Effects of disturbances on microbial community composition and activity of biofilms from the Great Barrier Reef

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STATEMENT OF ORIGINALITY

I herewith certify that this thesis does not incorporate without acknowledgement any material previously submitted for any degree or diploma in any University; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person, except where due reference is made in the text.

Verena Witt, 17. April 2012

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ZUSAMMENFASSUNG

Tropische Korallenriffe weltweit werden zunehmend durch globale (z.B., Erwärmung, Versauerung der Meere) und lokale (z.B., Eutrophierung, Überfischung) Störungen bedroht. Besonders betroffen sind Riffökosysteme nahe urbanisierter Küstengebiete, die durch extreme Wetterereignisse während der Regenzeit terrestrischen Abfluß empfangen, der zu reduzierter Licht- und erhöhter Nährstoffverfügbarkeit für benthische Korallenriff-Gemeinschaften führt. Terrestrischer Nährstoffeintrag verschlechtert die Küstenwasserqualität entlang des Großen Barriere-Riffs (GBR) und könnte somit die Dynamik, Produktivität und Zusammensetzung der Korallenriffe beeinflussen. Mikrobielle Biofilme reagieren rapide auf Veränderungen der Umweltbedingungen und könnten daher nützliche Bioindikatoren für Wasserqualität darstellen. Um dieses Potential zu untersuchen, wurden Biofilmassoziierte mikrobielle Gemeinschaften mit terminalem Restriktionsfragmentlängenpolymorphismus (T-RFLP) und durch Sequenzierung des 16S rRNA Gens analysiert. Des Weiteren wurden Sauerstoffflüsse durch simultane licht/dunkel Inkubationen bestimmt, und auf die Biomasse (C und N) und den Chlorophyll *a* (Chl *a*) Gehalt der Biofilme bezogen.

Zunächst erfolgte die Bestimmung eines geeigneten standardisierten Siedlungssubstrats für Biofilme (Kapitel 2), wofür Biofilmgemeinschaften *in situ* auf verschiedenen Substraten (d.h., Glasobjektträger, Keramikfliesen, Korallenskelette und Riffsedimente) in einem räumlichen (innere and äußere küstennahe Riffe) und zeitlichen (Regen- und Trockenzeit) Rahmen untersucht wurden. Die Ergebnisse zeigten, dass die Art des Substrats wenig Einfluss auf die Zusammensetzung der mikrobiellen Gemeinschaft hatte. Dabei wiesen insbesondere Glas und Korallenskelette sehr ähnliche Gemeinschaften auf. Daher eignen sich standardisierte Glasobjektträger gut für Biofilm-Indikator Langzeitstudien in tropischen Korallenriffökosystemen und wurden daraufhin als Siedlungssubstrat in allen darauffolgenden Studien verwendet. Jedoch zeigten Biofilme deutliche Veränderungen in der Zusammensetzung der Gemeinschaft auf einer räumlich-zeitlichen Ebene, welche zu weiteren vertieften Untersuchungen führten (Kapitel 3).

Um die Auswirkungen von Wasserqualität auf die mikrobielle Gemeinschaften zu ermitteln, wurden Glasobjektträger an fünf Saumriffen (drei innere und zwei äußere küstennahe Riffe) entlang eines Wasserqualitätsgradienten gelegen in der Whitsunday Inselgruppe, GBR, Australien während wiederholten Jahreszeiten (Regen- und Trockenzeit) über einen Zeitraum

von zwei Jahren, ausgebracht. Es konnten deutliche Unterschiede in den Gemeinschaften an inneren und äußeren Standorten sowie in denen der Regen- und Trockenzeit, festgestellt werden. Die Ergebnisse deckten auf, dass mikrobielle Gemeinschaften mit Wasserqualitätsparametern, insbesondere mit gelöstem organischen Kohlenstoff (DOC) und Chl *a* Konzentrationen, stark korrelierten. Mikrobielle Gemeinschaften an niedrig belasteten (äußere küstennahe Riffe mit hoher Licht- und niedriger Nährstoffverfügbarkeit) im Vergleich zu hoch belasteten (innere küstennahe Riffe mit niedriger Licht- und hoher Nährstoffverfügbarkeit) Riffen, wiesen überwiegend *Cyanobacteria* und *Roseobacter* (*Alphaproteobacteria*) auf, welche mit niedrigen Chl *a* Konzentrationen korrelierten. Folglich könnten diese Mikroorganismen Indikatoren für oligotrophe Bedingungen darstellen. An inneren Riffen häufig auftretende *Alteromonadaceae* (*Gammaproteobacteria)*, *Bacteroidetes* and Diatomeen Plastiden, die stark mit hohen Chl *a* Konzentrationen korrelierten sowie *Flavobacteriaceae* die mit niedrigen DOC Konzentrationen korrelierten, könnten somit denkbare Eutrophierungsindikatoren darstellen. Die beobachtete räumlich-zeitliche Verbreitung häufig auftretender Mikroben lassen sich sehr wahrscheinlich auf deren Funktion und deren Wettbewerbsvorteil unter bestimmten Bedingungen zurückführen.

Saisonale und räumliche Unterschiede in der Intensität des terrestrischen Eintrags könnten wechselwirkende Auswirkungen mit bestehender Meereserwärmung haben und Veränderungen in Korallenriff-Gemeinschaften fördern (Kapitel 4). Um die wechselwirkenden Effekte solcher Parameter auf Biofilme zu untersuchen, wurden Biofilme Kombinationen manipulierter Wassertemperaturen, Nitrat- and Lichtverfügbarkeiten in einem Durchfluß-Aquarienexperiment ausgesetzt. Kombinationen dieser Faktoren dienten zur Simulation innerer küstennaher Bedingungen (niedrige Licht-/ hohe Nitratverfügbarkeit) die *in situ* während der Trockenzeit (niedrige Temperatur), beziehungsweise während eines Hochwasserereignisses in der Regenzeit (hohe Temperatur) anzufinden sind, wohingegen die Kombination hohe Licht-/ niedrige Nitratverfügbarkeit ein küstenferneres Szenario darstellte. Unabhängig von Lichtverhältnissen, verringerten sich die Netto-O_2 Produktion, C-, N- und Chl *a* Gehalte in Biofilmen signifikant bei erhöhten Temperaturen. Temperatur-induzierte mikrobielle Gemeinschaftsveränderungen, die sich durch eine Zunahme der relativen Abundanz von *Oceanospirillum* (*Gammaproteobacteria*) beziehungsweise der Abnahme von *Cyanobacteria* zeigten, waren ebenfalls lichtunabhängig. Lediglich in hohen Lichtverhältnissen wurde die Netto-O_2-Produktion von additiven Effekten hoher Temperatur und hohen Nitratkonzentrationen signifikant reduziert. Des Weiteren wurden Temperatur-

induzierte Gemeinschaftsveränderungen von gehäuftem Auftreten der *Flavobacteriaceae*, und Nitrat-induzierte Gemeinschaftsveränderungen von vermehrtem Auftreten der Diatomeen Plastide, gesteuert. Darüber hinaus wurden Biofilme unter hohen Lichtbedingungen Nitrat-limitiert, wie die signifikant ansteigende Netto-O_2-Produktion bei intermediärer Nitratkonzentration erklärte. Im Gegensatz dazu waren Biofilme unter niedrigen Lichtbedingungen vermutlich Licht-limitiert, denn weder die Gemeinschaftszusammensetzung noch die Produktivität veränderten sich unter erhöhter Nitratverfügbarkeit. Hohe Temperaturen veränderten die mikrobielle Gemeinschaftszusammensetzung, Biomasse und Produktivität von Biofilmen, und interagierten mit terrestrischem Abfluss. Folglich, unter vorhergesagten Szenarien küstennaher Riffe (niedrige Licht-/ hohe Nitratverfügbarkeit), werden Biofilme durch Sedimenteintrag aus terrestrischem Abfluss Licht-limitiert sein. Allerdings werden Biofilme an äußeren küstenferneren Riffen (hohe Licht-/ niedrige Nitratverfügbarkeit), Nitrat-limitiert verbleiben, jedoch nur unter der Annahme, dass sich durch klimatische Beeinträchtigungen Wetter- und Hochwasserereignisse nicht verschlechtern und terrestrischer Abfluss diese Riffe nicht erreichen wird. Außerdem unterstützten Daten aus Aquarienuntersuchungen die in Felduntersuchungen nachgewiesenen Biofilmgemeinschaftsveränderungen, die durch Reaktionen ähnlicher bisher identifizierten mikrobiellen Schlüsselgruppen auf Umweltstressfaktoren, hervorgerufen wurden (Kapitel 3 und 4).

Effekte eines weiteren Klimawandelaspekts (d.h., Versauerung der Meere) auf marine Mikroben sind wenig erforscht und kaum bekannt (Kapitel 5). Deshalb wurden Biofilme einer Reihe von vier kontrollierten pCO_2 Konzentrationen ausgesetzt, welche Szenarien der Vorindustriellenzeit bis zum Ende dieses Jahrhunderts repräsentierten. Die Ergebnisse zeigten, dass erhöhte pCO_2 Konzentrationen die das späte Jahrhundert simulierten, die Zusammensetzung von Algen- und Bakteriengemeinschaften signifikant veränderten. Auch erhöhten diese Konzentrationen den organischen und anorganischen C und N Gehalt der Biofilme signifikant, und führten so zu erhöhten C:N Verhältnissen die das Redfieldverhältnis überschreiten. Algengemeinschaften verschoben sich zu einer von filamentösen Grünalgen-dominierten Gemeinschaft mit einer deutlichen Abnahme von Rotalgen und pCO_2-induzierte Bakteriengemeinschaftsveränderungen wurden durch die Zunahme der relativen Abundanz von *Bacteroidetes*, insbesondere *Flavobacteriaceae*, und Abnahme der *Alphaproteobacteria*, verursacht. Jedoch, beeinflusste erhöhtes pCO_2 weder die Photosynthese noch die Respiration.

Allumfassend, führte eine kurzfristige Exposition der Biofilme gegenüber erhöhtem pCO_2 zu signifikanten Veränderungen der mikrobiellen Gemeinschaftsstruktur mit einer sichtbaren Empfindlichkeit spezifischer Bakteriengruppen. Biofilmgemeinschaften reagieren rapide auf hohes pCO_2 durch Umstrukturierung der Gemeinschaft, begleitet von Veränderungen des C:N Verhältnisses, um Aktivitäten wie die Sauerstoffproduktion aufrechtzuerhalten. Im Großen und Ganzen verändern sich die auf Glas entwickelten mikrobiellen Biofilmgemeinschaften bei erhöhter Temperatur, Nitrat-, pCO_2- und Lichtverfügbarkeit und folglich, reflektieren sie effizient Veränderungen der umgebenden Wasserqualität. Die Ergebnisse zeigten übereinstimmend überwiegend *Gammaproteobacteria* (z.B., *Oceanospirillum, Altermonadaceae*), *Bacteroidetes*, (vorwiegend *Flavobacteriaceae*) und Diatomeen Plastide *in situ* und in simulierten küstennahen Bedigungen, während *Cyanobacteria* und *Alphaproteobacteria* (vorwiegend *Roseobacter*) häufiger unter Bedigungen an küstenferneren Riffen nachgewiesen wurden. Besonders relevant ist der wiederholte Nachweis ansteigender *Bacteroidetes*, vorwiegend *Flavobacteriaceae*, und eine begleitende Verringerung der *Alphaproteobacteria* auf veränderte Umweltbedingungen. Saisonal- und Wasserqualitätsgesteuerte Verschiebungen mikrobieller Schlüsselgruppen zeigen, dass sich mikrobielle Biofilme als nützliche Bioindikatoren tropischer Küstenwasserqualität eignen. Dies könnte Implikationen für zukünftiges Küstenmanagement haben. Des Weiteren, konnten die Untersuchungen zeigen, dass Klimawandel und Eintrag terrestrischen Materials sehr wahrscheinlich Auswirkungen auf die Produktivität, Zusammensetzung und Funktion zukünftiger Korallenriffökosysteme haben werden.

THESIS ABSTRACT

Tropical coral reefs worldwide are increasingly threatened by disturbances on both global (i.e., global warming, ocean acidification) and local (i.e., eutrophication, overfishing) scales. Of particular concern are reef ecosystems in close proximity to urbanized coastal areas, where extreme weather events in the summer wet seasons result in terrestrial runoff leading to reduced light and increased nutrient availability for benthic coral reef communities. Such runoff may deteriorate water quality along the Great Barrier Reef coast and hence affect coral reef dynamics, productivity and composition. Microbial biofilms respond rapidly to changing environmental conditions and may be useful bioindicators for water quality. To test this potential, microbial communities associated with biofilms were analysed by terminal restriction fragment length polymorphism (T-RFLP) and sequencing of 16S rRNA genes. Further, oxygen fluxes, determined by simultaneous light and dark incubations, were related to biofilm biomass (C and N) and chlorophyll *a* (Chl *a*) contents.

Initially, a standardised substrate for biofilm settlement was determined (Chapter 2). Biofilm communities were established *in situ* on different settlement substrates (i.e., glass slides, ceramic tiles, coral skeletons and reef sediments) and investigated on a spatial (inner and outer nearshore sites) and temporal (wet and dry seasons) scale. Findings revealed that substrate type had little influence on microbial community composition and, in particular, glass slides and coral skeletons exhibited very similar communities. This suggests the suitability of standardised glass slides for long-term biofilm indicator studies in tropical coral reef ecosystems. Hence, glass slides were used as the settlement substrate for biofilm formation in all of the following studies. However, biofilms clearly exhibited community composition changes on a spatio-temporal scale, leading to more in-depth investigations (Chapter 3).

To study the effects of water quality on microbial biofilm communities, glass slides were deployed at five fringing reefs (three inner and two outer nearshore) along a water quality gradient in the Whitsunday Islands, Central Great Barrier Reef, Australia, during repeated (wet and dry) seasons over the course of two years. Clear dissimilarities of microbial communities at inner and outer locations, and during wet and dry seasons could be identified. Our findings also revealed that microbial communities were strongly correlated by water quality parameters, in particular dissolved organic carbon (DOC) and Chl *a* concentrations.

Microbial biofilm communities at low impacted (outer nearshore reefs with high light/ low nutrient availability) compared to highly impacted (inner nearshore with low light/ high nutrient availability) reefs, displayed a predominance of members of the *Cyanobacteria* and *Roseobacter* (*Alphaproteobacteria*) correlated with low Chl *a* concentrations, and hence these groups may be indicators of oligotrophic conditions. The predominance of *Alteromonadaceae* (*Gammaproteobacteria)*, *Bacteroidetes* and diatom plastids at inner nearshore sites, closely correlated with high Chl *a* concentrations, and *Flavobacteriaceae* correlated *w*ith low DOC concentrations, could therefore represent conceivable indicators of eutrophication. The observed spatio-temporal distribution of predominant microbes was likely related to their function and competitive advantages under certain conditions.

Seasonal and spatial differences in terrestrial runoff intensity may have interactive effects with ocean warming and promote shifts in coral reef communities (Chapter 4). To examine the interactive effects of such parameters on biofilms, they were exposed to various combinations of manipulated water temperatures, nitrate and light availabilities in a flow-through aquarium experiment. These simulated inner nearshore conditions (low light/ high nitrate availability) found *in situ* during the dry season (low temperature) and during flood plumes in the wet season (high temperature), while the combination high light/ low nitrate availability represented the situation further offshore. Irrespective of light availability, high temperatures significantly decreased net O_2 production, C-, N- and chlorophyll *a* contents of biofilms. Similarly, temperature-induced microbial community shifts were independent of light availability and were driven by increases in the relative abundance of *Oceanospirillum* (*Gammaproteobacteria*) and decreases in members of the *Cyanobacteria*. Only under high light, additive effects of high temperature and high nitrate significantly reduced net O_2 production. Further, under high light temperature-induced microbial shifts were driven by increases in the relative abundance of members of the *Flavobacteriaceae*, and nitrate-induced community shifts were caused by increases in diatom plastids. Further, high light-exposed biofilms became nitrate-limited, as illustrated by significantly increasing net O_2 production at intermediate nitrate concentrations. In contrast, biofilms under low light availability were presumably light-limited, as neither community composition nor productivity changed under increased nitrate availability. High temperatures altered microbial biofilm community composition, biomass and productivity and interacted with terrestrial runoff. Thus, under predicted near-future inshore reef scenarios (low light/ high nitrate availability), biofilms are predicted to become light-limited due to increased sediment and particulate matter loads from

runoff. However, at offshore reefs (high light/ low nitrate availability), biofilms will remain nitrate-limited, yet only under the assumption that climatic effects will not worsen weather events and that flood plumes will not reach these reefs. Moreover, data from these aquarium investigations further supported detected community shifts driven by similar key dominant microbial groups in biofilm communities in response to environmental stressors determined in field investigations (Chapters 3 and 4).

Effects of another aspect of climate change (i.e., ocean acidification) on marine bacteria are poorly investigated and barely understood (Chapter 5). Therefore, biofilms were exposed to four controlled pCO_2 concentrations representing scenarios ranging from preindustrial to late century. Findings indicate that elevated pCO_2 simulating end of century concentrations, significantly altered algal and microbial community composition. These also significantly increased organic and inorganic C and N contents, and resulted in C:N ratios that were significantly higher than the Redfield ratio. Algal communities under elevated pCO_2 shifted towards filamentous green algae dominance with a decrease in red algae, while elevated pCO_2-induced microbial community shifts were driven by an increase in the relative abundance in *Bacteroidetes*, in particular *Flavobacteriaceae*, and decrease in *Alphaproteobacteria*. However, elevated pCO_2 did not affect photosynthesis or respiration. Overall, these results show that short-term exposure to high pCO_2 significantly shifts microbial biofilm communities and shows the sensitivity of specific microbial groups to these conditions. However, biofilm communities seemingly rapidly adapt and reorganise in response to high pCO_2 in order to maintain activity such as oxygen production, accompanied by changes in net production, respiration and C:N ratios.

Overall, microbial communities in biofilms established on glass slides shift in response to high temperature, nitrate, pCO_2 and light availability, and thus, efficiently reflect changes in ambient water conditions. Our results concordantly show a predominance of *Gammaproteobacteria* (e.g., *Oceanospirillum, Altermonadaceae*), *Bacteroidetes*, (in particular *Flavobacteriaceae*) and diatom plastids *in situ* and in simulated inner nearshore conditions, while *Cyanobacteria* and *Alphaproteobacteria* (in particular *Roseobacter*) are more frequently detected in outer nearshore conditions. Of particular relevance is the repeated finding of increases in *Bacteroidetes*, in particular *Flavobacteriaceae*, and a concomitant decrease in *Alphaproteobacteria* in response to changing environmental conditions (e.g., heat stress, rising pCO_2). Seasonal and water quality driven shifts in the relative abundances of key

microbial groups in biofilms suggest they are useful bioindicators for tropical coastal water quality and may have implications for future coastal management. Further, it is suggested that climate change and terrestrial runoff will likely impact productivity, composition and functioning in future coral reef ecosystems.

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GENERAL INTRODUCTION

CORAL REEF ECOSYSTEMS

Coral reefs are marine structures formed by the accumulation of biogenic calcium carbonate ($CaCO_3$) secreted by reef-building organisms such as corals, molluscs and coralline algae, whereby scleractinian corals, that produce up to 10 kg $CaCO_3$ m^{-2} y^{-1}, act as the main contributors to reef formation (Kleypas et al., 1999). Coral reefs cover a total area of 6 x 10^5 km^2 (Smith, 1978) and hence occupy less than 0.17 % of the world's ocean surface. Yet, reefs are important habitats for a variety of tropical organisms, providing shelter for 25 % of all marine species (Mulhall, 2007), thus representing 'biodiversity hotspots' (Devantier et al., 2006). Warm water coral reefs are mainly distributed in shallow tropical waters (0 - 30 m) in a zone extending from the Tropic of Cancer to the Tropic of Capricorn, 30°N to 30°S of the equator. Apart from tropical waters, cold water corals (40 - 850 m depth) also exist widely distributed around the world (Freiwald et al., 2004; Roberts et al., 2006).

Fig. 1 Map of the distribution of coral reefs around the world (courtesy of H. Ahlenius, UNEP/GRID-Arendal http://www.grida.no/graphicslib/detail/distribution-of-coldwater-and-tropical-coral-reefs_1153)

Coral reefs are of various types in relation to their location relative to land (fringing reefs, barrier reefs and remote atoll reefs). Fringing reefs are the most common reef type (Kennedy and Woodroffe, 2002), consisting of a reef belt parallel and close to the coast. Barrier reefs occur in the shallow areas of the continental shelf and are also located parallel to the coastline and separated from the coastline by a lagoon that can contain deeper areas. Barrier reefs can reach enormous extensions of more than 1000 km in length. A typical example is the Great Barrier Reef found along the coast of Queensland, Australia. Another reef type is the atoll

reef, forming a circular (annular) reef system surrounding a closed lagoon, and is an abundant reef type in the Indo-Pacific region (e.g., Maledives, French Polynesia).

CORAL REEF PRODUCTIVITY

Tropical coral reef habitats are characterised by very low inorganic nutrient concentrations (e.g., < 1.5 µM organic nitrogen and 0.5 µM phosphate) (Johannes et al., 1983). Paradoxically, coral reefs thrive in clear, oligotrophic waters with extremely low inorganic nutrient concentrations, yet with a mean annual gross primary production of 1500-5000 g C m^{-2} y^{-1}, coral reefs are highly productive (Sommer, 1998). Mean annual net primary production of coral reefs (2200 g C m^{-2} y^{-1}) exceeds primary production rates of adjacent open oceans (190 g C m^{-2} y^{-1}) (Abdey, 1998) by one order of magnitude, and further, is much higher than in its terrestrial counterparts, the rainforests (1600 g C m^{-2} y^{-1}) (Garrison, 2009). Whereas coral reefs are called the 'rainforests of the sea' with respect to their high productivity (and diversity), the surrounding waters are often referred to as 'nutrient deserts'. This contradiction is referred to as the Darwin's paradox and can be explained by the fact that the few available nutrients are recycled rapidly through a variety of mechanisms, particularly the internal cycling of nutrients between heterotrophic and autotrophic organisms, as seen in many cnidarians (e.g., hard and soft corals, anemones) and molluscs (e.g., clams and nudibranchs) living in symbiosis with dinoflagellates (zooxanthellae) (Muscatine and Porter, 1977; Schlichter et al., 1983). Nutrients are mainly processed and recycled in microbial food chains, e.g., rapid turnover of organic and inorganic nutrients within the reef framework (Crossland and Barnes, 1983), in sediments (Rasheed et al., 2002; Wild et al., 2005b; Wild et al., 2005a) and benthic biofilms (Battin et al., 2003). Moreover, another recycling mechanism is particle feeding by filtering organisms such as sponges that are typical for the reefs' framework system (Richter et al., 2001). In addition, N-fixation by *Cyanobacteria* (Larkum et al., 1988) and possibly by non-*Cyanobacteria* (Riemann et al., 2010) contributes importantly to nutrient availability in coral reefs.

Benthic communities are highly diverse and often dominate ecosystem processes and metabolism, especially in shallow coastal waters e.g., reef lagoons. Benthic communities receiving high levels of irradiance supporting photosynthesis typically comprise of photoautotrophs such as invertebrates (e.g., corals) with photosynthetic symbionts (Kühl et al., 1995), but mainly microalgae forming highly productive communities on the sediment

surface (Cahoon, 1999). In addition to sunlight photosynthetic organisms also require inorganic nutrients such as nitrate and phosphate, and these are made available by bacterial degradation. Hence, coral reefs are important sites of carbon cycling (e.g., C-fixation) and bacterial activity, and are important contributors to ecosystem photosynthesis and respiration. Coral reefs are an exception to the general rule that high primary productivity is strongly dependant on high availability of N and P. Gross production of coral reefs is as large as that of kelp forests (Garrison, 2009). However, most of the generated energy is not available to other reef organisms, but is tightly recycled leaving small net nutrient availability, which is a unique feature of coral reefs.

IMPORTANCE OF CORAL REEFS

Equivalent to rainforests, coral reefs are highly productive biodiversity hotspots. Therefore, coral reefs represent a valuable resource and revenue for humans (Moberg and Folke, 1999) with a global economical value estimated to 29.8 billion US dollars annually (Cesar and Pet-Soede, 2003). In addition, by reducing wave energy, coral reefs play an important role in coastal protection against storm damage, flooding and erosion.

TROPICAL CORAL REEFS UNDER GLOBAL AND LOCAL THREAT

Coral reef biodiversity hotspots occur where optimal equilibrium benefits from land-associated ecosystems and the fluxes generated. The association of coral reefs with coastal ecosystems is beneficial, but also poses problems when the above equilibrium is imbalanced (Dinsdale et al., 2008). However, this revenue is already being heavily compromised due to overexploitation (Cesar, 2002; Lough, 2008). Global (e.g., ocean acidification and global warming) and local (e.g., eutrophication) disturbances increasingly exert pressure on coral reef health and ecological balance.

GLOBAL CLIMATE CHANGE

Global warming refers to the average temperature rise of the Earth's oceans and atmosphere caused by greenhouse gases (e.g., carbon dioxide, methane, ozone, nitrous oxide) produced by human activities such as the burning of fossil fuels and changes in land use. Since the industrial revolution, human activity has increased concentrations of greenhouse gases in the atmosphere up to critical levels that are currently of global concern (IPCC, 2007). Increasing

global temperatures cause the sea level to rise, consequently altering precipitation volumes and patterns. This includes changes in frequency and intensity of extreme weather events such as drought, heavy rainfall and cyclones (IPCC, 2007). Climate change has already increased sea surface temperatures (SSTs) on the Great Barrier Reef (GBR) by an annual average of 0.7 °C within the last century (Lough, 2001; Lough et al., 2006). Furthermore, forecasts by the Intergovernmental Panel on Climate Change (IPCC, 2007) predict that GBR waters may warm by a further 1 - 3 °C by 2100.

Since the industrial era, the world's oceans have absorbed 30 - 40 % of anthropogenically emitted CO_2 from the atmosphere (Feely et al., 2004; Sabine et al., 2004). The uptake of CO_2 alters the seawater carbon chemistry, resulting in a reduction in pH and carbonate ion saturation (Caldeira and Wickett, 2003; Raven, 2005). Atmospheric CO_2 dissolves in the surface ocean so that it forms weak carbonic acid (H_2CO_3), which then dissociates into bicarbonate (HCO_3^-) and H^+ and then further into carbonate (CO_3^{2-}) and H^+ ions, as summarized in the equation below:

$$CO_2 + H_2O \rightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

This increased CO_2 input leads to a chemical equilibrium shift towards an increased concentration of H^+, consequently lowering the pH of the surface ocean and this is termed ocean acidification. Furthermore, reduced pH results in a decreased Ω aragonite saturation and reduced availability for the calcification of several reef organisms including cnidarians, molluscs and echinoderms (Kleypas et al., 1999). Thus, enhanced pCO_2 may erode the structural foundation for coral reef growth (Kleypas et al., 1999; Langdon et al., 2000; De'ath et al., 2009; Ries et al., 2009; Silverman et al., 2009; Fabricius et al., 2011). Recent reports have shown that ocean acidification reduces coral larval settlement and development (Albright et al., 2010; Suwa et al., 2010; Doropoulos et al., 2012). Further, under ocean acidification scenarios, it has been demonstrated that macroalgae have a competitive advantage over corals (Diaz-Pulido et al., 2011). It is well established, that ocean warming promotes frequency and intensity of coral bleaching events (Hoegh-Guldberg, 1999) and associated mortality (Anthony et al., 2007). More critical are the interactive effects of rising SSTs and ocean acidification lowering coral reef resilience (Anthony et al. 2011) that may shift future reefs from coral- to algal-dominance in (Hoegh-Guldberg et al., 2007; Anthony et

al., 2011). By reducing the growth potential and survivorship of corals, ocean warming and acidification likely negatively affect coral-macroalgae dynamics, by reducing the ability of corals to maintain or rapidly colonize available space following disturbances (Carilli et al., 2009). A shift in macro-organisms will also mean a concomitant shift in the associated microbes. Responses of coral reef-associated microbes to climate change will be explored in the current study.

LOCAL WATER QUALITY DETERIORATION

Water quality in coastal areas worldwide is declining because of high intensity of human activities such as land clearing, soil erosion and fertilizer use (Vitousek et al., 1997; Smith and Veenstre, 2003). One percent of the earth's surface area is cleared annually (GESAMP, 2001), N fertilizer use has increased by 6-fold since 1960 (Matson et al., 1997) and coastal urbanization expands disproportional in relation to human population growth.

For example, the Great Barrier Reef (GBR) receives substantial amounts of terrestrially derived nutrients, sediments and pollutants imported from river runoff (Bell, 1991). River catchments close to the GBR have experienced significant modifications since European settlement of Queensland's coast in the mid 1880s (Neil et al., 2002; Furnas, 2003; McCulloch et al., 2003). This has led to an increase of nutrient inputs by 400 % (Moss et al., 1992; Neil et al., 2002) and sediment inputs by 500 – 1000 % (McCulloch et al., 2003). Nitrogen stimulates pelagic microbial growth and thus has the capacity to influence photosynthetic rates and carbon dioxide levels. Increasing urbanization and human land-based activities (e.g., fertilizer input) lead to eutrophication. This can stimulate macroalgal growth rates (Schaffelke and Klumpp, 1998). Herbivorous fish control macroalgae growth through grazing (Mumby et al., 2007), however, these fish populations are dramatically reduced by overfishing. Runoff and eutrophication consequently promote shifts from coral-dominated towards algal-dominated reefs (Schaffelke and Klumpp, 1998). Hence, local disturbances may also have significant implications for micro communities due to impacts on the microbial loop, symbiotic relationships and disease processes. Overall, global and local disturbances threaten coral reefs and therefore the preservation of coral reef biodiversity is becoming a central ecological concern.

WATER QUALITY IN THE GREAT BARRIER REEF- STATE OF THE ART

This study focuses on investigating microbial ecology and functioning of the Great Barrier Reef. The Great Barrier Reef (GBR) is the world's largest known coral reef and World Heritage Area (UNEP, 1997). It is located off the coast of Queensland comprising of 2800 single, but often connected, coral reefs covering an area of 345 000 km^2, and extending 2600 km along north-eastern Australia (10.5 - 24.6°S). The GBR is of high biological- and economical importance, as its annual economic value is estimated to five billion Australian dollars with a highly lucrative tourism-, and commercial and recreational fishing industry (Hoegh - Guldberg and Hoegh - Guldberg, 2004). However, ocean acidification (De'ath et al., 2009; Wei et al., 2009), ocean warming (Lough et al., 2006), and terrestrial runoff (De'ath and Fabricius, 2010) put this valuable and unique coral reef at great risk.

A region of particular concern is the Proserpine river catchment in the Whitsunday Island area located in the Central GBR. Hence, this is also the main area of investigation of this thesis. The Whitsunday region has experienced extensive land clearing to facilitate agriculture (i.e., 33 % for beef grazing and 23 % for sugar cane cropping (van den Berg et al., 2007). These land-use practices facilitate the export of sediment and nutrient-enriched river runoff, which significantly alters water quality in the adjacent coastal reef environments (Fabricius, 2005; Wooldridge et al., 2006). In particular during the summer wet season, the O'Connell and Proserpine river flood plumes deliver varying levels of freshwater, sediments and nutrients onto coastal inshore reefs (Bell, 1991; Devlin and Schaffelke, 2009). For example, nutrient discharge by rivers is the largest source of new nitrate (Furnas, 2003) and has increased up to 10-fold since pre-agricultural times (Wooldridge et al., 2006). Terrestrial runoff inshore of the Whitsunday Islands area leads to higher concentrations of nutrients, chlorophyll *a* (Chl *a*) and suspended sediments, which decrease gradually with distance from the coast, forming a distinct cross-shelf water quality gradient (van Woesik et al., 1999; Furnas, 2003; Fabricius, 2005; Brodie et al., 2007; Cooper et al., 2007). Resulting eutrophication and increased turbidity further leads to low light availability at inner nearshore reefs compared to outer inshore reefs. During the summer wet season reduced salinity and increased SSTs further influence water quality at these coastal coral reefs. This water quality gradient in the Whitsunday Island area has been confirmed to be persistent and meanwhile has become a permanent site for coastal water quality monitoring (Cooper et al., 2007; Schaffelke et al., 2010; Uthicke, 2010).

Recent reef monitoring studies on deteriorating water quality along inshore sites of the Great Barrier Reef World Heritage Area (GBRWHA) indicate the necessity to investigate the responses of marine organisms to declining water quality. Natural- and anthropogenic disturbances including wet season flood events and associated fresh water plumes, suspended sediment loads, nutrients and agricultural pesticide residues being transported onto the reef can have adverse effects on marine communities. Significant variations in different reef community structure and water quality parameters with increasing distance from the Proserpine and O'Connell Rivers have already been demonstrated (Cooper et al., 2007; Fabricius et al., 2007; Uthicke and Altenrath, 2010). For example, coral reefs affected by eutrophication and sedimentation show significantly reduced coral growth and recruitment (van Woesik et al., 1999; McCook, 2001; Fabricius et al., 2003) and increasing algae-coral habitat space competition with consequent shifts in coral reef dynamics (Szmant, 2002; Fabricius, 2005; Schaffelke, 2005). Further, inshore locations show an increased occurrence of pathogenic bacteria in corals (Haapkyla et al., 2011). Hence, the Whitsunday region is considered a priority coastal management region (Furnas, 2003).

MARINE MICROBES

BIOFILMS - PROPERTIES AND APPLICATION

Bacterial abundance is estimated to 3.6×10^{29} cells residing in the Earth's ocean (Whitman et al., 1998) with over 200 phylotypes ml^{-1} seawater (Sogin et al., 2006). Tropical coral reefs harbour abundant microorganisms (5.0×10^{3} - 2.0×10^{6} cells ml^{-1} seawater) that predominantly form surface-attached communities of microorganisms embedded in an extracellular polymeric matrix termed biofilms (Mihm et al., 1981). The preference of microorganisms to adhere to surfaces was first noted 1943 by Claude Zobell. Biofilms are ubiquitous in medicine (An and Friedman, 2000), in nearly all technical assets (Costerton et al., 1987) and in the environment (Stal and Caumette, 1994). Biofilms are found in both fresh- and marine aquatic ecosystems colonizing rocks, wood and sediments. For this purpose, aquatic bacteria undergo an irreversible transition from the planktonic stage to the surface-attached biofilm stage forming complex biological systems (O'Toole et al., 2000). Biofilm formation can be divided into three main developmental stages (Characklis, 1990). The first stage is colonization, during which cells adhere to a surface by the excretion of polysaccharides, proteins, lipids and nucleic acids to form what is referred to as the extracellular polymeric substances (EPS). These substances have several functions and are

critical/ decisive factors for the structures and properties of the growing biofilm (Wingender et al., 1999). For example, biofilms developing under oligotrophic conditions form a highly complex system of channels and conduits through the biofilm (Wimpenny and Colasanti, 1997). Secondly, bacterial colonisation is followed by biofilm growth until the mature state and in the final development stage, single cells or larger fragments of the mature biofilm detachment from the substrate.

Environmental biofilms are complex communities comprised of photo- and heterotrophic microorganisms including bacteria, microalgae (dinoflagellates, diatoms), fungi, protists and small metazoans. Bacteria and diatoms commonly excrete extracellular substances, forming a matrix, to improve conditions for microorganism survival (e.g., increased access to nutrients and protection against toxins, antibiotics, UV-radiation, heavy metals and dehydration) (reviewed in Sutherland, 2001; reviewed in Thornton, 2002). Microbial biofilm communities are essential components of oligotrophic coral reef systems, contributing importantly to ecosystem productivity (e.g., biofilms can produce oxygen levels as high as 2 - 3-fold greater than atmospheric concentrations (Fenchel et al., 1998)), large-scale biogeochemical nutrient fluxes (Lock et al., 1984; Battin et al., 2003) and coral reef resilience (i.e., invertebrate larval settlement and metamorphosis) (Wieczorek and Todd, 1998; Webster et al., 2004).

BIOFILMS AND THEIR POTENTIAL AS BIOLOGICAL INDICATORS

In order to study water quality, regular sampling and surveying of water quality parameters is required. This includes physical (e.g., temperature, salinity, secchi disk depth, turbidity), chemical (e.g., pH, dissolved oxygen, dissolved nutrients) and biological (e.g., copeopods, microorganisms) characteristics. The determination of physical and chemical water quality parameters are well established. However, physical and chemical testing fails to determine the cumulative effects of pollutants or the duration of an environmental problem. Therefore, scientists are constantly searching for organisms to be used as biological indicators that can support water quality monitoring and help understand ecosystem integrity. A bioindicator is defined as a metric ecological indicator that is designed to inform about spatial and temporal changes in the condition of relevant ecosystem properties (Bortone, 2005). Biological indicators are species or species assemblages of known environmental requirements that respond to alterations of their habitats with changes in abundance, morphology, physiology or behaviour. Suitable bioindicators preferably show high sensitivity to environmental

disturbances, and are low cost and maintenance to allow rapid inferring of the environmental status.

In the past, studies aiming at developing and applying aquatic bioindicators to monitor ecological changes in response to changing environmental conditions have focused on the monitoring of algae (Foissner et al., 1992; Foissner and Berger, 1996), protists (Madoni and Bassanini, 1999; Wu et al., 2004) and higher animals (reviewed in (Fichez et al., 2005)), and have successfully been used in biological monitoring programs and surveys (McCormick and Cairns, 1994; Whitton and Kelley, 1995). However the search for a faster, more effective indicator continues.

Previous research suggested the use of biofilms as indicators for water quality and nutrient enrichment in agricultural, urban and industrial areas (Chessman, 1985; Chessman et al., 1992). Due to the biofilm property of concentrating and integrating organic and inorganic substances, they represent sites of intensified biogeochemical processes relative to their environment (Zobell, 1943). For this reason, biofilms are more sensitive indicators of ambient conditions than water column parameters. In recent years, there has been increasing evidence that biofilms possess many attributes that make them useful as biological indicators for the changes in water quality in riverine and estuarine systems (Burns and Ryder, 2001; Bauer et al., 2006). Therefore, in the past decade, microbial communities within biofilms and their response to environmental conditions have been increasingly investigated (Guckert et al., 1992; Manz, 1999).

The application of microbiological indicators targeting stress proteins, RNA/DNA ratios or phylogenetic community compositions provides various significant advantages over simple chemical contaminant indicators in the environment (Devereux et al., 2006). The discovery of correlations between the appearance of specific microbial organisms and various environmental factors has been recognized in early stages of microbial ecology (Dobell, 1958). Microbial biofilm communities are highly sensitive to changes in water quality due to their high surface to volume ratios, short generation times, high species and functional diversity, (Paerl and Pinckney, 1996), and ability to structurally self-organize (Tolker-Nielsen and Molin, 2000). These features increase the chance for biofilms to become valuable bioindicators for specific conditions (Fabricius et al., 2007). Despite being highly diverse and

abundant, very little is known about microbes as environmental indicators in marine environments. Therefore, microbial community structures in tropical and subtropical coastal, and estuarine sediments represent preliminary efforts of finding potential bacterial bioindicators for water quality (Wu et al., 2004; Hewson and Fuhrman, 2006; Uthicke and McGuire, 2007; Vieira et al., 2008). Understanding the microbial community structure in the tropical coastal regions is essential in order to develop fast responding indicators for water quality.

Recently, the effectiveness of microbial biofilms as potential indicators has been explored in aquatic systems and hence, biofilms have found application in bioindication and bioremediation of water quality. Several studies have investigated the effects of various environmental factors on bacterial community structure and suggest that bacterial communities differ along water quality gradients concerning abundance (Andrade et al., 2003) and diversity of phylotypes revealing many singularities within the bacterial communities between retrieved samples (Fuhrman et al., 2006; Hewson and Fuhrman, 2006; Newton et al., 2006). Bacterial biofilms have previously been tested as indicators for water quality conditions in riverine (Araya et al., 2003) and estuarine systems (Snyder et al., 2005; Moss et al., 2006; Jones et al., 2007; Nocker et al., 2007) along with polar (Webster and Negri, 2006) and temperate coastal marine environments (Dang et al., 2008). However, coral reef water quality has largely been neglected. Nevertheless, as in other aquatic environments, marine coastal biofilms associated with coral reefs may also find application as a biomonitoring tool of transient spatial and temporal variability and more persistent ecosystem change due to large-scale catchment management decisions or global climate change.

MICROBIAL COMMUNITY SHIFTS

MICROBIAL BIOFILMS AND SETTLEMENT SUBSTRATES

The types of settlement substrata, have shown to influence the microbial composition of biofilms, as suggested in studies on natural substrata in wetlands (Silyn-Roberts and Lewis, 2003) and on artificial substrata in rivers (Manz, 1999; Kröpfl et al., 2006). Of great importance for marine studies are microbial community compositions of biofilms with respect to larval settlement assays and bioindicators for water quality. Therefore, microbial biofilm composition on several artificial and natural substrates with differing surface properties have been investigated (Huggett et al., 2009; Chung et al., 2010; Sweet et al., 2011). These studies

showed that although bacteria initially settling on substrates may differ greatly between substrates (Sweet et al., 2011), yet bacterial communities in biofilms undergo temporal shifts from more different communities during colonisation and early developmental stages to more similar communities over time irrespective of the initial substrate type (Huggett et al., 2009; Chung et al., 2010). However, neither long-term studies (several weeks), nor investigations of biofilms deployed at various sites and seasons are available.

EFFECTS OF GLOBAL CLIMATE CHANGE ON MICROBIAL COMMUNITIES

Elevated SSTs may significantly impact marine microbes, potentially altering microbial diversity, function and community dynamics (Webster and Hill, 2007). Several studies have demonstrated shifts of bacterial community composition in biofilms in response to increased SST (Boivin et al., 2005; Lau et al., 2005; Chiu, 2006). This may change microbial succession of the biofilm and/or alter the ability of microbes to produce morphogenic signalling compounds. This may adversely (or positively) affect subsequent recruitment of macro-organisms, including corals, hence could have serious implications for reef-building, maintenance and recovery processes. Environmental conditions that adversely affect the distribution and abundance of microbes involved in settlement and metamorphosis of reef invertebrates could therefore have large-scale impacts on ecosystem structure and the distribution and reproductive fitness of some keystone species.

Bacterial communities associated to coral reef organisms have shown a decrease in *Alphaproteobacteria* was detected in coralline algae associated-biofilms in response to elevated temperature with a concomitant increase in *Bacteroidetes* (Webster et al., 2011) in particular *Flavobacteria* with anti-oxidative properties (Shindo et al., 2007). Similarly, diseased corals and sponges host a much greater abundance of *Bacteroidetes* than their healthy counterparts (Pantos and Bythell, 2006; Webster et al., 2008). There is much evidence that high water temperatures shift marine bacterial communities, but whether such shifts alter bacterial metabolism, such as oxygen fluxes, remains unknown.

Bacterial communities play a critical role in the health of coral reef ecosystems (Ritchie, 2006; Mouchka et al., 2010). However, responses of microorganisms, such as bacteria, to ocean acidification are largely unexplored (Liu et al., 2010; Joint et al., 2011). The few reports that are available have revealed that elevated pCO_2 causes bacterial community shifts

with an increase in diversity (Meron et al., 2011) and increased pathogenic microbiota in corals (Vega Thurber et al., 2009). Elevated pCO_2 further induced bacterial community shifts in bacterioplankton, while particle-attached bacterial communities remained stable, and bacterial abundance and activity remained unaffected (Allgaier et al., 2008). Microalgae in biofilms acidified by an *in situ* volcanic CO_2 vent have displayed increases in Chl *a*, diatom abundance and shifts of diatom species (Johnson et al., 2012). Recent publications have doubted the sensitivity of marine microbes (Hendriks and Duarte, 2010; Hendriks et al., 2010) and have hypothesised that acidification may have little to no effect on biogeochemical processes other than calcification (Joint et al., 2011). However, far too little is known about marine microbes under pCO_2 to draw precipitate conclusions. Marine microbes under ocean acidification still raise many unexplored questions and hypotheses.

EFFECTS OF LOCAL EUTROPHICATION AND SEDIMENTATION ON MICROBIAL COMMUNITIES

Nutrient availability is one of the major factors influencing biofilm diversity and composition (reviewed by (Costerton et al., 1995)), and this factor can vary with seasons (Claret et al., 1998; Lau et al., 2005). Microbial community shifts have been observed in response to other environmental disturbances, such as local anthropogenic nutrient impacts (Meyer-Reil and Koster, 2000; Lawrence et al., 2004; Chenier et al., 2006; Nocker et al., 2007; Chiu et al., 2008). Higher nutrient availability generally cause a shift from autotrophic to heterotrophic character of biofilms and to sulphur-reducing bacteria as a response to decreased light availability and increased load in organic material (Meyer-Reil and Koster, 2000; Webster and Negri, 2006; Uthicke and McGuire, 2007), while the overall biofilm diversity has been found to either remain on the same level (Moss et al., 2006) or to increase (Ford, 2000; Nocker et al., 2004). Seasonal runoff imports high nutrient and sediment loads onto coastal inshore reefs, thus promoting eutrophication and reduced light availability for benthic communities. Recently, an increased occurrence of pathogenic bacteria in corals has been detected at inshore locations (Haapkyla et al., 2011). Further, inshore disturbances have shown to affect benthic microbial communities (sediments and biofilms) along a water quality gradient in the GBR, demonstrated by a higher abundance of diatoms at inner nearshore (5 km distance from the coast) compared to outer nearshore (> 30 km distance from the coast) (Gottschalk et al., 2007; Kriwy and Uthicke, 2011). Further, more commonly found at inner nearshore reefs were the *Gammaproteobacteria*, while at outer nearshore reefs *Cyanobacteria*

and *Alphaproteobacteria*, in particular *Roseobacter*, were dominant (Uthicke and McGuire, 2007; Kriwy and Uthicke, 2011). Preliminary biofilm research in this study area showed distinct microbial assemblages at inner and outer nearshore locations; a finding that is likely linked to typical water quality parameters found inshore (high dissolved inorganic nitrogen, Chl *a*, particulate organic phosphorus, total suspended solids and particulate organic carbon).

SCIENTIFIC GAPS, RESEARCH GOALS AND HYPOTHESES

Microbial biofilms have been used as bioindicators for water quality in several aquatic environments. However, coral reef water quality has largely been ignored. Nevertheless, as in other aquatic environments, it is postulated that marine coastal biofilms associated with coral reefs may also find application as a biomonitoring tool of transient spatial and temporal variability. Generally, the effect of substrate type for biofilm settlement is known to affect microbial composition. However, the literature barely provides recommendations for a suitable substrate for biofilm indicator studies, especially not for marine and coral reef water quality studies. Additionally, the reports that are available lack long-term studies (several weeks) and investigation of location and season variability. Therefore, the first aim of this study is to reveal differences of microbial biofilm communities on several natural and artificial substrates on spatio-temporal scales. It is hypothesised, that substrates may have little effect on microbial biofilm composition, but rather seasonal and location effects will alter microbial community composition.

Previous field-based approaches to examine the suitability of biofilms for bioindication in coral reefs have indicated specific water quality parameters being responsible for the variation in microbial communities and trends of certain microbial groups to changes in water quality. However, no long-term or seasonal studies along the water quality gradient providing better insight into microbial distribution and diversity, are available. Thus, the next goal is to reveal microbial community changes in response to season and location, to find the decisive water quality parameter responsible for microbial community changes, and to determine trends in dominant location and/or season specific groups.

Previous research has shown that microbial communities associated with benthic communities e.g., sediments and invertebrate hosts in tropical coastal areas shift in response to elevated SSTs and eutrophication. Bacteria in biofilms from other aquatic habitats (e.g.,

freshwater streams) have also shown such responses. However, whether this also applies to bacteria in biofilms from tropical coral reefs is unknown. Further, interactions between specific parameters altered by climate change (elevated temperature) and runoff (light, nitrate) on aquatic microbial biofilms remain poorly understood. Therefore, quantitative (C-, N- and chlorophyll *a* contents) and qualitative (changes in microbial community composition) parameters in response to climate change (elevated temperature) and runoff (light, nitrate) were investigated. Also remaining barely investigated, is whether microbial community shifts also alter metabolism of microbial biofilms. Therefore, metabolic responses (O_2 fluxes) to different combinations of manipulated temperature, nitrate and light availabilities were investigated, and it was hypothesised that microbial community shifts will alter O_2 fluxes. Research conducted on effects of pCO_2 on marine microbes is scarce. Microbes under ocean acidification raise many unexplored questions and hypotheses. It will be investigated whether pCO_2 alters microbial community composition, biomass and O_2 fluxes. The main question is whether biofilms buffer the effects of elevated pCO_2 or will enhance/ reduce processes such as e.g., photosynthesis.

In summary, the objectives of this study are:
1) Investigating the effect of artificial and natural settlement substrata on bacterial biofilm community composition over longer spatio-temporal scales (six weeks, one wet and one dry season) and determine a suitable substrate for bioindicator application.

2) Investigating whether microbial biofilm communities in coral reefs shift in response to water quality and determination of the most decisive water quality parameter contributing to the community changes over different seasons.

3) Determining predominance and trends of bacterial groups, identifying potential target indicator species and evaluating the suitability of microbial biofilms as indicators for coral reef water quality and perhaps provide suggestions for coastal management.

4) Quantifying metabolism (O_2 fluxes), biomass, chlorophyll *a* and microbial community composition of biofilm communities in response to climate change (elevated temperature, pCO_2) and terrestrial runoff (elevated nitrate concentrations) and interactions of these factors,

in order to see whether changes in community composition are linked to metabolic functioning.

PUBLICATIONS OUTLINE

This thesis consists of four articles. Of these, two articles have been published in international journals, one article has been accepted for publication and is currently in press, and one further is in review.

In **Chapter 2**, 'Effect of substrate type on bacterial community composition in biofilms from the Great Barrier Reef', the aim was to determine a standardised substrate for biofilm settlement that is suitable for long-term biofilm indicator studies in tropical coral reef ecosystems. Bacterial biofilm communities will be established *in situ* on different settlement substrata (i.e., glass slides, ceramic tiles, coral skeletons and reef sediments). Further, bacterial biofilm communities are characterised on a spatial (inner and outer nearshore sites, one representative reef for each) and temporal (wet and dry seasons) scale to gain first insights into whether season and/or location with the corresponding water quality cause bacterial community shifts. Findings will put forward a hypothesis for further research.

Chapter 3, 'Terrestrial runoff controls bacterial community composition in biofilms from the Great Barrier Reef' elaborates on the hypothesis put forward in **Chapter 2** and will demonstrate seasonal, water quality and location effects on a larger scale (repeated surveys over two years at five fringing reefs). It will be determined whether season, location and/or water quality affect bacterial communities in biofilms and if, which water quality parameter is the most decisive inducing those community changes.

Chapter 4, 'Interactive climate change and runoff effects alter O_2 fluxes and bacterial community composition of coastal biofilms from the Great Barrier Reef' aims to confirm field results from **Chapters 2 and 3** and further disentangle single effects and interactions of relevant environmental factors found to be determining in the field (i.e., temperature, light and nitrate availability). Further, we aim to reveal whether changes in bacterial communities also consequently alter biofilm function (oxygen fluxes).

In **Chapter 5**, 'Effects of ocean acidification on microbial community composition of, and oxygen fluxes through, biofilms from the Great Barrier Reef' further explores climate change disturbances (i.e., ocean acidification) on oxygen fluxes, biomass (C- and N-contents) and microbial community composition in biofilms. As marine microbes in response to ocean acidification are poorly explored and predictions are controversial, this chapter may provide some valuable new insights into whether biofilm-associated microbes are sensitive, adapt or buffer the effects of elevated pCO_2 and may contribute to understanding future coral reefs under climate change scenarios.

Overall, the suitability and potential of the application of bacterial biofilms as bioindicators of water quality is evaluated. Apart from community shifts, it is aimed to identify dominant target species and trends of those species in response to various environmental disturbances in an effort to suggest key bacterial bioindicator groups and/or species for future monitoring and coastal management programmes. Further, it is attempted to gain an understanding of whether changes in bacterial community composition also affect metabolic activity of biofilms. Finally, the effects of climate change and terrestrial runoff on biodiversity, community composition and productivity of bacterial biofilms and implications on future coral reef ecosystem health will be discussed.

Publication 1) **Witt, V.**, Wild, C. and Uthicke, S.
Effect of substrate type on bacterial community composition in biofilms from the Great Barrier Reef. This article has been published in FEMS Microbiology Ecology (2011) 323:188-195

Contributions: The project on biofilms on different substrata (MTSRF) was initiated by S. Uthicke. The particular idea was developed by V. Witt. Data sampling was conducted by V. Witt with the help of S. Uthicke. Data analyses and writing of the manuscript were conducted by V. Witt with improvements by C. Wild and S. Uthicke.

Publication 2) **Witt, V**., Wild, C. and Uthicke, S.
Terrestrial runoff controls bacterial community composition in biofilms from the Great Barrier Reef. This article is in review for Applied and Environmental Microbiology.

Contributions: The project on biofilms and terrestrial runoff (MTSRF) was initiated by S. Uthicke. The particular idea was developed by S. Uthicke. Data sampling and analyses were conducted by V. Witt with the help of S. Uthicke. Writing of the manuscript was conducted by V. Witt with improvements by C. Wild and S. Uthicke.

Publication 3) **Witt, V.,** Wild, C. and Uthicke, S.
Interactive climate change and runoff effects alter O_2 fluxes and bacterial community composition of coastal biofilms from the Great Barrier Reef. This article is in press in Aquatic Microbial Ecology.

Contributions: The project effects of terrestrial runoff and climate change on biofilms (MTSRF) was initiated by S. Uthicke. The particular idea was developed by S. Uthicke. Data sampling, data analyses and writing of the manuscript were conducted by V. Witt with improvements by C. Wild and S. Uthicke.

Publication 4) **Witt, V.**, Wild, C., Anthony, K.R.N., Diaz-Pulido G., and Uthicke, S.
Effects of ocean acidification on microbial community composition of, and oxygen fluxes through, biofilms from the Great Barrier Reef. This article has been published in Environmental Microbiology (2011) 13:2976-2989

Contributions: The project on biofilms and ocean acidification (DFG, and MTSRF) was initiated by C. Wild. The particular idea was developed by C. Wild, S. Uthicke, K.R.N. Anthony and G. Diaz-Pulido. Data sampling was conducted by V. Witt with the help of C. Wild. Data analyses and writing of the manuscript were conducted by V. Witt with improvements by C. Wild, S. Uthicke, K.R.N. Anthony and G. Diaz-Pulido.

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- 2 -

EFFECT OF SUBSTRATE TYPE ON BACTERIAL COMMUNITY COMPOSITION IN BIOFILMS FROM THE GREAT BARRIER REEF

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ABSTRACT

Natural and anthropogenic impacts such as terrestrial runoff influence the water quality along the coast of the Great Barrier Reef (GBR) and may in turn affect coral reef communities. Associated bacterial biofilms respond rapidly to environmental conditions and are potential bioindicators for changes in water quality. As a prerequisite to study the effects of water quality on biofilm communities, appropriate biofilm substrates for deployment in the field must be developed and evaluated. This study investigates the effect of different settlement substrates (i.e., glass slides, ceramic tiles, coral skeletons and reef sediments) on bacterial biofilm communities grown *in situ* for 48 d at two locations in the Whitsunday Island Group (Central GBR) during two sampling times. Bacterial communities associated with the biofilms were analysed by terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of 16S rRNA genes. Findings revealed that substrate type had little influence on bacterial community composition. Of particular relevance, glass slides and coral skeletons exhibited very similar communities during both sampling times, suggesting the suitability of standardised glass slides for long-term biofilm indicator studies in tropical coral reef ecosystems.

INTRODUCTION

Similar to coastal regions worldwide, local natural and anthropogenic impacts such as land runoff from agriculture deliver inorganic nutrients, sediments, freshwater and pesticides to the coastal and coral reef waters of the Great Barrier Reef (GBR) (Bell, 1991), and thereby influence the water quality of this ecosystem. Coral reefs harbour abundant bacterial biofilms that are crucial catalysts of biogeochemical nutrient cycling (Battin et al., 2003) and are therefore critical to reef ecosystem functioning. This underlines the necessity to understand community composition and function of microorganisms within coral reef-associated biofilms.

Marine biofilms are complex microbial communities comprising of surface-attached microorganisms embedded in an extracellular polymeric matrix (Mihm et al., 1981). The bacterial communities within biofilms respond rapidly to changing environmental conditions, and therefore bacterial community composition of artificially and field grown biofilms have previously been used as bioindicators for water quality in freshwater (Campbell et al., 2011), estuarine (Jones et al., 2007; Nocker et al., 2007) and temperate and polar coastal marine environments (Moss et al., 2006; Webster and Negri, 2006; Dang et al., 2008). In addition, biofilms may also be potential bioindicators for water quality in tropical coastal coral reef ecosystems (Kriwy & Uthicke 2011).

Previous research addressed the composition of bacterial communities in marine biofilms in response to various environmental parameters such as the effects of nutrients (Chiu et al., 2008), tides (Dobretsov and Qian, 2006), water depth (Webster et al., 2004), salinity and temperature (Lau et al., 2005; Chiu, 2006). These studies have neglected to examine the effect that the settlement substrate has on the composition of the developing bacterial community and used artificial substrates i.e. polystyrene dishes or glass slides only. Only two invertebrate larval settlement studies from harbour waters investigated the effect of different substrates and showed that bacterial communities in biofilms undergo temporal shifts from more different communities during colonisation and early developmental stages to more similar communities over time irrespective of the initial substrate type (Huggett et al., 2009; Chung et al., 2010). These studies were, however, limited to only artificial substrates i.e., glass slides coated in different chemicals to simulate different ‘wettability’ properties, deployed at one site only (Huggett *et al*. 2009), or subtidal biofilms on two substrates, i.e., granite and petri

dishes, at one deployment time only (Chung *et al*. 2010). Therefore, although these studies have shed some light onto the effects of substrates on bacterial community compositions in marine biofilms, inferences on the suitability of various substrates for future studies cannot be drawn. This is especially the case for water quality bioindicator research, where substrates are required which on the one hand simulate or reproduce naturally occurring biofilm assemblages, but on the other hand are easy to deploy and sample and provide a standardised surface.

This study therefore evaluates the effects of various substrates on the bacterial community composition in biofilms from tropical coral reef ecosystems with the aim of providing better rationale for future bioindicator studies of water quality in these types of ecosystems. The criteria for the choice of substrate include ease of handling and removal of biofilm from the substrate, standardised size and resemblance of developed bacterial communities to those found on 'natural' substrates. We specifically examined bacterial community compositions using the molecular fingerprinting method terminal restriction fragment length polymorphism (T-RFLP) on two 'artificial' substrates, i.e., ceramic tile and glass slides, which are frequently used in aquatic biofilm studies, and two 'naturally occurring' substrates that were collected directly from the coral reef sampling area, i.e., coral skeletons and reef sediments. Further, the study extends previous knowledge by covering a more realistic time period for indicator biofilm development (i.e., 48 d), by incorporating temporal and spatial variability. Biofilms samples were collected in summer and winter (representing the annual water temperature extremes) at two locations (each at the end of a described inshore to offshore water quality gradient) to ensure that findings are not restricted to a single location or season. This investigation therefore results in recommendations on the best biofilm substrate for long-term water quality monitoring studies in coral reefs.

MATERIALS AND METHODS

STUDY SITE AND BIOFILM DEVELOPMENT

Four different substrates (glass slides, coral skeletons, reef sediments and ceramic tiles) were deployed for biofilm development. Glass microscope slides (Sail Brand) were pre-cleaned with 70% ethanol and fixed in polyvinyl chloride frames. Reef sediment (approximately 50:50 carbonate, silicate mixture) was collected from near-shore islands (Long, Lindeman,

Repulse) at 8 m depth in the Whitsunday Islands and sieved to a grain size of <100 µm >63 µm. The sediment was autoclaved and dried at 60 °C over night. Sediment was glued onto microscope glass slides with aquarium grade silicone (Selleys), dried for 24 h and fixed onto PVC frames. Coral cores from *Porites* sp. (cylinders of 2 x 2 cm) were autoclaved and unglazed ceramic tiles were sterilised by a 30 min UV treatment on each side. This study followed a hierarchical sampling design. Each substrate was deployed in duplicates at two replicate sites (25 m apart) at both Daydream Island (inshore, S 20°15.345′ E 148°48.729) and Deloraine Island (offshore, S 20°09.457′ E 149°04.183) (Supporting Information Fig. S1), therefore making 4 samples per substrate for each island. These two islands were positioned at each end of a previously described water quality gradient in the Whitsunday Islands of the central GBR (van Woesik et al., 1999; Cooper et al., 2007; Uthicke and Nobes, 2008; Uthicke and Altenrath, 2010; Kriwy and Uthicke, 2011b, a). Daydream Island (a permanent site of the long-term Reef Plan Marine Monitoring Program) was positioned inshore in 'low' water quality and Deloraine Island was positioned offshore in 'high' water quality (Table 1).

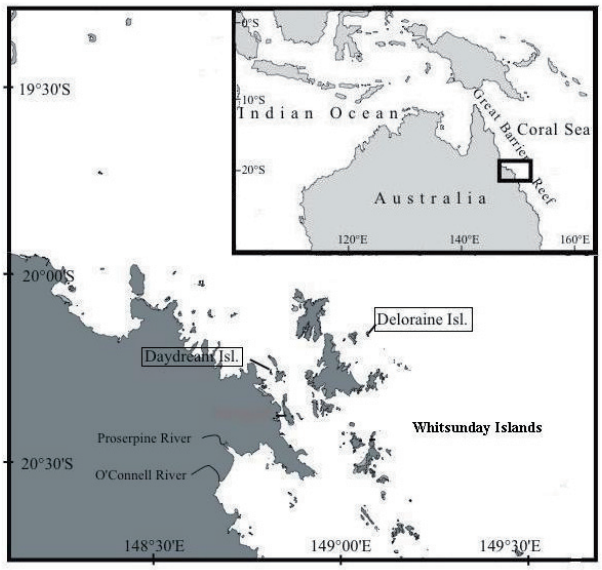

Fig. S1. Study site located in the Whitsunday Islands, Central Great Barrier Reef.

Table 1. Summary of analyses from the water column, irradiance variables and nearest distance from the coast of the Whitsunday Island sites. Water quality parameters for each year and season shown are chlorophyll *a*, turbidity, total suspended solids (TSS), temperature, light, salinity, dissolved inorganic nitrogen (DIN, includes NO_3, NO_2 and NH_4), dissolved inorganic phosphorus (DIP). Mean (standard deviation).

Island	Distance from Coast (km)	Season and year	Chlorophyll a ($\mu g\ l^{-1}$)	Turbidity (FLNTU)	TSS (mg l^{-1})	Temperature (°C)	Light (mmol m^{-1} d^{-1})	Salinity (ppt)	DIN ($\mu mol\ l^{-1}$)	DIP ($\mu mol\ l^{-1}$)
Daydream	3.0	Dry 2008	0.54 (0.12)	1.31 (0.56)	0.78 (0.21)	22.13 (1.21)	5.49 (17)	36.09 (0.59)	0.13 (0.07)	0.11 (0.04)
		Wet 2009	0.84 (0.11)	2.80 (1.96)	2.29 (0.41)	28.43 (0.41)	1.52 (30)	32.63 (1.27)	0.31 (0.12)	0.08 (0.04)
Deloraine	31.25	Dry 2008	0.34 (0.20)	-	0.78 (0.45)	21.6 (0.58)	8.39 (31)	35.36 (0.43)	0.08 (0.07)	0.16 (0.05)
		Wet 2009	0.53 (0.14)	-	1.84 (0.60)	27.71 (0.40)	6.34 (30)	35.20 (0.54)	0.14 (0.09)	0.10 (0.07)

All parameters measured were generally lower during the winter dry season than the summer wet season and higher inshore at Daydream Island compared to offshore at Deloraine Island, except light and salinity which showed the inverse trend. The water quality measurements are consistent with data obtained from the same monitoring sites along the water quality gradient from previous years (Cooper et al., 2007; Schaffelke et al., 2010). Substrates were deployed on two separate times (48 d during austral winter of August - October 2008, average temperature 21 °C and austral summer of January - February 2009, average temperature 29 °C) to represent annual water temperature extremes. In summary, there were two islands with two sites each where duplicate substrates were deployed. These were sampled at two different times giving a total of 16 samples per substrate. Substrates were deployed at 6 m water depth (below the lowest astronomical tide level) for ~48 d, and were vertically mounted approximately 40 cm from the underlying sediment on steel pickets (covered by ziplock bags to avoid effects from leached iron) and secured by cable ties. For sample collection after ~48 days of deployment, as much as possible of the biofilm material was carefully scraped off the substrates into cryovials using sterile No. 11 scalpel blades (yield was usually > 2 g) and snap-frozen in liquid nitrogen and stored at -80 °C until further processing.

WATER QUALITY

Water quality samples were obtained and analysed as described in detail in (Schaffelke et al., 2010) and (Cooper et al., 2007). In short, duplicate samples from two depths at each location per sample time were analysed for dissolved inorganic nutrients (DIN, includes NH_4, NO_2, NO_3), dissolved inorganic phosphorus (DIP), total suspended solids (TSS), chlorophyll *a* and salinity. For particulate nutrients and chlorophyll *a* (Chl *a*) analysis, water samples were collected on pre-combusted glass fibre filters and analysed after acetone extraction. Samples for determining total suspended solids (TSS) were collected on pre-weighed 0.4 µm polycarbonate filters and TSS concentrations determined gravimetrically. Salinity was determined using a Portasal Model 8410A Salinometer (Guildline). Autonomous water quality instruments (Eco FLNTUSB Combination Fluorometer and Turbidity loggers; WET Labs, Philomath, USA) recorded turbidity (optical backscatter) and in situ temperature data. Light was measured with Odyssey light loggers equipped with wiping units as described in Uthicke & Altenrath (2010).

GENOMIC DNA EXTRACTION

Total DNA was extracted from 0.5 g (wet weight) of each biofilm sample using the MoBio UltraClean Soil Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol with the following modifications. Bead-beating (Mini-Bead-Beater, Biospec Products, Bartleville, OK, USA) (2 x 30 s) cycles were performed, 900 ml of S3 buffer was used, and DNA was eluted from the column with 2 x 50 µl of 1 x TE buffer. DNA extracts were examined by standard 1% agarose gel electrophoresis and quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR AMPLIFICATION, CLONING AND SEQUENCING

Bacterial 16S rRNA genes were amplified by PCR using the general bacterial 16S rRNA gene primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) (Sigma-Proligo, The Woodlands, TX, USA) (Marchesi et al., 1998). Each sample was amplified in triplicate 25 µl reactions containing 2.5 µM non-acetylated bovine serum albumin (New England Biolabs, USA), 2 µM (2 mM each) dNTP (Astral Scientific, Australia), 2.5 µM forward primer 63F, 1.25 µM reverse primer 1389R, 1 µM $MgCl_2$ (Qiagen), 1.25U HotStar Taq (Qiagen), 2.5 µl HotStar Buffer (Qiagen, Germany)

and ~2 ng of template DNA. Amplification was performed with an initial incubation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 90 sec, and a final extension at 72 °C for 10 min.

Since T-RFLP profiles from glass slides and coral skeletons were very similar, only communities from glass slides were cloned. Four clone libraries of bacterial 16S rRNA genes amplified from DNA extracted from biofilms grown on glass slides were constructed, and represent one library for each season at each location. Therefore, the clone libraries represent a) inshore at Daydream Island during summer, b) inshore at Daydream Island during winter, c) offshore at Deloraine Island during summer, and d) offshore at Deloraine Island during winter. Triplicate PCR reactions were performed for each of the four replicate biofilm samples from each of these representative 2 sampling locations (total of 8) and 2 sampling times (overall total 16), and were pooled accordingly for construction of the four clone libraries. Samples were then purified using the MinELUTE PCR Clean-Up Kit (Qiagen, Germany) and cloned using a TOPO-TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions. After blue-white screening colonies were checked for correct insert size using a colony PCR method using primers 63F/1389R. Per clone library, 96 randomly picked clones were then dispersed in LB media and 10% glycerol in 96-well plate format and sent to the Australian Genome Research Facility Ltd. (Brisbane, Australia) for purification and sequencing by an ABI3730 XL Automatic DNA Sequencer.

Retrieved sequences were trimmed and analysed manually using Chromas Lite 2.33 (Technelysium Pty Ltd., Australia), and submitted to the Greengenes NAST Aligner (DeSantis et al., 2006) for alignment of sequences to the Greengenes database. Greengenes NAST-aligned 16S rRNA gene sequences were checked for chimeras using Bellerophon Version 3 (Huber *et al*. 2004), and identified chimeras were excluded from further analysis. The NAST-aligned 16S rRNA gene sequences were submitted to the Greengenes batch sequence classifier [http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi], and taxonomic assignments for each sequence were recorded using NCBI taxonomy. All sequences were submitted to the GenBank Database (Accession numbers: JF261700-JF262029)

TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS

Bacterial 16S rRNA genes were PCR amplified using the same reaction mixture and

conditions as outlined for clone libraries, except that fluorescently labelled 5′-Cy5-labelled 63F (Sigma-Aldrich) was used (adapted from Wilson *et al*. 2008). Each individual biofilm sample was amplified in three replicate PCR reactions. The amplicons were pooled, purified and quantified as above. Each purified product (150 ng) was digested with the restriction enzyme MspI (New England Biolabs) according to the manufacturer's instructions. Digested fragments were desalted using the DyeEx 2.0 Spin Kit (Qiagen, Germany) and vacuum dried for 40 min at low temperature in the dark. Terminal restriction fragments (T-RFs) were resolved and visualized using the CEQ 8800 Genetic Analysis System (Beckman-Coulter, Fullerton, CA, USA) with a 600 bp size standard (Beckman-Coulter). Replicate samples were compared using the software T-align (Smith, 2005) with a range of 0.5 bp peak area to determine the consensus peaks between duplicates. The relative fluorescence intensity of the peak area of T-RFs was used as a relative abundance measure of dominant T-RFs in further statistical analyses detailed below. For verification of T-RFs, purified DNA from individual clones were analysed by T-RFLP using the same protocol as for environmental samples, except that 75 ng of digested PCR products generated from each clone was used. Each clone produced a single peak (T-RF), which was then manually matched to T-RFs identified from whole community T-RFLP analyses. Prior to statistical analyses, T-RF peak area values were third root transformed and standardised. Principal Component Analysis (PCA) was used to determine whether bacterial assemblages in samples grouped by substrate, location and/or season. The significances of assemblage dissimilarities between substrates, seasons and locations were tested, by applying one-way Analysis of Similarity (ANOSIM) based on permutation procedures using the Bray-Curtis distance measure. The contributions of each taxon to the total dissimilarities of treatments were analysed using the Similarity Percentage (SIMPER) routine. All analyses were performed using the PAST statistical software (Hammer et al., 2001). One-way Analysis of Variance (ANOVA) was performed using the NCSS 2007 (NCSS, USA) statistical software to determine significant differences between relative abundances (peak area) for taxa at different locations.

RESULTS

The effect of substrate type on bacterial community structure in biofilms was examined by T-RFLP for the whole dataset (pooled from both sampling times and locations). Biofilm communities were very similar, regardless of the settlement substrate. PCA analysis showed that bacterial communities were largely overlapping for all substrates. PCA analyses also

suggested that biofilms grown on glass slides and coral skeletons were most similar to each other, while the reef sediments displayed the highest variability between replicate samples (Fig. 1).

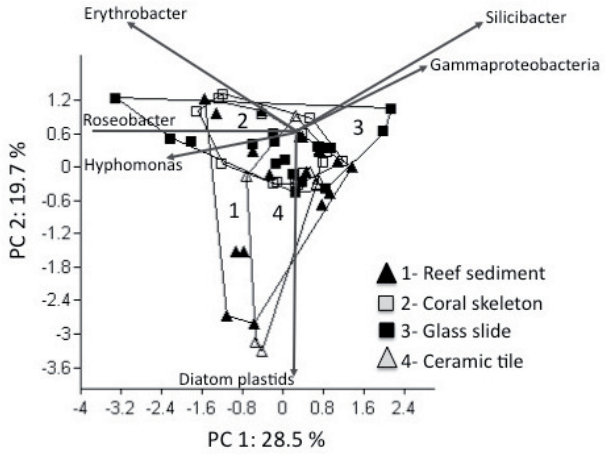

Fig. 1 Principal Component Analysis (PCA) incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing bacterial assemblages of each substrate independent of location and season (1, reef sediment: filled triangle; 2, coral skeleton: square; 3, glass slide: filled square; 4, ceramic tile: triangle). 15 % of the vectors are shown in the biplot and are labelled with the bacterial taxon.

For the global dataset, no significant differences in community structure among substrates could be detected by ANOSIM analysis (R= 0.039, p= 0.090). PCA analyses also suggested similar community structures occurred among different substrates when sampling times were analysed separately (Fig. 2A and 2B), although small but significant differences in bacterial community structures on different substrates within both sampling times were determined (ANOSIM summer: R= 0.122, p= 0.0316; winter: R= 0.175, p= 0.0059).

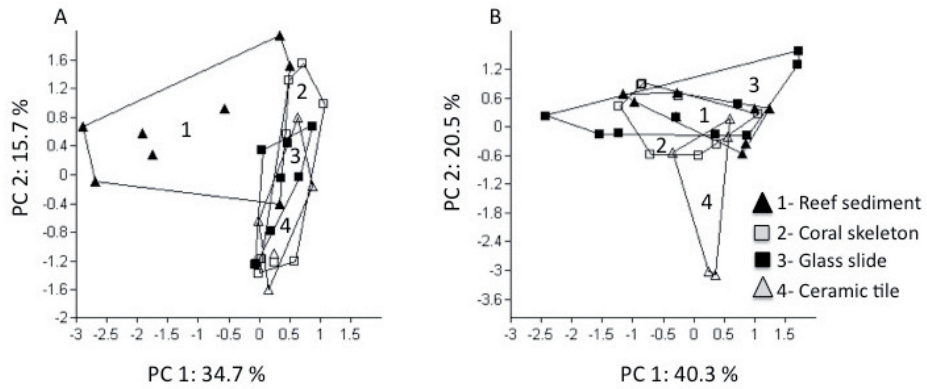

Fig. 2 Principal Component Analysis (PCA) incorporating relative abundances of T-RFs (using the

relative fluorescence peak intensity matrix) showing bacterial assemblages of each substrate at different sampling events A) winter and B) summer. (1, reef sediment: filled triangle; 2, coral skeleton: square; 3, filled glass slide: square; 4, ceramic tile: triangle).

For samples collected in winter, post hoc tests revealed that the only significant difference was between ceramic tile in comparison to reef sediments and coral skeletons (Table 2). Although the overall ANOSIM test of different substrates for the summer was significant (R= 0.122, p= 0.037), post hoc tests showed no significant effect between individual substrates (p > 0.05) (Table 2). When the substrate data was compared for each location, the four substrates were statistically indistinguishable (ANOSIM p= 0.0949) offshore at Deloraine Island, while inshore at Daydream Island significant differences were found for reef sediments compared to the remaining three substrates (p= 0.0009) (Fig. 3A and 3B).

Table 2. Table showing R and p values of the Analysis of Similarity (ANOSIM) on the relative abundance of terminal restriction fragments (T-RFs) (using the relative fluorescence peak intensity matrix) as derived by terminal restriction fragment length polymorphism (T-RFLP) grouped by substrate type for each sampling time (summer and winter). R and p values for the summer are above the diagonal, those within the winter are below. Overall ANOSIM for the summer: R= 0.122, p= 0.030; Winter R= 0.175, p= 0.006. P-values < 0.05 are highlighted for clarity.

	Reef sediment	**Coral skeleton**	**Glass slide**	**Ceramic tile**
Reef sediment		R= 0.154 p= 0.065	R= 0.146 p= 0.080	R= 0.064 p= 0.239
Coral skeleton	R= 0.146 p= 0.063		R= 0.054 p= 0.198	R= 0.093 p= 0.163
Glass slide	R= 0.073 p= 0.165	R= 0.077 p= 0.147		R= 0.203 p= 0.102
Ceramic tile	**R= 0.371** **p= 0.009**	**R= 0.416** **p= 0.004**	R= 0.131 p= 0.1044	

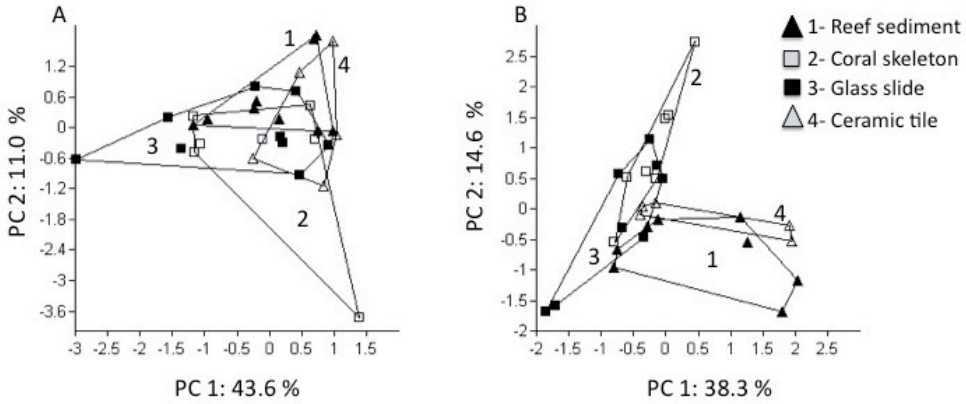

Fig. 3 Principal Component Analysis (PCA) incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing bacterial assemblages for all substrates at one A) inshore location (Daydream Island) and one B) offshore location (Deloraine Island). (1, reef sediment: filled triangle; 2, coral skeleton: square; 3, glass slide: filled square; 4, ceramic tile: triangle).

Although it was not the focus of the study, differences in bacterial community structures between the two sampling locations were examined in order to determine if the T-RFLP method is able to detect differences among bacterial assemblages, which are assumed to be due to differences in water quality. A PCA clearly separated the bacterial assemblages between the two locations and the two sampling times (Fig. 4). Replicates from each location were more variable during summer than winter, and more variable offshore than inshore (Fig. 4).

This result was confirmed by ANOSIM, which revealed significant differences between locations ($R= 0.544$, $p= 0.0177$) and sampling times ($R= 0.299$, $p < 0.0001$). The length of the species-vectors in the PCA biplot and a SIMPER analysis consistently indicated that T-RFs representing the *Roseobacter* clade *(Roseobacter* and *Silicibacter*), *Erythrobacter*, *Hyphomonas*, *Gammaproteobacteria*, and diatom plastids contributed mostly to the dissimilarities (54.9%) between substrates at different seasons and locations (Fig. 1) and between locations and sampling times despite substrate type (Fig. 4).

Overall, 37 terminal restriction fragments (T-RFs) were identified, of which, 89.2 % could be assigned to clones that were taxonomically identified from the clone libraries (within ± 0.5 bp) (Supporting Information Table S1), and thus could be assigned to a bacterial taxon. All T-RFs detected were present in the glass slide profiles.

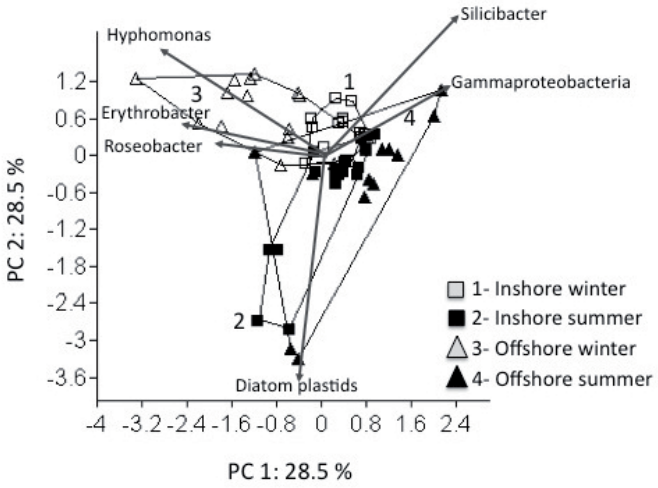

Fig. 4 Principal Component Analysis (PCA) incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing bacterial assemblages for different locations (inshore Daydream Island and offshore Deloraine Island) and sample times (winter and summer). (1, inshore winter: square; 2, inshore summer: filled square; 3, offshore winter: triangle; 4, offshore summer: filled triangle). 15 % of the vectors are shown in the biplot and are labelled with the bacterial taxon.

DISCUSSION

T-RFLP, cloning and sequencing of 16S rRNA genes revealed that coral reef associated biofilms comprised of complex bacterial and microalgal communities. Relatively similar, although not always identical, bacterial community structures were present on different substrate types over two sampling times (during a summer and a winter). Bacterial community composition on reef sediments differed significantly from the other substrate types at the inshore location that was influenced by pronounced changes in water quality during different seasons. Reef sediments also showed the largest variability in bacterial community composition among all investigated substrates. This suggests that reef sediments may have low reproducibility and is therefore not suitable for bioindicator studies in coral reefs in comparison to other more ideal substrates. Relatively variable bacterial community compositions were also identified on ceramic tiles in comparison to the other substrates during winter, suggesting that ceramic tiles are also not ideal substrates for bacterial biofilm bioindicator studies. In contrast, glass slides and coral skeletons substrates produced comparably stable and highly reproducible community compositions independent of sampling time and/or location.

Another aspect of substrate choice is the practical requirement for a simple method for the

removal of total and/or near complete biofilm biomass from the actual substrate. Removal of biofilms from rough, uneven surfaces such as those of the ceramic tile, reef sediment and coral skeleton is rather difficult. Although coral skeletons represent the most natural of all tested substrates, when regarding the ease of handling and removal of the biofilm, glass slides have the clear advantage in that their smooth, flat surfaces enable simple and rapid removal of most of the biofilm biomass. Considering that bacterial community structures on coral skeletons and glass slides were not significantly different, we propose the use of glass slides for future bioindicator studies.

Both spatial and seasonal influences (i.e., changes in water quality including light, salinity, turbidity, chlorophyll α) on bacterial community structures may have been responsible for some of the variability among certain substrates, rather than the actual substrate type. We suggest that all of the substrate types used in this study have relatively little influence on the bacterial community composition when examined after the relatively long deployment period (~48 d). Types of bacteria initially colonising and settling on specific substrates may be different depending on the surface properties of the substrate, however, biofilms undergo distinct temporal shifts, where the effect of substrate type diminishes, and tend to form more similar community structures over time (Huggett et al., 2009; Chung et al., 2010). In the present study, distinct bacterial communities were identified at the two different locations suggesting that discrete bacterial communities develop in response to the different environmental parameters found at the different locations rather than different substrates. Since our study sites were positioned at either ends of a clearly formed water quality gradient that is known from a continuous long-term monitoring program (Uthicke, 2010; Uthicke and Altenrath, 2010; Kriwy and Uthicke, 2011b, a) and from recently measured data (Table 1), we propose that this response was caused by differences in water quality at the two locations. The rationale to collect samples from two islands (representing extremes of a previously studied water quality gradient) and at two sampling times (representing the annual extremes in water temperature) was merely to test for substrate differences under a variety of environmental conditions and thus extends the validity of this study.

Given that differences between the bacterial community compositions at different sites could be easily detected, reproducible patterns among replicates were produced, and tentatively 89.2 % of the taxonomic affiliations of the T-RFs after comparison to sequence data produced

from clone libraries were identified. This study therefore suggests that T-RFLP is a suitable and rapid, high-throughput fingerprinting method for detecting spatio-temporal and water quality-induced bacterial community shifts. Further support is given by the fact that dominant bacterial taxa identified by this method (e.g., *Roseobacter*, *Rhodobacteraceae*) were similar to those found in previous aquatic biofilm studies using glass slides (Dang and Lovell, 2000; Jones et al., 2007).

In summary, this study suggests that when biofilms are subjected to long-term deployment (weeks to months), as presented here, simple glass slides enable the formation of bacterial biofilm communities that are highly similar to other 'natural' substrates such as coral skeletons or reef sediment grains. Additional advantages for the use of glass slides include a standardised size, low cost, ease of handling and the formation of relatively reproducible bacterial community structures among replicates. This study therefore also provides further evidence that monitoring bacterial communities associated with coastal biofilms may find application as a bio-monitoring tool for environmental management for examining local and regional changes in water quality in the long-term. Future work should include more in-depth studies of the bacterial communities grown in different water qualities over replicate seasons.

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SUPPORTING INFORMATION

Supporting Information Table S1. Terminal restriction fragments (T-RF) are shown with their phylogenetic affiliation (as classified using the greengenes batch sequence classifier [http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi] using the NCBI taxonomy) and accession number as derived from the 16S rRNA gene clone libraries from Daydream and Deloraine Island, Whitsunday Islands, Australia. T-RF sizes (bp), the Greengenes OTU ID, the 16S rRNA identification of the nearest phylogenetic neighbour (with accession number) and sequence similarity (%) to each corresponding T-RF as determined by BLAST from the Greengenes database are shown.

Clone and accession number	Greengenes classifier OTU ID	T-RF size (bp)	Nearest relative with accession number	Sequence similarity (%)
Alphaproteobacteria				
Hyphomonas (JF261762)	2120	116	Coral clone Gven_P10 (GU118388.1)	98.3
Erythrobacter (JF261950)	2325	113	*Erythrobacter* sp. D3043 (DQ480144.1)	99.4
Roseobacter (JF261797)	2190	401	Sponge clone B24 (FJ999598.1)	97.0
Rhodobacteraceae (JF261798)	1731	402	Coral clone SGUS538 (FJ202078.1)	97.2
Ruegeria (JF261782)	2196	400	Diseased coral tissue clone SHFG537 (FJ203135.1)	99.7
Pseudorugeria (JF261850)	2174	404	Dinoflagellate clone (FJ644602.1)	98.2
Phaeobacter (JF261709)	2169	403	Marine hydrocarbon seep sediment clone EthaneSIP4-6-33 (GU584519.1)	98.3
Gammaproteobacteria				
Glaciecola (JF261723)	2801	513	Seawater isolate E3marine str. E3 (EU183316.1)	96.6
Pelagiobacter (JF261854)	3123	107	Coastal water clone ARTE4_238 (GU230322.1)	98.5
Oceanospirillum (JF261814)	3089	96	Sediment clone LC3-5 (DQ289914.1)	96.6
S-oxidising symbiont (JF261830)	3163	125	Sediment clone T12d-oil (FM242420.1)	97.2
Alteromonadaceae (JF261844)	2777	84	Sediment clone Ucc1549 (AM997955.1)	96.9
Thermodesulfovibrio (JF261783)	1911	124	Sediment clone (GQ246343.1)	90.1
Bacteroidetes				
Psychroserpens (JF261771)	814	509	*Winogradskyella* sp. Str. K7-7 (FJ425226.1)	99.7
Flexibacter (JF261796)	929	106	Sponge cortex clone TAA-5-44 (AM259882.1)	97.3
Flexibacteraceae (JF261789)	883	437	Sponge cortex clone TAA-5-103 (AM259881.1)	97.2
Aquimarina (JF261820)	742	93	*Aquimarina* sp. MOLA (AM990846.1)	92.5

Cytophaga (JF261832)	792	507	Deep-sea sediment clone BD2-17 (AB015545.1)	94.7
Candidatus Amoebophilus (JF261845)	711	55	Sponge clone Hg92C2 (EU236396.1)	96.3
Actibacter (JF261793)	736	54	*Crocinitomix catalasitica* str. IFO 15977 (AB078042.1)	93.2
Flavobacteriaceae (JF261869)	821	510	Sphingobacteriales clone B2706_C7 (EF092231.1)	95.0
Flavobaceriaceae (JF261811)	735	118	Flavobacteriaceae str. SW062 (AF493684.1)	96.9
Deltaproteobacteria				
Desulfuromonas (JF261784)	2672	126	Marine sediment clone (GU302422.1)	98.2
Desulfomonile (JF261788)	2730	127	Tidal flat sediment clone MSB-1B5 (EF125392.1)	99.2
Cyanobacteria				
Synechococcus (JF261753)	1921	456	*Oscillatoria rosea* str. M-220 (AB003164.1)	98.0
Diatom plastids				
Stramenopiles (JF261711)	3909	458	*Aureoumbra lagunensis* str.CCMP1507 (GQ231542.1)	95.5
Phaeodactylum (JF261837)	3608	459	Chloroplast clone Dstr_E19 (GU119626.1)	99.6
Odontella (JF261851)	3614	458.5	*Fucus vesiculosus* (DQ307678.1)	97.6
Nanofrustulum (JF261843)	3649	460	*Pylaiella littoralis* Pla. (X14873.1)	97.0
Gyrosigma (JF261839)	3609	464	*Amphora coffeaeformis* str. CCAP 1001/1 C107 (FJ002183.1)	99.1
Haslea (JF261789)	3606	461	*Haslea nipkowii* (AF514850.1)	96.1
Firmicutes				
Carboxydothermus (JF261862)	1582	270	Sediment clone 35-49 (DQ833489.1)	90.5
Unclassified				
Unclassified bacterium (JF261785)	641	117	Sphingobacteriales clone B2706_C7 (EF092231.1)	95.1

- 3 -

TERRESTRIAL RUNOFF CONTROLS BACTERIAL COMMUNITY COMPOSITION OF BIOFILMS ALONG A WATER QUALITY GRADIENT IN THE GREAT BARRIER REEF

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ABSTRACT

Terrestrial runoff from land-based activities influences water quality along the Great Barrier Reef (GBR) coastline, thereby leading to reduced light and increased nutrient availability for coral reef communities. Microbial biofilms are potential indicators for changes in water quality because they integrate environmental changes. We investigated bacterial community composition of biofilms from repeated surveys during two distinct seasons over two years established for ~48 d at five reefs along a water quality gradient in the Whitsunday Islands, GBR. Microbial communities were profiled using terminal restriction fragment length polymorphism (T-RFLP), after which a PERMutational Multivariate ANalysis Of VAriance (PERMANOVA) was conducted that indicated significant dissimilarities between inner and outer nearshore communities during both seasons. In a distance-based redundancy analysis (dbRDA) of T-RFLP data, dissolved organic carbon (DOC) and chlorophyll *a* (Chl *a*) concentrations explained most of the community data variation. Outer nearshore reefs with high light and low nutrient availability displayed a predominance of *Cyanobacteria* and *Roseobacter* (*Alphaproteobacteria*) correlated with low Chl *a* concentrations, hence these groups may be indicators of oligotrophic conditions. Predominance at inner nearshore sites of *Alteromonadaceae* (*Gammaproteobacteria*), *Bacteroidetes* and diatom plastids, closely correlated with high Chl *a* concentrations and *Flavobacteriaceae* with low DOC concentrations, may be indicators of eutrophication. The observed spatio-temporal distribution of predominant microbes may be related to their function. Season- and water quality-driven shifts in key microbial groups suggest microbial biofilms are good bioindicators for water quality. Further, we suggest that terrestrial runoff influences microbial biodiversity likely having implications for coral reef ecosystem functioning.

INTRODUCTION

Tropical coral reefs are facing global climate change and local-scale disturbances at an increasing rate (Wilkinson, 2004; Fabricius et al., 2007; Hoegh-Guldberg et al., 2007). From the global scale, warming sea surface temperatures and ocean acidification affect coral-algal dynamics in tropical coastal regions worldwide (Anthony et al., 2011; Diaz-Pulido et al., 2011; Fabricius et al., 2011). Yet, from the local scale, natural (e.g., monsoonal rainfalls, cyclones) and anthropogenic (e.g., land-based activities, overfishing) impacts result in terrestrial runoff which deteriorate water quality, putting coastal regions at greater risk (GESAMP, 2001).

The Proserpine river catchment in the Whitsunday Island area located in the Central GBR in Australia has experienced extensive land-clearing to facilitate agriculture (i.e., sugar cane farming and grazing) and hence is considered a priority coastal management region (Furnas, 2003). In particular during the summer wet season, the O'Connell and Proserpine river flood plumes deliver varying levels of freshwater, sediments and nutrients (Bell, 1991; Devlin and Schaffelke, 2009) onto coastal inshore reefs. For example, nutrient discharge by rivers is the largest source of new nitrate input (Furnas, 2003) and has increased up to 10-fold since pre-agricultural times (Wooldridge et al., 2006). Terrestrial runoff inshore of the Whitsunday Islands area leads to higher concentrations of nutrients, Chl *a* and suspended sediments, which decrease gradually with distance from the coast, forming a distinct cross-shelf water quality gradient (van Woesik et al., 1999; Furnas, 2003; Fabricius, 2005; Brodie et al., 2007; Cooper et al., 2007). Resulting eutrophication and increased turbidity further leads to low light availability at inner nearshore reefs compared to outer inshore reefs (Brodie et al., 2007; Cooper et al., 2007). During the summer wet season reduced salinity and increased sea surface temperatures further influences water quality at these coastal coral reefs. This water quality gradient in the Whitsunday Island area has been confirmed to be persistent and meanwhile has become a permanent site for coastal water quality monitoring (Cooper et al., 2007) (Schaffelke et al., 2010; Uthicke, 2010). Significant variations in different reef community structure and water quality parameters with increasing distance from the Proserpine and O'Connell Rivers have been demonstrated (Cooper et al., 2007; Fabricius et al., 2007; Uthicke and Altenrath, 2010). For example, coral reefs affected by eutrophication and sedimentation show significantly reduced coral growth and recruitment (van Woesik et al., 1999; McCook, 2001; Fabricius et al., 2003) and increasing algae-coral habitat space competition with consequent shifts in coral reef dynamics (Szmant, 2002; Fabricius, 2005; Schaffelke, 2005). Further, inshore locations show an

increased occurrence of pathogenic bacteria in corals (Haapkyla et al., 2011).

Tropical coral reefs harbour abundant microorganisms, predominantly forming surface-attached micro colonies coalesced into extracellular polymeric matrix enclosed architectures (Mihm et al., 1981). Microbial biofilm communities are essential components of oligotrophic coral reef systems, contributing importantly to ecosystem productivity, large-scale biogeochemical fluxes (Lock et al., 1984; Battin et al., 2003) and coral reef resilience (i.e., invertebrate larval settlement and metamorphosis) (Wieczorek and Todd, 1998; Webster et al., 2004). Bacterial communities are highly responsive indicators of changing environmental conditions as a consequence of their rapid life cycle (Paerl and Pinckney, 1996) and ability to structurally self-organize (Tolker-Nielsen and Molin, 2000). Therefore, bacterial biofilms have previously been tested as indicators for water quality conditions in riverine (Araya et al., 2003), estuarine systems (Snyder et al., 2005; Moss et al., 2006; Jones et al., 2007; Nocker et al., 2007), polar regions (Webster and Negri, 2006) and temperate coastal marine environments (Dang et al., 2008). As in other aquatic environments, marine coastal biofilms associated with coral reefs may also find application as a biomonitoring tool of transient spatial and temporal variability as well as more persistent ecosystem change due to large-scale catchment management decisions or global climate change.
Despite the suitability of bacterial biofilm communities as environmental biondicators, biofilms in tropical coral reefs have barely been investigated. The few available field-based reports have documented changes in bacterial community structure in sediments (Uthicke and McGuire, 2007) and biofilms (Kriwy and Uthicke, 2011; Witt et al., 2011a), but these were not replicated across various seasons or years (Kriwy and Uthicke, 2011; Witt et al., 2011a). These studies suggested a higher abundance of *Cyanobacteria*, *Cytophaga-Flavobacteria-Bacteroides* and *Alphaproteobacteria*, including the *Roseobacter* clade, at outer nearshore reefs (Uthicke and McGuire, 2007; Kriwy and Uthicke, 2011; Witt et al., 2011a) due to high light and low nutrient availability, while opposite water quality conditions at inner nearshore reefs displayed lower bacterial diversity with significantly higher abundances of diatoms (Gottschalk et al., 2007) and *Gammaproteobacteria* (Kriwy and Uthicke, 2011).

In an effort to better understand how coastal marine ecosystems are influenced by anthropogenic (i.e., land runoff), climatic (i.e., seasonal increases in sea surface temperature) factors and terrestrial run off, we investigate how bacterial biofilm communities shift. This study therefore investigated the effects of water quality and, in particular, addressed seasonality on coastal

bacterial biofilms, by the deployment of glass slides for ~48 d during two summer wet (Jan 2009 and 2010) and two winter dry seasons (Aug 2008 and 2009) at five nearshore islands with increasing distance from the coast in the Whitsundays Islands. We used multivariate statistical techniques to test for effects of location and season, and to identify which of the water quality parameters explain variance in the dataset. Bacterial community data from terminal restriction fragment length polymorphism (T-RFLP) analysis and 16S rRNA gene clone libraries was used to unravel the most decisive water quality parameters for the detected community changes and evaluated whether coastal bacterial biofilms may serve as an early warning system and sensitive monitoring tool relevant for future tropical coral reef health.

MATERIALS AND METHODS

STUDY SITE SELECTION AND BIOFILM SUBSTRATE DEPLOYMENT

The study area included a previously well-described water quality gradient (van Woesik et al., 1999; Cooper et al., 2007) comprising of two inner nearshore (< 10 km from the coast) and two outer nearshore (> 30 km) islands in the Whitsunday Islands, Central GBR (Table 1). Daydream, Pine and Double Cone Island (permanent sites of the long-term Reef Plan Marine Monitoring Program) are positioned inner nearshore subjected to higher nutrients and suspended sediments, and Deloraine and Edward Island, which are positioned outer nearshore less exposed to land runoff (van Woesik et al., 1999; Cooper et al., 2007; Uthicke and Nobes, 2008; Uthicke and Altenrath, 2010; Kriwy and Uthicke, 2011).

Table 1. Location of sites in the Whitsunday Islands, central Great Barrier Reef, Queensland, Australia with GPS coordinates, location category (inner or outer nearshore), distances to shore, and depths where biofilm samples were deployed.

Site	Nearshore location (distance from shore in km)	Coordinates	Depth (m)
Pine	inner (5)	S 20°22.679′ E148°53.302	6
Daydream	inner (3)	S 20°15.345′ E 148°48.729	6
Double Cone	inner (10)	S 20°06.295′ E 148°43.305	6
Edward	outer (37)	S 20°14.693 E 149° 10.302	6
Deloraine	outer (31.25)	S 20°09.457′ E 149°04.183	6

Standard glass microscope slides (75 x 25 mm) were used for biofilm settlement as these allow growth of very similar bacterial communities as those established on natural coral skeleton substrata, as discussed in (Witt et al., 2011a). Initial settlement of bacteria as biofilm communities on different substrate types differs due to the physical properties of the substrata (Huggett et al., 2009; Sweet et al., 2011), however, the effect of substrate type diminishes and bacterial communities become more similar over time (Huggett et al., 2009; Chung et al., 2010). Glass microscope slides were pre-cleaned with 70 % ethanol, rinsed with sterile water and fixed in polyvinyl chloride frames. Three replicate glass slides were deployed at two replicate sites (25 m apart) at each of the five islands. By SCUBA, frames were vertically mounted at six m water depth (below the lowest astronomical tide level) approximately 30 cm from the underlying sediment on steel pickets (covered by zip lock bags to avoid effects from leached iron) and secured by cable ties. Biofilms were developed repeatedly for ~48 d during two replicate dry seasons (August, average seasonal temperature 22 °C) and two replicate wet seasons (January, average seasonal temperature 29 °C) over the course of two years (2008 - 2010). At the end of the study, this setup yielded six replicate samples per island and 60 per season (total of 120 samples). After ~48 d, biofilms were sampled for subsequent microbial community analyses by carefully scraping off the biofilm material from the glass substrate into cryovials using sterile No. 11 scalpel blades (yield was usually ~4 g), immediately frozen in liquid nitrogen and stored at -80 °C until further processing.

WATER QUALITY MEASUREMENTS

Water quality here is defined by Chl *a* concentration, total suspended solids (TSS), Secchi depth, dissolved inorganic nitrogen (DIN, the sum of NH_4, NO_2, NO_3), dissolved organic carbon (DOC) and the physical characteristics of temperature and salinity. Water quality samples were obtained and analysed as described in detail in (Cooper et al., 2007) and (Schaffelke et al., 2010). In short, duplicate samples from two water depths at each location per sample time were analysed for DIN, TSS, Chl *a* and salinity. For particulate nutrients and Chl *a* analysis, water samples were collected on pre-combusted glass fibre filters and analysed after acetone extraction. TSS samples were collected on pre-weighed 0.4 µm polycarbonate filters and concentrations were determined gravimetrically. Salinity was determined using a Portasal Model 8410A Salinometer (Guildline). Light was measured with Odyssey light loggers equipped with wiping units as described in Uthicke & Altenrath (2010) and as light permitted, a Secchi disk depth reading was taken at each sampling site.

GENOMIC DNA EXTRACTION, PCR AMPLIFICATION, CLONING AND SEQUENCING

Total DNA was extracted from 0.25 – 0.5 g of the total biofilm (wet weight) sample using the MoBio UltraClean Soil Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol with the following modifications as in Witt et al. (2011a). Bacterial 16S rRNA genes were amplified by PCR using 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R primers (5′-ACGGGCGGTGTGTACAAG-3′) (Sigma-Proligo, The Woodlands, TX, USA) (Marchesi et al., 1998). Each biofilm sample was amplified in triplicate 25 µl reactions containing 2.5 µM non-acetylated bovine serum albumin (New England Biolabs, USA), 2 µM (2 mM each) dNTP (Astral Scientific, Australia), 2.5 µM forward primer 63F, 1.25 µM reverse primer 1389R, 1 µM $MgCl_2$ (Qiagen), 1.25U HotStar Taq (Qiagen), 2.5 µl HotStar Buffer (Qiagen, Germany) and ~2 ng of template DNA. Amplification was performed with an initial incubation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1.30 min, and a final extension at 72 °C for 10 min.

Eight clone libraries of bacterial 16S rRNA genes amplified from DNA extracted from biofilms grown on glass substrate were constructed, and represent one library for each island and season. DNA extracted from each biofilm sample from all six replicates of each island was then subject to PCR in each triplicate PCR reactions to avoid PCR bias. Triplicate amplicons of each of the six samples island were then individually pooled back to the original six replicates. Each of the six replicates from each island was then pooled according to island and season for construction of the eight clone libraries. Pooled samples were purified using the MinELUTE PCR Clean-Up Kit (Qiagen) and cloned using a TOPO-TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions. After blue-white screening, colonies were checked for correct insert size using a colony PCR method with the specific sequencing primer 63F. Randomly picked clones were dispersed in Luria-Bertani (LB) media and 10 % glycerol in 96-well plate format and sent to the Australian Genome Research Facility Ltd. (Brisbane, Australia) for purification and sequencing by an ABI3730 XL Automatic DNA Sequencer.

ANALYSIS OF CLONE SEQUENCES

Retrieved sequences were edited using Chromas Lite 2.33 (Technelysium Pty Ltd., Australia), saved as fasta files and submitted to the Greengenes NAST Aligner (for alignment of sequences to the Greengenes database) (DeSantis et al., 2006). Greengenes NAST-aligned 16S rRNA gene sequences were checked for chimeras using Bellerophon Version 3 (Huber et al., 2004), and

identified chimeras were excluded from further analysis. The NAST-aligned 16S rRNA gene sequences were submitted to the Greengenes batch sequence classifier [http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi] (McDonald et al., 2011), and taxonomic assignments for each sequence were recorded using the NCBI taxonomy system. All sequences were deposited under GenBank Accession numbers: JQ726882-JQ727208.

TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) ANALYSIS

Bacterial 16S rRNA genes were PCR amplified using the same reaction mixture and conditions outlined for clone libraries, except that fluorescently labelled 5′-Cy5-labelled 63F (Sigma-Aldrich) forward and unlabelled 1389R reverse primers were used as stated in Witt et al. (2011). DNA template was diluted 1:10 in nuclease free water and cycling conditions were 32 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 90 sec. After PCR, samples were prepared for T-RFLP analysis according to Witt et al. (2011a). Terminal restriction fragments (T-RFs) were resolved and visualized using the CEQ 8800 Genetic Analysis System (Beckman-Coulter, Fullerton, CA, USA) with a 600 bp size standard (Beckman-Coulter). Replicate samples were compared using the software T-align (Smith, 2005) with a range of 0.5 bp peak area to determine the consensus peaks between duplicates. The relative fluorescence intensity of the peak area of T-RFs was used as a relative abundance measure for T-RFs in further statistical analyses detailed below.

STATISTICAL ANALYSIS

Bacterial community data (T-RF values) were square root transformed and standardised to relative abundances. Principal component analysis (PCA) was used to determine whether bacterial assemblages group by location or season. The assemblage dissimilarities between location and season were tested by applying two-way Permutational Multivariate Analysis of Variance (PERMANOVA) based on permutation procedures (9999 permutations) using the Bray-Curtis distance measure and *p* values derived from Monte-Carlo (p(MC)) simulations. Pairwise t-tests were used as post hoc test. The contributions of each taxon to the total dissimilarities of treatments were analysed using the Similarity Percentage (SIMPER) routine and represented by vectors in the PCA. Double Cone Is. was excluded from the PCA, as according to the water quality data we cannot clearly categorise this as an inner or outer nearshore island.

Prior to analysis, environmental water quality data was averaged over sample seasons, locations and years and z-transformed (average = 0, sd = 1) to accommodate different measurement units of these parameters. To determine the relationship between water quality parameters and bacterial communities a distance-based redundancy (dbRDA) analysis was performed. The dbRDA was constrained by the statistically significant explanatory variables from multivariate multiple regression model (DistLM) marginal tests using a Bray-Curtis distance matrix based on permutation procedures (9999 permutations). As this procedure does not discriminate between location (or season) the data from Double Cone Island was included. Principal Component Analysis, PERMANOVA, DistLM and dbRDA (McArdle and Anderson, 2001; Clarke, 2006) were performed using the Primer 6.0 statistical software (Clarke, 2006). To further determine significant differences between relative abundances (peak area) of contributing T-RFs, two-way Analysis of Variance (ANOVA) was performed using the Number Cruncher Statistical System 2007 statistical software (NCSS, USA) (Hintze, 2007).

RESULTS

WATER QUALITY DATA

All parameters measured were generally lower during the winter dry season than the summer wet season, and higher at inner (Pine, Daydream and Double Cone Islands) compared to outer (Deloraine and Edward Islands) nearshore locations (e.g., DIN, more than 2-fold higher at inner nearshore reefs), except for salinity showing the inverse trend due to diluting effects of heavy seasonal rainfall during the wet season (Supplementary Table 1).

COMMUNITY ANALYSIS USING T-RFLP AND CLONE LIBRARIES

Analysis of sequences obtained from the 16S rRNA gene clone libraries demonstrated that coral reef-associated biofilms comprised of complex bacterial and microalgal communities. *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* were consistently the most frequent groups found in bacterial biofilm communities independent of location and season. *Bacteroidetes*-affiliated sequences were consistently more frequent inshore (20 - 30 %) than offshore (13 - 23 %) (Table 2) and at each location more frequent in the wet than the dry season. One exception was Pine Is. that displayed the opposite trend. Similarly, *Gammaproteobacteria*-affiliated sequences were more frequent in the wet than the dry season and overall where equally abundant at inner and outer nearshore reefs, while *Alphaproteobacteria*-affiliated sequences were more frequent in the dry compared to the wet season at each location, with Daydream Is. as

an exception. *Cyanobacteria* were more abundant at outer than inner nearshore reefs and more frequent during the wet than the dry season (Table 2) and diatom plastids were predominantly found more at inner than outer nearshore reefs.

Table 2. Numbers indicate relative proportions of the indicated phylogenetic groups in percent (%) of the total clone library (n)

Island	Pine		Daydream		Deloraine		Edward	
Nearshore location	inner				outer			
Season	dry	wet	dry	wet	dry	wet	dry	wet
Clones sequenced	n= 84	n= 78	N= 85	n= 86	n= 84	N= 82	n= 85	n= 81
Bacteroidetes	32.14	20.51	25.41	31.4	13.1	23.17	14.12	18.50
Cyanobacteria	2.38	6.41	0	1.60	2.38	8.54	3.53	19.75
Firmicutes	0	0	1.176	2.30	3.57	0	2.35	0
Alphaproteobacteria	13.10	10.26	18.8	25.60	64.3	20.73	15.29	9.80
Betaproteobacteria	0	0	0	1.60	0	0	0	0
Deltaproteobacteria	4.76	0	5.88	0	1.19	0	1.17	1.23
Gammaproteobacteria	14.29	34.62	20	23.30	10.7	20.73	24.71	24.69
Acidobacteria	0	0	0	0	0	0	0	1.23
Actinobacteria	2.38	0	0	0	0	0	0	0
Oceaniserpentilla	0	1.28	0	0	0	0	0	0
Nitrospirae	0	0	1.176	0	0	0	0	0
Unclassified bacteria	7.14	3.85	4.70	10.77	1.19	17.07	10.59	11.04
Diatom plastids	23.81	23.07	17.65	3.49	3.57	9.76	28.24	13.76

Bacterial community fingerprinting data combined with clone library data showed a total of 60 T-RFs, of which 80 % could be assigned to clones from the clone libraries, apart from 12 T-RFs that could not be confirmed by any of the clones (Supplementary Table S2). Permutational Multivariate Analysis of Variance (PERMANOVA) of the overall community data using the factors season (wet/dry) and location (inner/outer nearshore islands, excluding Double Cone Is. from this analysis), revealed that bacterial communities at inner and outer nearshore location were significantly different in community composition between season and island with a significant interaction term ($p < 0.05$) (2-way PERMANOVA Table 3A). Post-hoc tests of that interaction revealed that communities from both locations significantly differed between seasons (p (MC) < 0.05) and this interaction may be explained by the high variability of the community data from the wet season (Tables 3B and 3C).

Table 3. Results of PERMANOVA analysis using two groups location (inner and outer nearshore) and season (wet and dry) (A) and posthoc test for the interaction term using two groups (B) of bacterial biofilm assemblages. Tests are based on 9999 permutations. P (MC): P value based on Monte Carlo random draws. P-values < 0.05 are highlighted for clarity.

A) Overall PERMANOVA of location and season

Source	Df	SS	MS	Pseudo-F	P (MC)
Location	1	9232.2	9232.2	3.646	**0.0018***
Season	1	16235	16235	6.412	**0.0001***
L x S	1	7084	7084	2.798	**0.0107***
Residual	92	2.33×10^5	2531.9		
Total	95	2.66×10^5			

B) Posthoc tests within the interaction term (season within location)

	dry/wet	
Groups	T	P (MC)
Inner	2.2361	**0.0002***
Outer	2.0197	**0.0031***

C) Posthoc tests within the interaction term (location within season)

	inner/outer	
Groups	T	P (MC)
Dry	1.8562	**0.0115***
Wet	1.7241	**0.0109***

Principal component (PCA) analysis grouped by location showed overlap between microbial assemblages between locations, while microbial assemblages were more segregated when grouped by seasons (Fig. 1). The PCA further illustrates that the seasons form distinct assemblages within each location, which may explain the significant interaction term of the PERMANOVA (Fig. 1). Similarity percentage (SIMPER) analysis revealed an overall average dissimilarity of 79.2 % between locations and seasons with *Alteromonadaceae* (*Gammaproteobacteria*) (contribution 7.1 %), *Cyanobacteria* (6.1 %), *Flavobacteriaceae* (5.0 %), diatom plastid (4.8 %) and *Roseobacter* (*Alphaproteobacteria*) (4.0 %) contributing the most to these dissimilarities of the overall community composition. These groups were confirmed by the length of the vectors in the PCA biplot of T-RFLP data. One-way Analysis of Variance (ANOVA) showed a significantly higher relative abundance of *Roseobacter* (12.2 % higher at outer than inner) and *Cyanobacteria* (19.3 %) at outer nearshore locations than inner nearshore locations (ANOVA $F = 4.04$, $p = 0.0473$ and $F = 6.12$, $p = 0.0403$). Comparing seasonal changes in the relative abundances of the most contributing phylogenetic bacterial groups, *Flavobacteriaceae* were significantly higher (by 24 %) during the dry compared to the wet

season (ANOVA F = 7.07, p = 0.0092), while the relative abundance of *Alteromonadaceae* increased (by 300 %) in the wet season (ANOVA F = 34.45, p < 0.0001).

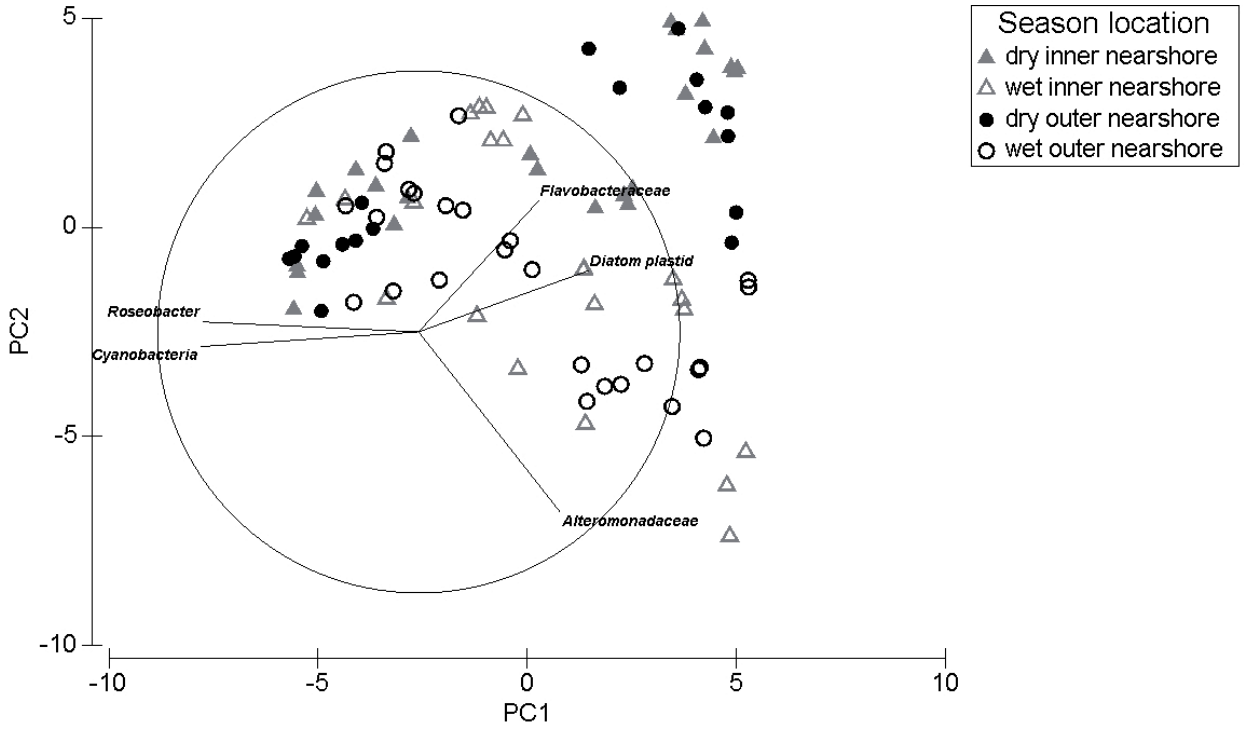

Fig. 1. Principal Component (PCA) analysis incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing bacterial assemblages at different locations and seasons (filled triangle = dry season/inner nearshore, triangle = wet season/ season/inner nearshore, filled circle = dry season/outer nearshore, circle = wet season/outer nearshore; PC1: 13.8 % PC2: 27.8 %). Vectors of the most importantly contributing T-RF (bp) as revealed by a Similarity percentage (SIMPER) analysis, are shown in the biplot and labelled with the corresponding taxon.

Distance-based redundancy analysis (dbRDA) was performed to test the relationship between microbial communities at from all five islands and water quality parameters. Seven environmental variables (total suspended solids (TSS), Salinity, dissolved inorganic nitrogen (DIN), dissolved organic carbon (DOC), chlorophyll *a* (Chl *a*), temperature and Secchi depth) explained a significant amount of the variation in the microbial community data (DistLM marginal test, Table 4). Concentrations of DOC (6.69 % of the explained variance) and Chl *a* (6.57 % variance) explained most of the total observed variation of the T-RFLP dataset and hence, were the most determining factors shaping microbial community at the investigated reef sites. Temperature and total suspended solids accounted for 4.84 % and 4.55 % of the total observed variation, while the remaining parameters accounted for < 4.00 % (Table 4 and Fig. 2).

Distance-based RDA illustrated that *Alteromonadaceae* and Diatom plastids were correlated with high Chl *a* concentrations (Fig. 2). In contrast *Cyanobacteria* and *Roseobacter* were correlated with low Chl *a* concentrations. To some extent, *Flavobacteriaceae* were correlated with low amounts of DOC.

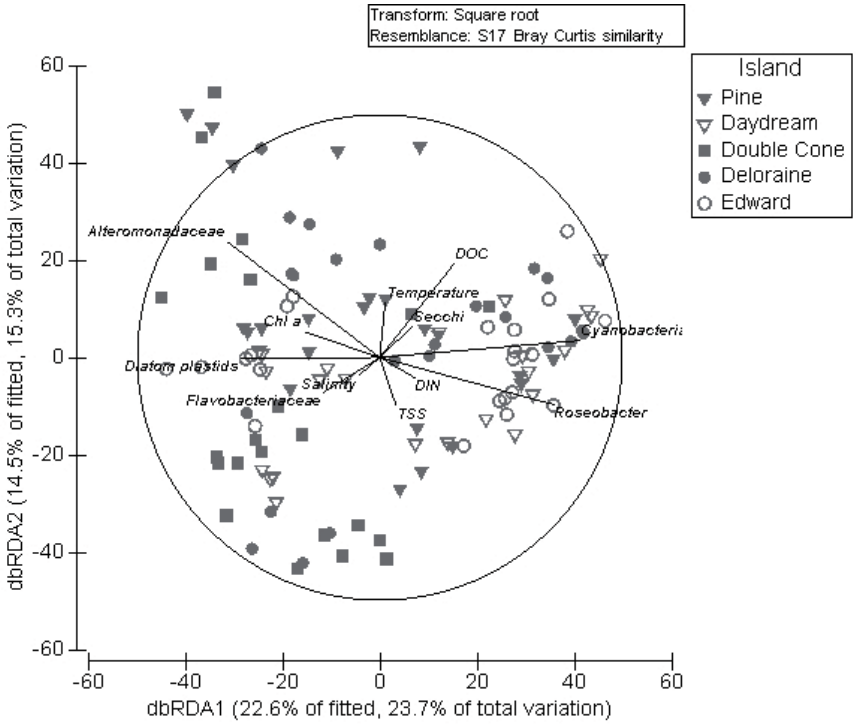

Fig. 2. Relationship between measured coastal coral reef water quality parameters and changes in bacterial biofilm community profiles from replicate glass substrata deployed within the reef.
Plot represents a distance-based redundancy analysis (dbRDA) ordination of T-RFLP trace data derived from biofilms generated from a Bray-Curtis distance matrix and with water quality variables chosen by significances of distance linear based model (DistLM) marginal tests.

Table 4. Distance-based linear model (DistLM) marginal tests based on permutation procedures (9999 permutations) showing the % variance of single water quality parameters: temperature, salinity, dissolved organic carbon (DOC), dissolved inorganic nitrogen (DIN), total suspended solids (TSS), chlorophyll *a* (Chl *a*) and Secchi depth. Residual degrees of freedom (df) = 116.

Variable	SS (trace)	Pseudo-F	P-value	% Variance
Temperature	16418	5.90	0.0001	4.84
Salinity	13574	4.84	0.0001	4.00
DOC	22692	8.32	0.0001	6.69
DIN	10609	3.74	0.0006	3.13
Chl *a*	22282	8.16	0.0001	6.57
TSS	15416	5.53	0.0001	4.55
Secchi depth	9545.2	3.36	0.0016	2.82

In summary, analyses of clone libraries and fingerprinting data were in agreement with each other, showing spatial and seasonal trends in dominant microbial groups. Spatial distributions showed increased *Bacteroidetes* at inner nearshore locations compared to outer nearshore locations, while *Cyanobacteria* and *Roseobacter* showed the inverse trend. Seasonal changes were illustrated by an increased presence of *Gammaproteobacteria*, in particular *Alteromonadaceae*, during the wet compared to the dry season, while *Alphaproteobacteria* and *Flavobacteriaceae* were found more dominant during the dry season.

DISCUSSION

We explored the spatio-temporal variation of bacterial biofilm communities in coral reefs in order to gain a better understanding of the responsiveness of marine ecosystems to terrestrial runoff and seasonal disturbances at pristine and human impacted coastal reefs. A molecular (T-RFLP and 16S rRNA gene clone libraries) and multivariate statistical (PERMANOVA, PCA and dbRDA) approach was used to identify the effects of location and season, and water quality parameter to determinant the main controls driving microbial community shifts. Finally, we evaluated the utility of bacterial biofilms as bioindicators for water quality in tropical coral reefs. The water quality measurements were consistent with data obtained from previous monitoring along the water quality gradient from previous years (Cooper et al., 2007; Schaffelke et al., 2010; Schaffelke et al., in press). Generally, our data followed previously recognized seasonal patterns with higher concentrations of most parameters (Chl *a*, suspended solids and nutrient species) during the wet season and spatial patterns with higher concentrations closer to the coast (e.g., at inner nearshore sites average Chl *a* concentrations increased by 120 % in the wet season and DOC by 25 %, and both increased ~15 % between inner and outer nearshore location). Our findings suggest that the parameters Chl *a* and DOC, both found at high concentrations inshore during the wet season, were the main drivers in spatio-temporal bacterial community shifts. Similarly, suspended sediments and organic carbon have previously been correlated with changes in GBR inshore coral reef community composition (Thompson et al., 2010; Uthicke, 2010). Linking bacterial communities with environmental parameters was achieved by the statistical tests PERMANOVA and dbRDA. These analyses show comparable results; PERMANOVA tests inner versus outer nearshore locations, under the presumption that environmental differences are mainly caused by water quality as illustrated by changes in nutrient concentrations (Table 2), whereas dbRDA does not differentiate between location

(inner/outer) and/or season (wet/dry), but takes into account the actual concentrations of each of the water quality parameters. Hence, the results from dbRDA analysis confirm and support our hypothesis that bacterial community differences are driven by water quality nutrients (e.g. DOC).

The *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were the most frequent groups detected in biofilms along the water quality gradient, which was in agreement with previous studies in this region (Kriwy and Uthicke, 2011; Witt et al., 2011a). Furthermore, these groups have been identified as major drivers of bacterial community differences in biofilms from local aquaria studies (Witt et al., in review) and mid-shelf reefs from the GBR (Webster et al., 2004). In addition, these groups were dominant in bacterioplankton in temperate marine coastal surface waters (Gilbert et al., 2012).

BACTERIAL COMMUNITIES AT INNER NEARSHORE REEFS

Gammaproteobacteria, in particular the relative abundance of *Alteromonadaceae,* showed a substantial increase during the wet season compared to the dry season. An increased relative abundance of *Gammaproteobacteria* and *Alteromonadales* has previously been detected at sites of reduced water quality inshore of the GBR (Kriwy and Uthicke, 2011; Witt et al., 2011a, in review), the Atlantic coast (Dang et al., 2008) and simulated inshore conditions in aquaria studies (Witt et al., in review). Chlorophyll *a* concentrations increased by 100 % at outer nearshore location and by 120 % at inner nearshore locations during the wet season compared to the dry season, but were generally higher at inner nearshore locations. Chlorophyll *a* concentration is highly correlated with particulate nitrogen, phosphorous, and suspended solids (Fabricius and De'ath, 2004) and hence, is commonly used as a proxy for nutrient status and a measure for water column productivity (Brodie et al., 2007). Mean annual values of Chl *a* concentrations for the inshore GBR are < 0.45 μg l^{-1} and these values are suggested to as water quality thresholds for coastal management (De'ath and Fabricius, 2010). Seasonal nitrogen inputs to the GBR lagoon poses a significant water quality issue, as summer flood events support high phytoplankton production leading to increased Chl *a* concentrations, while phytoplankton biomass remains N-limited throughout most of the year (Furnas et al., 2005). During the inner dry season N concentrations are well below the threshold, while at the inner wet season there is a 2-fold increase, exceeding the aforementioned threshold levels). This seasonal change explains the close correlation of *Alteromonadaceae* and Chl *a*, as this group rapidly responds to

disturbance and profits from organic carbon as illustrated by the rapid increase of *Alteromonadaceae* found naturally after disturbances in the field (Alonso-Saez and Gasol, 2007; Weinbauer et al., 2010) and in a nutrient-manipulating mesocosm study (Allers et al., 2007). Also positively correlated with high Chl *a* concentrations inshore, were the diatom plastids, which dominated inner nearshore locations. Such diatom dominance at nearshore sites has previously also been demonstrated in sediments (Gottschalk et al., 2007; Uthicke and McGuire, 2007), biofilms (Kriwy and Uthicke, 2011) and phytoplankton (Relevante and Gilmartin, 1982) from the GBR. This was most likely linked to elevated nutrient- and decreased light availability in nearshore waters, as diatoms have relatively high growth rates and are generally adapted to low light levels in high nutrient waters (Reynolds, 2006).

The *Bacteroidetes* were frequently found from the inner than outer nearshore sites and more during the wet than the dry season. In contrast, the *Flavobacteriacae* were consistently found in greater proportion in the dry season and were clearly correlated with low DOC concentrations. This inverse correlation may be explained by the fact that *Flavobacteriacae* are important heterotrophs involved in organic carbon cycling in aquatic habitats through their ability to breakdown high molecular weight organic matter through the secretion of extracellular enzymes (Cottrell and Kirchman, 2000; Kirchman, 2002), hence these are often abundant in nutrient-rich waters where biomacromolecules accumulate (Reichenbach, 1989). Moreover, planktonic *Flavobacteriacae* are demonstrated to be in higher abundance during the decay of phytoplankton blooms in a mesocosm experiment (Riemann et al., 2000). Furthermore, planktonic *Flavobacteriacae* are remarkably responsive to phytoplankton blooms and tightly coupled to diatom species (Pinhassi et al., 2004) indicating that *Flavobacteriacae* could be of particular importance in processing organic matter during such events. However, DOC is an indicator for decaying plankton blooms leaching organic nutrients, and hence correlations between *Flavobacteriacae* and low DOC, and negative correlations between DOC and Chl *a* raise further questions. With regard to bacterial species at inner nearshore locations, similar results have been obtained along a temperate river (Columbia River) to ocean gradient. The relative abundance of planktonic *Bacteroidetes* and *Gammaproteobacteria* were higher in a river plume compared to the coastal water (Fortunato et al., 2012).

BACTERIAL COMMUNITIES AT OUTER NEARSHORE REEFS

In agreement with recent studies, *Cyanobacteria* were significantly more abundant at outer

nearshore locations in the GBR (Uthicke and McGuire, 2007; Kriwy and Uthicke, 2011; Witt et al., 2011a), possibly explained by their preference of high light environments (Paerl, 1985; Robarts and Zohary, 1987). Hence, *Cyanobacteria* were negatively correlated with Chl *a* and this correlation is well in agreement with *Cyanobacteria* adapted to oligotrophic waters with high light availability (and high water clarity) as found at outer nearshore GBR sites.

Moreover, another group predominantly found at inner than outer nearshore sites were the *Alphaproteobacteria* (Kriwy and Uthicke, 2011; Witt et al., 2011a), which were more dominant during the dry season. In particular, *Hyphomonas*, *Erythrobacter*, and *Roseobacter* were more abundant during the dry than the wet season. The *Roseobacter* group was the most abundant of these genera and, like *Cyanobacteria*, was negatively correlated with Chl *a*. This indicates a potential competitive advantage of *Roseobacter* (to a certain extent *Erythrobacter*) due to their mixed metabolic strategies, for example aerobic anoxygenic phototrophy (Allgaier et al., 2003) and their ability to rapidly absorb dissolved nutrients in coastal waters (Denner et al., 2002; Alonso-Saez and Gasol, 2007). *Roseobacter* in surface-attached biofilm communities are good competitors under nutrient low conditions (Pinhassi and Berman, 2003; Pinhassi et al., 2004). The same trends have been observed in the previously mentioned bacterioplankton study along the Columbia River to ocean gradient, where *Cyanobacteria* and *Alphaproteobacteria* were more abundant in the coastal surface water than in the river plume (Fortunato et al., 2012).

Taken together, we focused on the identification of opportunistic bacterial groups to changing water quality conditions, growing to population sizes that can be measured by culture-independent techniques. Five dominant bacterial groups were identified in correlation with various water quality conditions found along a cross-shelf gradient. Along this gradient, *Gammaproteobacteria*, diatom plastids and *Bacteroidetes* are characteristic of inner nearshore biofilm communities, while *Cyanobacteria* and *Alphaproteobacteria* are the primary bacterial groups in the outer nearshore biofilm communities. In addition, we identified clear seasonal patterns as shown by the prevalence of *Flavobacteriaceae* and *Roseobacter* in the dry season and *Bacteroidetes, Cyanobacteria* and *Altermonadaceae* have a competitive advantage after disturbances in the wet season. Hence, both inner and outer bacterial groups and their water quality determinant displayed a clearly negative correlation. More specifically, *Alteromonadaceae* and diatom plastids were positively correlated with eutrophication (Chl *a*), *Flavobacteria* were correlated with low DOC concentration. Within individual microbial groups,

all three aforementioned were negatively correlated with *Cyanobacteria* and *Roseobacter*.

We detected specific bacterial groups under certain water quality conditions and suggest that *Alteromonadaceae* and diatom plastids may serve as indicators of eutrophication, while *Cyanobacteria* and *Roseobacter* may be indicators of oligotrophic waters with high light availability. In a series of studies on bacterial biofilms as indicators, specific bacterial groups were repeatedly reconfirmed to be similarly responsive (Kriwy and Uthicke, 2011; Witt et al., 2011b; Witt et al., 2011a, in review). Of particular relevance is a decrease in the relative abundance of *Alphaproteobacteria* (mostly *Roseobacter*) and concomitant increase in *Bacteroidetes* (mostly *Flavobacteriaceae*) that has been repeatedly detected in biofilms, e.g., in response to increased water temperature (Webster et al., 2011), increased pCO_2 (Witt et al., 2011b), nitrate and temperature-nitrate interactions (Witt et al., in review). As previously suggested, these groups should be studied in more depth, as these might be valuable bioindicator groups to be integrated into long-term coastal health monitoring programmes (Witt et al., in review). Finally, fingerprinting techniques (i.e., ARISA, T-RFLP) have shown to be capable of detecting significant bacterial variation in biofilms across spatial and temporal scales, representing a useful tool for water quality biomonitoring in streams (Lear et al., 2008; Lear and Lewis, 2009). Here we propose that T-RFLP may provide a cost-effective and rapid approach to monitor land use impacts on coastal water quality in tropical coastal coral reef ecosystems (Witt et al., 2011a). Furthermore, terrestrial runoff alters bacterial community diversity and abundance that may in turn affect coral ecosystem functioning.

CONCLUSION

Our findings contribute to new insights on the distribution of surface-attached coastal coral reef bacterial communities. Bacteria within a biofilm respond to environmental stress over short time periods (weeks) and can therefore serve as bioindicator to detect early warning response to changes in water quality and consequent changes in ecosystem integrity. Moreover, terrestrial runoff shifts microbial biofilm diversity and may affect invertebrate settlement of (e.g., corals). Hence, altered settlement and metamorphosis of corals may influence coral reef recruitment and resilience. To further support that the identified potential bacterial indicator groups consistently and reliably reflect water quality, further steps towards application should be undertaken. Future research should therefore include qualitative (e.g., 454-sequencing) and quantitative (e.g., Quantitative real-time PCR and Fluorescence in situ hybridisation) determination of bacterial biofilm communities over long-term spatio-temporal investigations (> 2 years). We further

suggest the application of more specific primer sets to effectively capture target species. Investigating the physiological and metabolic variability accompanying bacterial community shifts would further contribute to understanding coral reef ecosystem functioning.

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SUPPLEMENTARY INFORMATION

Table 1. Summary of analyses from the water column, irradiance variables and nearshore location from the coast. Water quality parameters for each season shown are dissolved inorganic nitrogen (DIN, includes NO_3, NO_2 and NH_4), dissolved organic carbon (DOC), salinity, Secchi disc depth, total suspended solids (TSS), chlorophyll *a* (Chl *a*) and temperature. Mean (standard deviation).

Island	Nearshore location	Season	DIN (µmol l^{-1})	DOC (µmol l^{-1})	Salinity (ppt)	Secchi depth (m)	TSS (mg l^{-1})	Chl *a* (µg l^{-1})	Temperature (°C)
Pine	inner	dry	0.21 (0.02)	60.13 (2.52)	35.71 (0.59)	3.75 (1.73)	3.45 (0.21)	0.48 (0.09)	23.90 (1.08)
		wet	0.63 (0.06)	70.74 (1.00)	33.60 (0.60)	7.50 (2.70)	2.85 (0.20)	0.82 (0.02)	28.95 (1.00)
Daydream	inner	dry	0.22 (0.07)	60.13 (2.34)	35.70 (1.20)	4.10 (1.23)	2.71 (0.45)	0.47 (0.04)	24.25 (2.19)
		wet	0.52 (0.23)	75.4 (2.19)	33.35 (0.22)	6.80 (3.01)	2.03 (0.40)	0.90 (0.17)	29.5 (1.92)
Double cone	inner	dry	0.22 (0.10)	63.47 (4.47)	35.20 (0.32)	5.50 (2.10)	1.50 (0.31)	0.33 (0.09)	23.25 (2.93)
		wet	0.48 (0.13)	62.81 (2.25)	33.60 (1.11)	5.52 (1.21)	1.55 (0.46)	0.94 (0.12)	29.35 (3.21)
Deloraine	outer	dry	0.09 (0.03)	69.40 (2.11)	35.12 (2.19)	7.75 (2.93)	1.50 (0.21)	0.29 (0.06)	22.60 (1.90)
		wet	0.17 (0.09)	70.60 (3.71)	35.10 (4.10)	9.25 (3.00)	1.14 (0.73)	0.60 (0.09)	28.80 (0.96)
Edward	outer	dry	0.06 (0.01)	69.15 (2.43)	35.05 (1.70)	8.00 (4.08)	1.35 (0.23)	0.36 (0.08)	22.55 (1.92)
		wet	0.24 (0.07)	69.15 (2.00)	34.85 (2.12)	7.50 (2.31)	1.85 (0.27)	0.49 (0.12)	28.60 (2.34)

Supplement Table S2. Terminal restriction fragments (T-RFs) are shown with their phylogenetic affiliation (as classified using the greengenes batch sequence classifier [http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi] using the NCBI taxonomy) and accession number as derived from the 16S rRNA gene clone libraries from each location. 16S rRNA identification of the nearest phylogenetic neighbour with accession number and sequence similarity (%) to each corresponding T-RF as determined by BLAST from the Greengenes database are shown.

Clone and accession number	Nearest relative with accession number	Sequence similarity (%)
Alphaproteobacteria		
Rhodobacteraceae (JQ726916)	Maribius sp. str. UDC481 (HM032012.1)	94.6
Rhodovolum (JQ726908)	Rhodovulum sp. str. SMB1 (DQ868668.1)	91.8
Roseobacter (JF261782)	Roseobacter sp. str. DSS-8 (AF098493.1)	99.2
Erythrobacter (JQ726904)	Erythrobacter sp. str. DG1288 (DQ486501.1)	99.3
Pseudorugeria (JF261850)	Pseudorugueria sp. str. HD-43 (FJ374173.1)	94.9
Gammaproteobacteria		
Alteromonas (JQ727104)	Alteromonas sp. str. UST981101-018 (EU982327.1)	99.4
Aestuariibacter (JQ726913)	Aestuariibacter halophilus str. JC2043 (AY207503.1)	95.8
Microbulbifer (JQ726945)	Marine str. HTCC2188 (AY386344.1)	95.0
Neptunomas (JQ727205)	Oleiphilus messinensis ME102 (AJ295154.1)	90.9
Oceanospirillum (JQ727083)	Oceanospirillum sp. str. P95 (EU195939.1)	90.9
Bacteroidetes		
Flavobacteraceae (JQ726884)	Aquimarina sp. str. antisso-27 (FJ882164.2)	98.1
Flexibacteraceae (JQ727194)	Flexibacter aggregans str. IFO 15974 (AB078038.1)	91.1
Cytophaga (JQ727183)	Cytophaga sp. 50.n (AB013834.1)	85.1
Flexibacter (JQ727172)	Flexibacter sp. str. SH9.43.1 (AB265187.1)	85.0
Marinicola (JQ726893)	Marinicola seohaensis str. SW-152 (AY739663.1)	90.2
Microscilla (JQ727102)	Fulvivirga kasyanovii (AB433335.1)	94.1
Muricauda (JQ726911)	Muricauda sp. str. 2PR55-3 (EU440997.1)	93.4
Roseivirga (JQ727140)	Roseivirga ehrenbergii str. UDC351 (GQ246639.1)	90.1
Flavobacterium (JQ727050)	Winogradskyella sp. str. 022-2-26 (FJ595484.1)	93.4
Gelidibacter (JQ727110)	Deep-sea sediment clone BD2-17 (AB015545.1)	94.3
Gaetbulibacter (JQ726897)	Owenweeksia hongkongensis str. UST20020801 (AB125062.1)	89.6
Winogradskyella (JQ726903)	Winogradskyella sp. str. DPG-24 (HM368527.1)	97.4
Cyanobacteria		
Synechococcus (JQ726906)	Synechococcus sp. str. PCC 8806 (AF448077.1)	91.1
Synechocystis (JQ726891)	Synechocystis sp. str. PCC 6308 (AB039001.1)	91.7
Leptolyngbya (JQ726882)	Limnothrix sp. str. B15 (GQ848190.1)	91.0

Phormidium (JQ726931)	Phormidium pristleyi ANT.PROGRESS2.6 (AY493585.1)	90.1
Prochlorothrix (JQ726928)	Acanthopleuribacter pedis str. NBRC 101209 (AB303221.1)	91.3
Diatoms		
Gyrosigma (JQ726985)	Amphiprora paludosa str. CCMP 125 C52 (FJ002240.1)	98.9
Lauderia (JQ726883)	Skeletonema pseudocostatum str. CSIRO CS-76	99.7
Thalassiosira (JQ726963)	Aureoumbra lagunensis str. CCMP 1507 (GQ231542.1)	94.0
Navicula (JQ727023)	Navicula sp. C21 (FJ002227.1)	98.6
Nanofrustulum (JQ727026)	Fucus vesiculosus (DQ307678.1)	96.7
Codium (JQ727051)	Rickettsia sp. str. IO1 (AF394906.1)	72.8
Haslea (JQ726946)	Fucus vesiculosus (DQ307678.1)	96.8
Firmicutes		
Carboxythermus (JF261862)	Thermosinus carboxydivorans str. Nor1 (AAWL01000009.1)	77.8
Acidobacteria		
Terriglobus (JQ726928)	Acanthopleuribacter pedis str. NBRC 101209 (AB303221.1)	91.3

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INTERACTIVE CLIMATE CHANGE AND RUNOFF EFFECTS ALTER O_2 FLUXES AND BACTERIAL COMMUNITY COMPOSITION OF COASTAL BIOFILMS FROM THE GREAT BARRIER REEF

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ABSTRACT

Global and local anthropogenic disturbances such as ocean warming and land runoff, affect coastal coral reefs worldwide. Terrestrial runoff, leading to reduced light and increased nutrient availability, may have interactive effects with existing ocean warming promoting shifts in benthic communities. As associated microbial biofilms rapidly reflect environmental changes, we investigated quantitative (C-, N- and Chl *a* contents), qualitative (microbial community composition) parameters and metabolic responses (O_2 fluxes) of biofilms established on glass slides to combinations of manipulated water temperatures (26, 29, 31 °C), nitrate (0.5, 1.0, 1.4 µM), and light availabilities (40, 200 µmol photons m^{-2} s^{-1}) in a 28 d flow-through aquarium experiment. Findings revealed that, independent of light availability, 31 °C significantly decreased 24 h net O_2 production, C-, N- and Chl *a* contents. Under high light, additive effects of 31 °C and 1.4 µM nitrate reduced 24 h net O_2 production. Terminal restriction fragment length polymorphisms (T-RFLP) of 16S rRNA genes showed light-independent temperature-induced microbial community shifts driven by increases in the relative abundance of *Oceanospirillum* (*Gammaproteobacteria*) and decreases of *Cyanobacteria*. In high light-exposed biofilms relative abundances of diatom plastids increased in response to elevated nitrate, while low light-exposed communities remained unimpaired. Overall, high temperatures altered microbial biofilm community composition, biomass and productivity. Under predicted near-future inner reef scenarios (low light/ high-nitrate availability) biofilms become light-limited through sedimentation, while outer (high light/ low-nitrate availability) inshore reef biofilms remain nitrate-limited. Understanding the interactive effects of environmental changes on microbial biofilm communities may contribute to bioindicator development and improved coastal management strategies for coral reefs.

INTRODUCTION

Several natural and anthropogenic impacts such as rising sea surface temperatures (SST) and increased inorganic nutrient imports from land may affect the functioning of coastal coral reef ecosystems worldwide. Climate change has already increased SSTs on the Great Barrier Reef (GBR) by an annual average of 0.7 °C within the last century (Lough, 2001; Lough et al., 2006). Furthermore, forecasts by the Intergovernmental Panel on Climate Change (IPCC, 2007) predict that GBR waters may warm by a further 1 - 3 °C by 2100. In addition to global warming, local terrestrial runoff from coastal agriculture (e.g., sugar cane) may contribute to the deterioration of water quality along coastal regions of the GBR. Terrestrial runoff is the largest source of new nitrate to the inshore GBR (Furnas, 2003), which drastically increases during the wet season (Devlin and Schaffelke, 2009), and these nutrient inputs have increased by a factor of 10 compared to the pre-agriculture baseline (Wooldridge et al., 2006). Runoff leads to reduced light and increased nutrient availability for coral reef communities at inner shelf reefs in comparison to outer nearshore reefs, where nutrient availability is low and light availability high. Such inner nearshore conditions shift benthic community composition, resulting in dominance by macroalgae that spatially compete with coral recruits, and consequently may promote decline in coral cover (Szmant, 2002; Fabricius, 2005; Schaffelke, 2005). Local chronic eutrophication continues to affect coral reefs and may have an additive interaction with global stressors such as rising SSTs, therefore posing a threat to coral reefs of the GBR (Uthicke and Altenrath, 2010; Schmidt, 2011; Uthicke et al., in press-b). Coastal water quality management of local disturbances may help preserve coral reefs from projected effects of climate change (Wooldridge, 2009; Negri, 2011; Schmidt, 2011).

Coral reefs harbour abundant microorganisms that predominantly exist as surface-attached communities (Costerton et al., 1995; Crump, 1998; Teske and Wooldridge, 2001). Surface-colonising biofilms are complex communities comprising of macromolecules and microorganisms embedded in an extracellular polymeric matrix (Mihm et al., 1981). These highly dynamic microbial biofilm communities respond rapidly to and are integrative of changing ambient environmental conditions (Paerl and Pinckney, 1996; Snyder et al., 2005). Microbial biofilm communities also contribute significantly to biogeochemical nutrient cycling in aquatic ecosystems such as coral reefs (Battin et al., 2003) and influence settlement as well as metamorphosis of important reef building organisms, such as corals, and therefore affect the establishment, recovery and resilience of coral reefs (Wieczorek and Todd, 1998; Webster et al.,

2004). The immense surface area available for biofilm colonisation and development highlights the important contribution of biofilms to coral ecosystem functioning.

Coral bleaching is promoted by increased SST (Hoegh-Guldberg, 1999) and, in combination with elevated nitrate availability, these effects may be exacerbated (Zhu et al., 2004; Wooldridge, 2009; Wooldridge and Done, 2009), leading to phase shifts from coral- to algae-dominated communities with concomitant reef decline (Fabricius 2005, Schaffelke, *et al*. 2005). Further, chronically disturbed inshore reefs of the GBR exhibit increased disease occurrence in sponges and corals (Webster et al., 2008; Haapkyla et al., 2011). Thus, shifts within biofilm communities may substantially alter bacterial community composition and function, subsequently affecting coral reef productivity (O_2 fluxes) and biomass.

Despite the importance of biofilm communities, the possible impacts of future environmental change (e.g. eutrophication, increased SSTs and ocean acidification) on these communities have rarely been investigated. Several studies have demonstrated shifts of bacterial community composition in biofilms in response to local anthropogenic nutrient impacts (Meyer-Reil and Koster, 2000; Lawrence et al., 2004; Chenier et al., 2006; Nocker et al., 2007; Chiu et al., 2008) and in response to increased SST (Boivin et al., 2005; Lau et al., 2005; Chiu, 2006). However, these investigations have either tested individual factors alone or one of these factors in combination with e.g. salinity, pollutants and their consequent effect on invertebrate larval settlement. Testing environmental parameters individually prevents determining additive or synergistic effects, making future predictions about the potential effects of climate change difficult. The environmental parameters temperature, light and nutrients (in particular nitrate from fertiliser input) are critical parameters that may affect biological entities in coral reef environments. Although effects of seasonal environmental inshore conditions on benthic organisms have been investigated in situ along a water quality gradient in the GBR (Uthicke, 2010; Uthicke and Altenrath, 2010; Kriwy and Uthicke, 2011; Witt et al., 2011a), interactions between these three specific parameters on aquatic bacterial biofilms remain poorly understood.
To disentangle interactive effects and determine disturbance tolerance levels, this study investigates metabolic (O_2 fluxes), quantitative (C-, N- and Chl *a* contents) and qualitative (bacterial community structure) changes in coastal marine biofilms in response to controlled combinations of elevated temperature and increased nitrate concentrations, under two different light availabilities, in a 28 d aquarium study. Hence, we directly measured metabolic activity (O_2

fluxes) and simultaneously investigated changes in community composition using molecular tools in marine biofilms in response to environmental factors (continued from a previous study on effects of pCO_2 (Witt et al., 2011b). Such investigations on the biofilm communities associated with coral reefs may elucidate the interactive effects of environmental stressors potentially leading to deleterious environmental change. These results can support future coastal management.

METHODS

AQUARIUM SET-UP AND SAMPLING

An indoor flow-through experiment for manipulation of nitrate concentrations, temperature and light conditions was conducted over 28 d in October 2009. The experimental setup consisted of three water temperatures (i.e., 26, 29, 31 °C) and three nitrate concentrations (i.e., 0.5, 1.0, 1.4 µM) each replicated in three separate tanks, resulting in a total of 27 aquaria (volume: 16 L).
Aquaria for each temperature were heated by 2 and 3 kW titanium-heating bars and connected to a computer control system (CR1000 Measurement and Control Datalogger, Campbell Scientific, Townsville, Australia). Automatic temperature sensors (Campbell Scientific) were placed randomly into each temperature treatment logging the temperature over the course of the experiment, and additionally manual measurements revealed constant temperatures of 26.4 ± 0.3, 29.2 ± 0.3 and 31.0 ± 0.3 °C (means ± SD) in the different treatments.

Prior to entering the aquarium system, inshore seawater directly pumped from the sea was stored in a settlement tank and was then filtered through a series of filters (pore width: 25 µm, 10 µm and 5 µm). Seawater flow rates of 500 ml min^{-1} ensured a steady supply of fresh seawater to each aquarium. Peristaltic pumps (Masterflex L/X multichannel Extech Equipment, Boronia, Australia) delivered nitrate into each aquarium at near constant flow rates of 0.8 – 1.0 ml min^{-1} to enhance the baseline nitrate concentration (average = 0.5 ± 0.3 µM NO_3^-, n = 9) which was very similar to that of typical local inshore seawater (about 0.5 µM, (Cooper et al., 2007). Nitrate concentrations were chosen according to naturally occurring nutrient concentrations during flood-plume events in the GBR (Devlin and Schaffelke, 2009) and baseline nitrate concentrations were therefore enhanced to 1.0 (average = 1.0 ± 0.4) and 1.4 (average = 1.4 ± 0.8) µM NO_3^- using potassium nitrate stock solutions (1 M KNO_3). Duplicate nutrient samples for dissolved inorganic nitrogen (DIN) were taken from each aquarium twice weekly and were analysed by the Australian Institute Laboratory Services by methods of (Ryle et al., 1981).

Salinity, determined daily using a handheld refractometer, remained constant (between 35-36 PSU) throughout the experiment. All aquaria were maintained under a diel light/dark cycle of 12:12 h. As an additional factor to the main setup, each aquarium was further divided into 'high' light (200 µmol photons m^{-2} s^{-1}) and 'low' light (40 µmol photons m^{-2} s^{-1}) conditions by covering half of the tank with
70 % shade cloth.

Glass microscope slides (75 x 25 mm) were used as settlement substrata for biofilm development, because they allow growth of biofilms with bacterial communities not significantly different from those grown on natural substrata such as coral skeletons or reef sediments (Witt et al., 2011a). Bacteria initially colonising and settling on specific artificial and natural substrates may be different depending on the surface properties of the substrate (Sweet et al., 2011), however, biofilms undergo distinct temporal shifts where the effect of substrate type diminishes, and biofilms tend to form more similar community structures over time (Huggett et al., 2009; Chung et al., 2010). Slides were fixed onto PVC holders, and biofilms were allowed to develop for 14 d in outdoor flow-through aquaria from the AIMS facility directly located at Cape Cleveland Bay, with untreated water pumped directly from the sea. At the start of the experiment, randomly selected glass slides (n = 6) with attached biofilms were sampled to represent initial communities. Two pre-conditioned biofilm slides were then immersed into each aquarium, one per light condition (total n = 108). At the end of the experiment (after 28 d), a total of 54 biofilm slides (one per treatment combination) was sampled for subsequent microbial community analyses by carefully scraping off the biofilm material from the glass substrate into cryovials using sterile No. 11 scalpel blades (yield was usually > 2 g). Samples were snap-frozen in liquid nitrogen and stored at -80 °C until further processing.

DETERMINATION OF O_2 PRODUCTION AND O_2 CONSUMPTION RATES

The remaining 54 biofilm slides were used for measurements of net O_2 production as described in Witt et al. (2011 a). Briefly, individual glass slides from each aquarium per treatment were transferred into airtight 50 ml glass vials filled with seawater from the corresponding aquaria. Dissolved O_2 (DO) concentrations were measured in the individual vials (using a hand-held luminescent optode HQ10-HQ20, HACH, USA) prior to carefully enclosing the biofilm slides and after incubation for 30 min at light conditions of 200 µmol photons m^{-2} s^{-1} and 40 µmol photons m^{-2} s^{-1}. Subsequently, the same procedure was repeated for a 60 min dark incubation.

Resulting hourly net O_2 production and consumption were calculated to 24 h net O_2 production and expressed as µmol O_2 cm^{-2} biofilm area d^{-1}. Production/respiration (P/R) ratios were calculated as follows: (hourly net production + hourly respiration) x 12/ (hourly respiration x 24) = P/R ratio. Subsequent to the O_2 measurements, half of the biofilm material from each slide (8.1 cm^2) was scraped into vials using scalpel blades, snap-frozen in liquid nitrogen and stored at -80 °C for Chl *a* analysis, while the other half was scraped onto pre-combusted GF/F filters (25 mm in diameter, Whatman, USA) for organic C and N analyses.

SPECTROMETRIC DETERMINATION OF BIOFILM CHLOROPHYLL A CONTENT

The analysis for the total Chl *a* content was adapted for biofilms from protocols for freshwater phytoplankton and marine sediments (Sartory and Grobbelaar, 1984; Uthicke, 2006). Samples were kept in low light conditions and on ice during analysis. The material collected from each slide (corresponding area: 8.1 cm^2) was extracted in hot (78 °C) ethanol (95 %) for 5 min and incubated for 24 h at room temperature in the dark and shaken occasionally. After 24 h, the biofilm extractions were centrifuged at 5200 rpm for 5 min. Subsequently, 320 µl of each extract was measured using a Synergy Plate Reader (Bio-Tek, USA) at wavelengths of 665 nm and 750 nm. To correct for phaeophytin content, 18 µl 0.1 N HCl was added to each sample after the first reading and samples were re-measured at both wavelengths. Chl *a* concentrations were calculated as described in Schmidt et al. (2011).

DETERMINATION OF CARBON AND NITROGEN CONTENTS IN BIOFILMS

GF/F filters with biofilm material were rinsed with freshwater to remove salts, then dried at 60 °C and homogenised using mortar and pestle. Total organic carbon (TOC) and total organic nitrogen (TON) were determined on a parallel sample for each slide on a Shimadzu elemental analyser (TOC5000A) using standard reference material (MESS-1 and Round 40).

DETERMINATION OF BIOFILM COMMUNITY COMPOSITION USING TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) ANALYSIS

Total DNA was extracted from 0.5 g of biofilm (wet weight) sample using the MoBio UltraClean Soil Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol with the following modifications. Bead-beating (Mini-Bead-Beater, Biospec Products, Bartleville, OK, USA) (2 x 30 s) cycles were performed and DNA was eluted with 2 x 50 µl of 1 x TE buffer. DNA extracts were examined by standard 1 % agarose gel

electrophoresis and quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Bacterial 16S rRNA genes were amplified by PCR using the 5′-Cy5-labelled 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R primers (5′-ACGGGCGGTGTGTACAAG-3′) (Sigma-Proligo, The Woodlands, TX, USA) (Marchesi et al., 1998). Each biofilm sample was amplified in triplicate 25 µl reactions containing 2.5 µM non-acetylated bovine serum albumin (New England Biolabs, USA), 2 µM (2 mM each) dNTP (Astral Scientific, Australia), 2.5 µM forward primer 63F, 1.25 µM reverse primer 1389R, 1 µM MgCl (Qiagen), 1.25U HotStar Taq (Qiagen), 2.5 µl HotStar Buffer (Qiagen, Germany) and ~2 ng of template DNA. Amplification was performed with an initial incubation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 90 sec, and a final extension at 72 °C for 10 min. After PCR, triplicate amplicons were pooled to avoid PCR bias and were subsequently purified using the MinElute PCR purification kit (Qiagen). PCR products were quantified using the NanoDrop Spectrophotometer (Thermo Fischer Scientific), and 150 ng of each purified product was digested with the restriction enzyme MspI (New England Biolabs) according to the manufacturer's instructions. Digested fragments were desalted using the DyeEx 2.0 Spin kit (Qiagen) and vacuum dried for 40 min at low temperature in the dark. Terminal restriction fragments (T-RFs) were resolved and visualized using the CEQ 8800 Genetic Analysis System (Beckman-Coulter, Fullerton, CA, USA) with a 600 bp size standard (Beckman-Coulter). Replicate samples were compared using the software T-align (Smith, 2005) with a range of 0.5 bp peak area to determine the consensus peaks between duplicates. The relative fluorescence intensity of the peak area of T-RFs was used as a relative abundance measure for T-RFs in further statistical analyses detailed below. To identify T-RFs, T-RFLP data were examined in relation to an extensive database of 16S rRNA genes identified from previous Great Barrier Reef biofilm studies (Witt et al., 2011b; Witt et al., 2011a).

STATISTICAL ANALYSIS

To assist the interpretation and to evaluate the effects of the two key stressors (nitrate and temperature) of this experiment, data obtained under two different light levels were analysed separately. Two-way Analysis of Variance (ANOVA) was applied to the 24 h net O_2 production data, C-, N- and Chl *a* contents at different temperatures and nitrate concentrations for each light condition. Homogeneity of variance was tested using Levene's test, and Tukey-Kramer test was used as a post hoc test. Linear regression analyses were used to determine relationships between 24 h net O_2 production data, C-, N- and Chl *a* contents. Linear regression and ANOVA analyses

were performed using the Number Cruncher Statistical System (NCSS) 2007 statistical software (Hintze, 2007).

Prior to statistical community analyses, T-RF peak area values were fourth root transformed and standardised to 100 %. Principle Component Analysis (PCA) was used to determine whether microbial assemblages in samples grouped by temperature and/or nitrate level. The significance of the overall assemblage dissimilarities between temperature and nitrate treatments were tested by applying two-way Permutational Multivariate Analysis of Variance (PERMANOVA) based on permutation procedures (9999 permutations) using the Bray-Curtis distance measure and p values derived from Monte-Carlo simulations. Pairwise t-tests were used as post hoc test. The contributions of each taxon to the total dissimilarities of treatments were analysed using the Similarity Percentage (SIMPER) routine and represented by vectors in the PCA. Principle Component Analysis and PERMANOVA (Clarke, 1993) were performed using the Primer 6.0 statistical software (Clarke, 2006). To further determine significant differences between relative abundances (peak area) for contributing T-RFs, two-way ANOVA analysis with the according post hoc test was applied as stated above.

RESULTS

Net oxygen production was measured for biofilms grown under both high- and low light conditions for each combination of nitrate and temperature treatments. Under high light conditions (200 µmol photons m^{-2} s^{-1}), biofilms were net autotrophic (Fig. 1A) (with P/R ratios ranging from 3.33 ± 0.91 to 7.19 ± 1.48 Supplementary Information Table S3). Twenty-four h net O_2 production in high light-exposed biofilms after 28 d was significantly affected by both temperature and nitrate levels (2-way ANOVA, Table 1, Fig. 1A, t_0 = 8.91 mol ± sd 0.72). Average 24 h net O_2 production of biofilms exposed to 31 °C was significantly (Tukey-Kramer post hoc test, $p < 0.05$, Table 1) lower (8.6 µmol O_2 cm^{-1} biofilm area d^{-1}) compared to biofilms exposed to 26 °C (16.6 µmol O_2 cm^{-1} biofilm area $d^{-1)}$ and 29 °C (13.7 µmol O_2 cm^{-1} biofilm area d^{-1}) (decrease by 48 % and 17 %, respectively). Biofilms at 1.0 µM NO_3^- produced significantly ($p < 0.05$) more O_2 compared to biofilms in 0.5 µM NO_3^- treatments (increase by 28 %) (Table 1). Percentage changes in 24 h net O_2 production from the control treatment (26 °C/ 0.5 µM NO_3^-) showed 72 % increase at 26 °C/ 1.0 µM NO_3^-, but only 20.6 % increase at 26 °C/ 1.4 µM NO_3^-. At 29 °C + NO_3^- net O_2 productions resulted in 8.8 - 19 % increase and 17 – 46 % decrease at 31 °C + NO_3^- from the control treatment (26 °C/ 0.5 µM NO_3^-). Nitrate concentrations averaged by

treatment were 0.53 (SD = 0.26) for the baseline, 1.00 (SD = 0.44) for the intermediate, and 1.42 (SD = 0.76) µmol L^{-1} NO_3^- for the highest nitrate concentration treatment. Although there was some variation, nutrient addition clearly resulted in the targeted nitrate concentrations and these differed between treatments.

Similar to high light-exposed biofilms, low light-exposed (40 µmol photons m^{-2} s^{-1}) biofilms were net autotrophic (with P/R ratios ranging from 3.31 ± 1.50 to 9.25 ± 1.40 Supplementary Table S3), but overall production was decreased by 56 % compared to high light-exposed biofilms (Fig. 1A). There was a significant effect of temperature on 24 h net O_2 production rates in low light-exposed biofilms after 28 d, however no effect of nitrate (2-way ANOVA Table 1). Twenty-four hour net O_2 production decreased significantly at 31 °C compared to 26 °C and 29 °C by ~40 % and ~48 %. Thus, for both light intensities, net O_2 production significantly decreased at 31 °C. However, in high light-exposed biofilms 31 °C combined with 1.4 µM NO_3^- significantly lowered 24 h net O_2 production with an additive effect.

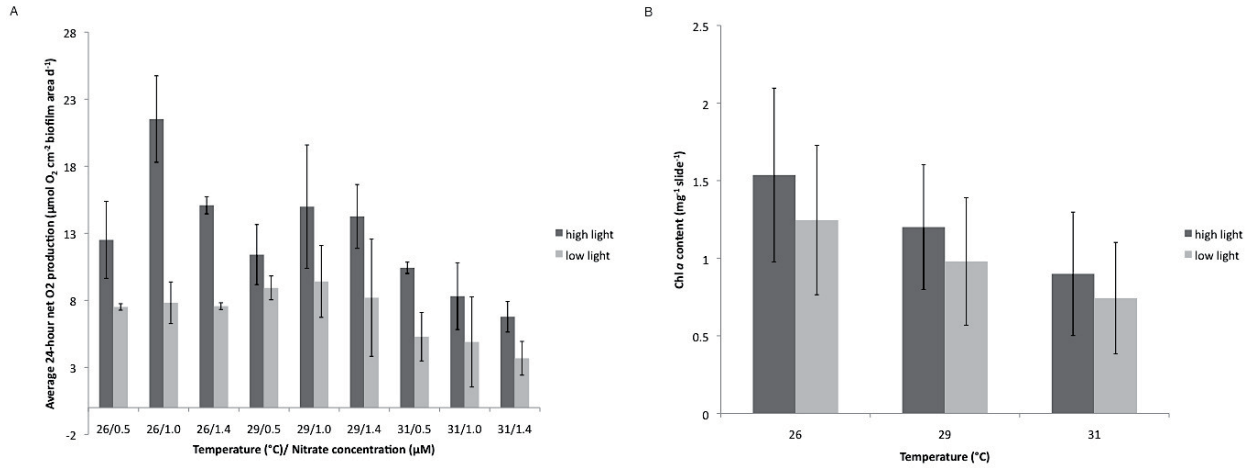

Fig. 1 A) Average 24 h net O_2 production of biofilm communities grown in different temperature and nitrate treatments under high light (200 µmol photons m^{-2} s^{-1}) and low light (40 µmol photons m^{-2} s^{-1}) availability. B) Average Chlorophyll *a* content of biofilm communities grown at different temperatures under high light (200 µmol photons m^{-2} s^{-1}) and low light (40 µmol photons m^{-2} s^{-1}) availability.

Table 1. Two-way ANOVA of 24 h net O_2 production of biofilms after 28 d in different temperature and nitrate treatments (generated with NCSS 2007). High light equals 200 µmol photons m^{-2} s^{-1} and low light 40 µmol photons m^{-2} s^{-1}. Temperature (T), nitrate (N) and interaction (T x N) Degrees of freedom (Df), mean square (MS), p (significance level), Tukey-Kramer (MC) post hoc test, significant p value (*).

Source of variation	Df	MS	F	P	Conclusion- MC post hoc test
High light					
Temperature	2	148.66	16.80	**< 0.0001***	26, 29 > 31
Nitrate	2	46.02	5.20	**0.0165***	0 < 1
T x N	4	18.04	2.04	0.1318	n.a.
Residual	18	8.85			
Low light					
Temperature	2	34.12	4.56	**0.0251***	26, 29 > 31
Nitrate	2	4.34	0.58	0.5703	n.a.
T x N	4	5.08	0.68	0.6156	n.a.
Residual	18	7.49			

CHLOROPHYLL A CONTENTS OF BIOFILM COMMUNITIES

Under both light conditions, water temperature had a significant effect (Table 2) on Chl *a* content of the biofilms, while nitrate concentration showed no significant effect. Under high light, the average Chl *a* concentration at 31 °C (0.8 ± 0.4 mg^{-1} cm^{-2}) was significantly lower than at 26 °C (1.5 ± 1.0 mg^{-1} cm^{-2}). Similarly, under low light the average Chl *a* concentration at 31 °C (average = 0.7 ± 0.4 mg^{-1} cm^{-2}) was significantly lower than at 26 °C (1.2 ± 0.8 mg^{-1} cm^{-2}) (Fig. 1B & Table 2). On average Chl *a* concentration in low light was about 20 % lower than in high light-exposed biofilms.

Table 2. Two-way ANOVA of chlorophyll *a* contents in biofilms after 28 d in different temperature and nitrate treatments generated with NCSS 2007. High light equals 200 µmol photons m^{-2} s^{-1} and low light 40 µmol photons m^{-2} s^{-1}. Temperature (T), nitrate (N) and interaction (T x N) Degrees of freedom (Df), mean square (MS), not significant (n.s.), p (significance level), Tukey-Kramer (MC) post hoc test, significant p value (*).

Source of variation	Df	MS	F	P	Conclusion- MC post hoc test
High light					
Temperature	2	1.060	6.49	**0.0075***	26 > 31
Nitrate	2	0.385	2.36	0.1228	n.a.
T x N	4	0.384	2.35	0.0927	n.a.
Residual	18	0.163			
Low light					
Temperature	2	0.601	5.29	**0.0156***	26 > 31
Nitrate	2	0.126	1.07	0.3620	n.a.
T x N	4	0.230	2.02	0.1340	n.a.
Residual	18	0.114			

CARBON AND NITROGEN CONTENTS OF BIOFILM COMMUNITIES

Total organic carbon (TOC) and TN contents of high light-exposed biofilms were both significantly affected by temperature, but not by nitrate (Table 3 and Supplementary Information Table S1). TOC was significantly different ($p < 0.05$, Table 3) between all temperatures, with TOC being highest at 26 °C and lowest at 31 °C. TN was significantly higher at 26 °C than 31 °C (Table 3).

In low light-exposed biofilm communities, TOC was affected by temperature and nitrate (Table 4). TOC was significantly lower at 31 °C than at 26 and 29 °C (by 59 and 68 %, respectively $p < 0.05$), and significantly lower at 1.4 µM NO_3^- than at 1.0 µM NO_3^- (by 71 %, $p < 0.05$) (Supplementary Table S1). Total nitrogen (TN) was solely affected by temperature ($F_{2,18} = 5.30$, $p = 0.0016$), whereby TN was lower in biofilms grown at 31 °C than at 26 and 29 °C (by 70 %, $p < 0.05$), while nitrate addition had no effect ($p > 0.05$, Table 3). TOC was on average 66 % lower and TN up to 80 % lower in low light compared to high light. A regression analysis showed a significant relationship between 24 h net O_2 production, Chl *a,* TOC and TN.

Table 3. Two-way ANOVA of carbon/nitrogen contents of biofilms at final measurements in different temperature, nitrate and light treatments. High light equals 200 µmol photons m^{-2} s^{-1} and low light equals 40 µmol photons m^{-2} s^{-1}. Total organic carbon (TOC) and total organic nitrogen (TN). Degrees of freedom (Df), mean square (MS), not significant (n.s.), significance level (p), Tukey-Kramer (MC) post hoc test, significant p value (*).

Source of variation	Df	MS	F	P	Conclusion- MC post hoc test
High light					
TOC					
Temperature	2	0.907	24.24	**<0.0001***	26 > 29 >31
Nitrate	2	8.095×10^{-2}	0.81	0.4617	n.a.
T x N	4	7.416×10^{-2}	0.74	0.5773	n.a.
Residual	18	0.100			
TN					
Temperature	2	8.849×10^{-3}	3.87	**0.0399***	26 > 31
Nitrate	2	4.017×10^{-3}	1.43	0.2642	n.a.
T x N	4	3.124×10^{-3}	1.11	0.3802	n.a.
Residual	18	2.802×10^{-3}			
Low light					
TOC					
Temperature	2	6.726×10^{-2}	18.15	**<0.0001***	26, 29 > 31
Nitrate	2	1.723×10^{-2}	4.66	**0.0233***	1 > 2
T x N	4	8.182×10^{-3}	2.21	0.1090	n.a.
Residual	18	3.706×10^{-3}			
TN					
Temperature	2	5.810×10^{-3}	11.28	**0.0007***	26, 29 > 31
Nitrate	2	8.155×10^{-4}	1.58	0.2325	n.a.
T x N	4	7.149×10^{-4}	1.38	0.2816	n.a.
Residual	18	5.149×10^{-4}			

T-RFLP OF BACTERIAL COMMUNITIES IN RESPONSE TO TEMPERATURE, NITRATE AND LIGHT

Across all treatments, molecular fingerprinting of bacterial communities with T-RFLP analysis revealed 37 terminal restriction fragments (T-RFs). Most of these fragments (36) were found in the low light communities, whereas only 65 % (24 T-RFs) were found in the high light communities (GenBank accession no. JF261709, JF261753, JF261762, JF261782, JF261789, JF261796, JF261798, JF261814, JF261820, JF261837, JF261845, JF261854, JF261881, JF261891, JF261915, JF261929; Supplementary Table S2). Therefore, 12 T-RFs (mainly *Gammaproteobacteria*) (Accession no. HQ601620, HQ601722, HQ601725, HQ601730, HQ601823, JF261867, JF261871, JF261872, JF261919, JF261935, JF261950, unidentified) were specific to the low light communities, but only one (Accession no. JF261929 *Flavobacteriaceae*, *Bacteroidetes*) was specific for the high light-exposed communities.

For the high light data set, significant differences in community structures were detected between temperature and nitrate treatments with a significant interaction term (2-way PERMANOVA Table 4A). Post-hoc tests of that interaction (presented as nitrate within temperature, Table 4B) revealed that communities with no nitrate addition were not different between temperature treatments (p (MC) < 0.05). In contrast, under enhanced nitrate levels (both under 1.0 and 1.4 µM NO_3^- addition) incubated biofilm communities were significantly different at 26 and 29 °C from those at 31 °C.

Table 4. Results of PERMANOVA analysis using three groups (A) and posthoc test for the interaction term using three groups (B) of bacterial biofilm assemblages. Tests are based on 9999 permutations. P (MC): P value based on Monte Carlo random draws. P-values < 0.05 are highlighted for clarity.

A) Overall PERMANOVA of temperature and nitrate groups in high light biofilms (200 µmol photons m^{-2} s^{-1})

Source	Df	SS	MS	Pseudo-F	P (MC)
Temperature	2	8101.6	4050.8	5.4017	**0.0001***
Nitrate	2	6456.8	3228.4	4.3051	**0.0008***
T x N	4	5697.9	1424.5	1.8995	**0.0357***
Residual	19	13498	749.91		
Total	27	33755			

B) Posthoc tests within the interaction term (temperature (°C) and nitrate (µM) groups)

	0.5		1.0		1.4	
Groups	T	P(MC)	T	P (MC)	T	P (MC)
26, 29	1.1688	0.2947	1.0688	0.3626	1.6667	0.0841
26, 31	1.4175	0.1611	3.2553	**0.0086***	2.5664	**0.0232***
29, 31	1.1407	0.309	2.0202	**0.0382***	2.2712	**0.0255***

C) Overall PERMANOVA of temperature and nitrate groups in low light biofilms (40 µmol photons m^{-2} s^{-1})

Source	df	SS	MS	Pseudo-F	P (MC)
Temperature	2	9066.7	4533.3	3.3335	**0.0125***
Nitrate	2	5418.3	2709.2	1.9921	0.084
T x N	4	6963.3	1740.8	1.2801	0.2516
Residual	19	23119	1359.9		
Total	27	44600			

Bacterial communities in high light-exposed treatments formed distinct community assemblages in response to different temperatures as demonstrated in a PCA analysis (Fig. 2). Within the temperature treatments, each nitrate concentration resulted in different community structures, as illustrated by each nitrate concentration forming distinct assemblages at 29 °C and 31 °C

compared to assemblages at 26 °C (Fig. 2). Vectors in the PCA and SIMPER analyses illustrated that community dissimilarities for the high light treatment at different temperatures were mainly driven by increasing relative abundance of *Oceanospirillum* (*Gammaproteobacteria*) and diatom plastids, and decreasing relative abundance of *Cyanobacteria* (Table 5). Further contributing groups were the *Roseobacter* clade and *Flavobacteriaceae* (Table 5).

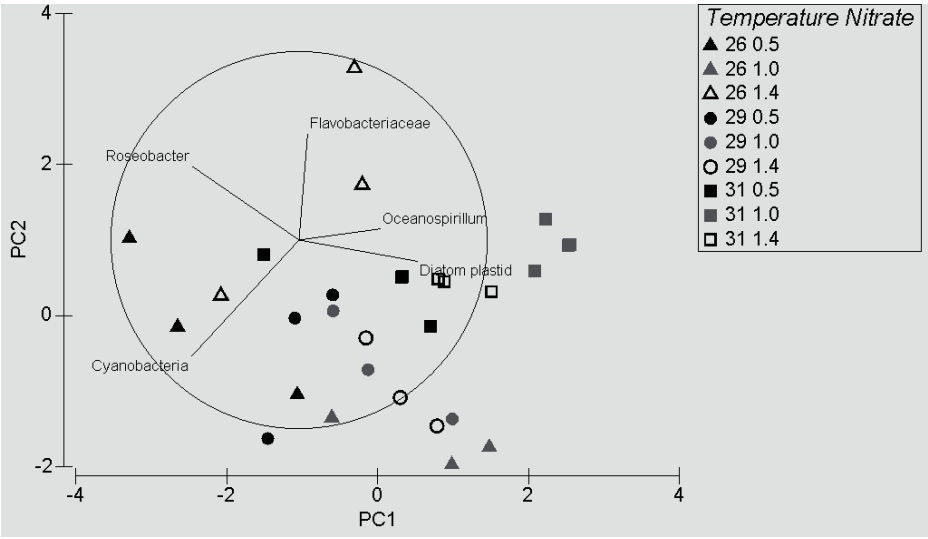

Fig. 2. Principal Component (PCA) analysis incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing bacterial assemblages at different temperatures in high light (200 µmol photons m^{-2} s^{-1}) availability (PC1: 57.2 % PC2: 16.5 %). Vectors of the most importantly contributing T-RF (bp) are shown in the biplot and labelled with the corresponding taxon.

Table 5. Similarity Percentage (SIMPER) analysis with overall average dissimilarity values of temperature treatment dataset in different light availability (high light: 200 µmol photons m^{-2} s^{-1}, low light: 40 µmol photons m^{-2} s^{-1}) and contribution (%) of 4 bacterial taxa (with T-RF length bp) contributing most to the community dissimilarities. Zero value (0) may indicate either absence from the T-RF or contribution is 1 or lower.

Temperature treatment	High light	Low light
Overall average dissimilarity (%)	56.6	64.4
Taxon (T-RF bp)	Taxon Contribution (%)	
Oceanospirillum (95)	13.3	6.0
Cyanobacteria (457)	10.7	10.7
Diatom plastid (461)	6.1	0
Synechococcus (456)	4.1	0
Roseobacter (401)	2.8	0
Vibrionaceae (469)	0	5.4
Flavobacteriaceae (55)	2.5	3.2
Gammaproteobacteria (451)	0	4.2

Under high light, differences in relative abundance of the species identified as important contributors to group differences by SIMPER were also investigated by separate 2-way ANOVA. *Oceanospirillum* was influenced by temperature only (2-way ANOVA Temperature: $F_{2,27}$ = 19.37, p < 0.0001; Nitrate: $F_{2,27}$= 1.97, p = 0.1687), its abundance being significantly lower at 26 °C than at 29 and 31 °C (Fig. 3). Similarly, T-RFs representing *Cyanobacteria* and *Synechococcus* were affected by temperature (ANOVA $F_{2,27}$ = 6.20, p = 0.0049; $F_{2,27}$ = 0.05, and p = 0.9468 and ANOVA $F_{2,27}$ = 8.08, p = 0.0021) and were significantly less abundant at 31 °C compared to 26 and 29 °C (Fig. 3). High temperature also affected *Flavobacteria* which increased significantly at 29 °C compared to 31 °C (ANOVA $F_{2,27}$ = 4.46, p = 0.0226). The relative abundance of *Roseobacter* was affected by temperature and nitrate (2-way ANOVA $F_{2,27}$ = 5.51, p = 0.0036; $F_{2,27}$= 6.22, p = 0.0210) and decreased significantly (p <0.05) in the high temperature treatments (29 and 31 °C) in comparison to the 26 °C treatments and were more abundant at 1.4 µM NO_3^- than at 1.0 µM NO_3^- (Fig. 3).

Diatom plastids were affected by nitrate only and showed a significant interaction (2-way ANOVA $F_{2,27}$ = 2.14, p = 0.1468; $F_{2,27}$= 3.90, p = 0.0391; $F_{4,27}$= 5.25, p= 0.0056). Although post hoc tests were negative, the relative abundance of diatom plastids increased with increasing nitrate addition, and in interaction with rising temperature the relative abundance of diatom plastids decreased at 1.0 µM NO_3^-. *Synechococcus* was only present at 0.5 µM NO_3^- (average relative abundance 5.28 %) and was not detected at both enhanced nitrate concentrations (1.0 and 1.4 µM NO_3^-).

For the low light data set, significant differences in community structures were detected between temperatures (2-way PERMANOVA Pseudo-$F_{2,27}$ = 3.33, p (MC) = 0.0125, Table 4C), while nitrate availability and the interaction showed no significant effect (p > 0.05 Table 4C). Post-hoc tests showed that low light biofilm communities were significantly different at 29 °C compared to those at 26 and 31 °C.

A PCA analysis of low light-exposed bacterial biofilm assemblages revealed pronounced overlap between 26 and 31 °C, while 29 °C separated from these two groups (Fig. 4), confirming patterns detected by PERMANOVA. SIMPER analysis revealed that community dissimilarities at different temperatures were mainly driven by increasing relative abundance of *Gammaproteobacteria* and *Oceanospirillum*, and *Cyanobacteria* being highest at 29°C (Fig. 3)

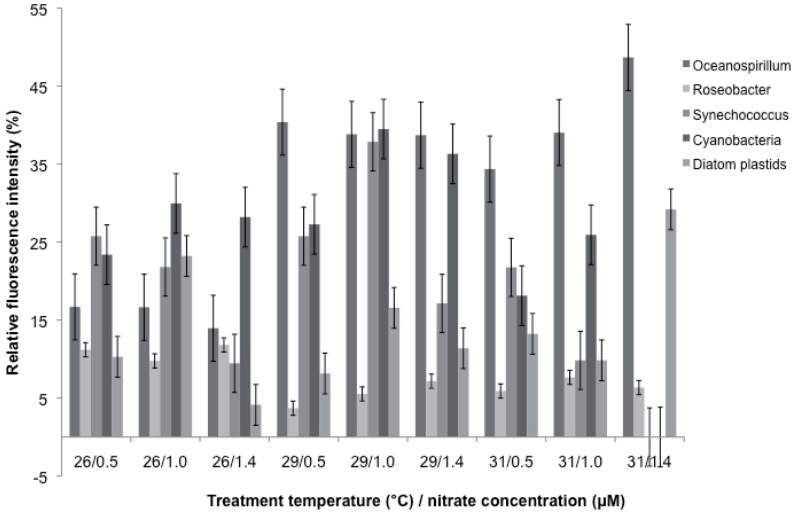

Fig. 3. Significant changes in the relative abundance of the most contributing T-RFs (bp) in the different combinations of temperature and nitrate treatments under high light (200 µmol photons m^{-2} s^{-1}) with standard errors.

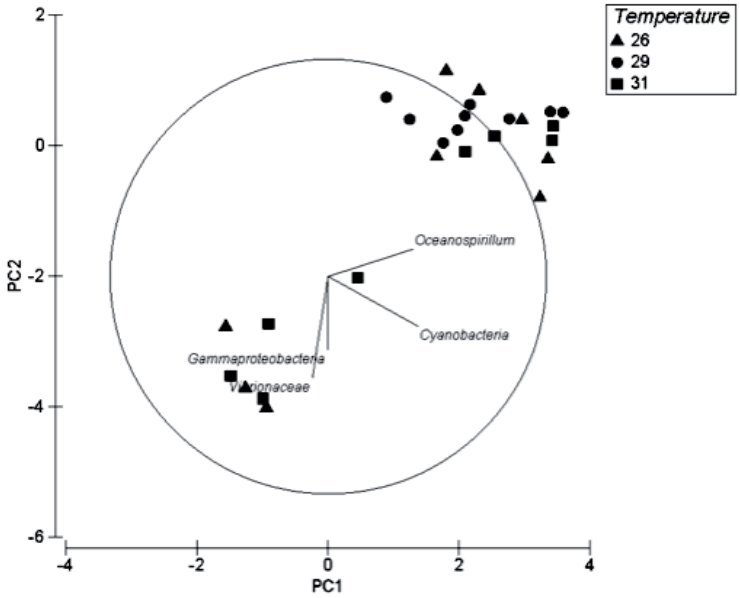

Fig. 4. Principal Component (PCA) analysis incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing bacterial assemblages at different temperatures in low light (40 µmol photons m^{-2} s^{-1}) availability (triangle = 26 °C circle = 29 °C, square = 31 °C; PC1: 57.2 % PC2: 16.5 %) with 40 % similarity level. Vectors of the most importantly contributing T-RF (bp) are shown in the biplot and labelled with the corresponding taxon.

As PERMANOVA showed no nutrient effects, abundances for individual taxa were only tested by one-way ANOVA using temperature as factor. The relative abundance of *Cyanobacteria* was significantly higher at 29 °C than at 31 °C and 26 °C (ANOVA $F_{2,26} = 6.66$, $p = 0.0052$, HSD

post hoc 26, 31 < 29). Both, relative abundance of *Oceanospirillum* and an unclassified *Gammaproteobacterium*, increased with rising temperature (ANOVA $F_{2,26} = 4.15$, $p = 0.0289$; $F_{2,26} = 6.73$, $p = 0.0050$) (Fig. 5). Finally, when comparing high contributing T-RFs occurring in both high- and low light treatments, *Oceanospirillum* significantly decreased in low light conditions ($F_{1,52} = 29.2$ $p < 0.0001$), while *Synechococcus* remained unaffected by light ($F_{1,52} = 3.9$, $p = 0.0601$).

In summary, changes in relative abundance of *Oceanospirillum*, *Cyanobacteria* and *Flavobacteriaceae* illustrated temperature-induced community shifts in high light-exposed biofilms, while microbial communities were affected by a temperature-nitrate interaction driven by diatom plastids and *Roseobacter*. In low light biofilms, temperature-driven microbial community shifts mainly resulted from changes in the relative abundance of *Gammaproteobacteria* and *Cyanobacteria*.

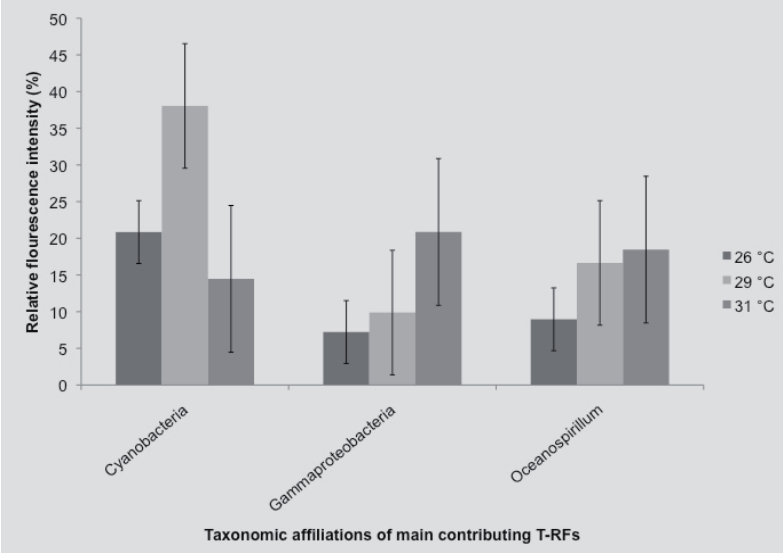

Fig. 5. Significant changes in the relative abundance of the most contributing T-RFs to differences in bacterial assemblages at different temperatures under low light (40 µmol photons m^{-2} s^{-1}) with standard errors.

DISCUSSION

We tested for interactive temperature and nitrate effects in a 28 d flow-through aquaria experiment mimicking inner (low light/ high-nutrient availability) and outer (high light/ low-nutrient availability) GBR inshore reef conditions (Uthicke, 2006; Cooper et al., 2007). Irrespective of nitrate and light availability, high (elevated by 2 - 3 °C over current summer

maxima) water temperatures of 31 °C significantly reduced biofilm community 24 h net O_2 production and biomass (organic C-, N- and Chl *a* contents). Experimental results suggested that under high light availability effects of high temperature and nitrate were additive. In addition, high light-exposed biofilms were nitrate-limited, illustrated by the 24 h net O_2 production increase at 1.0 µM NO_3^-. However, high temperatures had the most distinct effect on bacterial community composition, as elevated nitrate concentrations were only important in conjunction with high temperature (31 °C). In contrast, biofilms under low light availability were presumably light-limited; neither community composition nor productivity changed under increased nitrate. Our findings suggest that an increase in water temperatures of about 1 - 2 °C above the current average summer temperatures within the study area (28 - 29 °C) (predicted to occur by 2100 (Lough, 2001; Lough et al., 2006)), have a detrimental effect on photosynthesis of local biofilms.

BALANCE BETWEEN AUTOTROPHY AND HETEROTROPHY

In addition to global warming, coastal water quality is influenced by periodical terrestrial runoff causing increased nitrate and reduced light availability, which has become a chronic disturbance to inshore coral reefs (Devlin and Schaffelke, 2009). In this study, both high- and low light-exposed biofilms were net autotrophic as illustrated by 24 h net O_2 productions and high P/R ratios (> 1). Over the 28 d period communities did not shift from autotrophy towards predominant heterotrophy under the experimental conditions, however under longer exposure to low light biofilms may turn heterotrophic. Biofilms exhibited significantly lower 24 h net O_2 production and Chl *a* contents at 31 °C, but effects of temperature were more pronounced under low- than high light (i.e., 31 °C reduced 24 h net O_2 production by about 40 % from 26 °C). Nevertheless, low light-exposed biofilms were very productive, 24 h net O_2 production being half of that of high light-exposed biofilms, but in comparison with only 20 % of the light availability. A regression analysis showed a significant relationship between 24 h net O_2 production, Chl *a*, TOC and TN, suggesting that at least part of the production decrease was coupled to a reduction in biomass (50 % reduction of TOC, TN and Chl *a*).

LIGHT AND TEMPERATURE ALTER PRODUCTIVITY AND NUTRIENT UPTAKE

In agreement with other studies, results of lowered productivity and Chl *a* contents at 30 °C were also detected in marine coastal (Chiu, 2006) and estuarine biofilms (Nayar et al., 2005). Similarly, endosymbiontic diatoms in benthic low light adapted foraminifera showed reduced photosynthetic rates and Chl *a* at 31 °C (Schmidt, 2011; Uthicke et al., in press-b), resulting

from photo-inactivation caused by thermal and oxidative damage of the photosynthetic apparatus (Warner 1999). In this study, at higher temperatures, 24 h net O_2 production decreased proportionally with Chl *a*. Additionally, 50 % reduced TOC content, meaning less photosynthetically-fixed C, indicated lower photosynthetic activity. Therefore, temperature-induced changes detected in biofilms may be a consequence of a malfunctioning photosynthetic apparatus, potentially caused by thermal damage distorting enzymes in the dark reaction (e.g. Ribulose-1,5-biphosphate-carboxylase/-oxygenase (Rubisco)), responsible for photosynthetic carbon fixation. As demonstrated in higher plants, Rubisco can become inhibited by temperatures > 30 °C (Feller et al., 1998), which likely may be the case in our study in the highly productive diatoms and *Cyanobacteria* (Underwood and Kromkamp, 1999).

Lower TN contents at high temperature may be explained by increased membrane permeability in microalgae, hence less fixed N is incorporated into cells. Additionally, N-uptake mechanisms are largely light activated (Pennock, 1987; Boyer et al., 1994; Tuchman et al., 2006), therefore we propose that fast-growing microalgae in biofilms may suffer N-limitation and outcompete slow growing microalgae at inner nearshore locations chronically affected by flood plumes. Contrastingly, slow growing microalgae usually remain unaffected by increased N availability, however their growth is restricted by shading instead (Underwood and Kromkamp, 1999).

ENHANCED NITRATE INFLUENCES BIOFILM PRODUCTIVITY

In addition to high temperature, high light-exposed biofilms were influenced by enhanced nitrate concentrations. Increased 24 h net O_2 production (photosynthetic production) in response to intermediate nitrate concentrations (1.0 µM NO_3^-) as used in this study, has previously been observed for corals (Marubini and Thake, 1999; Zhu et al., 2004). While compared to increased 24 h net O_2 production at 26 and 29 °C/ 1.4 µM NO_3^- and decreased 24 h net O_2 production at 31 °C/ 1.4 µM NO_3^- in high light biofilms, in coral symbionts photosynthesis at 2 µM NO_3^- remained unaltered (Ferrier-Pages et al., 2001). Our findings indicate that temperature and nitrate tolerance limits of the investigated microbial biofilm communities range between 29 - 30 °C and ~1 µM NO_3^-. Past these levels, their photosynthetic functioning (O_2 fluxes) is impacted or inhibited. Given the additive effect under high light, elevated temperatures become more relevant under high nitrate regimes. When calculating percentage changes of each factor from the control treatment (26 °C / 0.5 µM NO_3^-), high temperature of 31 °C reduced 24 h net O_2 production by 17 %, and additive effects of 1.4 µM NO_3^- by an additional 28 % (thus total

reduction of 45%). Similarly, warming effects became more pronounced under elevated nutrient conditions in river biofilms suggesting that high temperatures may promote faster biofilm re-colonisation after disturbances (Diaz Villanueva, 2011). As also demonstrated in the present study, higher nutrient availability did not reflect in higher biomass. This was due to increased presence of ciliates at high temperature, hence increased grazing on biofilm bacteria. Therefore, grazing effects are to be investigated in future studies.

BIOFILM COMMUNITIES AT INNER NEARSHORE SITES

Community shifts induced by increasing temperature and nitrate concentrations in high light-exposed biofilms were driven by changes in the relative abundance of *Cyanobacteria*, *Gammaproteobacteria* and diatom plastids. *Alphaproteobacteria* and *Cytophaga-Flavobacteria-Bacteroides* were further contributing phylogenetic bacterial groups, previously identified as major contributors to community shifts in biofilms in temperate estuaries (Jones et al., 2007), at mid-shelf reefs (Webster et al., 2004) and, in response to nutrient and light stress at inshore GBR sites (Kriwy & Uthicke 2011).

Planktonic diatoms frequently dominate nearshore waters (Relevante and Gilmartin, 1982) with high nutrient input and low light availability. This has also been demonstrated for benthic diatoms (Gottschalk et al., 2007; Uthicke and McGuire, 2007) and biofilms from the GBR (Kriwy and Uthicke, 2011). However, diatoms have also previously been identified as a key driver of community composition in high light-exposed biofilm in response to elevated pCO_2 (Witt et al., 2011b). This is possibly due to phototrophic diatoms using light to produce exudates (extracellular polymeric substances) fuelling other microbes (reviewed in (Thornton, 2002)).

The high contribution of *Gammaproteobacteria*, in particular *Oceanospirillum*, in biofilms from simulated inner inshore conditions could be confirmed in field-grown biofilms at inner inshore locations (5 km distance from the coast) of reduced water quality on the GBR (Kriwy and Uthicke, 2011; Witt et al., 2011a) and the Atlantic coast (Dang et al., 2008). In estuarine biofilms increasing relative abundance of *Gammaproteobacteria*, in particular sulphate-reducing species, were correlated with decreasing dissolved O_2 availability in the water column, while enhanced nutrient availability had a secondary effect (Nocker et al., 2007). Increasing relative abundance of *Oceanospirillum* with rising temperature in high- and low light conditions, may also possibly be correlated with high temperatures resulting in reduced dissolved O_2 availability and heat-

stressed or decaying microalgae cells, hence providing more detritus availability to heterotrophic bacteria. Lower relative abundance of *Oceanospirillum* (by 50 %) at low- compared to high light availability indicated higher competition of bacteria under low light availability. *Gammaproteobacteria* species also contributed the most to microbial community shifts in field-grown biofilms at shallow water sites (high light availability) along a water depth gradient on the GBR (Webster et al., 2004).

BIOFILM COMMUNITIES AT OUTER NEARSHORE SITES

Cyanobacteria prefer warm water temperatures and high light environments (Paerl, 1985; Robarts and Zohary, 1987), and although dominant in outer inshore locations, light only has a limited effect. *Cyanobacteria* are able to adapt to growth in altered light quality and quantities through gene regulation of their chromatic phycobilisome structure (Grossman, 1990; Grossman et al., 1993), and control of their photosystem {Kulkarni, 1992 #174;Kulkarni, 1994 #173;Bhaya, 2002 #153}. Increasing relative abundance of *Cyanobacteria*, in particular *Synechococcus*, at 29 °C and decrease at 31 °C with a concomitant decrease in net O_2 production and Chl *a* content has also been observed in *Synechococcus*-dominated tropical estuarine biofilm communities grown on glass substrata (Nayar et al., 2005). These findings imply that 30 - 31 °C may also exceed the temperature optimum of *Cyanobacteria* species found in our study. Temperature-induced differences in the relative abundances of *Synechococcus* were only found within the 0.5 µM NO_3^- treatments, as at higher NO_3^- concentrations these were absent. Further, the absence of *Synechococcus*, at higher nutrient concentrations may be explained by the N-fixing ability and this decreases at > 30 °C (Breitbarth et al., 2006). Therefore, a consequent selection towards N-fixers at low N regimes leads to the predominant occurrence in tropical oligotrophic offshore waters (Crosbie, 2001a, 2001b; Moisan et al., 2010; Nelson et al., 2011), such as the outer inshore GBR (> 30 km distance from the coast) (Kriwy & Uthicke 2011).

Several bioindicator studies have confirmed *Roseobacter* as a dominant group in microbial biofilms in estuarine (Jones et al., 2007), polar (Webster and Negri, 2006), temperate (Dang et al., 2008) and coral reef environments (Kriwy and Uthicke, 2011). *Roseobacter* clade members were more abundant at outer than inner nearshore sites in the GBR (Kriwy and Uthicke, 2011; Witt et al., 2011a), possibly because of a competitive advantage due to rapid absorbance of nutrients dissolved in coastal waters (Alonso-Saez and Gasol, 2007) and anoxygenic phototrophy (Allgaier et al., 2003). A decrease in *Alphaproteobacteria* was also detected in biofilms in

response to elevated temperature {Webster, 2011 #201} and elevated pCO_2 (Witt et al., 2011b) with a concomitant increase in *Bacteroidetes*, in particular *Flavobacteria*. These findings were reconfirmed in this study, indicating that *Alphaproteobacteria*, in particular *Roseobacter*, and *Bacteroidetes* respond to environmental stress and may serve as indicator species for future biofilm bioindicator application.

Finally, unconsidered factors which have previously been demonstrated to influence bacterial community composition such as the impact of grazers on bacteria (Pernthaler, 2005; Pernthaler and Amann, 2005; Weinbauer et al., 2010) and viral infection (Hewson et al., 2003; Weinbauer et al., 2010) are worth exploring in further studies.

Overall, biofilms at both inner (5 km distance from coast) and outer nearshore (> 30 km distance from the coast) locations in the study area are primarily affected by warming SSTs, as illustrated by the changes in 24 h net O_2 production and bacterial community compositions. Inner reefs, adapted to high-nitrate regimes, are additionally affected by light reduction resulting from eutrophication and sedimentation. Hence, biofilms harbour more heterotrophic bacterial species (i.e., *Gammaproteobacteria*) limited by light, while outer reef biofilms are mainly composed of nitrate-limited autotrophic species (i.e. diatoms and *Cyanobacteria*).

Inner nearshore reefs are suffering from chronic low light availability. From a historical point of view, these reefs may either have always experienced higher sediment loads or have been altered through increased sediment load since European settlement (mid-18th century) and intense agriculture development of the Queensland coastline. Historical data on coral communities or biofilms is difficult to obtain. However, sediment cores have demonstrated that benthic foraminifera communities on some outer nearshore reefs have remained persistent over nearly two millennia, while inner reef communities remained stable, but changed since European settlement (Uthicke et al., in press-a).

In conclusion, both global warming and eutrophication simultaneously affect microbial biofilms established in coral reef habitats. Our findings add to the growing body of evidence that runoff alters community composition of coral reefs. As inner reefs are already experiencing light reduction due to increased sediment input, it appears that associated biofilms are less vulnerable to nitrate, but clearly vulnerable to temperature increase. Hence our findings are consistent with

a runoff-temperature interaction for inshore reefs. Unexpectedly, this was not an interaction with nitrate but with sedimentation (leading to light reduction). Yet, rising SSTs are difficult to influence, as long-lasting reductions in greenhouse gas emissions can only be reached by a concerted global management effort. In contrast, local nitrate and sediment input through farming can be controlled by sustainable regulations and coastal management. Therefore, a reduction of sediment (increasing light availability) input needs simultaneous reduction of nitrate load to prevent temperature-vulnerable outer reef scenarios. Although biofilms mimicking outer (presumed high light) reef conditions were affected by additive effects of temperature and nitrate, current flood plumes are unlikely to reach these reefs, unless under cyclonic conditions (Cooper et al. 2007). This suggests higher resilience to temperature at outer than inner reefs, assuming that runoff does not worsen in the near future.

Therefore, suggested stringent control over land-based pollution to ameliorate water quality, may be equivalent to reducing SSTs by 1 - 2 °C. Extending knowledge of qualitative and metabolic responses (i.e., O_2 fluxes) of biofilms contributes importantly to the future development of coastal management and bioindicator systems for coral reef health.

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SUPPLEMENTARY INFORMATION

Supplementary Information Table S1. Total organic carbon and total organic nitrogen for biofilms from different temperatures, nitrate concentrations and light availabilities. High light equals 200 μmol photons m^{-2} s^{-1} and low light equals 40 μmol photons m^{-2} s^{-1}. Mean (standard deviation).

Light	Temperature (°C)	Nitrate concentration (μM)	Total organic carbon (mg^{-1} cm^{-2})	Total organic nitrogen (mg^{-1} cm^{-2})
High light	26	0.5	0.94 (0.14)	0.18 (0.02)
	26	1.0	0.95 (0.49)	0.19 (0.09)
	26	1.4	0.82 (0.54)	0.15 (0.07)
	29	0.5	0.60 (0.30)	0.11 (0.04)
	29	1.0	0.66 (0.12)	0.14 (0.04)
	29	1.4	0.96 (0.29)	0.16 (0.04)
	31	0.5	0.27 (0.12)	0.07 (0.04)
	31	1.0	0.52 (0.15)	0.14 (0.03)
	31	1.4	0.58 (0.12)	0.13 (0.05)
Low light	26	0.5	0.33 (0.04)	0.08 (0.02)
	26	1.0	0.31 (0.12)	0.07 (0.03)
	26	1.4	0.19 (0.04)	0.05 (0.02)
	29	0.5	0.21 (0.03)	0.05 (0.02)
	29	1.0	0.31 (0.07)	0.09 (0.04)
	29	1.4	0.13 (0.10)	0.03 (0.02)
	31	0.5	0.14 (0.00)	0.02 (0.00)
	31	1.0	0.10 (0.06)	0.02 (0.01)
	31	1.4	0.10 (0.01)	0.02 (0.00)

Supplementary Information Table S2. T-RFs with clone and accession number detected from biofilms from different temperature, nitrate and light treatments. High light equals 200 µmol photons m^{-2} s^{-1} and low light equals 40 µmol photons m^{-2} s^{-1}. Filled boxes represent the presence of a T-RF in at least one of the three replicates per treatment.

T-RF size (bp)	Clone and GenBank accession no.	Light	Temperature (°C) Nitrate concentration (µM)								
			26 0.5	26 1.0	26 1.4	29 0.5	29 1.0	29 1.4	31 0.5	31 1.0	31 1.4
55	Flavobacteriaceae (HQ601837)	high							■	■	■
		low			■			■	■	■	■
56	Flavobacteriaceae (JF261845)	high	■	■	■	■	■	■	■		■
		low	■	■	■	■	■	■	■	■	■
58	Flexibacter (HQ601790)	high	■			■			■		
		low	■	■	■	■			■	■	
88	Rhizobiales (JF261891)	high		■		■	■	■	■	■	■
		low							■		■
90	Alphaproteobacteria (HQ601722)	high									
		low	■	■						■	■
91	Alcanivorax (HQ601742)	high	■	■	■	■					
		low		■	■		■	■		■	■
94	Rhodobacteraceae (JF261820)	high	■		■	■			■	■	
		low	■								
95	Oceanospirillum (JF261814)	high	■	■	■	■	■	■	■	■	■
		low	■	■	■	■	■	■	■	■	■
106	Flexibacter (JF261796)	high	■								
		low	■	■				■			
107	Pelagiobacter (JF261854/ HQ601633)	high			■	■					
		low	■		■	■		■			■
113	Erythrobacter (JF261950)	high									
		low			■	■		■	■		■
116	Hyphomonas (JF261762)	high			■						
		low		■	■			■			■
125	S-oxidising symbiont (JF261830)	high	■			■		■	■		
		low	■	■		■	■	■	■		■
126	Desulfuromonas (JF261784)	high			■			■			
		low		■	■		■	■	■		■
127	Nannocystaceae (HQ601730)	high									
		low		■		■	■				
168	Flavobacteriaceae (HQ601823)	high									
		low	■	■					■	■	■
400	Ruegeria (JF261782)	high	■		■	■		■	■		
		low	■	■	■	■		■	■		
401	Roseobacter (JF261798)	high	■	■	■	■	■	■	■		■
		low	■	■	■	■	■	■	■	■	■
404	Pseudoruegeria	high	■		■	■	■		■	■	

	(JF261709)	low	■	■	■	■	■	■	■	■	■
416	Unidentified	high									
		low						■	■		■
451	Gammaproteobacteria	high									
	(JF261871)	low		■					■	■	■
453	Gammaproteobacteria	high									
	(JF261872)	low	■	■	■				■	■	■
456	Synechococcus	high			■						
	(JF261753/ HQ601728)	low				■			■		
457	Cyanobacteria	high	■	■	■	■	■	■	■		
	(JF261915)	low	■	■	■	■	■	■	■	■	■
459	Diatom plastid	high	■		■						
	Phaeodactylum (JF261837)	low	■	■		■	■	■	■	■	■
461	Diatom plastid Haslea	high	■	■	■	■	■	■	■	■	■
	(JF261789)	low	■	■	■	■		■	■	■	■
462	Unidentified	high	■	■	■						
		low						■	■		■
469	Vibrionaceae	high									
	(JF261867)	low	■	■	■				■	■	■
471	Vibrio	high									
	(HQ601620)	low	■						■	■	
472	Polyangium	high									
		low							■		■
	(HQ601725)										
485	Unidentified	high									
		low	■							■	
510	Olleya	high	■								
		low							■		■
	(HQ601810)										
512	Flavobacteriaceae	high	■		■						
	(JF261929)	low									
581	Diatom plastid	high									
	(JF261919)	low	■						■		■
583	Diatom plastid	high									
	(JF261935)	low	■							■	
585	Diatom plastid	high	■			■		■			
	(JF261881)	low		■	■	■	■	■			■

EFFECTS OF OCEAN ACIDIFICATION ON MICROBIAL COMMUNITY COMPOSITION OF, AND OXYGEN FLUXES THROUGH, BIOFILMS FROM THE GREAT BARRIER REEF

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ABSTRACT

Rising anthropogenic CO_2 emissions acidify the oceans, and cause changes to seawater carbon chemistry. Bacterial biofilm communities reflect environmental disturbances and may rapidly respond to ocean acidification. This study investigates community composition and activity responses to experimental ocean acidification in biofilms from the Australian Great Barrier Reef. Natural biofilms grown on glass slides were exposed for 11 d to four controlled pCO_2 concentrations representing the following scenarios: A) preindustrial (~300 ppm), B) present-day (~400 ppm), C) mid century (~560 ppm), and D) late century (~1140 ppm). Terminal restriction fragment length polymorphism (T-RFLP) and clone library analyses of 16S rRNA genes revealed CO_2-correlated bacterial community shifts between treatments A, B and D. Observed bacterial community shifts were driven by decreases in the relative abundance of *Alphaproteobacteria* and increases of *Flavobacteriales* (*Bacteroidetes*) at increased CO_2 concentrations, indicating pH sensitivity of specific bacterial groups. Elevated pCO_2 (C+D) shifted biofilm algal communities and significantly increased C and N contents, yet O_2 fluxes, measured in light and dark incubations, remained unchanged. Our findings suggest that bacterial biofilm communities rapidly adapt and reorganise in response to high pCO_2 to maintain activity such as oxygen production.

INTRODUCTION

The world's oceans have absorbed 30 - 40 % of anthropogenically-emitted CO_2 from the atmosphere (Feely et al., 2004; Sabine et al., 2004) since the beginning of the industrial era. The uptake of CO_2 alters the seawater carbonate chemistry including a reduction in pH (Caldeira and Wickett, 2003; Raven, 2005). Such ocean acidification has already been detected in the Australian Great Barrier Reef (Wei et al., 2009). Ocean acidification may erode away the structural foundation for coral reef growth (Kleypas et al., 1999; Langdon et al., 2000; De'ath et al., 2009; Ries et al., 2009; Silverman et al., 2009), inducing future shifts from coral- to algal-dominated reefs (Hoegh-Guldberg, 1999; Hughes et al., 2003; Pandolfi et al., 2005; Anthony et al., 2011).

Bacterial communities play a critical role in the health of coral reef ecosystems (Ritchie, 2006; Meron, 2010; Mouchka et al., 2010; Meron et al., 2011). However, little is known about how microorganisms such as bacteria respond to changing ocean carbon chemistry (reviewed in (Liu et al., 2010; Joint et al., 2011). A recent study on the effects of pH on bacterial communities associated with corals has revealed community shifts and increased bacterial diversity with decreasing pH (Meron et al., 2011). Further, previous mesocosm experiments in Norway suggest that heterotrophic planktonic free-living bacterial communities shifted in response to high CO_2 levels, while the communities of particle-attached bacteria, bacterial abundance and activity remained unaffected by high CO_2, and were rather linked to a phytoplankton bloom (Allgaier et al., 2008). In addition to planktonic life styles, bacteria within marine environments often exist as surface-attached biofilm communities (Costerton et al., 1995; Crump, 1996, 1998; Teske and Wooldridge, 2001; Thornton, 2002), which are defined as complex surface-attached microbial communities comprised of photo- and heterotrophic microorganisms embedded in an extracellular polymeric matrix (Mihm, 1981). Biofilms contribute significantly to primary production, rapid nutrient recycling and the efficient degradation of organic matter (Lock, 1984; Battin et al., 2003), and are hence essential components of oligotrophic coral reef ecosystems.

Bacterial biofilm communities have the ability to structurally self-organise and respond rapidly to changing environmental conditions (Tolker-Nielsen and Molin, 2000). By providing surfaces for larval settlement of marine invertebrates, microbial biofilms also influence settlement cues and the induction of metamorphosis of important reef building organisms, such as corals, and therefore affect coral reef establishment, recovery and resilience (Wieczorek and Todd, 1998;

Webster et al., 2004). Further, coral reefs provide an immense surface area for biofilm colonisation and development and therefore, shifts in biofilm communities may therefore substantially affect reef productivity, biomass, composition and ecosystem functioning.

Studies have shown effects of high CO_2 concentrations on the photosynthetic productivity of microalgal and bacterial communities, but results are conflicting or scarce, precluding predictions of general patterns about possible future ecological impacts (Rost et al., 2008; Doney et al., 2009b; Doney et al., 2009a; Ries et al., 2009; Hendriks et al., 2010; Liu et al., 2010). As ocean acidification poses potential threats to coral reefs, understanding how primary reef colonising biofilms may respond to future ocean acidification is essential. Acidification may impact biofilm communities by shifting towards algal dominance, affecting productivity and nutrient cycling (reviewed in Rost et al. 2008) and an increased occurrence of disease causing bacteria (Meron et al., 2011). Ocean acidification has been investigated on various invertebrates and plankton, however despite the importance of biofilm communities, impacts of ocean acidification on these communities have not been studied in biofilms. This study therefore explores the potential effects of ocean acidification on the activity (expressed as O_2 fluxes) and community composition of tropical coral reef-associated biofilms from the Great Barrier Reef, Australia.

RESULTS

SEAWATER CHEMISTRY

As expected when using CO_2 bubbling, the total alkalinity only varied slightly between treatments (Table 1). In contrast, dissolved inorganic carbon (DIC) concentrations increased markedly from 1882 in the preindustrial treatment to 2123 in the late century treatment due to the CO_2 additions. Based on these measurements actual pCO_2 concentrations in the treatments were calculated as 305, 402, 564 and 1140 ppm (Table 1).

Table 1. pH, pCO_2 (partial pressure CO_2), TA (total alkalinity), DIC (dissolved inorganic carbon) and Ω_{Arag} (aragonite saturation state) values are means of 8 replicates (SE). pH and TA were measured while pCO_2, DIC and Ω_{Arag} were calculated for 24 - 25 ^{o}C. Samples collected in May 2009.

Treatments	pH	pCO_2 µatm	TA µmol kg^{-1}	DIC µmol kg^{-1}	Ω_{Arag}
Preindustrial	8.12 (0.02)	305 (11)	2193 (13)	1882 (16)	3.37 (0.06)
Today	8.02 (0.02)	402 (20)	2170 (27)	1918 (29)	2.78 (0.07)
Projected mid century under A1Fi scenario	7.85 (0.01)	564 (12)	2208 (22)	2012 (17)	2.26 (0.07)
Projected late century under A1Fi scenario	7.63 (0.02)	1140 (52)	2212 (20)	2123 (24)	1.32 (0.04)

CHANGES IN THE MACRO-COMMUNITY COMPOSITION IN BIOFILMS

The frequency of phototrophic flora components (e.g., diatoms and algae) of the biofilm macro-communities at 305 ppm significantly differed from the groups found at 402 ppm, and both were significantly different compared to 1140 ppm (Wilcoxon test, $p > 0.05$) (Table 2 and Supporting Information Table S1). At 1140 ppm the phototrophic community members exclusively comprised of diatoms (Bacillariophyceae), green filamentous and green algae (Chlorophyta), and the green algae decreased with rising CO_2. Further, compared to the biofilms in the other treatments, red algae, filamentous red algae and calcareous red algae (Rhodophyta), were completely absent at 1140 ppm (Table 2).

Table 2. Macro-community analysis of the algal component of biofilm replicates (n = 6) after 11 d for different pCO_2 treatments. The sum of rank numbers: (4) dominant, (3) frequent, (2) occasional, (1) rare, (0) absent, are listed for each algae category in each pCO_2 treatment.

***p*CO₂ treatment**	305	402	564	1140
Algae				
Diatoms	23	24	24	22
Filamentous green algae	15	15	15	15
Green algae	18	18	9	2
Filamentous red algae	6	6	2	0
Calcareous red algae	6	6	3	0
Fleshy red algae	6	6	2	0

METABOLIC ACTIVITY OF BIOFILM COMMUNITIES

During the period under high light conditions all biofilms were net O_2 producers and oxygen production (average = 2.270 μmol^{-1} cm^{-2} h^{-1} ± 0.009) and consumption rates (average = 0.069 μmol^{-1} O_2 cm^{-2} h^{-1} ± 0.0361) of biofilms between the four pCO_2 treatments were statistically indistinguishable (one-way ANOVA production $F_{3,60}$ = 1.35, p= 0.2668; consumption $F_{3,40}$ = 1.11, p= 0.3561).

CARBON AND NITROGEN IN BIOFILMS

Total organic carbon and inorganic carbon in biofilms after 11 d were significantly higher at 1140 ppm pCO_2 compared to 305 ppm (ANOVA $F_{3,20}$= 5.50, p= 0.0006; $F_{3,20}$= 4.70, p= 0.0121, respectively). Nitrogen content increased significantly (ANOVA $F_{3,20}$= 3.32, p= 0.0407) (Table 2) with increasing pCO_2 levels between 305 ppm and 1140 ppm (Tukey-Kramer test). C:N molar ratios (8.196 ± 2.499) remained statistically indistinguishable between pCO_2 treatments (ANOVA $F_{3,20}$= 2.14, p= 0.4644) (Table 3).

Table 3. Total organic carbon, total inorganic carbon and total nitrogen are shown with error bars representing one standard deviation of the mean (mean ± SD) at each pCO_2 treatment at the end of the experiment (11 d).

Treatment (pCO_2)	Total inorganic carbon (mg^{-1} cm^{-2})	Total organic carbon (mg^{-1} cm^{-2})	Total nitrogen (mg^{-1} cm^{-2})	C:N ratio (mol)
305 ppm	0.084 (0.026)	0.137 (0.019)	0.018 (0.003)	7.767 (3.588)
402 ppm	0.128 (0.238)	0.132 (0.009)	0.023 (0.065)	7.172 (1.816)
564 ppm	0.157 (0.295)	0.157 (0.050)	0.020 (0.096)	7.987 (2.225)
1140 ppm	0.189 (0.476)	0.175 (0.022)	0.025 (0.086)	8.245 (0.556)

T-RFLP OF BACTERIAL BIOFILM COMMUNITIES

A total of 37 peaks have been identified using T-RFLP. Thereof, 91.9 % could be successfully assigned to a clone from the clone libraries (within ± 0.5 bp) (Supporting Information Table S2). The most T-RFs were affiliated with the *Alphaproteobacteria* (35.1 %), *Bacteroidetes* (27 %) and *Gammaproteobacteria* (21.6 %). The family *Rhodobacteraceae* (16 %) was dominant in the *Alphaproteobacterial* T-RFs, and families *Flavobacteriaceae* (13.5 %) and *Flexibacteraceae* (8.1 %) were dominant in *Bacteroidetes* T-RFs in all pCO_2 treatments. Profiles of initial communities were significantly different from those at the end of the experiment (data not shown). At 1140 ppm more T-RFs belonging to the *Flavobacteriaceae* were identified than at

305 ppm. Pooling the relative abundances of the T-RFs (bacterial taxa) for each phylum in each treatment revealed that *Bacteroidetes* increased with rising pCO_2, being significantly more abundant (ANOVA $F_{3,230}$= 4.40, p= 0.0048) at 1140 ppm compared to the other treatments (Fig. 1). Conversely, the *Alphaproteobacteria* showed a decreasing trend, as their relative abundance was significantly lower (ANOVA $F_{3,230}$= 4.43, p= 0.0093) at 1140 ppm. *Cyanobacteria* showed a significantly lower relative abundance at 402 ppm than 305 ppm ($F_{3,81}$= 4.15, p= 0.0124). Relative abundances of other groups including *Gammaproteobacteria*, Diatom plastids and *Deltaproteobacteria* were statistically indistinguishable. A principle component analysis (PCA) of T-RFLP data revealed that distinct bacterial community assemblages were present in both high pCO_2 treatments compared to both lower pCO_2 treatments (Fig. 2). Principle component analysis showed that the bacterial biofilm community assemblages at 305 ppm CO_2 had the highest variability between replicate samples within a treatment, followed by 402 ppm CO_2, while the bacterial community assemblages within both high CO_2 treatments (especially the highest 1140 ppm) showed much less community variability (Fig. 2). This same pattern was revealed by re-analysis using nMDS (not shown).

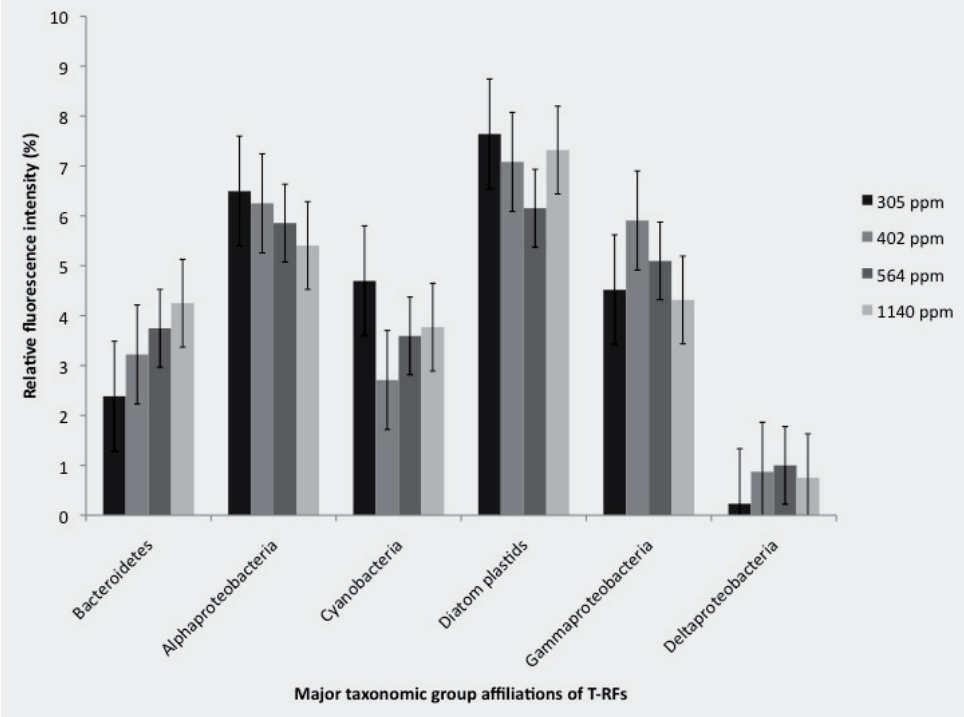

Fig. 1. Average relative abundance of T-RFs of the major phylogenetic groups revealed for each pCO_2 treatment determined by T-RFLP with standard error of the mean (mean ± SE).

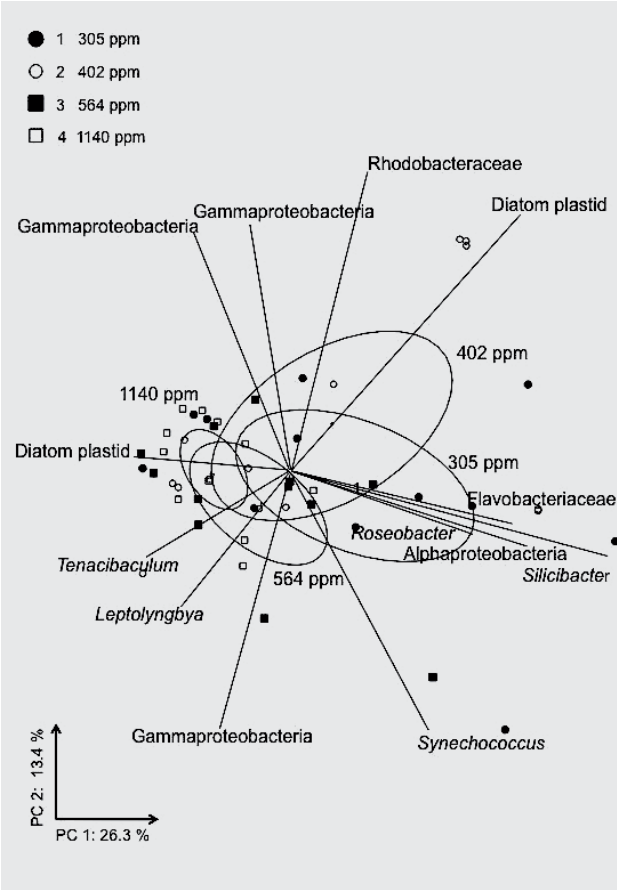

Fig. 2. Principle component (PCA) analysis incorporating relative abundances of T-RFs (using the relative fluorescence peak intensity matrix) showing microbial assemblages for different pCO_2 treatments. Vectors show 40 % of the species contributing the most to the differences between pCO_2 treatments and ellipses represent 95 % confidence around the centroid of each treatment (305 ppm, 402 ppm, 564 ppm and 1140 ppm).

One-way ANOSIM of T-RFLP data revealed global dissimilarities ($p < 0.0001$) of the treatments and post hoc tests indicated significant differences between both 305 ppm ($p= 0.0035$) and 402 ppm ($p= 0.0106$) compared to 1140 ppm. A SIMPER analysis of T-RFLP data showed an overall average dissimilarity of 40% between treatments. In agreement with the PCA biplot, T-RFs contributing the most to the dissimilarities between 305 ppm and 402 ppm to 1140 ppm were Diatom plastids, *Silicibacter*, *Tenacibaculum* and *Roseobacter* (Fig. 3 and Table 4). The average relative abundance of Diatom plastids, *Roseobacter* and *Silicibacter* T-RFs showed a decreasing trend with rising pCO_2, while the *Tenacibaculum* T-RF increased notably (one-way ANOVA $F_{3,40}= 6.62$, $p= 0.0009$) in both elevated pCO_2 treatments compared to 305 ppm (Fig. 3). Two further taxa contributing to the dissimilarities in the bacterial assemblages of 305 ppm and 402 ppm compared to the other treatments were T-RFs from the family *Rhodobacteraceae*, the order *Flavobacteriales* and the genus *Synechococcus*.

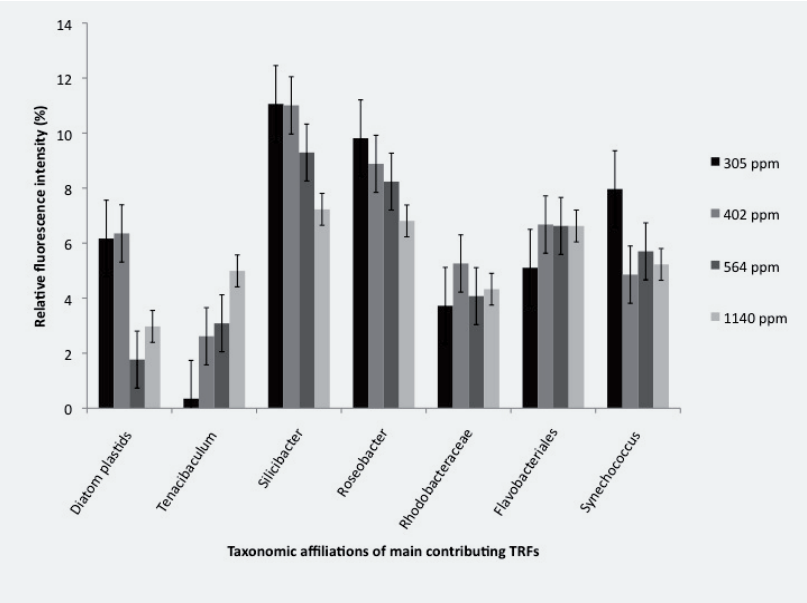

Fig. 3. Average relative abundance with standard error of the mean (mean ± SE) of T-RFs determined by T-RFLP contributing the most to the differences between pCO_2 treatments as revealed by SIMPER analysis.

Table 4. Similarity Percentage (SIMPER) analysis (overall average dissimilarity of 40 %) showing the contribution (%) of the bacterial taxa most responsible for the overall dissimilarities and dissimilarities between the significantly different (ANOSIM) microbial assemblages at the respective pCO_2 treatment.

Taxon	Contribution (%)			
	Overall	305 vs. 402	305 vs. 1140	402 vs. 1140
Diatom plastids	2.4	2.5	2.4	2.5
Silicibacter	2.3	2.6	2.2	2.2
Roseobacter	2.2	2.4	2.6	2.3
Synechococcus	1.9	2.2	2.1	2.0
Tenacibaculum	1.8	>1	2.8	1.9

CLONE LIBRARIES OF BACTERIAL BIOFILM COMMUNITIES

Analysis of sequences obtained from the 16S rRNA gene libraries from biofilms demonstrated that sequences affiliated with the *Alphaproteobacteria* were most abundant in all pCO_2 treatments, except in the control (402 ppm), where *Bacteroidetes* affiliated sequences were most frequent (Fig. 4). Diatom plastid affiliated sequences (~10%) was the only group showing a treatment related trend in the clone library analysis, and increased with rising pCO_2. The cyanobacterial sequences (~10%) belonged to three different orders, *Chroococcales*,

Oscillatoriales and *Nostocales* at 305 ppm, while at 1140 ppm *Chroococcales* sequences were found exclusively (data not shown).

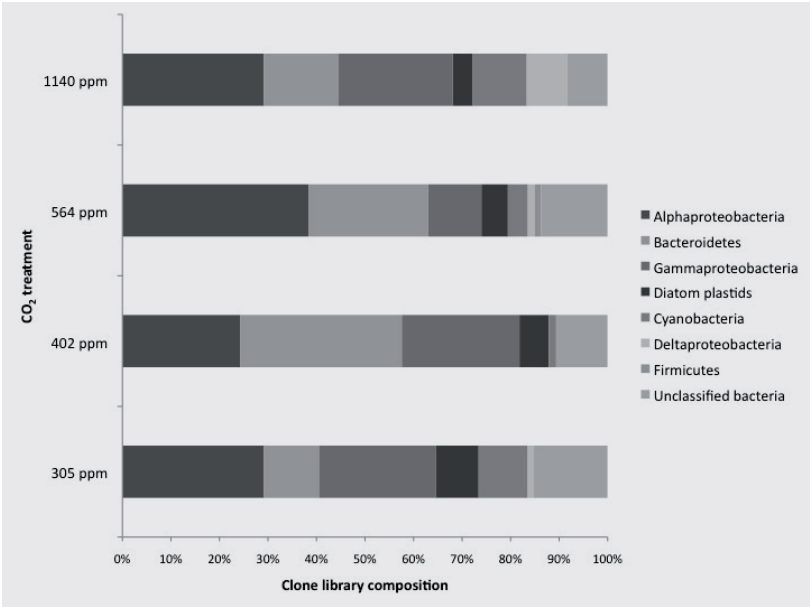

Fig. 4. Affiliations of 16S rRNA gene sequences retrieved from clone libraries from the different pCO_2 treatments (305 ppm n = 79, 402 ppm n = 66, 564 ppm n = 75, 1140 ppm n = 74). Bacterial sequence affiliations were grouped into dominant ribotype at phylum and phylum sub-divisions. Only groups representing 5 % or more of the clone library are represented in the Fig. Category 'other' shows minor groups showing < 5 % of each library.

The LIBshuff test revealed statistically significant differences in the bacterial community compositions between the libraries derived from 305 ppm and 402 ppm, and 305 ppm and 564 ppm (Supporting Information Table S3). Further, statistically significant differences were detected between 305 ppm and 402 ppm when compared to 1140 ppm, while the bacterial communities from 564 ppm and 1140 ppm were statistically indistinguishable. All libraries significantly differed from the initial community (T = 0).

DISCUSSION

The present study is the first to investigate the effects of ocean acidification on oxygen production and microbial community composition in marine biofilms associated with tropical coral reefs. The microbial community in the investigated biofilms was rapidly (after 11 d) and significantly affected by simulated ocean acidification. Despite an increase of carbon and nitrogen content and a significant change in the algal component of the biofilm community, photosynthetic and respiration activity remained on a similar level. T-RFLP and clone library

analysis illustrated that high CO_2 concentrations caused significant shifts in the microbial community composition. These findings add to the growing evidence that microbes are sensitive to disturbance by high CO_2 and are able to adapt on short-term scales to maintain a constant level of activity.

MACRO-COMMUNITY CHANGES AND OXYGEN FLUXES IN BIOFILMS

Changes in the macro-communities of investigated biofilms imply that exposure during the early stages of biofilm development to short-term high CO_2 levels significantly decreases algal diversity and promotes a shift towards diatom and filamentous green algae dominated biofilm communities. Similarly, previous studies under elevated CO_2 showed that phytoplankton communities became diatom-dominated (Tortell, 2002) and promoted an increase in filamentous algae (Kuffner et al., 2008). Further, high CO_2 has shown to promote non-calcareous turf algal growth with a significant increase in biomass (Russell et al., 2009; Connell and Russell, 2010) and such an increase in biomass was also observed in the present study. The abundance of red algae, especially calcareous red algae, was significantly decreased at high CO_2, which agrees with similar studies indicating a decrease in production, biomass or calcification rates of calcareous red algae under elevated pCO_2 conditions (Anthony et al., 2008; Kuffner et al., 2008; Russell et al., 2009). In addition, negative effects on photosynthesis and growth in red seaweeds in response to CO_2 enrichment have been observed (Zou and Gao, 2009).

Although only hourly production rates under full sunlight were measured, the average respiration rates in the dark were about one order of magnitude lower than the average production rates. It is therefore conservative to assume that the investigated biofilm communities were net autotrophic over a 24 h period, indicating a dominant phototrophic component in the biofilms. Despite alterations in the algal community, no significant changes in metabolic activity could be detected, indicating adaption mechanisms of phototrophic members of the macro-community in biofilms to high CO_2 conditions.

CARBON AND NITROGEN CONTENTS IN BIOFILMS

Although not linear, organic nitrogen and carbon contents in the investigated biofilms generally increased under higher pCO_2 (564 ppm and 1140 ppm). In the present study, C:N ratios did not vary with pCO_2 treatment, but were slightly higher (~8) than the Redfield ratio (6.6), supporting the assumption of algal dominated communities. Values exceeding the Redfield ratio may

indicate nitrogen limitation (Healey, 1979), which is typical for primary producers in oligotrophic environments such as the GBR. Increased organic C and N may be due to higher production rates of extracellular polymeric substances (EPS), as often seen in bacteria (reviewed in (Sutherland, 2001) and diatoms (reviewed in (Thornton, 2002) under stress due to nutrient limitation. Thus, we hypothesize that the release from potential CO_2 limitation may have increased N limitation, thus leading to enhanced EPS production binding more calcareous reef sediment particles in the biofilm matrix, hence also elevating the inorganic C. Further, generally elevated C:N ratios may also be explained by enhanced EPS production as a protection against harmful UV-radiation (Elasri, 1999), which is an important factor to consider in Australia and hence should be tested in future studies.

On the one hand, elevated C:N ratios exceeding the Redfield ratio (~8) were also detected in phytoplankton in response to high CO_2, and were speculated to lead to an excess CO_2 sequestration potential through the biological carbon pump in future oceans (Riebesell et al., 2007). Further, a study combining phytoplankton growth with TEPC formation has proposed that constant C:N ratios are no longer appropriate for estimating new production of POC from DIN uptake due to carbon overconsumption (Schartau et al., 2007), which might be applicable in a high CO_2 environment. Alternatively, CO_2 bubbling in the treatments may have formed transparent exopolymer particles (TEP) from dissolved matter (Zhou et al., 1998), which may have also potentially contributed to the increased C:N contents of biofilms within high pCO_2 treatments compared to the remaining treatments without or little bubbling. However, if increased C:N contents in biofilms are in fact linked to high CO_2, increased EPS production may therefore lead to a change in dissolved organic matter bioavailability with elevated pCO_2 that may change particle aggregation and substrate availability for marine microbes. Although this mechanism is plausible and may have far-reaching impacts for biofilm functioning, no direct measurements were conducted and further research is required to investigate this hypothesis.

BACTERIAL COMMUNITY COMPOSITION

Statistical tests (ANOSIM and LIBshuff) of fingerprinting and clone library data suggest that rising pCO_2 levels significantly change community composition in biofilms. Sequences affiliated with the *Rhodobacteraceae* (*Alphaproteobacteria*) and *Cytophaga-Flavobacterium-Bacteroides* (*CFB*) (*Bacteroidetes*) contributed most importantly to the differences between bacterial communities from the different treatments. The frequent detection of these two groups was not

surprising as *CFB* are primarily found on surfaces (McBride, 2001; Nocker et al., 2004; Webster and Negri, 2006), as well as members of the *Roseobacter* that are ubiquitous and rapid colonizers of surfaces, and both are therefore commonly found in marine biofilms (Dang and Lovell, 2000; Dang et al., 2008). Overall, the relative abundance of *Bacteroidetes*, in particular the genus *Tenacibaculum* of the *CFB*, increased with rising pCO_2, while *Alphaproteobacteria*, specifically members of the *Roseobacter* clade, appeared to show the inverse trend. Similar trends of *Flavobacteria* and *Alphaproteobacteria* were also observed in crustose coralline algae associated biofilms in response to other climate change factors such as elevated sea surface temperatures of 32 °C {Webster, 2011 #201}. Further, a substantial increase in *Bacteroidetes*, predominantly *Flavobacteria*, was observed in corals in response to low pH (Vega Thurber et al., 2009). This community shift may therefore be a response to high CO_2. Further, the high relative abundance of *Bacteroidetes* in the biofilms may be due to the fact that many members of the *CFB* cluster excrete exoenzymes to decompose high molecular weight organic material from detritus (Reichenbach, 1991; Keil, 1999; Cottrell and Kirchman, 2000; Kirchman et al., 2000), which may be an advantageous trait in oligotrophic waters as found on the GBR. The degradation rate of marine organic matter by bacterial extracellular enzymes is accelerated by high CO_2 (Piontek et al., 2010) which may be a possible reason for the increase in *CFB* with increasing pCO_2. A potentially higher EPS production under higher pCO_2 (see above) may give members of the *CFB* group a selective advantage due to the more effective acquisition of catabolic substrates.

Furthermore, little changes in the apparent relative abundances of diatoms as revealed by both T-RFLP and clone library analysis suggested little impact of pCO_2 levels on the frequency of diatoms. Previous findings showed that diatoms appear to be insensitive to pCO_2 regarding silification processes (i.e., building of frustules) (Milligan et al., 2009), and that pCO_2 only caused small changes in diatom populations (Kim et al., 2006). Further, Cyanobacterial sequences of the order *Chroococcales*, such as *Synechococcocus,* were detected exclusively in the 1140 ppm treatment in clone libraries and appeared to be more abundant in comparison to other *Cyanobacteria* in this treatment as determined by T-RFLP. *Synechococcus* strains have higher growth rates at elevated pCO_2 levels (Fu et al., 2007), suggesting that this group may also obtain a selective advantage over other *Cyanobacteria* in high CO_2 conditions.
Interestingly, T-RFLP showed low community variability among replicates at high pCO_2 (1140 ppm) compared to 305 and 402 ppm (Fig. 4) that may therefore suggest that communities

become more specialised and adapted to lower seawater pH. A study by Takeuchi and colleagues on the effects of seawater acidification on the growth rates of cultured marine microorganisms found that bacteria were relatively resistant to high concentrations of CO_2. Impacts on bacterial growth were observed only at pH values as low as 5.5 to 6.0 (Takeuchi, 1997). However, Takeuchi's study solely focused on bacterial growth rates and not community composition or diversity and artificial cultivation may cause a different response.

In the current study, both molecular techniques were in agreement and suggested that rising pCO_2 significantly changed community composition of biofilms. Low variability may indicate that there is less competition among bacterial groups. Taken together, the detected shifts in marine bacterial biofilm communities in this study may suggest the ability of these communities to structurally reorganise in response to increased levels of pCO_2.

Further, these community shifts in response to short-term exposure may also be stress-related. We expected to observe adaption via physiological adjustment such as increased O_2 production rates as previously observed in e.g. diatoms (Tortell et al., 2008) to compensate increased pCO_2 availability, but this was not the case. Community changes involved elevated C:N ratios, possibly due to enhanced EPS production as part of a stress response to short-term (11 d) high pCO_2 exposure as proposed above. However, Allgaier et al. (2008) also observed C:N ratios of ~8 after 24 d exposure to high pCO_2 with unaltered bacterial abundance and activity. As no further long-term exposure results are available, the assumption persists that community shifts display adaption mechanisms rather than short-term stress responses.

CONCLUDING REMARKS

Here, we propose that overall, short-term exposure (11 d) to rising pCO_2 levels was sufficient to significantly alter the algal community and the bacterial community composition of biofilms adjacent of tropical coral reefs. These findings indicate sensitivity, but at the same time the adaptive ability of such communities. This study aimed to provide insights into the impacts of elevated pCO_2 in bacterial biofilms and revealed possible target groups, such as *CFB*, diatoms, *Cyanobacteria* and the *Roseobacter* clade for future studies investigating the emerging field of ocean acidification on marine biofilm microbes. Future work should also focus on microbial processes and interactive effects of acidification and other direct or indirect changes of global change (such as temperature and nutrients), as well as exposure to these parameters for longer-

periods. The sensitivity of some bacterial biofilm species in response to altered ocean chemistry may lead to shifts in their abundance and/or function. As biofilms affect biogeochemical cycling the potential effects on future reef ecology needs further investigation.

In recent publications, the sensitivity of marine organisms to ocean acidification has been questioned (Hendriks and Duarte, 2010; Hendriks et al., 2010) and a null hypothesis has been put forward that responses of marine microbes are negligible and that acidification will have little effect on biogeochemical processes other than calcification (Joint et al., 2011). In contrast, a meta-analysis by (Liu et al., 2010) rejected this null hypothesis, but pointed out that microbes in response to ocean acidification have been investigated far too little to draw conclusions for future ecological scenarios. Ocean acidification is suggested to have worse effects on the development of larval stages of marine organisms (Dupont et al., 2010) and was shown to affect juvenile corals (Albright et al., 2010; Suwa et al., 2010). As biofilms are primary reef colonisers, facilitating invertebrate larval settlement and development, changes in these communities may have detrimental effects on future coral reefs.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL DESIGN

We conducted an experiment simulating ocean acidification at the Heron Island Research station, located in the Southern Great Barrier Reef, Australia (23°27'S, 151°55'E), during May 2009 (Austral autumn) using an outdoor flow-through aquarium system. The aquaria setup used a computer-controlled CO_2 dosing system (Aquatronica-AEB Technologies, Italy). Target pCO_2 values were set and the corresponding pH values were continuously monitored in the CO_2 200 L mixing tanks which supply individual tanks, using pH probes (Mettler-Toledo polarographic sensors) which automatically logged pH values. The pH probes used were of high precision and were routinely calibrated to the total seawater scale throughout the experiment at temperatures of 24-25°C, providing high confidence in the target values. The pH readings controlled the open/close status of a solenoid valve (Dupla Australia, Littlehampton, Australia) to regulate the pure CO_2 (analytical grade) supply (bubbling) in each mixing tank of each treatment (set up as in (Diaz-Pulido et al., 2011)). The experimental design consisted of three CO_2 dosing regimes and a control treatment, representing A: preindustrial levels of 300 ppm (pH 8.1- 8.2) (CO_2 scrubbing using soda lime as described in (Reynaud et al., 2003); IPCC, 2007 2007 #153; Uthicke, 2010)B: present-day control 400 ppm (pH 8.0) (reflecting the diurnal variability of the intake water from

the reef ranging from pH 7.9-8.2); C: projected mid-century 560 ppm (pH 7.9) and D: projected late-century under the A1FI scenario 1140 ppm (pH 7.6) by the Intergovernmental Panel on Climate Change (IPCC, 2007). Each treatment was replicated by 6 individual tanks (total of 24 tanks) with a volume of 10 L each at a flow-rate of 2 L^{-1} min^{-1} over 11 d. The aquaria were organized randomly, and shade screens were used to reduce the natural sunlight by 30% to average noon levels of about 1200 (max: 1700) µmol photons m^{-2} s^{-1}. Additionally, the pH of individual tanks was monitored daily using a hand-held pH electrode (HQ10-HQ20 Meters, Hach, USA). Samples for dissolved inorganic carbon (DIC) and total alkalinity (TA) were taken throughout the course of the experiment (reported in Diaz-Pulido et al. 2011). The calculated pCO_2 values were very close to the target values (Table 1). Light (Odyssey, Christchurch, New Zealand) and temperature (UA-001, Onset, USA) loggers were used over the duration of the experiment. As a suitable substrate type for colonisation, glass microscope slides (as proposed by Witt et al. in review) were fixed vertically in custom-built PVC holders (20 x 11 cm) holding 6 slides each. For pre-conditioning, pre-cleaned (washed in ethanol 70 % and rinsed in sterile water) glass slides were immersed into a flow-through tank with natural seawater from the lagoon for 24 d. Subsequently, established biofilm slides were introduced to the experimental tanks and exposed to 4 different CO_2 treatments for 11 d. Biofilm slides were sampled on the initial day (T = 0) and thereafter every third d for oxygen production measurements (see section below). For sample collection, as much as possible of the biofilm material was carefully scraped off the substrates into cryovials using sterile No. 11 scalpel blades (material yield was usually > 2 g, sufficient to extract a high yield of high quality DNA for PCR amplification see section below) and snap-frozen in liquid nitrogen and stored at -80°C until further processing.

DETERMINATION OF MACRO-COMMUNITIES IN BIOFILMS

The algal component of the macro-communities of biofilm replicates from six replicate tanks were examined every 3 days after the production measurements, under a binocular microscope (Olympus, Japan). Visualised organisms were photographed and then assigned to a category for each specimen (diatoms, filamentous green algae, green algae, filamentous red algae, calcareous red algae, fleshy red algae) and their frequency of occurrence (% coverage) was determined. Five categories were scored with the following cut-off values: dominant
(90 %), frequent (70 %), occasional (50 %), rare (20 %) and absent (0 %). The categories were given a rank number in decreasing value: (4) dominant (3) frequent, (2) occasional, (1) rare and

(0) absent. A Wilcoxon-Mann-Whitney rank sum test (U-test) was used to test for significant differences in average ranks between pCO_2 treatments.

DETERMINATION OF OXYGEN FLUXES IN BIOFILM COMMUNITIES

Oxygen production by the biofilm slides was measured in a time series every 3 d under both light and dark conditions for biofilm slides. For the light incubation, replicate (n = 6) biofilm slides were enclosed with water of the corresponding treatment in custom-made airtight glass vials (50 ml), with glass lids that were fixed with plastic clips. The vials were secured horizontally in custom-made holders to enable equal light distribution onto individual slides that were incubated in a transparent outdoor flow-through seawater tank at *in situ* seawater temperature of ca. 23 °C and natural light conditions at noon for 30 min. The dark incubations occurred correspondingly in an opaque tank for 1 h. Test experiments revealed that the chosen incubation times were sufficient to measure a clear response (at least a change of 10 % in dissolved O_2 concentrations). Vials were gently mixed before measuring dissolved oxygen (DO) concentrations using a hand-held luminescent dissolved oxygen optode (HQ10-HQ20 Meters HACH, Hydrolab oxygenmeter, USA) at start and end of incubations. Triplicate blank controls (seawater only) were run simultaneously with all incubations. Changes in O_2 concentration in the blanks were small (< 1 %) compared to the biofilm incubations and were subtracted from the biofilm measurements. Oxygen production and consumption rates were calculated in µmol O_2 cm^{-2} h^{-1}. As this was an outdoor experiment with natural photon flux density variability due to cloud cover, average values during measurements on all sampling days (every 3 d) at noon of 1406 at initial measurements, 1301 on 3 d, 982 on 7 d and 1054 µmol photons m^{-2} s^{-1} at final measurements were used.

CARBON AND NITROGEN MEASUREMENTS

For the measurement of total carbon and nitrogen concentrations in the samples, one half of each biofilm on the microscope slide (8.125 cm^2) was scraped off and transferred onto pre combusted GF/F filters (Whatman, 25 mm in diameter). Filters were dried for 48 h at 40 °C and analysed as described in (Wild et al., 2008). Measurements were performed with a THERMO NA 2500 elemental analyzer (standard deviations of C and N concentration measurements of replicates of the laboratory standard peptone were < 3 %) to derive total carbon and nitrogen concentrations. Biofilms were rinsed with freshwater to remove salts, then dried at 60 °C and homogenised using mortar and pestle. The percentage of organic carbon (treated with 200 µl of 1 M HCl) of

total carbon was determined on a parallel sample for each slide on a Shimadzu elemental analyser (TOC5000A) using standard reference material (MESS-1 and Round 40).

GENOMIC DNA EXTRACTION

Total DNA was extracted from 0.5 g of the total biofilm (wet weight) sample using the MoBio UltraClean Soil Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's protocol with the following modifications. Bead-beating (Mini-Bead-Beater, Biospec Products, Bartleville, OK, USA) (2 x 30 s) cycles were performed and DNA was eluted with 2 x 50 µl of 1 x TE buffer. DNA extracts were examined by standard 1% agarose gel electrophoresis and quantified using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

PCR AMPLIFICATION, CLONING AND SEQUENCING

Bacterial 16S rRNA genes were amplified by PCR using the general bacterial 16S rRNA gene primers 63F (5′-CAGGCCTAACACATGCAAGTC-3′) and 1389R (5′-ACGGGCGGTGTGTACAAG-3′) (Sigma-Proligo, The Woodlands, TX, USA) (Marchesi et al., 1998). Each biofilm sample was amplified in triplicate 25 µl reactions containing 2.5 µM non-acetylated bovine serum albumin (New England Biolabs, USA), 2 µM (2 mM each) dNTP (Astral Scientific, Australia), 2.5 µM forward primer 63F, 1.25 µM reverse primer 1389R, 1 µM $MgCl_2$ (Qiagen), 1.25U HotStar Taq (Qiagen), 2.5 µl HotStar Buffer (Qiagen, Germany) and ~2 ng of template DNA. Amplification was performed with an initial incubation at 95°C for 15 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 90 sec, and a final extension at 72°C for 10 min.

Five clone libraries of bacterial 16S rRNA genes amplified from DNA extracted from biofilms grown on glass substrate were constructed, and represent one library for each treatment (T = 0, 305 ppm, 402 ppm, 564 ppm and 1140 ppm). DNA extracted from each biofilm sample from all six replicate tanks of each treatment was then subject to PCR in each triplicate PCR reactions to avoid PCR bias. Triplicate amplicons of each of the six samples per treatment were then individually pooled back to the original six replicates. Each of the six replicates from each treatment was then pooled according to treatment for construction of the five clone libraries. Pooled samples were purified using the MinELUTE PCR Clean-Up Kit (Qiagen) and cloned

using a TOPO-TA Cloning Kit (Invitrogen, USA) according to the manufacturer's instructions. After blue-white screening, colonies were checked for correct insert size using a colony PCR method with the specific sequencing primer 63F. Randomly picked clones were dispersed in LB media and 10 % glycerol in 96-well plate format and sent to the Australian Genome Research Facility Ltd. (Brisbane, Australia) for purification and sequencing by an ABI3730 XL Automatic DNA Sequencer.

PHYLOGENETIC ANALYSIS OF CLONE SEQUENCES

Retrieved sequences were edited using Chromas Lite 2.33 (Technelysium Pty Ltd., Australia), saved as fasta files and submitted to the Greengenes NAST Aligner (DeSantis et al., 2006) (for alignment of sequences to the Greengenes database). Greengenes NAST-aligned 16S rRNA gene sequences were checked for chimeras using Bellerophon Version 3 (Huber et al., 2004), and identified chimeras were excluded from further analysis. The NAST-aligned 16S rRNA gene sequences were submitted to the Greengenes batch sequence classifier [http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi], and taxonomic assignments for each sequence were recorded using the NCBI taxonomy system.

TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) ANALYSIS

Bacterial 16S rRNA genes were PCR amplified using the same reaction mixture and conditions outlined for clone libraries, except that fluorescently labelled 5´Cy-5 63F (Sigma-Aldrich) and 1389R primers were used. Each of the six duplicate biofilm samples per treatment (12) (in triplicate PCR) was purified using the MinElute PCR purification kit (Qiagen). PCR products were quantified using the NanoDrop Spectrophotometer (Thermo Fisher Scientific), and 150 ng of each purified product was digested with the restriction enzyme MspI (New England Biolabs) according to the manufacturer's instructions. Digested fragments were desalted using the DyeEx 2.0 Spin Kit (Qiagen) and vacuum dried for 40 min at low temperature in the dark. Terminal restriction fragments (T-RFs) were resolved and visualised using the CEQ 8800 Genetic Analysis System (Beckman-Coulter, Fullerton, CA, USA) with a 600 bp size standard (Beckman-Coulter). The threshold for relative peak height was set at 20 % of the height of the second highest peak to remove any spurious artefact peaks from the analysis. Replicate samples were compared using the software T-align (Smith, 2005) with a range of 0.5 bp peak area to determine the consensus peaks between duplicates. The relative fluorescence intensity of the peak area of T-RFs was used as a relative abundance measure of dominant T-RFs in further

statistical analyses detailed below. For verification and identification of taxonomic identity of T-RFs, purified DNA from individual clones (provided by AGRF), taxonomically identified as above, were subject to PCR. The samples were analysed by T-RFLP using the same protocol as for environmental samples, except that 75 ng of digested PCR products generated from each clone was used. Each clone produced a single peak (T-RF), which was then manually assigned to T-RFs identified from whole community T-RFLP profile analyses.

STATISTICAL ANALYSIS

One-way Analysis of Variance (ANOVA) was used to determine significant differences between pCO_2 treatments for the response parameters: relative abundance of T-RFs, total organic and inorganic carbon, total nitrogen and carbon/nitrogen ratio. Two-way ANOVA was performed on production and respiration data to determine effects of the fixed factors time and treatment. Homogeneity of variances was tested using the Levene's Test, and the Tukey-Kramer Test was used as post-hoc tests to investigate the differences between the individual time points and treatments. These analyses were performed using the NCSS 2007 (NCSS, USA) statistical software.

LIBshuff in MOTHUR was used to determine the percentage coverage of the populations and whether there were significant differences in bacterial community composition between clone libraries. All sequences were submitted to the GenBank Database (Accession numbers: HQ601614- HQ601616, HQ601619- HQ601701, HQ601703- HQ601707, HQ601709- HQ601744, HQ601746- HQ601786, HQ601788- HQ601791, HQ601793- HQ601797, HQ601799-601814, HQ601816- HQ601900).

T-RF values were third root transformed and standardised prior to analysis. This is a standard transformation for PCA, to remove variance-mean relationships and remove over-emphasis of extremely rare or extremely abundant taxa (peaks). Principal Component (PCA) and Non-metric Multidimensional Scaling (nMDS) analyses using the Bray-Curtis distance measure were applied to determine whether bacterial assemblage in samples grouped by treatment. The significance of assemblage dissimilarities between pCO_2 treatments was tested by one-way Analysis of Similarity (ANOSIM) based on permutation procedures also using the Bray-Curtis distance measure. The contributions of each taxon to the total dissimilarities of treatments were analysed

using the Similarity Percentage (SIMPER) routine. All analyses were performed using PAST statistical software (Hammer, 2001).

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SUPPLEMENTARY INFORMATION

Supplementary Table S1 Wilcoxon-Mann-Whitney rank sum test (U-test) of the macro-community analysis of the algal component of six replicates after 11 d for different pCO_2 treatments. Different algae groups were observed and grouped into categories (diatoms, red algae, filamentous green algae etc.). The frequency of each algae category was assigned with an index: (4) dominant, (3) frequent, (2) occasional, (1) rare, (0) absent. Significances are shown as p values and $p < 0.05$ are highlighted for clarity.

pCO_2	305 ppm	402 ppm	564 ppm	1140 ppm
305 ppm	----------	**p= 0.0154**	**p= 0.0050**	**p= 0.0072**
402 ppm		----------	p= 0.0671	**p= 0.0031**
564 ppm			----------	p= 0.0601
1140 ppm				----------

Supplement Table S2. Terminal restriction fragments (T-RFs) are shown with their phylogenetic affiliation (as classified using the greengenes batch sequence classifier [http://greengenes.lbl.gov/cgi-bin/nph-classify.cgi] using the NCBI taxonomy) and accession number as derived from the 16S rRNA gene clone libraries from each treatment. 16S rRNA identification of the nearest phylogenetic neighbour with accession number and sequence similarity (%) to each corresponding T-RF as determined by BLAST from the Greengenes database are shown.

Phylogenetic group and accession number	T-RF (size in bp)	16S rRNA identification of nearest phylogenetic neighbour and accession number	Sequence similarity (%)
Alphaproteobacteria			
Microvirga (HQ601817)	86.5	Clone 300G-A04 (AY662029.1)	94.3
Alphaproteobacteria (HQ601722)	90	BBD coral tissue clone LK_36bf (EF123357.1)	96.7
Rhodobacteraceae (HQ601653)	400	Rhodobacteraceae clone MD2.18 (FJ403067.1)	94.1
Rhodobacter (HQ601832)	402	Seawater isolate A30A30 (AB302381.1)	94.0
Roseobacter (HQ601619)	401	Roseobacter sp. str. JC2219 (JC2219)	98.4
Silicibacter (HQ601746)	459	Silicibacter sp. Str. PaD1.36a (GQ391987.1) Clone SHAB655 (GQ348734.1)	99.7
Stappia (HQ601774)	404	Coral tissue clone BBD_217_35 (DQ446160.2)	91.3
Oceanicola (HQ601700)	403	Hyphomonas clone MD3.38 (FJ425626.1)	99.9
Hyphomonas (HQ601672)	394		97.0
Gammaproteobacteria			
Aestuariibacter (HQ601835)	454	Aestuariibacter sp. NT-2008 str. sd 2-38 (AB473549.1)	96.5

Alteromonadaceae (HQ601782)	113	Haliea rubra str.41_15a (EU161717.1)	97.0
Alcanivorax (HQ601742)	91	Sediment clone 61 Td-oil (FM242293.1)	88.3
Oceanospirillum (HQ601620)	138	Salt marsh clone SIMO 4305 (DQ421670.2)	91.3
Pelagiobacter (HQ601633)	107	Sponge clone TAA-10-62 (AM259849.1)	98.7
Vibrio (HQ601620)	471	Clone 401AA11 (EU188117.1)	92.5
Cyanobacteria			
Synechococcus (HQ601728)	456	Synechococcus sp. str. PCC 8807 (AF44	98.1
Anabaena (HQ601717)	460	Spirulina sp. str. CCC snake P.Y-85 (Y18793.1)	92.9
Leptolyngbya (HQ601759)	117	Leptolyngbya sp. str. LLi18 (DQ786166.1)	91.4
Cyanothece (HQ601643)	116	Cyanothece sp. str. WH8904 (AY620239.1)	93.1
Diatom plastids			
Isochrysis (HQ601779)	58	Eukaryote chloroplast clone (FJ425634.1)	92.3
Amphora/Haslea (HQ601760)	461	Amphora sp. C10 (FJ002217.1)	98.9
Bacteroidetes			
Bacteroidetes (HQ601687)	56	Sponge symbiont clone Hg92G12 (EU236422.1)	88.7
Flavobacteriaceae (HQ601816)	455	Gilvibacter sediminis str. Mok-1-36 (AB255368.1)	93.7
Flavobacteriaceae (HQ601823)	168	Coccinimonas marina str. IMCC1846 (EF108213.1)	97.2
Flavobacteriaceae (HQ601837)	54	Marine sponge symbiont (AY372916.1)	99.5
Flavobacteriales (HQ601775)	93	Marine water clone Vis_st3_73 (FN433358.1)	93.8
Olleya (HQ601810)	509	Flavobacterium sp. str. 5N-3 (AB01797.1)	94.6
Cytophaga (HQ601823)	507	Marine macro-alga clone DPC168 (DQ269107.1)	92.0
Flexibacter (HQ601790)	166	Sponge clone TAA-5-44 (AM259882.1)	94.4
Cytophagaceae (HQ601785)	106	Coral Flexibacteraceae clone MD3.32 (FJ425620)	98.5
Tenacibaculum (HQ601847)	108	Flavobacteriaceae 04PA2	88.7
Deltaproteobacteria			
Polyangium (HQ601725)	472	Wastewater clone (CU466766.1)	94.6
Bacteriovorax (HQ601828)	475	Marine clone S25_746 (EF574402.1)	89.3
Nannocystaceae (HQ601730)	127	Reef sand sediment clone AO27 (FJ358875.1)	92.4

Supplementary Table S3. LIBshuff statistical analysis for differences between 16S rRNA gene sequences in clone libraries and clone library coverage at different pCO_2 treatments (305, 402, 564 and 1140 ppm), generated using the program MOTHUR are shown. Coverage (C) values of libraries are given in ΔC_{AB} (top diagonal) and ΔC_{BA} (lower diagonal) scores and significances are given in p values Significant values for this analysis are those when p <0.05. C_A and C_{AB} represent the coverage within community A and the coverage of community A onto community B. *P*-values for the observed ΔC_{AB} and ΔC_{BA} values are determined by determining the fraction of 10 000 matrix permutations resulting in ΔC_{AB} and ΔC_{BA} values e.g., AB is significantly different, but BA is insignificant this means that A is a subset of B.

pCO_2	305 ppm	402 ppm	564 ppm	1140ppm
305 ppm		C= 0.0039 **P= 0.0154**	C= 0.0035 **p= 0.0050**	C= 0.0020 p= 0.0622
402 ppm	C= 0.0033 p= **0.0261**		C= 0.0024 **p= 0.0027**	C= 0.0034 **p= 0.0106**
564 ppm	C= 0.0018 p= 0.0671	C= 0.0005 P= 0.4881		C= 0.0015 p= 0.0602
1140 ppm	C= 0.0025 p= **0.0318**	C= 0.0051 **P= 0.0031**	C= 0.0002 p= 0.9421	

- 6 -
GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

Microbial biofilms respond rapidly to changing conditions in their environment and therefore have previously been examined for their potential as bioindicators in aquatic environments such as rivers, lakes and estuaries. Biofilms should therefore also be useful as bioindicators in marine ecosystems, however, they have barely been investigated in this regard. The main objective of this thesis therefore, was to determine if the detection of changes in microbial communities within biofilms in response to environmental parameters could be used as a bioindicator system for tropical coral reef ecosystems.

i) The first goal was to identify a suitable substrate for bioindicator application.
ii) Further, it was determined whether microbial biofilm communities in coral reefs shift in response to water quality and which water quality parameter is the most decisive in contributing to microbial community changes at inner (5 km distance from the coast) and outer (> 30 km distance from the coast) nearshore reefs over different seasons (wet and dry). The next aim was to then identify predominance and trends of key microbial functional groups that could serve as indicator species.
iii) From the main findings of studies i) and ii) microbial biofilms were evaluated for their potential and suitability as bioindicators.
iv) Disentangling parameters that are only found in combination with each other *in situ* and then targeting the most decisive water quality parameter, should contribute to the understanding of the responses of marine microbes in the course of climate change and thus could help predict future scenarios.
v) An interesting aspect of this study was to elucidate whether changes in the microbial community composition due to climate change factors (i.e., ocean acidification and warming, eutrophication) consequently affect microbial activity (oxygen fluxes).

Finally, the aim of the thesis was to investigate responses of microbial biofilm communities to local anthropogenic and global climate change disturbances. These were then evaluated and the gained knowledge was used to provide suggestions for improved future coastal management programmes.

I) EFFECT OF SUBSTRATE TYPE ON MICROBIAL BIOFILM COMMUNITIES

Firstly, the effect of artificial and natural settlement substrata on microbial biofilm community composition was investigated over longer spatio-temporal scales (48 d, one wet and one dry season) (Chapter 2). Microbial communities established on glass slides did not vary significantly from those grown on natural substrata at the time points in which the samples were taken for analyses of microbial communities. Of particular relevance was the finding of glass slides and coral skeletons substrata producing comparably stable and highly reproducible community compositions independent of sampling season and/or location. Although bacteria initially settling on surfaces may differ greatly between natural and artificial substrata due to surface properties and substrate shape (Sweet et al., 2011), yet, microbial communities in biofilms undergo distinct temporal shifts. The effect of substrate type diminishes and communities become more similar over time (Huggett et al., 2009; Chung et al., 2010). In addition, glass slides fulfil practical requirements, such as low cost and the ease of handling (deployment and biomass removal). Therefore, the use of glass slides under the aspect of future bioindicator studies in coral reefs is proposed, and hence glass slides were used as standardised settlement substrata for biofilm development for the following studies.

II) MICROBIAL BIOFILMS AS INDICATORS FOR WATER QUALITY
KEY MICROBIAL GROUPS

A hypothesis was put forward that microbial communities shift in response to spatial and seasonal water quality differences (i.e., light, salinity, turbidity, chlorophyll *a*, temperature). This was therefore tested *in situ* along a water quality gradient (Chapter 3). As hypothesised, communities shifted in response to water quality, in particular, dissolved organic carbon (DOC) and chlorophyll *a* (Chl *a*) concentrations explained most of the community data variation. Outer nearshore reefs with high light and low nutrient availability displayed a predominance of the phyla *Cyanobacteria* (*Synechococcus*) and *Alphaproteobacteria* (mostly members of the family *Rhodobacteraceae:* genera *Erythrobacter*, *Hyphomonas*, *Roseobacter* and *Silicibacter*) correlated with low Chl *a* concentrations, while at inner nearshore sites a predominance of *Gammaproteobacteria* (mostly the family *Alteromonadaceae*), *Bacteroidetes* and diatom plastids closely correlated with high Chl *a* concentrations was detected. A predominance of the family *Flavobacteriaceae* was detected at inner nearshore, correlated with low DOC concentrations. The observed spatio-temporal distribution of

predominant microbes is likely related to their function and metabolic properties, as well as competition amongst microbes.

MICROBIAL BIOFILM COMMUNITIES AT OUTER NEARSHORE REEFS

The spatial distribution of *Cyanobacteria* and *Alphaproteobacteria* in biofilms at outer nearshore reefs was in concert with results of recent studies investigating sediments and biofilms from the GBR (Uthicke and McGuire, 2007; Kriwy and Uthicke, 2011). Further, the distribution was very likely linked to the higher light- and lower nutrient availability at these sites, as further supported by the close correlation of these microbial groups with low Chl *a* concentrations (Chapter 3).

Cyanobacteria are adapted to oligotrophic waters with high light availability, which is a result of higher water clarity at locations further away from land runoff (Paerl, 1985; Robarts and Zohary, 1987). However, light manipulating aquaria experiments showed that light only had a limited effect on the relative abundance of *Cyanobacteria* (Chapter 4), which may be explained by their ability to adapt to growth in altered light quality and quantities (Grossman, 1990; Kulkarni et al., 1992; Grossman et al., 1993; Kulkarni and Golden, 1994; Bhaya et al., 2002). *Cyanobacteria* should therefore also be abundant at inner nearshore sites, however, these are outcompeted by other microbial groups that have a competitive advantage under high nutrient availability. This leads to a consequent selection towards N-fixing *Cyanobacteria* at low N regimes and hence to a predominant occurrence in tropical oligotrophic offshore waters (Crosbie, 2001a, 2001b; Moisan et al., 2010; Nelson et al., 2011; Kriwy and Uthicke, 2011) The differences in seasonal distribution (higher abundance during the wet season) may be explained by higher water temperatures during the wet season (average 28 - 29 °C) favouring *Cyanobacterial* growth (Paerl, 1985; Robarts and Zohary, 1987), as also reflected by an aquarium experiment where the relative abundance of the phylum *Cyanobacteria* and genus *Synechococcus* increased at higher temperature (29 °C) (Chapter 4).

The high relative abundance of *Roseobacter* at outer nearshore sites, may be because they have a potential competitive advantage due to their mixed metabolic strategies, for example, their ability to perform aerobic anoxygenic phototrophy (Allgaier et al., 2003) and ability to rapidly absorb dissolved nutrients in coastal waters (Denner et al., 2002; Alonso-Saez and

Gasol, 2007). *Rhodobacteraceae* harbour many N-fixing species and, in particular *Roseobacter* in surface-attached biofilm communities, are good competitors under low nutrient conditions (Pinhassi and Berman, 2003; Pinhassi et al., 2004). Considering the spatio-temporal distribution of these specific groups, *Cyanobacteria* and *Roseobacter* (*Alphaproteobacteria*) may be indicators of oligotrophic water conditions.

MICROBIAL BIOFILM COMMUNITIES AT INNER NEARSHORE REEFS

Contrastingly, the phyla *Gammaproteobacteria* and *Bacteroidetes*, and diatom plastids were were correlated with high Chl *a* concentrations, and were predominantly found at inner nearshore locations characterised by low light and high nutrient availability. A predominance of *Gammaproteobacteria*, in particular members of the marine heterotrophic *Alteromonadaceae* and helical marine heterotrophic *Oceanospirillum* group, has also previously been detected at sites of reduced water quality on the GBR (Kriwy and Uthicke, 2011), the Atlantic coast (Dang et al., 2008) and estuaries (Jones et al., 2007; Nocker et al., 2007). The spatial distribution of *Gammaproteobacteria* can be due to the physiological diversity and more efficient use of organic matter among these microorganisms (Gupta, 2000). The relative abundance of the genus *Oceanospirillum* increased with rising temperature under high and low light conditions, however was 50 % lower at low compared to high light availability, indicating higher competition amongst bacteria under low light availability. This has previously been observed in field-grown biofilms, where at shallow water sites (high light availability) abundant species of the phylum *Gammaproteobacteria* promoted community shifts along a water depth gradient on the GBR (Webster et al., 2004).

Planktonic diatoms are frequently more abundant in nearshore waters with high nutrient input and low light availability (Relevante and Gilmartin, 1982). This has also been demonstrated for diatoms in the benthos (Gottschalk et al., 2007; Uthicke and McGuire, 2007) and in biofilms from the GBR (Kriwy and Uthicke, 2011). Of particular relevance was the increase of diatoms in response to elevated nitrate, indicating that diatoms are potentially N-limited, and are hence often associated with nutrient-rich inshore waters, which also have significantly reduced light availability. However, in both aquarium studies described in this thesis, diatoms were also abundant under high light levels similar to those found at outer nearshore sites (Chapters 4 and 5). Under high light conditions diatoms exploit light availability to produce exudates (extracellular polymeric substances) fuelling other microbes (reviewed in (Thornton,

2002)). If diatoms in irradiance-limited habitats are not able to sequester light by e.g., motility to higher light environments for photoautotrophy (Johnson et al., 1997), some species switch to their heterotrophic metabolism (Tuchman et al., 2006). Further, in the case of terrestrial runoff importing sediment and nutrient loads, it has been demonstrated that diatom communities do not respond to short-term changes in light and nutrients, but rather to sediment type (Grinham et al., 2010).

Flavobacteriacae were consistently detected in greater relative abundance at inner compared to outer nearshore sites and were surprisingly correlated with low DOC concentrations. Heterotrophic *Flavobacteriacae* play an important role in organic carbon cycling in aquatic habitats and are able to breakdown high molecular weight organic matter through the secretion of extracellular enzymes (Cottrell and Kirchman, 2000; Kirchman, 2002). Hence, these are often abundant in nutrient-rich waters where organic macromolecules are abundant (e.g., during phytoplankton bloom decay) (Reichenbach, 1989; Riemann et al., 2000). However, high DOC can be an indicator for decaying plankton blooms that leach organic nutrients and hence correlations between *Flavobacteriacae* and low DOC are unclear. It is suggested that *Flavobacteriaceae* may therefore respond more to particulate than dissolved organic carbon. Moreover, in the current study *Flavobacteriacae* also increased at offshore reefs in response to elevated pCO_2, hence higher C availability (Chapter 5). It was speculated that *Flavobacteriacae* likely thrived on the increased C and N availability from presumed increased excretion of extracellular polymeric substances. Further, *Flavobacteriacae* are often tightly coupled to diatom species (Pinhassi et al., 2004), which further explains both of these groups being more frequently found together, e.g., at inner nearshore sites, but also under ocean acidification scenarios.

Nevertheless, given their spatio-temporal distribution, the three groups *Gammaproteobacteria, Flavobacteriacae* and diatom plastids may be indicators of eutrophication. Comparably, the same trends in the distribution of microbial groups have been observed in bacterioplankton along a gradient from the Columbia River Estuary to the open ocean, where *Cyanobacteria* and *Alphaproteobacteria* were more abundant in the coastal surface water than in the river plume, where *Gammaproteobacteria*, *Bacteroidetes* and diatoms were dominant (Fortunato et al., 2012). Findings of this study revealed significant spatio-temporal differences in the distribution of microbial biofilm communities (i.e., at inner

and outer nearshore sites, wet and dry seasons) due to influences of terrestrial runoff deteriorating water quality and seasonal temperature influences. Further, specific functional microbial groups such as autotrophs, N-fixers and heterotrophs could be identified in relation to specific environmental conditions (Table 1).

Table 1. A summary of key phylogenetic microbial groups and their abundance responses to environmental factors linked to their properties and function

Phylogenetic group	**High Temperature**	**High Nitrate**	**High pCO_2**	**Low Light**	**Properties and Function**
Cyanobacteria (*Synechococcus*)	increase	decrease	increase	no effect	autotrophy, N-fixation, light and temperature tolerance
Alphaproteobacteria (*Rhodobacteraceae, Roseobacter clade*)	decrease	decrease	decrease	decrease	auto- and heterotrophy, N-fixation, aerobic anoxygenic photosynthesis,
Diatom plastids	no effect	increase	little effect	no effect	auto- and heterotrophy, nitrate limitation
Bacteroidetes (*Flavobacteriaceae, Flavobacteria*)	increase	increase	increase	no effect	heterotrophy, decomposition of organic material from detritus
Gammaproteobacteria (*Alteromonadaceae, Oceanospirillum, Tenacibaculum*)	increase	increase	increase	decrease	heterotrophy, sulfur-oxidation, nitrate reduction

III) BIOFILMS AS INDICATORS

Taken together, the structure of microbial communities was determined in response to various environmental parameters, the dominance and trends of functional groups and potential indicator species were identified, and microbial communities within marine biofilms were successfully evaluated as suitable bioindicators for coral reef water quality. Five dominant microbial functional groups could be identified in correlation with water quality parameters (temperature, pCO_2, nitrate, light, DOC, Chl *a*) in a series of *in situ* and microcosm

experiments, and their dominance was likely explained by competitive advantages due to their function and metabolism. A predominance of *Gammaproteobacteria*, diatom plastids and *Bacteroidetes* characterise inner nearshore biofilm communities, while a predominance of *Cyanobacteria* and *Alphaproteobacteria* largely characterise outer nearshore biofilm communities. Of particular relevance is the detection of specific microbial groups under certain water quality conditions. For example the families *Alteromonadaceae* and *Flavobacteriaceae*, and diatom plastids may serve as indicators of eutrophication (also enhancement through elevated pCO_2) and predominant heterotrophy, while the phyla *Cyanobacteria* and *Alphaproteobacteria* (family *Rhodobacteraceae* and genus *Roseobacter*) may be indicators of oligotrophic waters, dominant phototrophy and N-fixation. The detected specific microbial groups were repeatedly reconfirmed in a series of experiments in the current thesis (Chapters 2 - 5) and in another GBR study (Kriwy and Uthicke, 2011). In particular, the finding of a decrease in the relative abundance of *Alphaproteobacteria* (mostly *Roseobacter*) and concomitant increase in *Bacteroidetes* (mostly *Flavobacteriaceae*) from 16S rRNA analyses that have been detected in biofilms in response to increased water temperature (Webster et al., 2011), increased pCO_2 (Chapter 5), nitrate and temperature-nitrate interactions (Chapter 4), is highly relevant. Therefore, more in-depth studies targeting these particular groups are recommended, as these might be valuable bioindicator groups to be integrated into long-term coastal health monitoring programmes. Finally, T-RFLP is a suitable method for detecting significant microbial variation in biofilms (microbial community shifts and relative abundance of microbial groups) across spatial and temporal scales. Hence, in this thesis it is proposed that T-RFLP may provide a cost-effective and rapid approach to monitor land use impacts on coastal water quality in tropical coastal coral reef ecosystems.

Microbial biofilms, like other indicators, nevertheless have their limitations that must be understood to effectively employ them as environmental sensors. As already suggested by Snyder et al. (2005) for estuarine microbial biofilms, it is unlikely that biofilms can be developed as an absolute indicator of a condition, i.e., if ‘x’ is detected from biofilm analysis, it means ‘y’ for that location. In this thesis it is suggested that this also applies to microbial biofilms in coral reefs, which are rather perhaps best employed as a relative indicator of differences across spatial and temporal scales. Comprising predominantly of single-cell organisms, biofilms are physiologically proximate to ambient conditions in which they

develop, and are integrative of conditions over the chosen incubation period. This characteristic makes biofilms reactive to environmental conditions and change in advance of other components of the ecosystem. The latter characteristic is perhaps most important in coastal coral reefs close to estuaries, where dynamics of the system often preclude adequate characterisation from single spatial and temporal point grab samples. Therefore, biofilms are applicable to analysis of spatial patterns of a condition or impact on site-specific to whole system scales. Biofilms bear large information contents and further research is recommended to realise their full potential.

IV) MICROBIAL BIOFILM COMMUNITIES UNDER FUTURE CLIMATE CHANGE

Five microbial target groups have been identified, correlated with certain environmental conditions. However, how do these microbial groups respond in the course of climate change and may the findings allow to predict future microbial coral reef-associated scenarios? Microbial biofilm communities in marine environments shifted in response to ocean acidification and elevated temperature. Only under high light, effects of elevated temperature and nitrate were additive (Chapters 4 and 5). Hence, climate change may shift microbial groups. Current *in situ* water temperatures in the central GBR during the wet season may favour the growth of *Cyanobacteria* as demonstrated in an aquarium study where the relative abundance of *Cyanobacteria*, in particular the genus *Synechococcus*, increased significantly at corresponding temperatures (Chapter 4). However, *Cyanobacteria* decreased drastically in response to predicted future elevated SSTs (IPCC, 2007; Lough, 2008), indicating that ocean warming may lead to altered biofilm microbial communities. Further, *Cyanobacteria* are sensitive to nitrate enhancement as found *in situ* during the wet season flood plumes. Depending on the intensity of weather conditions, such as cyclones, rainfall, wind and wave action, and water retention times, remnants of nutrient-rich flood plumes may reach outer nearshore reefs (Cooper et al. 2007). As demonstrated in an aquarium experiment, the relative abundance of *Synechococcus* decrease at elevated nitrate concentrations (Chapter 4). Thus, future reef scenarios may likely involve a shift within *Cyanobacteria* community compositions, possibly shifting away from N-fixing species. In contrast, elevated pCO_2 shifted towards *Synechococcus* dominance (Chapter 5). Interesting future research could be directed at studying interactive effects of acidification, temperature and nitrate enhancement. As N-fixation by *Cyanobacteria* importantly contribute to nutrient availability in coral reefs

(Larkum et al., 1988), terrestrial runoff and ocean warming interactions may impact the N cycle.

Further, terrestrial runoff imports increased nitrate and sediments to reef waters. *Roseobacter* are usually more abundant at outer nearshore reefs and responded to a temperature-nitrate interaction under high light availability; they were less abundant at elevated temperatures, but more abundant under highly elevated nitrate (flood plume concentrations) (Chapters 3 and 4). Therefore, ocean warming could perhaps decrease *Roseobacter*. Further, *Roseobacter* are adapted to high light and low nutrient availabilities, and therefore reduced light availabilities caused by increased sediment loads in the water column, have adverse effects on this functional group. Hence, this genus may either significantly decrease in the future or, along with other abundant photoheterotrophs (e.g., *Rhodobacteriaceae*), switch their metabolism to predominant heterotrophy and take advantage of excess nitrate availability. However, whether these flood plumes reach outer reefs, depends on tides and currents, and grain size deposition (e.g., fine-grained particles settle out of the water column slower than large particles, and are transported over longer distances by currents) (Cooper et al., 2007).

The increased abundance of *Gammaproteobacteria* and *Bacteroidetes* under poor water quality indicates that heterotrophs may have competitive advantages in future oceans due to their functional diversity (Gupta, 2000). However, high temperatures, acidification and nutrient inputs cause diseases in coral reef organisms, and, as shown by diseased corals and sponges, host much greater abundances of *Bacteroidetes* and *Gammaproteobacteria* than their healthy counterparts (Pantos and Bythell, 2006; Webster et al., 2008). These findings suggest that future coral reefs under climate change may be increasingly threatened by pathogens. Finally, diatoms seem relatively well adaptable to different environmental changes as demonstrated in previous experiments and, similar to *Rhodobacterales*, may switch to predominant heterotrophy.

V) MICROBIAL BIOFILM COMMUNITY PRODUCTIVITY UNDER CLIMATE CHANGE

Climate change disturbances (i.e., ocean warming and acidification), throughout the experiments, resulted in obvious microbial community shifts. However, do these changes in microbial community composition also alter biofilm functioning? Microbial community shifts in biofilms showed contradictory productivity and biomass responses under different

environmental conditions. For example, short-term exposure to elevated pCO_2 resulted in an increase in C and N contents, yet had no effect on oxygen production or respiration rates. Biofilm communities rapidly adapted and reorganised on short-term scales in response to high pCO_2 in order to maintain a constant level of activity (i.e., O_2 production and respiration) (Chapter 5). In contrast, temperature- and nitrate-induced microbial community shifts in biofilms also involved a consequent change in microbial activity. Although independent of light availability, C-, N-, and Chl *a* contents decreased in response to high temperature and oxygen production decreased linearly with biomass and Chl *a* contents. Only under high light availability additive effects of elevated temperatures and nitrate concentrations reduced oxygen productions (Chapter 4). This may indicate that microbial activity, in particular O_2 fluxes, in biofilms is more sensitive to temperature than pCO_2 levels. However, either pCO_2 may not affect O_2 fluxes, but may alter other metabolic processes that were not measured in this study or exposure of biofilms to elevated pCO_2 conditions were simply not long enough. To further evaluate effects of pCO_2 on biofilm metabolism, long-term exposure and other metabolic processes of biofilms will need to be investigated.

Overall, biofilms at both inner (5 km distance from coast) and outer nearshore (> 30 km distance from the coast) locations in the study area are primarily affected by warming SSTs. Inner reefs, adapted to high-nitrate regimes, are additionally affected by light reduction resulting from eutrophication and sediment loads. Hence, biofilms harbour more heterotrophic microbial species (i.e., *Gammaproteobacteria*) limited by light, while outer reef biofilms are mainly composed of nitrate-limited photoautotrophic species (i.e., Roseobacter, diatoms and *Cyanobacteria*). However, in comparison to other coral reef communities under inshore conditions, microbial biofilm community composition and activity did not, as suspected, shift from dominant phototrophy to heterotrophy. Even under simulated inshore conditions with low light availability, biofilms were still net productive with P/R ratios exceeding 1. Nevertheless, a shift towards heterotrophy is still likely to occur after long-term exposure to such conditions and should be subject to further research.

RESEARCH IMPLICATIONS

SUGGESTIONS FOR COASTAL MANAGEMENT

In conclusion, both global warming and eutrophication simultaneously affect microbial biofilms established in coral reef habitats. The findings add to the growing body of evidence that runoff alters microbial community composition in biofilms associated with coral reefs. Biofilms at outer reef conditions were affected by additive effects of temperature and nitrate. However, current flood plumes are unlikely to reach these reefs, suggesting higher resilience to temperature at outer than inner reefs, but only under the assumption that runoff does not worsen in the near future. As inner reefs experience light reduction due to increased sediment load, it appears that associated biofilms are less vulnerable to nitrate, but clearly vulnerable to temperature increase, indicating a runoff-temperature interaction for inshore reefs. However, the interaction of temperature is not with nitrate but with sediment loads in runoff. Yet, rising sea surface temperatures are difficult to influence, as long-lasting reductions in greenhouse gas emissions can only be reached by a concerted global management effort. However, with the current overpopulation, further exploitation of the Earth's resources leading to continuously rising CO_2 emissions, and rather slowly implemented use of alternative energy sources global management poses a great challenge. Contrastingly, local nitrate and sediment input through farming can be better controlled by sustainable regulations and coastal management. According to the results of this study, terrestrial runoff (simultaneous sediment and nitrate input) needs to be reduced to prevent temperature-vulnerable outer reef scenarios. Therefore, stringent control over land-based pollution to ameliorate water quality is suggested, as this may be equivalent to reducing SSTs and thus counteract adverse effects.

IMPROVEMENTS FOR FUTURE RESEARCH AND PERSPECTIVES

The findings contribute to new insights on the distribution of microbes within surface-attached communities in coastal coral reefs and to understanding the factors driving community changes and their concomitant metabolism. Microbes within biofilms respond to environmental stress and hence may serve as indicator species for microbial biofilms as bioindicators for the application in long-term water quality monitoring and future coastal management. To further support that the identified microbial groups are consistent indicators of water quality, further steps towards application to be undertaken should include qualitative (e.g., next-generation sequencing, application of specific primer sets to effectively capture target species) and quantitative (e.g., quantitative real-time PCR) determination of functional

microbial groups in biofilm communities over long-term spatio-temporal investigations (> 2 years). Of additional interest would be to study deeper into the rare biosphere and identify single differences in the community composition using next-generation sequencing. A quantitative physiological study on rare species could also give insights into some numerically outnumbered but physiologically highly active species. Finally, further suggestion of more in-depth investigation of the physiological and metabolic variability accompanying community shifts and monitoring of gene expression patterns is suggested. Specifically the relatively unexplored effects of pCO_2 on marine microbes should involve future investigations of interactions of pCO_2 with other factors such as elevated temperature and nutrients. This may importantly contribute to a better understanding of climate change effects on microbial composition, productivity and functioning in coral reef ecosystems, knowledge that can further protect the world's coral reefs.

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