

## Brief report

# Bacteria associated with the rapid tissue necrosis of stony corals

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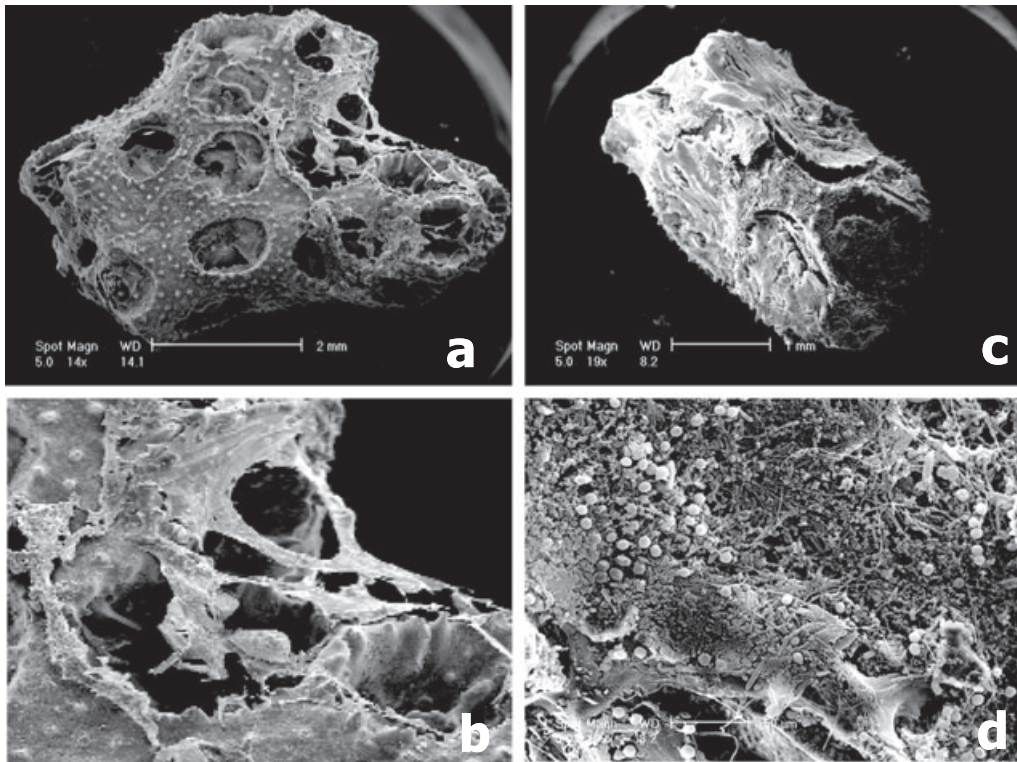
### Summary

The rapid tissue necrosis (RTN) is a common disease of both wild and captive stony corals, which causes a fast tissue degradation (*peeling*) and death of the colony. Here we report the results of an investigation carried out on the stony coral *Pocillopora damicornis*, affected by an RTN-like disease. Total abundance of prokaryotes in tissue samples, determined by epifluorescence microscopy, was significantly higher in diseased than in healthy corals, as well as bacterial counts on MB2216 agar plates. Further experiments performed by fluorescent *in situ* hybridization using a 16S rDNA *Vibrio*-specific probe showed that vibrios were significantly more abundant in diseased than in healthy corals. Accordingly, bacterial counts on TCBS agar plates were higher in diseased than in healthy tissues. 16S rDNA sequencing identified as *Vibrio* colonies from diseased tissues only. Cultivated vibrios were dominated by a single ribotype, which displayed 99% of similarity with *Vibrio harveyi* strain LB4. Bacterial ribotype richness, assessed by terminal-restriction fragment length polymorphism analysis of the 16S rDNA, was significantly higher in diseased than in healthy corals. Using an *in silico* software, we estimated that a single terminal restriction fragment, putatively assigned to a *Vibrio* sp., accounted for >15% and < 5% of the total bacterial assemblage, in diseased and healthy corals respectively. These results let us hypothesize that the RTN in stony corals can be an infectious disease associated to the presence of *Vibrio harveyi*. However, further studies are needed to validate the microbial origin of this pathology.

### Introduction

Over the past decades, coral reefs have experienced significant decline due to the interaction of many factors and alteration of several variables (Weil *et al.*, 2006), such as global warming, ozone depletion and increased UV radiation, overfishing, eutrophication, land-use practices and increased contamination related with human activities (Harvell *et al.*, 1999). Such coral reef decline is exacerbated by the spread and appearance of emerging coral diseases, which have increased in frequency in recent years and have been shown, in several cases, to be caused by bacterial pathogens (Kushmaro *et al.*, 1998; Ben-Haim *et al.*, 1999; Patterson *et al.*, 2002; Denner *et al.*, 2003; Barash *et al.*, 2005). Most known diseases are, however, only described for their symptoms, but have not been characterized in terms of aetiology and pathogenesis (Richardson, 1998). For most of them, detailed microbiological investigations have been complicated by the difficulties in pathogens' cultivation, only recently surmountable with the development of molecular methodologies (Amann *et al.*, 1995; Rohwer *et al.*, 2001; 2002; Pantos *et al.*, 2003). This is the case of several pathologies, such as those referred to as yellow blotch, dark spots, dark bands and particularly the so-called rapid tissue necrosis (RTN). Rapid tissue necrosis (also defined 'shut down reaction' or 'rapid tissue degradation') is a rapidly progressing syndrome, characterized by fast tissue degradation (Borneman, 2001; Anthony, 2004). This disease is responsible for a large fraction of stony corals mortality, both in the natural reef and in aquaria (Anthony, 2004). Despite the large number of cases documented in the web and in non-scientific literature, there are no specific studies aimed at identifying its origin and possible causative agents. Although field studies reported that coral tissue degradation occurred concomitantly with the appearance of a specific pathogenic bacterium, i.e. *Vibrio coralliilyticus* (Ben-Haim and Rosenberg, 2002; Ben-Haim *et al.*, 2003), the disease aetiology and pathogenesis have not been elucidated yet. The evidence that, in aquaria, the disease is easily transmitted from sick corals to the healthy ones supports the hypothesis that pathogenic microorganisms are involved, but other causes, such as a coral reaction to

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**Fig. 1.** Scanning electron microscopy analysis of the tissues of diseased (A and B) and healthy (C and D) corals. Small fragments of fresh corals were fixed in 2.5% glutaraldehyde in autoclaved artificial seawater (AASW) for 18 h and then dehydrated by immersion in ethanol at increasing concentrations (10%, 30%, 50%, 70%, 80%, 95% and 100%). Dehydrated samples were critical-point-dried (CO<sub>2</sub>), coated with gold and examined under a scanning electron microscope (Philips XL20).

stressful conditions (Borneman, 2001), or a combination of infection and climate change, cannot be excluded *a priori*. A better understanding of the causes, as well as of the specific traits of RTN, is needed to protect wild and captive hard corals and to identify possible therapy and remedial actions. The aim of the present study was to investigate RTN-associated bacteria in the stony coral *Pocillopora damicornis*.

## Results and discussion

Samples of tissue were collected from both unhealthy and healthy (i.e. corals not displaying signs of RTN) small coral colonies (five and three, respectively, c. 10 × 8 × 8 cm in size) coming from the same aquarium tank (capacity 20 000 l) hosted in a large public aquarium. The tank, containing natural seawater, displayed the following characteristics: temperature: 26°C, pH: 8.0–8.2, salinity: 36–37 PSU, oxygen concentrations: 6.0–6.6 mg l<sup>-1</sup>, nitrate concentrations: 8–15 mg l<sup>-1</sup>, nitrites concentrations: 0–9 µg l<sup>-1</sup>, ammonium concentrations: 0–20 µg l<sup>-1</sup>, phosphate concentrations: 0.7–1.0 mg l<sup>-1</sup>, calcium concentrations: 400–430 mg l<sup>-1</sup>. All diseased samples displayed typical signs of RTN-like disease, and

a fast evolution of the pathology that spreads over the entire coral branch within hours to few days.

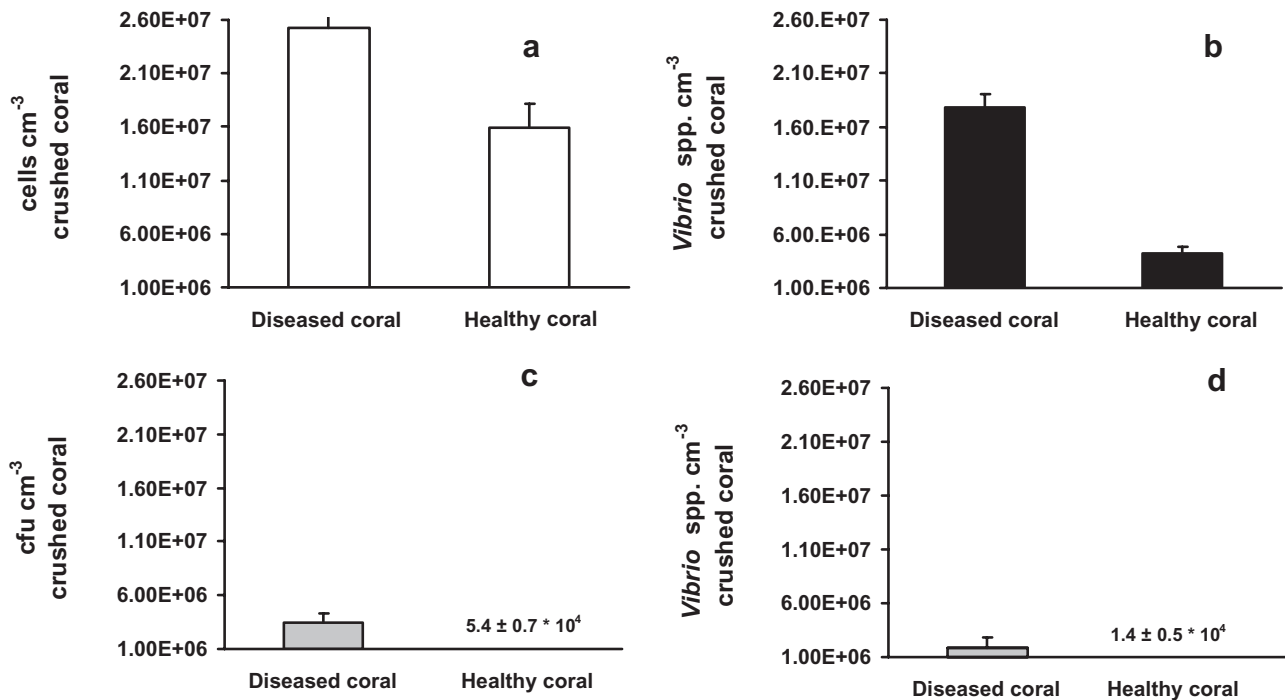
### Scanning electron microscopy

Diseased corals analysed by scanning electron microscopy revealed the presence of necrotic tissue interspersed with nude skeleton (Fig. 1A and B), whereas healthy corals were characterized by the presence of living tissue covering the entire skeleton and with zooxanthellae clearly visible on its surface (Fig. 1C and D).

### Prokaryotic and *Vibrio* spp. abundance

Total prokaryotic abundance on diseased and healthy coral tissues was determined by both culture-independent (epifluorescence microscopy) and culture-dependent (cultivation on MB2216 agar) approaches. In both cases, total prokaryotic abundance was higher (ANOVA,  $P < 0.01$ ) on diseased than on healthy corals (Fig. 2A and C).

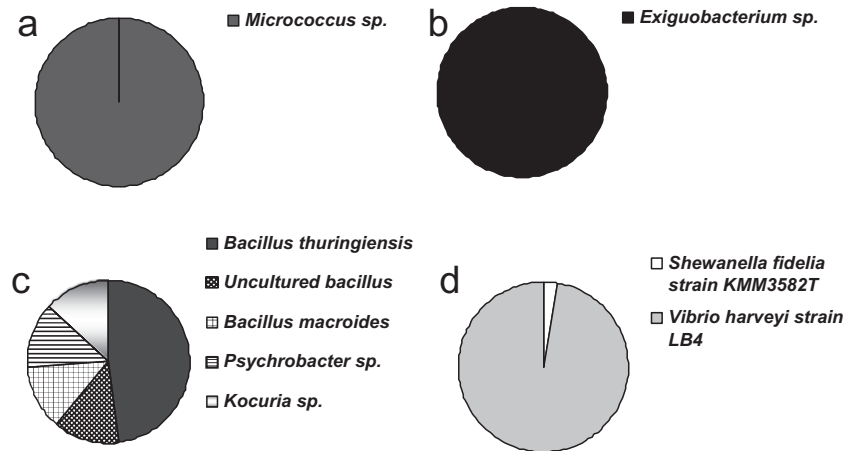
The genus *Vibrio* includes some of the most important pathogens for aquatic organisms (Thompson *et al.*, 2004; Baffone *et al.*, 2006), such as penaeid shrimps (Lui *et al.*, 1996) and several species of fishes and molluscs



**Fig. 2.** Abundance of total and culturable prokaryotes (A and C respectively) and of *Vibrio* spp. (B and D) associated with diseased and healthy corals. Total number of prokaryotes (A) was estimated by Sybr Green I staining and epifluorescence microscopy; total number of *Vibrio* spp. was estimated by FISH of 16S rRNA (B), whereas the abundance of culturable cells was estimated by plating on MB2216 (C) or TCBS (D) agar plates. Before analysis, each coral fragment was weighted and measured for length, width and height in sterile conditions to avoid any contamination, then washed twice with 5 ml of artificial and sterile seawater. Corals were crushed in a sterile mortar and pestle, homogenized for 1 min and left for 1 min to allow sedimentation of the coral material. Aliquots of the suspension were then collected and diluted using sterile artificial sea water. For total prokaryote counting, subsamples were fixed with 5 ml of 4% formalin, previously buffered with sodium tetraborate (20 g l<sup>-1</sup>) and filtrated through a 0.2 µm pore size filter. The number of total prokaryotes was determined by epifluorescence microscopy after staining with Sybr Green I (20 µl, Molecular Probes) diluted 1:20 in sterile milliQ water. Filters were incubated for 20 min in the dark, then washed twice with 1 ml of sterile milliQ water, mounted on microscopic slides and viewed using epifluorescence microscopy under blue excitation. For each filter, a minimum of 400 cells were counted. For the enumeration of *Vibrio* spp., coral tissues extracted as described above were diluted with PBS : ethanol (1:1 v/v) in sterile conditions and filtered through 0.2 µm black polycarbonate filters (Nuclepore). The filters were hybridized using a Cy3-labelled probe GV for *Vibrio* sp. (Eilers *et al.*, 2000), stained with DAPI (0.5 µg ml<sup>-1</sup>) and counted under epifluorescence microscopy. Positive and negative controls were obtained using the probes: EUB338 and NON338 respectively (Pernthaler *et al.*, 2001). Fluorescent *in situ* hybridization was also performed using a probe more specifically targeting the 16S rRNA of *Vibrio harveyi*, by using the VH-2 probe designed by Oakey and colleagues (2003). This probe is not highly specific for this target species, as it can detect also other phylogenetically related *Vibri*os. The FISH protocol was therefore tested on bacterial cultures as described in the study by Hugenholtz and colleagues (2001). We found that the use of 40% formamide provided the best results, also when the probe was tested for pure cultures of *V. harveyi*, *V. cholerae*, *V. vulnificus*, *V. parahaemoliticus*, *V. fluvialis* and *Escherichia coli* JM109. Among these species, only *V. harveyi* and *V. parahaemoliticus* were identified, so that counts presented here could be overestimated. For cfu counts, coral fragments were crushed and homogenized as described above. The obtained suspensions were serially diluted (up to 10<sup>-6</sup>) and 100 µl aliquots of the different dilutions plated onto MB2216 or TCBS (OXOID) agar for total heterotrophic and vibrios counts respectively. Bacterial colonies were counted after 48 h of aerobic incubation at 26°C. For a proper statistical treatment, only plates containing 30–300 colonies were considered. Prokaryote abundance was expressed as cells cm<sup>-3</sup> (for total prokaryote abundance) and cfu cm<sup>-3</sup> (for total culturable bacterial abundance) of coral. Differences in bacterial abundance between diseased and healthy corals were statistically analysed using one-way analysis of variance (ANOVA).

(Lightner, 1993; Lightner and Redman, 1998; Gomez-Gil *et al.*, 2004). Moreover, previous studies indicated *Vibrio* spp. as potential pathogens of stony corals (Ben-Haim and Rosenberg, 2002; Cervino *et al.*, 2004). We thus subsequently determined the abundance of bacteria belonging to *Vibrio* spp. by fluorescent *in situ* hybridization (FISH) (using a genus-specific probe) and by plating suitable amounts of tissue samples on TCBS agar. Fluorescent *in situ* hybridization counts were 10- to 300-fold higher than colony counts. However, in both cases, *Vibrio*

spp. abundance was higher in diseased than in healthy specimens: fourfold when evaluated by FISH (Fig. 2B) and more than 100-fold when evaluated by colony count (Fig. 2D). These results indicate that diseased corals hosted a significantly higher number of bacteria (ANOVA,  $P < 0.01$ ) than healthy corals, a large fraction of which was accounted by culturable *Vibrio*. The observed discrepancy between FISH (fourfold) and TCBS (100-fold) results could be explained by a higher abundance of culturable vibrios in diseased compared with healthy



**Fig. 3.** Diversity of culturable bacteria recovered from healthy (A and B) and diseased (C and D) specimens. A and C indicate colonies grown on MB2216 agar plates and B and D colonies grown on TCBS agar plates. Bacterial colonies of different colour and shape were picked up and amplified, according to Cervino and colleagues (2004). Each selected colony was re-plated twice to ensure complete isolation of the strain and stored at  $-80^{\circ}\text{C}$  in 20% glycerol. A total of 50 colonies were identified. For DNA extraction, isolates were grown overnight in liquid MB medium, aliquots transferred to a 2 ml eppendorf tube, centrifuged and the pellet was re-suspended in 200  $\mu\text{l}$  of milliQ sterile water. Cells were lysed at  $95^{\circ}\text{C}$  for 5 min and the test tubes were centrifuged to separate cell material from supernatant DNA. Two microlitres of DNA from each isolate was used as template for the polymerase chain reaction (PCR) amplification of the 16S rRNA gene. The PCR was carried out using Eubacterial universal primers 27F and 907R (Lane, 1991) as previously described (Danovaro *et al.*, 2006). At each amplification, negative and positive controls were run, using PCR reagents and the reaction mixture without DNA template and genomic purified DNA of *E. coli* (Sigma). Amplified products were screened by agarose gel electrophoresis (0.8% in TBE 1 $\times$ ) and sequenced after cleaning with the ExoSAP-IT $\text{\textcircled{R}}$  kit (USB Corporation). The identity of the isolates was determined by comparison with the NCBI database using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). The percentages reported here were calculated as fraction of colonies corresponding to each morphotype (identified to species level by sequencing) on the total number of colonies grown on agar plates. The 16S rDNA sequences obtained from all sequenced isolates are currently being deposited in the GenBank database.

corals. All microbiological variables displayed significant differences between diseased and healthy corals, but it is worth noticing that the largest differences were observed for *Vibrio* abundance.

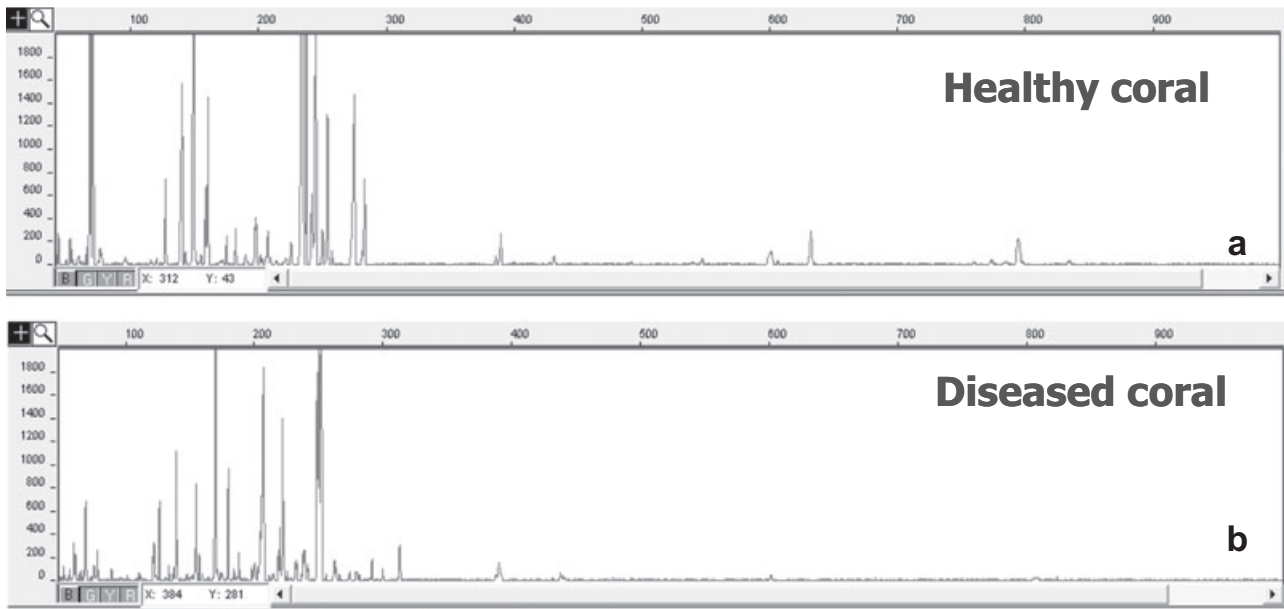
As a subsequent step, in order to better evaluate the possible pathogenic potential of the different species of *Vibrio*, colonies grown on TCBS were further identified to the species level.

#### Colony identification

Representatives of the dominant bacterial morphotypes on MB2216 and TCBS plates were identified using 16S rDNA sequencing. Twenty colonies (10 from healthy and 10 from diseased samples) grown on MB2216 and 30 (10 from healthy and 20 from diseased samples) grown on TCBS agar plates were picked and sequenced. The 16S rDNA sequencing of bacteria grown on MB2216 revealed that all those recovered from healthy corals belonged to the genus *Micrococcus* (Fig. 3A; 98% similarity). Our data are in agreement with those of Wahbeh and Mahasneh (1988), who found that *Micrococcus* was the most abundant bacterial genus associated with four different types of healthy stony corals. These results suggest that the involvement of the genus *Micrococcus* in the RTN disease is unlikely. Accordingly, we show here that diseased corals contained bacterial colonies belonging to other bacterial

genera such as *Bacillus* (86–99% similarity), *Psychrobacter* (99%) and *Kocuria* (99%; Fig. 3C). However, all these genera have been previously reported as typically associated with healthy corals (Rohwer *et al.*, 2001; Bourne and Munn, 2005; Lampert *et al.*, 2006) and the genus *Bacillus* has been already demonstrated to be not directly involved in the yellow blotch/band coral disease (Cervino *et al.*, 2004). All these findings let us hypothesize that these genera are not directly responsible of the observed disease. The 16S rDNA sequencing of bacteria grown on TCBS revealed that most (97%) of those isolated from diseased corals were closely related (99% similarity) to *Vibrio harveyi* strain LB4 (Fig. 3D), whereas this species was never encountered in any of the three healthy samples investigated. Conversely, from healthy corals, on TCBS we isolated only bacteria related to *Exiguobacterium* (98% similarity; Fig. 3B). The isolation of *Exiguobacterium* from healthy specimens is consistent with previous studies reporting the association of this genus with healthy stony corals (Lewis *et al.*, 2004). Keeping together, our results provide compelling evidence that *V. harveyi* was the dominating species in tissues of coral displaying the RTN.

Despite *V. harveyi* has been previously reported to be a serious pathogen for a wide range of marine animals (Austin and Zhang, 2006) and hypothesized as a possible pathogen of corals (Gomez-Gil *et al.*, 2004; Thompson



**Fig. 4.** Terminal-restriction fragment length polymorphism analysis of bacterial diversity. Reported are typical electropherograms obtained for healthy (A) and diseased specimens (B). For T-RFLP analysis, DNA was extracted from frozen coral fragments using the procedure described by Rohwer and colleagues (2001), previously utilized for the analysis of bacterial diversity on corals. Terminal-restriction fragment length polymorphism analysis of the 16S rDNA was carried out using the universal primers 27F (5'-HEX labelled) and 1492r and the enzyme AluI, as described elsewhere in more detail (Danovaro *et al.*, 2006; Luna *et al.*, 2006).

*et al.*, 2004; 2005), to our knowledge this is the first time that this bacterium has been isolated from diseased stony coral specimens.

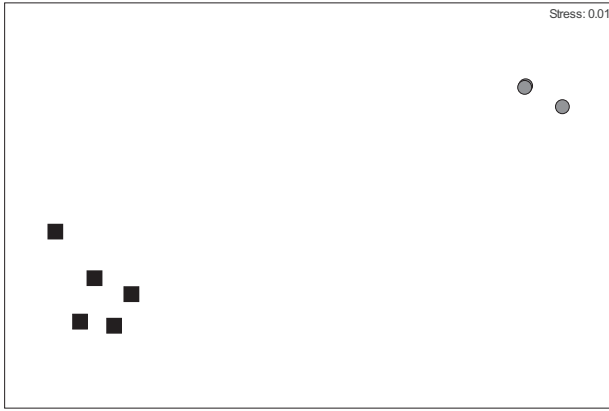
#### *Vibrio harveyi* abundance

To confirm the presence of high abundances of *V. harveyi* on diseased corals, we subsequently performed FISH experiments using a probe directed towards a 16S rRNA sequence specific to *V. harveyi*. The results indicated that diseased corals contained  $1.23 \pm 0.12 \times 10^7$  cells  $\text{cm}^{-3}$  of *V. harveyi*. Conversely, the same analysis, did not evidenced *V. harveyi* in any of the three healthy coral samples. Fluorescent *in situ* hybridization counts of *V. harveyi* on diseased corals accounted for *c.* 69% of counts obtained using the *Vibrio* genus-specific FISH probe. The finding that *V. harveyi* was the dominating *Vibrio* on diseased corals is consistent with the results obtained using cultivation-based methods. In addition, using the same FISH probe, *V. harveyi* was also identified and counted ( $2.54 \pm 0.42 \times 10^6$  cells  $\text{l}^{-1}$ ) in the aquarium seawater, showing an abundance higher than that normally reported in coastal seawater (Ramesh *et al.*, 1989).

#### Bacterial diversity

Bacterial diversity (as richness of bacterial ribotypes) and community composition on diseased and healthy corals (Fig. 4A) were investigated by terminal-restriction frag-

ment length polymorphism (T-RFLP) analysis (Fig. 4A and B). This rapid fingerprinting technique, previously utilized for analysing the biodiversity of complex prokaryotic communities (Danovaro *et al.*, 2006; Luna *et al.*, 2006), has been recently also applied to the characterization of bacterial assemblages on coral samples affected by black band disease (Frias-Lopez *et al.*, 2004), as it allows a putative identification of the dominant bacterial genera (Hewson and Fuhrman, 2004). Terminal-restriction fragment length polymorphism analysis revealed the presence of a significantly (*t*-test,  $P < 0.01$ ) higher number of bacterial ribotypes in diseased (48 ribotypes, as average of the five samples) than in healthy corals (38 ribotypes, as average of the three samples). Terminal-restriction fragment length polymorphism analysis revealed a higher bacterial diversity than that observed by cultivation on agar media; however, both techniques showed a higher diversity of taxa in diseased than in healthy coral specimens. Such a higher bacterial diversity in diseased than in healthy areas has been reported in previous studies on corals displaying other diseases, such as black band disease (Cooney *et al.*, 2002) and plague-like disease (Pantos *et al.*, 2003). The analysis of bacterial community composition also displayed that bacterial ribotypes from diseased and healthy corals were significantly different (ANOSIM,  $P < 0.01$ ). Multi-dimensional scaling ordination analysis clearly distinguished bacterial communities composition of diseased and healthy corals (Fig. 5). According to previous studies, our results



**Fig. 5.** Multi-dimensional scaling (MDS) ordination of bacterial community composition (b). Squares represent the five diseased samples, while circles represent the three healthy ones. On MDS plots, samples possessing the highest similarity in terms of community composition are grouped together. Differences in bacterial richness between diseased and healthy corals were statistically analysed using *t*-test. Differences in bacterial community composition were tested for statistical significance using ANOSIM (Analysis of Similarity), while MDS ordinations were carried out to represent such differences on a 2-D scatter plot. Both ANOSIM and MDS were carried out, on Bray–Curtis similarity values, with the Primer software, as previously described (Luna *et al.*, 2006).

suggest that tissues of diseased corals are rapidly colonized by saprophytic bacteria, so that the higher bacterial diversity observed in diseased areas is probably related to a secondary colonization following a previous attack by *V. harveyi*. However, at present, we cannot exclude that *V. harveyi* itself could be a secondary colonizer and not the primary cause of this disease.

Moreover, we found that a single bacterial ribotype (fragment length: 250 bp), identified as a *Vibrio* sp. using TAP-TRFLP (Marsh *et al.*, 2000), accounted for *c.* 15% of the total assemblage in all diseased corals and for < 5% in the healthy corals. Although T-RFLP does not allow inferring on the actual abundance of these bacteria in coral tissues, these results further confirm the presence of large numbers of vibrios in diseased corals.

## Conclusions

This investigation on healthy and RTN-affected coral tissues, carried out using both culture-dependent and culture-independent techniques, indicated the presence of a high abundance of *V. harveyi* associated with the diseased specimens, suggesting a possible role of this bacterium in the observed disease. Further studies are needed to clarify the ecology of *V. harveyi* and its involvement in the pathogenesis of RTN (i.e. testing the Koch's postulate), as well as to test its antimicrobial susceptibility pattern in order to find potential treatments for curing this coral disease.

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