Coral Reefs
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REPORT

A novel sponge disease caused by a consortium of micro-organisms

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Abstract In healthy sponges, microbes have been shown to account for up to 40 % of tissues. The majority of these are thought to originate from survivors evading digestion and immune responses of the sponge and growing and residing in the microenvironments of the mesophyll. Although a large percentage of these microbes are likely commensals, they may also include potentially pathogenic agents, which under specific conditions, such as temperature stress, may cause disease. Here we report a novel disease (sponge necrosis syndrome) that is severely affecting populations of the sponge *Callyspongia* (*Euplacella*) aff *biru*. Both ITS fungal and 16S rDNA bacterial diversities were assessed in healthy and diseased individuals, highlighting six potential primary causal agents for this new disease: two bacteria, a *Rhodobacteraceae* sp. and a cyanobacterium, *Hormoscilla spongeliae* (formally identified as *Oscillatoria spongeliae*), and four fungi, a *Ascomycota* sp., a *Pleosporales* sp., a *Rhabdocline* sp., and a *Clasosporium* sp. Furthermore, histological analysis showed the dominance of fungal hyphae rather than bacteria throughout the disease lesion, which was absent or rare in healthy tissues. Inoculation trails showed that only a combination of one bacterium and one fungus could replicate the disease, fulfilling Henle–Koch's postulates and showing that this sponge disease is caused by a polymicrobial consortium.

Keywords Maldives · Bacteria · Fungi · Koch's postulates · Necrosis · Syndrome

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Introduction

Marine sponges (Porifera) are sedentary, filter-feeding invertebrates that grow in many different ecological niches around the world (Fieseler et al. 2004). They have been shown to harbour both prokaryotic and eukaryotic microorganisms, including viruses, archaea, bacteria, cyanobacteria, microalgae, fungi, and protozoa (Osinga et al. 2001; Webster and Taylor 2012). Many of these micro-organisms, particularly the fungi and bacteria, have been shown to have significant biotechnological potential as sources of biologically active natural products. For this reason, the number of studies related to the microbial diversity associated with sponges has grown in recent years (Bungni and Ireland 2004; Aly et al. 2011; Debbab et al. 2011).

Some sponge species have been shown to harbour numbers of microbes in their tissues two to four orders of magnitude higher than can be detected in the neighbouring seawater (Weisz et al. 2008). Indeed, in some instances, these microbial associates have been shown to account for up to 40 % of the sponge tissue (Vacelet 1975; Imhoff and Truper 1976; Thirunavukkarasu et al. 2012). However, the origin of these microbes is still under some debate, although it is thought that many originate from survivors that have evaded the digestion and immune responses of

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the sponge and subsequently grow and reside in the microenvironment of the sponge mesophyll (Taylor et al. 2007). Generally, sponges are believed to benefit from these microbial associates through the provision of nutrition, transportation of waste products or active metabolites, chemical defence, and contributions to mechanical structure through sponge microbe symbiosis (Taylor et al. 2007; Weisz et al. 2008; Webster and Blackall 2009). However, the exact roles of many of these symbiotic associates are still largely unknown. Marine fungi, in particular, have been described as being the most underexplored group in the marine environment (Wang et al. 2008; Li and Wang 2009).

Disease incidence in many organisms is increasing worldwide, a large proportion of which has been attributed to changes in the climate (Peters 1997; Hoegh-Guldberg 2004; Hoegh-Guldberg et al. 2007; Miller and Richardson 2014). Although healthy sponges have been studied relatively intensively, diseases in sponges have received considerably less attention (Webster 2007). Despite this lack of information with regard to sponge diseases, studies have shown the dramatic effect diseases can have on these organisms. For example, in some cases, diseases have led to certain species being brought to the brink of extinction (Cerrano and Bavestrello 2009). A major example occurred in 1938 throughout the Caribbean, where an epidemic of sponge wasting disease affected 70–95 % of the total sponge populations studied (Galstoff 1942). In the Mediterranean, disease coupled with over-exploitation in the 1980s similarly led to devastating consequences for other sponge species such as *Spongia officinalis* and *S. zimocca* (Gaino et al. 1992; Cerrano and Bavestrello 2009). As with coral diseases (Sweet and Bythell 2012), the frequency of reports of sponge diseases and epidemics is increasing at a rapid rate (Webster 2007). However, despite the obvious impact on the ecosystem, only six sponge diseases have been assigned names (Table 1). Furthermore, only half of these have had likely aetiological agents assigned (Webster 2007). A filamentous cyanobacterium, *Hormoscilla* sp. has been implicated in the case of mangrove sponge disease (MSD) (Rüetzler 1988), and a novel member of the Alphaproteobacteria, distantly related to *Sulfitobacter pontiacus*, has been shown to be associated with samples exhibiting both sponge boring necrosis (SBN) and sponge white patch (SWP) (Vacelet et al. 1994; Webster et al. 2002). However, only one sponge disease, SBN, has had Henle–Koch's postulates successfully fulfilled, highlighting the urgent need for further studies on sponge diseases in general (Webster et al. 2002). Furthermore, despite the well-documented association of a large diversity of fungi present within healthy sponges (Li and Wang 2009; Thirunavukkarasu et al. 2012), few studies have screened for potential fungal pathogens in combination with bacteria. This study describes a novel sponge disease as well as documents the microbial communities associated with healthy and diseased sponges (both fungi and bacteria) and, finally, fulfils Henle–Koch's postulates with the proposed pathogens.

Materials and methods

Study site

Diseased sponges were first observed in field surveys running from the 2nd to the 23rd of April 2013 at Vavvaru Island, Lhaviyani Atoll, in the Maldives. The reef has a maximum depth of 15 m and consists of a wide diversity of hard scleractinian corals that provide substrate for other corals, gorgonians, and sponge species. The sponge species infected by this disease was identified as being closely related to *Callyspongia biru.* Previously, *C. biru* has been described only in Indonesia (Voogd 2004). In the Maldives, the studied specimens had the same general morphology and colour; however, the spicules and fibre sizes were smaller than those described for the holotype species, and therefore, we refer to our samples as *C.* (*Euplacella*) aff

Table 1 Highlighting the six 'named' sponge diseases at the time of writing, which species they have been shown to affect and the relevant studies discussing them

Disease name	Species of sponge affected	References
Mangrove sponge disease (MSD)	*Geodia papyracea*	Rüetzler (1988)
Sponge boring necrosis (SBN)	*Rhopaloeides odorabile*, *Hippospongia communis*, *Ircinia variabilis*, *Sarcotragus spinosula*, and *Spongia officiinalis*	Vacelet et al. (1994); Webster et al. (2002)
Aplysina red band syndrome (ARBS)	*A. cauliformis*	Olson et al. (2006)
Sponge orange band (SOB)	*Xestospongia muta*	Cowart (2006)
Brown lesion necrosis (BLN) or brown spot syndrome (BSS)	*Ianthella basta*	Cervino et al. (2006) and Luter et al. (2012)
Sponge white patch (SWP)	*Amphimedon compressa*	Angermeier et al. (2012)

biru until molecular analysis can be conducted to assert its true taxonomy.

Disease prevalence

Visual characteristics of the disease were determined, and surveys were conducted to assess the prevalence of the disease around Lhaviyani Atoll. Five 30-m randomly chosen transects were swum per site at a depth of between 10 and 15 m, where *C.* aff *biru* was prevalent. We surveyed two different reef sites at Vavvaru and Kamandoo Island. A total of ten transect surveys were therefore conducted. The total number of *C.* aff *biru* sponges was recorded together with the number of affected individuals within each transect. Since this species has a meandering, rope-like growth form, individual sponges were characterised as having separate attachments to the substrate as reported for *Aplysina cauliformis* by Olson et al. (2006). Diseased sponges were tagged and monitored in the field using scaled photographs to assess progression of the disease lesion.

Sample collection

Samples (10–15 cm) were collected from healthy, apparently healthy, and diseased *Callyspongia* (*Euplacella*) aff. *biru.* 'Apparently healthy' in this instance refers to the area of tissue directly adjacent to the disease lesion, where no visual signs of the disease itself are observed. Samples were removed underwater using scissors and placed into individual Ziploc bags. Samples were kept at ambient temperature and processed immediately upon return to the laboratory. Two individual sets of samples were collected. One set for microbial analysis was stored in 100 % molecular grade ethanol and kept at −20 °C until extraction and further analysis. The other set was used for immuno-histology and preserved in 5 % paraformaldehyde for 24 h, then in 100 % ethanol, and stored in the fridge (Sweet et al. 2014). After analysis of the samples at the molecular laboratories at the University of Derby, UK, a return field trip was conducted in April/May 2014 (from 7 April to 5 May), where three fungal isolates and two bacterial isolates were cultured and inoculation trials conducted (see below for details of inoculation trials). Sponges utilised in the inoculation experiment (see below) were sampled in the same manner for microbial and histological analysis. These sponges, which were experimented on in aquaria, were compared (based on microbial community profiles and immuno-histology) to those collected from the reef to assess for any tank effects. See below for specific methodologies used for the microbial and histology analyses and the inoculation trials.

It must be said that, at the time of sampling, no seawater controls were taken. As many of the bacteria associated with sponges at any given time are likely associated with the surrounding water column (discussed in the "Introduction"), such controls are important in studies such as this one. In retrospect, this was an oversight, and therefore, interpretation of the results should be taken into account with this in mind. Future studies attempting to replicate this study or describe new diseases in other sponge species should therefore take note.

Microbial analysis

Small segments of the sponges were removed using sterile techniques, placed into a microcentrifuge tube with 50 µL of filtered sterile artificial seawater, and gently macerated. DNA was subsequently extracted from all samples using QIAGEN DNeasy Blood and Tissue kits following the protocol described in Sweet and Bythell (2012).

ITS fungal diversity

For fungi, primers ITS3F (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4F-GC (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGC-CCCCGCCCCTCCTCCGCTTATTGATATGC-3′) were utilised following the protocols described in Huang et al. (2006). Thirty-µL PCR mixtures containing 1.5 mM $MgCl_2$, 0.2 mM dNTP (PROMEGA), 0.5 µM of each primer, 2.5 µL of Taq DNA polymerase (QBiogene), incubation buffer, and 20 ng of template DNA (Sweet et al. 2011) were carried out on a Hybaid PCR Express thermo cycler. PCR products were verified by agarose gel electrophoresis [1.6 % (w/v) agarose] with ethidium bromide staining and visualised using a UV transilluminator.

To screen all samples, DGGE was performed using the D-Code universal mutation detection system (Bio-Rad). PCR products were resolved on a 10 % (w/v) polyacrylamide gel that contained a 30–60 % denaturant gradient for 13 h at 60 °C with a constant voltage of 50 V. Gels were stained as per Sweet et al. (2010) and visualised using a UV transilluminator. Bands of interest (those which explained the greatest differences/similarities between samples) were excised from DGGE gels, left overnight in Sigma molecular grade water, vacuum-centrifuged, re-amplified with the same original primers, labelled using Big Dye transformation sequence kit (Applied Biosystems), and sequenced. Operational taxonomic units (OTUs) were defined from DGGE band-matching analysis using Bionumerics 3.5 (Applied Maths BVBA). Standard internal marker lanes were used to allow for gel-to-gel comparisons. Tolerance and optimisation for band matching was set at 1 %.

16S rRNA gene bacterial diversity

Bacterial 16S rRNA genes were amplified using standard prokaryotic primers: (357F; 5′-CAGCACGGGGGGCC-TACGGGAGGCAGCAG-3′) and (518R; 5′-ATTACCGC GGCTGCTGG-3′). Thirty PCR cycles were performed at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min (Sanchez et al. 2007). Two types of non-culture molecular techniques were utilised: DGGE and 454 analyses. Although the primers were standardised, a GC-rich sequence was attached to the 5′ end of the forward primer (357F) for use with DGGE (Sweet et al. 2011). For 454 sequencing, PCR protocols were the same as above with the only variation being the substitution of HotStarTaq polymerase (Qiagen, Valencia, CA), which offers greater specificity. PCR products for 454 were cleaned using AMPure magnetic beads (Beckman Coulter Genomics, Danvers, MA). Amplicon samples were quantified using the Qubit flourometer (Invitrogen, Carlsbad, CA, USA) and pooled to an equimolar concentration. Sequences were run on 1/8th of a 454 FLX Titanium picotiter plate at Newgene in the Centre for Life, Newcastle, UK.

Pyrosequences were processed using a QIIME pipeline (version 1.5.0; Caporaso et al. 2010). Sequences were filtered based on the following criteria: (1) sequences of <50 nucleotides, (2) sequences containing ambiguous bases (Ns), and (3) sequences containing primer mismatches. Any fitting into these criteria were discarded. Analysis using 2 % single-linkage pre-clustering (SLP) and average-linkage clustering based on pairwise alignments (PW-AL) was performed to remove sequencing-based errors. The remaining sequences were de-noised within QIIME. The resulting reads were checked for chimeras and clustered into 98 % similarity OTU using the USEARCH algorithm in QIIME. All singletons (reads found only once in the whole data set) were excluded from further analyses. After blast searches on GenBank, we retained the best BLAST outputs, i.e. the most complete identifications, and compiled an OTU table, including all identified OTUs and respective read abundances.

Histology

Samples were collected as for the microbial analysis. However, tissue samples were preserved with 5 % paraformaldehyde for 24 h then stored in 100 % EtOH until resin embedding in LR white (r). For each tissue type, the location of microbes was recorded using 0.01 % acridine orange (Schippers et al. 2005). The stain nigrosin (Bjorndahl et al. 2003) was used for evaluating the extent of mass tissue necrosis; necrotic cells appear black/brown in colouration. All sections were viewed under epifluorescence microscopy with an FITC-specific filter block (Nikon UK Ltd, Surrey, UK), and images recorded using an integrating camera (Model JVC KY-SSSB).

Samples for scanning electron microscopy were dehydrated using 25, 50, and 75 % ethanol (30 min each), then a further 2 × 1 h in 100 % ethanol, with final dehydration using carbon dioxide in a Baltec Critical Point Dryer. Specimens were then mounted on an aluminium stub with Achesons Silver Dag (dried overnight) and coated with gold (standard 15 nm) using a Polaron SEM Coating Unit. Specimens were examined using a Stereoscan 240 scanning electron microscope, and digital images collected by Orion 6.60.6 software.

Samples for transmission electron microscopy were dehydrated using 25, 50, and 75 % acetone, (30 min each) and 100 % acetone (2 × 1 h). They were then impregnated with 25 % LR White resin in acetone, 50 % resin/acetone, 75 % resin/acetone (1 h each), then 100 % resin for a minimum of three changes over 24 h, with final embedding in 100 % resin at 60 °C for 24 h. Survey sections of 1 µm were cut and stained with 1 % toluidine blue in 1 % borax. Ultrathin sections (approx 80 nm) were then cut using a diamond knife on a RMC MT-XL ultramicrotome. These were then stretched with chloroform to eliminate compression and mounted on Pioloform filmed copper grids. Staining was with 2 % aqueous Uranyl Acetate and Lead Citrate (Leica). The grids were then examined using a Philips CM 100 Compustage (FEI) transmission electron microscope, and digital images are collected using an AMT CCD camera (Deben) at the Electron Microscopy Research Services Laboratory, Newcastle University.

Inoculation trials

Due to culturing limitations at the field site, freshly collected diseased tissues were snap-frozen on the first trip to the Maldives and transported on ice to the UK. Bacteria were cultured using Marine Agar 2216 (Difco Laboratories), and fungi were cultured using malt extract agar. Single isolates were separated and cultured further. Representative subsets of these microbes were extracted using the QIAGEN DNeasy Blood and Tissue kits following the protocol described in Sweet and Bythell (2012) and run on the DGGE (as above). Those showing different banding patterns were subsequently sequenced using the same primers as the initial molecular screening above. These were subsequently matched to the sequences retrieved using both the DGGE and 454 analyses. Only, three fungal ribotypes (*Pleosporales* sp., *Rhabdocline* sp., and *Clasosporium* sp.) and one bacterial ribotype (*Rhodobacteraceae* sp.) matched those we were originally targeting for the inoculation trials. In an attempt to isolate the cyanobacteria associated with the diseased sponge, metabolically active trichomes were isolated from diseased sponge samples on site. Tissue was chopped with a

razor blade, and the trichomes were squeezed into a seawater-based medium containing polyvinylpyrrolidone, bovine serum albumin, dithiothreitol, glycerol, KCl, and Na_2CO_3 as described in Hinde et al. (1994). The isolated cyanobacteria were then concentrated by centrifugation and washed several times in fresh medium. Only one cyanobacterium was able to be cultivated (based on morphological identification). However, to confirm this, individual isolates were stored in ethanol and transported back to the UK for sequencing.

During the second field trip (7 April to 5 May), five apparently healthy sponge clones were experimentally infected with each of the five different bacterial or fungal isolates. Further sets of five apparently healthy sponges were infected with different combinations of these potential pathogenic bacteria and fungi with the aim of determining their aetiological role in the disease process. For the inoculation experiment, individual healthy sponges (15 cm^3) were maintained in purpose-built, flow-through aquaria. A single sponge in each tank was used for the pathogenicity trial to avoid possible confounding genetic variability or inter-sponge fluctuations in health status. The individual specimens were threaded onto flat plates of bleached coral skeleton following Webster et al. (2002) and secured in place using cable ties. These sponge specimens were allowed to heal for 2 weeks prior to the infection experiment. Sponge clones were placed in 80-L glass aquaria for each experimental dose. The majority of the putative pathogenic fungi and bacteria were infected in Marine Broth 2216 (Difco) at low, medium, and high concentrations corresponding to 10^2, 10^4, and 10^6 colony-forming units (CFU) mL^{-1}, as determined from direct plate counts and comparison of culture turbidity with McFarland standards. The cyanobacteria were infected in the same seawater-based medium as they were cultured in. Controls were established for all treatments. During the infection process, the water flow was stopped, the appropriate inoculum of cells added to each treatment, and the aquaria individually aerated for 5 h to allow the sponges to filter the 80 L volume of seawater. During this time, there was no detectable change in temperature of the aquarium water according to Hobo Data Loggers (29–30 °C). After a 5-h exposure, the water flow was restarted. The trial was conducted over 14 d, and throughout the experimental process, visual observations of the extent of external necrosis and epifouling were recorded and matched to that occurring in the wild types of the disease. Control samples ($n = 5$ sponges which had no inoculation, yet were treated in the same way regarding the water flow changes) were run alongside those being inoculated to assess signs of stress during the trials. All water utilised during the experiment was filtered through sand at the centre of the island to prevent any of the inoculated microbes from returning back to the sea.

At the end of the inoculation trials, the cyanobacteria were isolated in the same way as above and samples ($n = 4$) of the tank diseased sponges were collected, snap-frozen, and transported back to the UK. Bacteria and fungi were cultured using the same media as for initial isolation. A DGGE was run to compare the newly isolated fungi and bacteria against those originally isolated and used for the inoculation trials. The bands matching the target isolates ($n = 10$ per target bacterium and fungi) were sequenced and matched to the original sequences of the inoculated isolates, fulfilling Koch's postulates.

Statistical analysis

An analysis of similarity (ANOSIM) was conducted to test differences and similarities in 16S rRNA gene bacterial assemblages between and within the different sample types. The same was conducted for ITS rRNA gene fungal assemblages. In addition, hierarchical cluster analysis and non-metric multi-dimensional scaling were performed on the bacterial assemblages, based on Manhattan distance measures, in order to visualise patterns in microbial communities and establish potential clusters in assemblage composition. These latter two methods were performed using the R statistical programming language (R Core Team 2014) using the vegan package (Oksanen 2013).

Results

Prevalence

On shallow water reefs in Lhaviyani Atoll in the Maldives, *Callyspongia* (*Euplacella*) aff. *biru* are among the most dominant sponges. To date, it appears only that this species is affected by this new disease. The prevalence of the disease at the two sites surveyed ranged from 36 % at Vavvaru Island to 30 % at Kamandoo Island.

Pathology

The disease was recorded in the field progressing at 0.34 ± 0.08 cm d^{-1}. The lesion is characterised by a sharp demarcation between apparently healthy tissue and the apparently necrotic edge of the disease lesion (Fig. 1). Scanning electron micrographs of the disease showed that at the lesion, the ectosome is completely disorganised, showing wide openings providing potential access for opportunistic organisms directly to the choanosome (Fig. 2). However, no micro-organisms were seen associated with the surface of either the healthy tissues, apparently healthy tissues in advance of the disease lesion, and the disease lesion itself (Fig. 2).

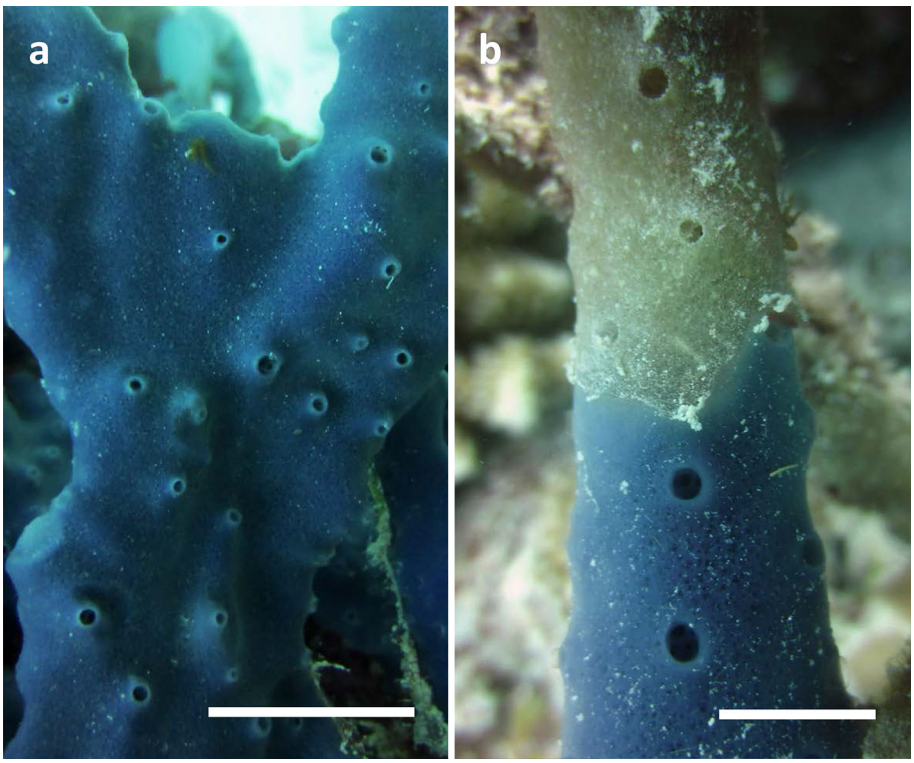


Fig. 1 Field signs of **a** a healthy specimen of the sponge and **b** the novel disease affecting the species. *Scale bars* 1 cm

Histology

All tissues showed the presence of symbiotic micro-organisms inside the spongin fibres (Fig. 3). This was evident in the sections stained with acridine orange (arrows in Fig. 3e–h). In some of the diseased sections, the presence of cyanobacteria (Fig. 3c) was detected. These specific types were not associated with healthy and/or apparently healthy tissues (Fig. 3). Diseased sections showed significantly damaged fibres (Fig. 3k); there were no signs of necrotic tissues in healthy samples (Fig. 3i) or within the apparently healthy tissues (Fig. 3j). The transmission electron micrographs supported the findings of the general histological sections, with signs of symbiotic bacteria and fungi associated with all tissues (Fig. 4). However, in the diseased tissues alone, there was a dominance of fungal hyphae (Fig. 4c, g, k). Furthermore, specific bacteria were also seen to burrow inside the spongin in diseased tissues (Fig. 4c, g, k). However, this was not observed in all replicate samples. A selection of the bacteria associated with the sponge tissues can be seen in Electronic Supplementary Materials, ESM, Fig. 1.

16S rRNA gene bacterial diversity

There was a significant difference between the 16S rRNA gene bacterial diversity associated with all tissue types (healthy, apparently healthy, and the diseased lesion). This trend was observed in both the DGGE profiles and the 454 analysis (ANOSIM, $R = 0.97$, $p = 0.001$; ANOSIM, $R = 0.86$, $p = 0.001$, respectively). Certain ribotypes were consistently detected in all samples. These included a *Thermotogales* sp. (GenBank Accession No: HQ416848), an uncultured gamma-proteobacterium (JF824778), *Pediococcus parvulus* (KC632077), a *Actinobacterium* sp. (KC702667), a *Propionibacterium* sp. (JQ516688), a *Chloroflexaceae* sp. (EU979454), an *Arcobacter* sp. (KC918190), *Shewanella benthica* (U91592), and an *Ascomycota* sp. (HF947897). Others were only associated with one type of tissue sample. However, there was often variation in the specific ribotypes found within replicate samples of the same tissue type. Only two bacterial ribotypes were found consistently in all replicates of apparently healthy and diseased tissues whilst being absent in healthy tissues. These included a ribotype similar to a *Rhodobacteraceae* sp. (FJ403063) and the cyanobacterium, *Hormoscilla spongeliae* (AY648943).

Interestingly, one individual sample (DL_3; Fig. 5) showed a much greater diversity of ribotypes than all other samples. It may have been the case that this particular sample represented a more advanced stage of the disease than the other three replicates. There was an overall reduction in total diversity in apparently healthy tissues compared to those associated with the healthy sponge (Fig. 5). However, this diversity increased markedly in all instances within the disease lesion. In particular, diseased

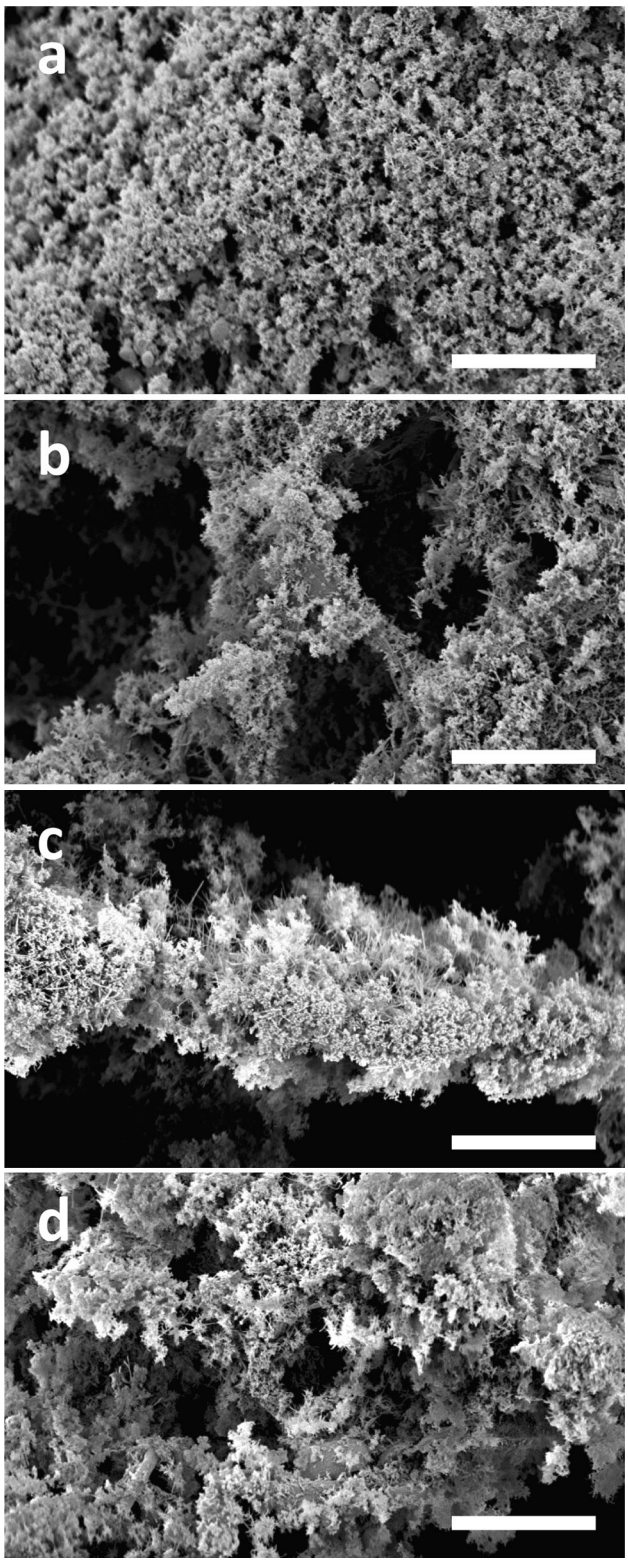


Fig. 2 Scanning electron micrographs of **a** healthy sponge, **b** apparently healthy tissue adjacent to the disease lesion, **c** the disease lesion itself, and **d** the inoculated sponge. *Scale bars* 25 μm

tissues showed an increase in ribotypes from the phyla Actinobacterium, Bacteroidetes, Firmicutes, and Verrucomicrobia (Fig. 5).

ITS rRNA gene fungal diversity

Only five ribotypes of fungi were detected in healthy sponge samples (Table 2); these included ribotypes related to *Helotiales* sp. (KC180696), *Thecaphore* sp. (EF200037), *Malassezia* sp. (AJ249956), *Glomus* sp. (JF439128), and a *Basidiomycota* sp. (JQ272345). Only the latter three were consistently present in all replicates and may likely be symbionts of *C.* aff *biru*. There was a significant difference (ANOSIM, $R = 0.98$, $p = 0.001$) between the fungal diversity associated with healthy, apparently healthy tissues, and those present in the diseased lesion. All the fungi present in healthy tissues remained present in the apparently healthy tissues and the diseased lesion (Table 2). However, an additional 15 ribotypes were detected in both apparently healthy tissues in advance of the lesion and the diseased tissues themselves (Table 2), yet were absent in healthy samples. In addition to those associated with healthy tissues, the fungi detected included four ribotypes of *Ascomycota* sp. (EU917100, AB725393, FJ440863, & FJ197199), another ribotype related to a *Malassezia* sp. (KC525789), a *Pleosporales* sp. (GU909793), a *Rhabdocline* sp. (U92292), a *Clasosporium* sp. (KC790536), a *Graphiopsis* sp. (EU009458), and a *Dothidiomycetes* sp. (AB746924). Four of these ribotypes increased in abundance in the disease lesion, indicative of a pathogenic organism. These included one of the *Ascomycota* sp. (a ribotype related to EU917100), the *Pleosporales* sp., the *Rhabdocline* sp., and the *Clasosporium* sp.

Inoculation trial

Healthy sponges were inoculated with single isolates of three fungal isolates (*Pleosporales* sp., *Rhabdocline* sp., and *Clasosporium* sp.) and two bacterial isolates (a cyanobacterium *Hormoscilla* sp. and a *Rhodobacteraceae* sp.). Separately, none of the isolates caused disease lesions. However, when a combination of both the fungus, *Rhabdocline* (Unique GenBank Accession No. KP001552), and the bacterium, *Rhodobacteraceae* (Unique GenBank Accession No. KP001553), was used as one inoculum, the resulting disease occurred in all replicate samples ($n = 5$ per dose rate; Fig. 6a). Rate of lesion progression ranged from 0.83 to 2.25 cm d^{-1} (Fig. 6a), with variation occurring between dose rates, replicates, and time periods. All three dose rates (10^2, 10^4, and 10^6 CFU mL^{-1}) resulted in

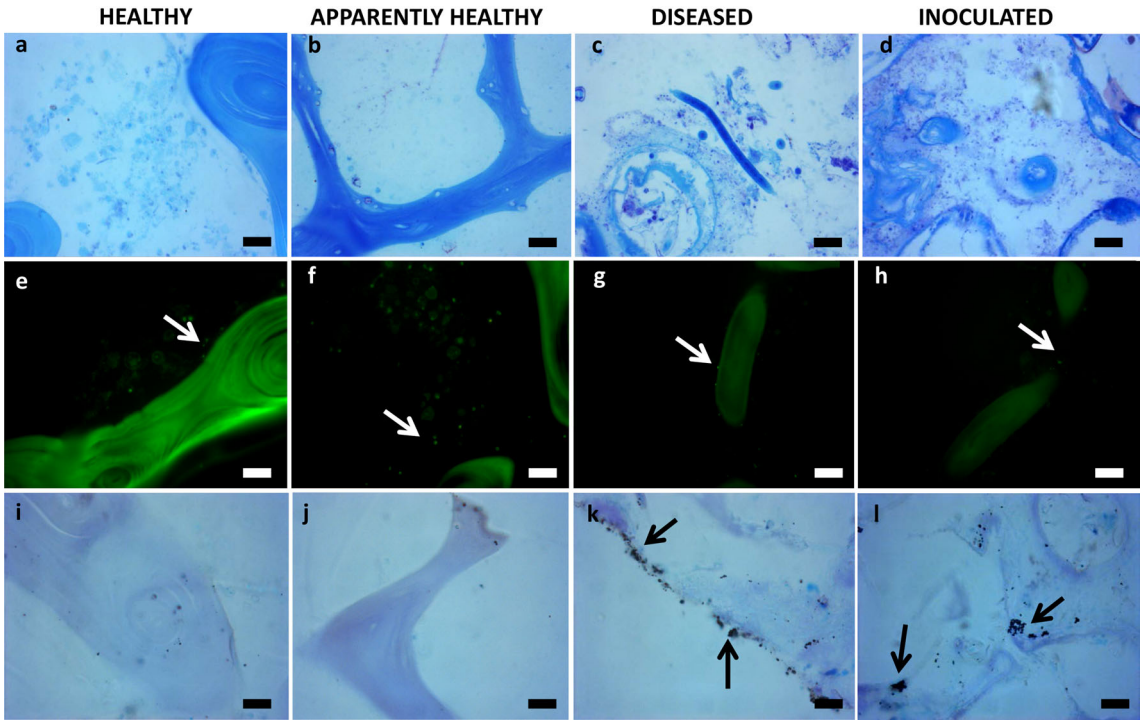


Fig. 3 Light micrographs of healthy, apparently healthy tissues, diseased, and inoculated sponge. **a**–**d** stained with toluidine blue, **e**–**h** stained with acridine orange, and **i**–**l** stained with nigrosin. *Scale bars* 10 µm. *Arrows* indicate the presence of bacteria within the tissues

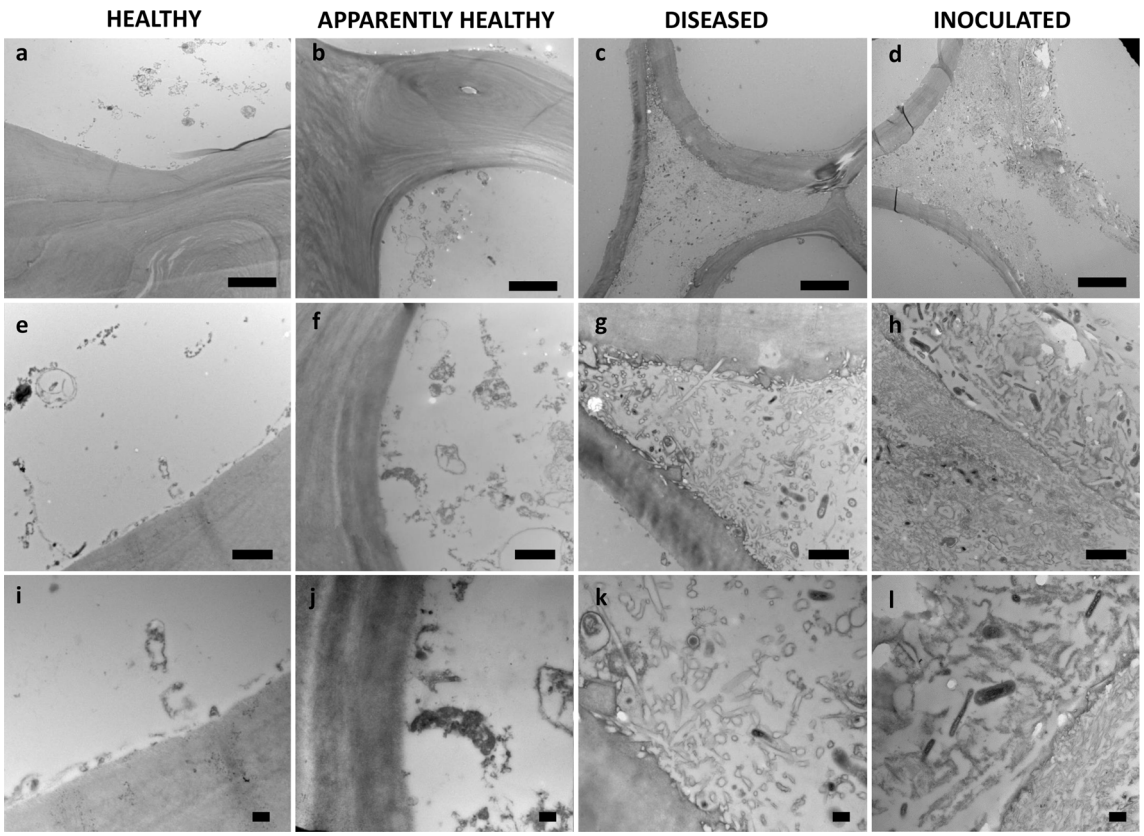


Fig. 4 Transmission electron micrographs of healthy, apparently healthy, and diseased tissues. **a**–**d** *scale bars* 10 µm, **e**–**h** *scale bars* 2 µm, and **i**–**l** *scale bars* 500 nm

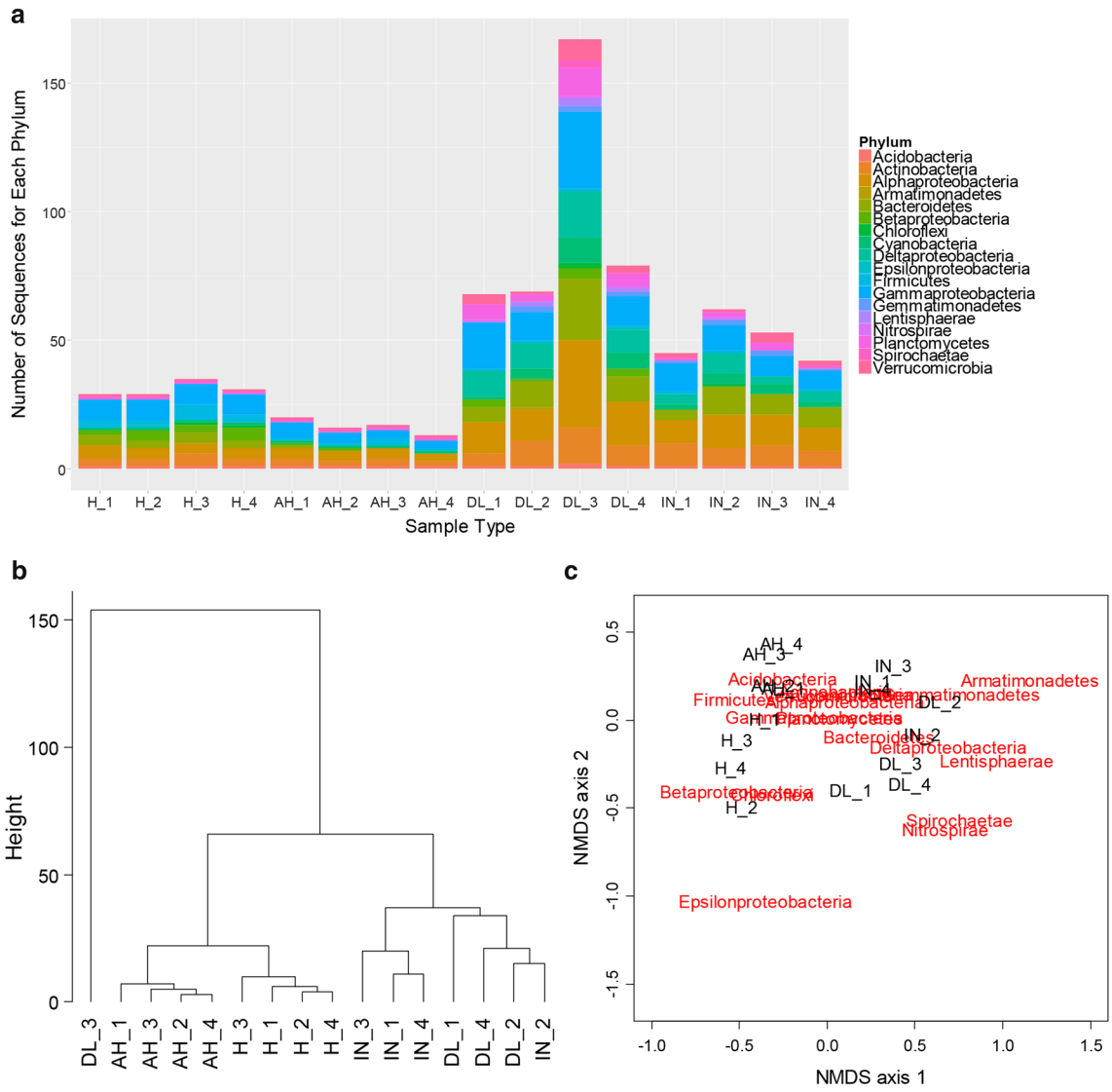


Fig. 5 **a** Stacked bar chart of the abundances of bacterial phyla associated with the various samples. **b** A cluster dendrogram resulting from the hierarchical clustering based on similarities of bacterial assemblages. **c** A non-metric multi-dimensional scaling plot based on similarities of bacterial assemblages. Stress, representing the goodness of fit of the data in the multi-dimensional ordination, was 0.084. *H* healthy, *AH* apparently healthy, *DL* diseased lesion, and *IN* inoculated. The associated *numbers* indicate the replicate number

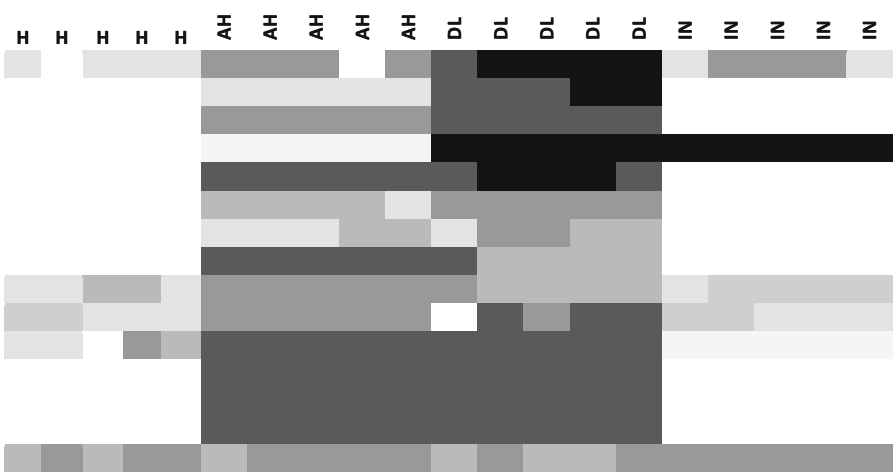
similar overall pathology with the same percentage of diseased tissue after the completion of the experiment (Fig. 6b). However, sponge inoculated with the lower dose rate (10^2 CFU mL^{-1}) took longer time to exhibit disease signs (132 h), compared to those inoculated with both 10^4 and 10^6 CFU mL^{-1}, which both showed disease lesions within 12 h of inoculation. The resulting pathology seen in all inoculated sponges resembled that seen in the wild type of the disease (Fig. 6c). Furthermore, the histopathology of the inoculated sponges that showed signs of the disease (Figs. 3, 4) was similar in appearance to that of the wild type. 16S rRNA gene diversity in inoculated samples was significantly different from all other samples collected from the wild (ANOSIM, $R = 0.54$, $p = 0.001$). However, overall diversity was similar to that seen in the diseased samples previously collected directly from the reef (Fig. 5). Only four of the samples that were inoculated were processed for 454 analysis due to cost. Both the fungi *Rhabdocline* (KP001552) and the bacterium *Rhodobacteraceae* (KP001553) were re-isolated from all replicate samples of experimentally diseased sponges, fulfilling the final steps of Koch's postulates.

Discussion

This is the first molecular and histological description of a highly prevalent yet novel sponge disease, occurring in the Indian Ocean, named here as sponge necrosis syndrome. We found that the disease occurred in approximately

Table 2 Heatmap created from DGGE analysis showing ITS rRNA gene fungal diversity associated with healthy (H), apparently healthy (AH), diseased sponges (DL), and those which were inoculated (IN)

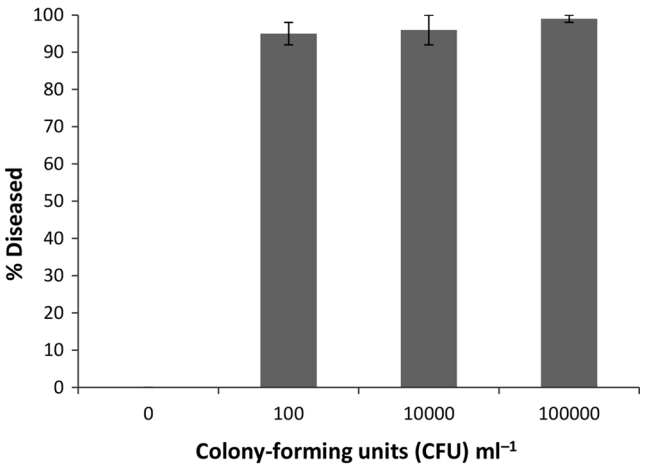
GenBank Acc. No.	Closest relative	Best Match
KC180696	*Helotiales sp*	100%
EU917100	*Ascomycota sp*	95%
GU909793	*Pleosporales sp*	100%
U92292	*Rhabdocline sp*	100%
KC790536	*Cladosporium sp*	100%
FJ440863	*Ascomycota sp*	100%
KC525789	*Malassezia sp*	100%
FJ197199	*Ascomycota sp*	97%
AJ249956	*Malassezia sp*	99%
JF439128	*Glomus sp*	100%
EF200037	*Thecaphora sp*	100%
EU009458	*Graphiopsis sp*	99%
AB746924	*Dothidiomycetes sp*	100%
AB725393	*Ascomycota sp*	99%
JQ272345	*Basidiomycota sp*	99%



a

Time	ND	Colony-forming units (CFU) ml^{-1}		
		10^2	10^4	10^6
0	0	0	0	0
12	0	0	1.8 ± 0.09	2.1 ± 0.15
24	0	0	1.2 ± 0.08	1.3 ± 0.19
36	0	0	2.1 ± 0.17	1.1 ± 0.11
48	0	0	1.8 ± 0.10	1.6 ± 0.09
60	0	0	1.9 ± 0.12	2.1 ± 0.15
72	0	0	1.2 ± 0.22	1.5 ± 0.15 **
84	0	0	*****	***
96	0	0		
108	0	0		
120	0	0		
132	0	1 ± 0.07		
144	0	1.0 ± 0.02		
156	0	1.4 ± 0.91		
168	0	1 ± 0.17		
180	0	1.2 ± 0.12		
192	0	*****		

b



c

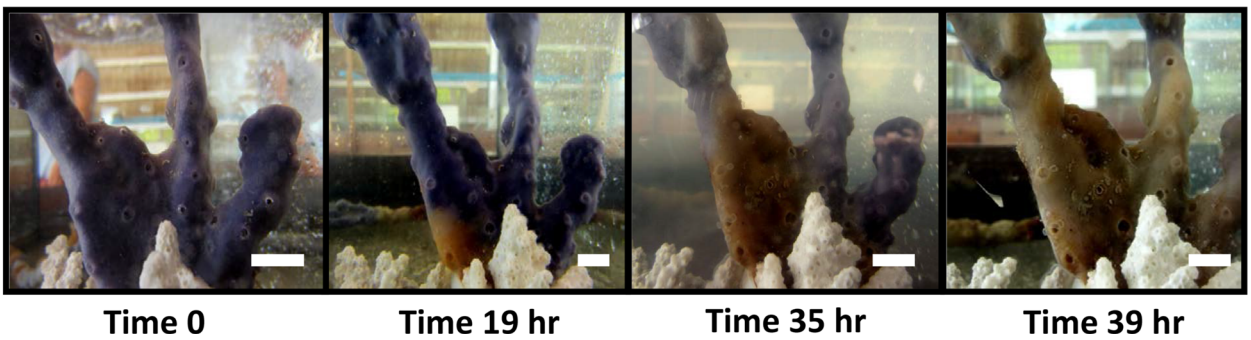


Fig. 6 Inoculation experiments. **a** Mean lesion progression rate (cm d^{-1}) with SE for the controls and the consortium inoculation with the fungus from the genus *Rhabdocline* and the bacterium *Rhodobacteraceae* sp. **b** Variations in percentage of sponge tissue showing the necrotic lesion with the varying concentrations of the microbial inoculums. **c** Visual panel illustrating tissue loss during the inoculation period. *Scale bar* 1 cm

30–33 % of all sponges surveyed (30-m transects). This phenomenon seems to be common in the area, being recorded on the same species also in July and August 2011 and 2012 around the island of Madhiriguraidhoo, which is in the same atoll as the present work (Turicchia E. pers. comm.). On the basis of morphological characters, the closest description is that available for *Callyspongia* (*Euplacella*) *biru* known from the Indonesian area (Voogd 2004), but the large distance from that area, along with some physical differences in the skeletal elements, keeps the species classification still open. A definitive taxonomic identification of this species should, however, be achieved after molecular analyses. The external pathology of the disease appears as a dark brown, apparently necrotic lesion on various locations of the sponge. At an advanced stage, the sponge fragments and the parts of the colony not attached to the substrate are observed to die rapidly. The disease can be spread from diseased individuals to healthy sponges by simple contact, and the disease shows pathological signs within 24 h from the first touch. Although the rate of the spread of the lesion within the inoculation experiments varied significantly from that recorded in the field (1.7 cm d^{-1} compared to 0.3 cm d^{-1}, respectively), we believe that the same pathology was observed in both instances. The increased advance rate observed in the tanks during inoculation trials may have occurred either as a result of additional stress experienced by the sponges due to being held within aquaria, or alternatively due to higher than natural concentrations of the pathogens being inoculated in the tank experiment. Furthermore, the lack of other organisms, such as fish within the aquaria that could potentially be acting as biological controls for the disease in the natural ecosystem, may have influenced the rate of disease progression in these controlled environments.

To date, only a few microbiological studies have been conducted on diseased sponges and only three have been assigned likely pathological agents, all three of which were highlighted as being bacterial (Webster 2007). The novel disease described here bares a similar pathology to one of these three sponge diseases, originally described as affecting the sponge *Rhopaloeides odorabile* off the east coast of Australia (Webster et al. 2002). SBN is characterised by soft and fragile lesions with large portions of pinacoderm eroded away revealing the collagenous skeletal fibres (Webster et al. 2002). Previously, SBN had been the only sponge disease which had had Henle–Koch's postulates fulfilled for it. It was experimentally induced by a single bacterial morphotype (strain NW4327), which after inoculation was seen to burrow through the collagenous spongin fibres causing severe necrosis (Webster et al. 2002). Later, Angermeier et al. (2012) found a similar alphaproteobacterium associated with a different disease, SWP, in the Caribbean. However, in this latter study, they could not confirm whether the sponge boring bacterium were true pathogens or merely opportunistic colonisers. In this study, we found a similar bacterium (99 % similar to the strain NW4327 identified by Webster et al. (2002), now identified as a *Rhodobacteraceae* bacterium; Unique GenBank Accession No. KP001553). However, we did not observe it burrowing into the spongin in all cases of the disease.

One further bacterium, which was previously associated with another sponge disease (MSD), was the cyanobacterium *H. spongeliae* (Rüetzler 1988). Although both bacteria were detected in the disease lesion of this novel disease, both failed to cause the onset of the disease during inoculation experiments. Therefore, we concluded that the bacteria were not the sole primary pathological agents associated with this new disease. Interestingly, with regard to *H. spongelia*, Vacelet et al. (1994) suggested that this bacterium and other members of the genus are more likely to be associated with the outside of necrotic sponge lesions and are therefore likely to represent secondary phenomena rather than primary pathological agents. In further support of the role of cyanobacteria in certain sponge diseases, a similar, but as yet undescribed, cyanobacterium has been associated with another sponge disease, *Aplysina* red band syndrome (ARBS) (Olson et al. 2006). Olson et al. (2006) suggested that this associated cyanobacterium could either be the aetiological agent, or alternatively, be a secondary, opportunistic pathogen taking advantage of the already health-compromised sponges. Our results support the latter theory, as the cyanobacterium was only present on diseased individuals, yet failed to induce lesions during the inoculation experiment. This strongly suggests that the cyanobacterium colonises already dead or dying tissues, rather than initially infecting healthy sponges.

In this study, both bacteria targeted were able to be cultured using standard techniques, whilst only three of the candidate fungal pathogens could be cultured. These included the *Pleosporales* sp., the *Rhabdocline* sp., and the *Clasosporium* sp. As with the two bacterial species, neither of the fungal ribotypes caused disease signs during the inoculation experiment, despite the histology showing a significant build-up of fungal hyphae associated with the lesion interface in the wild-type samples. However, during the mixed inoculation trials, a microbial consortium consisting of the *Rhabdocline* fungus (Unique GenBank Accession No. KP001552) and the bacterium *Rhodobacteraceae* (Unique GenBank Accession No. KP001553) elicited the same pathology as that found in the wild. This further supported preliminary trials in which contact caused the spread of the disease between diseased and healthy individuals, suggesting a microbial agent was associated with the disease lesions. Furthermore, ribotypes isolated from these experimentally disease-induced sponges matched (100 %) the sequences of the inoculated microbes. Therefore, this makes this study only the second in which Koch's postulates have been fulfilled for a sponge disease.

In general, we observed clear changes in the composition and structure of communities in both bacteria and fungi in the transition from healthy to diseased. For bacteria specifically, there was an initial drop in diversity in the apparently healthy stage, followed by marked increases in diversity in the disease stage, also mirrored in the inoculated sponges. Although an interesting result, further work is needed in order to elucidate the form of interactions between specific micro-organisms and their roles in infecting disease progression. For example, if we look at bacteria which are reduced during the transition (instead of those increasing, as is the case for potential pathogens), one bacterium from the genus *Pseudovibrio* stands out. This ribotype was dominant in healthy tissues, found to be reduced in abundance in apparently healthy tissues, and was absent in the disease lesion of all samples. This genus of bacteria has previously been shown to play an important role in the host defences of many other sponge species (Enticknap et al. 2006; Webster et al. 2008). Specifically, the genus has been shown to harbour strong antimicrobial capabilities (Penesyan et al. 2011). This result may highlight the importance of healthy symbiotic micro-organisms in sponges, potentially being even more important than the presence of specific pathogenic bacteria or fungi eliciting diseases.

Finally, the disease recorded in this study follows similar patterns to other sponge disease outbreaks, whereby the infection is constrained to a particular sponge species (often within the same higher taxon). This is indicative of neighbouring species being able to protect themselves from infection in some way (Wulff 2006). Sponges have been shown in previous studies to have the ability to isolate damaged tissue from the healthy sponge biomass by constructing tissue barriers (Smith 1941; Rüetzler 1988). Tissue reorganisation excludes the damaged areas and allows the water currents to flow to healthy regions of the sponge tissue. Isolation of necrotic tissues could be easier in sponges with a siliceous reticulate skeleton than a skeleton built by a spongin fibre network. Sections of reticulate siliceous skeleton may act as walls containing the spread of the disease more effectively than a simple network of spongin fibres. However, we do not know whether the sponges studied here are capable of this protective measure, although those inoculated in the tanks were observed to die quickly after succumbing to the disease and showed no signs of being able to isolate the damaged tissue. This result also highlights another very important issue with many marine disease studies in which the majority are conducted in aquarium systems. The results we witnessed within the controlled setting are likely to be different to those in the wild. The rate of tissue loss was considerably quicker in the tank system, suggesting that the stress of the collection, containment, and/or inoculation processes had an additional effect on the sponge, although the control sponges (those not inoculated with any potential pathological agent) did survive for the duration of the experiment.

In conclusion, this study is only the second to fulfil Henle–Koch's postulates for a sponge disease. Furthermore, it illustrates that sponge diseases, similar to those of corals (Miller and Richardson 2014), may be more likely caused by a consortium of micro-organisms rather than a single pathogenic agent. Here we show that a bacterium and a fungal pathogen work in conjunction to illicit a highly prevalent, yet species-specific sponge disease, highlighting the urgent need for further work on sponge diseases in the future.

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