

## ORIGINAL ARTICLE

# Temperature thresholds for bacterial symbiosis with a sponge

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**The impact of elevated seawater temperature on bacterial communities within the marine sponge *Rhopaloeides odorabile* was assessed. Sponges were exposed to temperatures ranging between 27 and 33 °C. No differences in bacterial community composition or sponge health were detected in treatments between 27 and 31 °C. In contrast, sponges exposed to 33 °C exhibited a complete loss of the primary cultivated symbiont within 24 h and cellular necrosis after 3 days. Furthermore, denaturing gradient gel electrophoresis (DGGE) and clone sequence analysis detected a dramatic shift in bacterial community composition between 31 and 33 °C. Within the first 24 h most of the DGGE bands detected in samples from 27 to 31 °C were absent from the 33 °C sponges whereas eight bands were detected exclusively in the 33 °C sponges. The 16S rRNA sequencing revealed that most of the microbes from sponges exposed to 27–31 °C had highest homology to known sponge-associated bacteria. In contrast, many of the microbes from sponges exposed to 33 °C were similar to sequences previously retrieved from diseased and bleached corals. The 16S rRNA clone library analysis also detected a significant shift in bacterial community structure. The 27 °C library was composed of *Proteobacteria*, *Actinobacteria*, *Nitrospira*, *Acidobacteria* and *Chloroflexi* whereas the 33 °C library contained sequences from the *Proteobacteria*, *Bacteroidetes* and *Firmicutes*. The clear shifts in community composition at elevated temperatures can be attributed to the loss of symbionts and to the establishment of alien microbial populations including potential pathogens. Breakdown of symbioses and stress in the sponge occurred at temperatures identical to those reported for coral bleaching, indicating that sponges may be similarly threatened by climate change.**

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## Introduction

Global climate change will have a direct and significant impact on marine systems including the iconic Great Barrier Reef (GBR) (Hughes *et al.*, 2003). The primary effect of climate change will be a projected 1.8–4 °C increase in global air temperature by 2100, along with similar rises in sea surface temperatures and sea level rises (range of best estimates from the six Special Report on Emission Scenarios (SRES) emissions marker scenarios) (IPCC, 2007). Other associated effects include increased ocean acidity, increased terrestrial inputs and changes to oceanic circulation. These outcomes of climate change will result in a significant impact on marine microbes, potentially altering microbial diversity, function and community dynamics (Webster and Hill, 2007). Although microbes constitute by far the largest

diversity and biomass of all marine organisms, the likely widespread effects of climate change on microbial communities has been largely overlooked.

Microbes are involved in a variety of important symbiotic relationships with marine invertebrates of almost all phyla. Proposed symbiotic functions for marine microbes include: nutrition (through direct incorporation of dissolved organic matter in the seawater or translocation of photosynthate) (Wilkinson, 1979), assistance with reproductive processes (Klussmann-Kolb and Brodie, 1999), enhancement of chemical defences (Unson *et al.*, 1994), contribution to structural rigidity (Wilkinson, 1983), metabolism of a wide range of waste compounds (Wickins, 1983) and production of secondary metabolites (Schmidt *et al.*, 2000). There are also many symbioses where the type of interaction between the host and its symbionts remains unknown. Environmental conditions that affect the distribution or abundance of symbiotic marine microbes could have significant effects on functional symbiosis and subsequently reduce the fitness and survival of invertebrate host species.

The relationship between sponges and their associated bacteria has received considerable

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research attention over the past decade (reviewed in Taylor *et al.*, 2007). Sponges represent a significant component of the benthic environment in terms of their biomass, diversity and their ability to influence benthic and pelagic processes. In the case of sponges, up to 40% of the biomass of the organism can be composed of bacteria (Vacelet, 1975). These are often remarkably complex symbioses with high microbial diversity, including novel species that have not been found in other ecosystems. There is evidence that some bacteria are ubiquitous in various sponges from different oceans and that some of the phylogenetic clades found in sponges are more similar to each other than to types from other environments (Hentschel *et al.*, 2002, Enticknap *et al.*, 2006, Taylor *et al.*, 2007). For example, the bacterial candidate phylum 'Poribacteria' has so far been found in numerous sponge species, yet these microbes have <75% 16S rRNA sequence homology to any previously described bacteria (Fieseler *et al.*, 2004). Many studies also report that sponges contain distinct microbial communities not found in the surrounding seawater (Webster and Hill, 2001, Hentschel *et al.*, 2002, Taylor *et al.*, 2004).

In the intensively studied GBR sponge *Rhopileides odorabile*, the cultivated bacterial community is dominated by an Alphaproteobacterium (Webster and Hill, 2001), which is closely related to symbionts in many other sponges from broad tropical locations (Lafi *et al.*, 2005, Enticknap *et al.*, 2006). However, the total microbial community in *R. odorabile* as detected by molecular techniques contains a great diversity of bacteria (Webster *et al.*, 2001c) as well as two archaea (Webster *et al.*, 2001a). The roles of many of the symbionts in this sponge remain enigmatic, although the culturable Alphaproteobacterium appears to be linked to sponge health (Webster *et al.*, 2002).

Considering the predominance of bacteria within sponges, the complexity of these symbioses and the evidence for vertical transfer of some sponge symbionts from the parent sponge to the larvae (reviewed in Taylor *et al.*, 2007), it is apparent that bacterial symbionts are important for their host sponges. It is also likely that many microbial symbionts have strict temperature thresholds, and a breakdown in symbiosis could result in reduced host fitness, shifts in host geographic range, increased disease, an increase in predation or grazing and host mortality. There is also the possibility that increased seawater temperatures may cause a shift from symbiotic to pathogenic function for some microbial species.

Microbes are ideal indicators for alerting us to stress in marine systems as they (1) exhibit very strict physiological thresholds and as a result can be sensitive to small environmental perturbations and (2) respond very rapidly to changing environmental conditions (Staley *et al.*, 1997). Significant research effort has been directed towards understanding the

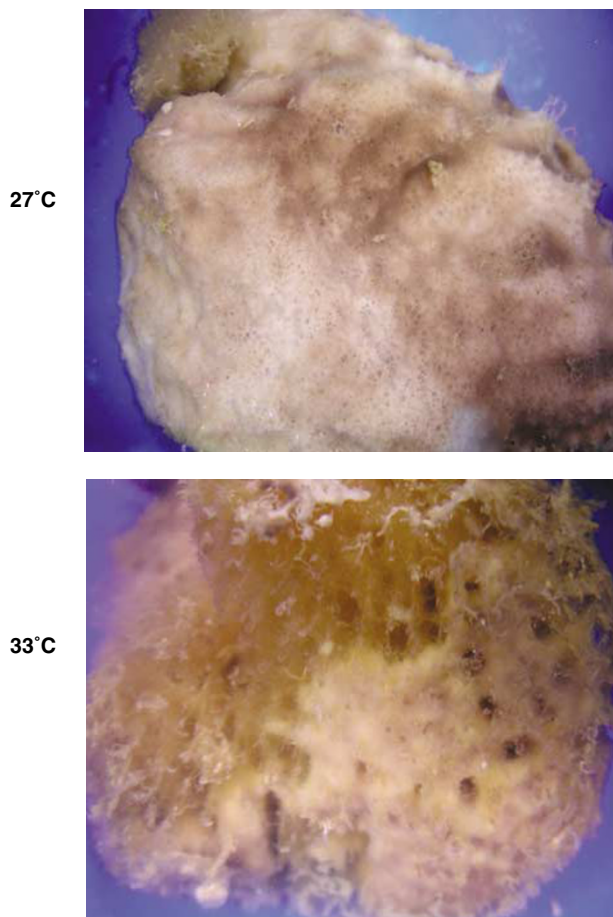
symbiotic partnership between coral and their symbiotic eukaryotes (zooxanthellae) and how this coral-algal partnership is impacted by elevated sea temperatures and other environmental stressors (Brown, 1997). In this article we introduce a different model to examine the potential impacts of temperature on symbiosis—that between a sponge and its bacterial symbionts.

## Methods

### Temperature exposure

Four large *R. odorabile* sponges were collected by scuba at 15 m from Pelorus island, North Queensland, Australia (18°32.710'S, 146°29.273'E). These donor sponges were cut into a total of 100 clones (approximately 15 cm<sup>3</sup>) using a sharp knife and transferred to plastic racks that were secured to the reef base near the original collection site. The sponge clones were allowed to heal on the reef for 8 weeks before collection and transportation to the indoor temperature-controlled aquarium at the Australian Institute of Marine Science, Townsville. Initial denaturing gradient gel electrophoresis (DGGE) screening of microbial communities in each of the four donor sponges revealed no inter-sponge variability (data not shown); hence all clones were pooled into a common stock. The experimental design incorporated four temperatures (27, 29, 31 and 33 °C; range ±0.2 °C) in three replicate 30 l flow-through aquaria per temperature, each holding eight sponge clones. Incoming seawater was filtered to 1 µm to remove large particulates yet provide the sponges with a sufficient nutritional supply in the form of small particulates and microorganisms. Sponges were maintained under a diel cycle of 12:12 h at 80 µmol quanta m<sup>-2</sup> s<sup>-1</sup>, reflecting light intensity at 15 m on the reef. Initially, all aquaria were left at 27 °C for 72 h and then temperatures were adjusted gradually (0.2 °C h<sup>-1</sup>) until reaching the final temperatures. Four sponge clones were randomly removed per temperature treatment at each time point (T = 0, 1, 3, 7, 14 and 28 days). These clones were photographed, frozen in liquid nitrogen for molecular analysis and processed for bacterial cultivation. In the 33 °C treatment, sponges exhibited major surface necrosis after 3 days (Figure 1). At this time the temperature in the 33 °C treatment was returned to 27 °C. In the other treatments, temperatures were maintained for the first 14 days and then all temperatures were returned to 27 °C for the final 14 days of the experiment as a recovery period.

Seawater samples (1 l) from each temperature at each time point were filtered through a 0.2 µm sterivex filter (Durapore; Millipore, North Ryde, New South Wales, Australia), which were filled with 1.8 ml of lysis buffer (40 mM EDTA, pH 8.0; 50 mM Tris and 0.75 M sucrose) and frozen at -80 °C.



**Figure 1** *Rhopaloeides odorabile* from T = 3 days showing intact pinacoderm tissue from sponges at 27 °C and large areas of surface necrosis in sponges at 33 °C.

#### Bacterial cultivation

All isolation procedures were performed aseptically as previously described in Webster and Hill (2001). Briefly, a 1 cm<sup>3</sup> portion of sponge tissue was excised, rinsed quickly in 70% ethanol and rapidly transferred to sterile artificial seawater. The tissue was removed from the seawater, cut into thin sections using a sterile scalpel and finely ground with a mortar and pestle. Serial dilutions of sponge homogenates were spread-plated in triplicate on Bacto Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA) for detection and enumeration of the previously described *R. odorabile* symbiont, strain NW001.

#### DGGE

Frozen sponge tissue (approximately 0.5 g per sample) was aseptically transferred to 1.5 ml Eppendorf tubes using sterile scalpels. Grinding buffer (0.5 ml) was added to each replicate sample (100 mM Tris, pH 9.0; 100 mM EDTA, pH 8.0; 1% SDS and 100 mM NaCl). Tubes were immersed in liquid nitrogen and ground with plastic pestles. Samples were incubated at 65 °C for 60 min before addition of

187 µl 5 M potassium acetate. Samples were incubated on ice for 30 min and centrifuged at 8000 g for 15 min. The supernatants were transferred to fresh tubes and DNA was precipitated with 0.8 volumes of isopropanol. DNA was extracted from seawater filters by addition of 200 µl lysozyme (10 mg ml<sup>-1</sup>), incubation at 37 °C for 45 min, addition of 200 µl of proteinase K (0.2 µg ml<sup>-1</sup>) and 1% SDS and incubation at 55 °C for 1 h. Lysates were recovered into fresh Eppendorf tubes and DNA was extracted with a standard phenol:chloroform:isoamyl alcohol procedure and precipitated with 0.8 volumes of isopropanol.

The 16S rRNA gene from each sponge clone and seawater sample was amplified by PCR with primers 1055f: 5'-ATGGCTGTCGTCAGCT-3' and 1406r: 5'-ACGGGCGGTGTGTAC-3' (Ferris *et al.*, 1996). The reverse primer was modified to incorporate a 40 bp GC clamp (Muyzer *et al.*, 1993). PCR reactions were performed as described by Ferris *et al.* (1996). Products from triplicate PCR reactions were combined and 15 µl applied to duplicate 8% w/v polyacrylamide (37.5:1) gels containing a 50–70% denaturing gradient of formamide and urea. Gels were electrophoresed at 60 °C for 17 h in 1 × TAE (Tris-acetic acid EDTA) buffer at 50 V using the Ingeny D-Code system. Gels were stained with 1 × Sybr Gold for 30 min, visualized under UV illumination and photographed. Duplicate gels were analysed using Fragment NT analysis application version 1.1a (Quantity One; Bio-Rad, Gladesville, New South Wales, Australia), and individual band numbers were assigned based on their migration relative to control bands. Bands assigned the same number had identical migration end points. Bands that did not migrate to the same point on duplicate gels were not considered in subsequent analyses. Representative bands from clones at each temperature and time point were excised, re-amplified by PCR and checked for correct mobility on a 50–70% DGGE gel. PCR products were sequenced using the forward primer, the PRISM Ready Reaction Kit (PE Applied Biosystems, Scoresby, Victoria, Australia) and the ABI 310 and 373 automated sequencers. Sequences retrieved from DGGE analysis were submitted to GenBank under the accession numbers EU335053–EU335082.

#### Cloning and sequencing

For a more comprehensive phylogenetic comparison between the two extreme temperatures (27 and 33 °C), the 16S rRNA gene from each sponge clone sampled at T = 3 days was amplified by PCR with primers 63f and 1387r (Marchesi *et al.*, 1998), and the PCR products were combined for all sponge clones per temperature and ligated into the TOPO TA cloning vector (Invitrogen, Mount Waverley, Victoria, Australia). Ligations were sent to the Australian Genome Research Facility (AGRF; University of Queensland, Brisbane) for transformation and sequencing of 192 clones for each library. AGRF

transformed the ligation into Genehog electrocompetent cells (Invitrogen), grew the cells in  $2 \times$  YT + ampicillin media, performed a standard alkaline lysis plasmid preparation and sequenced the clones with ABI BigDye v3.1 chemistry on the AB3730  $\times$  1 (standard 50 cm array run module). Clone sequences were submitted to GenBank under the accession numbers EU183744–EU184009.

#### Phylogenetic analysis

DGGE sequences were compared to available databases using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) to determine the nearest relatives and per cent homology. Clone sequences were submitted to BLAST to determine approximate phylogenetic affiliations and chimeric sequences were identified using the program CHECK\_CHIMERA (Maidak *et al.*, 1999). Partial sequences were compiled, automatically aligned and manually edited in the ARB software package (<http://www.arb-home.de> (Ludwig *et al.*, 1998)). Initially, trees were calculated with almost complete 16S rRNA (1400 bp) sequences for all close relatives of target sequences using the neighbour-joining and maximum parsimony methods in ARB. Partial sequences were subsequently imported to the tree without changing branch topology using the ARB parsimony-interactive method. The robustness of inferred tree topologies was evaluated after 1000 bootstrap re-samplings of the neighbour-joining data in the Phylip program (Felsenstein, 1993). *Thermotoga maritima* was used as an outgroup.

#### Data analyses

Variability in the relative per cent of strain NW001 in the total cultivated bacteria was assessed using Arcsin transformed data in a two-way analysis of variance with time and temperature as the independent variables (Statistica 6.0; StatSoft Inc., Tulsa, OK USA). Post-hoc differences were examined with the Tukey's HSD test.

Principal components analysis (PCA) was performed to analyse similarity in microbial community composition using DGGE data (Statistica 6.0; StatSoft Inc.). A matrix was constructed using the presence (1) or absence (0) of a band in each sample. Mean scores from each of the time and temperature treatments ( $n=4$  clones) were used in the PCA. Clone library rarefaction curves were produced using the analytical approximation algorithm of Hurlbert (1971). Calculations were performed using the freeware program aRarefactWin (<http://www.uga.edu/~strata/Software.html>). Curves were plotted using Sigmaplot (V7.101, SPSS Inc.) and regressions were performed using the algorithm for an exponential rise to a maximum:  $y = a(1 - e^{-bx})$ . The asymptote of the curve ( $a$ ) describes the estimated total diversity of the clone library. Various indices and models were also used to analyse the variation

of microbial diversity within each clone library (Magurran, 1988). Richness indices: the Shannon–Weiner diversity index (Shannon and Weaver, 1963) was calculated as:  $H' = -\sum_{i=1}^s pi \ln(pi)$  where  $pi$  is the proportion of clones belonging to the  $i$ th operational taxonomic unit (OTU), and  $S$  is the total number of OTUs (sequences with  $<98\%$  identity were considered as discrete OTUs). Abundance models: Fisher's  $\alpha$ -log series richness index (Fisher *et al.*, 1943). Coverage ( $C$ ) values are calculated by the equation:  $C = 1 - (n/N) \times 100$ , where  $n$  is the number of unique clones and  $N$  is the total number of clones examined (Good, 1953). The Chao1 estimates total species richness as:  $S_{\text{Chao1}} = S_{\text{obs}} + a^2/2b$  where  $S_{\text{Chao1}}$  is the estimate of the total number of classes in the population,  $S_{\text{obs}}$  is the number of classes observed in the sample,  $a$  is the number of classes with exactly one individual (singletons),  $b$  is the number of classes with exactly two individuals (doubletons) and where 1 is substituted for  $a$  or  $b$  if either has no singletons or doubletons (Bohannan and Hughes, 2003).

## Results

#### Temperature treatment observations

All sponge clones survived in each of the 14 day exposures at 29–31 °C and subsequent 14 day recovery periods and were visibly indistinguishable from controls maintained at 27 °C. However, clones exposed to 33 °C began ejecting small amounts of cellular material and exhibited minor surface necrosis ( $<10\%$  surface area) after 24 h. Major necrosis of sponge tissue (50–70%) was apparent after 3 days, revealing protrusions of skeletal fibres from the tissue and demonstrating significant stress to the host (Figure 1). Despite the seawater temperature being returned to 27 °C in the 33 °C treatment after 3 days, the sponge clones showed no further necrosis or signs of cellular recovery during the remainder of the 28-day experiment.

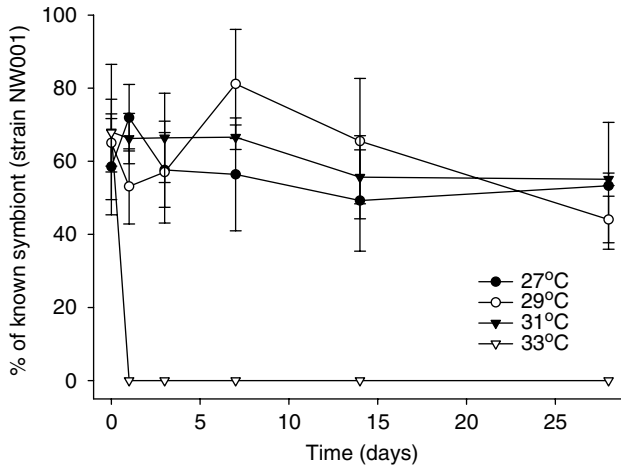
#### Shift in symbiosis

The culturable Alphaproteobacterium symbiont of *R. odorabile* (strain NW001) was highly sensitive to 33 °C within the first 24 h (Figure 2). The proportion of strain NW001 at 33 °C declined to 0% after 24 h which was a significant reduction from all other time periods and temperatures ( $F(2.7, 15) = 4.4$ ;  $P < 0.001$ ). The proportion of the culturable fraction comprised by strain NW001 averaged 65% throughout the 28 days exposure/recovery period for the 27–31 °C clones showing no other significant variability with temperature or time (Supplementary Table I).

#### Shifts in community composition

The bacterial communities detected by DGGE were highly conserved throughout all replicate clones, time periods and temperatures up to and including

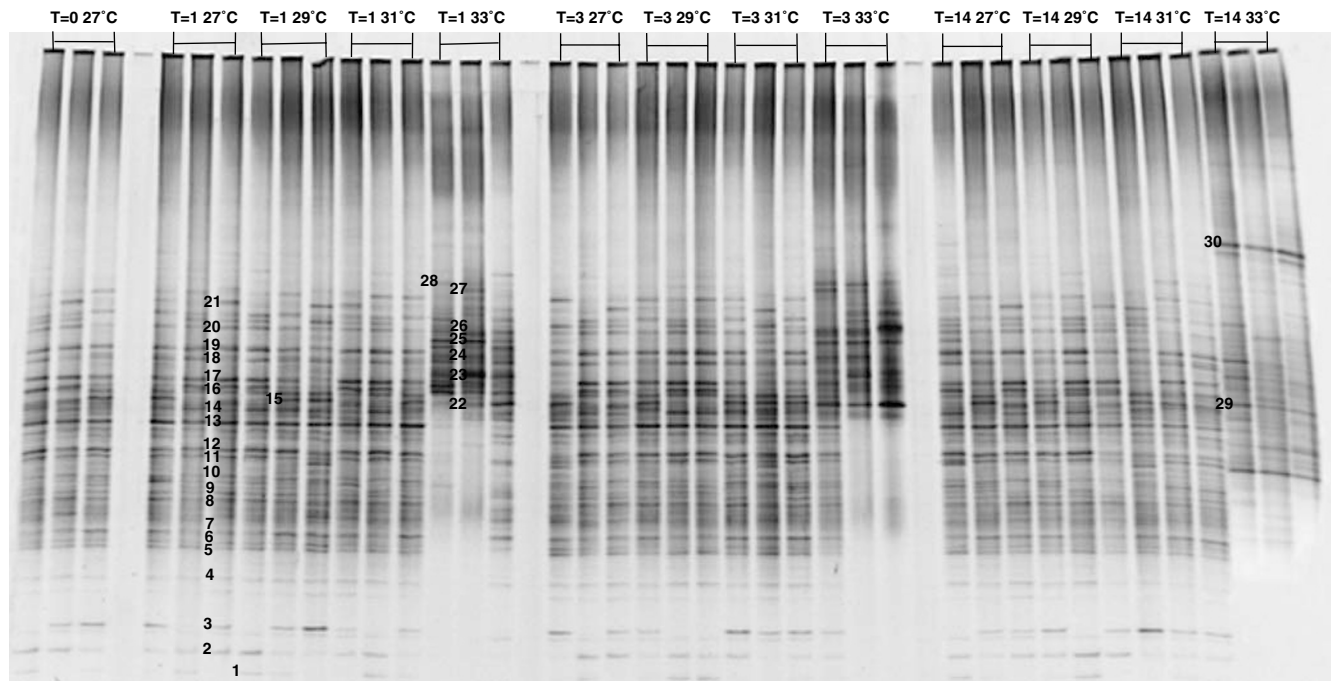
31 °C (Figure 3; Supplementary Figure 2). In contrast, 33 °C appears to be a critical threshold temperature for many of the sponge-associated microbes. Within the first 24 h most of the bands detected in samples from 27 to 31 °C were absent from the 33 °C sponges. In addition, we detected the appearance of bands (22–30) that were exclusively present in the sponges exposed to 33 °C. The 16S rRNA sequencing of excised DGGE bands revealed



**Figure 2** The proportion of strain NW001 relative to total cultivated bacteria (percentage of total) isolated from *R. odorabile* exposed to 27, 29, 31 and 33 °C seawater over a 28-day trial. Counts are expressed as colony-forming units (CFU) per ml of sponge tissue.  $N=4$ , bars represent  $\pm$  s.e.

that (with the exceptions of bands 4, 15 and 16) all microbes corresponding to bands from sponges exposed to 27–31 °C had highest homology to known sponge-associated bacteria or sponge symbionts (Table 1). In contrast, the closest known relatives to some of the microbes corresponding to bands seen exclusively at 33 °C were sequences previously retrieved from coral disease (band 25) and coral bleaching (bands 23 and 28) studies. Only one of the sequences from 33 °C had highest homology to a sponge-associated bacterium (band 24). To more effectively visualize this shift in community composition at 33 °C, PCA was performed to examine the level of similarity in DGGE banding patterns between samples. PCA explained 59% of the variability in the first two factors and clearly demonstrated a very high level of similarity in detectable bacterial bands in sponges between 27 and 31 °C (Figure 4; Supplementary Figure 1). Although there is a distinct shift along the x axis of the PCA plot in all 33 °C samples, PCA revealed relatively high degrees of variability in DGGE banding patterns at different time points throughout the experiment at this elevated temperature. When the temperature of the 33 °C sponges was returned to 27 °C after 3 days, the microbial communities remained distinct from the communities of the 27–31 °C sponges.

The seawater DGGE profiles showed a large number of bands that were largely conserved within all samples (Supplementary Figure 3). The primary exception was a dense band exclusively present in  $T=1$  day and  $T=3$  days, 33 °C seawater samples.

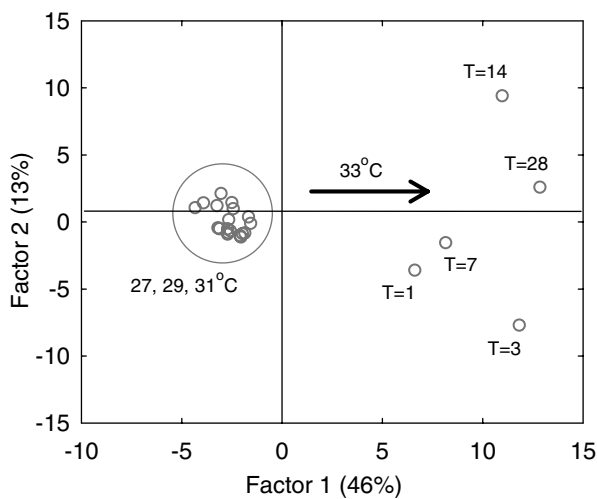


**Figure 3** Denaturing gradient gel electrophoresis (DGGE) profile of 16S rRNA-defined bacterial populations from replicate specimens at times 0, 1, 3 and 14 days from sponges exposed to 27, 29, 31 and 33 °C. Three replicates were used per treatment and individual bands excised for sequencing are labelled 1–30 on the left-hand side of the lane.

**Table 1** Nearest relatives for each of the sequences retrieved from excised DGGE bands

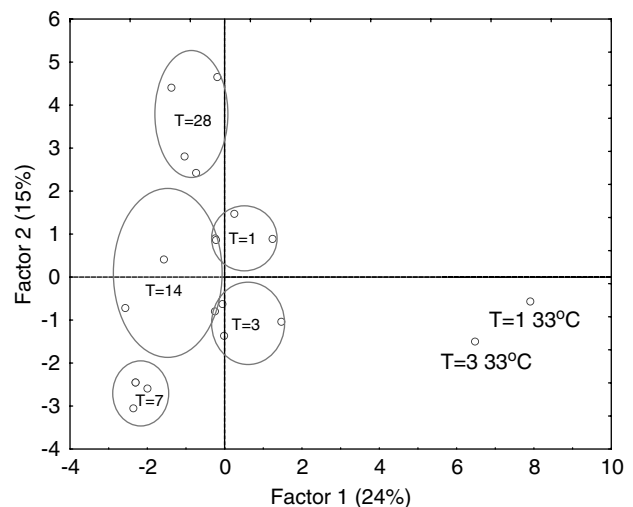
Band ID	Nearest relative	Homology (%)
Band 1 (EU335053)	Sponge Actinobacterium (AM259924)	97
Band 2 (EU335054)	Sponge Bacterium TK66 (AJ347047)	94
Band 3 (EU335055)	Sponge Bacterium Cnuc56 (DQ079049)	92
Band 4 (EU335056)	Mangrove Planctomycete sp. (DQ811899)	83
Band 5 (EU335057)	Sponge Acidobacterium (AJ850100)	98
Band 6 (EU335058)	Sponge Bacterium PAUC26f (AF186410)	93
Band 7 (EU335059)	Sponge Chloroflexi PK010 (EF076074)	97
Band 8 (EU335060)	Sponge Poribacterium 64K2 (AY713479)	97
Band 9 (EU335061)	Sponge Actinobacterium C-129 (DQ180146)	95
Band 10 (EU335062)	Sponge Chloroflexi PK040 (EF076113)	89
Band 11 (EU335063)	Sponge Chloroflexi AnCha11f (EF076229)	94
Band 12 (EU335064)	Sponge Poribacterium 236 (AY485289)	97
Band 13 (EU335065)	Sponge Poribacterium 64K2 (AY713479)	99
Band 14 (EU335066)	Sponge Poribacterium 96 (AY485282)	97
Band 15 (EU335067)	Coral Bacterium sluc1 (DQ416275)	94
Band 16 (EU335068)	Gamma proteobacterium I3K-0272 (AY867959)	97
Band 17 (EU335069)	Sponge Poribacterium 64K2 (AY713479)	97
Band 18 (EU335070)	Sponge Poribacterium 214 (AY485288)	96
Band 19 (EU335071)	Sponge Chloroflexi AD084 (EF076173)	95
Band 20 (EU335072)	Sponge Bacterium PK019 (EF076072)	99
Band 21 (EU335073)	Sponge Symbiont PAUC32f (AF186411)	94
Band 22 (EU335074)	<i>Microscilla</i> sp. DG946 (AY258124)	91
Band 23 (EU335075)	Bleached Coral Bacterium b3ub4 (EF207026)	90
Band 24 (EU335076)	Sponge Shewanella UST040317 (DQ180743)	96
Band 25 (EU335077)	Coral BBD Gamma proteobacterium (AY038559)	90
Band 26 (EU335078)	Coral Chloroflexi Cobs2TisF2 (EU246818)	98
Band 27 (EU335079)	Coral Gamma proteobacterium EC167 (DQ889884)	96
Band 28 (EU335080)	Coral Bacterium W3Ca9 (DQ416657)	97
Band 29 (EU335081)	Gamma proteobacterium ISO-0527 (AY868111)	89
Band 30 (EU335082)	<i>Nitzschia frustulum</i> (AY221721)	98

From Figure 3 using the basic local alignment search tool BLAST.



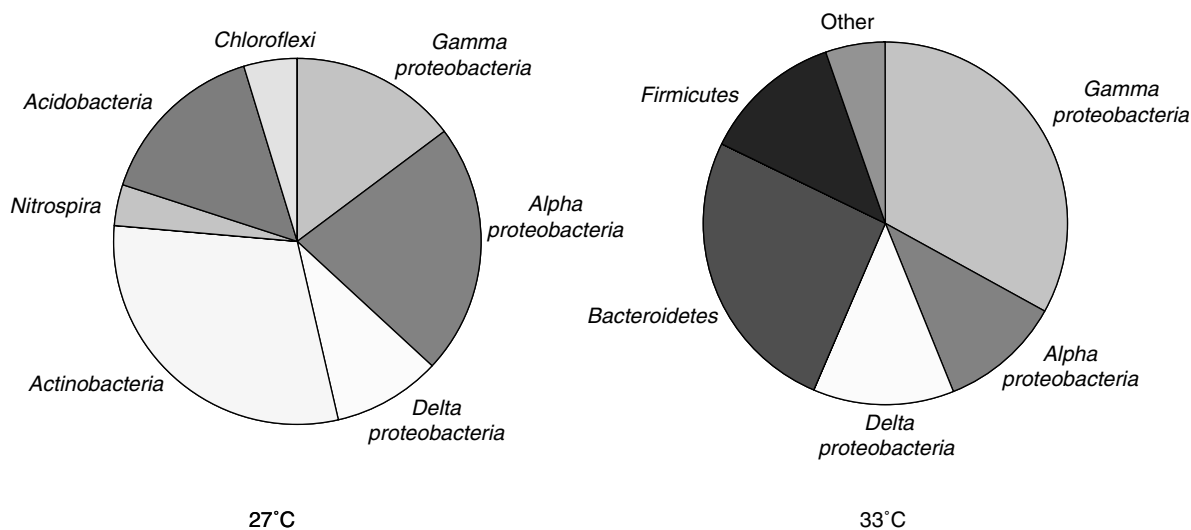
**Figure 4** Principal components analysis (PCA) of sponge bacterial community composition at 27, 29, 31 and 33 °C throughout the 28 day temperature experiment. Denaturing gradient gel electrophoresis (DGGE) banding pattern data were used to construct a similarity matrix for PCA analysis. Each point represents the mean score from four sponge clones.

This band (EU342352) was excised, sequenced and identified as having highest homology (97%) to an Alphaproteobacterium isolated from the bleached coral *Oculina patagonica* (EF207075). Surprisingly,



**Figure 5** Principal components analysis (PCA) of seawater bacterial community composition at 27, 29, 31 and 33 °C throughout the 28 day temperature experiment. Denaturing gradient gel electrophoresis (DGGE) banding pattern data were used to construct a similarity matrix for PCA analysis.

this sequence was not detected in any of the sponge DGGE sequences or the comprehensive clone library analysis of the T = 3, 33 °C sponges. PCA of the seawater DGGE data explained 39% of



**Figure 6** Pie charts showing the differences in bacterial community composition between 27 and 33 °C clone libraries at the phyla and class taxonomic levels. The 'other' category contains sequences from the 'TM7, OP1 and OP11' candidate phyla. Graphs were constructed using the frequency of 16s rRNA sequences belonging to each bacterial group from an extensive clone library analysis ( $n = 149$  for 27 °C and 111 for 33 °C).

the variability in the first two factors and again revealed clear separation of the 33 °C, T = 1 and T = 3 samples (Figure 5). All other samples, including the seawater in 33 °C treatments that were readjusted to 27 °C after 3 days, showed clear clustering according to sampling time rather than seawater temperature, although no clustering can be considered after day 14 when all temperatures were returned to 27 °C.

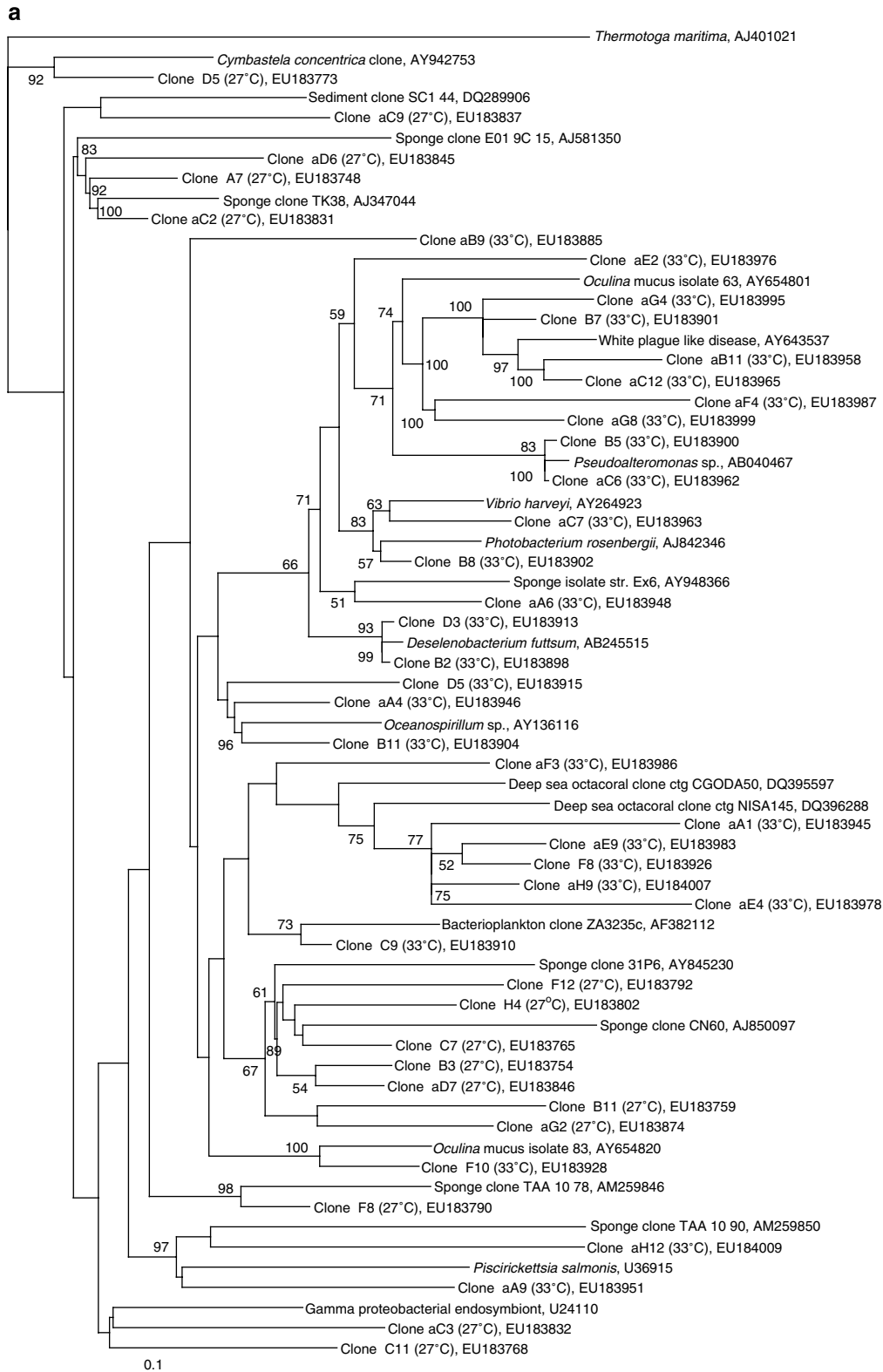
A more comprehensive phylogenetic comparison between the 27 and 33 °C sponge samples at 3 days using clone sequence data showed major differences in the microbial community composition (Figure 6). Bacterial phyla such as the *Actinobacteria*, *Nitrospira*, *Acidobacteria* and *Chloroflexi* were only detected in the 27 °C sponges and comprised over 50% of the total sequences analysed. In contrast, the *Firmicutes* and the *Bacteroidetes* were only present in the 33 °C sponges and made up 38% of the total sequences analysed. This indicates that elevated sea temperatures can cause a major shift in prokaryote community structure at a very high taxonomic level. When examined in closer detail, it was clear that the 27 and 33 °C sequences could be further differentiated within individual bacterial classes. The phylogenetic trees of the Alpha (Figure 7a) and Gamma (Figure 7b) proteobacterial classes revealed distinct temperature-dependent grouping of the sequences. The clone sequencing corroborated results from DGGE, with many of the sequences retrieved from sponges at 27 °C displaying highest homology to known sponge-associated bacteria/symbionts, whereas many of the sequences recovered from sponges at 33 °C had highest homology to microbes associated with diseased and bleached corals.

#### Shifts in diversity

Rarefaction analysis was performed to determine the number of unique bacterial clones (sharing <98% sequence similarity) as a proportion of the estimated total diversity. Neither the 27 °C nor the 33 °C rarefaction curves reach a clear asymptote (data not shown), indicating that further sampling of both clone libraries would have revealed additional diversity. For the 27 °C library the asymptote ( $a$ ) = 97.1 indicated that 74% of the estimated diversity was sampled and for the 33 °C library the asymptote ( $a$ ) = 138.9 indicated that 53% of the diversity was sampled. The number of clones analysed and the coverage was higher for the 27 °C library than the 33 °C library, whereas the observed number of sequence types was almost identical in both libraries (Table 2). Had exhaustive cloning been possible, the projected diversity of the 33 °C sponges (139 microbial species) would be 43% higher after 3 days than for sponges cultivated at 27 °C (97 microbial species). A range of diversity and evenness indices (Table 2) also indicated that bacterial diversity and evenness were both higher in the 33 °C sponges than in the 27 °C sponges.

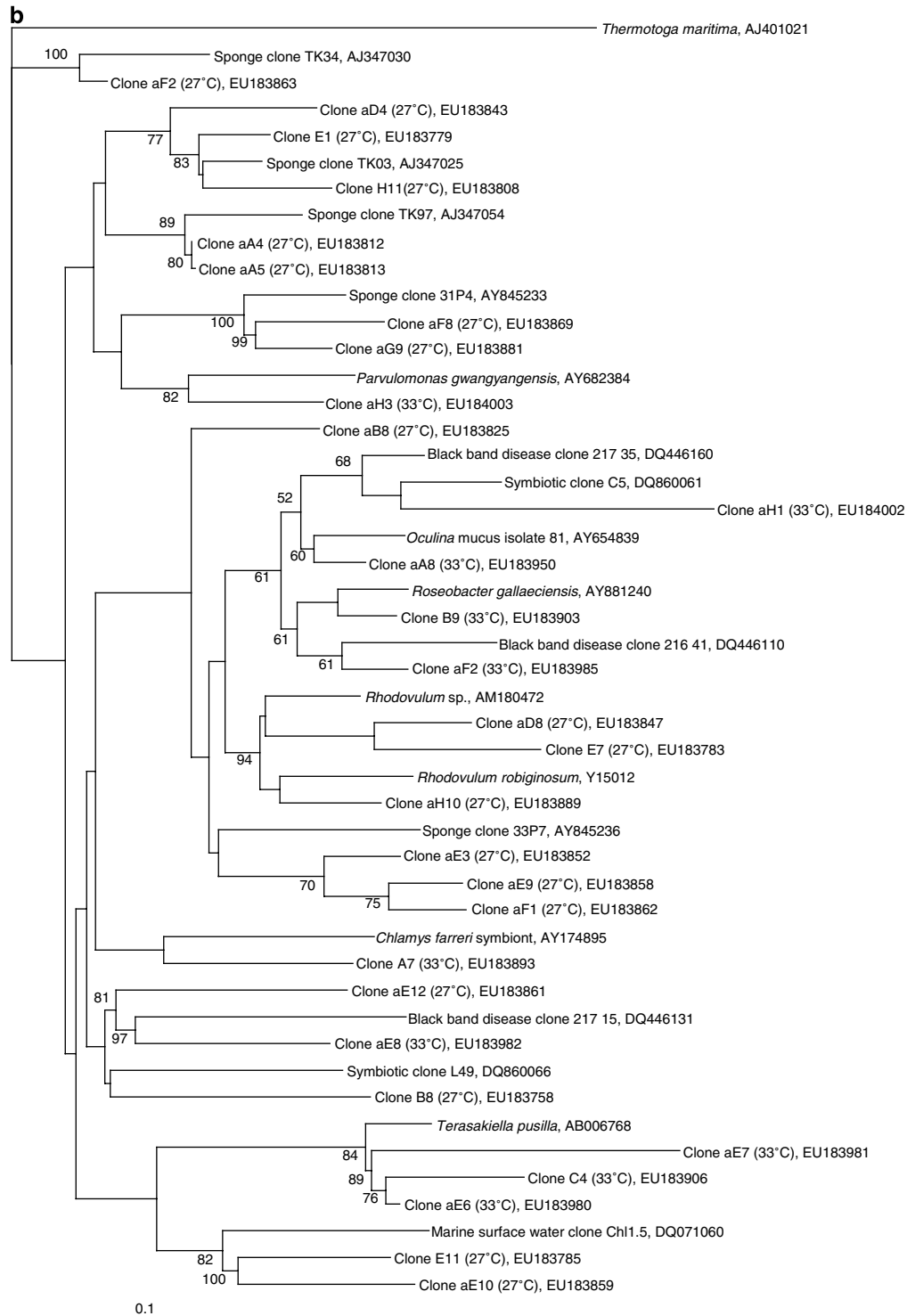
#### Discussion

This study revealed that the tropical reef sponge, *R. odorabile*, and its associated microbial communities (including symbionts) do not tolerate seawater temperatures of 33 °C, only 2–4 °C above the mean maximum annual temperature at Orpheus island, nearby Pelorus island from where the sponges were collected (Berkelmans and Willis, 1999). After 3 days at 33 °C, both the host sponge and their



**Figure 7** Neighbour-joining phylogenetic trees from analysis of all 16S rRNA gene library sequences within the *Gammaproteobacteria* (a) and *Alphaproteobacteria* (b). The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbour-joining analysis of 1000 re-sampled data sets. Only values > 50% are shown. Scale bar represents 0.1 substitutions per nucleotide position.





**Figure 7** Continued.

associated microbial communities exhibited clear signs of stress, including gross tissue necrosis of the sponge surface, accompanied by large shifts in microbial community structure. The *Firmicutes* and *Bacteroidetes*, absent at 27 °C, became dominant at 33 °C, and only sponges exposed to 33 °C possessed *Proteobacteria* previously associated with coral bleaching and disease. It is likely that increas-

ing sea surface temperature directly affects both the host and microbial components of sponges along with symbiotic partnerships, critical to the health of the holobiont (host–symbiont complex).

Numerous other biochemical and molecular markers have been used to study the impact of thermal stress on sponges. These include increased expression of the heat shock protein 70 and the DnaJ-like

**Table 2** Diversity indices calculated from sequences of 16S rRNA genes

Parameters	27 Degrees	33 Degrees
No. of clones analysed	149	111
Coverage of clone libraries (%)	51.7	34.3
Observed no. of sequence types	72	73
Shannon–Weaver diversity (H')	3.93	4.07
Chao1	160	238
Fisher's $\alpha$	54.0	92.8
Simpson's evenness (D)	46.1	72.7

protein in response to a 7 °C increase above ambient seawater temperature (Koziol *et al.*, 1997). A down-regulation of the Rab GDP-dissociation inhibitor (Kraske *et al.*, 1997), a reduction in trehalose and glutathione and a decline in the enzyme activity of glutathione *S*-transferase in response to a 10 °C increase above ambient seawater temperature have also been reported (Bachinski *et al.*, 1997). However, the current study showed significant responses (both necrosis and a shift in the symbiotic microbial community) with temperatures only 2–4 °C above ambient seawater.

The stress response (necrosis and cell loss) in *R. odorabile* at 33 °C combined with the lack of any gross tissue damage in these sponges at 31 °C indicate an acute temperature threshold for this sponge species between 31 and 33 °C. This temperature range was also critical to the partnership between the sponge and a known *Alphaproteobacteria* symbiont (strain NW001), which was no longer able to be cultured from 33 °C sponges within the first 24 h. This result suggests that strain NW001 is either highly sensitive to thermal stress or that aggressive, alien microbial populations were able to outcompete the native bacterial species over this short time period. The implications of the breakdown in symbiosis at 33 °C are unclear, and further investigations are required to determine whether this symbiosis is as critical to sponges as the mutualistic relationship between corals and their dinoflagellate symbionts (zooxanthellae).

The temperature threshold observed for *R. odorabile* was identical to the bleaching threshold (expulsion of zooxanthellae) for the corals *Pocillopora damicornis* and *Acropora elseyi* collected from the nearby inshore location at Orpheus island. (Berkelmans and Willis, 1999). Summer seawater temperatures on inshore reefs of the central GBR periodically exceed 30 °C and our results indicate that short-term increases above 31 °C, which can cause mass coral bleachings (Berkelmans *et al.*, 2004), may also affect sponge symbiosis. Thermal stress thresholds for sponges are, however, likely to vary along the GBR, being governed by the mean maximum seawater temperature encountered on each reef and also influenced by long-term adaptation to their native environments. The short-term rate of change in seawater temperature is also likely to influence stress responses in sponges and their

symbionts. In the present study, a relatively slow linear adjustment of the experimental seawater temperatures of 0.2 °C h<sup>-1</sup> was applied to the sponges. This rate is equivalent to rates commonly experienced on the reef slope due to tidal cycles at Orpheus island, near the site of collection of the sponges (Berkelmans, 2001).

The dramatic shift in bacterial community structure between 31 and 33 °C occurred at the class, phyla, genus and species levels. At the phyla level, the 27 °C clone library was composed of *Proteobacteria* (Alpha, Gamma and Delta classes), *Actinobacteria*, *Nitrospira*, *Acidobacteria* and *Chloroflexi*. The sponges maintained at 33 °C contained sequences from the *Proteobacteria* (Alpha, Gamma and Delta classes), *Bacteroidetes* and *Firmicutes*, hence the only bacterial phyla common to both libraries was the *Proteobacteria*. The phyla detected in the 27 °C library contained many of the sponge symbionts that have previously been described in studies of sponge microbial diversity (Taylor *et al.*, 2007). In fact, community composition in sponges maintained at 27 °C closely reflected a previous study that investigated the microbial diversity in *R. odorabile* on the GBR (Webster *et al.*, 2001c). In particular, the large number of distinctive *Actinobacteria* and *Gammaproteobacteria* sequences was consistent between both studies. Both the *Firmicutes* and *Bacteroidetes* phyla (detected only in the 33 °C library from the present study) have also been reported from numerous sponge species including *R. odorabile* (Webster *et al.*, 2001c). Sequencing additional clones from each library may have revealed either fewer or greater differences in community composition at the phyla level.

Within the *Proteobacteria* classes there was clear separation of the sequence affiliation depending on what temperature the host sponge had been subjected to. In the *Gammaproteobacteria* nearly all of the 27 °C clones had highest homology to previously described sponge-associated bacteria. For example 27 °C clones F12, H4, C7, B3, B11 and aG2 all had highest homology to clone 31P6 (AY845230) from a Dictyoceratid sponge and clone CN60 (AJ850097) from the Mediterranean sponge *Chondrilla nucula*. In contrast, 33 °C clones aG4, B7, aB11, aC12, aF4 and aG8 all had highest homology to *Thalassomonas loyana* sp. nov., the causative agent of white plague disease in corals (AY643537) (Thompson *et al.*, 2006). In the *Alphaproteobacteria* class most of the 27 °C sponge sequences had highest homology to previously described sponge-associated bacteria. There were fewer 33 °C sequences in the *Alphaproteobacteria* but again many of them had highest homology to coral disease sequences. For instance, the 33 °C clones aE8 and aF2 had highest homology to the black band disease clones 217 15 (DQ446131) and 216 41 (DQ446110), respectively (Sekar *et al.*, 2006). Further investigation is required to determine whether the new 'disease' strains that appear in the 33 °C sponges are primary pathogens or mere

opportunists that proliferate when the normal microbial communities are disrupted and the host immunity compromised.

The microbial community of sponges exposed to 33 °C changed after 24 h and did not return to that of the 27 °C sponges over the next 27 days. This contrasts with the microbial communities of the seawater, which were distinct at 33 °C for days 1 and 3 but returned to their original community composition when the seawater temperature was re-adjusted to 27 °C. This indicates that the shift in community composition within the sponges was not driven by variation in the seawater communities. The variation in the seawater microbes detected by DGGE was primarily influenced by sampling time, regardless of temperature. The exceptions were at T = 1 day, 33 °C and T = 3 days, 33 °C, which contained a dominant band with highest sequence homology to an Alphaproteobacterium from the bleached coral *Oculina patagonica*. However, this bacterium was not detected in any of the sponge DGGE bands or clone libraries, suggesting it was not a major driver of the observed microbial shifts within the sponges.

The broader impacts of this shift in sponge community composition relate to both the breakdown in symbiotic functions caused by the disappearance of many symbionts and also to the establishment of alien microbial populations that may include potential pathogens. Both of these outcomes are likely to have had seriously adverse effects on host health. The clustering of sequences from the 27 °C sponges with known sponge symbionts, in combination with the clustering of 33 °C (3 days) sequences with known pathogens indicates likely shifts in microbial function within the host sponge. The failure of microbial communities in sponges exposed to 33 °C for 3 days to return to their original compositions during the recovery period indicates a permanent breakdown of associations (including symbiosis). Instead, these communities transformed further over time, which was likely to be caused by both the return to 27 °C along with rapid succession of communities colonising recovering sponge tissue, dead cells and skeleton.

The clone libraries revealed a 53% higher microbial diversity at 33 °C compared with the diversity at 27 °C whereas the number of bands detected by DGGE was lower at 33 °C. This is probably due to the fact that DGGE is based on visual detection of bands and hence not particularly useful for visualizing rare components of the total community. Although there is limited data on how marine microbial diversity responds to temperature, previous studies have shown both increases (Nogales *et al.*, 2007) and decreases (Webster *et al.*, 2001b) in bacterial diversity in response to anthropogenic disturbance. A decrease in diversity at higher disturbance/stress levels may be due to exceeding physiological thresholds of a broad range of microorganisms, therefore selecting for a more narrow range of bacterial species. An increase in diversity at higher disturbance/stress levels may be

due to elevated nutrient levels (which may also happen *in situ* if stressed animals start sloughing off decaying cells) that actually provide a more abundant and varied source of compounds for microbial metabolism. In sponges exposed to elevated temperatures there is also the possibility that as the animals become stressed, their defence mechanisms become compromised which facilitates the establishment of bacterial populations that would not normally survive within the host. The increase in bacterial diversity detected at elevated temperatures may have significant implications for overall microbial resilience in the face of climate change. The 'rare biosphere' of seawater bacteria recently identified by Sogin *et al.* (2006) might provide some resilience to environmental change, as this enormous diversity of low-abundance populations could take over ecological niches that become available due to environmental perturbations. However, bacterial symbionts are often highly specialized and host-specific so the concept of functional redundancy may not apply to these more specific host–bacteria relationships.

## Conclusion

The thermal threshold for *R. odorabile* tissue necrosis was between 31 and 33 °C. Once this threshold was reached, a breakdown in sponge–bacterial symbiosis also occurred. The thermal threshold of *R. odorabile* and its symbiosis with the Alphaproteobacterium strain NW001 was identical to that of corals and their zooxanthellae symbionts collected from a similar location on the GBR (Berkelmans and Willis, 1999). This result indicates that sponges may have a similar vulnerability as corals to changes in sea surface temperatures. We also detected a dramatic shift in the overall bacterial community composition with elevated temperature. This shift occurred at all levels of phylogenetic resolution and likely involved a significant change in microbial function. In *R. odorabile* this was signified by a move away from known sponge symbionts towards known coral pathogens. Further research is required to assess to what extent the loss of important symbionts and establishment of putative pathogens facilitated the decline in sponge health.

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