

# *Vibrio harveyi* as a causative agent of the White Syndrome in tropical stony corals

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## Summary

We investigated bacterial assemblages associated with corals displaying symptoms of the ‘White Syndrome’ (WS), a general term used for indicating the appearance of bands, spots or patches of tissue loss, which is devastating wide areas of tropical ecosystems worldwide. We collected WS-diseased ( $n=15$ ) and healthy ( $n=15$ ) corals from the natural reef (Indonesia, Indian Ocean) and from four large public aquaria. By using culture-dependent and culture-independent techniques, we found that a large fraction (73%) of the investigated WS events was associated with the presence of a high bacterial abundance and, specifically, of *Vibrio* spp. *Vibrio harveyi*, a pathogen of many marine organisms and recently involved in coral Yellow Band disease, was the most represented species, being recovered from five out of 15 diseased corals. In experimental infection assays, two *V. harveyi* strains, isolated from diseased corals, were inoculated on a total of 62 healthy colonies of *Pocillopora damicornis*. WS signs appeared in 57 corals, confirming the ability of *V. harveyi* strains to induce the disease. We conclude that *V. harveyi* is one of the coral pathogens involved in the appearance of WS. However, not all of the investigated WSs were associated to *V. harveyi* detection, nor to other *Vibrio* species (such as *V. coralliilyticus*), which supports the hypothesis that WS is not caused exclusively by *Vibrio* spp., but rather can have a multifactorial aetiology, or can represent a group of diseases caused by a variety of agents. Further investigations

to identify specific virulence traits will contribute to the understanding of the role of *V. harveyi* in WS pathogenesis.

## Introduction

Increasing evidence indicates that stony corals are degraded, as a result of synergistic impacts of overfishing, increases in CO<sub>2</sub> levels, sea surface temperature warming, eutrophication, sedimentation and pollution (Hughes *et al.*, 2003; Weil *et al.*, 2006; Hoegh-Guldberg *et al.*, 2007; Anthony *et al.*, 2008). Reports on coral diseases continue to rise, with currently 29 reported disease and syndromes in the Caribbean and 7 reported from the Indo-Pacific (Sussman *et al.*, 2008). Many of them have been associated with bacterial infections (Harvell *et al.*, 2002; Denner *et al.*, 2003; Ben-Haim *et al.*, 2003; Barash *et al.*, 2005; Cervino *et al.*, 2008); however, the role of bacteria as primary infectious agents has been recently questioned (Lesser *et al.*, 2007; Work *et al.*, 2008).

In the last decade, the term White Syndrome (WS; Bythell *et al.*, 2004; Willis *et al.*, 2004) has been associated with a range of different diseases including ‘White Band’, ‘White Plague’ and ‘Shut Down Reaction’ (Ainsworth *et al.*, 2007) and a variety of WS outbreaks has been reported from several tropical areas, under various names (Bruno *et al.*, 2007; Sussman *et al.*, 2008). The term has been initially introduced to address difficulties in relating white signs of unknown aetiology in the Indo-Pacific to a multitude of white diseases observed in the Caribbean (Bythell *et al.*, 2004). WS include diseases manifesting as white bands, spots or patches as a consequence of tissue loss exposing white skeleton. The white ‘band’ or ‘area’, after which these syndromes are named, is the result of the rapid sloughing of coral tissue (Bythell *et al.*, 2004). Their typical features include a rapid tissue loss (rates of loss varying from 1 to 100 cm<sup>2</sup> day<sup>-1</sup>) and a clear lesion boundary between apparently healthy tissue and exposed skeleton. Previous studies provided contrasting results about the possible role of bacteria in the development of WS (Ainsworth *et al.*, 2007; Sussman *et al.*, 2008; 2009), which highlights the need for more extensive investigations. WS-like events, well documented in the field, are now apparently evident also in aquaria (Hörmansdorfer *et al.*, 2000; Luna *et al.*, 2007), where live stony corals are maintained for ornamental,

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educational, scientific or biomedical purposes (Borneman and Lowrie, 2001; Clode and Marshall, 2003). Diseased corals in aquaria displaying WS-like symptoms are also referred to as affected by Rapid or Slow Tissue Necrosis (RTN or STN) or Shut Down Reaction (SDR; Borneman, 2001). Recent studies in wild or aquaria have observed a relationship between the appearance of WS and the presence of *Vibrio* species (Luna *et al.*, 2007; Sussman *et al.*, 2008).

In order to confirm the role of vibrios in WS development in stony corals, we collected several specimens displaying the typical symptoms of the disease, from both natural reefs and aquaria. To do this, we conducted both culture-dependent and culture-independent (FISH) analyses, coupled with SEM (Scanning Electron Microscopy) observations and followed by inoculation trials to test the Koch's postulates for the most frequently recovered bacterial strains, according to Sussman and colleagues (2008).

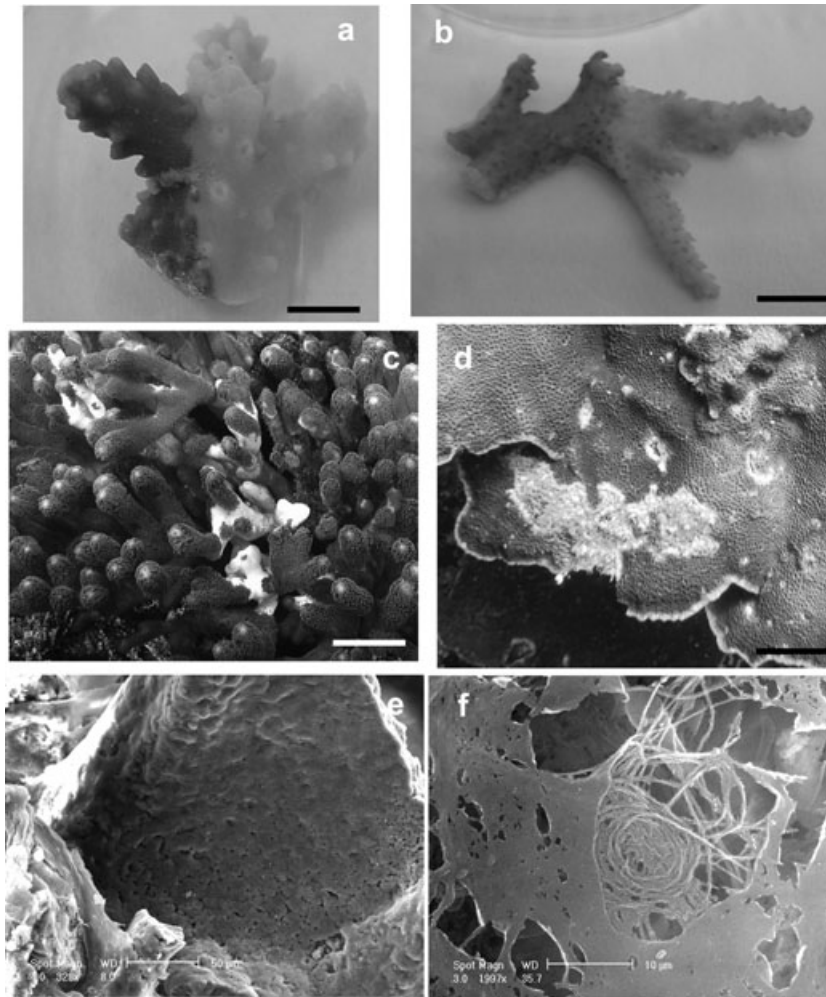
## Results and discussion

All of the investigated diseased corals displayed symptoms of tissue loss corresponding to those described by Work and Aeby (2006) as typical of WS. Out of the 15 corals investigated, the six diseased *Acropora* samples (from either aquaria and reef) revealed consistent symptoms, i.e. a large area of tissue loss (starting from the base or the tip), distinctly separated from intact tissue and revealing an intact skeleton (Fig. 1A and B). In some cases, the nude skeleton appeared green due to algal coverage (data not shown). The *Stylophora pistillata* colony from Aquarium 2 displayed multifocal and apical patches of tissue loss (Fig. 1C), while the *Pocillopora damicornis* from Aquarium 1 and the *Seriatopora histrix* from Aquarium 3 displayed multifocal and basal tissue loss, which moved fast to the apical portion of the colony. Finally, all the *Montipora* and *Echinopora* colonies (either from aquarium or reef) displayed one or more peripheral areas of tissue loss (Fig. 1D), revealing white intact skeleton. Detailed visual inspections of the immediate surroundings of the corals did not reveal the presence of snails or other coral predators, which could have been responsible for the tissue loss.

All of the WS-affected corals analysed by SEM revealed the presence of necrotic tissue (Fig. 1F). In the same samples, the presence of bacterial cells on the tissue surface was often observed. Conversely, SEM analysis of healthy samples provided evidence of an healthy-looking tissue (Fig. 1E), with sometimes sparse adherent material on the surface, such as flocs of detritus, elongate ropes made up of multiple flocs or filaments reminiscent originating from other marine organisms, as already reported in healthy corals (Johnston and Rohwer, 2007).

Epifluorescence microscopy analyses indicated that 11 diseased specimens (seven from the aquaria and four from the reef), corresponding to 73% of all samples analysed, displayed a significantly higher abundance of total prokaryotes when compared with healthy specimens (Fig. 2A and B; ANOVA,  $P < 0.01$ ). When compared with healthy corals, the same 11 diseased corals displayed a significantly higher *Vibrio* spp. abundance, as determined by the FISH approach (Fig. 2C and D; ANOVA,  $P < 0.01$ ). These findings let us to hypothesize a possible association between the presence of high abundances of *Vibrio* and tissue lesions. The abundance of aerobic culturable bacteria displayed the same patterns of total prokaryotic abundance, with significantly higher values on diseased than on healthy corals (Table 1; ANOVA,  $P < 0.01$ ). Culturable bacterial abundance from corals in both wild reefs and aquaria was on average four and 13 times higher on diseased than on healthy corals respectively. Such a higher bacterial abundance on diseased corals is consistent with previous studies (Luna *et al.*, 2007; Sussman *et al.*, 2008).

Identification of the most abundant bacterial colonies formed on MA2216 and TCBS agar revealed a wide diversity of species on both healthy and diseased tissues. Culturable bacteria from healthy corals belonged to the genera *Acinetobacter*, *Bacillus*, *Corynebacterium*, *Kocuria*, *Micrococcus*, *Pseudomonas*, *Psychrobacter*, *Roseomonas*, *Salinibacter*, *Shewanella* and *Vibrio*. Most of these bacterial genera have been reported to be associated with mucus and tissues of stony corals (Kooperman *et al.*, 2007; Cervino *et al.*, 2008), where they are believed to play an important role in the holobiont physiology and health. Bacteria isolated from diseased corals belonged to the genera *Acinetobacter*, *Aerococcus*, *Agrococcus*, *Arthrobacter*, *Bacillus*, *Knoellia*, *Jeotgaliococcus*, *Microbacterium*, *Micrococcus*, *Pseudomonas*, *Paracoccus*, *Staphylococcus*, *Shewanella* and *Vibrio*. The number of different bacterial genera was higher on diseased than on healthy corals, as already reported (Luna *et al.*, 2007; Sunagawa *et al.*, 2009). Culturable *Vibrio* spp. were only rarely isolated from healthy coral tissues. Conversely, in five of the 15 diseased corals (two from aquaria and three from the wild; Fig. 2C and D), *Vibrio harveyi* was abundant and even dominant (up to 100% of isolates on TCBS agar). It is worth noting that this *Vibrio* species was exclusively associated with diseased tissues, as previously observed for *Pocillopora damicornis* (Luna *et al.*, 2007). The five corals colonies from which *V. harveyi* was isolated were the *Pocillopora damicornis* from Aquarium 1 and four *Acropora* colonies (Fig. 2C and D), collected from Aquarium 3 and from the three reef sites. The lesions observed in the four *Acropora* were similar, but different from those observed in the *P. damicornis* colony. These findings indicate that *V. harveyi* could be associated with

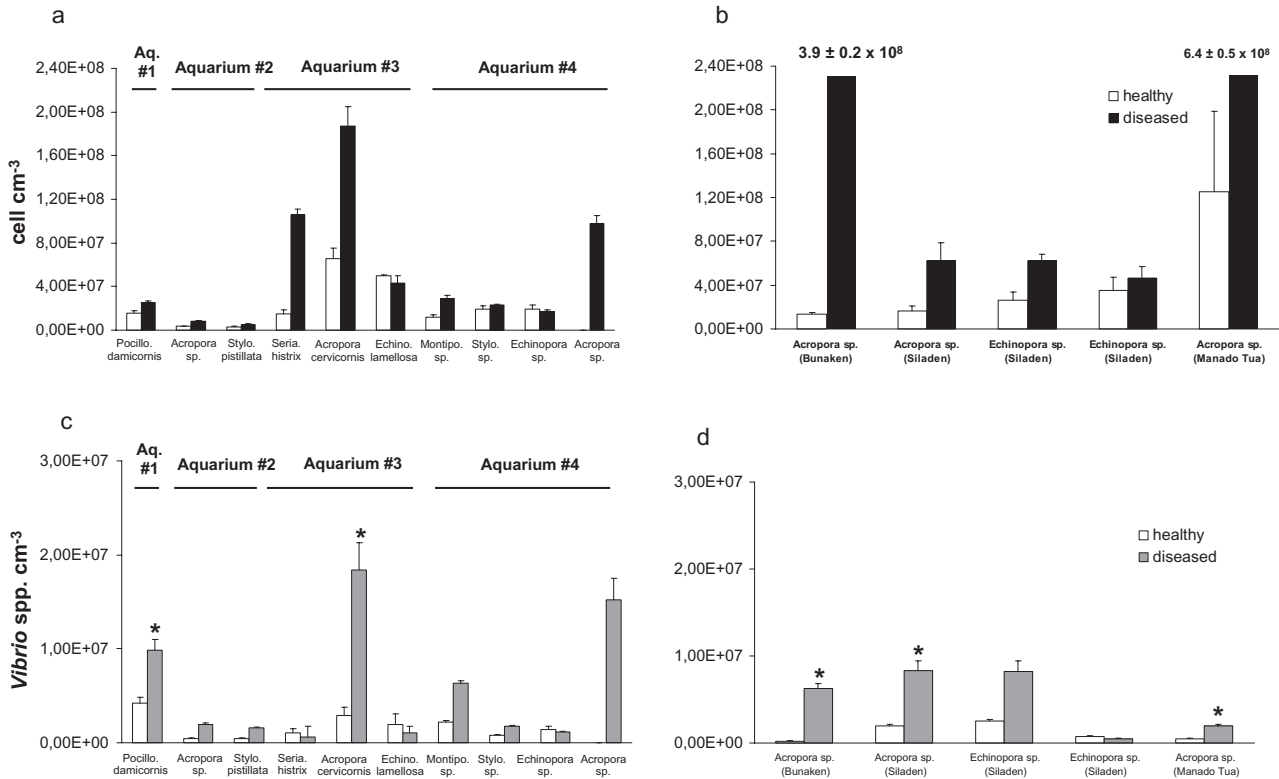


**Fig. 1.** Lesions occurring on the affected corals (A–D) and Scanning Electron Microscopy (SEM) of healthy (E) and diseased (F) tissues. (A) *Acropora* from Aquarium 3; (B) Wild *Acropora* sp. from Bunaken; (C) *Stylophora pistillata* from Aquarium 2; (D) Wild *Echinopora* sp. from Bunaken (Indonesia). For SEM analyses, small fragments of corals were washed to remove the mucus, processed as previously described (Luna *et al.*, 2007) and observed by a Philips XL20 SEM. Diseased Indo-Pacific corals (five colonies), collected in August 2007 (depths between 1 and 3 mt) at reefs located in Bunaken, Manado Tua and Siladen (Indonesia), were represented by three large tabular *Acropora* sp. and two plate *Echinopora* sp. The water temperature was 28°C. Aquarium diseased corals (10 colonies) were collected from four large public aquaria, located in Italy (Aquarium 1), France (Aquariums 2 and 4) and Portugal (Aquarium 3). From Aquarium 1, we collected samples from a *Pocillopora damicornis* colony, hosted in a 20 m<sup>3</sup> tank having the following characteristics: temperature 26°C, pH 8.1, salinity 36 PSU, oxygen concentration 6.0–6.3 mg l<sup>-1</sup>, nitrates 8–11 mg l<sup>-1</sup>, nitrites 0–3 mg l<sup>-1</sup>, ammonium 0–5 mg l<sup>-1</sup>, phosphate 0.8–1.1 mg l<sup>-1</sup> and calcium 400–420 mg l<sup>-1</sup>. From Aquarium 2, we collected samples from a large *Acropora* sp. and a large *Stylophora pistillata*, which were both hosted in a large 60 m<sup>3</sup> tank (temperature 25°C, pH 7.7–7.9, salinity 34–35 PSU, oxygen concentration 6–8.5 mg l<sup>-1</sup>, nitrates 1 mg l<sup>-1</sup>, nitrites and ammonia absent, phosphates 0.04 mg l<sup>-1</sup> and calcium 400 mg l<sup>-1</sup>). From Aquarium 3, we collected samples from a large *Acropora cervicornis*, a *Seriatopora hystrix* colony and a plate *Echinopora lamellosa*, hosted in a 3 m<sup>3</sup> tank (temperature 24–25.5°C, pH 8.15–8.33, salinity 32–34 PSU, oxygen concentration 6.5–7.5 mg l<sup>-1</sup>, nitrates 0–0.5 mg l<sup>-1</sup>, nitrites and ammonia 0–0.1 mg l<sup>-1</sup>, phosphates 0–0.01 mg l<sup>-1</sup> and calcium 325–400 mg l<sup>-1</sup>). Finally, from Aquarium 4, we collected samples from small colonies of *Montipora* sp., *Echinopora* sp., *Stylophora* sp. and a large tabular *Acropora* sp. The colonies were maintained in a tank at temperature 25°C, pH 8.1–8.3, salinity 33–34 PSU, oxygen concentration 6.5–7 mg l<sup>-1</sup>, nitrates 0–0.5 mg l<sup>-1</sup>, nitrites and ammonia absent, phosphates 0–0.01 mg l<sup>-1</sup> and calcium 325–370 mg l<sup>-1</sup>. Diseased corals were sampled at the border between exposed skeleton and healthy tissue. For each diseased coral, 'control' samples were collected from healthy colonies (i.e. corals not displaying symptoms) from the same site (i.e. the same reef for wild corals or the same tank for aquarium corals) or, alternatively, from areas of the same colony not displaying disease signs of the disease. From all corals, each sample was collected in triplicate from different lesions or different areas of the healthy colony. Scale bar (approx.): (A) and (B) = 1 cm, (C) = 4 cm, (D) = 20 cm.

different types of lesions and different environments (aquarium and reef).

To confirm *V. harveyi* identification, crushed material from the same five samples were plated on VH agar, a

selective and differential medium specifically developed for this species (Harris *et al.*, 1996). The colonies displaying the typical morphology described for *V. harveyi* (diameter 2–5 mm, light green with dark green centers



**Fig. 2.** Abundance of tissue-associated total prokaryotes on aquarium (A) and wild corals (B) and total *Vibrio* spp. on aquarium (C) and wild corals (D). Total abundance of prokaryotes was estimated under epifluorescence microscopy on crushed corals using Sybr Green I as described by Koren and Rosenberg (2006) and Luna and colleagues (2007). Briefly, coral fragments were washed twice with 5 ml of Artificial Autoclaved Sea Water (AASW) to remove the mucus and fixed with 5 ml of 4% formalin in AASW, previously buffered with sodium tetraborate (20 g l<sup>-1</sup> of formaldehyde) and sterilized using a 0.2 µm syringe filter. Before fixing, each coral fragment was weighted and its size measured (as maximum length, width and height) using a vernier calliper and under sterile conditions to avoid contamination. The crushing process consisted in a mechanical disruption of each sample using a sterile mortar and pestle, then vortexing for 1 min at maximum speed. Once crushed, each suspension was left for 1 min (to allow sedimentation of the coral material), and then small aliquots collected from the upper phase of the liquid were diluted using AASW and filtered through 0.2 µm Anodisc filters. Cells were stained by putting each filter into a Petri dish containing a drop (20 µl) of Sybr Green I solution (Molecular Probes) diluted 1:20 in sterile milliQ water. Filters were incubated for 20 min in the dark, and then washed twice (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 to achieve an appropriate statistical significance. Prokaryotic abundance was expressed as cells cm<sup>-3</sup> of crushed coral sample. The estimates were expressed as an average of all counted replicates plus a standard deviation. Fluorescence *in situ* hybridization (FISH) analyses were carried out for the enumeration of total *Vibrio* spp. using the probe 'GV' (Eilers *et al.*, 2000) and for the specific enumeration of *Vibrio harveyi* using the probe 'VH-2' targeting the 16S rRNA of *V. harveyi*, as described by Oakey and colleagues (2003) and Luna and colleagues (2007). Coral tissues crushed 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 Cy3-labelled probes, counterstained with DAPI (0.5 µg ml<sup>-1</sup>) and counted under epifluorescence microscopy. Positive and negative bacterial controls were obtained using the probes EUB338 and NON338 respectively (Pernthaler *et al.*, 2001). Differences in the investigated bacterial parameters between diseased and healthy corals were statistically analysed using one-way analysis of variance (ANOVA). The asterisk '\*' indicates diseased samples characterized by the presence of *V. harveyi*. As for total prokaryotic counts, estimates were expressed as an average of all counted replicates plus a standard deviation.

and showing a yellow halo) were randomly analysed by biochemical tests, which confirmed their *V. harveyi* identity. To further validate these results and to provide information on the abundance of *V. harveyi* on the five diseased corals, additional FISH analyses were carried out on crushed coral samples using the 'VH-2' *V. harveyi* species-specific probe (Luna *et al.*, 2007). The results of these analyses, when compared with those performed with the general *Vibrio* spp. probe, indicated that *V. harveyi* accounted from 65 to 82% of the total *Vibrio*. More in detail, *V. harveyi* abundance was

6.34 ± 1.24 × 10<sup>6</sup> cells cm<sup>-3</sup> on the *P. damicornis* (Aquarium 1), 1.25 ± 0.84 × 10<sup>7</sup> cells cm<sup>-3</sup> on the *A. cervicornis* (Aquarium 3), 5.12 ± 1.24 × 10<sup>6</sup> cells cm<sup>-3</sup> on the *Acropora sp.* from Bunaken, 6.35 ± 1.12 × 10<sup>6</sup> cells cm<sup>-3</sup> on the *Acropora sp.* from Siladen and 1.30 ± 0.24 × 10<sup>6</sup> cells cm<sup>-3</sup> on the *Acropora sp.* from Manado Tua. Both approaches (culture-dependent and molecular) confirmed the presence of a high abundance of *V. harveyi* on all the five diseased corals. Conversely, *Vibrio coralliilyticus*, previously reported as causative agent of bleaching in *Pocillopora damicornis* (Ben-Haim

**Table 1.** The abundance of culturable bacteria was estimated by plating crushed coral tissues on MA2216 (for aerobic heterotrophic bacteria).

Aquarium	Coral sample	Healthy		Diseased	
		cfu cm <sup>-3</sup>	± SD	cfu cm <sup>-3</sup>	± SD
1	<i>Pocillopora damicornis</i>	5,38E+04	6,66E+03	3,45E+06	8,34E+05
2	<i>Acropora</i> sp.	5,99E+03	1,42E+02	1,09E+04	2,41E+03
2	<i>Stylopora pistillata</i>	4,17E+03	5,13E+02	6,18E+03	4,21E+02
3	<i>Seriatopora histrix</i>	2,35E+03	6,30E+01	5,90E+02	1,21E+02
3	<i>Acropora cervicornis</i>	1,84E+03	2,14E+02	5,73E+04	0,00E+00
3	<i>Echinopora lamellosa</i>	4,23E+03	1,42E+03	3,58E+03	7,55E+02
4	<i>Montipora</i> sp.	1,33E+05	2,77E+04	2,31E+05	2,32E+04
4	<i>Stylophora</i> sp.	1,89E+05	4,87E+04	2,85E+05	5,43E+04
4	<i>Echinopora</i> sp.	2,84E+05	5,30E+04	3,72E+05	1,87E+04
4	<i>Acropora</i> sp.	–	–	5,14E+06	6,79E+05
Reef					
Bunaken (Indonesia)	<i>Acropora</i> sp.	1,63E+05	2,40E+04	7,54E+05	4,24E+04
Siladen (Indonesia)	<i>Acropora</i> sp.	5,34E+04	8,07E+03	4,05E+05	8,79E+04
Siladen (Indonesia)	<i>Echinopora</i> sp.	1,35E+05	3,52E+04	3,41E+05	7,45E+04
Siladen (Indonesia)	<i>Echinopora</i> sp.	3,66E+04	2,01E+03	7,23E+04	1,53E+04
Manado Tua (Indonesia)	<i>Acropora</i> sp.	2,41E+04	4,25E+03	1,06E+05	1,52E+04

In addition, crushed coral samples were plated on TCBS agar for *Vibrio* spp. isolation. Briefly, small pieces of corals were measured (as described above), washed twice with 5 ml of AASW, crushed and vortexed for 1 min. The obtained suspensions were serially 10-fold diluted in AASW and 100 µl aliquots plated in triplicate, followed by aerobic incubation at 28°C for 48–96 h (for MA2216) and 30°C for 24–48 h (for TCBS). The most abundant morphotypes (sharing the same colour and shape of the colony), as assessed also using a standard stereomicroscope (Cervino *et al.*, 2004), were then isolated from the plates. Each selected colony was streaked twice, grown in Marine Broth and stored at –80°C in 20% glycerol. For phylogenetic identification, isolates were grown overnight in Marine Broth 2216, the crude DNA extracted and used as template for the PCR amplification of the 16S rRNA gene. The PCR was carried out using bacterial universal primers 27F/907R and the purified amplicons sequenced as described (Luna *et al.*, 2007). A total of 120 colonies were identified. Identification was performed by comparing the obtained sequences with those deposited in the NCBI database using BLAST (<http://blast.ncbi.nlm.nih.gov/>). We used, as cut-off for species identification, a homology equal or more than 99%. Moreover, for species identification of *Vibrio* isolates, additional analyses were performed, which included standard morphological analyses, physiological (growth and morphology on the VH medium; Harris *et al.*, 1996) and biochemical assays (API 20E galleries, Biomerieux). –, sample not available.

*et al.*, 2003) and WS in Indo-Pacific corals (Sussman *et al.*, 2008) and *Vibrio alginolyticus*, previously associated with tissue necrosis in several aquarium *Acropora* (Hörmansdorfer *et al.*, 2000), were never isolated in this study.

In the five samples displaying the presence of culturable *V. harveyi*, it always accounted for the most abundant culturable bacterial population, indicating a dominance over all other bacterial species. In two of these samples (two *Acropora* sp., one from Bunaken and one from Manado Tua), it represented the only culturable *Vibrio*, and in the remaining three (samples), even if other vibrios could be isolated (such as *Vibrio splendidus* and *Vibrio littoralis*), its abundance was markedly higher.

Two *V. harveyi* isolates from the diseased *P. damicornis* from Aquarium 1 (namely isolates 4 and 6) were subsequently used to verify the Koch's postulates in the framework of three separate inoculation experiments, whose results are summarized in Table 2. Coral colonies exposed to the two strains (at concentration of 1 × 10<sup>6</sup> cells ml<sup>-1</sup>) consistently displayed WS symptoms (Table 2 and Fig. S1). Conversely, un-inoculated corals did not develop any sign of disease (Fig. S1). *Vibrio harveyi* (displaying 100% 16S rRNA gene sequence identity to the inoculated strains) could be re-isolated from all the inoculated corals displaying the symptoms (Fig. S2).

SEM analyses on infected corals provided evidence for lysing tissue and high numbers of bacterial cells adhering to the tissue surface (Fig. S3). Thus, the Koch's postulate was fulfilled and the involvement of *V. harveyi* in the development of the WS was unequivocally demonstrated. *Vibrio harveyi* is a potentially important pathogen of many marine organisms, such as fishes (e.g. flounders, groupers, sharks, seabream, seabass and turbot; Gauger *et al.*, 2006), molluscs and prawns (Nicolas *et al.*, 2002; Alavandi *et al.*, 2006). It has been recently included in the *Vibrio* core group responsible for the Yellow Band disease in stony corals of the Caribbean and Indo-Pacific (Cervino *et al.*, 2008). In addition, Gil-Agudelo and colleagues (2007) reported the unique association of *V. harveyi* with dark-spot diseased corals. This is, however, the first time that the fulfillment of the Koch's postulates unequivocally demonstrates the role of *V. harveyi* in the development of the WS in tropical stony corals.

Despite this, of the 11 diseased corals showing high abundances of *Vibrio* spp., only five were characterized by the presence of *V. harveyi*. Within the remaining six, the diseased *Acropora* sp. from Aquarium 4 was dominated (> 90% of colonies) by an hypothetical *Vibrio* sp. sharing only 95% 16S rDNA sequence identity with other *Vibrio*; two corals (a *Montipora* sp. from Aquarium 4 and a wild *Echinopora* sp. from Siladen) were characterized by

**Table 2.** Results summarizing the inoculation experiments, carried out at the facilities of Acquario di Genova (Italy).

Experiment	Experiment 1	Experiment 2	Experiment 3
Period	23 April–21 May 2007	16–22 September 2008	21 October–14 November 2008
Number of infected colonies	12	20	30
<i>V. harveyi</i> strains inoculated	4	4 and 6	4
Colonies showing symptoms	9/12	20/20	28/30
First symptoms appearance	After 7 days	After 2 days	After 2 days
Infectivity (%) <sup>a</sup>	75	100	93

a. 'infectivity' was calculated as described by Sussman and colleagues (2008).

Prior to bacterial inoculation, coral fragments were acclimatized for 5 days to allow recovery from handling. In Experiment 1, *Vibrio harveyi* was added to two 25 l tanks, each containing 6 coral colonies not displaying WS symptoms. The final inoculum concentration in each tank was  $1 \times 10^6$  cells ml<sup>-1</sup>. The bacterial inoculum was grown at 30°C in Marine Broth 2216 up to exponential growth phase. Cell density was determined by plating triplicates of serial dilutions on agar plates and/or by measuring 600 nm absorbance. Each cell culture was resuspended in 5 ml of sterile sea water prior bacterial inoculation into the aquarium water. Only in Experiment 1, an alternative way of bacterial inoculum (i.e. using sterile cotton balls squares, soaked into the bacterial suspension and cabled tied to the base of the corals without touching the living tissue). Each of the inoculation treatments included a negative control tank, which contained 6 coral colonies with no bacteria added. Each tank contained an immersion heater (for temperature control) and an airline with a small airstone bubbler to provide water circulation. Inoculated and control tanks were maintained at the same temperature (25–26°C) and light intensity throughout the experiment. Corals were monitored and/or photographed on a daily basis, in order to detect and record the development of disease signs. The experiments were terminated following mortality of corals in the infection tanks or, alternatively, after 1 month. At the end, infected and non-infected coral fragments were crushed to check the presence/abundance of *Vibrio harveyi* using TCBS and VH agar and 16S rDNA sequencing. In the Experiment 2, two *V. harveyi* isolates were tested (4 and 6) on a more abundant set of colonies (two 25 l inoculation tanks, 10 *Pocillopora damicornis* in each tank). Experimental set-up, controls tanks and analysis/interpretation of symptoms were the same as described in Experiment 1. In the Experiment 3 (October 2008), only *V. harveyi* isolate 4 was used to infect 30 *P. damicornis* colonies, hosted in 7 l tanks, each containing 5 colonies. Experimental conditions and set-up were identical to previous experiments, including a negative control tank (no inoculum) containing 5 coral colonies.

*Vibrio splendidus* and *Vibrio littoralis* while, from the remaining three specimens, no tissue-associated *Vibrio* isolate could be obtained. Such a discrepancy in *Vibrio* spp. cultivation versus FISH enumeration could be explained by the presence of vibrios in an unculturable state, already reported in coral-associated bacteria (Koren and Rosenberg 2006; Luna *et al.*, 2007).

Our results indicate that *V. harveyi* acts as a causative agent of WS in stony corals living in both aquaria and natural reefs. However, the inability of identifying this bacterium in all of the diseased corals also suggests that WS can be caused by other (non *Vibrio*) pathogens, as well as by other stress factors (Sussman *et al.*, 2009). In fact, it cannot be excluded that, in those diseased corals in which we failed to identify *Vibrio* pathogens, the WS could be due to a programmed cell death induced by non-infective causes (Ainsworth *et al.*, 2007). Our results, in agreement with recent findings (Sussman *et al.*, 2009), support the hypothesis of a multifactorial aetiology of WS and suggest that the 'White Syndrome' definition may include a group of different diseases caused by a variety of infectious agents. At the same time, an important role of *V. harveyi*, possibly triggered by specific environmental or physiological conditions, can be hypothesized. The pathogenicity of *V. harveyi* can be the result of the expression of still-undescribed virulence factors, likely involved in coral tissue adhesion and proteolysis and responsible for their necrosis. Further investigations aimed at identifying specific virulence factors will contribute to the understanding of the role of *V. harveyi* in WS pathogenesis.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** *Pocillopora damicornis* colonies un-inoculated (A) and inoculated (B) with *V. harveyi* in aquarium experiments and photographed after 2 days from the inoculum. (C) is a zooming on an infected colony at the end of the experiment and shows the multifocal and basal tissue loss, which moved fast to the apical portion of the colony. All images refer to the Experiment 2 (Table 2). Scale bars dimensions (approximately): (A) and (B) = 3 cm, (C) = 1 cm.

**Fig. S2.** Cultures on TCBS plates of un-inoculated ('control') and inoculated *P. damicornis* samples, showing the absence

of growth and a high abundance of isolates (respectively). Biochemical (API galleries) and genetic (16S rDNA sequence analysis) assays confirmed that the bacterial isolates corresponded to the inoculated *V. harveyi* strain.

**Fig. S3.** SEM images displaying *P. damicornis* corals after experimental infection with *Vibrio harveyi*. (A) Necrotic coral tissue; (B) and (C) Zooming at different magnifications, revealing the lysing tissue (B) and a high number of bacterial cells adhering to the tissue surface (C).

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