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ORIGINAL ARTICLE

New insights into *Oculina patagonica* coral diseases and their associated *Vibrio* spp. communities

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Bleaching of *Oculina patagonica* has been extensively studied in the Eastern Mediterranean Sea, although no studies have been carried out in the Western basin. In 1996 *Vibrio mediterranei* was reported as the causative agent of bleaching in *O. patagonica* but it has not been related to bleached or healthy corals since 2003, suggesting that it was no longer involved in bleaching of *O. patagonica*. In an attempt to clarify the relationship between *Vibrio* spp., seawater temperature and coral diseases, as well as to investigate the putative differences between Eastern and Western Mediterranean basins, we have analysed the seasonal patterns of the culturable *Vibrio* spp. assemblages associated with healthy and diseased *O. patagonica* colonies. Two sampling points located in the Spanish Mediterranean coast were chosen for this study: Alicante Harbour and the Marine Reserve of Tabarca. A complex and dynamic assemblage of *Vibrio* spp. was present in *O. patagonica* along the whole year and under different environmental conditions and coral health status. While some Vibrio spp. were detected all year around in corals, the known pathogens *V. mediteranei* and *V. coralliilyticus* were only present in diseased specimens. The pathogenic potential of these bacteria was studied by experimental infection under laboratory conditions. Both vibrios caused diseased signs from 24 °C, being higher and faster at 28 °C. Unexpectedly, the co-inoculation of these two *Vibrio* species seemed to have a synergistic pathogenic effect over *O. patagonica*, as disease signs were readily observed at temperatures at which bleaching is not normally observed.

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Introduction

Over the past two decades, the increase in prevalence and severity of coral disease outbreaks has seriously impacted reef-building corals throughout the oceans worldwide (Goreau *et al.*, 1998; Harvell *et al.*, 1999). The most severe disease affecting coral reefs is bleaching, which is the loss of colour from affected corals caused by the disruption of symbiosis between coral hosts and photosynthetic microalgal endosymbionts, known as zooxanthellae (Brown, 1997). Since the early 1980s, mass coral bleaching events have increased in extent and frequency and have been directly correlated with increasing sea temperatures (Hoegh-Guldberg, 1999). This rise of global average temperatures has been about 0.74 °C in the later 100 years and approximately 0.13 °C/decade over the past 50 years (Salomon *et al.*, 2007). Environmental changes associated with climate warming have been linked to an expansion of coral pathogen ranges and to changes in host susceptibility as a result of increasing environmental stress (Harvell *et al.*, 2007). Several putative agents have been identified as the cause of mortality outbreaks worldwide, including eight coral pathogens that have been implicated in the onset of coral disease lesions. Half of these pathogens belong to the Vibrionaceae family (Rosenberg *et al.*, 2007).

In the Northwestern Mediterranean Sea, two large mass mortality events of sessile epibenthic invertebrates were recorded in 1999 and 2003 (Cerrano *et al.*, 2000; Garrabou *et al.*, 2009) and two less marked events in 2006 and 2008

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(Bensoussan *et al*., 2010; Vezzulli *et al*., 2010; Huete-Stauffer *et al*., 2011). This increasing of mass mortality of marine life may be linked with the warming trend in the Mediterranean Sea (Garrabou *et al*., 2009; Vargas-Yañez *et al*., 2010), where temperatures have risen from 1982 to 2005 at an average rate of $0.04 \pm 0.01\,^{\circ}$C/year (Diaz-Almela *et al*., 2007). Together with the temperature, microbial pathogens have been associated with coral mass mortality episodes. For example, *Vibrio coralliilyticus* was linked to the disease of the purple gorgonian *Paramuricea clavata* (Ben-Haim *et al*., 2003; Bally and Garrabou, 2007; Vezzulli *et al*., 2010), being upregulated at higher temperatures (Kimes *et al*., 2012) and other *Vibrio* species with diseases of *Oculina patagonica* (see below). These two *Vibrio* spp. have been retrieved from *Pocillopora damicornis*, a major reef-building coral found throughout the Great Barrier Reef, suggesting that they may be opportunist pathogens (Bourne and Munn, 2005). The scleractinian coral *O. patagonica* is a putative alien species in the Mediterranean Sea where it was observed for the first time in 1966, on the Ligurian coast of Italy (Zibrowius, 1974). In the Spanish coast, it was first recorded in the Alicante Harbour in 1973 (Zibrowius and Ramos, 1983; Zibrowius 1992), and nowadays it is widely spread from Algeciras to Catalonia and the Balearic Islands (Zibrowius and Ramos, 1983; Ramos, 1985; Ballesteros, 1998; Izquierdo *et al*., 2007; Coma *et al*., 2011; Serrano *et al*., 2012, 2013; Rubio-Portillo *et al*., 2014). Bleaching of *O. patagonica* has been studied extensively, although there is a considerable controversy on the nature of its principal cause, due to the different existing hypothesis about whether microorganisms have a role or not in the bleaching process. *O. patagonica* bleaching was first observed along the Israeli coastline in the summer of 1993 and *Vibrio shilonii* (originally spelled *shiloni*), a later synonym of *Vibrio mediteranei* (Thompson *et al*., 2001), was identified as the causative agent of the disease (Kushmaro *et al*., 1996, 1997). In this case, increased seawater temperature was related to virulence, as this was the environmental factor that triggered the disease (Kushmaro *et al*., 1998). The adhesion of the pathogen to the coral mucus layer (Banin *et al*., 2001a), the production of toxin that inhibits algal photosynthesis (Banin *et al*., 2001b) and the expression of superoxide dismutase that protects *Vibrio* from oxidative stress into coral (Banin *et al*., 2003) are all temperature-dependent reactions. Accordingly, the infection occurred during summer (water temperatures from 25–30 °C), but not in winter (16–20 °C). Sussman *et al*. (2003) demonstrated that the marine fireworm *Hermodice carunculata* was a winter reservoir and spring–summer vector for *V. mediteranei*.

Although *O. patagonica* bleaching has continuously occurred in the Mediterranean sea during the summer months, *V. mediterranei* has not been related to bleached or healthy colonies of this coral since 2003 (Reshef *et al*., 2006) nor has caused experimental infection in aquaria. In fact, Ainsworth *et al*. (2008) stated that *V. mediteranei* was not involved or was not the primary cause of the annual bleaching of *O. patagonica* in the Eastern Mediterranean. These authors stated that bacteria do not have a primary but rather a secondary role during coral bleaching because of an increase in susceptibility to microbial attack experienced by corals during environmental stress. However, a recent study by (Mills *et al*., 2013), carried out in Israel, suggest that even though *O. patagonica* might have developed resistance to infection by *V. mediteranei*, bacteria are responsible for bleaching, being *V. coralliilyticus* one possible causative agent.

In order to shed some light about the relationship between *Vibrio* spp., seawater temperature and coral diseases, we have carried out an analysis of seasonal patterns of abundance and diversity in *Vibrio* spp. communities associated with healthy and diseased *O. patagonica* colonies. Two sampling points located in the Western Mediterranean sea were chosen for this study: Alicante Harbour and the Marine Reserve of Tabarca. Our results provide support for the bacterial bleaching hypothesis, showing that two species of *Vibrio*, namely *V. mediteranei* and *V. coralliilyticus*, are related to natural diseases of *O. patagonica*. The pathogenic potential of these two infectious bacteria was further investigated under laboratory conditions. Tissue damage increased with temperature and, remarkably, the co-inoculation of these two *Vibrio* species seemed to have a synergistic pathogenic effect over *O. patagonica*, as disease signs were readily observed at 20 °C.

Material and methods

Sample collection and environmental variable measurements

Samples were taken from two different locations in Alicante coast (Spain), separated by a distance of 22 kilometres: Alicante Harbour (38°20′11.1″N, 00°29′11.8″9 W) and the Marine Reserve of Tabarca in Alicante Bay (38°09′59″N, 0°28′56″W). Nine fragments of *O. patagonica* and three samples of surrounding water were randomly collected from each location every 3 months from September 2010 to December 2011. The colonies, located at 3 m depth, were removed by SCUBA diving using a hammer and chisel, placed in plastic bags underwater and transported to the laboratory in a cooler within the next 2 h.

Ambient seawater temperatures were recorded *in situ* with Madgetech Temperature Loggers (0.1 °C), with data points taken automatically every 1 h. Chlorophyll a (*Chl a*) concentration in water samples was determined by filtering 5 l of seawater onto a Whatman GF/F glass fibre filters (25 mm); pigment extraction was performed with 10 ml of 90% acetone, at 4 °C during 24 h in the dark,

followed by centrifugation at 13 000 × g for 10 min (Biofuge-pico, Heraeus instruments) and absorbance reading at 750, 664, 647 and 630 nm according to Jeffrey and Humphrey (1975).

Circular sediment traps (86 mm in diameter and 96 mm high) were used to estimate sedimentation rates during 24 h. Collected sediments were separated by grain size using 2.0 and 0.0063 mm and GF/F glass fibre filters. The three sediment fractions obtained (gravel, sand and mud) were dried for 48 h at 110 °C to obtain the total suspended solids (g/l) and then were calcined at 550 °C for 1 h to get the organic matter (ESS method, 1993).

Bleaching estimations

The spatial extent of bleaching (white colouration) was visually estimated as a percentage of colony surface area. Colonies were grouped into five categories according to bleaching extent: 1 normally pigmented, 2 lightly bleached (<25% white), 3 moderately bleached (25–50% white), 4 severely bleached (>50% white) and 5 tissue necrosis (Bourne *et al.*, 2008).

For *Chl a* measurements, 10 ml of the crushed tissue homogenate (obtained as described below) was centrifuged at 5000 × g for 10 min at 4 °C (Labofuge 400R, Heraeus instruments) and the supernatant was discarded leaving the algal pellet. Pigments were extracted from the algal pellet as described above.

Mucus and tissue extraction

Two coral fractions were used in this study: mucus layer and coral tissue. In order to separate these fractions, the colonies were gently washed three times with 50 ml of sterile filtered seawater (SFSW) to remove nonassociated bacteria, broken into ~2 × 2 cm^2 pieces, placed in 50-ml centrifuge tubes and centrifuged for 3 min at 2900 g (Labofuge 400R, Heraeus instruments) to obtain the secreted mucus from the supernatant. After centrifugation, the coral pieces were crushed in SFSW using a mortar and pestle, the $CaCO_3$ skeleton was allowed to settle for 15 min and the supernatant (that is, crushed tissue) was removed and kept for further analyses.

Isolation of microorganisms

For plate counts of *Vibrio* spp., 10-fold serial dilutions of seawater, coral mucus and crushed tissue were prepared in SFSW and plated on thiosulphate citrate bile sucrose (TCBS) agar (Pronadisa, Spain) and marine agar (MA) (Pronadisa). All plates were incubated at 30 °C for 48 h. Different colony morphotypes were identified on the basis of colour, size and morphology, and were re-streaked onto fresh TCBS, incubated for further 18 h at 30 °C, and the process was repeated three times until pure cultures were obtained. Colonies isolated in MA were tested for Gram-negative staining and fermentative glucose metabolism by O/F test (Pronadisa, Spain), in order to analyse only the isolates, which could belong to *Vibrio* genus.

DNA extraction and PCR

DNA was extracted for each bacterial isolate by boiling. In brief, a colony was resuspended in 400 μl sterile Milli-Q water, heated to 99 °C in a dry block (Thermomoxer compact, Eppendorf) for 10 min and centrifuged at 13 000 × g for 10 min (Biofuge-pico, Heraeus instruments). DNA in the supernatant was used as a template for PCR amplification with primers 8F and 1492R, specific for bacterial 16S rRNA genes (Lane, 1991). The reaction mixtures (50 μl) contained 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), each deoxyribonucleotide triphosphate (Invitrogen) at a concentration of 200 μM, 1 U of *Taq*I DNA polymerase (Invitrogen), each oligonucleotide primer at a concentration of 2 mM and 20 ng of template DNA. Amplification conditions included an initial denaturation step at 95 °C for 3 min followed by 30 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 2 min, and a final extension step at 72 °C for 7 min. Negative controls to which no template DNA was added were included. PCR products were checked on 1% agarose gels (LE; SeaKem) in 1 × Tris-acetic acid-EDTA (TAE) buffer and visualised under UV light after ethidium bromide staining.

Analysis of isolates by amplified rDNA restriction analysis

PCR-amplified 16S rRNA genes were compared by amplified rDNA restriction analysis (Vaneechoutte *et al.*, 1992) with enzymes *Hinf*I and *Mbo*I (New England Biolabs). Enzymatic digestion was carried out by incubating (37 °C, 16 h) 10 μl of the PCR products with 5 U of enzyme and the corresponding enzyme buffer. Digestion products were analysed by electrophoresis on 2% agarose (LE; SeaKem) gels in 0.5 × Tris-boric acid-EDTA buffer (TBE), stained and visualised as described above. At least two isolates were selected from each restriction profile for sequencing.

16S rRNA gene sequencing and analysis

PCR products were purified using the GeneJET PCR purification kit (Fermentas, EU) and sequenced using an ABI 3730xl sequencer (Applied Biosystems). All sequences were preliminary classified using BLAST (Basic Local Alignment Search Tool) and the reference NCBI database (http://www.ncbi.nlm.nih.gov). Sequence anomaly tests, clustering and rarefactions were carried out with MOTHUR (Schloss *et al.*, 2009). More sequence analyses were conducted using the ARB software package (Ludwig *et al.*, 2004) with the reference databases

SSU Ref 111 (SILVA project, Quast *et al.*, 2013; http://www.arb-silva.de) and LTP s111 (LTP project, Munoz *et al.*, 2011; http://www.arb-silva.de/projects/living-tree). All sequences were automatically aligned using the SINA software (Pruesse *et al.*, 2012), followed by a manual inspection of misplaced bases using the ARB sequence editor and taking into account the secondary structure of the rRNA. Three phylogenetic reconstructions were performed using Neighbour Joining, Maximum Likelihood (RaxML, Stamatakis, 2006; model: GTRGAMMA) and Maximum Parsimony methods. In all cases, a 40% maximum frequency filter was applied to remove noise from the alignment and to further guarantee positional orthology. The partial sequences were added *a posteriori* using the ARB parsimony tool. The selected tree represents a consensus topology between the different reconstructions; multifurcations have been manually introduced where the phylogeny could not be unambiguously resolved according to the current data.

Laboratory infection experiments

Fragments (about 5 cm of diameter) of healthy *O. patagonica* were collected as described above from the Marine Reserve of Tabarca in June 2013. Each fragment was transferred to the laboratory and acclimated for 3 days in aquaria (20 l) before being placed in separate aquaria (500 ml) for inoculation experiments. Afterward, fragments were slowly acclimated to the experimental temperature by increasing the temperature by 0.5 °C per day, until the final temperature was reached. Fragments showing disease signs were immediately removed from the experiment. Before inoculation, the corals were maintained at the experimental temperature for 3 days in SFSW. Water was replaced every 3 days during the infection experiment.

Corals were infected with two representative *Vibrio* strains isolated from diseased *O. patagonica* colonies collected in June 2011: isolate Vic-Oc-068 that showed a 99.5% 16S rRNA gene sequence identity to *V. coralliilyticus* and isolate Vic-Oc-097 that showed a 99.5% sequence identity to *V. mediteranei*. A third *Vibrio* strain, Vic-Oc-027, isolated from healthy corals and showing a 100% 16S rRNA gene sequence identity to *V. gigantis* was used as a control. These bacteria were grown at 30 °C in Luria Bertani broth with 3% NaCl for 4 h under agitation, collected by centrifugation ($4000 \times g$, 10 min) and washed twice with SFSW. Infections were carried out at three different temperatures: 20 °C, 24 °C and 28 °C. For each temperature, four replicate aquaria (500 ml) were inoculated with 0.5 ml of each *Vibrio* strain and a mixture of the three strains at a final concentration of 10^5 cfu ml^{-1} in the innoculum. In addition, four replicate uninfected corals were inoculated with 0.5 ml of SFSW and kept in the same conditions as the infected specimens. Thus, a total of 20 aquaria (including the 4 controls) were set. All infection experiments lasted for 10 days.

During the course of all experiments, the percentage of damaged tissue was estimated visually and recorded every day for each colony in the infected and control aquaria. At the end of the experiments, *Vibrio* spp. abundance and *Chl a* concentration were measured as described for the environmental samples.

Data analysis

Environmental data (temperature, seawater *Chl a*, sedimentation rate, organic matter and mud) were taken as the independent variables, whereas biological parameters (*Vibrio* spp. diversity, *Vibrio* spp. plate counts, bleaching and *Chl a* from tissue) were taken as dependent variables. First, a principal component analysis (PCA) (using correlation) was carried out to determine which environmental variables were important in driving differences between samples taken from every sampling location. Then, ordination methods were used to analyse the variation of the phylotypes according to the environmental data using canonical correspondence analysis (CCA) to study the relationship between environmental variables and biological parameters. The resulting ordination biplots approximated the weighted average of each phylotypes with respect to each of the environmental variables, which were represented as arrows. The length of these arrows indicated the relative weight of that environmental factor, while the angle between arrows indicated the degree of correlation between two environmental factors. A Monte Carlo test with 999 permutations was carried out to ensure the significance of the canonical axes. These analyses were performed using the software package CANOCO 4.5 (ter Braak and Smilauer, 2002).

Differences among *Vibrio* assemblages were assessed with UniFrac analysis (Lozupone and Knight, 2005), which is a β-diversity measure (differentiation at diversity level among habitats) that uses phylogenetic information to compare environmental samples, which were plotted in two dimensions with the UniFrac-based principal coordinate analysis (PCoA) To further identify environmental factors explaining differences among *Vibrio* assemblages, each sample was classified into four different categories based on sampling location (Harbour and Tabarca) and coral bleaching status (healthy and unhealthy). A relatively small UniFrac distance implies that two communities are similar, consisting of lineages sharing a common evolutionary history.

For each temperature (20, 24 and 28 °C) in the infection experiment, differences in *Vibrio* abundance and *Chl a* concentration were tested using two-factor (temperature and infection treatment) ANOVA (analysis of the variance). Before calculating ANOVA, heterogeneity of variance was tested using

Cochran's C-test, and vibrio abundance data were transformed (Ln(x)) as the variances were significantly different at $P = 0.05$. When ANOVA indicated a significant difference for a certain factor, the source of differences was identified using Student–Newman–Keul (SNK) tests (Underwood, 1997).

Results

Environmental parameters, coral health status and seasonal variations of Vibrio *spp.*

The distribution of the samples according to their physicochemical characteristics was studied by PCA of the environmental data. Two components were required to explain 89.1% of the total variance among the samples. The first component, C1, had a very strong contribution of sedimentation rate and mud fraction and, to a lesser extent, of organic matter and *Chl a* concentration, while C2 was mainly related with temperature (Table 1). According to these two components, both Harbour and Tabarca samples were organised according to C1, which could be an indicator of trophic status, whereas C2 (that is, the temperature) determined the segregation of summer, spring-autumn and winter samples (Figure 1).

The state of *O. patagonica* colonies (Figure 2) in the sampling sites was monitored every 3 months from September 2010 to December 2011. The highest bleaching indexes were recorded in September 2011 in Tabarca when the sea temperature reached 28.2 °C. Bleached colonies showed an extensive tissue loss, leading to necrosis and mortality of affected areas. In the Harbour samples, the bleaching indexes were higher in December 2010, after sea temperature had reached 28.8 °C during the summer; tissue loss was not detected in these bleached colonies (Figure 3a). In December 2010, the Harbour sampling site was shaded by a large ship, and this could have an effect on coral disease as we checked experimentally that the lack of light could induce bleaching (Rubio-Portillo *et al.*, submitted).

The temporal variation of culturable *Vibrio* counts in seawater, mucus and coral tissue during the study period is shown in Figure 3. *Vibrio* spp. ranged from $1 \times 10^{\circ}$ to 1.8×10^3 CFU l^{-1} in seawater and from 9.8×10^1 to 2×10^5 and from 3×10^1 to 7.6×10^5 CFU g^{-1} in the mucus and coral tissue, respectively. In seawater (Figure 3b), culturable *Vibrio* spp. were only detected from June to December, while they could be detected in all the coral samples taken along the year (Figure 3c), although the counts were lower from January to March. In both localities, culturable counts in coral samples collected during the bleaching event were consistently higher in diseased ($1 \times 10^5 \pm 2 \times 10^4$ CFU g^{-1}) than in healthy corals ($1 \times 10^3 \pm 1 \times 10^2$ CFU g^{-1}) (*t*-test, $P < 0.05$). Culturable *Vibrio* accounted for less than 0.02‰ of the total DAPI counts in mucus and coral tissue (unpublished results).

Diversity and dynamics of Vibrio *community*

The screening of the 296 *Vibrio* isolates obtained by using amplified rDNA restriction analysis yielded a total of 53 different patterns. At least one isolate per restriction pattern was selected for complete

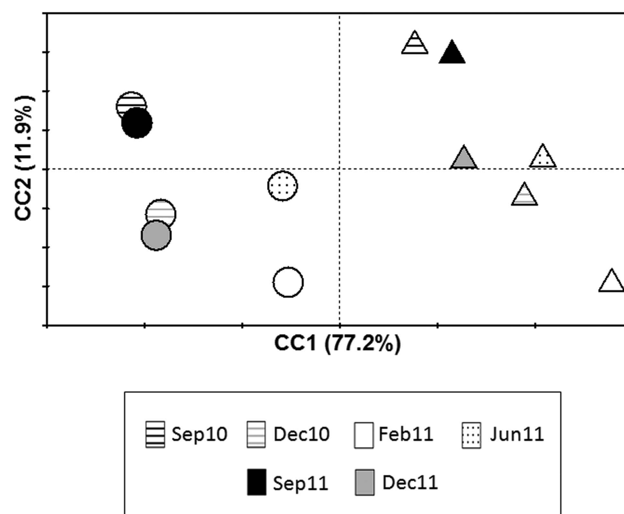

Figure 1 PCA ordination diagram of *O. patagonica* samples from Harbour (circles) and Tabarca (triangles) collected at the different sampling times.

Table 1 Enviromental variables used in PCA analisys and their contribution to canonical axes

Variables	*Location*	*Sampling time*						*C1*	*C2*
		Sep 10	*Dec 10*	*Feb 11*	*Jun 11*	*Sep 11*	*Dec 11*		
Temperature (°C)	Harbour	25.75 ± 0.67	16.23 ± 0.87	13.56 ± 0.20	23.40 ± 1.60	25.52 ± 0.87	16.95 ± 0.65	0.094	0.926
	Tabarca	25.53 ± 0.46	14.47 ± 0.45	13.27 ± 0.18	22.86 ± 1.20	25.81 ± 0.64	17.15 ± 0.82		
Sedimentation rate (g m^{-2} per day)	Harbour	35.50 ± 7.15	35.12 ± 5.98	35.11 ± 6.71	33.18 ± 6.12	34.87 ± 7.38	38.72 ± 6.79	0.903	− 0.216
	Tabarca	17.12 ± ± 6.08	24.24 ± 5.25	32.29 ± 4.5	18.95 ± 6.47	16.76 ± 5.9	30.10 ± 6.73		
Mud fraction (%)	Harbour	45.24 ± 6.72	37.87 ± 5.02	33.45 ± 5.11	35.24 ± 7.06	46.82 ± 5.65	39.48 ± 3.75	0.901	0.208
	Tabarca	29.59 ± 5.07	10.33 ± 3.58	9.44 ± 4.08	18.66 ± 4.60	31.22 ± 4.76	22.81 ± 5.14		
Organic Matter (%)	Harbour	39.58 ± 7.07	22.87 ± 7.11	15.23 ± 5.54	24.7 ± 5.92	41.67 ± 6.04	20.77 ± 7.39	0.853	0.345
	Tabarca	11.37 ± 4.70	6.21 ± 2.61	4.50 ± 2.04	8.86 ± 4.02	10.79 ± 3.35	6.88 ± 3.98		
Chl a (μg l^{-1})	Harbour	0.61 ± 0.02	1.68 ± 0.29	2.65 ± 0.42	0.69 ± 0.04	0.70 ± 0.03	2.15 ± 0.31	0.797	− 0.473
	Tabarca	0.17 ± 0.01	0.28 ± 0.02	0.29 ± 0.05	0.14 ± 0.03	0.15 ± 0.01	0.27 ± 0.01		

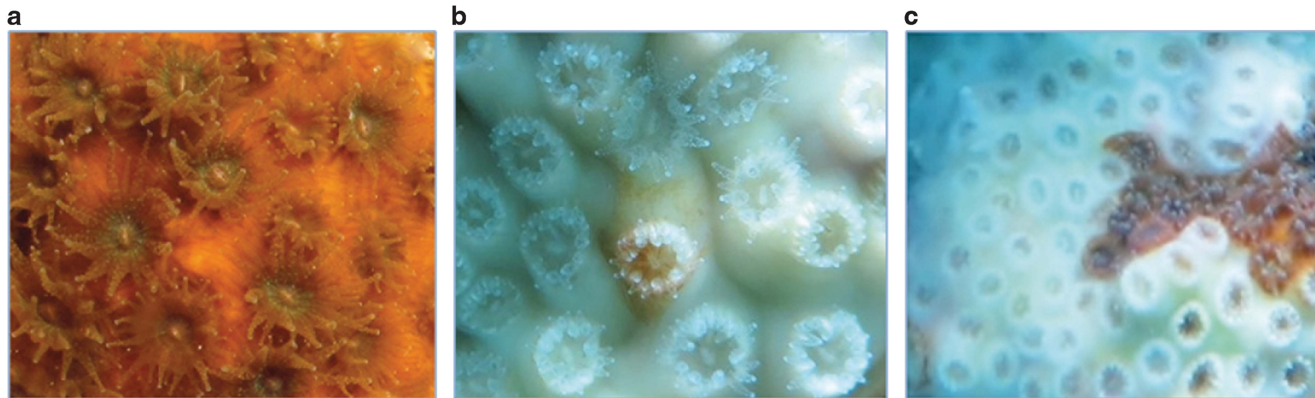

Figure 2 Different *O. patagonica* status. Shown are under water photographs of a healthy coral (**a**), bleached coral in Alicante Harbour (**b**) and coral displaying tissue necrosis in the Marine Reserve of Tabarca (**c**).

sequencing, producing in total 133 sequences of adequate quality (1458 bp and 0% ambiguities as median values) (See Supplementary Figure S1). The operational taxonomic unit saturation observed in the rarefaction curves (Supplementary Figure S2) indicated that the sequencing effort undertaken was sufficient to detect nearly all the distinct species (98.7% similarity in the whole 16S rRNA gene, Stackebrandt and Ebers, 2006) and genera (94.5% similarity, Yarza *et al.*, 2008) present in the samples. The analysis of the available 19 complete genomes of *Vibrio* species (Yarza, unpublished) indicated that the number of ribosomal operons varies from 6 to 12, with an average of 8. The maximum divergence among copies of 16S rRNA genes present in the different operons ranges from 98.3% in *Vibrio splendidus* LGP32 to 99.9% in *Vibrio* spp. EJY3, with an average of 99.3%. In particular, only 2 of the 19 species have similarities above 98.7% among the different 16S rRNA gene copies. Thus, our cutoff for species delineation seems appropriate for *Vibrio* strains. This is in agreement with previous studies (Yarza *et al.*, 2010) that showed that interoperonic 16S rRNA differences are very seldom higher than 2%.

For classifying purposes, we considered a phylotype as a monophylethic group of sequences with similarities of 98.7% or above. Among the 133 sequenced 16S rRNA genes, 22 distinct phylotypes were observed (Table 1), which were named according to the known taxa found within the clades (Supplementary Figure S1). *Vibrio communis—Vibrio owensii* (22.22% of samples) and *Vibrio harveyi—Vibrio rotiferianus* (11.11%) were the phylotypes most frequently retrieved in the seawater, appearing both in the Harbour and Tabarca. Some phylotypes like *Providencia vermicola*, *Photobacterium lutimaris* and *Shewanella fidelis* were only found in the Harbour water. Most coral samples harboured strains closely related to *V. splendidus—V. gigantis—V. atlanticus—V. pomeroyi* (50%), *V. harveyi*-like—*V. rotiferianus* (24.07% of the samples) and *Vibrio comitans–Vibrio rarus–Vibrio breoganii* (17.59%), which were detected all year around in corals. In contrast, some phylotypes like the known pathogens *V. mediteranei* and *V. coralliilyticus* were only present in warm months (Supplementary Figure S1 and Supplementary Table S1).

CCA was used to investigate the relationship between environmental factors and the presence of different phylotypes in the coral samples. Environmental variables appear in the CCA biplot as arrows; their length and orientation indicate the relative influence of each axis, and the angle between arrows indicates the degree of correlation between variables. All arrows represent a gradient, with the mean value located at the origin and the arrow pointing to the direction of increase. For clarity, results from mucus and coral tissue are shown together as they displayed very similar trends (data not shown). As shown in Figure 4, there was a succession of phylotypes along the organic matter gradient that was accompanied by a decrease in diversity. More *Vibrio* phylotypes were retrieved from Tabarca (16 phylotypes, 4 unique of this site) than from Harbour samples (14 phylotypes). Three phylotypes, *V. splendidus—V. gigantis—V. atlanticus—V. pomeroyi* (number 1 in the figure, we will refer to this group as *V. splendidus* super clade), *V. harveyi*-like (10) and *V. comitans* (14) appeared at almost every sampling time and location, and thus could be part of the 'constitutive' coral microbiota. Phylotypes appearing together in the CCA space (such as 15 and 17 in Tabarca, and 20 and 21 in the Harbour) displayed the same dependence of physicochemical parameters (Figure 4a) and could thus have the same growth requirements. Finally, the potential pathogens *V. mediteranei* (2) and *V. coralliilyticus* (4) were only present from June to December and always in diseased corals. As shown in Figure 4a, *V. mediterranei* was more frequently retrieved from Harbour corals and V. *coralliilyticus*, from Tabarca's.

The *Vibrio* community was different in healthy and diseased corals (Supplementary Figure S1) as was also indicated by UniFrac-based principal coordinate analysis (Figure 5). *Vibrio* communities

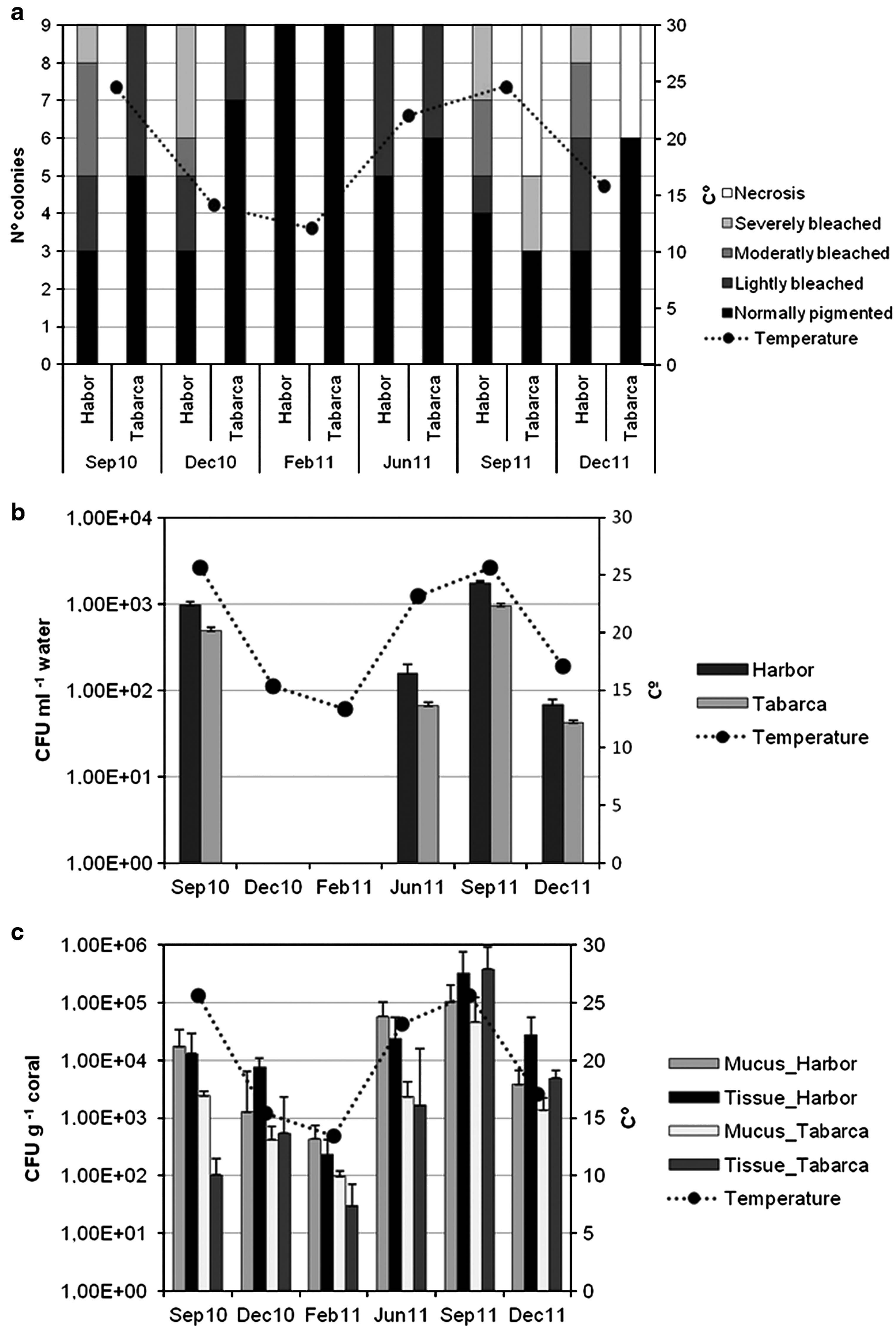

Figure 3 Seasonal fluctuations of *O. patagonica* bleaching (**a**) and *Vibrio* spp. counts in seawater (**b**) and in mucus and coral tissue (**c**) in both sampling locations. Temperature changes (averages calculates for each sampling time) are displayed in the three panels. (CFU: colony-forming units).

in healthy corals were similar between the two locations, both dominated by *V. splendidus* super clade (70.37% of the healthy corals in Harbour and 72.22% in Tabarca). Bleaching events seemed to disrupt this equilibrium leading to a decrease in *V. splendidus* super clade (below 30% in Harbour and 20% in Tabarca diseased corals) and an increase of potential pathogens. Thus, *V. mediterranei* could be isolated from 59.25% of diseased corals in the Harbour, while *V. coralliilyticus* was detected in 81.25% of the unhealthy corals in Tabarca. The different distribution of these pathogens, together with their different locations in the CCA space (Figure 4), suggests that they may

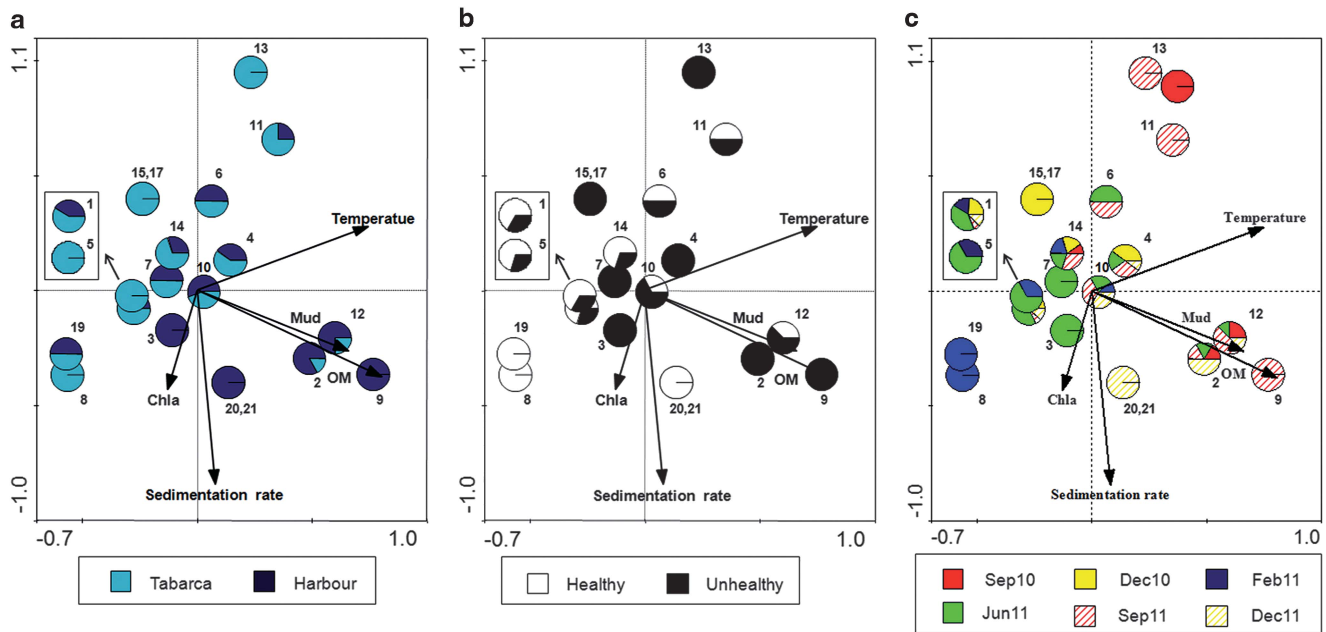

Figure 4 Biplot of the CCA axes 1 and 2 for *Vibrio* phylotypes and environmental parameters. Every phylotype is represented by a circle that is divided into different sections according to their proportions in the sampling locations (**a**), coral status (**b**) and sampling times (**c**). Phylotypes are labelled as in Table 3.

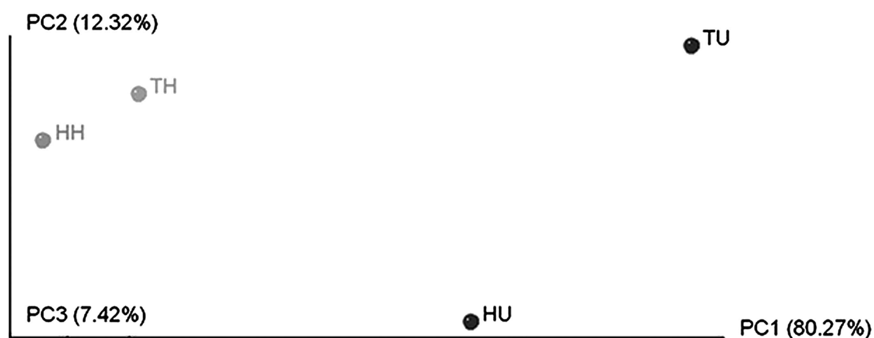

Figure 5 Communities clustered using principal coordinate analysis of the unweighted UniFrac distance matrix. TH, healthy *O. patagonica* colonies from Tabarca; HH, healthy colonies from the Harbour; TU, unhealthy colonies from Tabarca; and HU, unhealthy colonies from the Harbour.

have different growth requirements in natural conditions.

Analysis of two potential Vibrio *pathogens by experimental infections*

Experimental infections were carried out at three different temperatures (20, 24 and 28 °C) simulating the seasonal fluctuations observed in the study sites, at the end of spring and during summer. Together with their respective uninfected controls, infections with four different inocula were set for every temperature: *V. mediterranei*, *V. coralliilyticus*, *V. gigantis* and a mixture of the three of them. The final concentration of vibrios in water was similar to that found in the environmental samples when seawater temperatures were between 15 and 20 °C (around 10^2 UFC ml^{-1}). Tissue damage on *O. patagonica* increased with temperature in all infection treatments (Figure 7), including controls, while concentrations of photosynthetic pigments decreased significantly (ANOVA, $P<0.01$; Table 2 and Figure 6a). In addition, significant differences were detected in the number of culturable vibrios from corals, with samples maintained at 28 °C showing higher abundances (ANOVA, $P<0.01$; Table 2 and Figure 6b).

At 20 °C, corals coinoculated with the mixture of *Vibrio* spp. developed signs of disease after 7 days and reached 78.3 ± 10.4% of tissue damage after 10 days based on visual assessment (Figure 6), together with reduced *Chl a* concentrations compared with the controls treatments (ANOVA, $P<0.01$; Table 2 and Figure 6a). Corals inoculated with either pathogen did not show significant differences in tissue damage at the end of the experiment compared with the controls (Table 2 and Figure 6b).

Table 2 Results of the two-factor ANOVA for: CFUs (colony-forming units) and Chlorophyll a concentration

		CFU g⁻¹		*Chl a g⁻¹*	
Source of variation	df	MS	*P*-value	MS	*P*-value
Temperature (T)	2	3.476×10^{14}	0.001	1.370	0.001
Infection treatment (IT)	4	2.328×10^{14}	0.001	0.386	0.001
T × IT	8	2.011×10^{14}	0.001	0.057	0.001
Residual	30	3.182×10^{12}		0.009	
Transformation		None		$\sqrt{x+1}$	
SNK		T: 28 > 24 = 20 IT: 5 > 4 = 3 = 2 = 1 TxIT: 20: 5 > 1 = 2 = 3 = 4 24: 5 > 4 > 1 = 2 = 3 28: 5 > 4 > 3 > 1 = 2		T: 20 > 24 > 28 IT: 1 = 3 > 2 > 4 > 5 TxIT: 20: 1 = 2 = 3 = 4 > 5 24: 1 = 2 = 3 > 4 > 5 28: 1 = 2 > 3 = 4 > 5	

Abbreviations: df, degrees of freedom; MS, mean square; *P*-value, level of significance. Infection treatments: 1 uninfected aquaria; 2 *V. gigantis*; 3 *V. coralliilyticus*; 4 *V. mediterraneii* and 5 mixture.

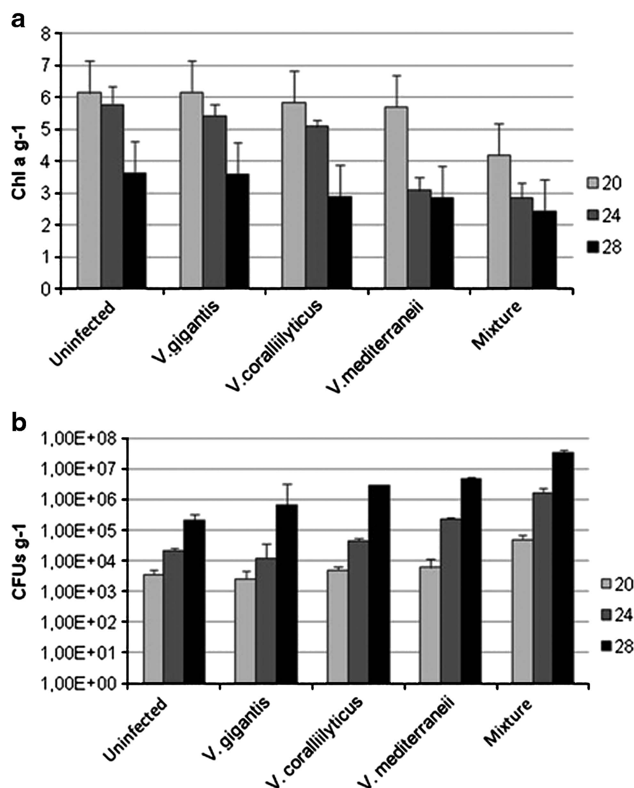

Figure 6 Chlorophyll a (**a**) and concentration of culturable Vibrios (**b**) in *O. patagonica* samples after 10 days of experimental infection with the indicated inocula at different temperatures (CFU, colony-forming units).

Corals maintained at 24 °C underwent a more pronounced development of disease signs, mainly in aquaria inoculated with the mixed culture in which all samples showed 100% of tissue damaged. Corals infected only with *V. mediteranei* or *V. coralliilyticus* reached 80 ± 7.6% and 36.6 ± 5.8% of tissue damaged, respectively. Accordingly, significant differences in *Chl a* concentration were also detected between the two distinct infections (ANOVA, $P < 0.01$; Table 2 and Figure 6a). At this temperature, *V. mediteranei* seemed thus to be more virulent than *V. coralliilyticus* (Figure 7).

Finally, at 28 °C all the corals were damaged indicating that this temperature could be lethal to *O. patagonica* if maintained for a long time. Damage extent reached 70 ± 10% and 76.6 ± 5.8% in uninfected corals and in those inoculated with *V. gigantis*, respectively. In contrast, while no significant differences were detected between single infections with either pathogen at this temperature; their mixture was more deleterious for the corals (Figure 7).

Discussion

Our results indicate that a complex and dynamic assemblage of *Vibrio* spp. is present in *O. patagonica* along the whole year and under different environmental conditions and coral health status. Thus, *Vibrio* spp. could be considered as a part of the autochthonous coral microbiota albeit, in our case, the culturable *Vibrio* spp. constitutes a very small fraction of the total microbiota. The association of *Vibrio* with healthy *O. patagonica* had been demonstrated before in specimens taken from the Israeli coast (Koren and Rosenberg, 2006). These authors showed that by 16S rRNA gene clone library analysis that *V. splendidus* was the most abundant bacteria in *O. patagonica* mucus both in summer and in winter. Later, Sharon and Rosenberg (2010) showed that *O. patagonica* taken from the Eastern Mediterranean maintained *Vibrio* spp. in the viable but nonculturable state. These authors speculate that this had a probiotic effect conferring protection against further infection. In other Mediterranean corals, such as the gorgonian *Paramuricea clavata*, *Vibrio* spp. constitute less than 1% of the healthy coral bacterial community as determined by partial 16S rRNA gene partial pyrosequencing (Vezzulli *et al.*, 2013).

Changes in environmental parameters were not only accompanied by changes in health coral status but also in the numbers and composition of their associated *Vibrio* spp. assemblages. Certainly, the occurrence of vibrios during the studied time frame was highly correlated with seawater temperatures,

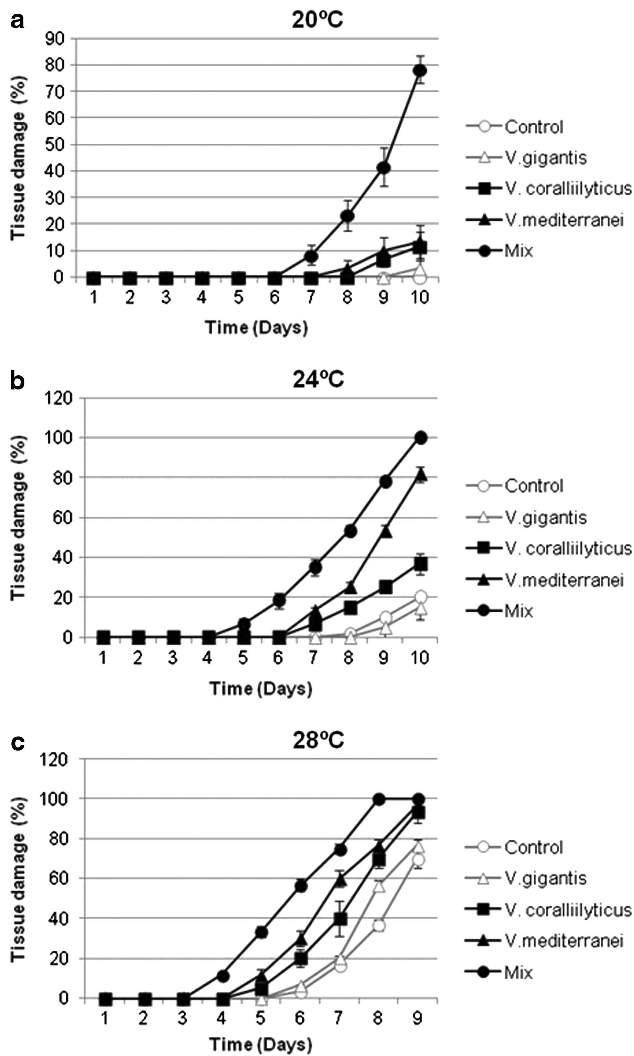

Figure 7 Development of tissue damage of *O. patagonica* colonies (average ± s.d. from three replicate colonies) following experimental infections in aquaria with the indicated inocula at different temperatures: 20 °C (**a**), 24 °C (**b**) and 28 °C (**c**).

with a sharp increase in *Vibrio* abundances during summer months when the temperatures reached around 28 °C. *Vibrio* concentrations were also linked to organic matter concentration and *Chl a* (which is an index of phytoplankton biomass), which could be due to the fact that phytoplankton blooms may support the proliferation of *Vibrio* spp. (Asplund *et al*., 2011) and thus would be an indirect effect of temperature.

Together with the changes in *Vibrio* spp. numbers along the year, there was a succession of phylotypes with time, as well as differences in the vibrios recovered from the two sampling sites (see Figure 4). Most importantly, Unifrac analysis indicated that diseased and healthy corals harboured distinct *Vibrio* spp. assemblages. The majority of isolates from healthy samples was closely related to *V. splendidus* super clade, while vibrios related to *V. mediterranei* and to *V. coralliilyticus* were recovered only from diseased samples (see Table 3) and were not detected in healthy colonies (although some diseased corals did not harbour either of these vibrios). There was a clear spatial distribution of these phylotypes, with *V. mediterraei* mainly found in the Harbour and *V. coralliilyticus* in Tabarca. Accordingly, and considering the different disease signs experienced by corals in both locations, these two *Vibrio* spp. seem to be related with *O. patagonica* diseases.

The presence of isolates associated with *V. mediterranei* in diseased corals contrasts with the results of Ainsworth *et al.* (2008) and Mills *et al.* (2013) that reported the absence of this bacterium from bleached *O. patagonica* in the Eastern Mediterranean. This is especially noteworthy if we consider that our sampling overlapped in time with that of Mills *et al.* (2013). Furthermore, while we detected *V. coraliilyticus*-like organisms only in diseased corals, it has been detected in healthy corals from the Eastern Mediterranean (Pollock *et al*., 2010). As differences between Eastern and Western Mediterranean basins are well known for planktonic microbes (Siokou-Frangou *et al*., 2010), it would not be surprising that corals from both sides of this sea could be harbouring different microbiotas. Caution should then be exerted when drawing general conclusions based on locally restricted studies.

The data on seasonal *Vibrio* spp. dynamics discussed above show the temporal coincidence between the presence in the corals of certain species of *Vibrio* and the development of disease symptoms that were clearly correlated with an increase in water temperature. The effect of temperature on coral disease and *Vibrio* numbers increase in marine environments has been widely reported before (Kushmaro *et al*., 1998; Ben-Haim *et al*., 2003; Bourne *et al*., 2008; Vezzulli *et al*., 2010), and there is still much controversy on the role of *Vibrio* spp. in causing disease (see above). We tried to get a new insight into this issue by analysing the response of *O. patagonica* to experimental infection under different conditions. This experimental approach has well-known limitations (Ainsworth and Hoegh-Guldberg, 2009). Furthermore, the use of SFSW in the experimental aquaria hampers the possibility of adaptive bleaching (the switching of associated zooxanthelae, Buddemeier and Fautin, 1993), and thus limits the response of corals to stressful conditions. However, experimental infection can still be useful for comparing coral responses to infection under different conditions.

Disease signs developed faster and to a higher extent in corals inoculated with 'pathogenic' vibrios than in untreated controls, with higher/faster disease signs in the corals inoculated with the mixed inoculum. These results are in agreement with those of Cervino *et al.* (2004). These authors observed that when four *Vibrio* spp. isolated from diseased specimens of the Caribbean coral *Montastrea* spp. were inoculated together onto healthy specimens, yellow band disease (YBD) symptoms developed faster than

Table 3 Phylotypes detected on *O. patagonica* (C, H, Healthy and U, Unhealthy) and seawater samples (W) collected from Alicante Harbour and the Marine Reserve of Tabarca

Phylotipe Number (N^o)	*Phylotype name*	*Location*	*Sep 10*			*Dec 10*			*Feb 10*			*Jun 11*			*Sep 11*			*Dec 11*		
			W	*C*		*W*	*C*		*W*	*C*		*W*	*C*		*W*	*C*		*W*	*C*	
				H	*U*		*H*	*U*		*H*	*U*		*H*	*U*		*H*	*U*		*H*	*U*
1	*Vibrio splendidus—Vibrio gigantis—Vibrio atlanticus—Vibrio pomeroyi*	Harbour	—	—	—	—	4	2	—	9	—	—	5	4	—	—	—	—	3	—
		Tabarca	—	2	—	—	4	—	—	9	—	2	6	3	—	2	—	—	3	—
2	*Vibrio mediterranei*	Harbour	—	—	3	—	—	—	—	—	—	—	—	4	—	—	5	—	—	4
		Tabarca	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
3	*Vibrio fortis*	Harbour	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—
4	*Vibrio coralliilitycus*	Harbour	—	—	—	—	—	3	—	—	—	1	—	2	—	—	1	—	—	—
		Tabarca	—	—	—	—	—	2	—	—	—	—	—	3	—	—	5	—	—	3
5	*Vibrio hepatarius—Vibrio tubiashii*	Tabarca	—	—	—	—	—	—	—	2	—	—	2	2	—	—	—	—	—	—
6	*Vibrio xuii*	Harbour	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—
		Tabarca	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—
7	*Vibrio maritimus*	Harbour	—	—	—	—	—	—	—	—	—	—	—	3	—	—	—	—	—	—
		Tabarca	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—
8	*Sp3*	Tabarca	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—
9	*Vibrio ponticus*	Harbour	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	—
10	*Vibrio harveyi –Vibrio rotiferianus*	Harbour	1	—	—	—	—	—	—	—	—	—	2	3	—	—	—	1	—	8
		Tabarca	—	—	—	—	—	—	—	3	—	—	—	—	2	3	3	—	—	3
11	*Vibrio natriegens*	Harbour	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	—
		Tabarca	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—
12	*Vibrio communis—Vibrio owensii*	Harbour	3	2	1	—	—	—	—	—	—	1	—	—	1	—	1	—	—	1
		Tabarca	1	—	—	—	—	—	—	—	—	2	—	2	—	—	—	—	—	—
13	*Vibrio agarivorans*	Tabarca	—	—	—	—	—	—	—	—	—	—	—	1	1	—	6	—	—	—
14	*Vibrio comitans–Vibrio rarus—Virbio breoganii*	Harbour	—	2	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	2
		Tabarca	—	—	—	—	6	—	—	5	—	—	—	—	—	—	2	—	—	—
15	*Photobacterium rosenbergii*	Harbour	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		Tabarca	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—
16	*Photobacterium lutimaris*	Harbour	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—
17	*Photobacterium swingsii*	Tabarca	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—
18	*Providencia vermicola*	Harbour	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—
19	*Agarivorans albus*	Harbour	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	—
		Tabarca	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	—
20	*Shewanella fidelis*	Harbour	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	1
21	*Shewanella waksmanii*	Harbour	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	2
22	*Sp2*	Harbour	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—

Six Samplings were carried in September 2010, December 2010, February 2011, June 2011, September 2011 and December 2011.

when they were inoculated individually, leading to the suggestion that these four vibrios were acting as a consortium. Subsequently, these authors confirmed that a consortium of different Vibrio species caused YBD in both Caribbean and Pacific Sea. The infection could be initiated at 25 °C and increased with rising seawater temperature (29–30 °C) (Cervino *et al.* 2008). Another coral disease, black band, is caused by a consortium of microorganism that includes cyanobacteria, sulphur reducers and oxidisers, as well as *Vibrio* spp. (Frias-Lopez and Klaus, 2004; Arotsker *et al.*, 2009). However, Vezzulli *et al.* (2010) observed that, when they inoculated three different Vibrio strains together on *Paramuricea clavata*, disease signs developed more slowly than when strains were inoculated separately, and therefore a clear role cannot be assigned to bacteria in the onset of coral diseases.

As expected, an increase in temperature produced an increase in disease symptoms. Indeed, at 28 °C, coral underwent bleaching even in the absence of inoculum. Although this could mean that temperature by itself is enough to induce disease, it does not rule out the possibility of damage being caused by infection as corals can harbour *Vibrio* spp. in viable but nonculturable state (Sharon and Rosenberg, 2010; Vezzulli *et al.*, 2013). However, for these authors, viable but nonculturable vibrios would be acting as a protection against pathogens, which is not the case shown here. Again, this would not solve the question of whether *Vibrio* spp. are primary or opportunistic pathogens (that is not a clear cut classification). Furthermore, although our results show a clear implication of *Vibrio* spp. in coral infection in aquaria, they do not rule out the possibility that bleaching symptoms developed at the different assayed temperatures correspond to different aetiologies. Indeed, it has been proposed (Lesser *et al.*, 2007) that coral diseases should rather be called syndromes.

Overall the most striking result from the experimental infections described above is the development of disease signs at 20 °C when mixed cultures of *V. mediterranei*, *V. coralliilyticus* and *V. gigantis* were inoculated into healthy corals. The fact that only the mixture of vibrios was inducing disease signs at low temperature indicated that these bacteria could be harmful to coral under conditions

in which the individual (putative) pathogens would not have any deleterious effect. In our environmental survey, *V. coralliilyticus* and *V. mediterranei* were seldom together in the same sampling site and seem to have different growth requirements. However, the experimental infection results show the pathogenic power of the mixture, which raises concerns about the possible deleterious effects of the dispersal of pathogens among different locations. This risk, if proven real such as for human pathogens transported by ballast water (Ruiz *et al*., 2000), could have consequences for coral health worldwide.

Conflict of Interest

Esther Rubio-Portillo holds a pre-doctorate grant from the University of Alicante. The remaining authors declare no conflict of interest.

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