Vol. 116: 47–58, 2015
doi: 10.3354/dao02911

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 17

Changes in the bacterial community associated with black band disease in a Red Sea coral, *Favia* sp., in relation to disease phases

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ABSTRACT: Changes of the black band disease (BBD)-associated microbial consortium on the surface of a *Favia* sp. coral colony were assessed in relation to the different disease phases. A number of highly active bacterial groups changed in numbers as the BBD disease signs changed. These included *Gamma*- and *Epsilonproteobacteria*, *Bacteroidetes* and *Firmicutes* groups. One cyanobacterium strain, BGP10_4S^T (FJ210722), was constantly present in the disease interface and adjacent tissues of the affected corals, regardless of disease phase. The dynamics of the operational taxonomic units (OTUs) of this BBD-specific strain provide a marker regarding the disease phase. The disease's active phase is characterized by a wide dark band progressing along the tissue-skeleton interface and by numerous bacterial OTUs. Cyanobacterial OTUs decreased in numbers as the disease signs waned, perhaps opening a niche for additional microorganisms. Even when black band signs disappeared there was a consistent though low abundance of the BBD-specific cyanobacteria (BGP10_4S^T), and the microbial community of the disease-skeleton interface remained surprisingly similar to the original band community. These results provide an indication that the persistence of even low numbers of this BBD-specific cyanobacterium in coral tissues during the non-active (or subclinical) state could facilitate reinitiation of BBD signs during the following summer. This may indicate that this bacterium is major constituent of the disease and that its persistence and ability to infiltrate the coral tissues may act to facilitate the assembly of the other BBD-specific groups of bacteria.

KEY WORDS: Coral · Black band disease · Cyanobacteria · Microbiota · Red Sea

INTRODUCTION

A global decline in world coral populations has been observed over the last 4 decades, and has been attributed to a general deterioration of the marine environment (Aronson et al. 1998, Loya 2004, Pandolfi & Jackson 2006). Recent studies cite increasing coral tissue losses as a result of diseases as one of the major causes of coral mortality (Harvell et al. 1999, Nugues 2002, Rosenberg & Loya 2004). While coral diseases currently include 29 syndromes in the Caribbean (Weil 2004) and 7 syndromes in the Indo-Pacific region (Willis et al. 2004), only few coral pathogens have been identified, and only for a limited number of diseases (Krediet et al. 2013).

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Coral black band disease (BBD) was the first coral disease to be documented, affecting a number of corals on the reefs worldwide (Antonius 1973, Garrett & Ducklow 1975). Four decades later, BBD is considered a well described but, as yet, unresolved disease plaguing stony corals (Antonius 1985, Al-Moghrabi 2001, Dinsdale 2002, Willis et al. 2004). To date, a primary pathogen has not been characterized for this disease, and it is thought to be caused by a complex and diverse microbial consortium with regional specificity (Carlton & Richardson 1995, Richardson 2004, Barneah et al. 2007, Arotsker et al. 2009, Miller & Richardson 2011, Aeby et al. 2015). Environmental parameters, such as water depth and clarity, as well as water temperature, have been correlated with this disease's prevalence and dispersal (Miller & Richardson 2015), though the roles of these parameters are as yet unclear (Rützler et al. 1983, Kuta & Richardson 2002, Zvuloni et al. 2009, Krediet et al. 2013). For example, Sato et al. (2011) found that high light intensity (440 µmol m^{-2} s^{-1}) significantly enhanced BBD progression in the coral *Montipora hispida*, while high temperatures were not found to statistically significantly affect disease progression.

A meta-analysis conducted on published clone libraries of BBD samples collected over the last decade from 10 different geographic locations found one cyanobacterial operational taxonomic unit (OTU) present in 71% of the samples, and 3 OTUs (one of *Cytophaga–Flavobacter–Bacteriodetes* and 2 of *Alphaproteobacteria*) present in 13% of the samples (Miller & Richardson 2011). Indeed, in the majority of BBD studies published to date, examination of the affected coral samples consistently revealed an abundance of non-heterocystous, filamentous cyanobacteria (Cooney et al. 2002, Frias-Lopez et al. 2003, Myers et al. 2007, Sato et al. 2009, Miller et al. 2011b) some of which were shown to penetrate into the coral tissues (Barneah et al. 2007, Miller et al. 2011b, Kramarsky-Winter et al. 2014). The cyanotoxin microcystin was found in several BBD samples (Richardson et al. 2007, Casamatta et al. 2012), and was cited as a possible cause of tissue damage and even death of corals and other organisms (Dow & Swoboda 2000). Thus it is likely that this cyanotoxin contributes to the complex pathogenicity mechanisms of BBD. Richardson & Ragoonath (2008) demonstrated the ability of the BBD-associated cyanobacterium *Geitlerinema* to use exogenous organic carbon to enhance survival in the dark, under both the anaerobic and illuminated aerobic, sulfide-rich conditions in BBD mats. These cyanobacteria were responsible for extensive skeleton boring and were also observed to penetrate into the overlying coral tissue (epidermis and gastrodermis) in the BBD-affected coral *Montastraea annularis*. A population of novel, as yet unidentified, small filamentous bacteria was also found at the leading edge of the migrating band emerging from within the skeleton and were present throughout the mesoglea between tissue layers in corals from the Florida Keys (Miller et al. 2011b). Their location in the BBD mat may also suggest a role in this disease etiology (Miller et al. 2011b). In Red Sea BBD-affected corals (*Favia* spp.) a similar bacterial strain BgP10_4S^T was isolated and characterized as a proposed novel species of cyanobacteria named *Pseudoscillatoria coralii* (FJ21072) (Rasoulouniriana et al. 2009). Filaments of this cyanobacterium were found both in the mat, and penetrating the coral tissues (Rasoulouniriana et al. 2009, Kramarsky-Winter et al. 2014). Aeby et al (2015) showed that the isolate from BBD-affected *Montipora capitata* in the Hawaiian archipelago was 99% similar to *P. coralii* (BgP10_4S^T) from the Red Sea. In the Caribbean a cyanobacterium *Roseofilum reptotaenium* was isolated from BBD-affected *Diploria strigosa* and *Siderastrea siderea* corals; this strain was shown to synthesize microcystin-LR and experiments showed that the strain is capable of initiating an infection on healthy coral that resembles *in situ* BBD (Casamatta et al. 2012).

In the Red Sea, as in other areas, the signs of BBD have a seasonal appearance: increasing in the summer with rising water temperatures and disappearing in winter with the decreasing water temperatures (Zvuloni et al. 2009, Miller & Richardson 2015). Often the disease signs reappear on the same coral colony the following year (Carlton & Richardson 1995, Zvuloni et al. 2009). Occasionally, the disease signs may persist year round depending on coral colony and environmental conditions (E. Kramarsky-Winter pers. obs.). BBD-infected corals have not been observed to recover, and the bare skeleton may remain uncovered by coral tissue for many years (Kuta & Richardson 1997, Edmunds 2000, Zvuloni et al. 2009). In the Red Sea corals BBD displays spatiotemporal waterborne transmission patterns (Zvuloni et al. 2009). Moreover, infected colonies surviving a disease season appear to play a role in the reintroduction of the disease to the coral reef in the following season (Zvuloni et al. 2009).

The persistence of this disease and the relatively low recovery rate of diseased colonies make elucidation of the temporal changes occurring in the different microbial members of the black band (facultative or not) an important tool for assessing this disease's

etiology. We therefore monitored the BBD-associated bacterial community throughout 1 yr, and during the different phases of the disease manifestation. We sampled the BBD community during the peak activity (summer), when disease signs were waning (fall) and during the quiescent or subclinical stage (winter), where no symptoms were visible. This enabled the assessment of possible changes occurring in the microbial community during the different disease phases.

MATERIALS AND METHODS

Sample collection

Samples from the surface of 2 tagged BBD-affected *Favia* sp. colonies were collected at 3 different time points (Table 1) from the coral reef near the Inter-University Institute (IUI) for Marine Science at Eilat (Northern Red Sea, 29° 51′ N, 34° 94′ E) from a depth of 5 m. The samples containing a mixture of mucus, surface microorganisms and coral cells were collected using 10 ml syringes and immediately transported to the laboratory for processing. The samples were taken from the area on the colony showing disease signs (the black band), as well as from the area on the same colonies with no disease signs (the apparently healthy tissue) at a distance of ≥10 cm from the lesion. During the non-active phase samples were taken from the interphase between the coral tissue and skeleton that had been bared by the band as well as from 10 cm from the lesion. The sampling time points represented 3 phases of the disease: highly active, waning and non-active phases; this resulted in a total of 12 samples (4 at each sampling period) that were sent for pyrosequencing. The highly active stage of the disease was characterized by thick black band signs and by ongoing loss of coral tissues and the appearance of newly exposed skeleton adjacent to the disease front. The waning stage was characterized by a reduction in band advance, and typified by the persistence of the band but with no newly exposed skeleton and the previously exposed skeleton being covered over by algae and other organisms. The non-active stage was characterized by the disappearance or loss of the band and no or minimal evidence of tissue recovery. When typical BBD signs were not evident, samples were collected from the interface area, the area between the apparently healthy tissue and the exposed skeleton of the previously marked corals. Water temperatures (Table 1) were measured *in situ* in areas adjacent to the colonies.

Table 1. Sampling of 2 black band disease (BBD)-affected coral colonies from Eilat, Northern Red Sea during the 3 disease phases, showing ambient water temperatures (data from the Israeli National Monitoring Program); 4 samples from each sampling date were sent for 454-pyrosequencing

Sampling time	Disease phase	Max. water temperature (°C)
August 2009	Highly active	27.8
October 2009	Waning	26.1
February 2010	Non-active	22.0

DNA extraction and pyrosequencing

Total genomic DNA was immediately extracted from the fresh samples using a MoBio Power Soil DNA isolation kit with protocol supplied by the manufacturer. Quality and concentration were assessed using a NanoDrop ND 1000 spectrophotometer. Two representative DNA samples from healthy and diseased tissues from each time point were submitted to Molecular Research (MR) DNA Laboratories for 16S rRNA gene pyrosequencing using Roche 454 (454 Life Sciences; Tables 1 & 2). The company's primer 27Fmod AGR GTT TGA TCM TGG CTC AG was used to obtain bacterial reads of the hypervariable regions V1-V2 from the coral samples.

Bacterial diversity analysis

In total, 151 626 bacterial sequence reads were recovered and analysed using mothur software version 1.9.1 (Schloss et al. 2011). The sequences were trimmed using mothur software for removal of primers, barcodes, ambiguous nucleotides, long homo-polymers, reads below a minimum quality score and sequences shorter than 150 bases. Sequences were aligned, checked for chimeras, filtered, and classified using the Ribosomal Database Project II website (RDP Release 10; Wang et al. 2007) and BLAST (NCBI Database). Reads classified as chloroplast, mitochondria or 'unknown' were removed. The 'sub-sample' function was used to randomly select equally sized reads from each library, further reducing data sets for subsequent analyses, leaving final equalized bacterial sub-samples of 1810 sequences (per sample) for further consideration.

Table 2. Average alpha-diversity indices (97%) of bacterial species (16S rRNA gene sequences) computed using mothur software based on 454-pyrosequencing of DNA samples from healthy (H) and diseased (blackband, BB) tissues from 2 representative BBD-affected *Favia* sp. corals at 3 time points during the progression of the disease: number of sub-sampled sequences, number of operational taxonomic units (OTUs), coverage, invsimpson diversity and Chao richness estimation. White: summer; grey: winter

Sample	No. of sequences	Avg. no. of OTUs	Coverage	Invsimpson	Chao
Aug 09 BB	3620	71	0.978	1.565	144.413
Aug 09 H	3620	130	0.964	3.459	230.045
Oct 09 BB	3620	342	0.888	9.523	723.824
Oct 09 H	3620	127	0.966	6.280	214.201
Feb 10 BB	3620	671	0.732	82.068	1770.278
Feb 10 H	3620	170	0.958	6.605	251.726

The pyrosequencing reads of the community data were also analyzed by multiple sequence alignments using the dist.seqs function; mothur OTU analyses were conducted (by a 0.03 distance level) to present alpha-diversity: Chao1 richness estimators, the inverse Simpson diversity index and rarefaction curves, which were plotted on a line chart using Microsoft Excel (see Fig. 1). PC-ORD 6 software (MJM Software Design) was used to calculate beta-diversity: nonmetric multidimensional scaling (NMS) ordination.

Raw sequencing data was deposited in the MG-RAST (https//:metagenomics.anl.gov) archive under accession numbers 4541470.3 to 4541481.3.

RESULTS

Bacterial diversity

Two BBD-affected coral colonies were monitored on the reef of Eilat at 3 different time points during 2009–2010, according to previously observed BBD activity level. Samples were taken during the highly active BBD periods, when the band was well developed, and water temperatures reached a maximum of 27.8°C in August 2009. They were then taken once more during disease waning at a time point when water temperature declined to a maximum of 26.1°C in October 2009. A third set of samples was collected during the non-active state of the disease, when maximal water temperature had decreased to 22.0°C in February 2010 (Table 1).

Empirical average OTU numbers of most samples as represented by rarefaction curves (Fig. 1) did not reach saturation and were lower than the theoretical Chao index (Table 2). Good's coverage of the BBD-originating reads decreased from 97.8% in highly active diseased colonies to 88.8% in those with waning disease signs and to 73.2% in non-active disease states. This implies that even during the waning of the disease there are only relatively low numbers of singletons. For some BBD samples, the rarefaction curve failed to reach a

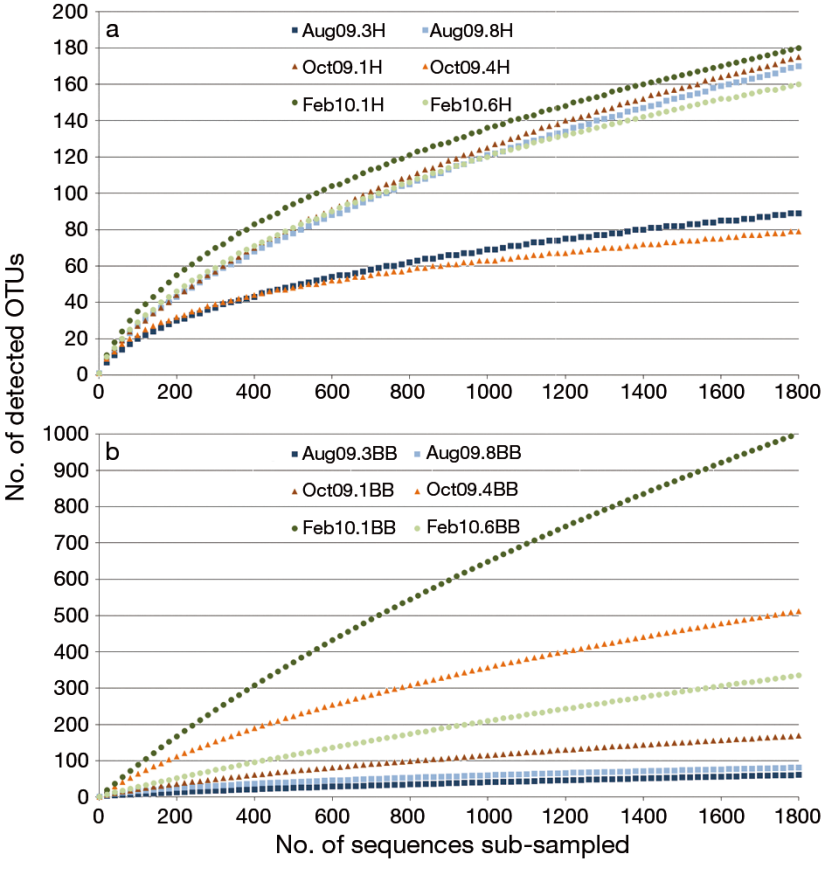

Fig. 1. Rarefaction curves indicating 16S rRNA gene richness of highly active, waning and non-active phases of coral black band disease (BBD), in (duplicate) samples collected in August 2009, October 2009 and February 2010, respectively, from Eilat, Northern Red Sea. (a) Samples from apparently healthy tissues (H); (b) samples from black band mats (BB). OTUs defined at a 0.03 distance cutoff (n = 1810 sequences per sample)

plateau (Fig. 1). This is especially true for the black band samples of February and October. In general, richness and diversity of the apparently healthy area of BBD-affected colonies showed small differences between samples. These indices for the black band communities were significantly inversely correlated with water temperature and disease state. Moreover, the rarefaction curve (Fig. 1) and NMS ordination plot (Fig. 2) revealed a clear separation between the BBD-originating bacteria and those of the apparently healthy tissue of the affected coral at all time points. In addition, samples from active BBD (Aug 09 and Oct 09) resembled each other more than they did the non-active samples (Feb 10) that lacked the BBD signs.

Microbial dynamics in BBD mats vs. apparently healthy area of BBD-affected colony

Reads of 16S rRNA genes that were retrieved from all samples belong to 10 different phyla: *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Gemmatimonadetes*, *OD1*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia*, in addition to unclassified bacteria (RDPII classification). An NMS ordination plot (Fig. 2) showed that although BBD and apparently healthy samples showed different distributions, on a class level both had high percentage of 'unclassified bacteria' (Fig. 3). Further analysis using NCBI/BLAST database revealed that 93.6% and 71.6% of the unclassified reads of Aug 09 and Oct 09 black band samples, respectively, had high similarity (99 to 100%) to the known BBD-specific cyanobacterium BgP10_4S^T (FJ210722) (Table 3). Only 0.4% of the Feb 10 black band's unclassified reads (0.22% of total reads) were found to resemble this cyanobacterial strain.

The BBD bacterial community changed with sampling date and with the ambient water temperature. During the highly active phase of the disease, in Aug 09, when ambient water temperatures peaked, the band was dominated by the cyanobacterium BgP10_4S^T, encompassing 80.1% of the OTUs. This was followed by classes of, *Gamma*-, *Alpha*- and *Epsilonproteobacteria* (3.62% 2.93% and 0.97% respectively), *Sphingobacteria* (1.52%), *Bacteroidetes* (2.70%), and *Clostridia* (2.18%) as well as by 5.98% other unclassified bacteria. During the waning stage of the disease in Oct 09, there was an almost 2°C drop in maximal water temperature (26.1°C), and the band communities changed with the abundance of BgP10_4S^T decreasing to 46.5% of the total sequences. Decreases in *Epsilonproteobacteria* (0.44%), *Sphingobacteria* (0.22%) and *Clostridia* (0.33%) were also evident. During this phase other communities appeared and/or increased in numbers. The *Alpha*-, *Gamma*-, *Delta*- and unclassified *Proteobacteria* increased (12.18, 6.32, 2.21 and 2.98%, respectively), as did *Flavobacteria* (3.12%), unclassified *Bacteroidetes* (4.06%), *Opitutae* (0.86%), *Bacilli* (0.39%) and unclassified *Firmicutes* (0.58%). In Feb 10 maximal water temperature had decreased to 22°C and the BBD signs were no longer visible. These coral colonies lost the major disease sign (black band), were characterized by partially bare skeleton at the lesion site and did not regenerate lost tissues (E. Kramarsky-Winter pers. obs.). During this period the samples collected from the interface of the apparently healthy tissue and the bare skeleton revealed bacterial communities more similar to the original BBD community than to the healthy looking tissue distant from the lesion in the affected colonies. This interface area contained a small amount of cyanobac-

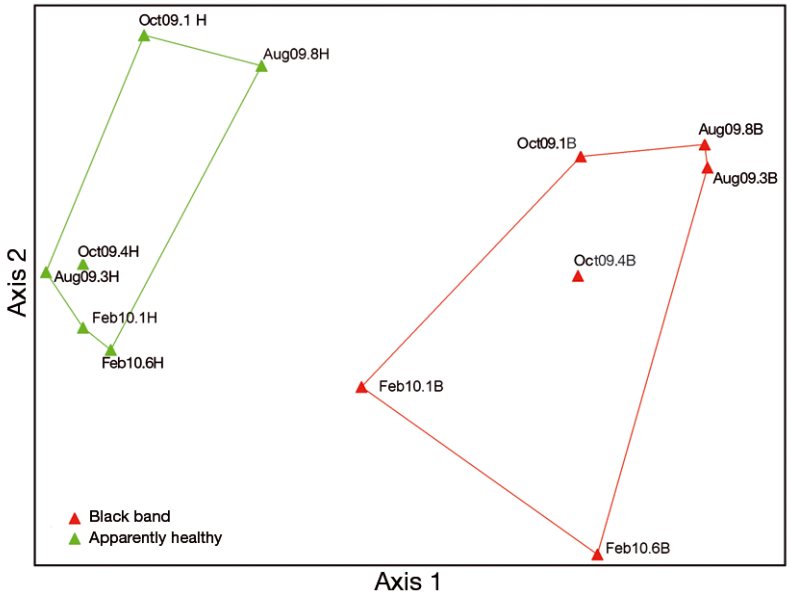

Fig. 2. Beta-diversity nonmetric multidimensional scaling (NMS) ordination plot of coral samples collected during highly active (Aug 09), waning (Oct 09) and non-active (Feb 10) phases of BBD. The plot gives a 2-dimensional representation of samples' relative distribution with final stress of 8.573 and final instability of 0.019 (500 iterations). The distance between symbols is equivalent to the dissimilarity of the samples (cutoff ≥0.03). Red triangles: black band samples; green triangles: apparently healthy tissue samples

Fig. 3. Bacteria diversity based on 16S rRNA sequence similarity, classified to a class level using RDPII, of (above) black band samples and (below) apparently healthy tissue samples collected from BBD-affected corals during highly active (Aug 09), waning (Oct 09) and non-active (Feb 10) phases of the disease. Bacteria with >1% representation are shown

Table 3. Cyanobacteria and other bacterial diversity using 16S rRNA gene of the black band (BB) and apparently healthy (H) tissue of BBD-affected corals, classified to class using NCBI/BLAST. Values are percentages of each sample. Note 'other cyanobacteria' include those with high similarities to X63141 *Prochloron* sp., HM217074 *Leptolyngbya* sp., and AY615507 *Oscillatoria spongeliae*

Species	Aug 2009		Oct 2009		Feb 2010	
	BB	H	BB	H	BB	H
Strain BgP10_4S^T (FJ210722)	80.08	0.5	46.55	0.08	0.22	0.06
Other cyanobacteria	0	0.03	2.4	0.28	38.2	0.58
Other bacteria	19.92	99.48	51.05	99.64	61.57	99.36

teria BgP10_4S^T (0.22%). In addition other bacterial groups such as *Alpha-*, *Gamma-*, *Delta-* and unclassified *Proteobacteria* dominate the niche (11.71, 8.23, 0.88 and 4.17%, respectively). *Flavobacteria* (5.25%), *Sphingobacteria* (0.75%) *Opitutae* (0.8%), and unclassified *Bacteroidetes* (5.28%) were also found and a large portion of unclassified bacteria (61.24%; RDP classification). Interestingly, the majority of the unclassified bacteria (36.74% of total reads) classified by NCBI/BLAST as cyanobacteria with 99% similarity to *Prochloron* sp. (X63141).

Anaerobic heterotrophs and opportunistic pathogens

The *Clostridia* class of the *Firmicutes* was found in the active black band in smaller amounts, forming 2.18% of the community during the active disease phase (Aug 09), and decreasing to 0.33% during the waning stage (Oct 09) and to 0.11% during the non-active phase (Feb 10). In addition, 0.28% of Aug 09 BBD samples revealed *Clostridia* reads highly similar (100%) to *Flavonifractor plautii* (GU968163) and other human pathogens (e.g. HQ820588, JN555371). Interestingly members of the *Gammaproteobacteria* class dominated the apparently healthy area of the coral throughout the year. As the BBD signs waned towards the winter, the proportion of this group in the BBD mat increased (from 3.62% to 8.23%). *Vibrio* spp. were consistent in the black band mat throughout this study, with changes in percentages from 0.60% during the active stage to 1.28% during the waning stage and to 1.15% during the inactive stage of the disease. In contrast in the apparently healthy area of the same colonies percentages of these *Vibrio* sp. changed from 0.06% during the active phase of the disease to 0.03% as the disease waned and to 0.28% when no disease signs were evident in the colonies. Reads of *Deltaproteobacteria* that contain several groups of anaerobic sulphate reducing bacteria (SRB), including the *Desulfobulbaceae* family, appeared in the waning diseased coral samples (2.21%) and remained in the non-active Feb 10 samples. Only traces of *Desulfovibrionaceae* (0.06%) were found in the highly active band. *Epsilonproteobacteria* decreased in numbers as the BBD signs weakened (0.97% to 0.44% to 0.11%). The highly active and waning (Oct 09) BBD mats contained reads highly similar (97 to 99%) to *Arcobacter* spp. (AP012048, JQ895021) and to clones associated with other diseased corals (99 to 100%; FJ203099, GQ455291, GU200373).

Cyanobacteria

The BBD-specific cyanobacterium, BgP10_4S^T (FJ210722), was persistent in these corals throughout the year. It was the sole cyanobacterial ribotype constantly retrieved, with numbers peaking during the highly active stage of the disease and decreasing as the activity of the disease waned (Table 3). As the ribotype numbers of BgP10_4S^T decreased, there was an increase in other cyanobacterial ribotypes (from 0 reads in Aug 09, to 2.4% in Oct 09, and to 38.2% in Feb 10) as well as of other bacteria. The amount of this ribotype retrieved from the healthy portion of the colony tissues were small throughout the year, and also changed with season, decreasing from 9 reads in Aug 09, to 2 in Oct 09 and to 1 read in Feb 10 (0.5, 0.08 and 0.06%, respectively; Table 3).

DISCUSSION

BBD is a dynamic disease whose signs wax and wane throughout the year. The changes in disease signs correlate with both season and water temperature changes (Rützler et al. 1983, Kuta & Richardson 1996, Zvuloni et al. 2009, Miller & Richardson 2015). In order to clarify the dynamics of this disease, we assessed and compared the bacterial communities found during the 3 stages of the disease, highly active black band stage (collected during the summer when water temperature reached an average of 28°C) the waning stage (fall when water temperature average decreased to 25°C) and the non-active stage (winter

when water temp average was 21°C). This allowed us to examine the constant and varying features of the BBD population at different times and throughout the disease process. We were able to demonstrate that the microbial communities associated with the black band and underlying necrotic tissue of BBD-affected coral colonies are diverse, dynamic and unique to specific seasonal time points and disease morphology, and differ greatly from the communities that populate the tissues that do not show disease signs in the same coral colony (Table 2, Fig. 2).

Interestingly, common microbial features of the black band lesion in the Red Sea *Favia* were observed throughout the year, the most significant of which was the cyanobacterium BgP10_4S^T strain (FJ210722) for which the name *Pseudoscillatoria coralii* was previously proposed (Rasoulouniriana et al. 2009). This may suggest the importance of this cyanobacterium strain in the characterization of the disease. The cyanobacterium strain BgP10_4S^T strain (FJ210722) reported here has not yet been identified according to valid provisions of the rules of International Codes of Nomenclature of prokaryotes due to lack of axenic monocultures in culture collections (Casamatta et al. 2012). Despite this, on the basis of its morphological, physiological and phylogenetic distinctiveness, this strain has been unofficially described as related to subsection III (formerly Oscillatoriales) (Rasoulouniriana et al. 2009). Interestingly, the cyanobacterium strain BgP10_4S^T is very closely related (99%) to the strain Cyano OCN074 (KJ-914890) from Hawaii BBD (Aeby et al. 2015) and also closely related but not identical (>97%) to *Roseofilum reptotaenium* (Oscillatoriales, Cyanobacteria) which was isolated and characterized from Caribbean BBD-affected corals (Casamatta et al. 2012). 16S rRNA gene sequence data place this strain into a highly supported clade with other strains identified as *Oscillatoria sensu lato*, yet clearly genetically distinct from the type, *O. princeps* (Casamatta et al. 2012).

The overall bacterial diversity in BBD affected corals differed between apparently healthy tissue and the BBD mat, as well as between seasons (Fig. 1, Table 2). During winter when there are no BBD signs, the average OTU number was almost tenfold higher than in summer at peak BBD activity. This implies that the bacterial consortium of the BBD may be dominating the specific community that is responsible for the appearance and progression of the black band (Fig. 1b). Alpha-diversity parameters (Table 2) indicate an increasing diversity in the black band microbial communities occurring when disease signs begin to disappear and as the environment cools down. Despite this, there are microbial groups that are persistent in the coral tissue-skeleton interface even when disease signs disappear. Beta-diversity parameters (Fig. 2) showed a separate clustering of the BBD microbial mat samples compare with the apparently healthy coral surface communities at all time points. Throughout the year healthy tissues showed domination of 2 groups, unclassified bacteria (43.84 to 33.56%) and *Gammaproteobacteria* (49.53 to 18.40%). Diseased tissues, on the other hand, were dominated by the cyanobacterium BgP10_4S^T strain (FJ210722) as well as by unclassified bacteria, and contained also a wider range of groups but including only small percentages of *Gammaproteobacteria*. Richness and diversity indices of the black band samples were inversely correlated with water temperature. These results indicate that the highly active BBD consortium is a specific and defined group that is characteristic of the active phase of the disease.

The cyanobacteria sequences of strain BgP10_4S^T (FJ210722), together with a number of *Vibrio* species (*Gammaproteobacteria*), *Sphingobacteria* (*Bacteroidetes*), *Clostridia* (*Firmicutes*) and *Epsilonproteobacteria*, compose the black band in the highly active phase, decreasing in numbers as the disease signs wane (Fig. 3). Interestingly, as described in previous studies, many of these groups of bacteria are consistent members not only of BBD, but of other coral diseases, including white syndrome and white plague (Frias-Lopez et al. 2002, Barneah et al. 2007, Sekar et al. 2008, Sussman et al. 2008, Arotsker et al. 2009, Sunagawa et al. 2009, de Castro et al. 2010, Ben-Dov et al. 2011). In general, *Gammaproteobacteria*, such as *Vibrio* spp., are often found in elevated numbers in immunocompromised aquatic animals (Sussman et al. 2008, Sunagawa et al. 2009, Vezzulli et al. 2010, Garcia et al. 2013) with certain species of vibrios found in association with bacterial bleaching in Mediterranean corals (Kushmaro et al. 1996), with white syndrome in Indo-Pacific corals (Sussman et al. 2008), and with BBD from the Caribbean (Sekar et al. 2008) and the Red Sea (Barneah et al. 2007, Arotsker et al. 2009). In BBD we found that the abundance of *Vibrio* spp. in the lesion actually increased as the disease state waned, going from 0.60% during the highly active state to 1.28 and 1.15% in the active and non-active state, respectively. In addition, apparently healthy tissues contained vibrios one order of magnitude lower than BBD affected tissues throughout the year (0.06, 0.03 and 0.28% in the 3 stages, respectively). These results may indicate that some of these *Vibrio* species are secondary pathogens affecting the corals once they are immunologically com-

promised and/or that other members of the mat community affect the *Vibrio* species composition. This though merits further study.

Numerous studies have shown that a cyanobacterium defines the active BBD of *Favia* sp. corals in the Red Sea (Barneah et al. 2007, Rasoulouniriana et al. 2009, Kramarsky-Winter et al. 2014); this strain has been recovered in several faviids over time (Rasoulouniriana et al. 2009, Kramarsky-Winter et al. 2014). Here we show that this strain significantly dominates the highly active samples representing 80.1% of the reads, decreases in abundance as the disease signs wane, and remains in only trace amounts (0.22%) in the corals when the disease signs disappear. Moreover, we show that the numbers of this cyanobacterium in the healthy tissues of diseased corals are negligible, ranging from 0.5% at peak disease activity to 0.06% during the non-active period (Table 3). Despite the low numbers of this cyanobacteium found on and in the diseased coral tissues during the non-active phase of the disease, its persistence throughout the year indicates that these remnants may infiltrate in low numbers into the healthy looking tissues (Kramarsky-Winter et al. 2014). Furthermore they were not found in completely healthy colonies (L. Arotsker pers. obs.). This suggests that these cyanobacterial cells provide a source that, under the right conditions, can pioneer the assembly of the consortium de novo. Therefore, it is likely that the relatively high numbers of cyanobacterial strain BgP10_4S^T in the band, together with their presence in the apparently healthy tissue of the affected coral during the highly active period, may have contributed to the rapid reassembly and progression of the disease during the summer.

Cyanobacterial species closely related to the BgP10_4S^T strain may not be restricted to Red Sea *Favia* sp. BBD alone. The high genetic resemblance (97 to 100%) to BBD-originating clones and isolates from different species from distant geographic areas, including Palau, the Indo-Pacific (AY839641, Sussman et al. 2006), GBR, Australia (GQ204970, Sato et al. 2009), and the Caribbean Sea, including St. Croix and Barbados (AF473936, Cooney et al. 2002, Sekar et al. 2009) and Curaçao, Netherlands Antilles (AY038527, Frias-Lopez et al. 2002) may indicate that this cyanobacterial genus is a primary and important constituent of BBD over large geographic ranges. Indeed the results of the meta-analysis of clone libraries (Miller & Richardson 2011) clearly indicate the presence of a dominant cyanobacterial OTU highly homologous (99%) to cyanobacterium BgP10_4S^T strain (*Pseudoscillatoria coralii*; FJ210722) in BBD from 9 out of 10 geographic locations, and in 12 of the 16 coral host species sampled. This strengthens the hypotheses that a specific cyanobacterium may be the primary pathogen of BBD (Miller & Richardson 2011).

During the highly active stage BgP10_4S^T represented the only cyanobacterial OTU revealed in the samples of Red Sea *Favia* BBD. As the disease signs began to wane, additional species of the cyanobacteria could be found in the lesion area. This indicates that sampling time is crucial to ascertaining the characteristic populations of microorganisms relevant to the disease. Thus the changes in microbial assemblage occurring throughout the disease cycle may be one explanation regarding the differences in cyanobacterial species previously reported in BBD from other geographic regions (Sekar et al. 2008, Myers et al. 2007, Sato et al. 2009). Differences in strains (Sekar et al. 2009, Sato et al. 2011) found in corals from different geographic regions may also be attributed to species specificity or to tolerance of certain cyanobacterial strains to water temperature fluctuations (5 to 10°C fluctuations in Eilat, the Red Sea). The persistence of cyanobacteria in affected corals throughout the year, even when signs are not visible during low temperatures of the winter/spring, provides further evidence for their importance in reinitiation of the disease when environmental and physiological conditions are right (Miller & Richardson 2015). Indeed in a study of the BBD in the Caribbean Casamatta et al. (2012) experimentally showed that the closely related BBD-associated cyanobacterial strain *Roseofilum reptotaenium* infected the corals.

In addition to cyanobacteria, other constituents of the microbial mat may play an important role in BBD dynamics. Sulfate-reducing bacteria (SRB) bacteria play an important role in BBD (Richardson 2004). SRBs such as *Clostridia* (known to contain anaerobic species) were reported on diseased corals including BBD-affected corals (GQ455274, Ben-Dov et al. 2011; EF089465, Barneah et al. 2007; AF441943, Frias-Lopez et al. 2002), coral white syndrome (EU780347, S. E. Godwin et al. unpubl.), white plague (FJ203247, Sunagawa et al. 2009) and *Mussimilia hispida* diseased coral from Brazil (GU199771, de Castro et al. 2010). In this study, reads highly similar (100%) to *Flavonifractor plautii* (GU968163) and other pathogens (e.g. HQ820588, JN555371) originating in human feces (Durbán et al. 2012, Li et al. 2012), were found in the samples from the highly active stage, indicating a possible anthropogenic origin of some of the BBD consortium. It is possible that the surge of *Clostridia* during the active phase of BBD is because

they thrive in the oxic/anoxic gradient microenvironment of the BBD (Richardson 2004). Thus they may represent a part of the anoxic community, prevalent during the highly active disease phase. Since this group of bacteria produce a variety of cytotoxins harmful to eukaryotic cells (Schirmer & Aktories 2004) it is possible that together with cyanobacterial cytotoxins and the sulfide-rich environment that they create, these bacteria contribute to the active coral tissue necrosis occurring during the warm summer months.

This study provides us with a glimpse at the dynamics of the BBD disease process and provides evidence regarding some of the major players in this disease. Further studies should include carefully controlled experiments under specific and differing conditions, and using, if possible, a number of isolated strains from the consortium.

BBD model

In order to illustrate our current understanding of the BBD in Red Sea *Favia* sp., we depict a model of the disease dynamics (Fig. 4) occurring in affected colonies. When water temperature and irradiance rise during the summer months, corals that are immunologically repressed become a good medium for the infiltration of cyanobacteria and accompanying bacteria such as vibrios and SRBs. These bacteria infiltrate and anchor into the coral tissues (Kramarsky-Winter et al. 2014) resulting in the necrosis of the coral tissues and in the development of a wide and highly active band dominated by the cyanobacterium. When water temperatures are high the disease progresses rapidly, degrades coral tissues and facilitates the assembly of additional BBD-specific groups of bacteria. Members of this consortium infiltrate the tissues further degrading the coral tissues. As the environmental conditions change and water temperatures begin to decrease, the progression rate of the band slows, and the BBD-specific communities change. This opens a niche for additional microbial communities to 'enjoy the feast' of debris and necrotic tissues, and as a result changes the community structure of the BBD consortium. Finally, during the periods of lower water temperature and irradiance occurring during the winter, the disease wanes, the numbers of the major players decrease and the black band signs disappear. Thus the diversity of the microbial community of the interface remains similar to the original BBD community but with different diversifying features. During this period, the numbers of some of the persistent members of the BBD may become so low as to be 'sub-clinical'. Despite their low numbers they can still provide a reservoir for resumption of the disease process when the environmental parameters or coral immunity are conducive. The effect of the coral's physiology on the ability of these pathogens to remain anchored to or inside the tissues is supported by the fact that following injury, little or no tissue regeneration occurs during winter or following environmental stress (Kramarsky-Winter & Loya 2000, Paz-García & Reyes-Bonilla, 2006, Denis et al. 2011). Thus, the persistence of BgP10_4S^T in low numbers in apparently healthy tissues during that time may facilitate reinitiation of the BBD once the environmental conditions allow. Understanding the changes occurring in the surface community microbiome of BBD affected corals provide an important insight into the dynamics of this disease as a first step in assessing possible modes of disease mitigation.

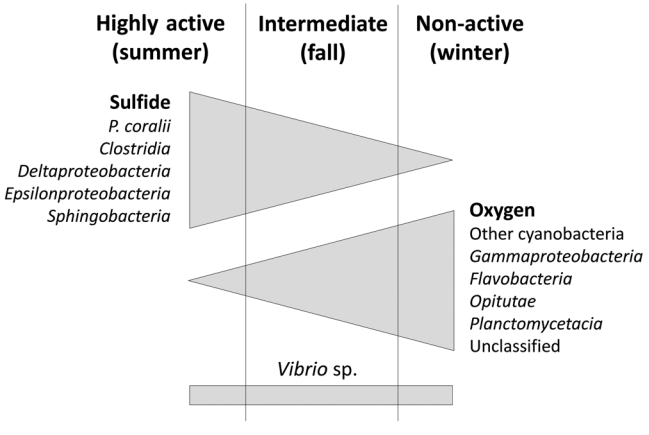

Fig. 4. Model of the dynamics of BBD-associated bacteria found in the band lesion of the Red Sea *Favia* sp. coral during the different disease phases

Acknowledgements. This research was supported by ISF grant no. 1167/07. In addition, we thank Dr. Karnit Bahartan for her assistance in the sample collection process and IUI in Eilat for use of their facilities.

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Editorial responsibility: Garriet Smith, Aiken, South Carolina, USA

Submitted: April 13, 2015; Accepted: July 29, 2015
Proofs received from author(s): September 5, 2015