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BACTERIAL DIVERSITY AND METABOLISM IN TROPICAL CORAL REEF BIOFILMS

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Abstract

Water quality in coastal regions around the world is declining in response to global (i.e., ocean warming and acidification) and local (i.e., land-based activities) anthropogenic impacts. Terrestrial runoff leads to increased nutrient and reduced light availability to nearshore coral reefs with adverse effects. Bacterial communities associated with biofilms shift rapidly in response to changes in water quality and hence may serve as bioindicators for reef health. However, the potential of microbial biofilm indicators and whether microbial communities shift also alter community functioning remain unexplored. This study investigated the recovery and adaption potential of biofilm-associated microbes and determined potential consequent changes in organism functioning. Biofilms were established on glass slides for 35 d in situ at inshore coastal sites exposed to runoff (low light, high nutrient availability) and more pristine sites (high light, low nutrient availability). Transplant experiments were performed with biofilm replicates (transplanted from impacted to pristine and vice versa) and sampled after 4 and 10 d exposure to the new site. Bacterial community composition was determined by applying the DNA fingerprinting technique of Automated (ARISA) of the 16s-23s ITS region and metabolism was determined by direct measurement of O_2 fluxes by dark/light incubations using a luminescent optode. The most diverse biofilm communities were found at inshore sites (352 operational taxonomic units OTUs), while both transplanted biofilm communities were least diverse (290 OTUs). OTUs were predominantly shared amongst communities, while purely site specific

associated OTUs were rare (maximum 6 OTUs). Multivariate statistics (i.e., Nonmetric multidimensional scaling analysis) of ARISA data showed clear segregation of inshore-, offshore- and transplanted communities at each sampling point. Analysis of Similarity (ANOSIM) showed significant differences between all four communities (p=0.0001). Although transplants developed into distinct communities, yet each transplanted community structure shifted towards that of biofilms at the new site rather than the original site. Offshore biofilms were twice as productive as inshore ones. Off- to inshore tranplants benefited from increased nutrient availability and hence doubled their productivity, while vice versa productivity remained unimpaired. Hence, despite microbial community shifts through tranplantation, only nutrient- instead of light availability also reflected in the respiratory metabolism and function of the biofilms. Findings may contribute to understanding consequences of terrestrial runoff on ecosystem functioning and resilience, and to the application of microbial biofilms in long-term water quality monitoring programmes and future coastal management.

Introduction

Water quality in coastal regions worldwide is declining in response to global (i.e., ocean warming and acidification) and local (i.e., land-based activities) anthropogenic impacts. In particular during the wet season, rivers import large amounts of nutrients, sediments and freshwater onto coastal tropical waters. Terrestrial runoff leading to eutrophication, reduced light availability and decreased salinity, consequently deteriorates water quality of coastal tropical coral reefs (Wilkinson, 1999). Coastal development and land-clearing to facilitate agriculture destroy natural barriers thus promote greater impacts of terrestrial runoff and flood events during the wet season on vulnerable coral reef communities in South-East Asia (Cruz*, et al.*, 2007), the Great Barrier Reef (Fabricius, 2005), Hawaii and French Polynesia (Chin*, et al.*, 2011). Recent reports document significant alterations in the composition of benthic communities in response to degrading water quality e.g., on the Great Barrier Reef (GBR) including corals (Fabricius, 2005, Fabricius*, et al.*, 2007, Haapkyla*, et al.*, 2011, Weber*, et al.*, 2012), algae (Schaffelke, 2005), foraminifers (Uthicke & Altenrath, 2010), sediments (Uthicke & McGuire, 2007) and bacterial biofilms (Kriwy & Uthicke, 2011, Witt*, et al.*, 2011, Witt*, et al.*, 2012). Another region of concern is French Polynesia in the

Pacific, where land use farming practices result in terrestrial runoff from fertilizer (pineapple farming) and livestock waste (piggeries) importing nutrients and pollutants onto coastal coral reefs thus altering benthic community structure (Adjeroud, 1997, Chin*, et al.*, 2011).

Terrestrial runoff has recently been discovered to alter microbial communities associated with corals with negative impacts on the host's health (Haapkyla*, et al.*, 2011, Weber*, et al.*, 2012), thus intensifying the research focus on marine microbes. Marine bacteria are the first line of defence in coastal ecosystems due to their potential to mitigate the detrimental effects of certain anthropogenic pollutants, through degradation and transformation of harmful compounds. Marine bacteria predominantly organize themselves in surface-attached biofilm communities that play essential roles in invertebrate larval settlement (Wieczorek & Todd, 1998) and nutrient turnover (Battin*, et al.*, 2003). As these communities are highly responsive indicators of changing environmental conditions as a consequence of their rapid life cycle (Paerl & Pinckney, 1996) and ability to structurally self-organize (Tolker-Nielsen & Molin, 2000), bacterial biofilms have been tested for their potential as indicators for water quality conditions in riverine (Araya*, et al.*, 2003), estuarine (Snyder*, et al.*, 2005, Moss*, et al.*, 2006, Jones*, et al.*, 2007, Nocker*, et al.*, 2007), polar (Webster & Negri, 2006) and marine (Dang*, et al.*, 2008) systems. Despite the importance of biofilm communities regarding coral reef resilience and functioning, only few studies have dealt with microbial biofilm bioindicators in coral reef ecosystems. These report that bacterial community composition in biofilms shift in response to local anthropogenic nutrient and consequent light impacts (Meyer-Reil & Koster, 2000, Nocker*, et al.*, 2007, Kriwy & Uthicke, 2011, Witt*, et al.*, 2011, Witt*, et al.*, 2012), increased sea surface temperatures (Witt*, et al.*, 2011, Witt*, et al.*, 2012) and ocean acidification (Witt*, et al.*, 2011). However, in-depth studies on microbial target indicator species and effects of anthropogenic impacts on biofilm functioning, to date, have been ignored.

Previous own work identified that increased water quality parameters (in particular elevated Chl *a* and DOC concentrations) during the wet season shift bacterial community composition in field-grown biofilms showing changes in relative abundances of bacterial functional groups (Witt*, et al.*, 2012). For example, bacterial biofilms displayed an increased abundance of *Flavobacteria* and decrease in *Roseobacter* clade members when exposed to nutrient-

(Witt*, et al.*, 2012) (Witt*, et al.*, 2012), thermal- (Webster*, et al.*, 2011, Witt*, et al.*, 2012) and acidification (Witt*, et al.*, 2011) stress. Further, bacterial community shifts have lead to changes in O_2 fluxes in response to nitrate, temperature and light (Witt*, et al.*, 2012), but not to ocean acidification (Witt*, et al.*, 2011). Further, GBR *in situ* inshore and offshore scenarios during the wet and dry seasons were simulated in aquaria by manipulating combinations of the factors temperature (26, 29, 31°C) nitrate (0.5, 1.0, 1.4 µM) and light (40 and 200 µmol photons $m^{-2}s^{-1}$). Biofilms in simulated 'offshore' conditions became nutrient-limited, while 'inshore' biofilms became light-limited. Consequently it was hypothesised that at inshore reefs, light reduction, through sediment input from runoff, has a stronger influence on triggering bacterial community shifts with a concomitant reduction in O_2 fluxes than increased nutrient input (Witt*, et al.*, 2012).

Despite the importance of bacterial communities, the knowledge of abiotic factors controlling the growth of specific functional groups within coral reef-associated biofilms and whether microbial community shifts consequently affect biofilm functioning (sponge larval settlement, biogeochemical processes) is presently very limited, but of great concern from a management aspect. Hence, it is important to investigate the diversity and function of bacteria in order to investigate their potential as bioindicators and furthermore to understand the resilience of coral reef ecosystems. Coral reefs are threatenend by human impacts such as coastal development and land-based pollution, in parallel with coral bleaching and crown-of-thorns outbreaks. Coral reefs in Australia, Hawaii and French Polynesia are the best monitored reefs: management and conservation strategies include long-term monitoring, creating local regulations and zoning, and addressing land-based threats. Although much financial and research effort is invested, for example, in monitoring and conserving the GBR in Australia, the reef degrades at rapid rates and there is still a need for bioindicator research and coastal management improvement. Understanding qualitative and metabolic responses of biofilms to terrestrial runoff will contribute importantly to understanding coral reef ecosystems and further, may contribute to future coastal management which may be readily adaptable to other tropical regions.

The project therefore aims to firstly investigate responses of biofilms along a water quality gradient with respect to bacterial composition, diversity and function (metabolic) on a

spatial scale (changes in nutrient- and light availability and salinity caused by terrestrial runoff from flooding events). Secondly, to reveal the resilience of bacteria, meaning to what extent biofilm communities retain and/or adapt their composition, diversity and/or function from their original environment to a new environment and investigate whether microbial community shifts also alter biofilm functioning. For this purpose, biofilms were established on glass substrates in situ along a water quality gradient comprising of sites in the bay highly exposed to terrestrial runoff (low light, high nutrient availability) and more pristine sites (high light, low nutrient availability). Biofilms were transplanted from impacted to pristine locations and vice versa. Biofilms may serve as bioindicators for water quality on coral reefs and on a greater ecological scale, bacterial biofilms affect invertebrate larval settlement, and may ultimately also alter coral reef recruitment and resilience. Knowledge on bacterial biofilms may therefore add to the development of monitoring impacts and conservation planning.

Methods

Sampling sites and experimental setup

The field experiment was conducted at the island of Moorea, French Polynesia in the South Pacific in September 2013. A total of 72 glass microscope slides for biofilm development (Witt*, et al.*, 2011) were fixed into custom-made holders anchored in the reef sediment at 3 m depth. Pickets were wrapped in plastic foil (to minimise metal contamination) were anchored in the reef at 2-3 m depth deployed as described in Witt et al. 2011) along a water quality gradient at the north end of Moorea for 40 d to enable biofilm formation. The gradient comprised of 2 ‘impacted’ sites exposed to terrestrial runoff (low light, high nutrient availability) in Opunohu Bay (Opunohu 1: S 17°31.047' W 149°51.061' Opunohu 2 : S 17°31.050' W 149°51.073') and 2 ‘pristine control’ sites at the long-term water quality monitoring site Tiahura (Tiahura 1 : S 17°29.227' W 149°53.994' Tihaura 2 : S 17°29.221' W 149°53.974') exhibiting high light and low nutrient availability with each 2 replicate sites 25 - 30m apart. Water quality background data acquired from loggers will be provided by Joachim Claudet and Gilles Siu. Water samples were taken in duplicates from each replicate site at each sampling point and analysed for nutrient content (nitrite, nitrate and phosphate) kindly by Benoit Espinau. In addition, light and temperature loggers Hobo monitored throughout the transplant experiment. Glass slides (36 per location i.e., 18 per site) were

deployed at the north end of Moorea comprising of 2 impacted sites near the bay head exposed to terrestrial runoff Oponohu Bay) and 2 control pristine sites at Tiahura (long-term water quality monitoring site) for 35 d to enable biofilm formation. Biofilm control triplicates at each site (n=12) were sampled at T=0 prior to exposure to the field sites. The remaining replicates (n=) were deployed at the selected field sites. After 4 d (T=1) control replicate biofilms (n=3) were sampled, replicates (n=6) were transplanted from each site (from impacted to control sites and vice versa), while 3 control replicates remained until the final sampling event. after 10 d (T=2) of exposure to the new site. One half of the biofilm replicates were sampled as described in (Witt*, et al.*, 2011) and stored in RNA later for molecular analyses. The other half of the replicate samples from each sampling point were used for O_2 flux measurements as described below.

Shading experiment

Glass slides (48) were also newly introduced in situ at one of each of the location sites (Tiahura and Opunohu) to the field at t_0 holder natural light condition at the concurrent sites and one additionally shaded using shade cloth. Biofilms were sampled after 7 and 14 d of incubation as in the transplant experiment triplicates for molecular- and triplicates for O_2 flux analysis. Additional light loggers (HoboTidbit) were deployed in the shaded area.

O_2 flux analyses

Oxygen flux experiments were conducted in a semi-shaded outdoor flow-through aquarium system with average light intensities of 136 µmol photons m^{-2} s^{-1} during measurements on the sampling days. Each biofilm (n = 3) was incubated with seawater in a separate glass container (160 ml) sealed air-bubble free. After transferring the biofilms into the glass containers, the initial dissolved oxygen concentrations (t_0) were determined using a dissolved oxygen sensor (Cyberscan PC 6500, Thermo Fisher Scientific). All glasses were then placed in flow-through aquaria to ensure constant ambient temperature conditions during the incubation procedure incubated for 90 min in light conditions (Witt*, et al.*, 2011) Light and temperature were logged in 10 min intervals (Hobo loggers). At the end of the incubation (t_1), glass jars were gently mixed, and the oxygen concentration in the glasses was measured: Productivity = O_2 * (t_1) – O_2 * (t_0). Immediately following the light incubation, the same biofilm was incubated again in darkened, PVC-foil wrapped glass chambers to

measure the dark respiration. The oxygen consumption was determined according to the following formula: Respiration = O_2 * (t_1) – O_2 * (t_0). In addition to both incubations, seawater-control chambers (without biofilms) were incubated in the light and in the dark to measure the planktonic metabolism in the water. The seawater-control values were subtracted from the P and R values (with organisms) to receive oxygen production or consumption from the incubated biofilm only. The resulting hourly net O_2 production and consumption were calculated as the 24 h net O_2 production and consumption rates and expressed as µmol O_2 cm^{-2} biofilm area d^{-1}. Production/respiration (P/R) ratios were calculated as follows: P/R ratio = (hourly net production + hourly respiration) 12/(hourly respiration * 24). To determined O_2 fluxes were related to the surface area and biomass of the organisms. Subsequently, the biofilm material from each slide was scraped off using scalpel blades and transferred onto GF/F filters (Whatmann 25 mm diameter) for carbon and nitrogen elemental analyses according to protocols specified in (Witt*, et al.*, 2011, Witt*, et al.*, 2012).

Molecular analyses

DNA was extracted from biofilms using the Power Soil DNA Isolation Kit (Mobio Laboratories, Inc., Carlsbad, US). PCR amplification of bacterial 16S-23S ITS region was performed using the FAM-labelled forward primer ITSF (5´-GTCGTAACAAGGTAGCCGTA-3´) and eubacterial ITSReub (5´-GCCAAGGCATCCACC-3´; Cardinale et al., 2004). 10 ng DNA were amplified in triplicate reactions per sample with 5X PCR buffer, 1.25 U of Go Taq polymerase (Promega)., of 40 mM dNTP mix (Promega), bovine serum albumin (3 µg μl^{-1}, final concentration), 1.5 mM of MgCl2, and 0.5 mM ITSF and ITSReub (each). The mixture was held at 94 °C for 3 min, followed by 30 cycles of 94 °C for 45 sec, 55 °C 1:30 min, 72°C for 1 min and a final extension step at 72 °C for 5 min. PCR products were cleaned using the Nucleospin PCR purification kit. After quantification with the NanoDrop spectrometer 100 ng of the purified products were mixed with a separation cocktail containing 0.5 µl of internal size standard LIZ (20-1200 bp), and 14 µl of deionized Hi-Di –formamide (Applied Biosystems, Foster City, CA, USA). Discrimination of the PCR-amplified fragments via capillary electrophoresis was carried out on an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems) and the ARISA profiles were analyzed using the GeneMapper Software v 4.0 (Applied Biosystems).

Statistics

A suite of multivariate analyses was used to identify relationships between different locations and different time points and to evaluate the variability in microbial communities. A similarity matrix for the ARISA data sets was calculated using Bray-Curtis distance matrix. Non-metric multidimensional scaling (MDS) was used to ordinate the similarity data, using 9999 random restarts for three data partitions, peak presence/absence, peak area and relative abundance. Statistical test ANOSIM to reveal significant difference in the groups and SIMPER to identify the peaks contributing the most to these community differences. Performed in PAST (Hammer et al.)

Preliminary results

145 *Environmental parameters*

All sites were equipped with light and temperature loggers (HoBoTidbit) throughout the experiment. Water temperature throughout the experiment was average of 26.8°C ± 0.058 during the day with a maximum of 29°C. Control sites read a maximum of 14000 lux at midday and a daily average of 8000 lux, at impacted sites a maximum of 9800 lux was recorded and a daily average of 7000 lux. Nutrient values at impacted sites were taken at each sampling point were an average of 0.1 µmol/L for NO2 0.08 µmol/L NO3 (µmol/L) Si(OH)4 (µmol/L) PO4 (µmol/L), Salinity 36.1 ± 0.011 PSU, Oxygen 2.603 (mL/L), ± 0.051, turbidity 0.525 NTU ± 0.557, Fluorometry chlorophyll 0.039 microg/L ± 0.064.

146 *Microscopy*

147 Binocular microscopy images showed the first differences between biofilms grown at
148 impacted and pristine sites. While biofilms from Opunohu Bay displayed a dominance by
149 sediment particles and nematodes, biofilms from Tiahura were dominated by green algae
150 and crustose coralline algae. Results indicate that there is a differences in O_2 fluxes between
151 control and impacted locations, as well as between light and dark treatments.
152

153 *Molecular fingerprinting*

154 Overall, 352 operational taxonomic units (OTUs) were detected in the inshore, offshore and
155 both transplanted biofilm communites at sample points T_0 and T_2. The most diverse
156 community was found in impacted communities (T2) with 347 OTUs, followed by the control
157 community with 318 OTUs. Both transplanted communities were equally diverse with 290
158 OTUs. The majority of the OTUs were shared amongst the 4 communites (207 OTUs) and
159 only few were completely site specific (i.e., inshore = 6) were shared amongst all 4
160 communities Amongst these OTUs were detected in than in Fig. 1. Transplantation of
161 biofilms resulted in a decrease in bacterial diversity, while the diversity loss of OTUs was
162 greater in impacted than control communites.

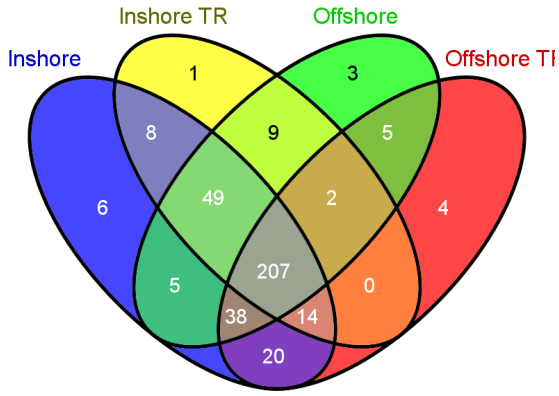

Fig. 1 Shared OTUs between inshore, offshore and tranplanted biofilm communitites. The Venn diagramm displays the number of OTUs unique to or shared between four major sample groupings. Inshore refers to impacted sites, offshore to control sites. Inshore TR means grown at control site and transplanted to impacted and offshore TR vice versa.

Molecular fingerprinting of bacterial communities from biofilm samples retrieved freshly from the impacted and control field sites clearly showed significant differences in community structure and sample dispersion. Control communities showed a higher sample dispersion than did those from impacted sites. ARISA data was visualised in a Non-metric multidimensional scaling plot using the Bray–Curtis distance matrix (nMDS) (Fig. 2) showing discrete groupings according to biofilm location. Biofilms from impacted and control sites and the transplanted sites were all significantly different from each other as confirmed by ANOSIM (overall R=0.509 p= 0.0001). The specimens contributing the most overall to these differences were revealed by SIMPER analysis 328bp (4.3%), 523bp (4.1%), 147bp (2.8%). Communities at T2 differed significantly from those at T0.

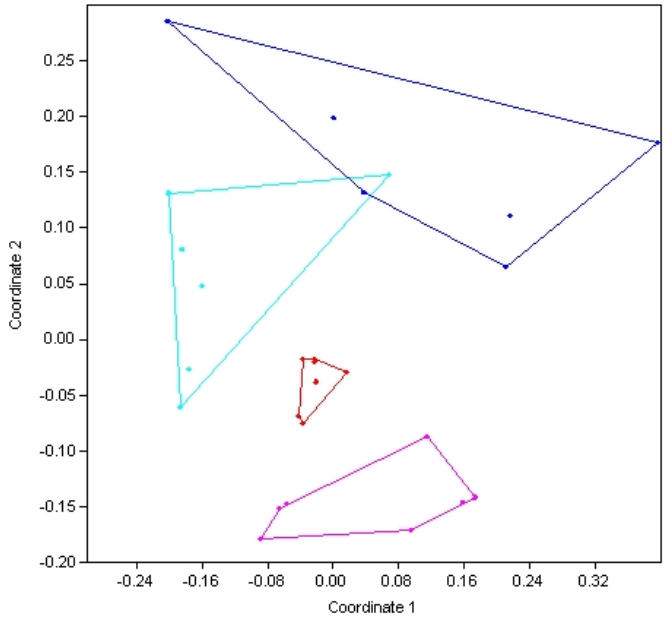

Fig. 2 Non-metric multidimensional NMDS ordination plot (Bray–Curtis distance matrix) of ARISA profiles for biofilm-derived samples. The proximity between samples in the plot corresponds to high-community similarity. Blue = control, turquoise = control transplant, red = impacted, pink = impacted transplant

O2 fluxes

Biofilms from control sites were twice as productive (249.2 µmol O_2 cm^{-2} biofilm area d^{-1}) as the ones from impacted sites (125.8 µmol O_2 cm^{-2} biofilm area d^{-1}). Transplants from control to impacted sites doubled their productivity to values of biofilms exclusively grown at control sites (227.4 µmol O_2 cm^{-2} biofilm area d^{-1}), while vice versa productivity remained unimpaired (249.8 µmol O_2 cm^{-2} biofilm area d^{-1}).

Outlook

The data showed a tendency of bacteria to adapt to their new environment over short time spans (10d). Communites retained a certain structure from their formation at their site of origin but with a loss in diversity in both transplant cases. Biofilms grown in nutrient limited environment metabolically took advantage of the nutrient availability, while light limited communites did not adapt as quickly to benefit from extra light availability. It is hypothesised that over time, transplanted biofilms will eventually fully adapt to their

tranplanted new site, but the time scale is further to be tested and was not as fast as expected as for instance seen in crustose coralline algae that recover from temperature changes within 7d (Webster et al. 2011) or more extreme, to convert bacterial communities from saltwater to fresh water communities thatto fully adapt within 6 months (Martiny et al. 2012). These results suggest that light and nutreints (and other covarying parameters) select for a distinctive bacterial biofilm composition and to a certain degree keep a core community. Changes in water quality influences bacterial biofilm communities, the existing taxa within these communities can specialize and coexist.

After optimisation of the ARISA technique the current data now enables to select for further next-generation sequencing techniques (V6 region) to give a deeper insight into the bacterial diversity. An aquarium experiment in our in-house facility was repeated with different light conditions and transplantation. Together with this data and the currently running next-generation sequences, the data is planned to be submitted to Coral Reefs or MEPS.

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