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Research article

Changes in microbial diversity associated with two coral species recovering from a stressed state in a public aquarium system

M.J. Sweet[1*], D. Smith[1], J.C. Bythell[1] and J. Craggs[2]

[1] Coral Health and Disease Laboratory, Newcastle Institute for Research on Sustainability, Newcastle University, NE1 7RU
[2]Horniman Museum and Gardens, Forest Hill, London, SE23 3PQ, UK
**Correspondence: michael.sweet@newcastle.ac.uk*

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Abstract

Coral diseases are a major factor in the decline of coral reefs worldwide, and a large proportion of studies focusing on disease causation use aquaria to control variables that affect disease occurrence and development. Public aquaria can therefore provide an invaluable resource to study the factors contributing to health and disease. In November 2010 the corals within the main display tank at the Horniman Museum and Gardens, London, UK, underwent a severe stress event due to reduced water quality, which resulted in death of a large number of coral colonies. Three separate colonies of two species of reef coral, *Seritopora hystrix* and *Montipora capricornis* showing signs of stress and acute tissue loss were removed from the display tank and placed in a research tank with improved water quality. Both coral species showed a significant difference in 16S rRNA gene bacterial diversity between healthy and stressed states (*S. hystrix*; ANOSIM, R=0.44, p=0.02 and *M. capricornis*; ANOSIM, R=0.33, p=0.01), and between the stressed state and the recovering corals. After four months the bacterial communities had returned to a similar state to that seen in healthy corals of the same species. The bacterial communities associated with the two coral species were distinct, despite them being reared under identical environmental conditions. Despite the environmental perturbation being identical different visual signs were seen in each species and distinctly different bacterial communities associated with the stressed state occurred within them. Recovery of the visually healthy state was associated with a return of the bacterial community, within two months, to the pre-disturbance state. These observations suggest that coral-associated microbial communities are remarkably resilient and return to a very similar stable state following disturbance.

Introduction

All coral species have a diverse community of microorganisms living within healthy tissues, the skeleton and the surface mucus layer. These bacterial communities have been shown to differ among and within species within healthy corals (Rohwer et al. 2002; Rosenberg et al. 2007). The physiochemical properties of the coral microhabitats (skeleton, tissues and mucus) appear to have the ability to select certain species from environmental pools, allowing some to grow and develop and eliminating others that initially colonise (Sweet et al. 2011a). Furthermore, certain properties of the coral or its healthy microbial associates, such as antimicrobial activity, have been shown to have a significant effect on the microbial communities within the holobiont (Ritchie and Smith 1995; Kooperman et al. 2007; Sharon and Rosenberg 2008). These microbial communities have been shown to vary between healthy and diseased or stressed tissues (Frias-Lopez et al. 2003; Pantos et al. 2003; Jokiel and Coles,2004; Williams and Miller 2005; Gil-Agudelo et al. 2007; Mydlarz et al. 2009). Specifically bacterial communities have been shown to change in advance of the visible disease signs, highlighting the importance of microbial communities and their role in coral disease (Pantos et al. 2003; Croquer et al. 2012).

Corals have a variety of mechanisms for defense against invasive pathogens, including a physical barrier protecting the epithelium in the form of the surface mucus layer (reviewed by (Brown and Bythell 2005; Bythell and Wild 2011), and the pro-duction of antimicrobial compounds by the host and/or other microbial associates (Ritchie 2006). This latter process is thought to be mediated to a large extent by the coral's natural microbial community, with over 20–30% of bacterial isolates in healthy corals possessing antibiotic activity (Gunthorpe and Cameron 1990; Kim 1994; Castillo et al. 2001; Rohwer and Kelley 2004; Ritchie 2006; Geffen et al. 2009; Rypien et al. 2010). If the bac-terial community associated with the coral is the primary source of this defense (via antibiotic production), then a disturbance of the healthy coral microbiota may allow opportunistic infection and the onset of specific disease signs

(Lesser et al. 2007a). Sweet et al. (2011b) and Garren et al. (2009) showed that ma-nipulation of the natural microbiota in healthy corals (through experimental manipulation and transplantation respectively), allowed opportunistic, potentially pathogenic bacteria to colo-nize the tissue. However, interestingly in both cases, the healthy bacterial community reverted to the natural state after the stress event had subsided and there were no visual signs of stress or disease in either of these manipulations. These results suggest that other factors must be at play to cause the onset of disease other than the availability of potential pathogens or the opening of available niches due to disturbance of the natural associated microbial communities and loss of potentially probiotic strains.

Several studies have shown a general trend towards *Vibrio* domination under stressful conditions (Kushmaro et al. 1997; Cervino et al. 2008; Luna et al. 2010) and therefore it may be expected that corals exposed to identical environmental perturbations may become more similar with their microbial communities. To date few studies have been able to monitor bacterial communities associated with corals recovering from symptoms of stress in controlled environments. In this study we were able to opportunistically sample the bacterial community associated with two species of coral as they recovered from a serious stress event over a period of four months in a public aquarium.

Methods

Sample collection

Corals at the Horniman Museum and Gardens public aquarium underwent a severe stress event in 2010. In total all nine species of scleractinian coral within the display where affected, with mortality ranging from 100% (in species such as *Hydnophora rigida*) to approximately 5% (in *Acropora formosa*). This trend was observed in multiple tanks within the system ruling out a single tank effect. Three colonies of two different coral species, *Seriatopora hystrix* and *Montipora capricornis* which were showing visual signs of stress and subsequently developed lesions were followed over four months and sampled throughout this time period. The three colonies of each species were originally from a single genotype, generated via asexual fragmentation. *S. hystrix* colonies showed signs of acute tissue loss whilst *M. capricornis* showed signs of bleaching and acute tissue loss (Fig 1). Stressed corals were removed from the display tank and placed in an experimental research aquarium for the period of the experiment. Here the water parameters were optimised and the corals recovered and began to regrow over a period of four months. Three approximately 1 cm^2 coral fragments were taken from the disease lesion from each colony initially, and once a month the same size samples were taken at the same part of the coral, where tissue was shown to be recovering and/or regrowing. It must be noted that there were no samples collected for *M. capricornis* at Month 1. Three healthy samples of a coral not affected by the disease were also taken at the start of the experiment for comparison. Each coral was photographed before removal from the aquarium and the subsequent sample taken. These samples were all then placed in sterile 50 ml falcon tubes and stored in 100% EtOH at -20° C until further analysis. Samples were then centrifuged at 13,000 g for 20 min to concentrate the tissue slurry, 1000 µl of which was subsequently used for DNA extraction using QIAGEN DNeasy Blood and Tissue kits with an added step to concentrate the lysate using a vacuum centrifuge for 2 h at 24° C.

Bacterial diversity, DNA extraction, amplification and DGGE analysis

Bacterial partial 16S rRNA gene fragments were amplified using standard prokaryotic primers (357F) (5′-CCTACGGGAGGCAGCAG-3′) and (518R) (5′-ATTACCGCGGCTGCTGG-3′). The GC-rich sequence 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA GCA CGG GGG G-3′ was incorporated in the forward primer 357 at its 5′ end to prevent complete disassociation of the DNA fragments during DGGE. Thirty PCR cycles were performed at 94°C for 30 seconds, 53° C for 30 seconds and 72° C for 1 min and a final extension at 72° C for 10 min (Sanchez et al. 2007). Three independent 10 µl PCR reactions were used, each containing 1.5 mM $MgCl_2$, 0.2 mM dNTP (PROMEGA), bovine serum albumin (BSA, 400 ng/µl), 0.5 µM of each primer, 2.5 U of Taq DNA polymerase (QBiogene), incubation buffer, and 20 ng of template DNA (Siboni et al. 2007). These replicate PCR's for each sample were then combined and cleaned using QIAGEN QIAquick PCR purification kits, reducing the final volume to 15 µl in Sigma molecular grade H_2O. All reactions were performed using a Hybraid PCR Express thermal cycler. PCR products were verified by agarose gel electrophoresis (1.6% (w/v) agarose) with ethidium bromide staining and visualized using a UV transilluminator.

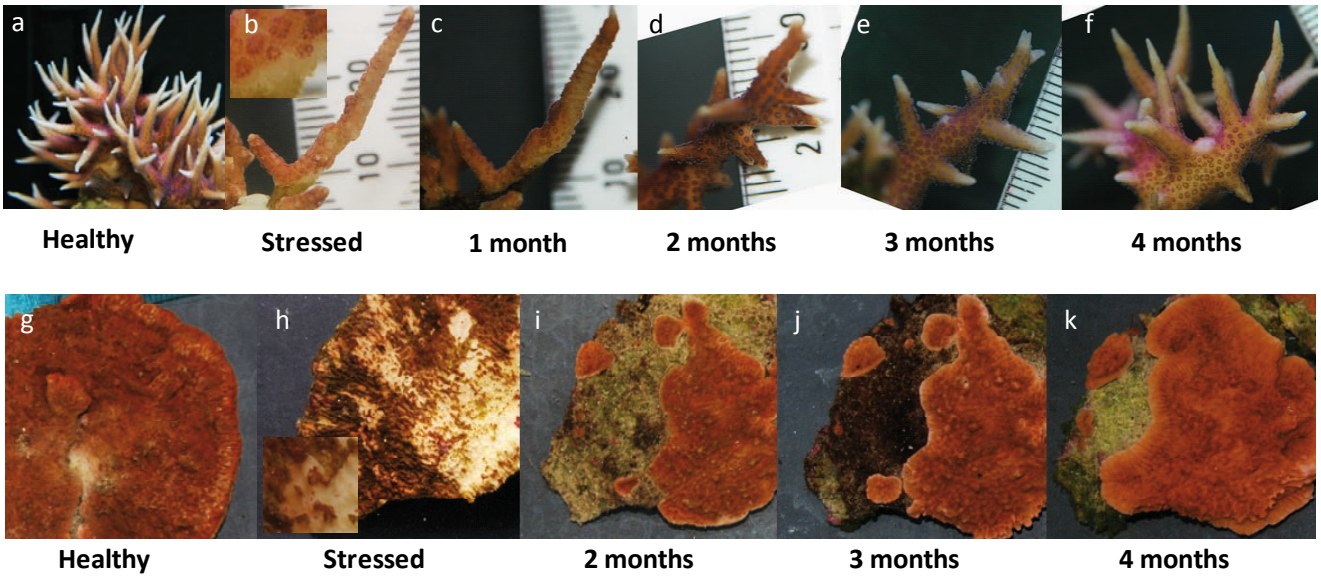

Figure 1. Corals showing signs of stress and subsequent recovery; S*eriatopora hystrix* (a-e), *Montipora capricornis* (f-k). The remaining live tissue in *M. capricornis* was severely bleached (white patches in stressed state, h).

DGGE was performed using the D-Code universal mutation detection system (Bio-Rad). Bacterial PCR products were resolved on 10% (w/v) polyacrylamide gels that contained a 30–60% denaturant gradient for 13 h at 60° C and a constant voltage of 50 V. Gels were stained with a concentrated solution of 9 µl Sybr® Gold (Sigma) in 50 µl of 1X TAE poured directly onto the gel surface, covered and left in the dark for 20 min then further washed in 500 ml 1X TAE for 30 min and visualised using a UV transilluminator. Bacterial operational taxonomic units (OTUs), were defined from DGGE band-matching analysis using Bionumerics 3.5 (Applied Maths BVBA) following methods described by (Guppy and Bythell 2006). Standard internal marker lanes were used to allow for gel-to-gel comparisons. Tolerance and optimisation for band-matching was set at 1%. OTUs of interest (those which explained the greatest differences/similarities between samples), were identified by sequence analysis. Bands were excised from DGGE gels, left overnight in Sigma molecular grade water, vacuum centrifuged, re-amplified with the appropriate primer set, labelled using Big Dye (Applied Biosystems) transformation sequence kits and sent to Gene-vision (Newcastle University, UK) for sequencing.

Total bacterial abundance

To estimate bacterial abundance, three filters per time period were sampled, similar to that for microbial analysis. 1000 µl of tissue slurry was collected, lyophilised to remove the ethanol and weighed to standardise the amount of tissue sampled between replicates. 100mg of lyophilised tissue extract was used for each sample to account for varying amounts of tissue remaining on the samples at time of collection. This was then resuspended in sterile filtered sea water and filtered through a 0.22 µm black polycarbonate filter and fixed with 100 µl of paraformaldehyde until analysis (Fuhrman et al. 2008). The filters were then stained with 100 µl DAPI solution (final concentration 5 µg/ml) for 10 mins rinsed with Phosphate Buffer Solution (Yu et al. 1995; Weinbauer et al. 1998; Yamaguchi et al. 2007), and viewed under epifluorescence microscopy using a DAPI-specific filter set. For each filter, 50 fields of view (FOV) were taken at X1000 magnification. These were then scaled up to the total area of the filter and calculated to give total bacterial abundance per cm_3 of stressed tissue. Total amount of stressed tissue rather than complete coral nubbin surface area was used to account for the varying amount of tissue on the stressed samples as this could not be standardised at time of collection. All images were analysed using an automatic cell counter (Cell C; Selinummi *et al.* 2005). The parameters were set to exclude any objects smaller than 0.0314 μm^2 and anything larger than 0.7 μm^2. Counts of three tissue sub-samples were taken from each coral and averaged to provide a cell density per sample.

Antimicrobial assays from aqueous coral extracts

Bacterial inhibition growth was determined from aqueous extracts of each sample similar to that reported by Mydlarz et al. (2009). For this, the ethanol used to preserve the coral fragments was transferred into new pre-weighted falcon tubes and these were lyophilized to determine the amount of coral extracts in each sample (weighed and standardised as above). Samples were then resuspended in 100% ethanol to reach a standard concentration of 100 mg/ml as a primary stock solution for all samples. This stock extract was further diluted to 3 mg/ml in 0.1 M phosphate buffer, pH 7.8 following the protocol by Mydlarz et al. (2009). In a 96-well microtitre plate, 10 µl of each extract were added to 105 µl of marine broth and 15 µl of bacteria culture. Positive controls using 0.05 mg/ml of tetracycline were utilised along with negative controls using 100% EtOH. The kinetic of bacterial growth was determined by reading the OD every 5 min for 24 h, in a Biotek power wave HT plate reader. The rate of bacterial growth during exponential phase was calculated by plotting the OD against time giving a growth curve. The gradient was then calculated from the linear part of the growth curve, all experimental samples could then be compared to the growth rate of the control sample (ethanol control), giving the relative growth rate.

To obtain the pure cultures of a bacterial pathogen known to affect corals, crushed coral sand from an aquarium where the corals had recently died from an outbreak of coral disease were suspended in filtered sea water and spread onto Thiosulfate Citrate Bile Sucrose (TCBS) Agar media in duplicates. Plates were incubated at 28° C for 24 h. Resulting bacterial colonies were isolated based on colony morphology, size, and shape. These were then picked and spread on individual plates to produce pure colonies. Representatives of each plate were sequenced using universal bacterial primers pA and pH. Gen-Bank BLAST searches of the 16S rRNA gene sequences were performed to determine the percentage of isolate relatedness to known bacteria. Each isolate was stored at -80°C in cryovials containing 30% glycerol and 70% TCBS media. Only one pure culture showed similarities to any known coral pathogen referenced in the literature and this was a ribotype with 100% match to *Vibrio harveyi*. An aliquot of the pure freezer stock from this isolate was streaked on TCBS agar and incubated at 26° C for 24 h. A single distinct colony was removed, put into sterilised marine broth and incubated again in a shaker at 26° C for 24 h. To standardise the bacterial cell density in this assay, the culture was adjusted to an optical density of 0.2 at 600 nm (5 X 107 cells/ml) using a spectrophotometer in a Biotek power wave HT plate reader (Mydlarz et al. 2009).

Water quality

The display and research tanks were monitored before, during and after the stress event, on a weekly basis. Parameters such as NH_3, NO_2, NO_3, (Hach DR890 colorimeter) PO_4 (D&D – The Aquarium Solutions High sensitivity test kit) Ca, Mg and Alkalinity (Salifert) were monitored. pH was monitored using a HQ11d Portable pH/ORP Meter with IntelliCAL™ PHC101 Standard Gel Filled pH Electrode.

Table 1. Tests of metals and other elements present within the water systems at the Horniman Museum and Gardens Aquarium. (i) Analysed by Cheshire Scientific, (ii) analysed by Horniman Museum and Gardens.*Copper readings can be masked by other elements such as aluminium, magnesium, iron and calcium. This problem with readings can be overcome using a calibration buffer (CuVer 2 Copper Reagent) these readings when done in house using a Hach DR/890 Colorimeter reported a higher amount of copper within the system equaling 0.11 mg/l in the display tank.

Test	Display tank	Research tank
i - Copper (mg/l)	0.01*	<0.01
i - Aluminum (mg/l)	<0.02	<0.02
i - Chromium (µg/l)	14	11
i - Selenium (µg/l)	2.8	0.8
i - Mercury (mg/l)	<0.01	<0.01
i - Manganese (mg/l)	<0.01	<0.01
i - Strontium (mg/l)	5.2	5.6
ii - NO3 (mg/l)	10.18	2.9
ii - PO4 (mg/l)	0.06	0.039
ii - Ca (mg/l)	473.61	420.55
ii - Alkalinity (dHK)	9.19	9.56
ii - Magnesium (mg/l)	1378	1310
ii - pH	8.03	8.16

Statistical analysis

Analysis of Similarity (ANOSIM) tests based on Bray-Curtis similarities (Clarke and Warwick, 2001) were used to test for differences in the bacterial 16S rRNA gene DGGE profiles associated with stressed and healthy corals. A non-metric multidimensional scaling (MDS) analysis was used to represent each sample type on a 2-D plot (Clarke and Warwick 2001). An analysis of contribution to similarities (SIMPER) was performed to determine which 16S rRNA gene OTUs best explained dissimilarities among sample types that were statistically different, these were then excised from the DGGE and sequenced to give the closest match according to the BLAST database. The abundance of bacteria (total bacterial counts) was compared between healthy and stressed samples. For *Montipora capricornis*, data met the assumptions of normality and equality of variances and a one way ANOVA was used. However for *Seriatopora hystrix*, Levenes test for homogeneity showed heterogeneity of variances and therefore variances were not equal ($F_{4.10} = 4.020$, $p = 0.034$). Therefore the nonparametric test Kruskal-Wallis was used.

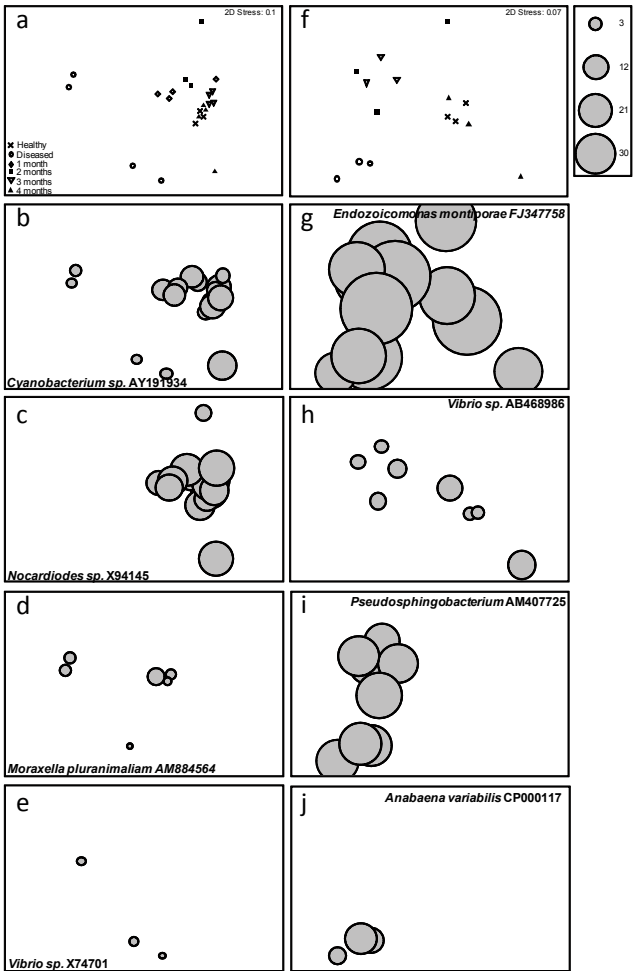

Figure 2. (a) Multidimensional scaling (MDS) plot showing changes in bacterial communities from n = 3 healthy, n = 4 stressed state and n = 3 recovering (4 different time periods) *Seriatopora hystrix* colonies; (b-e) show 16S rRNA gene bacterial ribotypes that caused greatest similarities or differences between sample types (closest relative and best match Table 3) (b) *Cyanobacterium* sp. AY191934 (c) *Nocardiodes* sp. X94145 (d) *Moraxella pluranimaliam* AM884564 and (e) *Vibrio* sp. X74701. (f) MDS plot showing changes in bacterial communities from n = 3 healthy, n = 3 stressed state and n = 3 recovering (4 different time periods) of *Montipora capricornis* colonies (g-j), show 16S rRNA gene bacterial ribotypes that caused greatest similarities or differences between sample types (closest relative and best match Table 2) (g) E*ndozoicomonas montiporae* FJ347758 (h) Vibrio sp. AB468986 (i) *Pseudosphingobacterium* AM407725 (j) *Anabaena variabilis* CP000117. Bubble size represents relative density of denaturing gradient gel electrophore-sis (DGGE) band of that particular sequence within individual samples. There were no samples collected for *M. capricornis* at Month 1.

Results

Water quality

Water quality in the main display tank was below optimal levels prescribed for coral husbandry in aquariums and zoos, with higher than normal readings of various metals and non-metals including copper, iron, aluminium and selenium (Table 1). In contrast, overall water quality was of a higher standard within the research tank behind the scenes. For example; levels of nitrate (NO_3) and phosphate (PO_4) were higher in the display tank compared to the research tank; NO_3; 10.2 mg/l and 2.9 mg/l; PO_4; 0.06 mg/l and 0.039 mg/l respectively. Conversely, pH values were lower in the display tank compared to the research tank; 8.03 and 8.16 respectively (Table 1).

There was a significant difference in 16S rRNA gene bacterial diversity (ANOSIM, R = 0.64, p = 0.001) between healthy coral colonies of *Seriatopora* hystrix and the healthy colonies of *Montipora capricornis*. Although there was a shift in bacterial diversity between healthy and stressed states, a significant difference in 16S rRNA gene bacterial diversity remained between the two species in this stressed state (ANOSIM, R = 0.56, p = 0.001).

Seriatopora hystrix

Bacterial communities were diverse in all samples of *S. hystrix*, with pairwise comparisons showing 16S rRNA gene bacterial diversity being significantly different between stressed and healthy corals and between the stressed corals and all the recovering time periods (ANOSIM, R > 0.44, p < 0.02) (Fig. 2a). However, there was no significant difference in 16S rRNA gene bacterial diversity between the communities associated with healthy *S. hystrix* and those in recovering colonies after four months (ANOSIM, R = 0. 57, p = 0.12). Two bacterial ribotypes dominated the community profile in the DGGE, these included a ribotype similar to a known nitrogen fixing *Cyanobacterium* sp. (AY191934) (Fig. 2b; Table 2) and a *Nocardioides* sp. (X94145) (Fig. 2c; Table 2). The *Cyanobacterium* sp. didn't vary between states and remained a dominant ribotype throughout, however interestingly the *Nocariodides* sp. was completely absent from the DGGE profile in stressed samples and only detectable in healthy corals and those recovering from the disease. Only three species of bacterium were detected in stressed corals and absent in healthy corals, these includes ribtoypes similar to previously identified potential pathogens from the genus's *Moraxella* (NR042666) (Fig. 2d), *Vibrio* (X74701) (Fig. 2e), and *Cyanobacterium* (EU780252) (Table 2).

Bacterial abundance

Although bacterial abundance did not increase significantly in stressed corals compared to healthy corals (Kruskal Wallis $\chi^2_{(4)} = 1.433$, $p = 0.838$). The average bacterial abundance increased in healthy and stressed corals (from $1.7 \times 10^7 \pm 0.76$ cells/cm_3 to $3.4 \times 10^7 \pm 0.15$ cells/cm_3 respectively). Over four months this abundance gradually reduced back to healthy levels (Fig. 3). After an initial drop in abundance after one month, it began to rise again in month 2 but remained below the total abundance of the stressed samples.

Antimicrobial activity

All coral tissues showed significant antimicrobial activity ($F_{6, 33} = 5.665$, $p < 0.008$), suppressing growth of the tested bacterium

Table 2. Heatmap derived from relative 16S rRNA gene DGGE operation taxonomic units (colors arranged by 0, 0.1-1.0, 1.1-2.0, 2.1-3.0, 3.1-4.0, 4.1-5.0, 5+), for *Seriatopora hystrix*. Replicate samples (n = 3 or 4 per time period) were combined to give an overall average for clarity within the display. The larger the number under each sample column the stronger the DGGE band intensity for that specific 16S rRNA gene ribotype.

Closest relative	Accession no.	GenBank	Class	Healthy	Stressed	1 month	2 months	3 months	4 months
Nocardioides sp.	X94145	98%	Actinobacteria	5.43	0	6.3	5.99	6.61	5.37
Unknown	NA	NA	NA	0.67	3.81	0	0	0	0.56
Desulfurococcus sp.	AY264344	100%	Archaea	1.21	3.33	2.7	4.46	0.3	1.05
Unknown	NA	NA	NA	0.62	2.53	0.73	1.26	0	0.32
Lactobacillus sp.	AB326350	99%	Firmicutes	2.61	0	3.5	1.16	2.56	2.41
Coral DNA	NA	NA	NA	2.54	0.52	0	0	1.21	2.7
Coral DNA	NA	NA	NA	2.67	0	0	1.17	2.22	2.27
Moraxella sp.	NR042666	100%	Gammaproteobacteria	0	2	1.84	0	0	0
Actinobacillus sp.	AY362899	98%	Gammaproteobacteria	1.56	1.71	0.63	0.34	1.64	1.09
Methanococcus sp.	DQ195164	96%	Archaea	1.12	0	0	0	0	1.4
Prochlorococcus sp.	BX548174	98%	Cyanobacterium	1.09	0.38	0.83	0	0.63	1.49
Pyrobaculum sp.	AJ277124	96%	Archaea	1.67	0.37	0	0.34	0.95	1.4
Methanocaldococcus sp.	AF547621	98%	Archaea	2.15	1.65	2.12	1.51	2.61	2.31
Ascoparia sp.	FR837678	100%	Ascopariidae - flat worm	0.98	1.07	0	0	0	0.44
Cyanobacterium sp.	EU780252	100%	cyanobacterium	0	1.11	0	0	0	0
Pseudomonas sp.	AM293567	98%	Gammaproteobacteria	0.88	0.65	1.61	2.13	2.19	0.76
Flagellimonas sp.	DQ191180	98%	Flavobacteriia	1.33	0.94	0.46	0.34	0.97	1.35
Cyanobacterium	AY191934	100%	Cyanobacterium	4.08	2.93	4.06	2.38	4.05	3.74
Vibrio sp.	X74701	98%	Gammaproteobacteria	0	0.94	0	0	0	0
Unknown	NA	NA	NA	0	0	0	2.37	1.81	0
Unknown	NA	NA	NA	0.86	0	0	3.16	1.25	0.76

Vibrio harveyi when compared to the EtOH control (Fig. 4). There was no significant difference (p = 1.0) in antimicrobial activity between stressed tissues and those in healthy corals. Interestingly, growth rate of *V. harveyi* was higher in some instances during the recovering periods than in the stressed coral, despite visual coral health appearing to return to normal and tissues beginning to re-grow (Fig. 1 d, e). Growth rate after 4 months was the same as that in healthy tissues (Fig. 4).

Montipora capricornis

Bacterial communities were again diverse throughout all samples (Table 3). Furthermore, there was a significant difference between time periods (ANOSIM, R = 0.707, p = 0.001), with pairwise comparisons showing that the 16S rRNA gene bacterial diversity remained significantly different between stressed corals and healthy corals and between the stressed corals and all the recovering time periods (ANOSIM, R > 0.33, p < 0.01) (Fig. 2f). There was again no significant difference in 16S rRNA gene bacterial diversity between the communities associated with healthy *M. capricornis* and those in recovering corals after a period of 4 months (ANOSIM, R = 0. 78, p = 0.10) (Fig. 2f). Bacterial diversity was higher overall in *M. capricornis* corals compared to *S. hystrix* (Table 2 and 3). Eight ribotypes were dominant in relative 16S rRNA gene abundance throughout all sample points including the coral symbiotic bacterium *Endozoicomonas* sp. (FJ347758) (Fig. 2g; Table 3), two potential coral pathogens from the genus *Thalassomonas* (AY194066 and AY643537), and an opportunistic pathogen from the genus *Bacteroidetes* (GQ204958). Similarly to *S. hystrix,* nitrogen fixing bacteria were also present from the genus *Paenibacillus* (X60625), along with a *Amycolatopsis* sp. (AJ293757) known to produce a weak antibiotic and a *Sphingomonas sp.* (JQ027711) a strong biodegrading species of bacteria (Table 3). A further eight ribotypes were only detected in stressed samples including; the nitrogen fixing filamentous cyanobacterium *Anabaena* (CP000117) (Fig. 2j) and a nitrite reducing *Paracoccus sp.* (HQ538757), two sulphur reducing bacterium, *Deferribacteres sp.* (GQ204938) and *Sulfurisphaera sp.* (D85507), and a potentially pathogenic Vibrio sp. (AB470931). Similar to *S. hystrix,* certain ribotypes were detected in healthy tissues, absent in the stressed lesion and present in the subsequent recovering samples (Table 3). These included ribotypes related to; a bacterium previously associated with marine macroalgae *Kiloniella sp.* (AM749667), a bacterium capable of degrading organic matter from the genus *Actinomyces* (AM084228), and a further *Vibrio sp.* (AB468986) (Fig 2h).

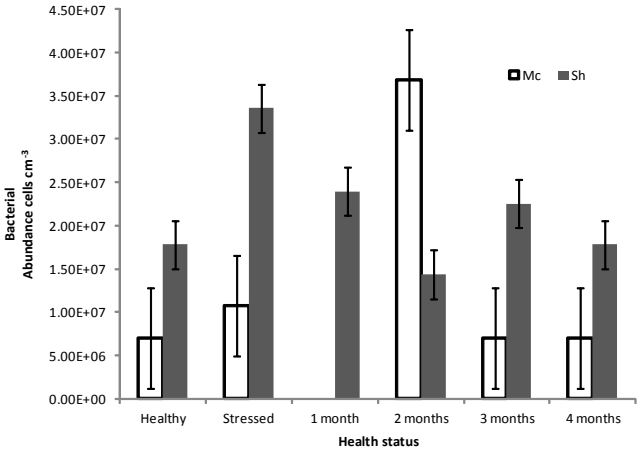

Figure 3. Mean bacterial abundance (cm-3) of n = 3 replicates of filtered coral tissue taken at all time periods. Mc = *Montipora capricornis*; Sh = *Seriatopora hystrix*. Error bars show standard error from collective mean. There were no samples collected for *Montipora capricornis* at Month 1.

Bacterial abundance

Bacterial abundance increased in stressed corals compared to healthy corals similarly to that seen in *S. hystrix*, however the increase again was not significantly different (ANOVA F = 2.259, p = 0.186). Total bacteria abundance in healthy corals was lower

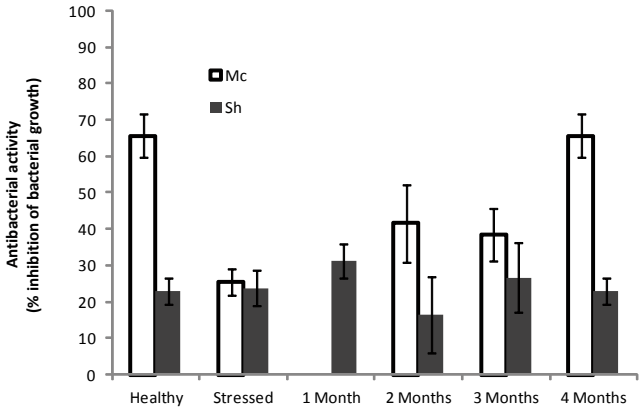

Figure 4. Antimicrobial assays, showing suppression of growth rate of the proposed coral pathogen *Vibrio harveyi* in different coral health states. The coral tissue samples show natural antimicrobial activity of the coral tissues during healthy state, stressed state and during recovery. Replicate samples were combined to give an average mean for each sample period. Error bars show standard error from collective mean. Mc = *Montipora capricornis*; Sh = *Seriatopora hystrix*. There were no samples collected for *M. capricornis* at Month 1.

than that found in *S. hystrix* (Fig. 3), however there was large variation between replicates (7.1 X 10_6 ± 1.11 cells/cm_3). Bacterial abundance increased to 1.1 X 10_7 ± 0.70 cells/cm_3 in stressed corals and in contrast to that of *S. hystrix* abundance carried on increasing after the coral started to recover (Fig 3). After two months, by which time the tissue appeared healthy, the bacterial load was on average three times greater than in the stressed state (3.7 X 10_7 ± 1.73 cells/cm_3), however these replicate samples again showed high amounts of variation within this time period (Fig 3). Furthermore although the tissue appeared to be of a healthy state, growth rate was relatively slow (Fig 1a). After 3 months, bacterial abundance reduced to similar levels seen in the healthy tissues (7.1 X 10_6 ± 1.11 cell/cm_3) and growth rate increased to pre-disturbance levels (Fig 1a).

Antimicrobial activity

Similar to S. hystrix, all coral tissues showed significant antimicrobial activity ($F_{5, 33}$ = 26.731, $p < 0.007$) (Fig 4). In contrast to S. *hystrix, M. capricornis* showed a significant increase ($p < 0.001$) in growth rate of the pathogenic bacterium *V. harveyi* in stressed tissue slurry's compared to healthy samples. Healthy corals inhibited

Table 3. Heatmap derived from relative 16S rRNA gene DGGE operation taxonomic units (colours arranged by 0, 0.1-1.0, 1.1-2.0, 2.1-3.0, 3.1-4.0, 4.1-5.0, 5+) for *Montipora capricornis*. Replicate samples (n = 3 or 4 per time period) were combined to give an overall average for clarity within the display. The larger the number under each sample column the stronger the DGGE band intensity for that specific 16S rRNA gene ribotype. There were no samples collected for *M. capricornis* at Month 1.

Closest relative	Accession no.	GenBank	Class	Healthy	Stressed	2 month	3 month	4 month
Pseudosphingobacterium sp.	AM407725	97%	Sphingobacteriia	0	3.01	2.03	2.82	0
Kiloniella sp.	AM749667	100%	Alphaproteobacteria	1.67	0	0.72	0.73	1.92
Anabaena sp.	CP000117	100%	Cyanobacterium	0	1.77	0	0	0
Cyanobacterium	AY191934	100%	Cyanobacterium	0	1.59	0	0	0
Deferribacteres sp.	GQ204938	100%	Deferribacteres	0	1.51	0	0	0
Streptomyces sp.	DQ462649	98%	Actinobacteria	2.13	0.92	0.82	0	2.33
Sulfolobus sp.	D85519	90%	Archaea	1.12	2.58	2.67	1.7	1.21
Thalassomonas sp.	AY194066	98%	Gammaproteobacteria	0.68	2.1	1.48	1.69	0.8
Marinobacterium sp.	AB006765	96%	Actinobacteria	2.41	1.01	0.12	1.25	2.21
Vibrio sp.	AB468986	100%	Gammaproteobacteria	1.38	0	1	1	1.28
Bacteroidetes sp.	GQ204958	98%	Bacteroidia	2.21	1	0.84	1.14	1.99
Paracoccus sp.	HQ538757	100%	Alphaproteobacteria	0	1.16	0	0	0
Mesorhizobium sp.	DQ310707	98%	Alphaproteobacteria	1.12	0.37	1	1.8	1.24
Pseudoalteromonas sp.	AB544013	100%	Gammaproteobacteria	0.34	1.57	0	0	0.47
Sphingomonas sp.	JQ027711	98%	Alphaproteobacteria	0.77	1.77	1.72	1.81	0.67
Sulfurisphaera sp.	D85507	100%	Archaea	0	1.1	0	0	0
Actinomyces sp.	AM084228	98%	Actinobacteria	1.1	0	0.38	1	1.08
Amycolatopsis sp.	AJ293757	100%	Actinobacteria	2.25	3.46	2.07	2.6	2.45
Endozoicomonas sp.	FJ347758	100%	Gammaproteobacteria	4.67	3.87	4.54	5.09	4.83
Halorubrum sp.	AY149598	98%	Archaea	0.52	0.69	0	0	0.67
Thalassomonas sp.	AY643537	97%	Gammaproteobacteria	2.45	1.87	2.51	2.54	2.77
Paenibacillus sp.	X60625	100%	Firmicutes	0.91	0.65	0.84	0.72	0.79
Unknown	NA	NA	NA	0.58	0.21	0.21	0.3	0.96
Methanococcus sp.	DQ195164	96%	Archaea	0.43	0	0.63	0.81	0.79
Cyanothece sp.	CP002198	100%	Cyanobacterium	0.98	1.67	0	0	0.9
Unknown	NA	NA	NA	0	0.77	0	0	0
Cyanobacterium	JN166514	97%	Cyanobacterium	1.54	1.2	1.52	0	1.82
Vibrio sp.	AB470931	100%	Gammaproteobacteria	0	0.66	0	0	0
Clostridium sp.	GQ204966	100%	Firmicutes	0.44	0	0	0	0.57
Corynebacterium sp.	AY226509	99%	Actinobacteria	0	0.55	0	0	0
Desulfovibrio sp.	AB353727	100%	Deltaproteobacteria	0.46	0	0	0	0.55

growth rate of the bacterium by 65%, whilst in the stressed state inhibition was as low as 25% (Fig. 4).

Discussion

The corals in this study underwent a severe stress event likely caused by changes in the water quality perturbation within the main aquarium display tank at the public aquarium. Increases in both heavy metals and nutrients such as selenium, chromium, copper, NO3 and PO4 were observed in the display tank. One or more of these changes in water quality variables was thought to have caused the onset of a severe stress event, leading to many of the coral species showing signs of stress such as bleaching and/or tissue loss.

Two dominant corals within the system; *Montipora capricornis* and *Seriatopora hystrix* showed dramatic declines in health. *M. capricornis* showed bleached tissues and large areas of tissue loss whilst *S. hystrix* showed an advancing band of tissue loss followed by denuded skeleton similar to that reported for WS seen in many corals in the Indo-Pacific (Willis *et al.* 2004). Once moved to more optimal conditions, the corals gradually showed improvement in their visible health status and by 1–2 months were showing signs of new tissue growth over bare skeleton. Microbial diversity was analysed during these periods to see if bacterial populations changed over time. There was a significant difference in bacterial communities between stressed and healthy individuals in both coral species, similar to that found in many previous studies on coral diseases in the natural environment (Pantos *et al.* 2003; Sekar *et al.* 2006; Sunagawa *et al.* 2009; Sweet and Bythell 2012). Similarly, there were differences between stressed bacterial associates and those present on corals showing signs of recovery.

A large proportion of bacterial ribotypes detected in this study are associated in some way with nitrogen fixation. These include ribotypes related to; *Mesorhizobium* sp*, Cyanobacteria* sp*, Desulfovibrio* sp*, Vibrio sp, Anabaena sp, Actinobacillus sp* and *Paenibacillus* sp. The presence and importance of nitrogen-fixing bacteria has been shown with regard to corals and their symbiotic dinoflagellates (Lesser *et al.* 2004; Lesser *et al.* 2007b; Olson *et al.* 2009; Lema *et al.* 2012). Specifically, studies have claimed that certain diazoptrophic bacteria provide the main nitrogen source to the corals algal symbionts (Muscatine and Kaplan 1994; Olson *et al.* 2009). Eight of the eleven nitrogen fixing bacteria in this study are diazotrophic and have been associated with corals in natural systems, these include three *Cyanobacteria* sp, three *Vibrio* sp, a *Desulfovibrio* sp and a *Mesorhizobium* sp. Although all of these species have been associated with fixing nitrogen compounds within the coral holobiont (Lesser *et al.* 2004; Lesser *et al.* 2007b; Olson *et al.,*2009; Lema *et al.* 2012), there is conflicting evidence as to which genus and/or species are more fundamental in this role.

Three other ribotypes, belonging to diazotrophic bacteria, including *Anabaena sp, Actinobacillus* sp and *Paenibacillus* sp, have not been associated with nitrogen fixation in corals before and thus could pertain to the aquarium system alone (a potential artefact brought about by the high nitrate levels in this system). Furthermore, another interesting note is that a significantly high proportion of the ribotypes sequenced in this study belong to *Archaea*, another group of organisms which have been associated with the recycling of ammonia into nitrite (Siboni *et al.* 2008).

This study is the first of its kind whereby microbial diversity has been followed over time in relation to recovery after a major stress event. Here we show how a shift in the bacterial community structure, one which includes potentially harmful bacterial associates can be reverted to that of healthy communities by improving environmental conditions. Similar results were found by Garren *et al.* (2009) where corals transplanted into waters exposed to fish farm effluents incorporated potentially pathogenic bacteria present within the water column at the new site. Although the corals in their study failed to show field signs of any disease during this period, they showed that the original bacterial community shifted from that of the natural micorbiota and subsequently recovered when returned to non polluted waters after a period of 22 days (Garren *et al.* 2009). One further study by Sweet *et al.* (2011b), showed a similar pattern of bacterial disturbance and recovery yet on a shorter time scale (over 96 h). In that study the antibiotic Ciprofloxacin was utilized to disrupt the natural microbiota on the coral simulating that observed in natural stress events. The treatment showed a significant shift in bacterial diversity between healthy and treated samples with an increase in relative abundance of potentially pathogenic bacteria such as *Clostridium* (Sweet *et al.* 2011b).

Approximately 30% of the natural microbiota in corals has been shown to possess some form of antimicrobial activity. Interestingly, several of the bacteria that were reduced in the stressed state in both coral species in this study are known to be antibiotic producers which target specific microbial species (Attia *et al.* 2009; Wiese *et al.* 2009). Bacterial ribotypes related to *Kiloniella sp.* and *Streptomyces sp.* in *M. capricornis* reduced in dominance or were completely absent in stressed samples, whilst *Moraxella sp.,* in *S. hystrix* is the opposite and increased in disease samples. This perturbation in the corals natural microbial community, may lead to further destabilisation of the community structure as a whole. This may in turn allow potentially pathogenic bacteria to proliferate and cause the onset of specific coral diseases. The two coral species studied in this instance showed that corals and their associated microbes react very differently to stress events despite being within the same environment. In the case of *M. capricornis* the corals natural antimicrobial capabilities are hampered during the disease state, whereby the corals ability of inhibiting potentially harmful bacteria are decreased during this time period. It is likely that the coral in this stressed state is also more susceptible to other potential microbial pathogens such as viruses and ciliates (Sweet and Bythell 2012) although further work analysing these microorganisms would need to be conducted. The antimicrobial capabilities were high during the recovery period but reduced to healthy levels after four months. These results suggest that either; 1) the coral itself has reduced capabilities to fight off potential pathogens during its stressed state or alternatively 2) specific bacterial associates which usually have antimicrobial capabilities are effectively knocked back and allow other potentially pathogenic bacteria to proliferate in their absence. In contrast, this doesn't appear to be the case with *S. hystrix*. The antimicrobial activity, although significantly different than that of the ethanol control, did not vary between healthy and stressed states. This result suggests that antimicrobial capabilities are varied between coral species and affected in different ways depending on species and/or type of disease. As the diseases could not be characterised any further, it is difficult to conclude more form these results, but further studies utilising the techniques in this study should be conducted when similar situations arise within aquariums in the future. Furthermore, we report the presence of potentially pathogenic bacteria such as two *Vibrio sp.* and an *Actinomyces sp*. in healthy tissues, yet absent in diseased tissues confirming many studies showing corals harbour these pathogenic bacteria in their healthy microbial communities (Luna *et al.* 2007; Luna *et al.* 2010; Sweet *et al.* 2011c; Sweet and Bythell 2012). Two further ribotypes, which were closely related to *Thalassomonas ioyana*, the reported causal agent of White Plague-like disease in the Caribbean (Thompson *et al.* 2006), were found in *M. capricornis* corals in all samples, one ribotype did increase in relative 16S rRNA gene diversity during the disease state, however was not a dominant member of the stressed bacterial community. This raises interesting questions in the role of all these bacteria within the healthy corals natural microbial associates.

In conclusion this study shows that distinct bacterial communities develop in different species under identical aquarium conditions, confirming many previous studies which showed that coral species are associated with unique bacterial communities. Contrary to expectation, identical environmental stresses produced different community responses and visual signs of stress between the two species. Interestingly, the bacterial communities associated with the coral show high resilience, returning to a virtually identical state once visibly healthy signs returned. In this case, removal of the environmental stress allowed recovery to a normal health status and normal bacterial communities within two months. However, whether bacterial community shifts are a contributing cause or a simple effect of the visible signs of stress cannot be determined.

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