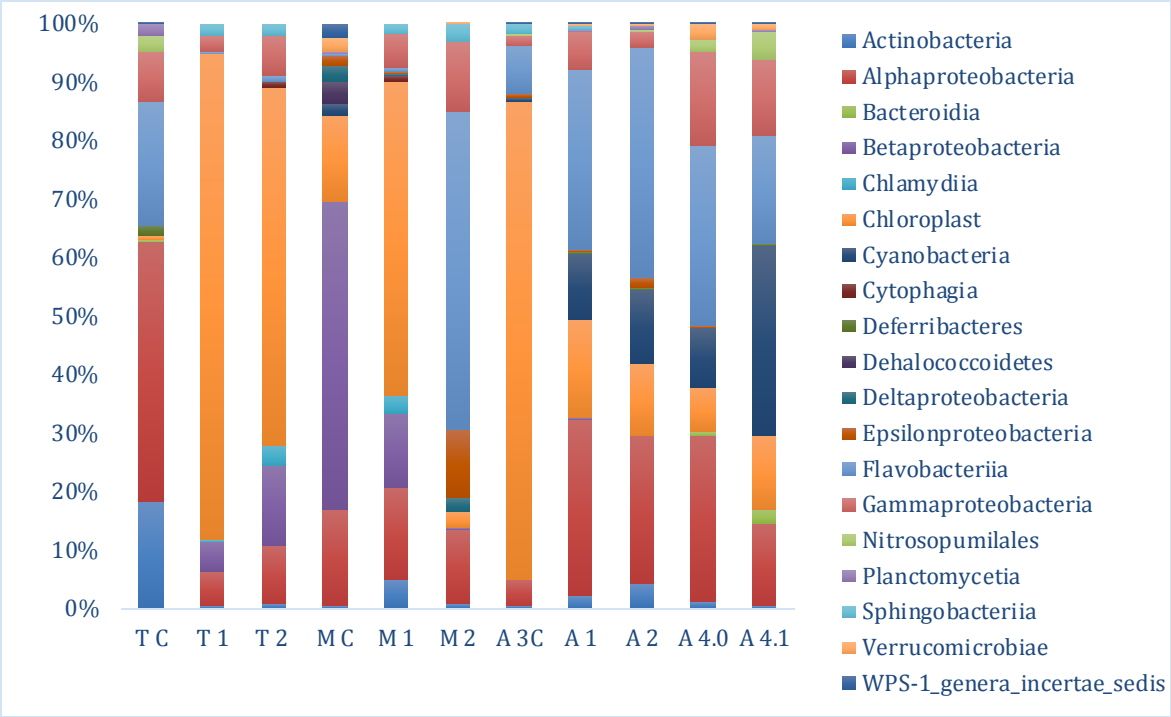

Figure 9: Class level community structure at each site. Bacterial species and % abundance.

KEY: TC = Tauranga control, T1 & T2 = Tauranga impacted sites, MC = Maketu control, M1 & M2 = Maketu impacted sites, A3C = Astrolabe reef control site, A1, A2, A4.0 & A4.1 = Astrolabe reef impacted sites.

The two sites most alike from statistical analysis of sequence data are M1 and T2 with both sites dominated by Eukaryota, Betaproteobacteria then Alphaproteobacteria.

In a consortium comparison of each site, shown in Table 2, M1 has additional Alphaproteobacteria to the T2 site. Table 2 indicates that the Maketu control site consortium is quite similar in composition to the Astrolabe Reef microbial communities.

3.3 Discussion

Overall, all environmental variables indicated significant influence over microbial Conductivity was the most influential factors, supporting findings from Pommier *et al.* (2006) and Powell *et al.* (2003), that suggested environmental factors influence bacterial community structures.

Elemental analysis found Sulphur, boron and chromium to be most influential with the heavy metal suite of zinc, arsenic, copper, cadmium, lead and mercury to have significant influence (*P*-value = (0.98) over microbial community structures. In identifying the elemental influence it is impossible to deduce from this study any one element which is acting more significantly than others as drivers of community structure. Further research on the impact of elements as a consortium would benefit understandings of microbial community interactions, response mechanisms and functional traits and their processes.

Overall the phylum of Cyanobacteria consisted of Eukaryota sequences and assigned both phyla as cyanobacterial OTU's, thus heavily weighting cyanobacterial dominance overall locations. The next predominate microbial consortia were *alpha* and *beta Proteobacteria* and *Flavobacteriia* overall sites.

Although the Eukaryota was the algal substrate, it was most dominant at the Tauranga location, it also had the least microbial heterogeneity of any of the communities see Table 1.

Two sites most similar, M2 and T1, have nearly identical microbial consortium with the exception of couple of additional genera at M2. However, dominant influencers measured in this research could not be confidently identified on the basis of their similarities.

The consortium composition at Maketu control is highly similar to the Astrolabe reef assemblages as shown in Table 1.

There are distinct bacterial assemblages at each site with *Cyanobacterial Proteobacteria* and *CFB* as the overall predominate phyla. Cyanobacterial dominance is this instance is skewed by the heavy presence of Eukaryota or chloroplast OTU's from the molecular analysis, as the biofilm substrate and particle attached communities targeted for sampling this was no surprise.

The low heterogeneity of diversity from the Tauranga locations is unexpected, considering the similarity of anthropogenic inputs over the same time span as the Maketu location (Cromarty & Scott, 1996; KRTA ltd, 1986). This could also suggest that the stressors on the Tauranga harbour area have had a more significant impact in comparison to the Maketu location. When examining the microbial community consortium at Tauranga, the bacterial community may have crossed a threshold from a previous stable state, where the natural microbial community has been thrust into a new recovery state in comparison to the other two sites. Sustained impact or the significant alterations which have occurred and are occurring in this harbour can account for the significantly lower diversity and heterogeneity found here.

The similarity of microbial heterogeneity from Astrolabe reef and Maketu was unanticipated considering that until the *Rena* oil spill, the Astrolabe reef area was considered to be in a pristine environmental condition with little to none anthropogenic inputs (Hamill, 2014; Robertson, 2014). Most interestingly the community composition similarities and what these may infer, so far as functionality and primary production of these communities, would be most useful for future marine natural products and biomedical research.

The original aim of this research was to document previously unknown microbial communities taking advantage of a catastrophic environmental event and to observe how these communities respond. This has been achieved at a broad level and has set a basis for continued more detailed research on the ecology of microbial community response in a recovery mode as the pollutant influences wain over time.

Chapter 4

Synthesis

Written for submission to: Plos ONE

NB: As this chapter was prepared for publication, some repetition will occur from previous chapters.

Bacterial community responses to anthropogenic disturbance of coastal habitats: the *MV Rena* shipwreck and urban pressures.

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Abstract

Understanding microbial community structures and their functional capabilities provides an overview of how these communities are responding in changes to stressors from anthropogenic or natural sources. The MV *Rena* Oil Spill was a significant anthropogenic stressor which impacted a large area of the Bay of Plenty Region. The aim of this research is to carry out a spatial survey of representative coastal biofilm bacterial communities near the MV *Rena* oil spill and wreck site, contrasted with a range of adjacent habitats to examine the effects of different types of anthropogenic contamination (ship versus urban), mindful of a general regional volcanic geological backdrop. Localised anthropogenic contaminated sites are represented at Tauranga harbour; urban city run off with an extended catchment

which is mostly dairy farming and horticulture and a rural township; and Maketu with intensive dairy farming and horticultural inputs in its catchment.

Microorganisms with potential to bioremediate are most abundant at the most impacted locations in this study. Sequencing of 16S rRNA gene PCR amplicons revealed that *Alpha-* and *Beta-proteobacteria*, *Cytophaga Flavobacterium Bacteroides* (CFB), and *Cyanobacteria* are the overall dominant taxa, potentially reflecting the influence of anthropogenic stressors. Most interestingly, the genera *Roseovarius* was found only at high impact Astrolabe Reef sites (the site of the *Rena* oil spill) and the Tauranga Harbour 'control'. This is a species which is considered to be a successional PAH degrader. The *Shewanella* genus was found at Astrolabe reef sites 1, 4.0 and 'control'; previous literature links this genera to having bioremediation qualities for metal and organic contaminants as well as it being a tetrodotoxin producing neurotoxin. The Astrolabe 'control' site has since been shown to also be influenced by the *Rena* pollution.

Our results suggest that anthropogenic environmental stressors are highly influential in determining the structure of bacterial communities. These results contribute to our understanding of the composition and structure of bacterial assemblages in coastal habitats and how microbial responses can inform ecological restoration programs.

4.1 Introduction

Microbial communities are the 'biomechanistic' (Burkart, 2001) nucleus of the earth, mainly due to the fact that they dominate primary production pathways and are vital in ecosystem functioning. Ubiquitous in dispersal and colossal in number, estimated at 5×10^{30} bacterial biomass globally (Paerl *et al*, 2002; Fuhrman 2009)

these organisms are essential in core nutrient cycling and biogeochemical processes in marine biomes. Bacteria by nature are sensitive bio-indicators of shifts in nutrients, pollutants, sediment loading, and hydrological changes in biospheres (Webster *et al.,* 2001; Paerl *et al.*, 2002).

Anthropogenic stressors on ecosystems have been widely documented in marine ecosystems (Webster *et al.*, 2001; Paerl *et al.*, 2002; Paerl & Huisman 2009), and of significant concern are anthropogenic sourced stressors such as agriculture, horticulture, construction development and contaminant events such as oil spills, plant or mine discharges (Webster *et al.,* 2001; Paerl *et al.,* 2002; Hamill, 2014; Ross & Battershill, 2014).

Characterising the complex interactive network of microbial communities and their functional capabilities has been advanced considerably with DNA sequencing (Fierer *et al.*, 2014).

The aim of this study is to characterise the taxonomic composition and phylogenic diversity of environmental samples taken from the sampled sites - Tauranga Harbour, Maketu Estuary and Astrolabe Reef, in order to compare the *Rena* grounding impact with the existing local anthropogenic influences. Using the 16S rRNA marker we were able to map the spatial heterogeneity of the microbial communities from our selected sites and compare the β-diversity previous literature. Using univariate statistics, hierarchical clustering and ordination analysis we were able to elucidate the community trends of the most dominant and rare bacterial assemblages.

4.1.1 Urban pressures

The two coastal sites (Tauranga Harbour and Maketu Estuary) were used for comparison with samples collected at Astrolabe reef. These sites were all affected by the *Rena* Oil spill to varying degrees. Maketu being the first mainland area hit by the spill and to a much lesser degree Tauranga harbor, due to its more northern location.

Maketu has had several decades of alterations and additions starting in late 1890's when the Kaituna River flooded the plains of the area. The farming community led successful campaigns to have the river diverted at a breech point where the river met the open ocean (KRTA ltd, 1986), with the addition of pump stations and various flood management strategies. This succession of interventions has led to significant sedimentation infilling of the estuary, increased eutrophication, and the cumulative spread of algae up the Kaituna river, decimation of the original kaimoana (seafood beds) and overall fisheries ecology which were once abundant in this area (Hamill, 2014). The local district council consents the primary industries of the area to discharge into the Kaituna River, these discharges include treated sewage, dairy company wastes, meat works wastes, and a fruit pack house waste products (KRTA ltd, 1986).

Tauranga Harbor is one of New Zealand largest ports, with future expansion under way, with channel dredging and widening in order to allow larger container and cruise ships to dock in the area, all supported by the local tourism and business industries (Priority 1 2011). Tauranga port includes the wharves, servicing areas and marina all located in the southern part of the harbour at Mt Maunganui and Sulphur Point. Extensive land reclamation has been done in response to the exponential growth of this region from ports operations, various marine industries

- recreational and commercial, tourism and transport requirements (a four way expressway along the Waikareao Estuary claimed 9 ha of tidal land) (Cromarty & Scott, 1996).

The surrounding catchment is farmed or in horticultural use, the impacts from the successive human development have occurred since occupation. These changes have been evidenced in changes in the harbour biota, which has declined in diversity and biomass from pollutant discharges such as herbicides, fertilisation, septic tanks, effluent, industrial waste spillages and other localised discharges (Cromarty and Scott, 1996).

4.1.2 The *Rena* grounding at Astrolabe Reef (Otaiti)

In October 2011 the Bay of Plenty region experienced a catastrophic environmental event with the cargo ship MV *Rena* grounding on Astrolabe reef, just 18kms off the coast line and closer to the relatively pristine island Motiti. The vessel was carrying an assortment of cargo including heavy metals, perishable foodstuffs and dangerous goods (Ross & Battershill, 2014; McSweeney, 2015). The most severe and immediate biological effect was in response to over 1,700,000 kg of heavy fuel oil (HFO380) that spilled into the coastal ocean gyre, and washed up on the shoreline of Motiti Island and all along the Bay of Plenty coastline Figure 10. Of additional concern was the 23000 kg of copper filings lost from some containers and now spread on the seafloor together with a debris field around the wreck of approximately 10 square kilometers (Brodie *et al.*, 2014; BOPRC, 2015b; Cary, 2013; McSweeney, 2015). Other pollutants which merit mention, are the 2.6 tonnes of fertilizer and over 4,000 tonnes of milk powder and similar products (MV *Rena* Cargo Manifest; Subsurface 2014) which was released into the area; such nutrients

have previously been correlated to eutrophication in marine, estuarine and freshwater bodies (Paerl *et al.*, 2002).

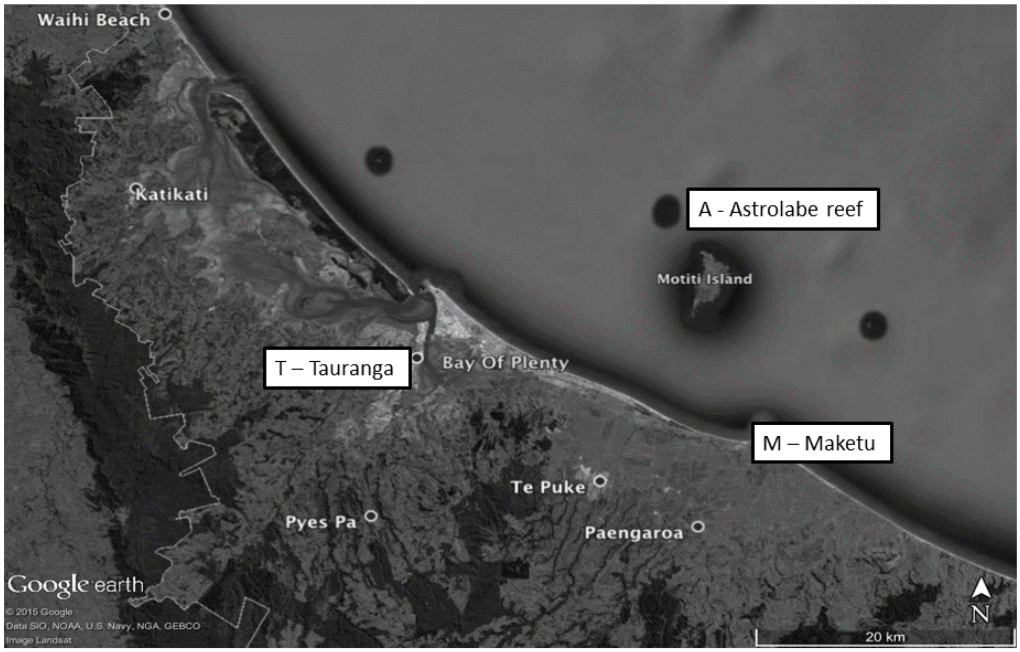


Figure 10: Bay of Plenty coastline and context of sampling sites, Astrolabe reef – (Otaiti) where the *Rena* grounding occurred.

Certain bacterial taxa are known to be associated specifically with biodegradation of hydrocarbons and pollutants (Leahy & Colwell, 1990; Redmond & Valentine, 2012). Several studies have found that from additions of crude oil and nutrients microbial populations with bioremediation capabilities surge in abundance (Redmond & Valentine, 2012; Reihana, 2013; Yakimov *et al.*, 2004). Communities which have had exposure to hydrocarbons or pollutants show a quicker response mechanism to additions than communities which have never been exposed (Atlas, 1991; Yakimov *et al.*, 2004). The process of bioremediation is dependent on hydrocarbon composition and bioavailability of the hydrocarbon to bacteria. Specific consortia associated with bioremediation are identified as various genera from the Alpha and Gamma Proteobacteria as well as *Cytophaga Flavobacterium Bacteroides* (CFB) (Kostka *et al.*, 2011; Yakimov *et al.*, 2004).

While a preliminary baseline community structure study has been done on Astrolabe reef by Cary (2011), an investigation of the effects at the larger whole reef and wider Bay scale has not been undertaken. In order to examine the full extent or a range of the anthropogenic impacts and how these communities may have been influenced, sites for this study were selected based on impact from the Rena oil spill. The sites chosen are Tauranga Harbour as a low impact zone, Maketu Estuary as residual medium impact zone, and Astrolabe reef as the high impact zone.

Marine substrates (natural and artificial) are known to encourage primary colonising communities which are characterised by macromolecules, attached bacterial biofilms, all enmeshed with extracellular polymers (Webster & Negri, 2006); these being the ideal signal communities after a trauma event such as the Rena oil spill. Based on anecdotal evidence that crude oil additions are contributing to the increase in algal blooms in the region, algal substrates were selected as a mechanism to investigate bacterial assemblages associated with the sites anthropogenic stressors (MacNaughton *et al.*, 1999; Kostka *et al.*, 2011; Paerl *et al.*, 2014).

Environmental samples aimed to depict the current state of the microbial communities are exemplified by many spatial and temporal surveys studies such as Archer *et al.* (2014), Green *et al.* (2008) Negri *et al.* (2006) and Zhang *et al.* (2014). Utilising DNA sequencing techniques paired with geochemistry spot characterisation of three sites within three locations, resulted in cyanobacterial diversity profiles being generated.

The aim of this research is to,

1) Characterise bacterial communities on natural and artificial substrates within the selected habitats across a pollution profile.

2) To construct a broad preliminary genera level library of microbial community composition within the Bay of Plenty coastal region reflecting various levels of anthropogenic disturbance (urban environments versus a major ship wreck).

Presented here is the first baseline spatial survey of bacterial communities from the MV *Rena* oil spill benchmarked against a range of adjacent habitats to examine combined effects of different types of anthropogenic contamination (ship versus urban).

4.2 Materials and methods

4.2.1 Sampling sites and locations

Samples were collected from the three locations, Tauranga Harbor, Maketu Estuary and Astrolabe reef,

. Tauranga and Maketu are coastal habitats with samples for this study taken in shallow embayment's, no deeper than 50cm as described by Hamill (2014). Astrolabe reef, (latitude 37º 33'37"S) and (longitude 176 º 23'47" E) samples were collected from the wreck and debris sites on and around the grounded *MV Rena*, by the monitoring divers. Samples were collected based specifically on visual assessment, indicated by algal blooms and in low residence locations (stagnant sites and debris field - impacted) and algal vegetation in high residence locations, (centre of channels, outside debris field - non impacted). A hierarchical sampling regime based on amended Kirchman (2002) and Rashid *et al*. (2013) protocols was designed. Physical characteristics of each location are varied, with several hundred kilometers separating each location. However, similar sampling sites within locations were chosen based on observational characteristics.

The Astrolabe reef sites were located in the sub tidal reef habitat characterised by an array of rock platforms, shelves, and sand and sediment boulder medley's. Samples were collected at ≤ 10m and 30m depths.

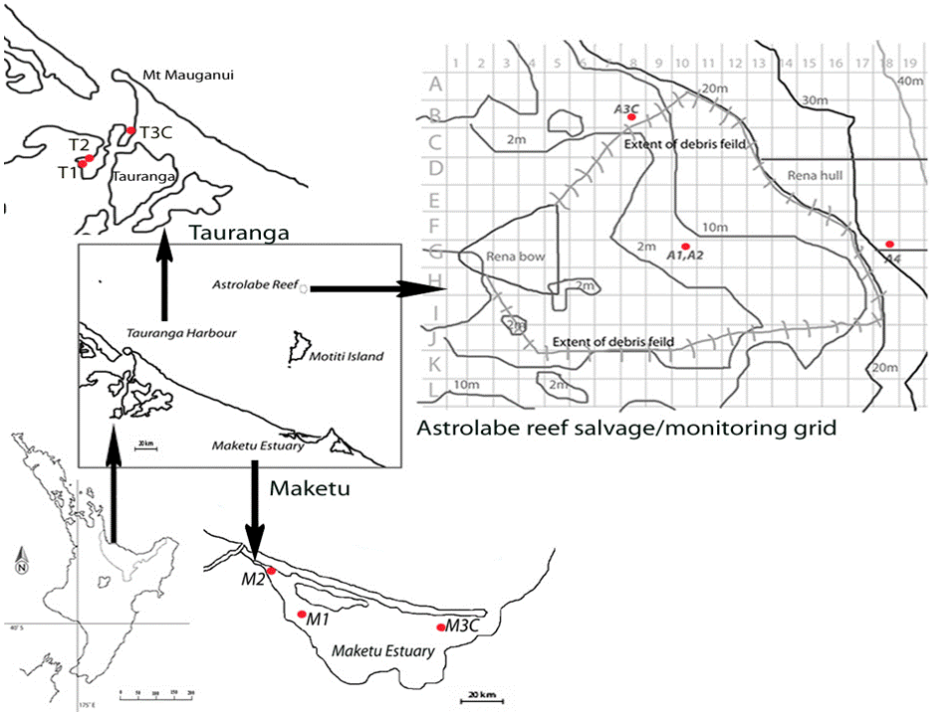


Figure 11: Location map of the sampling sites and the MV Rena Wreck on Astrolabe reef. Red dots are the locations of each sampling site. The extent of the debris field from the MV Rena grounding is outlined in the top right insert.

Tauranga Harbour is a natural lagoon enclosed with spits and an island barrier; it essentially contains two hydrological basins formed by the entrances at each end of the harbor. Tauranga city sits at one end, flanked by a major commercial cargo port, surrounded by a catchment dominated with cattle farms and horticulture. Impacted samples (T1 & T2) were collected in one of the embayment estuaries,

and the control was retrieved from marine fish tanks located at the Sulphur point coastal research centre. The source water of these tanks was collected from the Tauranga main channel (T3C). Over a period of three months algae has grown in the tank and is used here as the harbor 'non-impacted' control.

Maketu Estuary is a human modified river outlet, with alterations transpiring since early 1922, to remediate flooding of the surrounding farmed plain lands. The estuary was essentially cut off as the river mouth outlet and subsequent degradation of the marine, wetland and river habitats has occurred,

.

4.2.2 Sampling protocol

Following the protocols of Garcia-Pichel *et al.* (1996) (natural samples of marine algal filaments) were collected from each site with water in 50cc falcon syringes, and one 15ml amber bottle of organic algal filament preserved in 10% formalin for taxonomic identification of algal genera.

Water samples were filtered with 0.2 µm and 0.45 µm Sartorius membrane filters into 50ml falcon tubes, and frozen at -20º within two hours of collection as per the protocols of Archer *et al.* (2014) and Powell *et al.* (2003).

Organic algal matter was collected from each location and placed in 50ml falcon tubes and frozen at -20˚ within two hours of field collection as described by Garcia-Pichel *et al.* (1996) and Powell *et al.* (2003)

At each site, the environmental parameters (pH, dissolved oxygen (DO), specific (SPC) conductivity and temperature were recorded using a hand held Aquaread AP 2000 meter (Aquaread ltd, UK) as described by Bottos *et al.* (2008), Larsen *et al.* (2012) and Pommier *et al.* (2006).

4.2.3 Trace element analysis

Trace metal analysis was performed on each water sample, which was firstly filtered with 0.2 µm and 0.45 µm Sartorius membrane filters into 15ml falcon tubes as per the protocols of Archer *et al.* (2014). Then inductively coupled plasma mass

spectrometry (ICP-MS), was performed using a Mass Spectrometer ELAN DRC II (Perkin Elmer Inc., Münster, Germany). Samples prepared for ICP-MS analysis were diluted with Type 1 water at 20%, then 9.8µl of the sample was measured out and 0.2µl of nitric acid to achieve a 0.02% concentration in samples as a preservative, following the protocols described in Archer *et al*. (2014) and Jenner *et al*. (1990).

4.2.4 DNA extraction

Samples from all sites were extracted with a standard CTAB lab extraction protocol as described by Archer et al (2014) and then compared to a commercial isolation MoBio Power soil (MoBio, Carlsbad, CA, USA) (Cary, 2011).

Weak amplifications of DNA as shown in SI App. 1**Error! Reference source not found.**, lead to amended protocols to eliminate inhibitors and extract higher quality DNA amplicons.

MoBio ultra clean 15, clean up extractions were used to process samples which were considered to have high carbon content (Astrolabe reef and some of the other weak amplifications) as per manufacturer's instructions with the exception of an increased supernatant volume of 700µL and 640µL for the lysate stages then centrifuged for 1mins, with this step repeated three times.

For the MoBio ULTRA CLEAN 15 kit the manufacturers' protocols were used as per the manuals instructions, with an increase in the ULTRABIND ratio of 12µL: 2µL to the DNA template for amplification.

Extraction efficiency was determined by diagnostic gels and PCR, trials gel images are shown in SI. App. 1. PCR reactions were performed in triplicate and combined to overcome later biases during DNA sequencing. To determine and compare

relative diversity between all sites, automated ribosomal intergenic spacer analysis (ARISA) was used. Amplification of the intergenic spacer of the rRNA operon was carried out in 25µl reactions containing 10mM of forward primer FAM labelled primer PET-CY-ARISA-F (50 - PET/TG GYC AYR CCC GAA GTC RTT A - 30) and 10mM of reverse primer 23S30R (50 - CHT CGC CTC TGT GTG CCW AGG T - 30) as described by Barrett et al (2006), Sokol *et al.* (2013) and Wood *et al.* (2008).

4.2.5 Ion Torrent sequencing

To prepare samples for Ion Torrent sequencing (ITs), the V4 region of the 16S rRNA gene was amplified using the forward primer linked with Ion adapter "F" sequence,

(5'CCATCTCATCCCTGCGTGTCTCCGACGATGTGCCAGCMGCCGCGGT AA '3, and Ion Xpress barcode sequences were inserted between adapter "F" and the primer. The reverse primer was linked to Ion adapter "p1" sequence 5'CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTA CHVGGGTWTCTAAT '3 as described by Fujimoto et al (2014). The Ion express bar codes table are shown in SI App. 2. PCR amplicons were performed in 25µL reactions, using 2µL of DNA template, BSA 1.0µL, 1.0µL of primers, 3.0µL of 10X buffer, dNTPS, $MgCL_2$ and 0.12µL of U Platinum Taq, with the remaining made up of MilliQ H_2O. PCR thermocycler conditions were 94º for 3mins, a cycle of 94º for 45s, 50º for 1min, then 30 cycles of 72º for 1.5min, a final extension of 72º for 10mins before holding at 4ºC until refrigerated overnight. Pooled DNA amplifications were viewed on a 2% TAE agarose gel, which had been run for 35mins at 75V and stained with 'SYBR safe' (Invitrogen ltd).

PCR products were then cleaned again with the SPRIselect process to remove primer dimers. SPRI was added to DNA template at a ratio of 0.8 to 1µL, mixing together by manual pipetting 10x. Incubated at room temperature for 1min, and then placed on a magnetic tube rack to allow settling for 2mins. The supernatant was discarded and the pellet washed with 180µL AR grade 85% EtOH, returned to the magnetic rack and allowed to incubate at room temperature for 30s. The EtOH was discarded and pellet allowed to dry at room temperature. Once dry elute pellet with 20µL 1 X TE and pipette to mix solution, then incubate at room temperature. Place tube on magnet again then transfer eluent to a new tube, repeat final step twice to remove any residue contaminate. All DNA products were quantified using Nanodrop ND-1000 at 260nm DNA product was checked on a Qubit Fluorometer (Invitrogen Ltd).

4.2.6 Data processing

Ion Torrent sequence and ARISA data was processed into their unique name files and cluster sequences of operational taxonomic units (OTUs) with Mothur 1.17.0 (Archer et al 2014). Phylogenetic assignments were analysed using Ribosomal Database Project (RDP) release 10, update 15 and converted into fasta files for processing as defined by Fujimoto *et al*. (2014).

Chimera purging was completed with RDP levels and a threshold criteria. First level criteria was Family confidence of <38%, Genus confidence at <30%, Class confidence level at < 90%, Phylum confidence level at <96%, then Domain at <99%. After each RDP level was verified, cyanobacteria were selected out due to uncertainty and mis-classification with macroalgae. Cyanobacteria had a threshold criteria of >97% (Zhang et al 2014), all cyanobacteria at <97% were assigned as macroalgae (Fujimoto et al 2014). Each OTU identified below the filtered threshold

was run through the Basic Logic Alignment Search tool (BLAST) (Webster and Negri 2006), firstly in a nucleotide BLASTN check, then a second check through a MEGA BLASTN as verification.

Abundant selection criteria had a cut off threshold of 1% to capture the centric core species and for the rare species threshold criteria was 0.05%.

Sequence analysis was accomplished with software package Primer 7 (Primer-E: Lutton, Ivy Bridge, United Kingdom) to investigate community patterns (Kostka *et al.*, 2011). Geochemical data was log (X+1) transformed and results were generated into a resemblance matrix worksheet to simplify data and examine the inter-relational dynamics between variables. Spearman's rank correlation coefficients were then calculated, resulting in a probability (*P*) of community differences being explained by the differences in geochemistry. The combination of geochemical variables whose Euclidean distance matrix gave the highest *P*-value was considered the most likely drivers of community dynamics see SI Table 1. PCA plots were generated from these findings to view overall and individual influences on community assemblages. To compare the sites and locations further, between the rare and abundant communities, CLUSTER analysis was used, and then SIMPROF to identify the resemblance relationships. ANOSIM (one way) was used to identify R statistics and pairwise significance levels for similarities between locations, with a principle coordinate ordination (PCO) cluster to assess the distance matrix between impacted and non-impacted sites.

The ARISA data was used to generate a Non Metric multidimensional (nMDS) diversity profile to represent distance matrices between samples. The stress levels of the nMDS plots were 0.06 for 2D, and 0.02 for 3D as a means of considering the

true representation of the dimensionality distance of the data. Then a SIMPER analysis was done on both rare and abundant species to quantify the similarity and dissimilarity between samples.

4.2.6.1 Environmental variables

ICP-MS trace elemental data was analysed with BEST to match probability of influence with variables and correlated with the Spearman rank method, measured by Euclidean distance. An element threshold criterion was selected at (0.9) for correlations of significance. All environmental variables above the threshold were then compared in a Principle component analysis (PCA) against all sites to elucidate elemental influences. The trace metal suite used to identify contamination in Negri *et al.* (2006) showed sediment contamination in Antarctica as a baseline comparative analysis. In this study the same suite of metals was analysed with PCA to correlate ecological influences (Negri *et al.*, 2006; Zhang *et al.*, 2007; Kostka *et al.*, 2011; Zhang *et al.*, 2014). RELATE analysis revealed *rho* statistics which gave linear correlation coefficients between the sites and factors of influence (Powell *et al*, 2003).

4.2.6.2 Community assemblages

Community assemblages were then compiled from the OTUs, these were converted into a representation percentage of the community at Phylum and Genera level. Overall comparisons were made between sites, and then within locations to establish average dominate bacterial community structures, similar to comparisons made in Aguiló-Ferretjans *et al.* (2008), Pommier *et al.* (2006) and Webster & Negri (2006). ANOSIM One way pairwise analysis elucidated similarity of the relative diversity from the abundant and rare species, then BOOTSTRAP alignments measured the data's dependability (Baldauf, 2003).

4.3 Results and discussion.

4.3.1 Sampling site characteristics.

At the Tauranga impacted sites (T1 & T2), algal substrates sampled consisted of fine green filaments which amassed in thick mats on the water surface. For the control site sample (TC) algal filaments were retrieved from the marine fish tanks located at the Sulphur point coastal research centre.

For the Maketu impacted sites sampled, M1 contained visible chlorophyte vegetation which exhibited cyanobacterial algal smothering with sediments sporadically anoxic, (from previous surveying for the following report (Hamill 2014)) with a slight sulphur odour (Plate 4). The M2 site sample was taken from an approximately 6cm thick algal mat, with associated anoxic black sediments which had a strong sulphuric stench on disturbance. For the M3C site there was significant *Ulva lactuca sp*. stands in the vicinity.

Plate 4: Top plate: M1 site, visual cue of anthropogenic pressure. Bottom plate: *Rena* monitoring divers collecting algal samples from the remnant of the hull.

At Astrolabe Reef samples A1 and A2 were collected off the MV *Rena* hull wreckage, Plate 4. The A4.0, A4.1 sites were located in the most impacted part of the debris field of the MV *Rena* container ship, while the A3C was located outside the debris field Figure 12.

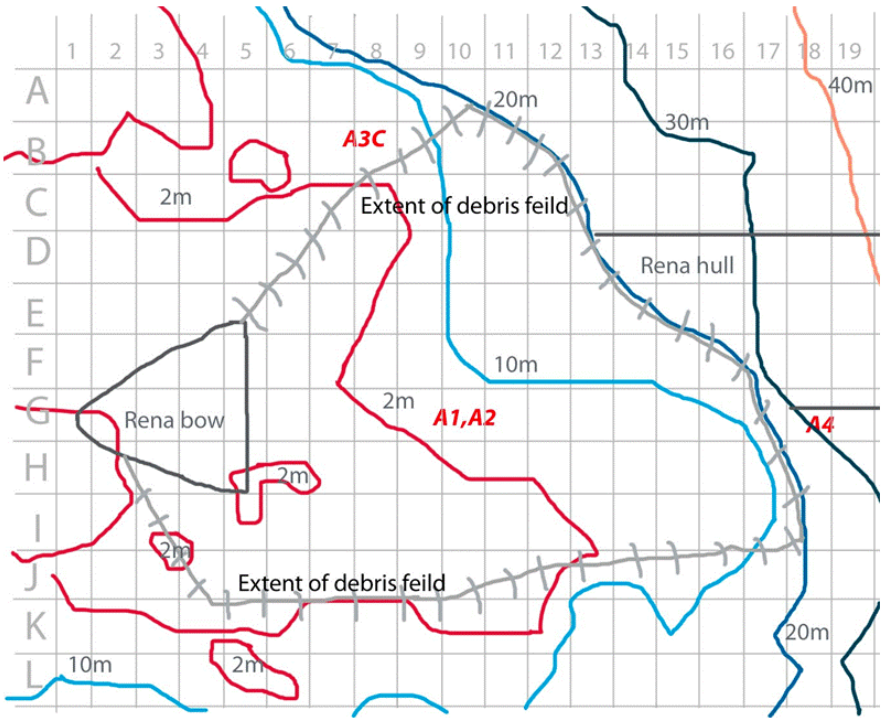


Figure 12: Astrolabe reef grid map of monitoring area and sampling sites, extent of debris field shown in grey crossed lines, in relation to remnant of ship hull and bow.

Given the varied geology in the Bay of Plenty region underpinning a range of anthropogenically sourced pressures (KRTA ltd, 1986; Cromarty & Scott, 1996; UoO, 2014) physiochemical variables were examined to ascertain the degree by which these influenced bacterial community structures.

4.3.2 Microbial community assemblages

Eleven site samples generated 14 666 reads, with 250+bp. ITs data verified OTUs used for rare species were 201 in total, and abundant species were 148 OTUs.

Based on rank frequency analyses the community assemblages at Astrolabe and Tauranga locations appear clearly dominated by the *Cyanobacteria* Phyla (A: 45.93%, T: 56.11%), then *Proteobacteria* (A: 25.98%, T: 28.65%) and *Bacteroidetes* (A: 24.13%, T: 7.60%). At Maketu the most dominant phyla was the *Proteobacteria* (53.28%), followed by *Cyanobacteria* (23.89%) and *Bacteriodetes* (15.66%) Figure 13.

4.3.2.1 Class level compositions

Class level histograms of each bacterial assemblage are in SI Fig. 2.

Further class level investigations revealed the *Cyanobacteria's* phylum fragmentation into macroalgae and assigned genuine *Cyanobacteria*, with ≤ 1% presence at Maketu and Tauranga, and a 12.38% presence at Astrolabe reef.

The *Proteobacteria* phylum are dominated by *Alphaproteobacteria*, a consistent 15-18% range across all sites, *Betaproteobacteria* dominating in Maketu with a 27.67% presence, and *Gammaproteobacteria* with a low range of 4.8 – 7.18% presence across all sites.

A notable presence of *Epsilonproteobacteria* at all Astrolabe and Maketu sites of ≤4%, *Flavobacteria* are most dominant with 22.92% at Astrolabe, 14.13% at Maketu and only 5.64% at Tauranga. There is a small representation of *Sphingobacteria* ≤ 2% at all sites with a *Cytophagia* presence at Tauranga 2 and Maketu 1 sites only.

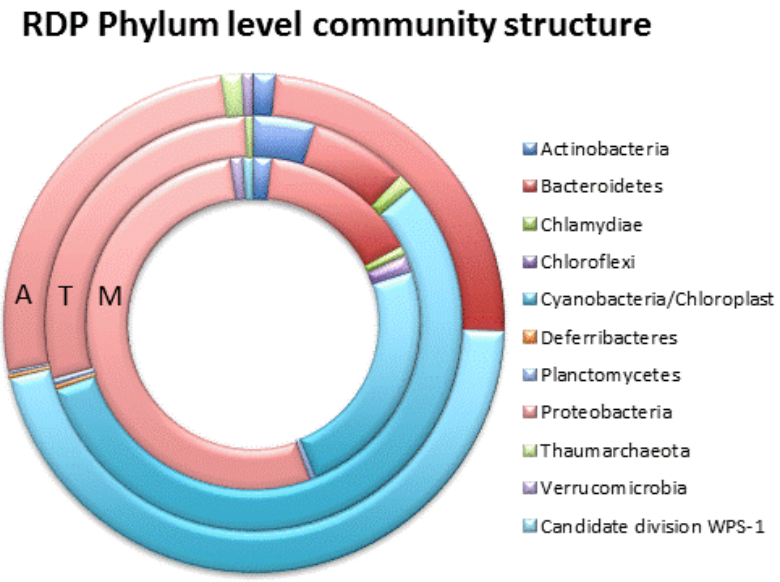


Figure 13: Phylum level location bacterial community compositions by location.
Key: A = Astrolabe Reef, T = Tauranga and M = Maketu

4.3.3 OTU enumeration

OTUs distinctions were determined using a 97% confidence limit in the NCBI data bank, SI App. 4. Sequences from this study grouped closely with a series of other bacterial phylotypes, which are known to undertake certain processes, therefore an assumption about the following results, is that the bacterium surveyed here have matching functional traits within their genera categorisation.

4.3.3.1 Cyanobacteria

At the Astrolabe reef hull sites 1 and 2, the cyanobacteria phylum *Lyngbya sp.* was present with the highest percent of (3.94%) at site 2. At all Astrolabe reef sites the *Oscillatoriales* order had *Synechococcus* present. While *Prochlorococcus marinus* was found at all Astrolabe reef sites as well as M2 and M3C with the highest occurrence of (1.41%) at M2. The remaining cyanobacteria OTUs were verified as *Eukaryota*, *Rhodophyta* genera of various red and green algal chloroplasts.

4.3.3.2 Proteobacteria

Proteobacteria was the next major represented phylum, and was dominated by representatives of the Class *Alphaproteobacteria*. The genera *Anderseniella* was found at all Maketu sites with the highest occurrence of (1.39%) at M1. *Hypomonas* was found at all Astrolabe reef sites, M1 and M3C as well as TC with the highest occurrence of (1.93%) at A1. The *Roseovarius* genus was identified at the Tauranga control site and all the Astrolabe reef sites. Another genera identified at M1, M2 and T2 was the *Sphingomonas* which is a entophytic bacteria associated with freshwater macrophytes (NCBI, 1993).

The *Betaproteobacteria* class occurred at all Maketu sites and T2, having the highest percentage of (7.11%) at T2. The genera *Methylophilus* was identified at

each of these sites and the only genus identified in this order. In the order *Burkholderiales*, the genera *Ralstonia* had 3 phylotypes at the M2 site only

The next dominant class at Tauranga sites was the *Gammaproteobacteria*, however, no verifiable DNA match could be found. At M3C site, *Alteromonas* and *Haliea* were highly dominant (36.32%), with a lesser representation of *Haliea* and *Marinomonas* of (0.06%) at M2. At Astrolabe sites 1, 4.0, 4.1 and C all three genera occurred with the addition of *Shewanella* at Sites 4.0 and 4.1 and the control, at (≤ 4.61%).

At Maketu the highest occurrence of *Deltaproteobacteri*a (≤ .01%) was at the control site, with the highest occurrence of *Epsilonproteobacteria* (≤ 4.49%) was at M2. The only genera of *Deltaproteobacteria* found at Maketu were the *Desulfobacterales Desulfopila.*

4.3.3.3 Bacteriodetes

Eight different genera of the *Flavobacteria* were detected, with seven being common components at all Astrolabe reef sites. The exception being the genus *Dokdonia*, which was only found at A1. However, the *Flavobacteria* were a dominant component at M2 (21.50%), (exclusively the genus *Tenacibaculum),* with the next highest incident at TC with (8.95%), which consisted of the consortia of three genera (*Tenacibaculum*, *Maritimimonas* and *Bizionia).* At the MC and all Astrolabe sites the *Flavobacteria* consortia consisted of the *Aquimarina*, *Dokdonia* and *Actibacter*. The *Flavobacterium* genus occurred at M1, T1 and T2.

4.3.4 Variables and Elemental composition

Site characteristics such as temperature, pH, DO and SPC conductivity of the water column for each site are presented in Table 4.

Site label	pH	Temp (°C)	SPC cond (mS cm-1)	DO %
T C	8	16.1	40.27	76.5
T 1	8	10.4	41.78	96
T 2	7.4	11.7	16.19	80
M 1	8.8	20.4	19.45	158
M 2	8.1	16.8	31.31	125
M 3C	8.7	23.3	37.16	164.8
A 1	8	15.7	19.6	96
A 2	8.1	15.6	39.6	110.5
A 3C	8.1	15.9	40.31	93.75
A 4	8.1	15.75	41.46	86.45

Table 4: Environmental variables for each site

For biogeochemical characterisation BEST analysis correlations of all physiochemical factors, suggest conductivity to be the primary factor of influence with a *P*-value (0.98), and the elements boron and chromium with a *P*-value of (0.97) overall the sites. Other elements with a *P*-value of (0.98) were nitrogen and cobolt.

From the comparative suite of metals analysed as defined by Negri *et al.* (2006), the *P*-value was (0.98) for copper, zinc, cadmium, lead, mercury and arsenic. Other elements with the same *P*-value were selenium, chlorine, sulphur and phosphorous.

At each site geochemical influence varied considerably. Sulphur had a significant presence at all sites as well as boron. A large *P*-value of (0.96) for arsenic was calculated for all Maketu and Astrolabe reef sites, while no trace effect was found at Tauranga sites. Analyses revealed high concentrations of nitrogen, lead, chlorine, cobolt and cadmium only at Astrolabe reef sites 4, Figure 14. Consistent levels of the suite of arsenic, zinc and copper across all Maketu and Astrolabe reef sites were observed. The other elements of notable interest were selenium (0.98) at the M3C, A3C and A4 sites and phosphorus (0.98) at A3C. All PCA plots showing influences over each site are shown in SI App. 3.

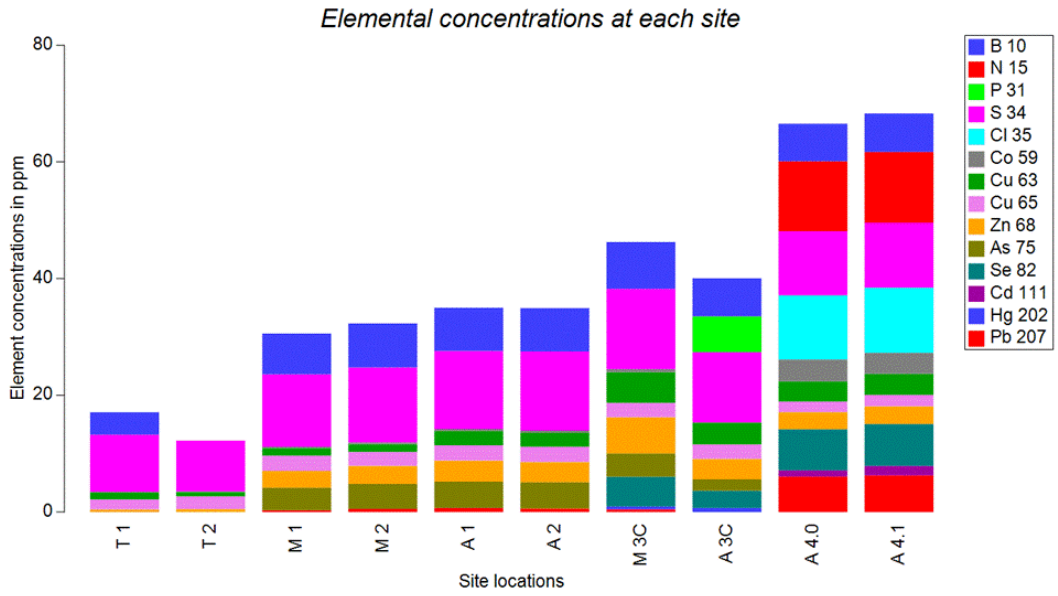


Figure 14: Elemental concentrations in ppm at each site, 14 top influential elements based on *P*-values of (>0.98) in SI App. 3.

To determine the relationship between the variables and their influence over the bacterial community assemblages, RELATE analysis was performed on environmental factors and as well as the suite of elements. For the environmental factors a *rho* statistic of (0.935) with a significance level of 0.1% provides a positive correlation co-efficient of the linear relationship between sites and the environmental factors.

The above results signal positive correlation co-efficient and the linear relationship between variables and the elemental components as influencers of the bacterial assemblages of each site.

4.3.5 Impacted sites vs non impacted.

A threshold of 0.5% overall relative abundance was the selection criteria to represent the centric reserve of rare and abundant species (Early & Thomas, 2007). To explore patterns which reflect community assemblages the abundance and rare species populations were compared using CLUSTER analysis. Bray Curtis

correlations of the community phylogeny show sites which are most similar to others Figure 15. From the CLUSTER plots distinct biogeographical patterns exist. Non impacted sites cluster in both data sets separately from the impacted sites, illustrating that the bacterial assemblages are significantly different at each site. At Astrolabe reef, the abundant species clearly clustered separately from the control, unexpectedly in the rare species there is no distinction with the control.

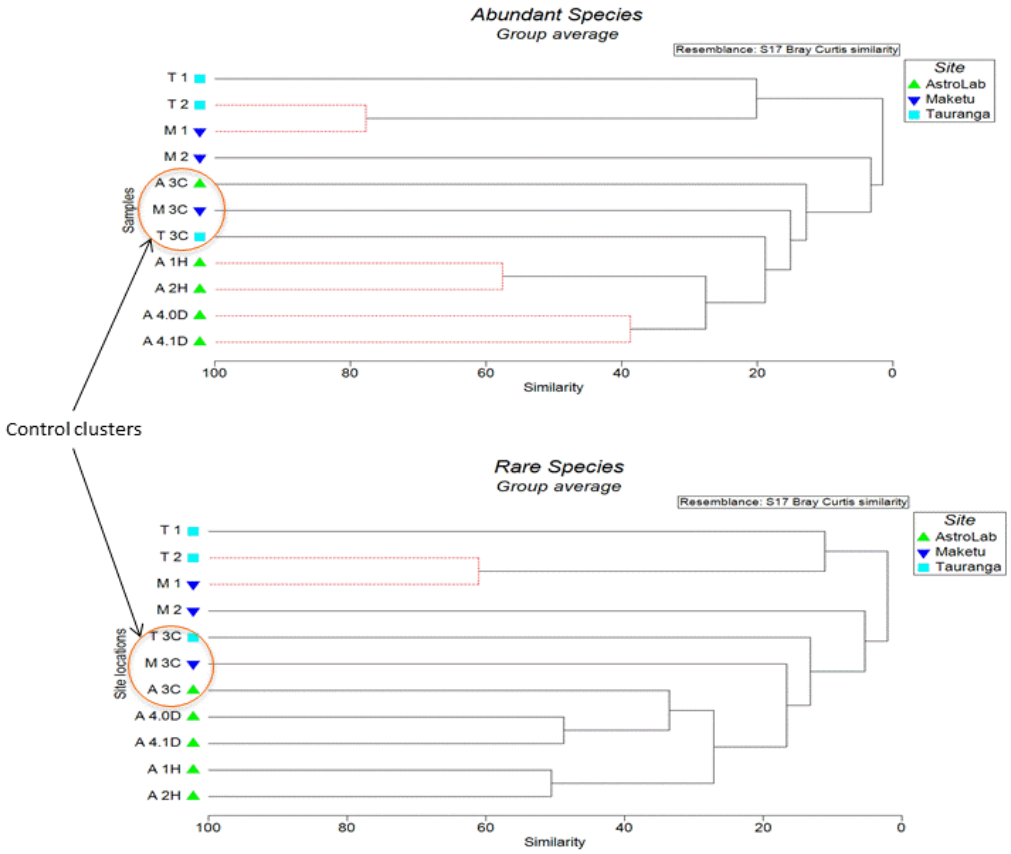


Figure 15: Hierarchical trees of rare and abundant bacterial phylotypes at each site based on Bray-Curtis similarities. KEY: T = Tauranga, M = Maketu, A =Astrolabe Reef, numbers are the site locations in Figure 1 and C = control. Red dotted lines represent samples that are not statistically distinct in phylotype composition.

In the abundant species three clusters were similar, A1 and A2 with a similarity significance of 60%, and the A4 sites with 40%. The third clusters from both rare and abundant species is the T2 and M1 sites with 78% in the abundant species and

60% similarity in the rare species. From the SIMPROF analysis a similarity value of 99.6% for the abundant and 98.6% for the rare species was determined (significance level of 5%) the entire result set is in SI Table 1.

To test the significance of the relationship between all sites, for similarities in phylogenetic compositions, both data sets were analysed with ANOSIM – one way which revealed pairwise R values (SI Table 1) supporting the linear relationship between bacterial composition and locations. R values of ≤1 indicate microbial structures were more similar to each other, with an R value of 0 indicating variation within the group is as significant as between the sites. The significance level was taken to be significant at ≤1. Overall the abundant species had a negative linear relationship between Tauranga and Maketu with a pairwise *r* value of -0.074% at 30%, with an overall level of significance of 2.1% and a sample statistic of 0.442. In the rare species a similar negative correlation with a pairwise *r* value of -0.111 at 30% with an overall level of significance of 0.5% and sample statistic of 0.603 between all locations was observed. Results for the linear relationships between Astrolabe and Maketu pairing and Astrolabe and Tauranga reflected the same negative trend see full results in SI Table 1.

In the principle coordinate ordination (PCO) analysis between abundant and rare species a similar trend in the distance matrix relationships between control and impacted sites was seen, Figure 16. Overall, Astrolabe reef sites have clear differentiation from Maketu and Tauranga sites. Again the curious duo of M1 and T2 pairing up in both rare and abundant species.

In the rare species only, Maketu and Tauranga had the clear differentiation, while the Astrolabe reef sites all clustered together. The Astrolabe reef clustering in the rare species suggests that this may be the endemic bacterial community at this site.

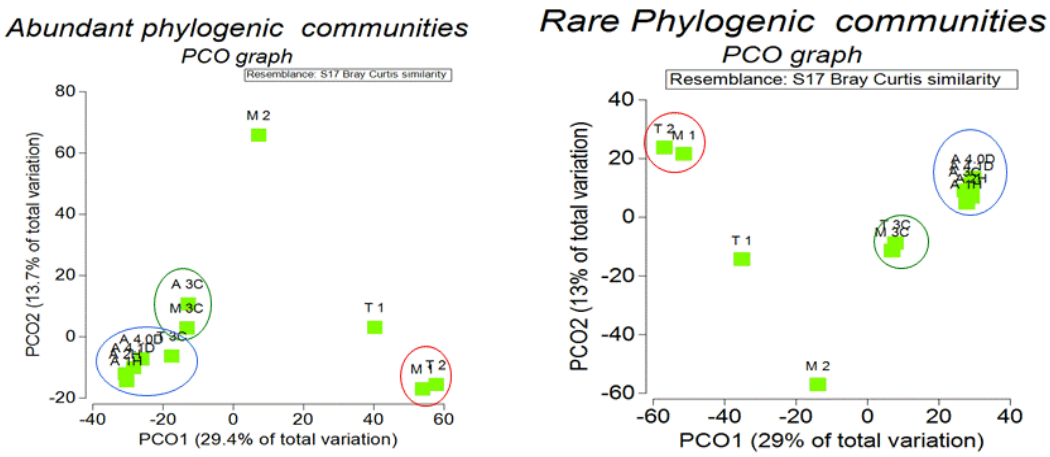


Figure 16: PCO plots from abundant and rare community, Blue circles are control clusters, green circles Astrolabe reef clusters, red circles 99.6% in abundant, and 98.6% in rare SIMPROF similarity between sites, Tauranga 2 and Maketu 1.

The ANOSIM analysis supported the trend of dissimilarity and similarity displayed in the PCO graph (full results in SI Table 1). The BOOTSTRAP alignment rho value was (0.992) with the Kruskal stress formula value of 1, see full results at SI Fig. 1.

Cyanobacterial as a common member component across sites was examined to identify a diversity profile within each site. The cyanobacterial diversity index is visualised in the MDS plot see Figure 17. Surprisingly the Cyanobacterial ARISA DNA fingerprinting analyses and physiochemistry showed that the cyanobacterial communities were clearly distinct at each site. The distance between sites indicates the differences of the diversity profiles. Two clear sets emerged, as well as three sites which had very low correlating similarities to any other diversity profiles generated.

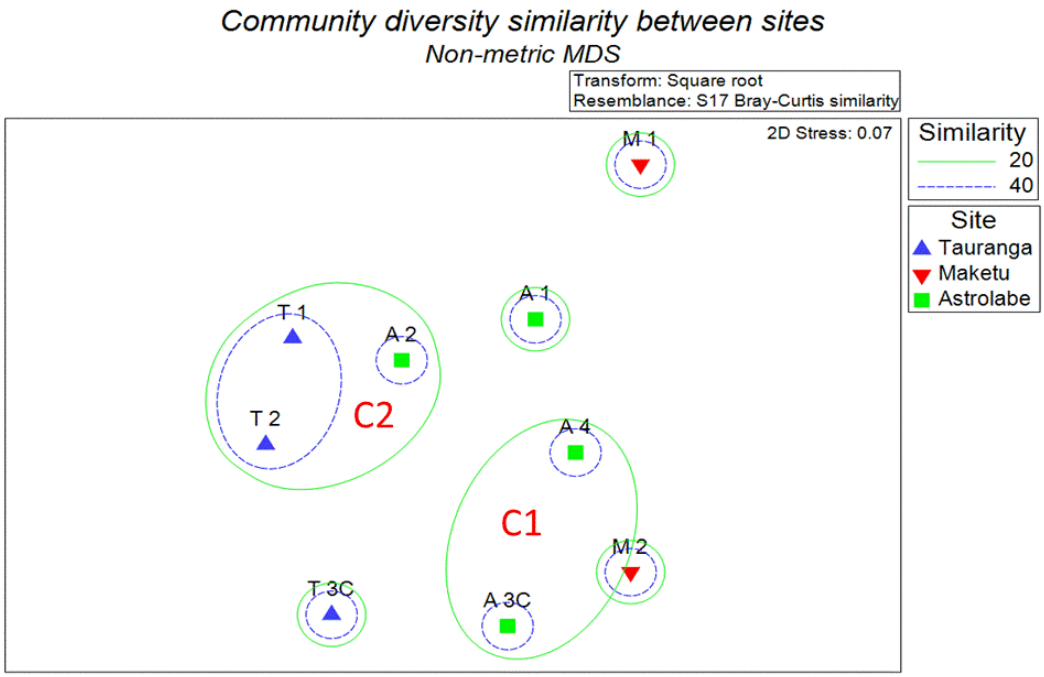


Figure 17: ARISA MDS clustering of sites with high diversity is shown, the greater the distances between sites the greater the diversities between the sites.

Key: C1=Cluster 1, C2 = Cluster 2.

In C1 – cluster one, Astrolabe reef sites 4 and 3C had a diversity profile similarity of <20%, while Maketu site 2 sat on the 20% line similarity the Astrolabe reef sites Figure 17. For C2 – cluster two Tauranga sites 1 and 2 had a <40% diversity similarity then a <20% with Astrolabe reef site 2. Significant dissimilarity of bacterial communities was identified at 40% similarity. The exceptions to this observation were Astrolabe reef site 1, Maketu 1 and Tauranga 3 control. The physical distance between sites in the MDS represents the distance between diversity relatedness.

A Bray Curtis similarity parameter with a 90% low cut off contribution, identified the total average dissimilarity between Astrolabe reef locations and Tauranga $\Sigma\delta_i$ = 94.06, Astrolabe and Maketu $\Sigma\delta_i$ = 92.82 and Maketu and Tauranga $\Sigma\delta_i$ = 87.03. For the complete rare species and average similarities results see SI Table 1.

The SIMPER also authenticated the dissimilarities within locations and between sites, with average similarities in abundant species being 26.80 at Astrolabe, 3.05 at Maketu and 6.85 at Tauranga, rare species reflected similar results SI Table 1.

DNA sequencing and microbial community assessment has identified the dissimilarity between locations as well as within sites, but most notably that these sites show clear clustering between impacted sites and non-impacted sites. In general the abundant bacterial community structures displayed significant dissimilarities indicating distinct patterns, however, their lifecycle processes may differ (Horner-Devine *et al.*, 2007; Nemergut *et al.*, 2011). Although distinct patterns were observed between locations, heterogeneity at impacted sites was more diverse than the control sites supporting findings by Powell *et al.* (2003). In the Powell *et al.* (2003) study the heterogeneity was higher at impacted sites than the non-impacted, their correlations indicated the total organic carbon (TOC) had significant influence over the microbial community structures. Powell *et al.*, (2003) showed that the infaunal communities surveyed in the same region correlated species associated with pollutants at identified impacted sites versus controls.

Across various biomes and analysis techniques a consistent hierarchy exists in microbial community structures Table 5. Precise factors which explain the assemblages are however, more complex than any singular theory has to date been able to empirically define. As shown in Table 5, soils and sediments of the Antarctic can show high similarities to dominate bacterial community from coastal biomes.

	Sites	Depth - m	Firmicutes	Deinococcus-Thermus	SRB- sulphate reducing	GSB - Green sulpur	Proteobacteria	Planctomycetes	Cyanobacteria	Bacteroidetes	Actinobacteria
This study	M	≥ 0.5	○	○	○	○		○			○
	T	≥ 0.5	○	○	○	○		○			○
	A	≥ 30	○	○	○	○		○			○
Zhang et al. 2007, Coastal	TLC-PA		○	○	○	○		○			○
	TLC-FL		○	○	○	○		○	○		
	VH-PA		○	○	○	○		○			○
	VH-FL		○	○	○	○		○	○		
	PC-PA		○	○	○	○		○			○
	PC-FL		○	○	○	○		○	○		
Alonso-Saez et al. 2007, Coastal			○	○	○	○		○	○		○
Suzuki et al. 2001, Coastal				○	○	○		○		○	○
Floyd et al. 2005, Coastal surface	SOI			○	○	○		○	○	○	
	HTP			○	○	○		○	○	○	
	HTM			○	○	○		○	○	○	
	FRL		○	○	○	○		○			○
	MAC		○	○	○	○		○	○	○	○
Bottos et al. 2008, Antartica	MARK		○	○	○	○		○	○		
	WARD		○	○	○	○		○	○		
Neiderberg et al. 2015, Antartic soils	NP4D		○	○	○	○		○			○
	NP2W		○	○	○	○		○			○
	MS1-3D		○		○	○	○	○	○		
	MS1-2W		○	○	○	○		○			○
	ML1-4D		○	○	○	○		○	○		
	ML1-2W		○	○	○	○		○		○	
	BL4D		○	○	○	○		○	○		
	BL2W		○	○	○	○		○			○
Webster & Negri 2006, Antartica	CA	12	○	○	○	○		○	○		○
		18	○	○	○	○		○	○		○
	SB-1	12	○	○			○	○	○	○	○
		18	○	○			○	○	○	○	○
	MM-I	12	○	○		○	○		○	○	○
		18	○	○		○			○	○	○

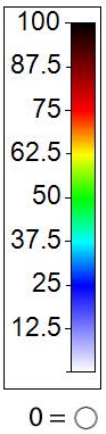


Table 5: Phylum dominance of bacterial communities from selected molecular studies. Phylum represented in percentage of relative abundance in sampled communities. All class and lower order genera from studies were assigned to phylum orders for consistency.

Shown in Table 5, is the predominant bacterial hierarchy from the surveyed studies, primarily *Proteobacteria*, *Bacteroidetes*, *Cyanobacteria* then *Actinobacteria* in the aqueous based biomes.

Of interest are findings from the two geographically disparate impacted sites; T2 and M1, which were significantly similar in bacterial assemblages Figure 18. Both sites were considered to be visually the most stagnant marine environs at all three locations, their physical environmental characteristics were considered to be highly similar having thick algal blooms on the surface waters, located ≤ 0.5m depth of water, and zero to nil water exchange on any given day. Factors which were not sampled for and may explain the assemblage may have been excluded from the testing such as O_2, Nh_4, NO_2, PO_4 or TOC.

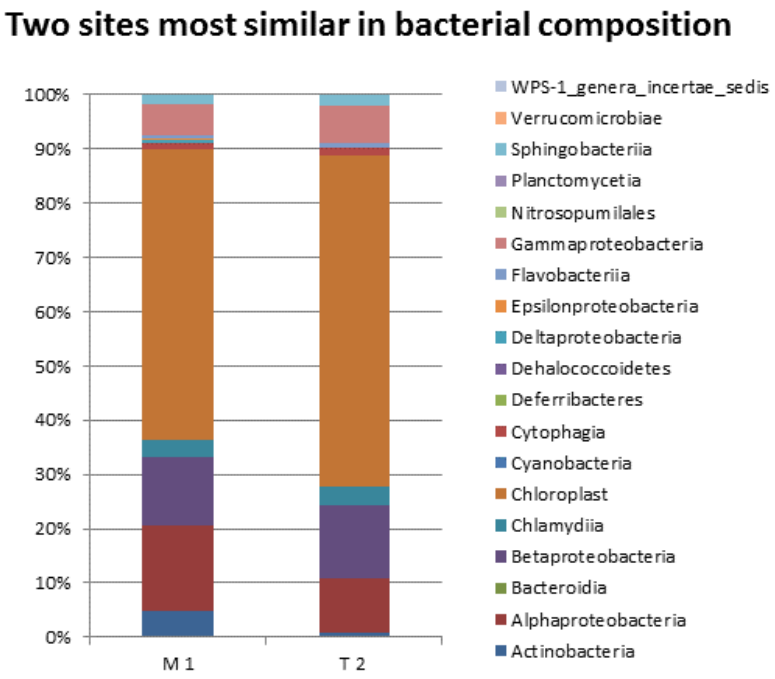


Figure 18: Bacterial community assemblages at sites M1 and T2

4.3.6 Ecophysiological influences

Ecophysiological characteristic patterns correlate to site environmental factors which is consistent with findings from Powell *et al.* (2003) and Pommier *et al.* (2006), supporting evidence that community patterns are influenced by environmental factors. These similarities and dissimilarities are supported by the statistical analysis used in this project. Overall, from BEST analysis all environmental variables were considered to be influential at (0.8), with secondary

influence being pH, DO and SPC conductivity, the latter two both indicators of anthropogenic influences above normal ecosystem capacity. Of these three factors, SPC conductivity and pH are recognised as the primary drivers of community structure in aquatic environments (Powell *et al.*, 2003; Archer *et al.* 2014). From BEST analysis SPC conductivity had a *P*-value of (0.98) indicating its significant position of influence overall. In this study phylotype profile at each location suggested a pH influence, at Astrolabe and Tauranga, yet less influence than Maketu; these are shown in the PCA plots of Dissolved oxygen SI App. 3.

Overall, environmental factors asserted varying influence over each location and site; however, no one factor could be identified as a primary driver at any site. Although indications of influence from the results of this thesis, other factors than those explored here may alter these findings (Zhang *et al.*, 2014). A general observation of the BEST analysis with a *P*-value of (0.98) shows that increased elemental composition, correlates to increased bacterial heterogeneity at sites- Maketu and astrolabe reef sites have larger elemental compositions and also more diverse bacterial heterogeneity.

The heavy metal suite tested by Negri *et al.* (2006) and replicated in this study, correlated with a *P*-value of (0.98), shows these findings support the Powell *et al.* (2003) study. These metals were contributing influencers over microbial community structures, however, it is surmised that environmental factors are probable influencers but not in isolation.

4.3.7 Ecophysiology and functional potential of abundant microbial taxa.

The overall ecophysiological characteristics of bacterial taxa reflect the functional potential of the community structures signalling what processes are significant in

the community based on the relative abundance. The complete bacterial consortia at each site are found in SI Table 2.

The bacterial community at Astrolabe reef had the *Prochlorococcus marinus sp.* and *Lyngbya sp.* two genera which are synonymous with anthropogenic stressors. The *Prochlorococcus marinus* genera found in this study is an oxyphotoautotrophic bacterium which is ubiquitous in the ocean and is often found in an ultraoligotrophic setting (Dufresne *et al.*, 2003). It is also a rare specimen providing a direct correlation between genomic characteristics and environmental factors (Swingley *et al.*, 2008). *Lyngbya sp.* in both fresh and marine environs are synonymous with increased nutrients, which cause algal blooms, usually associated with anthropogenic sourced increases (Paerl *et al.*, 2002; Paerl & Huisman, 2009).

The *Synechoccous sp.* found only at Astrolabe reef sites, is a unicellular cyanobacteria and are abundant in the world's oceans as a primary producers of the marine food web and core biogeochemical (carbon, nitrogen, sulphur) processes of the ocean (Flemming, 2007). Molecular techniques did not identify *Lyngbya* at the M2 site. However, microscopic observations of *Lyngbya sp.* from Maketu site 2 are shown, see Plate 5.

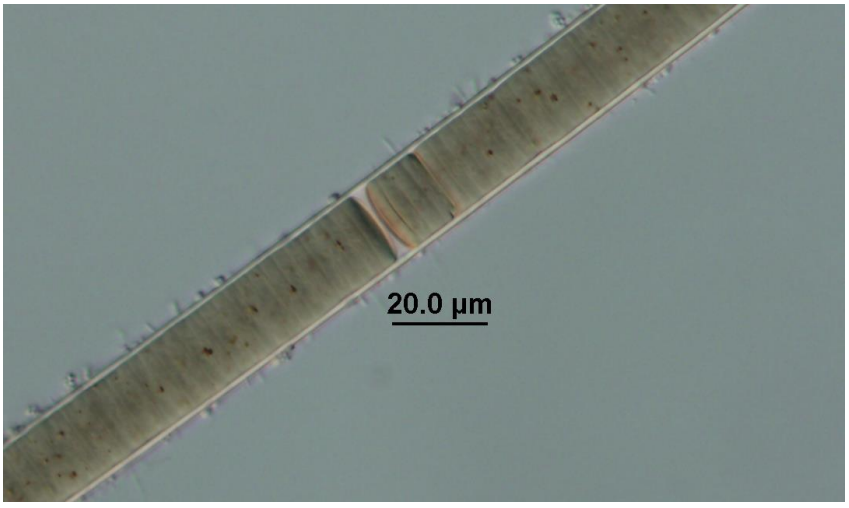


Plate 5: *Lyngbya sp.* found at Maketu site 2, microscopic identification

For the *Proteobacterial* phylum, the *Roseovarius* genus identified at the Tauranga control site and all the Astrolabe reef sites is considered an indicator species of successional PAHs degradation (Wang *et al.*, 2009, 2014; Kostka *et al.*, 2011).

From the *Gammaproteobacteria* one of the known hydrocarbon bioremediation phyla, found at Astrolabe reef and Maketu sites only was the consortia consisting of Marinomonas, *Haliea* and *Alteromonas*. *Marinomonas* are a gram negative, strictly aerobic, catalase positive and oxidase negative organism associated with anaerobic phenol metabolism and is thought to have aromatic degradation pathways (Rabus *et al.*, 2005; Lucas-Elio *et al.*, 2011). This genera is a known degrader of ethylbenzene - a colourless flammable liquid hydrocarbon, used in the manufacture of styrene, also an indigenous oil degrading bacteria found in crude oil contained water from the Yellow Sea in China (Lucas-Elio *et al.*, 2011).

The *Bacteroidetes* another phylum which has been reclassified due to advances in molecular techniques is known as *Cytophaga-Flavobacteria Bacteroidetes*, or *CFB* or vice versa (Kirchman, 2002) are all anaerobes, most notably found in the microflora of the human colon. *Cytophaga-Flavobacteria* is chemoorganotrophic and proficient in degrading various biopolymers such as cellulose, chitin, and pectin. Heterotrophic bacterium, which metabolises dissolved oxygen as one of the largest organic carbon sources in the biosphere (Kirchman, 2002). The majority of the consortia are discovered at Astrolabe reef and Maketu with only one genus found at the Tauranga sites.

However, the exact gauge of resilience or the stable state of these microbial communities was beyond the scope of this project. Had regular microbial monitoring been undertaken in the months after the Rena oil spill, a temporal survey

of primary colonisers and successional species could give valuable data on bioremediation capabilities.

4.4 Conclusion

The dynamics of the in-situ bacterial communities and their responses to environmental stimuli such as increased nutrients and oil contamination revealed significant differences between impacted and no impacted sites. Relative abundances of *Gamma, Alpha Proteobacteria* and CFB were more abundant at the Astrolabe reef and Maketu sites, which was the grounding location and the first coastal settlement to be in impacted by the *Rena* oil spill, these findings support previous research by (Kostka *et al.*, 2011). Other genera associated with anthropogenic stressors were the cyanobacterial and chloroplast or Eukaryota genera which were quite dominant at all sites. Although Tauranga is a major port of New Zealand the heterogeneity of the bacterial communities was significantly lower than the other two sites, this does reflect the current and historical alterations at this site. While the character of the information is observational, site dissimilarities were evident between all sites, except for two, T2 and M1 which were significantly similar to any other sites. From variables and elements measured correlations could not empirically identify why these two sites were so similar, however, variables which could have correlated these similarities may not have been surveyed such as O_2, NH_4, NO_2 or PO_4 or TOC.

From the results a bias was observed with the weighting of relative abundance being identified as chloroplast, showing up as cyanobacteria in the phyla analysis. On further examination at the genera level, they were revealed to be a variety of red and green *Rhodophyta* Figure 18. As the Eukaryota were also the algal substrate utilised as a mechanism for this research, these results are not surprising.

Highlighted in this study was the inconsistency between taxonomic identification and molecular techniques, the specific example in this study being the *Lyngbya sp.* which appeared at Maketu in microscopy, but not in molecular analysis (Paerl *et al.*, 2002, 2014). However, the discrepancy here could also be attributed to sampling or PCR primer bias. While the debate about identification and its physical presence has been going since molecular developments, these are not the only questions around prejudice in scientific techniques. As an example to avoid bias in molecular techniques triplicate pooling of samples is used as a mechanism to overcome it in analysis procedures as illustrated by Archer *et al.* (2014), Webster and Negri (2006) and Wood *et al.* (2008). However, further developments in these areas are still required.

The inference of known bacterial ecophysiology was identified in their functional characteristics, which indicate the bioremediation capabilities of the consortia found at the Astrolabe reef and Maketu sites, and interestingly the genera *Roseovarius* indicating successional recovery of the communities at Astrolabe reef, two years on. Another microbe of interest was the *Shewanella* with its bioremediation capabilities of organic contaminants and possibly as a byproduct of its functionality, it produces a tetrodotoxin, a neurotoxin.

What has been most fascinating is that although Maketu has been stressed by anthropogenic alteration and landuse, for over 70 years the assemblages at Astrolabe reef were very similar in composition, suggesting that temporal stressors at Maketu did not show a significant difference from the one off pollutant event of the *Rena* Oil Spill. Of note was the Maketu control site consortium which was almost identical in composition to any of the Astrolabe reef sites composition SI Table 2.

Due to the selection of sites and sample size it is difficult to empirically ascertain how representative these results are of the studied areas, however, this study has now provided as a baseline mapping of taxonomic composition and phylogenic diversity of the differences in the communities and indications of factors of influence.

In summary this work underscores the experimental mapping of bacterial communities as a means of collating data to inform ecosystem restorative ecology paradigms.

Chapter 5

Conclusion

Microbial community consortium are previously unrecorded in the Bay of Plenty area. Anthropogenic stressors are considered to be the main sources of pollutants and contamination in the marine biome, one known example of nature's response are algal blooms (Paerl *et al.*, 2002; Paerl & Huisman, 2009). Algal substrates encourage primary colonising microbial communities which signal invertebrate larval settlement and aid in establishing higher trophic web recruitment and colonisation (Webster & Negri 2006).

In this Master's project spatial mapping of these communities was successfully completed at the selected sites, with statistical data and observational analysis illuminating some interesting findings.

Impacted sites showed higher heterogeneity than non-impacted sites, with bacterial community diversity more abundant at Maketu and Astrolabe reef sites than at Tauranga Harbour. With the exception of two sites one at Tauranga and one at Maketu, why these two sites were so similar could not conclusively be identified in the scope of this research.

The univariate analysis indicates that environmental variables did correlate to influencing bacterial communities, most influential was DO and SPC conductivity. Elemental influence also correlated as highly probable drivers of community assemblages, and each site was influenced by a differing metal or element. Therefore elemental consortia may play a bigger role in influencing communities than any isolated elements investigated in this study. Therefore future research in

the area of elemental consortia and its influence over bacterial community's in-situ may reveal insights lab based studies are not currently detecting, in regards to bioremediation, bioenergy mining and nutrient bacterial processing.

The techniques used from sample collections through to processing and analysing all come with their own bias, and precautions are taken for example, triplicate pooling of PCR amplicons to overcome bias, however no processes are empirically protected. As with the yield quality of DNA, several variations of clean up protocols were trialled in order to achieve a higher quality DNA product within the PCR assays, and ultimately a better quality DNA yield was achieved.

Other considerations within the processing of samples was 'primer appropriateness', and the target region the optimal for analysis in this thesis. From the trials it was identified that specific cyanobacterial primers did not appear to capture cyanobacterial diversity in its entirety. Therefore a bacterial primer was deemed more appropriate, however upon closer investigation of the OTUs at class and genera level, the original primer may have indeed shown a truer representation of cyanobacteria at the selected sites. Overall the sites revealed very low cyanobacterial populations which challenges the original assumptions made for this research, that cyanobacteria are abundant at the selected sites. The results prove that this is not the case, the dominate cyanobacterial OTUs were in fact heavily biased to Eukaryota, the substrate to which the research premise was associated to.

The challenge here is how this data can inform, habitat restoration programs and biogeochemical energy and mining prospecting. Data such as this catalogue with its taxonomic identification and diversity profile can potentially map future sources of biochemical resources.

Communities which have been sequenced and functional potential identified can make future aquaculture and biogeochemical energy research or prospecting much easier to eliminate or focus future expeditions at these sites. Future mapping of more of these areas in the Bay of Plenty can built a more comprehensive library for upcoming use.

Other potential opportunities are using this data to inform district or regional council environmental policies, using the qualities, these communities' possess to predict habitats shifts and earmark milestone recovery achievements. Signal bacterial consortium can indicate anthropogenic pressures which are pushing a habitat or community over a threshold, into a collapse or a shift in its stable state, to a new state. These kinds of shifts are visibly seen as algal and diatoms blooms, whereas bacterial consortia monitoring could indicate community shifts prior to the visual cues, enabling predictions to mediate problems before they occur.

Cultural implications are now considered at every legislative level, from the RMA 1991 (NZ Government, 1991), to the '*Rena* recovery - Long term Environmental recovery plan' (MfE, 2012), in the latter document, a whole section is dedicated to measuring and considering the impact on Māuri. Seldom does the opportunity arise to explore these notions in a lateral sense, by contributing with contemporary knowledge and technology. Fusing with Mātauranga, here this study can contribute on the marine basal food web, information which can signal recolonisation of bacterial communities and how these communities may indicate the overall health of the particular marine biome (Astrolabe reef). Couple this data with the other scientific research on kaimoana, fisheries, water quality etc and whole ecosystem mapping pictures can be built to gauge current and future recovery stages. While

all indications are that Astrolabe reef is recovering, the implications of the *Rena* oil spill will resonate for some time yet.

While undertaking an analysis on the impact of the Mauri has not been done here, in the Bennette, (2015) report which did do an assessment on Māuri for the Maketu area, entails years of consultation, reassessment and refinement of the data to produce such a report, that was beyond the scope of this research. As the total area of impact, covered several different iwi (tribes), protocols and boundaries that such an assessment would have been difficult to embark on in a thesis context. However, it was essential to keep the context of impact across all dimensions, so that the data presented here does have avenues for various conduits.

The experimental mapping exercise undertaken for this research has defined what bacterial consortia exist at the selected sites and highlighted potential future research opportunities.

Appendices

Appendix 1: White Island data

Site Information

			Latitude	Longitude
Site 1	Site 1, WHI 36	2M	S37 31.015	E177 11.645
Site 2 - Champagne bay	044; WHI 18	1M	S37 31.190	E177 11.596
Site 3.1	031; WHI 1	-1M	S37 31.144	E177 11.678
Site 3.2	032; WHI 2	-2M	S37 31.177	E177 11.666
Site 3.3	033; WHI 3	-3M	S37 31.191	E177 11.646
Site 4 - Kiri Bay 1st dive on 5th	Site 4 Chris site	10.0 m - 30.0 m	S37 30.670	E177 11.280
Site 5 - scum off surface	floating scum	10.0 m max depth	S37 30.841	E177 10.178
Site 6 -	Site 6	10.0 m depth	S37 30.602	E177 11.062
Site 7 -	Site 7	10.0 m - 30.0 m	S37 32.184	E177 11.107
Site 8 - Work bay	Site 8 +Julien1	10.0 m max depth	S37 31.633	E177 11.480

ANOSIM results

Analysis of Similarities
One-Way - A

Resemblance worksheet
Name: Resem13
Data type: Similarity
Selection: All

Factors

Place	Name	Type	Levels
A	Factor 1	Unordered	4

Factor 1 levels
T = Tauranga
M = Maketu
W = White Island
A = Astrolabe Reef

Tests for differences between unordered Factor 1 groups
Global Test
Sample statistic (R): 0.208
Significance level of sample statistic: 1.4%
Number of permutations: 999 (Random sample from a large number)
Number of permuted statistics greater than or equal to R: 13

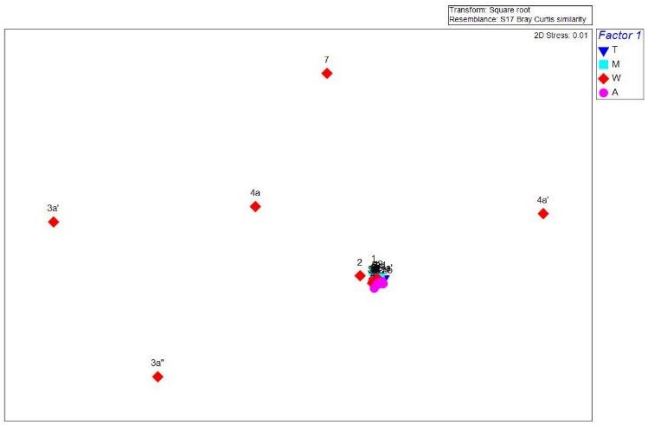
Pairwise Tests

Groups	R Statistic	Significance Level %	Possible Permutations	Actual Permutations	Number >= Observed
T, M	0.143	33.3	15	15	5
T, W	0.143	13.7	495	495	68

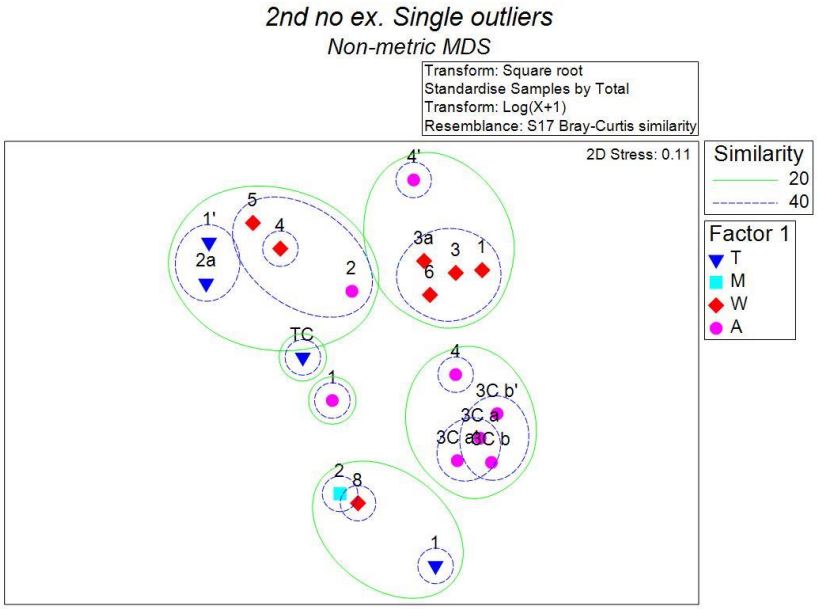
T, A	0.305	3.2	495	495	16
M, W	0.228	17.8	45	45	8
M, A	0.366	11.1	45	45	5
W, A	0.173	5.1	6435	999	50

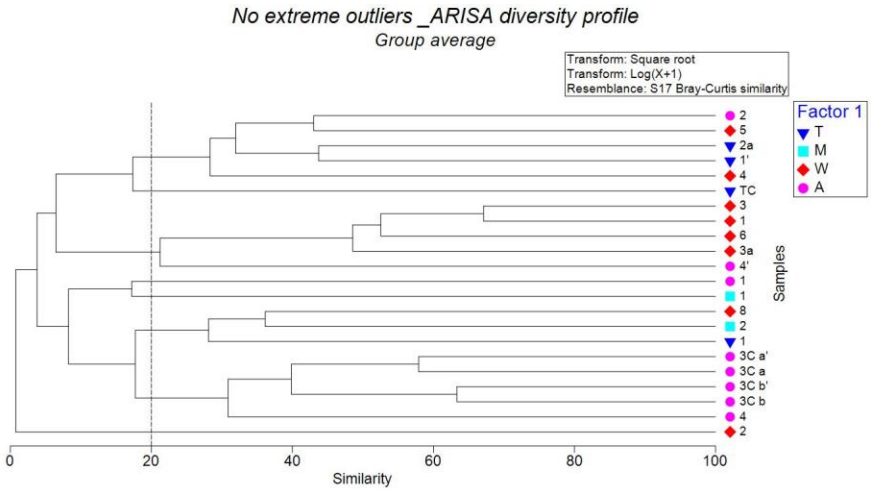
Outputs

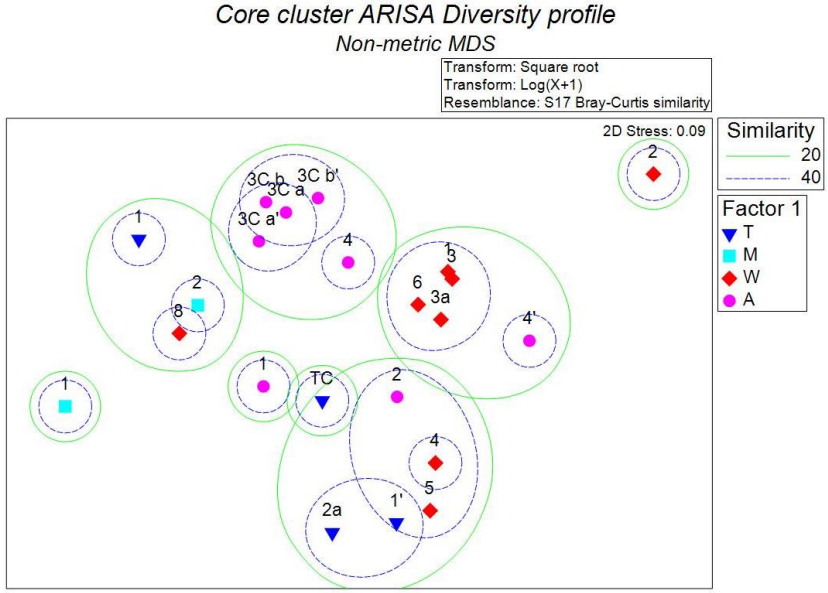
Second screen outliers 6th of January 2015



Second screen analysis with outliers




No extreme outliers _ARISA diversity profile
Group average
Transform: Square root
Transform: Log(X+1)
Resemblance: S17 Bray-Curtis similarity
Factor 1
T
M
W
A
2
5
2a
1'
4
TC
3
1
6
3a
4'
1
1
8
2
1
3C a'
3C a
3C b'
3C b
4
2
Samples
0
20
40
60
80
100
Similarity


Core cluster ARISA Diversity profile
Non-metric MDS
Transform: Square root
Transform: Log(X+1)
Resemblance: S17 Bray-Curtis similarity
2D Stress: 0.09
Similarity
20
40
Factor 1
T
M
W
A
2
3C b
3C b'
3C a
3C a'
4
1
3
6
3a
4'
1
2
8
1
1
TC
2
4
5
2a
1'

Supplementary information for manuscript

7.1 SI S 1 – PCR protocols

Polymerase Chain reaction (PCR) was performed on CTAB and MoBio DNA extractions in 25µL reactions containing 2ng/µL of DNA and 23 µL of master mix. Master mix compromised of MgC12 (50mM) 1.5µL, dNTPs (2µm) 2.5µL, 10X buffer 2.5µL, milliQ- H_2O (UVed) 12.05µL and Taq Platinum (5U/ µL) 0.2 µL. The region amplified was V4 of the 16S rRNA.

Amplification was performed using an initial denaturation step at 94˚C for 2 mins followed by 34 cycles of 94ºC, 20s; 55ºC, 15s; 72ºC, 1.5 mins and a final extension step of 72ºC for 7 mins. Triplicate PCR reactions were pooled together and diluted to 1:20 and stored at -20ºC until being sent to the University of Waikato DNA sequencing facility at the University of Waikato, New Zealand, for fragment size determination using an ABI 3130 xl sequencer (PE Applied Biosystems, Foster City, USA) as described by Sokol et al. (2013).

All PCR were run on a BIO RAD Peltier thermocycler. DNA concentrations and quality were measured by Nano drop ND-1000 at 260 nm and Qubit (Invitrogen fluorometer, V2) in ng/µL before ITS sequencing. Each PCR stage was visualised on diagnostic gels in a BIO RAD Gel tank to check PCR reactions, each sample was loaded alongside a ladder and a positive and negative for diagnosis. Gels were run on a 1% TAE agrose gel stained with 'SYBR Safe'(Invitrogen Ltd) at 75 volts, for 25mins as per protocols described by Garcia-Pichel (2008), Wood et al. (2008) and Archer et al. (2014) .

PCR protocol for the ITS DNA library has a denaturing stage at 94˚ at 3 min, then 94˚ at 45s, an annealing stage of 50˚ for 1min 30 times, an extension phase at 72˚ for 1.5min, another extension phase of 72˚ at 10 mins, to complete with a hold phase of 4˚ until removed and stored until use. The Solid Phase Reversible

Immobilisation (SPRI) protocol was used to purify PCR amplicons prior to sequencing.

PCR Trials

First CTAB trials revealed clear bands for 11 of the 22 samples, , 5 samples were visible but questionable, with 6 samples yielding weak or no bands SM Appendix 1-1. Samples 4 & 5 were added to the 5 samples with no bands for a second extraction trial, the results were mixed and known human error occurred SM Appendix 1-2. To enhance PCR reactions and limit inhibitors a reagent adjustment of 2µl of BSA was added to master mix, the results amplified all samples with exception of the previously identified difficult samples SM Appendix 1-3. No real difference was observed in yield or quality of DNA for the successful extracted samples. MoBio extraction protocols were trialed on the samples, which didn't respond to amended CTAB protocols with MV RENA samples SM Appendix 1-6, 1-7. DNA yields of previously difficult samples were less inhibited, with this protocol. Triplicates were run so that the successful reactions could be pooled and sent for ARISA. From the ARISA samples were selected for ITS processing.

ITS primer CYANO specific vs 16S rRNA was compared to see which set would yield the best results SM Appendix 1-4. When FASTA files were checked through the NCBI library, BLASTN check, the 16S rRNA gave greater hits of genuine DNA, than the CYANO primers, although the CYANO primers gave clear bands in the PCR. Another trial was done to check the cleaned (SPRI) DNA against the uncleaned DNA samples SM Appendix 1-5, the SPRI cleaned samples yielded clearer bands in the PCR reaction. Selected ITS PCR samples were run in triplicate and pooled for processing SM Appendix 1-8.

7.2 SI Table 1- Analysis summary and significance

Analysis type	Significance
Geochemistry	**Results missing Tauranga control data** **All data (Log x+1) transformed**
BEST analysis SI App. 3 Environmental variables	• DO, pH, spc cond, Temp all correlation equally @ .78 overall sites, with DO and Spc Cond consistently having higher affiliations with influence over sites than pH and Temp.
• ICP-MS and Env. Variables combined	• Correlations @ .98 are spc cond, B10, N15 Co59, As75, Cr 52 • PCA CHECKS FOR SITE INFLUENCES • B10 overall sites, exception T2, • N15 A4 sites only, • Co59 strong influence over A4, less on A1, A2 and all M sites, weak correlations on T1,T2 and A3C • As 75 high correlations at all M sites, A2, A1 and A3, nothing at T1, T2 and A4 • Cr 52 strong influence over all A & M sites, nothing over Tauranga
• ICP-MS Suite of 6 metals analysis	• Correlations at .98 are spc cond, ~~B~~, ~~N~~, P, S, Cu 65, Zn, ~~As~~, Se, Cd 11, Hg, Pb, Cl • PCA CHECKS FOR SITE INFLUENCES • P 31 influence over A3 only • S 34, highest influence over all A & M sites, less at T • Cu 65, highest influence over all M sites largest at M1 and A1, 2 less at A3, less at A4 sites, large influence at T2 and less at T1 • Cu 63, Significant influence at M3C, A3 and A4 sites, less at A3, A2 and M1, M2 sites. T1, T2 sites less influenced but still quite high. • Zn 68, extremely high at M3, then M1, M2, and all A sites, small influence over T sites. • Se 82 large influence at A4, A3 sites and M3, nothing at any others. • Cd 111 influence at A4 sites only • Hg 202, large influence at M3 and A3 sites only. • Pb 207, large influence at A4 sites smaller influence at all M sites and A1, 2 sites. Nothing at control and T sites. • Cl 35 huge influence at A4 sites only
RELATE ANALYSIS Supple. Appendix 1	**ENVIRONMENTAL VARIABLES** • Sample statistic (Rho): 0.935 • Significance level of sample statistic: 0.1% **ENVIRONMENTAL VARIABLES & ICP-MS** • Sample statistic (Rho): 0.585 • Significance level of sample statistic: 1.4%

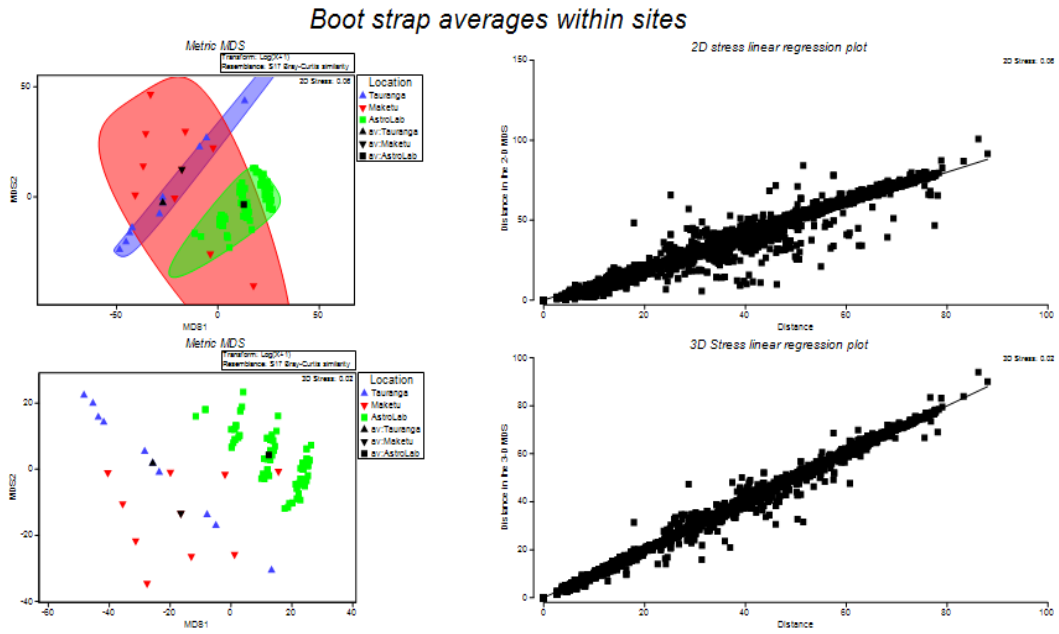
Similarity between sites and locations	
CLUSTER analysis – between sites Bray Curtis similarity Figure 5: chapter 4	**Abundant species** **Similarity of:** **T2 & M1 = Significance at 78%** **A1 & A2 = Significance at 60%** **A4.0 & A4.1 = Significance at 40%** **SIMPROF test : Resemblance** **Bray Curtis similarity** **Significance level 5%** **M1 & T2 – significance of 99.6%** **A1 & A2 - significance of 71.2%** **A4.0 & A4.1 – significance of 70.9%** **Rare species** **Significance level 5%** **SIMProf test : Resemblance** **Similarity of:** **T2 & M1 = significance at 60%** **SIMPROF test : Resemblance** **Bray Curtis similarity** **Significance level 5%** **M1 & T2 – significance of 98.6%** **A4.0 & A4.1 - significance of 3.9%**
ITS: Abundant species between locations ANOSIM – ONE way site similarities	• Pairwise test R value for A & M .549 at 1.8% • Pairwise test R value for A & T .579 at 3.6% • Pairwise test R value for T & M -0.074 at 30% • Overall level of significance = 2.1% • Sample stat 0.442
ITS: Rare species between locations ANOSIM – ONE way site similarities	• Pairwise test R value for A & M .846 at 1.8% • Pairwise test R value for A & T .744 at 1.8% • Pairwise test R value for T & M -0.111 at 30% • Overall level of significance = 0.5% • Sample stat 0.603
PCO cluster analysis Figure 6:Chapter 4	• Abundant – all control sites cluster , all Astrolabe reef sites cluster, then M1 & T2 cluster, then singles - T1, M2 • Rare – Maketu and Tauranga control site cluster, all astrolabe reef site cluster, same M1& T2 cluster, and same single clusters T1, M2.
Class level RDP site comparison	
ANOSIM – between locations	• Sample stat. @ 0.778 • Significance level 12.7%

	• R level statistics A4 & A1, A4 & A2 66.7% • All other site comparisons 33.3%
Bootstrap averages – between sites Similarity Figure 7: chapter 3	• Rho 0.992, Kruskal stress formula 1, minimum stress 0.01 • Stress dimension 2D = 0.06, 3D = 0.02 • Boot strap regions 95%
ARISA – CLUSTER similarity between sites Cophenetic correlation 0.95%	• A1 & A2 = 86.25% • T2 & M1 = 84.68% • A4.0 & A1, A2 (12) = 78.36% • T1 & A3 = 76.33% • T2, M1 & T1, A3 = 66.75% • A4.1 & A4.0, A1, A2 = 66.69% • TC & A4.0, A4.1, A1, A2 = 54.92% • M2 & TC, A4.0, A4.1, A1, A2 = 53.05% • MC & T2, M1, T1, A3 = 34.61%
ARISA – MDS similarity between sites Figure 7: Chapter 4 To reveal the monotonic relationship between dissimilarities of the sites, and the Euclidean distance between them, it locates the low dimensional space between them. 2D and 3D stress gradients. Stress being a dimensionless quality, of distances which are relative not absolute. 3D ordination is a satisfactory representation to consider the true dimensionality of the data.	• Clusters C1 = A4, A3 and M2 outside the 20% highly similar • C2 = A2 at 20% with T1, T2 then at T1, T2 at 40% to each other highly similar • M1, A1 and TC not similar to any other, distinctively not similar to any other site. • Kruskal formula : 1, min stress 0.01 • Dim stress 2D = 0.06 and 3D = 0.02 •
SIMPER – analysis between rare and abundant species at OTU level between locations Bray Curtis similarity – cut off for low contribution at 90%	Abundant species • Astrolabe average similarity = 26.80 • Maketu average similarity = 3.05 • Tauranga average similarity = 6.85 • Astrolabe & Maketu average dissimilarity = 92.82 • Astrolabe & Tauranga average dissimilarity = 94.06 • Maketu & Tauranga average dissimilarity = 87.03 Rare • Astrolabe average similarity = 32.92 • Maketu average similarity = 7.94 • Tauranga average similarity = 4.43 • Astrolabe & Maketu average dissimilarity = 91.32 • Astrolabe & Tauranga average dissimilarity = 95.10 • Maketu & Tauranga average dissimilarity = 89.80

7.3 SI Table 2 – Dominate bacterial consortia components

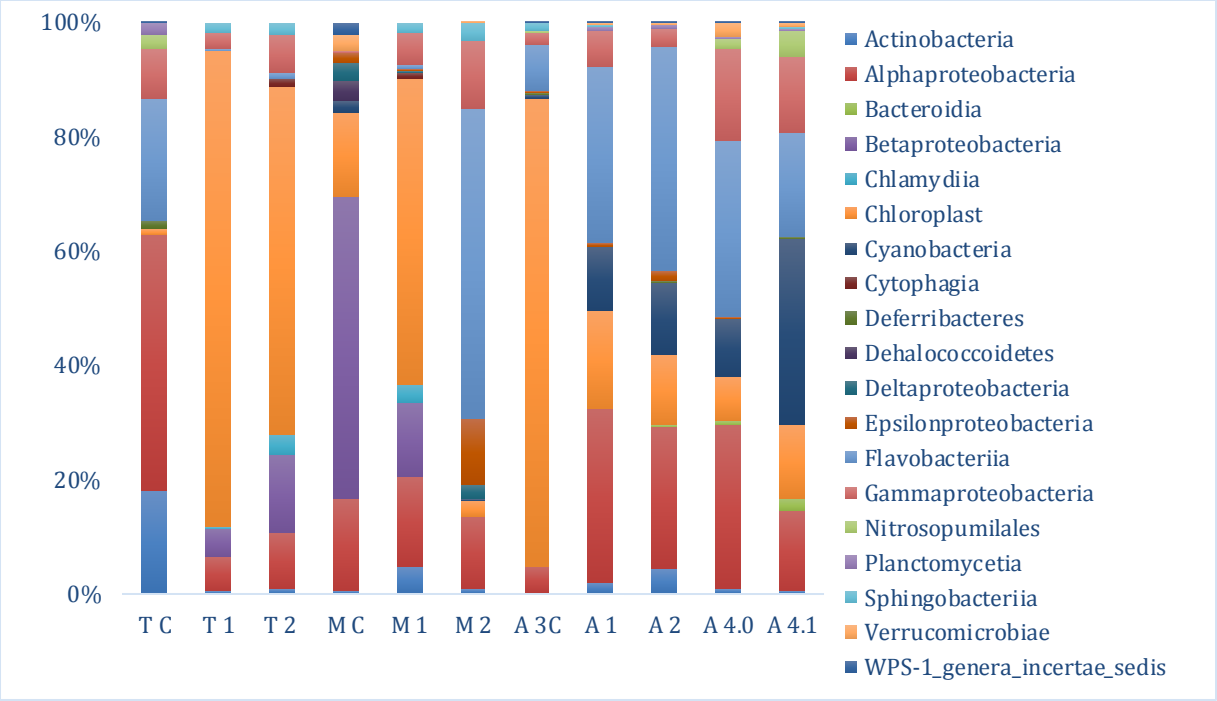
	M1	M2	MC	T1	T2	TC	A1	A2	A3C	A4.0	A4.1
Alpha	Hyphomonas	Methylobacterium	Andersenieila	Erythrobacter	Hyphomonas	Hyphomonas	Jannaschia	Hyphomonas	Hyphomonas	Jannaschia	Hyphomonas
	Andersenieila	Methylobacterium	Hyphomonas	Ruegeria	Sphingomonas	Roseovarius	Hyphomonas	Roseovarius	Roseovarius	Hyphomonas	Roseovarius
	Sphingomonas	Hyphomonas		Hyphomonas		Andersenieila	Roseovarius	Andersenieila	Andersenieila	Roseovarius	Andersenieila
		Andersenieila					Andersenieila			Andersenieila	
		Sphingomonas									
Beta	Methylophilus	Ralstonia	Methylophilus		Methylophilus		Methylophilus				
		Ralstonia									
		Ralstonia									
		Methylophilus									
Gamma		Marinomonas	Alteromonas				Marinomonas		Hallea	Marinomonas	Hallea
		Hallea	Hallea				Alteromonas		Shewanella	Hallea	
			Alteromonas				Hallea			Alteromonas	
							Alteromonas			Shewanella	
							Shewanella				
Delta	Desulfopila	Desulfopila	Desulfopila								
Bacteroidetes											
Flavobacteriia/Cytophagia											
	Flavobacterium	Tenacibaculum	Bizionia	Flavobacterium	Flavobacterium	Bizionia	Bizionia	Bizionia	Bizionia	Bizionia	Bizionia
		Tenacibaculum	Aquimarina				Aquimarina	Aquimarina	Aquimarina	Aquimarina	Aquimarina
			Dokdonia				Dokdonia	Dokdonia	Tenacibaculum	Tenacibaculum	Tenacibaculum
			Tenacibaculum			Tenacibaculum	Tenacibaculum	Tenacibaculum	Tenacibaculum	Tenacibaculum	Tenacibaculum
			Tenacibaculum			Maritimimonas	Tenacibaculum	Tenacibaculum	Maritimimonas	Actibacter	Maritimimonas
			Actibacter				Actibacter	Actibacter	Aquimarina	Maritimimonas	Aquimarina
			Aquimarina				Maritimimonas	Maritimimonas		Aquimarina	
							Aquimarina	Aquimarina			
Epsilonproteobacteria											
	Sulfurovum	Sulfurovum	Sulfurovum				Sulfurovum	Sulfurovum	Sulfurovum	Sulfurovum	Sulfurovum
	Sulfurovum	Sulfurovum	Sulfurovum				Sulfurovum	Sulfurovum			
Cyanobacteria											
		Prochlorococcus	Prochlorococcus	Chroococcales, synechococcus	Chroococcales, synechococcus	Chroococcales,	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta
				Oscillatoriales	Oscillatoriales	Oscillatoriales					
		Rhodophyta	Rhodophyta	cryptic	cryptic	cryptic	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta	Rhodophyta
		Prochlorococcus	Prochlorococcus	Oscillatoriales	Oscillatoriales	Oscillatoriales	Lyngbya	Rhodophyta	Prochlorococcus	Prochlorococcus	Rhodophyta
				cryptic	cryptic	cryptic					
				Oscillatoriales	Oscillatoriales	Oscillatoriales	Prochlorococcus	Lyngbya	Rhodophyta	Rhodophyta	Prochlorococcus
				cryptic	cryptic	cryptic					
						Oscillatoriales	Rhodophyta	Prochlorococcus	Chroococcales, synechococcus	Chroococcales, synechococcus	Rhodophyta
						cryptic	Chroococcales, synechococcus	Rhodophyta	Prochlorococcus	Prochlorococcus	Chroococcales, synechococcus
							Prochlorococcus	Chroococcales, synechococcus			
								Prochlorococcus			

7.4 SI Fig. 1 – Bootstrap plots



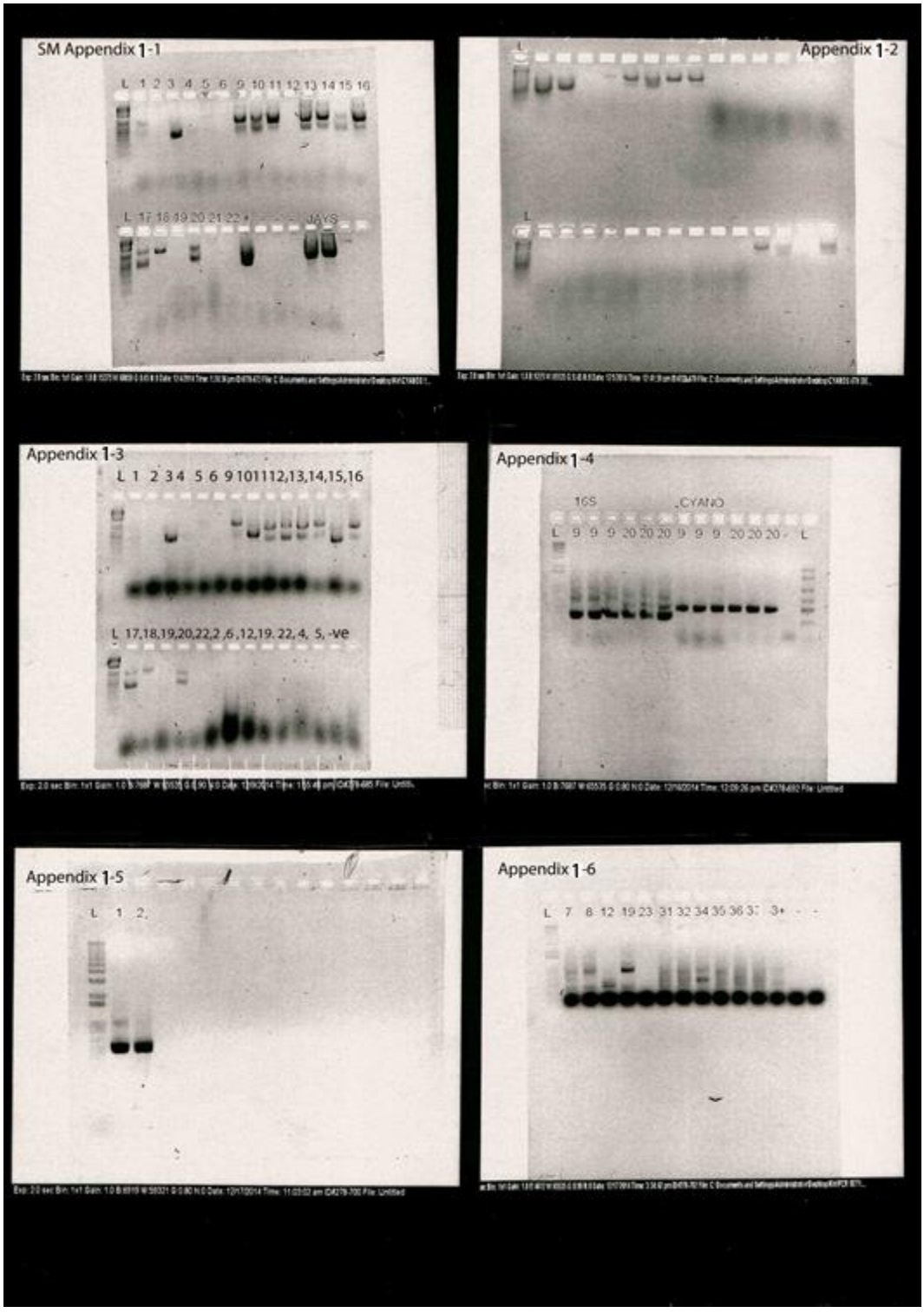
Boot strap plots, non metric MDS bray curti, plots shwoing class level relatedness. Linear regression 3D and 2D plots indicating dimensional stress gradients.

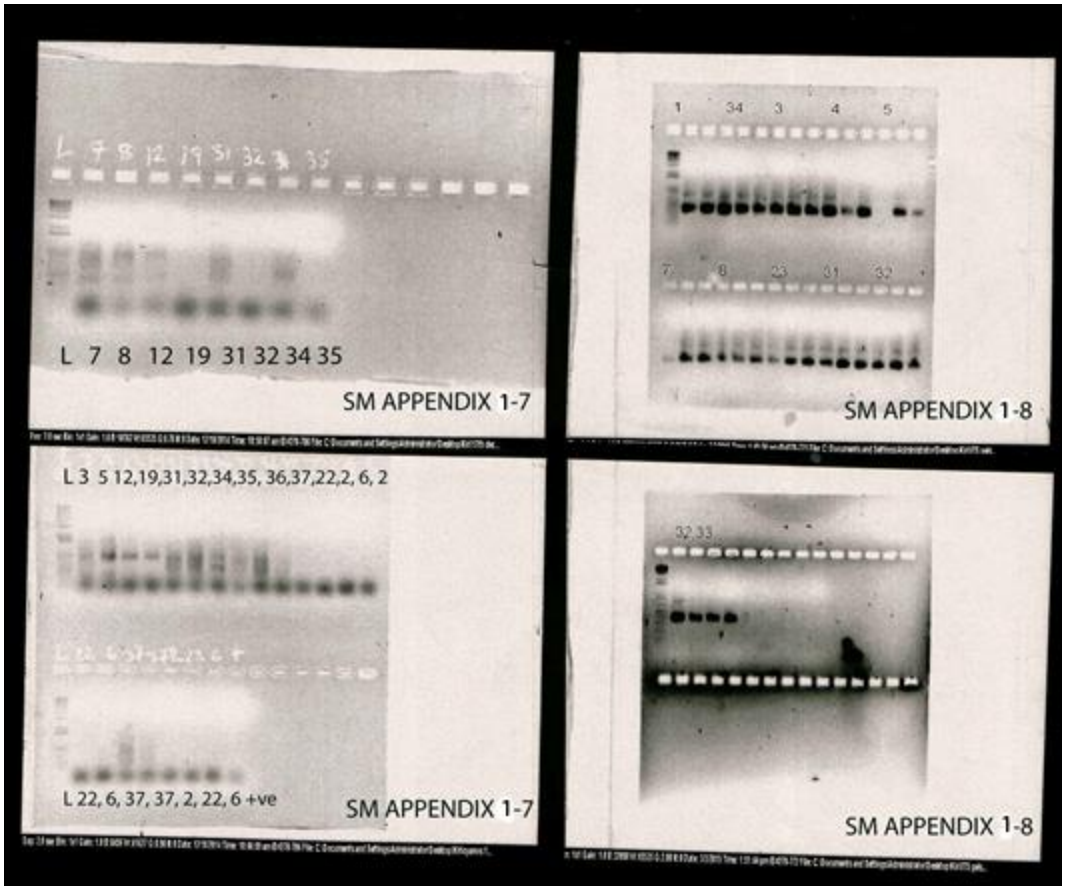
7.5 SI Fig. 2 – Class level community structure at each site



Key T = Tauranga, M = Maketu, A = Astrolabe reef, numbers are site (impacted) and C = control sites.

7.6 SI App. 1 – PCR inhibition trial gels




L 7 8 12 19 31 32 34 35
SM APPENDIX 1-7
1 34 3 4 5
7 8 23 31 32
SM APPENDIX 1-8
L 3 5 12,19,31,32,34,35, 36,37,22,2, 6, 2
L 22, 6, 37, 37, 2, 22, 6 +ve
SM APPENDIX 1-7
32,33
SM APPENDIX 1-8

7.7 SI App. 2 – Ion Torrent express bars and codes

Work flow for Ion Torrent suite.

1. Quanititate eDNA with Qubit fluromoeter HS assay

Master mix :

N Samples + 2 standards * (198µL buffer + 1µL Qubit reagent)
Prepare the following in 1.5ml Eppendorf tubes

Standards
10µL each standard into seperate tubes + 190µL master mix = total volume 200µL

Samples
2µL DNA + 198µL Mastermix = total volume 200µL

2. Amplify V4 Region of 16S with PCR

Amplify DNA with fusion rpimers, adding 2ng DNA per reaction. The sequencing primers are in **Error! Reference source not found.**. PCR reactions are performed n triplicate and subsequently pooled. Primer sequences are as follows:

The forward primer consisted of, the adaptor **library key** + IonXpress barcode + Barcode adapter + F seq Primer 5'

CCATCTCATCCCTGCGTGTCTCCGAC**TCAG**XXXXXXXXXXX

GATGTGCCAGCMGCCGCGGTAA '3

reverse primer ITR-V4 which consisted of P1 + R Seq primer

5'CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTA

CHVGGGTWTCTAAT '3

Ion Torrent express bars codes

ITS Data sheet									Ro's columns					
PCR products					ITS products							300 pg/µL dilution Calcs.		
Sample Name	UOW Primer No.	Ion Express barcode No.	Assay conc. ng/mL 6/3/15	Stock Conc. ng/µL 6/3/15	Assay Conc. ng/mL 9/3/15	Stock conc. ng/µL 9/3/13	Stock averages	pg	divided by 300	pg 9/3/15	divided by 300 9/3/15	(DF)	DNA µL qty	1XTE µL qty
KR1	52	24	2.58	0.516	18.567	3.71	2.113	2113	7.04333	3710	12.3667	12.37	1	11.367
KR3	55	77	41.8	8.35	39.3	7.86	8.105	8105	27.0167	7860	26.2	26.2	1	25.2
KR4	56	81	19.1	3.82	17.2	3.45	3.635	3635	12.1167	3450	11.5	11.5	1	10.5
KR5	57	82	13.7	2.75	13.1	2.62	2.685	2685	8.95	2620	8.73333	8.7	1	7.7
KR6	51	23	18.6	3.72	13.4	2.67	3.195	3195	10.65	2670	8.9	8.9	1	7.9
KR7	58	85	17.6	3.51	23	4.59	4.05	4050	13.5	4590	15.3	15.3	1	14.3
KR8	59	86	43.1	6.83	28.7 av.	5.74 av.	6.83	6830	22.7667	5740	19.1333	19.1	1	18.1
KR23	60	96	16.4	3.27	11.5	2.31	2.79	2790	9.3	2310	7.7	7.7	1	6.7
KR31	61	1	36.1	7.23	33.3	6.66	6.945	6945	23.15	6660	22.2	22.2	1	21.2
KR33	63	18	23.8	4.75	19.4	3.89	4.32	4320	14.4	3890	12.9667	12.9	1	11.9
KR34	49	5	26	5.2	17.8	3.55	4.375	4375	14.5833	3550	11.8333	11.8	1	10.8

7.8 SI App. 3 – PCA plots & BEST, RELATE analysis

BEST – Environmental variables

Biota and/or Environment matching

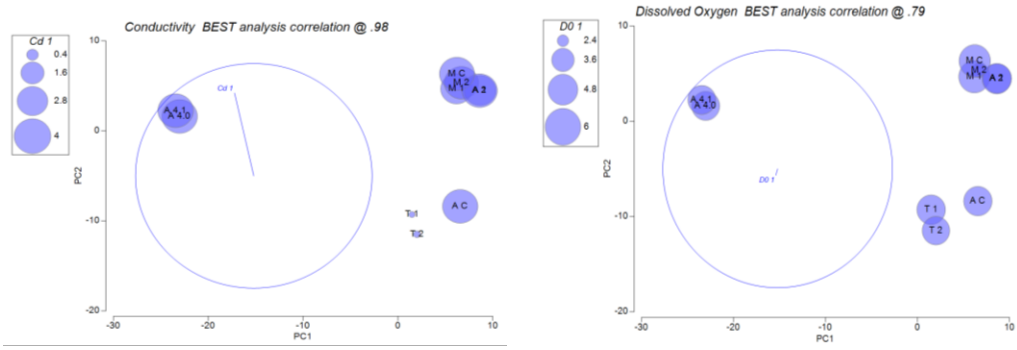
Data worksheet
Name: Data3
Data type: Environmental

Parameters
Rank correlation method: Spearman
Method: BIOENV
Maximum number of variables: 5
Resemblance:
Analyse between: Samples
Resemblance measure: D1 Euclidean distance

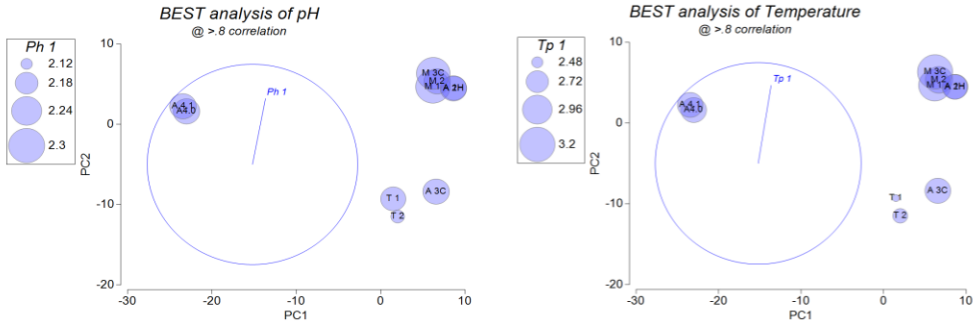
Variables
1 pH
2 Temp
3 Cond
4 DO

Global Test
Sample statistic (Rho): 0.793
Significance level of sample statistic: 1%
Number of permutations: 99 (Random sample)
Number of permuted statistics greater than or equal to Rho: 0
Best results

No.Vars	Corr.	Selections
4	0.793	All
3	0.791	1,3,4
3	0.789	2-4
2	0.788	3,4
3	0.728	1-3
2	0.724	1,3
1	0.715	3
2	0.712	2,3
1	0.604	2
2	0.417	1,2



Overal influence over sites for Conductivity and Dissolved oxygen



Overal influence over sites for pH and Temperature

BEST – combined environmental variables

Biota and/or Environment matching

Resemblance worksheet
Name: Resem4
Data type: Similarity

Parameters
Correlation method: Spearman rank
Method: BIOENV
Maximum number of variables: 5
Analyse between: Samples
Resemblance measure: D1 Euclidean distance

VARIABLES

Ph	Ph 1	Trial
Temp	Tp 1	Trial
Cond 1	Cd 1	Trial
D0	D0 1	Trial
B	B 10	Trial
N	N 15	Trial
Na	Na 23	Trial
Mg	Mg 24	Trial
Al	Al 27	Trial
Si	Si 28	Trial
P	P 31	Trial
S	S 34	Trial
Cl	Cl 35	Trial
K	K 39	Trial
Ca	Ca 43	Trial
V	V 51	Trial
Cr	Cr 52	Trial
Fe	Fe 54	Trial
Mn	Mn 55	Trial
Co	Co 59	Trial
Ni	Ni 60	Trial
Cu 63	Cu 63	Trial
Cu 65	Cu 65	Trial
Zn	Zn 68	Trial
As	As 75	Trial
Se	Se 82	Trial
Br	Br 79	Trial
Sr	Sr 88	Trial
Rb	Rb 85	Trial
Zr	Zr 90	Trial
Mo	Mo 98	Trial

Ag	Ag 109	Trial
Cd 11	Cd 111	Trial
Sn	Sn 118	Trial
Ba	Ba 137	Trial
La	La 139	Trial
Hg	Hg 202	Trial
Tl	Tl 205	Trial
Pb	Pb 207	Trial
U	U 238	Trial
Fe-	Fe-NH3 57	Trial
Co-	Co-NH3 59	Trial

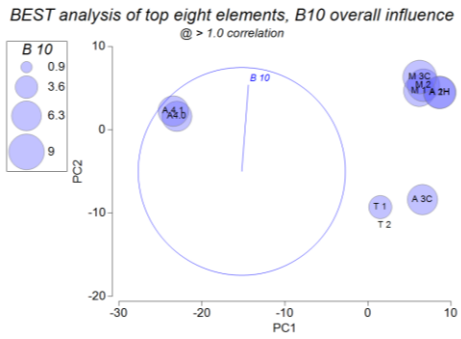
Best result for each number of variables

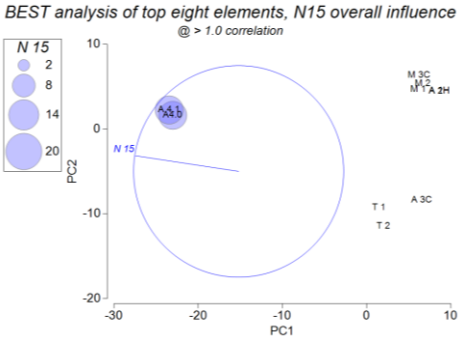
No. Vars	Corr.	Selections
1	0.909	K
2	0.947	B ,N
3	0.956	Cr,Co ,Hg
4	0.963	Cd 1,N ,S ,Sn
5	0.968	Cd 1,B ,N ,Co ,As

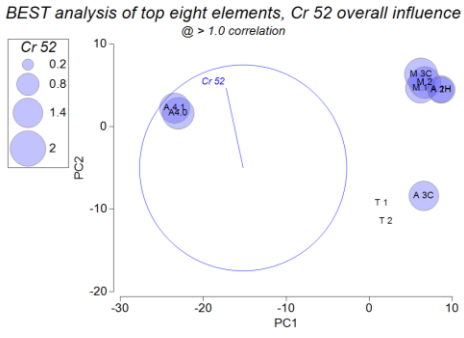
Best results

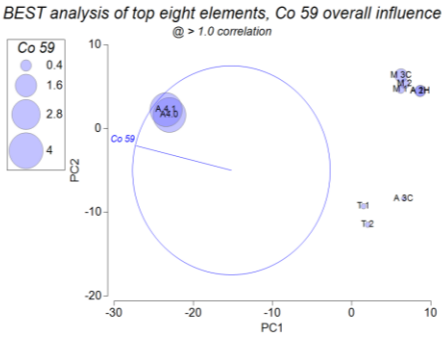
No. Vars	Corr.	Selections
5	0.968	Cd 1,B ,N ,Co ,As
5	0.968	Cd 1,B ,Cl,Co ,As
5	0.967	Cd 1,B ,N ,As,Hg
5	0.967	Cd 1,B ,N ,Cr,As
5	0.967	Cd 1,B ,Cl,Cr,As
5	0.966	Cd 1,B ,N ,S ,Se
5	0.966	Cd 1,N ,S ,Co ,Sn
5	0.966	Cd 1,S ,Cl,Co ,Sn
5	0.966	Cd 1,B ,N ,As,Mo
5	0.966	Cd 1,B ,Cl,As,Mo

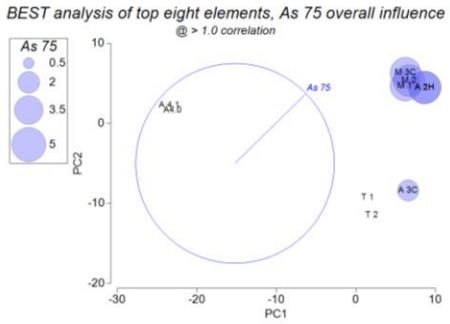
Elements overall site Influence











BEST – Suite of 6

Biota and/or Environment matching

Resemblance worksheet

Name: Resem4

Data type: Similarity

Parameters

Correlation method: Spearman rank

Method: BIOENV

Maximum number of variables: 6

Analyse between: Samples

Resemblance measure: D1 Euclidean distance

VARIABLES

Ph	Ph 1	Trial
Temp	Tp 1	Trial
Cond 1	Cd 1	Trial
D0	D0 1	Trial
B	B 10	Trial
N	N 15	Trial
Na	Na 23	Trial
Mg	Mg 24	Trial
Al	Al 27	Trial
Si	Si 28	Trial
P	P 31	Trial
S	S 34	Trial
Cl	Cl 35	Trial
K	K 39	Trial
Ca	Ca 43	Trial
V	V 51	Trial
Cr	Cr 52	Trial
Fe	Fe 54	Trial
Mn	Mn 55	Trial
Co	Co 59	Trial
Ni	Ni 60	Trial
Cu 63	Cu 63	Trial
Cu 65	Cu 65	Include
Zn	Zn 68	Include
As	As 75	Include
Se	Se 82	Trial
Br	Br 79	Trial
Sr	Sr 88	Trial
Rb	Rb 85	Trial
Zr	Zr 90	Trial
Mo	Mo 98	Trial
Ag	Ag 109	Trial
Cd 11	Cd 111	Include
Sn	Sn 118	Trial
Ba	Ba 137	Trial
La	La 139	Trial
Hg	Hg 202	Include

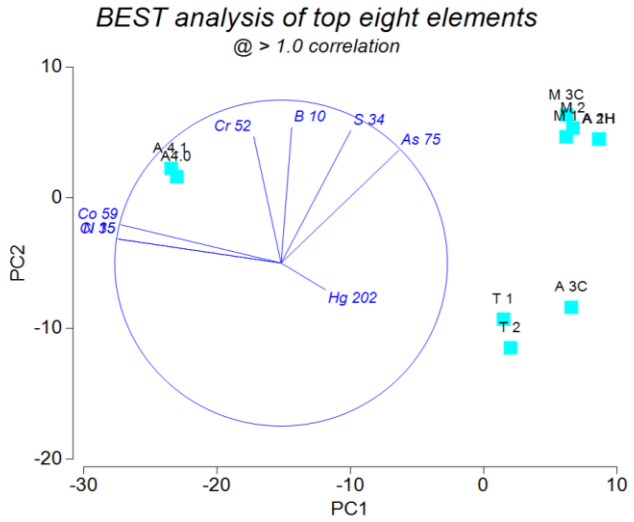
Tl	Tl 205	Trial
Pb	Pb 207	Include
U	U 238	Trial
Fe-	Fe-NH3 57	Trial
Co-	Co-NH3 59	Trial

Best result for each number of variables

No.Vars	Corr.	Selections
7	0.909	Mg,Cu 65,Zn,As,Cd 11,Hg,Pb
8	0.942	B ,N ,Cu 65,Zn,As,Cd 11,Hg,Pb
9	0.956	B ,N ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
10	0.969	Cd 1,B ,N ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
11	0.976	Cd 1,B ,N ,P ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
12	0.984	Cd 1,B ,N ,P ,S ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb

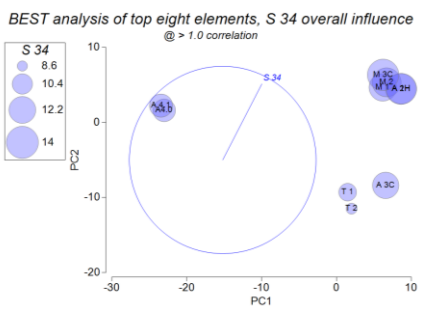
Best results

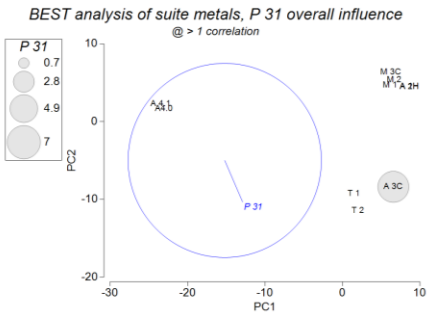
No.Vars	Corr.	Selections
12	0.984	Cd 1,B ,N ,P ,S ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
12	0.984	Cd 1,B ,P ,S ,Cl,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
12	0.978	Cd 1,B ,N ,P ,Cr,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
12	0.978	Cd 1,B ,P ,Cl,Cr,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
12	0.977	Cd 1,B ,N ,P ,Cu 65,Zn,As,Se,Mo,Cd 11,Hg,Pb
12	0.977	Cd 1,B ,P ,Cl,Cu 65,Zn,As,Se,Mo,Cd 11,Hg,Pb
12	0.976	Cd 1,B ,N ,P ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb,U
12	0.976	Cd 1,B ,P ,Cl,Cu 65,Zn,As,Se,Cd 11,Hg,Pb,U
11	0.976	Cd 1,B ,N ,P ,Cu 65,Zn,As,Se,Cd 11,Hg,Pb
11	0.976	Cd 1,B ,P ,Cl,Cu 65,Zn,As,Se,Cd 11,Hg,Pb

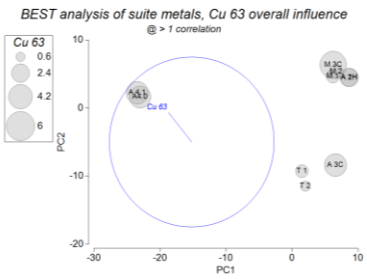


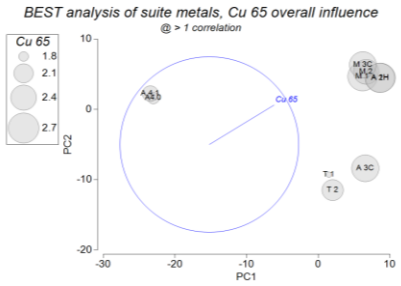
Top 8 elements of influence

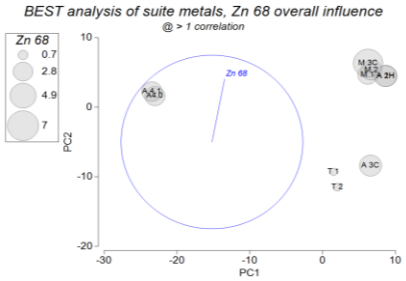
Site influences

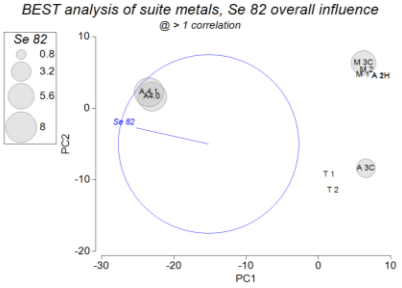


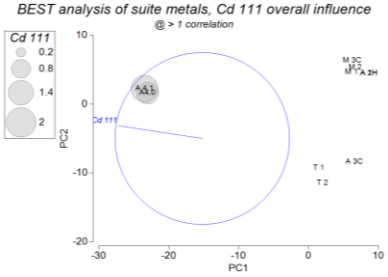


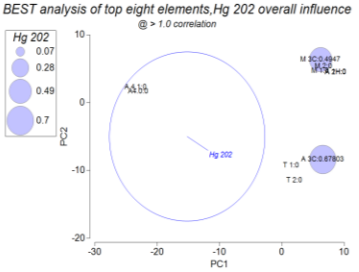


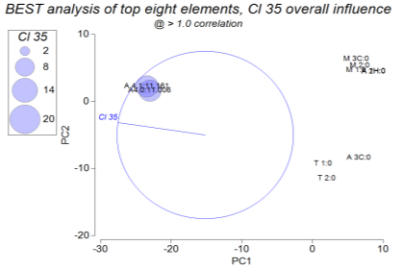


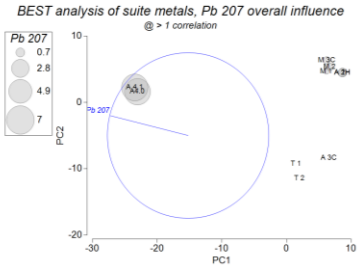












RELATE analysis 1 = Environmental variables ; Temp, pH, DO and Conductivity

Testing matched resemblance matrices
Data type: Similarity
Selection: All
Secondary data: Resemblance/model matrix

Parameters
Rank correlation method: Spearman

Sample statistic (Rho): 0.935
Significance level of sample statistic: 0.1 %
Number of permutations: 999
Number of permuted statistics greater than or equal to Rho: 0

RELATE analysis 2 = Environmental variables combined with ICP-MS

Testing matched resemblance matrices
Data type: Similarity
Selection: All
Secondary data: Resemblance/model matrix

Parameters
Rank correlation method: Spearman

Sample statistic (Rho): 0.585
Significance level of sample statistic: 1.4 %
Number of permutations: 999
Number of permuted statistics greater than or equal to Rho: 13

7.9 SI App. 4 – Dominate OUT results, community genera data

OTUId	Kiri31_01	Kiri34_05	Kiri33_18	Kiri06_23	Kiri01_24	Kiri03_77	Kiri04_8_1	Kiri05_82	Kiri07_85	Kiri08_86	Kiri23_96	Filter	No	Domain	DominCon	Phylum	PhylumCo	Class	ClassCon	Order	OrderConf	Family	FamilyC	Genus
OTU_51	0.00%	0.00%	0.00%	0.16%	0.04%	58.36%	0.23%	0.70%	0.00%	0.00%	0.00%	59.50%	16S	Bacteria	99%	Cyanobact	92%	Chloropla	88%	Chloroplas	88%	Bacillariophyta	86%	
OTU_1	0.00%	0.00%	0.00%	0.00%	0.01%	4.86%	28.11%	20.19%	0.00%	0.00%	0.00%	53.17%		Bacteria	100%	Cyanobact	99%	Chloropla	92%	Chloroplas	92%	Bacillariophyta	86%	
OTU_2	41.59%	1.46%	0.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	43.12%	16S	Bacteria	100%	Cyanobact	89%	Chloropla	75%	Chloroplas	75%	Bacillariophyta	35%	
OTU_8	0.07%	0.03%	0.02%	8.91%	0.01%	0.00%	0.05%	0.06%	0.84%	0.86%	0.13%	10.97%	16S	Bacteria	100%	Cyanobact	99%	Chloropla	99%	Chloroplas	99%	Bacillariophyta	99%	
OTU_10	0.68%	1.05%	0.88%	0.24%	0.19%	0.00%	0.00%	0.00%	2.31%	1.88%	0.53%	7.77%	16S	Bacteria	100%	Cyanobact	100%	Chloropla	100%	Chloroplas	100%	Bacillariophyta	100%	
OTU_4	0.00%	5.36%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.03%	0.00%	5.39%	16S	Bacteria	100%	Cyanobact	78%	Cyanobac	28%	Family VIII	20%	GpVIII	20%	
OTU_5	0.00%	4.95%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	4.95%	16S	Bacteria	100%	Cyanobact	93%	Cyanobac	59%	Family IX	22%	GpIX	22%	
OTU_3583	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	2.38%	1.78%	0.00%	0.00%	0.00%	4.16%	16S	Bacteria	99%	Cyanobact	93%	Chloropla	88%	Chloroplas	88%	Bacillariophyta	75%	
OTU_70	0.15%	0.20%	0.16%	0.02%	0.00%	0.61%	1.54%	1.14%	0.11%	0.03%	0.04%	4.00%	16S	Bacteria	100%	Cyanobact	100%	Chloropla	100%	Chloroplas	100%	Bacillariophyta	100%	
OTU_56	0.06%	0.35%	0.52%	0.84%	0.00%	0.00%	0.00%	0.00%	0.29%	0.25%	0.04%	2.35%	16S	Bacteria	100%	Cyanobact	100%	Cyanobac	100%	Family II	100%	GpIIa	100%	
OTU_23	0.21%	0.76%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.27%	1.10%	0.00%	2.35%	16S	Bacteria	100%	Cyanobact	73%	Cyanobac	34%	Family VIII	23%	GpVIII	23%	
OTU_2360	0.53%	0.41%	0.26%	0.77%	0.00%	0.00%	0.02%	0.01%	0.07%	0.09%	0.11%	2.26%	16S	Bacteria	100%	Cyanobact	100%	Chloropla	100%	Chloroplas	100%	Bacillariophyta	100%	
OTU_29	0.00%	0.01%	2.06%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	2.07%	16S	Bacteria	100%	Cyanobact	84%	Cyanobac	34%	Family X	19%	GpX	19%	
OTU_77	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.77%	1.15%	0.00%	1.92%	16S	Bacteria	100%	Cyanobact	84%	Cyanobac	45%	Family VIII	34%	GpVIII	34%	
OTU_112	0.07%	0.01%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.46%	1.27%	0.00%	1.84%	16S	Bacteria	100%	Cyanobact	99%	Cyanobac	99%	Family IV	98%	GpIV	98%	
OTU_44	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.75%	0.06%	0.00%	1.81%	16S	Bacteria	100%	Cyanobact	100%	Cyanobac	100%	Family XIII	99%	GpXIII	99%	
OTU_7239	0.12%	0.27%	0.40%	0.00%	0.02%	0.00%	0.00%	0.00%	0.80%	0.06%	0.07%	1.74%	16S	Bacteria	100%	Cyanobact	100%	Chloropla	100%	Chloroplas	100%	Bacillariophyta	100%	
OTU_66	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.20%	0.20%	0.00%	1.40%	16S	Bacteria	100%	Cyanobact	92%	Chloropla	90%	Chloroplas	90%	Chlorophyta	84%	
OTU_10462	0.07%	0.70%	0.21%	0.04%	0.03%	0.00%	0.00%	0.00%	0.09%	0.01%	0.19%	1.34%	16S	Bacteria	100%	Cyanobact	100%	Chloropla	100%	Chloroplas	100%	Bacillariophyta	100%	
OTU_1817	1.21%	0.04%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	1.26%	16S	Bacteria	100%	Cyanobact	75%	Chloropla	65%	Chloroplas	65%	Bacillariophyta	48%	
OTU_94	0.00%	0.32%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.71%	0.00%	1.03%	16S	Bacteria	100%	Cyanobact	89%	Chloropla	49%	Chloroplas	49%	Chlorophyta	34%	
OTU_9132	0.03%	0.10%	0.15%	0.57%	0.00%	0.00%	0.00%	0.00%	0.06%	0.08%	0.03%	1.01%	16S	Bacteria	100%	Cyanobact	100%	Cyanobac	100%	Family II	100%	GpIIa	100%	
	44.80%	16.02%	4.75%		0.30%	63.83%	32.33%	23.88%	9.04%	7.78%	1.13%	203.85%												

Example of Cyano/chloroplast dominate OTU results.

Phlya breakdowns			
			NOTES
Sites present	Order	Genus	
Proteobacteria			
Alpha			
M2, OTU 12741	Rhizobiales	Methylobacterium	MEGA - 98% id match, 2e-122 E value Kato et al 2008, Kato et al 2005
T1, OTU 6	Sphingomonadales	Erythrobacter	99% id match, 8e-121 E value. Erythrobacter sp. Marine environ, California, MEGABLAST South China sea Xu et al 2010.
T1, OTU 16	Rhodobacterales	Ruegeria	100% id match, 2e-123 E value, strain, Cultured sponges bacterial diversity in Mediterranean sponges, Rio de jeaniero sponge and its associated hetertrophic bacterial community, marine environment - sponge symbiote. MEGA 99% 6e-127 E value, Seashore sand in Korea-Kim et al 2008
M2, OTU 9	Rhizobiales	Methylobacterium	99% id match, 8e-121 E value. Strain pure water, plants, rice from thailand and Japan and barley. Floating pumice microbial community. Freshwater isoalte- Kato et al 2008
A 4.0, A 1, OTU 19	Rhodobacterales	Jannaschia	99% id match, 8e-121 E value, Jannaschia helgolandensis strain, hydrothermal sediments of the south atlantic ocean, Chin and Japan strain from oceanography uni's,
ALL SITES, OTU 38	Caulobacterales	Hyphomonas	100% id match, uncultured alpha, from mediterranean sea, bio film from Canada, 100% marine bacteria from Israel MEGA - 97% id match 1e-118 E value Badger et al 2006, Weiner 2000
ALL A sites and TC, OTU 24	Rhodobacterales	Roseovarius	99% id match, 7e-122 E value, marine costal community spain, tidal flat in yellow sea korea, marine sponge China, MEGA 99% id match, 1e-128 E value, Yoon et al 2008
All A sites, all M sites and TC, OTU 63	Rhizobiales	Anderseniella	89% id match, 6e-122 E value Brettar et al 2007,
M2, M1, T2, OTU 41	Sphingomonadales	Sphingomonas	100% id match, 2e-123 E value, endophytic bacteria Turkey, MEGA 100% id, 3e-130 E value ; Mueller 1997, neutral mineral water - Lee et al 2001
Beta			
M2, OTU 3	Burkholderiales	Ralstonia	MEGA - 99% id match, 1E-124 E value, Coyne et al 2003, Anzai et al 2000
M2, T2, M1, MC, A1, OTU 34	Methylophilales	Methylophilus	Lapidus et al 2011, Kalyuzhnaya et al 2006, Doronina et al 2005
M2, OTU 2002	Burkholderiales	Ralstonia	MEGA 97% match, 2e-117, Coyne et al 2003, Anzai et al 2000
M2, OTU			

	A	B	D	F
22	**Gamma**			
23	A4.0, M2, A1, OTU 27	Oceanospirillales	Marinomonas	100% id match, 2e-123 E value, indigenous oil degrading bacteria in crude oil contaminated sea water of the yellow sea China, Usa Lucas- ellio 2014, MEGA 100% ID, 3e-130 E value, Lucas-ellio 2011 seagrass
24	MC, A1, OTU 21	Alteromonadales	Alteromonas	99% id match, 1e-119 E value, China marine lab, Potrugal biological dept. coral symbiotic algae calcify ex hospite in partnership with bacteria. Sediments from Indonesia, hydrothermal sediments of south atlantic ocean. MEGA 99% id match, 3e-125 E vlaue Yoon et al 2003
25	AC, A4.0, A4.1, A1, M2, MC, OTU 91	Alteromonadales	Halles	98% id match, 3e-120 E value, Maediterranean marine environ, Lucena et al 2010
26	A4.0, A1, MC, OTU 8480	Alteromonadales	Alteromonas	MEGA 99% id match, 2e-126 E value, Vandecandelaere et al 2008, Yoon et al 2003
27	AC, A4.0, A1,OTU 1007	Alteromonadales	Shewanella	MEGA 99% match, 2e-127 E value, Wang et al 2008, Yoon et al 2004
28				
29	**Delta**			
30	All M sites, with M2 largest, OTU 158	Desulfobacterales	Desulfopila	MEGA - 96% id match, 3e- 113 E value, Suzuki et al 2007 , Gittel et al 2008.
31				
32	**Bacteroidetes**			
33	**Flavobacteriia/Cytophagia**			
34	All A sites, TC, MC, OTU 28	Flavobacteriales	Bizionia	99% id Match, 7e-122 E value, Antartic marine habitat - Bowman & Nichols 2005, MEGA 99% 1e-128 E value, Bowman Nichols 2005
35	All A sites and MC, OTU 37	Flavobacteriales	Aquimarina	100% id match, 2e-123 E value, Intertidal coast of Portugal , gut contents of MUSSEL KOREA, Sediment Japan: Aquimarina macrocephali sp. Article Miyazaki 2010. MEGA 100% id match, 3e-130 E value - Park et al , 2012, and Miyazaki again
36	MC, A1, A2, OTU 22	Flavobacteriales	Dokdonia	100% id match, 2e-123 E value, aerobic bacteria from marine hydrothermal vents fields, rio grande rise region; south atlantic, sea water from korea, MEAGA 100% Match 3e-130 E value, Yoon et al 2005
37	All A sites, MC, TC, M2, OTU 5359	Flavobacteriales	Tenacibaculum	99% id match, 3e-125 E value Park and Yoon 2013, Heindl et al 2008
38	All A sites, MC, TC, M2, OTU 86	Flavobacteriales	Tenacibaculum	99% id match 6e-127 E value, Pinerio-Vidal et al 2008, Heindl et al 2008, Jung et al 2006.
38	All A sites, MC, TC, M2, OTU 86	Flavobacteriales	Tenacibaculum	99% id match 6e-127 E value, Pinerio-Vidal et al 2008, Heindl et al 2008, Jung et al 2006.
39	A4.0, A1, A2, MC, OTU 35	Flavobacteriales	Actibacter	100% id match, 8e-121 E value, Sediment tidal flat USA, Kim et al 2008. MEGA 99% id match, 6e-127 E value, Kim et al 2008
40	All A sites, TC, OTU 46	Flavobacteriales	Maritimimonas	16S Megablast - 100% id match, 3e-130 E value, Park et al 2009 MEGA - 100% match, 3e130 E value, Park et al 2009.
41	All A sites and MC, OTU 3242	Flavobacteriales	Aquimarina	99% id match, 1e-128 E value, Kennedy et al 2013, Oh et al 2010
42	T1, T2, M1, OTU 219	Flavobacteriales	Flavobacterium	MEGA 97% 6e-117 match, LaFrentz et al 2014, Warm spring, freshwater, feshwater shrimp culture pond, Nupur 2013 coastal seawater,
43				
44	**Sphingobacteriia**			
45	M2, T1, T2, M1, OTU 39	Sphingobacteriales	Sediminibacterium	
46	T1, T2, M1, OTU 347	Sphingobacteriales	Ferruginibacter	100% id, 3e-130 E value, Lim et al 2009
47	AC, A4.1, A1, A2, OTU 93	Sphingobacteriales	Lewinella	100% id Match 3e-130 E value, Lee SD, 2007
48				
49	**Epsilonproteobacteria**			
50	All A sites and All M sites, largest at MC, OTU 7	Campylobacterales	Sulfurovum	Uncultured saltmarsh, Baltic sediments degrading crude oil, anthropogenic pollution in coastal waters Bhavnagar Indai, seabed sediments Leghorn, floor sediment mdeserrian sea, sediment in aquaculture farm souther coast of Korea , seafloor surface , 99% ID, 2e-121 E value, Marine Zero valence sulfur oxidizers (S^0). 98% MEGA id match, 3e-120 E value Nakagawa et al 2007
51	All M Sites, A1, A2 M2 largest presence, OTU 137	Campylobacterales	Sulfurovum	MEGA - 96% Match, 3e-115 E value, Deep sea hydrothermal vent - Mino et al 2014, Nakagawa et al 2007
52				
53	**Archaea**			
54	**Thaumarchaeota**			

55	AC, A4.0, A4.1, A2, TC, OTU 9807	Nitrosopumilaceae	Nitrosopumilus	MEGA 98% id match, 4e-124 E value, Walker et al 2010, Park 2010
56	AC, A4.0, A4.1, A2, TC, OTU 340	Nitrosopumilaceae	Nitrosopumilus	98% id match 1e-123 E value, walker et al 2010, Park 2012,
57	AC, A4.0, A4.1, OTU 62	Nitrosopumilaceae	Nitrosopumilus	98% id match 6e-122 E value, walker et al 2010
58				
59	**Verrucomicrobia**			
60	**Verrucomicrobiae**			
61	All A Sites, MC, TC, OTU 169	Verrucomicrobiales	Rubritalea	MEGA 98% id match, 1e-123 E value tangeria strain Yoon et al 2007, 97% id match, 6e-117 E value Scheuermayer et al 2006.
62				

62				
63	**Cyanobacteria**			
64		order	genus	
65	A4.1, A1, A2, OTU 4	Eukaryota	Rhodophyta	97% chloroplast Neosiphonia harveyi, red algae
66	A4.1, OTU 5	Eukaryota	Rhodophyta	97% plastid Antithamnionella spirographidis,red algae
67	A1, A2, A3C, A4.1, OTU 23	Eukaryota	Rhodophyta	95%, Environmental generic - Cyano/bacteria
68	A4.0, A4.1, OTU 29	Eukaryota	Rhodophyta	94% chloroplast Calliarthron tuberculosum, Red algae
69	A1, A2, OTU 44	Oscillatoriales	Lyngbya	Lyngbya aestaurii strain,98% MEGA match 3e-120 value, Tomitani et al 2006, Garcial-Pichel, Nubel, Muyzer 1998
70	ALL A Sites, M2, MC, OTU 56	Prochlorales	Prochlorococcus marinus subs	Prochlorococcus marinus subs. Marinus strain 97% match, 6e-117 e value, Dufresene 2003, Rippke 2000.
71	ALL A Sites, M2, MC, OTU 77	Eukaryota	Rhodophyta	100% chloroplast Corallina officinalis, red algae
72	A1, A2, A4.0, A4.1, A3C, OTU 112	Oscillatoriales	Chroococcales, synechococcus	Cyanobacteria, 91% match, 1e-93 e vaule, swingley et al 2008, synochococcus ernst et al 2003
73	assigned macro, OTU 135	Bacteria	Planctomycetes	99% Algisphaera; environmental samples,
74	ALL A Sites, M2, MC, OTU 9132	Prochlorales	Prochlorococcus	Prochlorococcus marinus subs. Marinus strain 94% match, 1e-104 e value, Dufresene 2003, 3e-103 Rippke 2000. Synechococcus rubescens, 92% 1e-94 e value.
75				

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