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Impact of environmental changes on Oculina patagonica coral holobiont

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Tesis **Doctorales**

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DOCTORADO EN CIENCIAS DEL MAR Y BIOLOGÍA APLICADA

Impact of environmental changes on *Oculina patagonica* coral holobiont.

Impacto de los cambios ambientales en el holobionte del coral *Oculina patagonica*.

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CERTIFICAN:

Que la memoria de Tesis doctoral titulada **"Impact of environmental changes on *Oculina patagonica* coral holobiont"**, presentada por **ESTHER RUBIO PORTILLO**, ha sido realizada bajo su dirección en el Departamento de Ciencias del Mar y Biología Aplicada y en el de Fisiología, Genética y Microbiología de laUniversidad de Alicante,. Y para que conste a los efectos oportunos, firman en Alicante a 23 de Febrero del año dos mil quince.

Fdo: Alfonso A. Ramos Esplá   Fdo: Josefa Antón Botella

A mis padres

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CONTENS

Abstract / Resumen

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Introduction

Corals belong to the metazoan phylum Cnidaria, Class Anthozoa and Subclass Hexacorallaria, which can be broadly divided ecologically, but not systematically, into non-reef-building (ahermatypic) corals and reef-building (hermatypic), with most hermatypic corals belonging to the Order Scleractinian. Until recently, corals have been considered as a product of a mutualistic interaction between the coral animal and unicellular algal symbionts commonly referred to as zooxanthellae; this view did not considered the symbiotic potential of a large and diverse assemblage of other coral-associated microorganisms such as bacteria, fungi or archaea, which all form a collaborative consortium named the coral holobiont (Rohwer *et al.*, 2001; Knowlton and Rohwer, 2003).

Phothosynthetic zooxanthellae associated to cnidarians are located in host-derived vacuoles (symbiosomes) within the gastrodermal host cells. These zooxanthellae were originally described as one single dinoflagellate species, *Symbiodinium microadriaticum* (Freudenthal, 1962), although

Introducción

Los corales son metazoos que pertenecen al filo Cnidaria, Clase Anthozoa y Subclase Hexacorallaria, el cual se divide desde el punto de vista ecológico, pero no sistemático, en corales no constructores de arrecifes (ahermatípicos) y en corales constructores de arrecifes (hermatípicos), perteneciendo los últimos al Orden Scleractinia. Hasta hace poco, los corales se han considerado un producto de una interacción mutualista entre el coral y las algas unicelulares simbióticas conocidas como zooxantelas; este punto de vista no considera la existencia de un gran y diverso número de microorganismos asociados, como bacterias, hongos y arqueas, que forman un consorcio colaborativo denominado el "holobionte coralino" (Rohwer *et al.*, 2001; Knowlton y Rohwer, 2003).

En la asociación entre cnidarios y zooxantelas, las algas fotosintéticas se localizan en vacuolas (simbiosomas) dentro de las células de la gastrodermis del coral hospedador. Estas zooxantelas al principio fueron descritas como una única especie de

subsequent studies have shown that this genus represents a large species complex, currently encompassed by nine clades (A-I) (Pochon *et al.*, 2004; Pochon and Gates, 2010), divided into multiple types, based on the nuclear internal transcribed spacer, region 2, (ITS2) (see Baker, 2003 for a review).

Besides zooxanthellae, other phototrophic eukaryotes have also been detected in corals, whose diversity increase under stress conditions and the subsequent loss of zooxanthellae (Lins-de-Barros *et al.*, 2013), which allows a deeper penetration of light within the coral skeleton and the secondary colonization of *Chlorophyta* and *Streptophyta* (Garcia *et al.*, 2013).

The presence and diversity of coral-associated bacteria has been widely studied in some coral species, although little is known about the role of these microorganisms in the coral holobiont. There are increasing evidences that coral microbiota is crucial in this consortium, at least, in biogeochemical cycling (Lesser *et al.*, 2004; Chimetto *et al.*, 2008; Raina *et al.*, 2009; Kimes *et al.*, 2010), and

dinoflagelado, *Symbiodinium microadriaticum* (Freudenthal, 1962), aunque estudios posteriores han demostrado que este género está representado por un complejo mayor de especies, que actualmente abarca nueve clados (A-I) (Pochon *et al.*, 2004; Pochon y Puertas, 2010), divididos a su vez en múltiples tipos, basándose en el espaciador interno transcrito 2 (ITS2) (ver Baker, 2003 para una revisión).

Además de las zooxantelas, se han descubierto otros organismos eucariotas fototrofos en corales, cuya diversidad aumenta cuando los corales pierden sus zooxantelas en condiciones de estrés (Lins-de-Barros *et al.*, 2013), lo que permite una mayor penetración de la luz dentro del esqueleto del coral y por tanto la colonización secundaria del mismo por algas clorófitas y estreptofitas (Garcia *et al.*, 2013).

La presencia y diversidad de las bacterias asociadas a corales ha sido ampliamente estudiada en algunas especies, pero se conoce poco sobre su papel en el "holobionte coralino". Hay evidencias de que la microbiota

resistance to diseases (Ritchie and Smith 2004; Reshef *et al.*, 2006) via competition for nutrients and/or production of antibiotics (Ritchie, 2006).

Coral reefs dominate coastal tropical environments, between the Tropic of Capricorn and Tropic of Cancer, 25ºS-25ºN, (Veron, 1986), where they find the environmental variables appropriate to their subsistence. In the Mediterranean Sea, which is a temperate and oligotrophic sea with a great marked seasonal variation in the main environmental parameters, the number of coral species represents less than 5% of those around the world. Among these species, *Cladocora caespitosa* (Linnaeus, 1767), a scleractinian coral belonging to the family Faviidae, is the only endemic reef-forming species (Zibrowius, 1980; Laborel, 1987; Pereiano *et al.*, 2004), which is endangered by increasing disturbances that are affecting coastal ecosystems, including elevated seawater temperatures (Rodolfo-Metalpa *et al.*, 2000, 2005, 2008a; Kersting *et al.*, 2013) or invasive species (Kersting *et al.*, 2014).

de los corales juega un papel crucial, al menos en los ciclos biogeoquímicos (Lesser *et al.*, 2004; Chimetto *et al.*, 2008; Raina *et al.*, 2009; Kimes *et al.*, 2010), así como en la resistencia ante enfermedades (Ritchie and Smith 2004; Reshef *et al.*, 2006), a través de la competencia por los nutrientes y/o por la producción de antibióticos (Ritchie, 2006).

Los arrecifes de coral dominan los ambientes costeros tropicales, entre el trópico de Cáncer y el de Capricornio, 25ºS-25ºN, (Veron, 1986), donde se dan las condiciones ambientales necesarias para su subsistencia. En el Mar Mediterráneo, que es un mar templado y oligotrófico con una marcada estacionalidad de los principales parámetros ambientales, el número de especies de corales representa menos del 5% de los existentes en el mundo. Entre estas especies, *Cladocora caespitosa* (Linnaeus, 1767), coral scleractinio perteneciente a la familia Faviidae, es el único endémico capaz de formar arrecifes (Zibrowius, 1980; Laborel, 1987; Pereiano *et al.*, 2004), que está en peligro por el aumento de perturbaciones que afectan los

The coral *Oculina patagonica* De Angelis, 1908 is an alien scleractinian coral in the Mediterranean Sea that was detected for the first time in 1966 on the Ligurian coast of Italy, near the Savona harbour (Zibrowius, 1974). It was described using Pleistocene fossil samples collected in northern Argentina, assuming that this species could have been accidentally transferred to the Mediterranean by transoceanic transport from the temperate South West Atlantic (Zibrowius, 1974; 1992; Zibrowius and Ramos, 1983). Although it was first recorded near Savona and then in the Alicante harbour in 1973, it seems that large extensions of the Spanish Southeast coast were initially colonized, considering the abundance and extent of its colonies (Zibrowius, 1992; Zibrowius and Ramos, 1983). Nowadays, this species is widely spread throughout the Mediterranean Sea, due to the intense intra-Mediterranean maritime traffic (Zibrowius, 1992) and the increase of anthropogenic activities (Serrano *et al.*, 2013; Salomidi *et al.*, 2013), as well as its ability to survive under different environmental conditions (Armoza-Zvuloni *et al.*, 2012) that have favored

ecosistemas costeros, incluyendo el aumento de la temperatura del agua del mar (Rodolfo-Metalpa *et al.*, 2000, 2005, 2008a; Kersting *et al.*, 2013) o las especies invasoras (Kersting *et al.*, 2014).

El coral *Oculina patagonica* De Angelis, 1908 es un coral scleractinio introducido en el Mar Mediterráneo que fue detectado por primera vez en 1966 en la costa de Liguria en Italia, cerca del puerto de Savona (Zibrowius, 1974). Fue descrito a partir de fósiles del Pleistoceno encontrados en el norte de Argentina, asumiéndose que esta especie fue accidentalmente transportada al Mediterráneo, por medio de barcos transoceánicos, desde el Suroeste Atlántico (Zibrowius, 1974; 1992; Zibrowius and Ramos, 1983). Aunque se observó primero cerca de Savona y después en el puerto de Alicante en 1973, parece que las costas del Sureste de España fueron anteriormente colonizadas debido a la abundancia y extensión de las colonias encontradas (Zibrowius, 1992; Zibrowius and Ramos, 1983). Hoy en día, esta especie está ampliamente distribuida a través de todo el Mediterráneo, debido al

its establishment and colonization of new coastal areas.

Coral reefs are probably the first marine ecosystem to suffer extreme damage and possible collapse as a consequence of climate change and the subsequent increase of seawater temperatures (Wilkinson, 2008). Temperatures in the Mediterranean Sea have risen at an average rate of 0.04±0.01 °C/year (Diaz-Almela *et al.*, 2007). It is known that the global warming also promotes the proliferation of *Vibrio* spp. particularly and accordingly a rise of *Vibrio*-associated coral diseases (Harvell *et al.*, 2002, Baker-Austin *et al.*, 2012, Vezzulli *et al.*, 2013). Among them one of the most important is the coral bleaching (breakdown of the symbiotic relationship between corals and zooxanthelae).

Bleaching of *O. patagonica* has been extensively studied, although there is a considerable controversy on the nature of its principal cause, due to the different existing hypothesis about whether microorganisms have or not a role in the bleaching process. This process was first observed along

intenso tráfico marítimo de este mar (Zibrowius, 1992) y al incremento de actividades antropogénicas (Serrano *et al.*, 2013; Salomidi *et al.*, 2013), así como a su habilidad para subsistir bajo diferentes condiciones ambientales (Armoza-Zvuloni *et al.*, 2012), lo que favorece su establecimiento y la colonización de nuevas áreas costeras.

Los arrecifes de coral son probablemente uno de los primeros ecosistemas marinos en sufrir daños, llegando incluso al colapso, como consecuencia del cambio climático y del aumento de las temperaturas de los océanos (Wilkinson, 2008). Este aumento en el Mar Mediterráneo ha alcanzado una media de 0.04±0.01 °C/año (Diaz-Almela *et al.*, 2007). El calentamiento global además promueve la proliferación de bacterias del género *Vibrio* y por tanto de las enfermedades de los corales asociadas a dichas bacterias (Harvell *et al.*, 2002, Baker-Austin *et al.*, 2012, Vezzulli *et al.*, 2013). Una de las más importantes es el blanqueamiento (ruptura de la simbiosis existente entre los corales y sus zooxantelas).

the Israeli coastline in the summer of 1993 and Koch's postulates were applied to demonstrate that *Vibrio shilonii* (=*Vibrio mediteranei*) was the causative agent of bleaching (Kushmaro *et al.*, 1996, 1997). The increasing of seawater temperature was the environmental factor that triggered the disease (Kushmaro *et al.*, 1998). Later studies suggested that V. *mediteranei* was not involved or was not the primary cause of the annual bleaching of *O. patagonica* in the Eastern Mediterranean (Ainsworth *et al.*, 2008), suggesting that bacteria have a secondary role during coral bleaching. It is due to an increase in susceptibility to microbial attack experienced by corals during environmental stress. However, a recent study (Mills *et al.*, 2013), showed that antibiotics inhibit temperature-induced bleaching of *O. patagonica* fragments and made the corals sensitive to *V. mediterranei* infection, providing support for the microbial hypothesis of coral bleaching.

El blanqueamiento de *O. patagonica* ha sido ampliamente estudiado, aunque existe una gran controversia sobre la naturaleza de su causa principal, debido a que existen diferentes hipótesis sobre el papel que juegan los microorganismos en este fenómeno. Este proceso fue observado por primera vez en las costas de Israel en el verano de 1993 y mediante la aplicación de los postulados de Koch se demostró que el agente causante era *Vibrio shilonii* (=*Vibrio mediteranei*) (Kushmaro *et al.*, 1996, 1997). El aumento de la temperatura del mar es el factor ambiental que lo desencadenaba (Kushmaro *et al.*, 1998). Estudios posteriores sugirieron que *V. mediterranei* no estaba involucrado o al menos no era la causa principal del blanqueamiento anual de *O. patagonia* en el Mediterráneo oriental (Ainsworth *et al.*, 2008), sugiriendo que las bacterias juegan un papel secundario en el blanqueamiento. Los corales en condiciones de estrés ambiental presentarían una mayor susceptibilidad a ser atacados por microorganismos. Sin embargo, un estudio reciente (Mills *et al.*, 2013), ha demostrado que el uso de antibióticos induce el

Within this frame, this PhD thesis aims to improve the knowledge about the response of the coral *O. patagonica* and its microbial community to different environmental conditions, as well as to elucidate the role of *Vibrio* spp. and environmental factors in bleaching events, particularly in the global warming scenario. To accomplish this goal this thesis has been structured in two parts, the first focused on the biological and ecological characteristics of the coral, including the study of the coral spatial distribution and its "invasive level" along the Valencian Region (South East of Spain, South Western Mediterranean Sea (chapter 3.1), as well as its growth and bleaching in two different sampling locations with different environmental conditions (Alicante Harbour and Marine Protected Area of Tabarca; chapter 3.2). In the second part of this thesis the characterization of the microbial community as well as its changes with environmental factors are shown. The results regarding Eukarya and Bacteria associated with the coral are shown in chapter 3.3, while in the chapter 3.4 the stress was placed on *Vibrio* spp. assemblages. Finally, the chapter 3.5

blanqueamiento de *O.patagonica*, haciendo a los corales más sensibles a la infección, dando soporte a la hipótesis del blanqueamiento microbiano de los corales.

Dentro de este marco, esta tesis doctoral trata de mejorar el conocimiento sobre la respuesta del coral *O. patagonica* y su comunidad microbiana ante diferentes condiciones ambientales, así como esclarecer el papel de los *Vibrio*s y los factores ambientales en los eventos de blanqueamiento, particularmente en el actual escenario de calentamiento global. Para lograr este objetivo la tesis está estructurada en dos partes, la primera centrada en las características biológicas y ecológicas del coral, estudiando su distribución espacial y "nivel de invasión" en la Comunidad Valenciana (Sureste de España, Suroeste del Mar Mediterráneo, capítulo 3.1) y su crecimiento en blanqueamiento en dos localidades de muestreo con diferentes condiciones ambientales (el puerto de Alicante y el área Marina Protegida de Tabarca, capítulo 3.2). En la segunda parte de esta tesis se muestra la caracterización de la comunidad microbiana asociada

shows the results obtained from infection experiments in aquaria to investigate the pathogenic potential of *V. mediterranei* and *Vibrio corallilyticus*, previously isolated from unhealthy samples of the coral, as a result of warmer seawater conditions.

Numerous methodologies have been used in order to achieve the different objectives exposed above, from sample collection and live monitoring to live corals in field experiments, needed to study the biological and ecological characteristic of *O. patagonica* (Part I), to the use of culture and molecular techniques applied to get a better insight into the temporal dynamics and spatial variation of *O. patagonica* microbial communities, including eukarya, bacteria and *Vibrio* spp. (Part II).

al coral, así como los cambios de la misma con los factores ambientales. En el capítulo 3.3 se muestran los resultados relativos a la comunidad de eucariotas y bacterias mientras que en el 3.4 se incide con más detalle en la caracterización de la comunidad de especies de *Vibrio*. Finalmente, en el capítulo 3.5 se muestran los experimentos en acuarios realizados con el objetivo de investigar el potencial patogénico de *V. mediterranei* y *Vibrio coralliilyticus*, previamente aislados en muestras enfermas, como resultado del aumento de temperatura del agua.

Para cumplir los objetivos de esta tesis se han utilizado numerosas metodologías. Desde la recolección de muestras de coral y el seguimiento de colonias en los experimentos de campo, para estudiar las características biológicas y ecológicas de *O. patagonica* (Parte I), hasta la utilización tanto de técnicas dependientes de cultivos como de técnicas moleculares para obtener un mayor conocimiento sobre las dinámicas tanto espaciales como estacionales de sus comunidades microbianas, incluyendo los eucariotas, bacterias y las especies del género

Results and discussion

The knowledge acquired in chapter 3.1 demonstrates that nowadays this species is widely distributed along the infralittoral shallow rocky bottoms in our study area, being found in 60.65% of the sampling points and being detected, from depths of 0 (pools on abrasion platforms) to 12 m, with the highest abundances at the breaker zone, decreasing its abundance below 4-5 meters depth. Four hot spots (defined as the sampling units that displayed the highest aggregation of colonies) of *O. patagonica* were identified: ¨La Zenia, Peñíscola, the Marine Protected Area of Tabarca and Alicante Harbour, which represented the sampling point with the highest values of abundance within the entire study area. The fact that *O. patagonica* has spread widely along the Mediterranean Spanish coast from 1973, when was first observed in the Alicante Harbour (Zibrowius and Ramos, 1983), as well as with the results obtained in the chapter 3.3 about its association with *Symbiodinium* B2, clade rare in the Mediterranean Sea and more common in the tropical Western Atlantic, where this species

Vibrio (Parte II).

Resultados y discusión

El conocimiento adquirido en el capítulo 3.1 demuestra que actualmente esta especie tiene una amplia distribución en los fondos infralitorales rocosos poco profundos de nuestra área de estudio, encontrándose en un 60.65% de los puntos muestreados, entre los 0 (plataformas de abrasión) y los 12 m de profundidad, detectándose las mayores abundancias en la zona de rompiente y decreciendo su abundancia por debajo de los 4-5 m de profundidad. Se identificaron cuatro puntos calientes (unidades de muestreo que mostraron las mayores agregaciones de colonias): La Zenia, Peñíscola, el Área Marina Protegida de Tabarca y el puerto de Alicante, donde se encontraron las mayores densidades de esta especie. El hecho de que *O. patagonica* haya aumentado su distribución considerablemente en las costas del Mediterráneo español desde 1973, cuando fue observada por primera vez en el Puerto de Alicante (Zibrowius and Ramos, 1983), junto con los resultados obtenidos en el capítulo 3.3 sobre su asociación con *Symbiodinium* B2, clado raro en el Mar

seems to come from, support that *O. patagonica* is an alien species in the Mediterranean Sea.

The factors that largely determine the presence of this species are the distance from the nearest harbour, since it has ever been detected at distances larger than 10 km of them, and the benthic macroalgae community, that determines its recruitment, which seems to be favored by filamentous algae turfs. Therefore, the increase of anthropogenic disturbances over coastal ecosystems such as the construction of man-made structures (Salomidi *et al.*, 2013; Serrano *et al.*, 2013), as well as eutrophication or physical destruction of habitats that can produce changes in algae community, could be providing new spreading areas for *O. patagonica*. In fact, Moyle and Light (1996) suggested that aquatic systems with high levels of human disturbance allow development of a much higher range of invasive species that in systems with low levels of human disturbance.

Mediterráneo y más común en el Atlántico tropical occidental de donde se supone que procede esta especie, apoyan la idea de que que *O. patagonica* es por tanto una especie introducida en el Mar Mediterráneo.

Los factores que determinan en mayor medida la presencia de este coral son la distancia al puerto más cercano, no siendo detectada a más de 10 km de los mismos, y la comunidad bentónica de macroalgas que condiciona su reclutamiento, el cual parece verse favorecido por los céspedes de algas filamentosas. Por tanto, el incremento de las perturbaciones antropogénicas sobre los ecosistemas costeros y la construcción de nuevas estructuras (Salomidi *et al.*, 2013; Serrano *et al.*, 2013), así como la eutrofización o la destrucción física de los hábitats que producen cambios en las comunidades algales, podrían estar proporcionando nuevas áreas de colonización para *O. patagonica*. De hecho, Moyle y Light (1996) muestran como en los sistemas acuáticos con una mayor influencia humana se pueden desarrollar un mayor rango de especies invasoras que en los sistemas con niveles menores de

Moreover, the results obtained in chapter 3.1 highlight the ability of this species to bear different environmental conditions such as ultraviolet radiation, water salinity, turbidity, hidrodynamism and pollution. In fact, in chapter 3.2, by monitoring marked colonies during 18 months, we demonstrated that this species shows similar growth rates in eutrophic and turbid environments (Alicante Harbour, 39±3% increase in surface area) than in oligotrophic and clear ones (Tabarca, 37±4%). The seawater temperature is the factor that more influence has over its growth. Growth rates of *O. patagonica* show a minimum during cold months (February, 13ºC) and enhancement during warm months (June to September, 18-28ºC). However, when seawater temperatures are maintained over 26ºC for prolonged periods of time not only *O. patagonica* growth rates are affected, decreasing considerably, but bleaching reached the maximum percentages, such as was observed in summer 2011 (September). Remarkably, bleaching recovery rates are different depending on environmental conditions such as organic matter and mud proportion in

perturbaciones antropogénicas.

Además, los resultados obtenidos en el capítulo 3.1 destacan la habilidad de esta especie de resistir diferentes condiciones ambientales como radiación ultravioleta, salinidad, turbidez, hidrodinamismo y contaminación. De hecho, en el capítulo 3.2, mediante el seguimiento de colonias marcadas durante 18 meses, se ha demostrado que esta especie presenta tasas similares de crecimiento en ambientes turbios y eutróficos (39±3% de incremento de su superficie) que en ambientes de aguas claras y oligotróficas (37±4%). La temperatura es el factor que más influye sobre su crecimiento. Las tasas de crecimiento de *O. patagonica* muestran un mínimo durante los meses fríos (febrero, 13ºC) y un incremento durante los meses cálidos (junio-septiembre, 18-28ºC). No obstante, cuando el agua se mantiene durante periodos prolongados por encima de los 26ºC no sólo se ve afectado el crecimiento de *O. patagonica*, que disminuye de forma considerable, sino que su blanqueamiento alcanza sus máximos porcentajes, tal y como se observó en el verano de 2011 (septiembre). Cabe

seawater. In this way most colonies affected by bleaching in the Harbour recovered their normal pigmentation, while in Tabarca most bleached colonies developed tissue necrosis (34.9±4.5% coral surface) at the end of the summer 2011, and three of them died as a result of algae overlaying.

In addition to seawater temperature, the light also has influence over *O. patagonica* bleaching, such as it was demonstrated by a field experiment carried out in the Alicante Harbour, that is shown in the chapter 3.2 of this thesis In this experiment we used methacrylate plates to cover coral colonies and to test the light attenuation effect over this coral, and confirmed that *O. patagonica* is able to acclimatise to slight light reductions of photosynthetic active radiation (15%) promoting an increase in Chlorophyll *a* concentration. The increase of Chlorophyll *a* allows the absortion of more light and consequent growth of this species in turbid environment. This agrees with previous studies, in other coral species, that showed how pigment concentrations can be influenced by environmental

destacar, que la recuperación del blanqueamiento es diferente en función de las condiciones ambientales, tales como la materia orgánica o la proporción de finos en el agua. De forma que la mayoría de las colonias blanqueadas en el puerto recuperan su pigmentación normal, mientras que en Tabarca presentaron necrosis del tejido (34.9±4.5% de la superficie del coral) al final del verano de 2011, muriendo tres de ellas como consecuencia del recubriendo algal.

Además de la temperatura del agua, la luz también tiene una gran influencia sobre el blanqueamiento de *O. patagonica*, como se ha demostrado mediante la realización de un experimento de campo en el puerto de Alicante, recogido en el capítulo 3.2 de esta tesis. En este experimento se utilizaron placas de metacrilato para cubrir los corales y testar el efecto de la atenuación de la luz sobre este coral, de forma que se comprobó que *O. patagonica* es capaz de aclimatarse a una leve atenuación de la radiación fotosintéticamente activa (15%) por medio del incremento de la clorofila *a* en sus tejidos: el aumento de la clorofila *a* permite al coral absorber

conditions (Dubinsky *et al.*, 1984), that result in changes in zooxanthellae density and/or in pigment concentration per zooxanthellae to optimise light capture (Anthony and Hoegh-Guldberg 2003). However, low-light conditions (reduction of photosynthetic active radiation around 70%) produced bleaching in *O. patagonica*, effect that is more evident with the increase of seawater temperature. In fact, a recent study provided evidences that heat stress (32 °C) and low-light conditions act on the zooxanthellae, via physical disruption of photosynthetic electron transport, causing coral bleaching (Downs *et al.*, 2013).

Environmental variables not only have an effect over coral host but also over its bacterial communities, which show changes in their composition (chapter 3.3.) depending mainly on seawater temperature and coral health status. The bacterial communities associated to *O. patagonica* are different among the three coral compartments or microhabitats (mucus, tissue and skeleton), that implies a compartmentalisation of bacterial communities within the coral

una mayor cantidad de luz, lo que le permitiría a esta especie crecer en ambientes turbios. Estudios previos, realizados con otras especies, han mostrado como la concentración de pigmentos en sus tejidos está determinada por las condiciones ambientales (Dubinsky *et al.*, 1984), siendo el resultado de cambios en la densidad de zooxantelas y/o de la concentración de pigmento por zooxantela para optimizar la captura de luz (Anthony and Hoegh-Guldberg 2003). Sin embargo, en condiciones de baja luminosidad (reducción de la luz fotosintéticamente activa alrededor de un 70%) se produce el blanqueamiento de *O. patagonica*, siendo este efecto más evidente con el aumento de la temperatura del agua. De hecho, un estudio reciente prueba que el estrés térmico y las condiciones de baja luminosidad actúan sobre las zooxantelas alterando la cadena de transporte de electrones, causando el blanqueamiento de los corales (Downs *et al.*, 2013).

Las variables ambientales no sólo afectan al coral hospedador, sino también a sus comunidades bacterianas, que muestran cambios en

holobiont. Our results show that the microbiota inhabiting mucus and skeletal compartments are more stable than that of coral tissue, which showed different composition depending mainly on season and coral health status variations and to a less extent on environmental conditions. The results from molecular approaches, show changes in tissue bacterial assemblages between cold and warm months, being the class *Alphaproteobacteria* dominant in cold months (44-83 % of the sequences obtained by Next generation Sequencing), while the proportion of *Gammaproteobacteria* increase considerably during warm months (69-91%), being the order *Vibrionale*s the main responsible for this increase. It is remarkable tthe presence of *Pseudovibrio*-related sequences, among *Alphaproteobacteria*, accounting for between 17% and 35% of Illumina libraries, being the dominant genus in healthy corals and decreasing with the increase of seawater temperature.

su composición (capítulo 3.3) en función de la temperatura y del estado de salud del coral. Las comunidades bacterianas asociadas a *O. patagonica* son diferentes entre los tres compartimentos o microhabitats presentes en el coral (mucus, tejido y esqueleto), existiendo por tanto una compartimentalización de las comunidades bacterianas en el holobionte. Nuestros resultados muestran que la microbiota del mucus y del esqueleto es más estable que la del tejido, que muestra diferencias en su composición dependiendo principalmente de la estación del año y del estado de salud del coral y en menor medida de las condiciones ambientales. Nuestros resultados, basados en técnicas moleculares, muestran cambios en la composición bacteriana encontrada en los tejidos entre los meses fríos y cálidos, siendo la clase *Alphaproteobacteria* la dominante en los meses fríos (44-83 % de las secuencias obtenidas mediante técnicas de secuenciación masiva), mientras que la proporción de *Gammaproteobacteria* aumenta en los meses cálidos (69-91%), siendo el orden *Vibrionales* el que más contribuye a este incremento. Destaca

In addition, corals from Alicante Harbour and Tabarca bored communities dominated by different taxa, such as *Rhodospiralles*, *Burkolderiales*, *Clostridiales*, *Bacilliales* and *Chlamydiales* in the eutrophic Alicante Harbour and *Sphingomonadales*, *Alteromonadales* and *Flavobacterales* in the pristine Tabarca, which suggest that the occurrence of certain bacterial orders in *O. patagonica* tissues could be regulated by external environmental parameters. Similarly, Lee *et al.* (2012) showed differences among bacterial communities associated with Red Sea corals from sites with different environmental conditions, such as water nutrient content that is the primary determining factor in bacterial coral communities changes.

la presencia de secuencias relacionadas con el género *Pseudovibrio*, representando entre el 17 y el 35 % de las *Alphaproteobacteria*, siendo el género dominante y decreciendo su presencia con el incremento de la temperatura del agua.

Además, los corales del puerto de Alicante y los de Tabarca albergan comunidades dominadas por diferentes taxones, como *Rhodospiralles*, *Burkolderiales*, *Clostridiales*, *Bacilliales* y *Chlamydiales* en el ambiente eutrofizado y *Sphingomonadales*, *Alteromonadales* y *Flavobacterales* en la prístina Tabarca, lo que sugiere que la presencia de ciertos ordenes de bacterias en *O. patagonica* podría estar regulada por los parámetros ambientales externos. De forma similar, Lee *et al.* (2012) mostraron diferencias en las comunidades bacterianas asociadas a corales del Mar Rojo que se encuentran en sitios con diferentes condiciones ambientales, como el contenido en nutrientes en el agua, que es el factor que determina los cambios en dichas comunidades bacterianas.

Changes in the dominant bacterial groups in bleached samples were detected in the mucus layer by Denaturing Gradient Gel Electrophoresis (DGGE), as well as in the coral tissue where differences were also identified by Illumina sequencing. In mucus layer *Rhodobacterales* were predominant in both healthy and unhealthy corals, while the proportion of sequences belonging to *Cytophaga* and *Acidobacteria* became higher in unhealthy corals. Bacterial communities associated to coral tissue were more homogeneous for unhealthy corals than in healthy ones and although the proportions of 16S rRNA gene sequences corresponding to the main bacterial groups that were recovered by the two techniques (DGGE and Illumina) were different; they both indicated the same trends in the shifts experienced by tissue bacteria assemblages when comparing healthy and unhealthy corals. The phylum *Proteobacteria* was always the most dominant and constituted from 58 to 96% of the qualified bacterial Illumina reads in coral tissues with a large proportion of *Gammaproteobacteria*, present in both

Se han detectado cambios en los grupos dominantes de bacterias presentes en el mucus de muestras blanqueadas, mediante Electroforesis en Gel de Gradiente Desnaturalizante (DGGE), así como en el tejido donde dichas diferencias fueron también identificadas mediante la secuenciación por Illumina. En la capa mucosa el orden *Rhodobacterales* fue el dominante tanto en muestras sanas como en blanqueadas, sin embargo la proporción de secuencias pertenecientes *Cytophaga* y *Acidobacteria* aumentó en corales blanqueados. Las comunidades bacterianas asociadas al tejido fueron más similares entre muestras blanqueadas que entre sanas y aunque las proporciones de secuencias obtenidas para los principales grupos fue diferente dependiendo de la técnica utilizada (DGGE e illumina), ambas técnicas mostraron las mismas tendencias al comparar las bacterias presentes en el tejido de muestras sanas y blanqueadas. El filo *Proteobacteria* fue siempre el dominante, constituyendo entre el 58 y el 96% de las lecturas de Illumina, con una gran proporción de *Gammaproteobacteria*, presentes tanto en

healthy and unhealthy corals. Within this class, a large number of sequences corresponded to the genus *Vibrio* that accounted for up to 53% (Harbour) and 81% (Tabarca) of the sequences in unhealthy corals.

Therefore, *Pseudovibrio* genus seems to be mainly related to healthy corals and cold months and *Vibrio* genus with warm months and mainly with unhealthy corals. These results suggest that the genus *Pseudovibrio* could play a key role in the *O. patagonica* holobiont system and in the coral health status. This fact has been demonstrated in other coral species where not only plays a role in carbon and nitrogen cycles within the coral (Bondarev *et al.*, 2013), but also inhibits the growth of the coral pathogens, *V. mediterranei* and *V. coralliilyticus,* by antimicrobial activity (Nissimov *et al.*, 2009; Rypien *et al.*, 2010). Accordingly, the increase of seawater temperature and consequent loss of possible beneficial bacteria such as *Pseudovibrio* genus could disrupt the coexistence holobiont equilibrium, inducing a shift to pathogen dominance (Mao-Jones *et al.*, 2010).

muestras sanas como en las que presentaban signos de enfermedad. Dentro de la clase *Gammaproteobacteria,* un gran número de secuencias se correspondían con el género *Vibrio* que representaba más de un 53% (Puerto) y un 81% (Tabarca) de las secuencias en corales blanqueados.

Por lo tanto, el género *Pseudovibrio* parece estar principalmente relacionado con corales sanos y meses fríos y el género *Vibrio* con los meses cálidos y los corales blanqueados. Por ello, consideramos que el género *Psudovibrio* podría tener un papel importante en el holobionte de *O. patagonica*, estando involucrado en la salud del mismo. Este hecho ha sido previamente demostrado en otros corales ya que este género no solo juega un papel en los ciclos del nitrógeno y el carbono del coral (Bondarev *et al.*, 2013), sino que también inhibe el crecimiento de posibles patógenos para el coral, *V. mediterranei* y *V. coralliilyticus*, por medio de su actividad antimicrobiana (Nissimov *et al.*, 2009; Rypien *et al.*, 2010). De acuerdo con esto, el incremento de la temperatura del agua y la consecuente pérdida de bacterias

This PhD thesis also shows that *O. patagonica* holobiont not only harbour *Vibrio spp.* in unhealthy corals but also in healthy ones, so that some of these species could be autochthonous members of their microbial communities, whose composition and abundance also depend on environmental conditions (seawater temperature and organic matter concentration), geographic location and coral health status (chapter 3.4). *Vibrio* assemblages associated with healthy coral samples are dissimilar among samples collected in different locations and become more similar in unhealthy ones. We identified two main *Vibrio* phylotypes that could be part of the coral "core" microbiota, since they were mainly in healthy samples, *Vibrio harveyi-like* and *Vibrio splendidus* super clade. This last clade has been previously identified as one of the most abundant taxa in samples from Israeli coast (Koren and Rosenber, 2006). However, *V. mediterranei* and *V. coralliilyticus,* which had been previously identified as coral pathogens (Kushmaro *et al.*, 1996; Ben-Haim *et al.*, 2003; Vezulli *et al.*, 2010), were only retrieved by

beneficiosas como el género *Pseudovibrio* podrían acabar con el equilibrio existente en el holobionte y hacer que el sistema cambie y pase a estar dominado por patógenos (Mao-Jones *et al.*, 2010).

Esta tesis doctoral también resalta el hecho de que el holobionte de *O. patagonica* no sólo alberga especies de *Vibrio* en muestras blanqueadas, sino también en sanas, por tanto las especies de este género podrían ser miembros autóctonos de sus comunidades microbianas, cuya composición y abundancia depende de las condiciones ambientales (temperatura y concentración de materia orgánica), de la localización geográfica y del estado de salud del coral (capítulo 3.4). Las comunidades de *Vibrio* asociadas a muestras sanas son diferentes entre sí dependiendo de la localidad de muestreo, volviéndose más similares en corales con signos de enfermedad. Dos filotipos de *Vibrio* han sido identificados como parte constituyente del núcleo de la microbiota de esta especie, *Vibrio harveyi-like* y el super-clado *Vibrio splendidus*. Este último clado había sido previamente identificado como uno de

culturable methods in unhealthy samples. However, we also detected these *Vibrio* spp. by Next Generation Sequencing in healthy samples, even during cold months, suggesting that could be part of the normal *O. patagonica* microbiota, maintained in viable but non-culturable state until the environmental conditions allowed their growth (increase of seawater temperature).

Moreover, *Vibrio* communities associated with the endemic coral *Cladocora caespitosa* were also characterised and compared with those from *O. patagonica* in the chapter 3.4, showing that phylotypes that constitute part of the "core" microbiota are different among the coral hosts. Accordingly, coral-associated microbial communities, at least at *Vibrio* level, could be coral species dependent, which has been demonstrated in previous studies (Rohwer *et al.*, 2001; Kvennefors *et al.,* 2010). However, *Vibrio* communities associate to unhealthy samples of both species are more homogeneous and similar between them, being also *V. mediterranei* and *V. coralliilyticus* present in necrotic tissue of *C.*

los taxones más abundantes en muestras de las costas de Israel (Koren and Rosenberg, 2006). Sin embargo, *V. mediterranei* y *V. coralliilyticus*, previamente identificados como patógenos de corales (Kushmaro *et al.*, 1996; Ben-Haim *et al.*, 2003; Vezulli *et al.*, 2010) fueron únicamente aislados de muestras enfermas. No obstante, estas species de *Vibrio* han sido también detectadas mediante secuenciación de nueva generación en muestras sanas, incluso en las recogidas durante meses fríos, lo que sugiere que podrían formar parte de la microbiota normal de *O. patagonica*, manteniéndose en estado viable pero no cultivable hasta que las condiciones fueran las adecuadas para su crecimiento (aumento de la temperatura del agua)

Además, se caracterizaron las comunidades de *Vibrio* asociadas al coral endémico *C. caespitosa* y se compararon con las presentes en *O. patagonica* en el capítulo 3.4, mostrando que los filotipos que forman parte del núcleo de sus microbiotas son diferentes entre los dos corales hospedadores. Sin embargo, las comunidades asociadas a muestras con

caespitosa. Therefore, the spreading of the alien *O. patagonica* in the Mediterranean Sea could increase the possibility of a secondary *Vibrio* horizontal transmission to other native coral species (in this case *C. caespitosa*) by physical contact between them. This possibility of contagion among corals has been previously demonstrated among colonies of *Pocillopora damicornis* (Ben-Haim and Rosenberg, 2002).

Finally, in this thesis the potential pathogenic of *V. mediterranei* and *V. coralliilyticus* over *O. patagonica* has been demonstrated (chapter 3.5), by an infection experiment carried out in aquaria at different temperatures (20, 24 and 28ºC). Disease signs developed faster and to a higher extent in corals inoculated with pathogenic *Vibrios* than in untreated controls, with an expected increase of disease symptoms with the raising of seawater temperature, as well as an increase of number of culturable *Vibrios* in coral tissue. Both pathogens began to produce disease signs in corals at 24ºC that were more evident in the infection with *V. mediterranei*, reaching percentages of tissue damage of 80%,

signos de enfermedad son más homogéneas y similares entre sí, encontrándose también *V. mediterranei* y *V. coralliilyticus* en *C. caespitosa* con necrosis en sus tejidos. Por tanto, la propagación de la invasora *O. patagonica* en el Mediterráneo podría incrementar la posibilidad de una transmisión horizontal de estos *Vibrios* hacia especies de corales autóctonos (en este caso *C. caespitosa*) mediante el contacto entre ambos. Esta posibilidad de contagio entre corales ha sido demostrada para la especie *Pocillopora damicornis* (Ben-Haim and Rosenberg, 2002).

Finalmente, en esta tesis se ha demostrado el potencial patogénico de *V. mediterranei* y *V. coralliilyticus* sobre *O. patagonica* (capítulo 3.5), mediante un experimento de infección realizado en acuarios a diferentes temperaturas (20, 24 y 28ºC). Los signos de enfermedad se desarrollaron más rápidamente, afectando a un mayor porcentaje de la colonia en los corales inoculados con los *Vibrios* patógenos que en los corales control, con un esperado incremento de estos signos al aumentar la temperatura, detectándose al mismo tiempo un aumento del

than in *V.coralliilyticus* (where the damage was of 36%). Nevertheless, at 28ºC both pathogens produced tissue damage in the totality of infected colonies. Surprisingly, when these pathogens were inoculated together, corals developed disease signs faster, even when corals were maintained at 20ºC, reaching 78% of tissue damage at the end of the experiment. Interestingly, at 28ºC untreated corals also showed disease signs at the end of the experiment, indicating that this temperature could be lethal to *O. patagonica* if maintained for a long time.

One sample of each treatment was randomly chosen, at the end of the experiment, to be sequenced using Illumina platform and observed by electron microscopy, in order to detect *Vibrio* pathogens and to elucidate the changes in their bacterial communities. Just as in coral samples collected from the sea, the phylum *Proteobacteria* was the most dominant in samples from this experiment, accounting between 56 and 92% of the qualified bacterial Illumina reads in coral tissues. Within *Proteobacteria* the class *Alphaproteobacteria*, with a

número de *Vibrio*s cultivables en los tejidos del coral. Ambos patógenos comenzaron a producir daño en los corales a 24ºC, siendo éstos más evidentes en el caso de *V. mediterranei*, que alcanzó porcentajes de daño del 80% en los tejidos, que en el caso de *V.coralliilyticus* (donde el daño fue de un 36%). Sin embargo, a 28ºC ambos patógenos causaron daños en la totalidad de los tejidos de los corales infectados. Sorprendentemente, cuando los patógenos se inoculaban de forma conjunta los corales desarrollaban signos de enfermedad de forma más rápida, incluso en las infecciones realizadas a 20ºC, alcanzando porcentajes de daño en los tejidos de alrededor del 78%. Cabe destacar que a 28ºC los corales control, sin infectar, también mostraron signos de enfermedad, indicando que esta temperatura es letal para *O. patagonica*.

Una muestra de cada tratamiento fue seleccionada al azar, al final del experimento, para ser secuenciada con Illumina, con el fin de evaluar los cambios en las comunidades bacterianas y detectar los *Vibrio*s patógenos. Tal como se observó en las

majority belonging to the order *Rhodobacterales,* was the dominant in samples at 20ºC (20-29%), with the exception of the sample inoculated with the mixed culture in which the percentage of *Alphaproteobacteria* (8.6%) was more similar to samples maintained at 24 or 28ºC. In contrast, the class *Gammaproteobacteria,* with the order *Vibrionales* as the most abundant taxon within this class, increased their percentages at higher temperatures, reaching more than 70% of the sequences retrieved in samples maintained at 28ºC. Noticeably, the coral sample inoculated with the mixed culture at 20ºC also showed percentage of *Gammaproteobacteria* (47%) considerably higher than the rest of samples maintained at 20ºC (3-10%). In these samples *Vibrio* strains present in the inocula were also detected. Both pathogenic strains (*V. mediterranei* and *V.coralliilyticus*), together with other sequences belonging to these phylotypes (with a 98.7% identity), were mainly detected in samples at 28ºC. In uninfected coral maintained at 20ºC no sequences related to pathogens were detected, and at 28ºC *Vibrio* sequences was not retrieved, probably due to the lower

muestras ambientales, el filo *Proteobacteria* fue el dominante en las muestras de los acuarios, representando entre el 56 y el 92% de las secuencias. Dentro de las *Proteobacteria* la clase *Alphaproteobacteria*, con una mayoría del orden *Rhodobacterales,* fue la dominante en las muestras mantenidas a 20ºC (20-29%), con la excepción de la muestra inoculada con el cultivo mixto de patógenos que presentó un porcentaje de *Alphaproteobacteria* (8.6%) similar al de las muestras mantenidas a 24 y 28ºC. Por el contrario, la clase *Gammaproteobacteria*, con el orden *Vibrionales* como el más abundante, alcanzó más del 70% de las secuencias obtenidas a partir de los corales mantenidos a 28ºC. Cabe destacar que el coral inoculado con el cultivo mixto a 20ºC también presentó un gran porcentaje de *Gammaproteobacteria* (47%), siendo considerablemanete superior al del resto de muestras mantenidas a 20ºC (3-10%). En estas muestras también se detectaron los aislados de *Vibrio* presentes en el inóculo. Ambos patógenos (*V. mediterranei* y *V.coralliilyticus*), junto con otras secuencias pertenecientes a sus filotipos (con un 98.7% de identidad

quality of this sample sequences. By contrast, in the control sample maintained at 24ºC sequences related with *V. mediterranei* and *V.coralliilyticus* were retrieved, including sequences with a 99.7% identity with the *V. coralliilyticus* strain used in the experiment. Therefore, these pathogens could be in viable but not culturable state inside the coral, as was observed in field samples, and with the increase of temperature increase their pathogenicity causing coral damage.

In summary, this PhD thesis highlights that one the most significant risks to *O. patagonica* is global warming and the consequent increase of seawater temperatures, as well as the necessity to study the changes produced at all levels in the coral holobiont. We have shown that there is a relationship between changes in normal *O. patagonica* microbiota and the increase of seawater temperature, as well as the presence of possible *Vibrio* pathogens and bleaching. These results reinforce the idea that global warming could be leading to the increase of coral diseases as consequence of the

con nuestros aislados), se detectaron principalmente en las muestras de 28ºC. En el coral control de 20ºC no se detectaron secuencias relacionadas con nuestros patógenos, y en el de 28ºC no se recuperó ninguna secuencia perteneciente al género *Vibrio*, probablemente debido a la menor calidad de las secuencias obtenidas en esta muestra. Por el contrario en el control a 24ºC ambos patógenos fueron detectados, incluyendo secuencias con un 99.7% de identidad con el aislado de *V. coralliilyticus* utilizado en el experimento. Por lo tanto, estos *Vibrio*s patógenos podrían estar en los tejidos en estado viable pero no cultivable y con el aumento de la temperatura aumentar su patogenicidad causando daño a los corales.

En resumen, en esta tesis doctoral se resalta el hecho de que uno de los mayores riesgos para *O. patagonica* es el cambio climático y el consecuente aumento de temperatura del agua, así como la necesidad de estudiar los cambios producidos a todos los niveles del holobionte. Se ha demostrado la existencia de una

interactions between *Vibrio* pathogens and corals.

relación entre los cambios de la microbiota de *O. patagónica* y el aumento de temperatura, así como la presencia de posibles *Vibrio*s patógenos y el blanqueamiento. Por tanto, se reafirma que la relación entre *Vibrio*s patógenos y las enfermedades en corales puede incrementarse como consecuencia del cambio climático.

1. Introduction

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Photo/Figure: *Oculina patagonica* colony in the Marine Protected Area of Tabarca.

1.1. CORAL HOLOBIONT

Until recently, corals have been considered a product of a mutualistic interaction among the coral animal and single cell algae symbionts belonging to the genus *Symbiodinium* (commonly referred to as zooxanthellae); this view did not consider the symbiotic potential of a large and diverse assemblage of other coral-associated microorganisms such as bacteria, fungi or archaea, which all form a collaborative consortium named the coral holobiont (Rohwer *et al.*, 2001; Knowlton and Rohwer, 2003).

1.1.1. The host

Corals belong to the metazoan phylum Cnidaria, which also includes hydroids, jellyfish and sea anemones, and Class Anthozoa and Subclass Hexacorallaria, which can be broadly divided ecologically, but not systematically, into reef-building (hermatypic) and non-reef-building (ahermatypic) corals. Most hermatypic corals, belong to the Order Scleractinia, and consist of colonies of individual polyps interconnected by complex and well-developed system of gastrovascular canals, allowing exchange of nutrients. These polyps are radially symmetrical with one opening, that serves as both mouth and anus, which is surrounded by tentacles used for capture zooplankton. Corals are composed of two cell layers (Fig. 1.1), separated by a thin connective-tissue layer (mesoglea):

- The epidermis contains stinging cells called cnidocyst (also known as cnida or nematocysts) characteristic of Cnidaria, and mucocytes that synthesise the surface mucus layer, which is in contact with seawater.
- The gastrodermis contains the symbiotic algae, zooxanthellae, which reside exclusively in membrane-bound vacuoles.
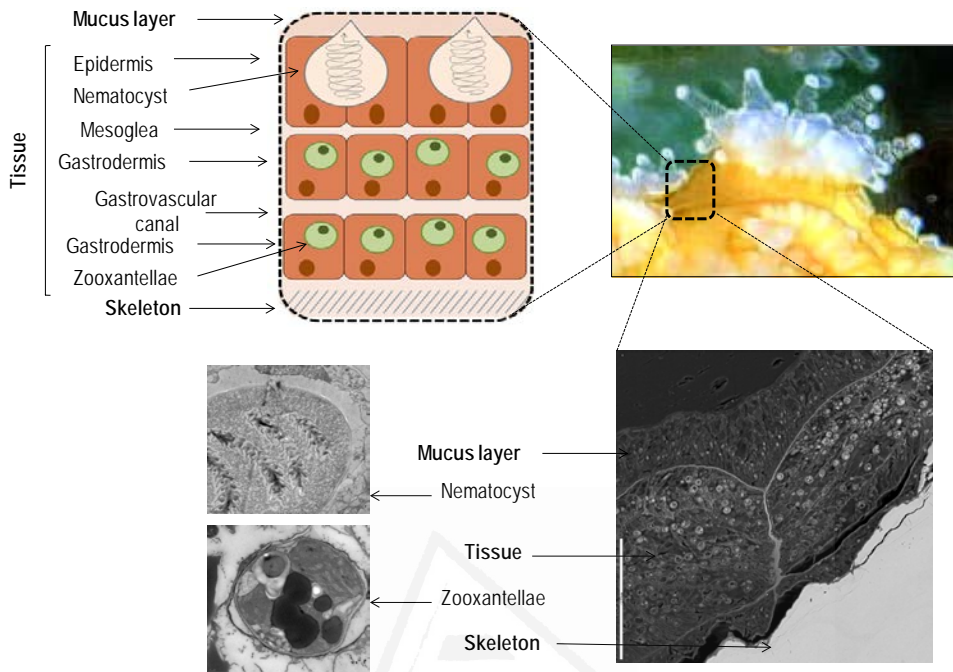
Figure 1.1. Coral structure. The right-up panel is a picture of the coral *Oculina patagonica* and the left-up panel is a schematic diagram showing the surface mucus layer, coral tissue layers and the skeleton. Down panels are microscopy images of internal structure of *O. patagonica*, showing nematocyst and zooxanthellaes (Microscopy images: Virginia Souza-Egipsy).

1.1.2. Zooxanthellae

In the cnidarian-zooxanthellae associations, photosynthetic algae are located in host-derived vacuoles (symbiosomes) within the gastrodermal layer (Fig. 1.1). These zooxanthellae almost without exception belong to the genus *Symbiodinium* (Trench, 1987) and are by far the best-understood microbial associates of corals. This is a mutualistic symbiosis, in which the symbiont provides up to 95% of its photosynthetic products (glycerol, glucose, amino acids or lipids) to the host (Muscatine, 1981), thus procuring to the coral of the energy (Gattuso *et al*., 1999) required for the growth and formation of coral reefs. In return, the host provides protection to the zooxanthellae as well as a source of inorganic nutrients, such as inorganic carbon, a suitable source of nitrogen or phosphate (see Yellowlees *et al*., 2008 for review).

Zooxanthellae were originally described as one single dinoflagellate species, *Symbiodinium microadriaticum* (Freudenthal, 1962), although subsequent studies have shown that this genus represents a large species complex, currently encompassed by nine clades (A-I) (Pochon *et al.*, 2004; Pochon and Gates, 2010). Furthermore, each clade is divided into multiple types based on the nuclear internal transcribed spacer, region 2, (ITS2), which is considered the most informative region for identifying *Symbiodinium* types (e.g., LaJeunesse, 2001; van Oppen *et al.*, 2005). *Symbiodinium* in clades A, B, C, D and F are known to associate regularly with scleractinian corals (Pochon and Gates, 2010).

Zooxanthella symbioses can be established either from free-living *Symbiodinium* of the ocean environment (horizontal acquisition; Trench, 1987), or directly from the parent egg or brooded larvae (maternal or vertical acquisition; Trench, 1987). Coral species that transfer zooxanthellae by vertical transmission show homogeneity in their symbiont clades (Karako-Lampert *et al.*, 2004); while in horizontal transmissions the host generally forms associations with a broad range of symbiotic genotypes (Douglas, 1998), which are acquired from the environment during the larval stage (Harii *et al.*, 2009).

Reef building coral species can harbour genetically different symbionts that show clear biogeographical patterns, being *Symbiodinium* clades A, B and F more common at higher latitudes and clade C at tropical latitudes. However, although there are latitudinal patterns, differences between oceans also exist. For instance, corals in the tropical Western Atlantic host *Symbiodinium* A and B much more commonly than those from the Pacific Ocean, where harbour clade C at comparable latitudes (see Baker, 2003 for a review). In the Mediterranean Sea, the clade "temperate A" or A is harboured by almost all the host cnidarian species studied so far (Savage *et al.* 2002, Visram *et al.*, 2006; Forcioli *et al.*, 2011), while only one anemone species, *Bunodeopsis strumosa*, which is endemic of the Mediterranean but of ancient tropical origin (Visram *et al.*, 2006), harbours clade B.

The symbiosis between *Symbiodinium* and corals is dynamic and can vary temporally and even in response to changes in environmental conditions. Little *et al.* (2004) hypothesized that the non-selective acquisition of symbionts and the maintenance of multiple symbiont types is an adaptive feature for corals, since it permits changes in the relative abundance of symbiont types with distinct physiological characteristics, which may be a mechanism for the holobiont to acclimatize to perturbations in the environment (Buddemeier and Fautin 1993). For example, *Acropora millepora* is able to increase its thermal tolerance (1.0–1.5°C) as a direct result of a change in the symbiont type dominating in their tissues from Clade C to D (Berkelmans and van Oppen, 2006). This strategy provides an advantage over other corals that exhibit high specificity or fidelity to one or a few closely related *Symbiodinium* types, in the face of a global warming scenario.

1.1.3. Other Eukaryotes

Besides zooxanthellae, other phototrophic eukaryotes have also been detected in corals. The diversity of such phototrophs can increase during times of stress and subsequent loss of zooxanthellae (Lins-de-Barros *et al.*, 2013), since the *Symbiodinium* loss allows a deeper penetration of light within the coral skeleton and the subsequent secondary colonization of *Chlorophyta* and *Streptophyta* (Garcia *et al.*, 2013). For instance, an increase of filamentous endolithic green algae of the genus *Ostreobium* has been observed in corals that loss their zooxanthellae (Fine *et al.*, 2002a; Littman *et al.*, 2011), together with a higher transference of photoassimilates to the overlying coral tissue, which can be used as an energy source for the coral (Schlichter *et al.*, 1996; Fine *et al.*, 2002b).

1.1.4. Bacteria

Most studies carried out to investigate the Bacteria community associated to corals examine the whole animal, without considering that corals are complex animals with different microhabitats (mucus layer, coral tissue and skeletal matrix), even being demonstrated that bacterial communities are different among coral compartments (Sweet *et al.*, 2011).The coral bacterial communities are also different from communities of the surrounding environmental samples, confirming that corals harbour and maintain their own distinct microbial flora; which can be really diverse. In fact, recent studies using pyrosequening approaches have detected up to 1000 bacterial species from a single Red Sea coral (Lee *et al.*, 2012), and around 4000 in Caribbean species (Sunagawa *et al.*, 2009).

Previous studies have reported the existence of unique bacterial communities for different coral species (Rohwer *et al.*, 2001; 2002), which are geographically consistent, suggesting that the coral host determine the composition of prokaryotes within the holobiont. However, the spatial and temporal stability of these interactions has been debated in other studies that show that coral-associated microbial species are site specific (Littman *et al.*, 2009); implying that environmental factors have some role influencing coral- associated microbial communities. Lee *et al.* (2012) showed how bacterial communities varied greatly between locations with different environmental conditions; being similar among species in disturbed sites and showing larger variations in pristine waters. Seasonal effects have also been reported to cause shifts in coral-associated bacterial diversity (Hong *et al.*, 2009).

While the presence and diversity of coral-associated bacteria has been widely studied, little is known about the role that these microorganisms play in the coral holobiont; although, there are increasing evidences that coral microbiota is crucial, at least in biogeochemical cycling. Previous studies have demonstrated the presence of bacteria involved in nitrogen (Lesser *et al.*, 2004; Chimetto *et al.*, 2008) and sulfur cycling (Raina *et al.*, 2009); which was later confirmed by Kimes *et al.* (2010) using a functional gene array to assesss the potential biogeochemical

cycling of microbial communities associated with the coral *Montastraea faveolata*, and detected genes involved in carbon, nitrogen, and sulfur cycling.

It has also been hypothesized that the normal microbiota associated with corals play a role in resistance to diseases (Ritchie and Smith 2004; Reshef *et al.*, 2006) via competition for nutrients and/or production of antibiotics (Ritchie, 2006). Changes or alterations in these microbial–coral associations due to environmental stressors can disrupt the coral´s immune system and increase coral susceptibility to bacterial infections and diseases (Ritchie, 2006; Lesser *et al.*, 2007).

1.2. CORALS IN THE MEDITERRANEAN SEA

Coral reefs dominate coastal tropical environments, between the Tropic of Capricorn and Tropic of Cancer, 25ºS-25ºN, (Veron, 1986). Their subsistence depend on many environmental variables including temperature, salinity, nutrients, light availability and aragonite saturation state of seawater (Kleypas *et al.*, 1999), which influence over physiological processes of photosynthesis and calcification, as well as coral survival. These variables are the first-order determinants of their distribution in select areas of the world's oceans. In the Mediterranean Sea, which is a temperate and oligotrophic sea with a great marked seasonal variation in the main environmental parameters, the number of coral species represents less than 5% of those being around the world. Another reason why hermatypic corals are scarce in the Mediterranean is the high competence with benthic algae that restrict the presence of corals on the illuminated rocky surfaces (Zabala and Ballesteros, 1989).

Among the few corals inhabiting the Mediterranean Sea, *Cladocora caespitosa* (Linnaeus, 1767), a scleractinian coral belonging to the family Faviidae, is the only endemic reef-forming species, which may forms hemispherical colonies (Fig. 1.2) from surface waters to 40 m depth (Zibrowius, 1980; Laborel, 1987; Peirano *et al.*, 2004). However, living banks (formed by the fusion of numerous colonies) of *C. caespitosa* are currently restricted to only a few Mediterranean locations (Zibrowius, 1980; Laborel, 1987; Morri *et al.*, 1994; Kruzic and Pozar-Domac,

2003; Kerstin and Linares, 2012). By the other hand, this species is endangered by increasing disturbances that are affecting coastal ecosystems, including elevated seawater temperatures (Rodolfo-Metalpa *et al.*, 2000, 2005, 2008a; Kersting *et al.*, 2013) or invasive species (Kersting *et al.*, 2014); being listed on CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Apendix II and on UICN (International Union for Conservation of Nature) red list of threatened species.

1.2.1. *Oculina patagonica* an alien coral in the Mediterranean Sea

The coral *Oculina patagonica* De Angelis, 1908 had been the only occurrence of an alien scleractinian coral in the Mediterranean Sea until now, that another non indigenous zooxanthellate scleractinian coral from the Indo-pacific, *Oulastrea crispata*, has been detected in shallow waters on the west coast of Corsica (Hoeksema and Ocaña, 2014). *Oculina patagonica* was detected for the first time in 1966 on the Ligurian coast of Italy, near of the Savona harbour (Zibrowius, 1974). It was described using Pleistocene fossil samples collected in northern Argentina, assuming that this species could have been accidentally transferred to the Mediterranean by transoceanic transport from the temperate South West Atlantic (Zibrowius, 1974; 1992; Zibrowius and Ramos, 1983). Although it was first recorded near of Savona harbour and then in the Alicante harbour in 1973 (Fig. 1.2); it seems that large extensions of the Spanish Southeast coast were initially colonized, considering the abundance and extent of its colonies (Zibrowius, 1992; Zibrowius and Ramos, 1983).
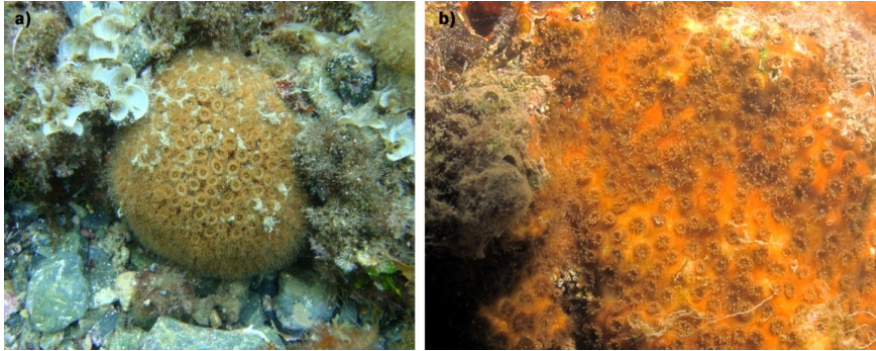
Figure 1.2. At left a *Cladocora caespitosa* colony at 5m depth in the Marine Protected Area of Tabarca and at right *Oculina patagonica* colony at 5m depth in the Alicante Harbour.

Nowadays, this species is widely spread throughout the Mediterranean: Ligurian Sea, in Italy, (Zibrowius, 1974); France (experimental transplantation of *O. patagonica* near Marseille; Zibrowius, 1974); Spain (from Algeciras to Catalonia and Balearic Islands) (Zibrowius and Ramos, 1983; Ramos-Esplá, 1985; Ballesteros, 1998; Izquierdo *et al.*, 2007; Coma *et al.*, 2011; Serrano *et al.*, 2012, 2013); Lebanon (Bitar and Zibrowius,1997); Israel (Bitar and Zibrowius, 1997; Fine *et al.*, 2001); Egypt, (Bitar and Zibrowius, 1997); Turkey (Çinar *et al.*, 2006); Greece (Salomidi *et al.*, 2006) Algeria, Tunisia (Sartoretto *et al.*, 2008) and Adriatic Sea (Cvitovic *et al.*, 2013). Most likely, this wide expansion has been caused by the intense intra-Mediterranean maritime traffic (Zibrowius, 1992); and the increase of anthropogenic activities (Serrano *et al.*, 2013; Salomidi *et al.*, 2013) as well as its ability to survive under different environmental conditions (Armoza-Zvuloni *et al.*, 2012) that have favored its establishment and colonization of new coastal areas.

1.3. IMPACTS OF GLOBAL WARMING ON CORALS

Coral reefs are probably the first marine ecosystem to suffer extreme damage and possible collapse as a consequence of climate change and the following increase of seawater temperatures (Wilkinson, 2008). The rise of global average temperatures has been about 0.74 ºC in the later 100 years and approximately 0.13 ºC/decade over the past 50 years (Salomon *et al.*, 2007); in the Mediterranean Sea temperatures has risen from 1982 to 2005 at an average rate of 0.04±0.01 ºC/year (Diaz-Almela *et al.*, 2007). In spite of the difficulty of making accurate predictions on the future of coral reefs given the variety of stresses and climate factors that affect corals, expert predictions are that 20% of the reefs will be under threat in 20-40 years (Wilkinson, 2008).

Reports of large-scale mass mortality of marine populations are becoming more frequent all over the world. In the Mediterranean Sea, two large mass mortality events of sessile epibenthic invertebrates have been detected in 1999 and 2003 (Cerrano *et al.*, 2000; Garrabou *et al.*, 2009) and two less marked events in 2006 and 2008 (Bensoussan *et al.*, 2010; Vezzulli *et al.*, 2010; Huete-Stauffer *et al.*, 2011); and may be linked with the warming trend observed in this sea (Garrabou *et al.*, 2009; Vargas-Yañez *et al.*, 2010).

1.3.1. Influence on coral growth

There are good evidences that average water temperatures are a major environmental factor controlling coral growth rates, as shown by the strong correlation between coral calcification rates and sea surface temperatures (Lough and Cooper, 2011). The optimum temperature for coral calcification is around 27ºC, which has been calculated from different coral species and locations, such as the Caribbean Sea (Marshall and Clode, 2004) or the Great Barrier Reef (Cooper *et al.*, 2008). Increasing seawater temperature can accelerate coral growth (generally 18-30ºC), but if rise beyond the optimal coral temperature range could cause an adverse impact on coral-zooxanthellae association, inhibiting the coral calcification (Marshall and Clode, 2004; Cooper *et al.*, 2008). Therefore, the increase of seawater temperatures as a consequence of climate change could be one of the causes of the coral decline in the following years.

In the Mediterranean Sea, corals suffer larger seawater temperature fluctuations, which cause seasonality in coral growth rates. For *C. caespitosa*, Kružić *et al.* (2012) noted a positive correlation between coral growth and sea temperatures, detecting the highest growth rates in the warmer periods. Nevertheless, it has been demonstrated that prolonged periods of high temperatures cause a decrease in its calcification rates (Rodolfo-Metalpa *et al.* 2006). It seems thus that although temperate corals are well adapted to marked temperature seasonality, extreme seawater temperatures can have an evident impact on the growth of this species, even leading to mortality events, as previously recorded during eventually warm years (Kersting *et al.,* 2013).

1.3.2. Coral bleaching

Environmental variables also affect the zooxanthellae that live in symbiosis with corals (Rowan, 1998; Warner *et al.,* 2006; Downs *et al.,* 2013), which confer most of the pigmentation to the holobiont. The loss of these zooxanthellae or their pigments are the cause of coral bleaching, i.e. corals appear white (or bleached) because the white calcium carbonate coral skeleton shows up through the transparent coral tissue (Brown, 1997). Since the early 1980s, mass coral bleaching events have increased in extent and frequency and have been directly correlated with increasing seawater temperatures (Hoegh-Guldberg, 1999). The coral bleaching observed worldwide following the 1998 El Niño was the most massive and devastating recorded up to now (Hoegh-Guldberg, 1999), when surface temperatures were more than 0.9ºC above normal in the warmest month (satellite-derived data; Goreau *et al.*, 2000). This phenomenon has as result the depressed growth of corals (Goreau and MacFarlane, 1990) and mass coral mortality (Hoegh-Guldberg, 1999; McClanahan, 2004); being one of the major impacts on coral status and health at regional and global scales.

There is no coherent or simple explanation of the causes of bleaching, despite the considerable research on this topic. One hypothesized mechanism of coral bleaching involves the production of reduced oxygen intermediates (toxic oxygen) from damaged photosynthetic symbionts and host cell mitochondrial membranes, that subsequently causes host tissue damage and expulsion of

zooxanthellae (Lesser, 1997); suggesting that bleaching is a host innate immune response (Weis, 2008). Another explanation would be the reduction in the antibiotic properties of mucus (derivative from its microbial communities) during thermal stress, making colonies more susceptible to pathogenic microorganisms (Ritchie, 2006); whose distribution range and virulence are also linked to climate change (Harvell *et al.*, 2007).

These different phenomena are not necessarily mutually exclusive. They may occur sequentially or in parallel, and vary in importance depending on the triggering factor of bleaching. Bleaching can, therefore, be considered as a linked group of ailments or diseases in the broadest sense of deleterious physiological responses (i.e. not restricted to pathogen-induced disease; Douglas, 2003).

1.3.3. Coral diseases

Over the past two decades, the increase in prevalence and severity of coral disease outbreaks has seriously impacted corals throughout the oceans worldwide (Goreau *et al.*, 1998; Harvell *et al.*, 1999). Coral diseases could be defined as the processes that result in coral tissue damage, breakdown of host–symbiont relationship or alteration of its physiological functions, producing visible symptoms; being caused by pathogenic microorganisms, environmental stress, and a debilitation of the host immune system, as consequence of changes in its normal microbiota. There are approximately 18 coral diseases identified thus far (Rosenberg *et al.*, 2007; Bourne *et al.*, 2009; Rosenberg and Kushmaro, 2011). Although most of the pathogens responsible for these diseases are unknown, several putative agents have been identified, belonging many of them to the *Vibrionaceae* family (Table 1.1).

Table 1.1. The best studied infectious diseases of corals, with their identified responsible pathogens (modified from Rosenberg and Kushmaro, 2011).

Disease	Species infected	Ocean	Pathogen	References
Bacterial bleaching	*Oculina patagonica*	Mediterranean Sea	*Vibrio shiloii (=Vibrio mediterranei)/ Vibriocoralliilyticus*	Kushmaro *et al.*, 1996/ Mills *et al.*, 2013
Bacterial bleaching and lysis (White syndrome)	*Pocillopora damicornis* *Paramuricea clavata*	Indo-Pacific, Red Sea Mediterranean Sea	*Vibrio coralliilyticus*	Ben-Haim *et al.*, 2003 Vezulli *et al.*, 2010
White plague	Mainly *Favia* and *Goniastrea* genus	Red Sea	*Thalassomonas loyana*	Thompson *et al.*, 2006
White Band	Acroporidae family	Caribbean Sea	*Vibrio carchariae (harveyi)*	Gil-Agudelo *et al.*, 2006
White pox	*Acopora palmata*	Caribbean Sea	*Serratia marcescens*	Patterson *et al.*, 2002
Yellow band disease (Yellow blotch)	*Montastrea* genus	Caribbean Sea and Indo-Pacific	*Vibrio* core group	Cervino et al. 2008
Black band disease	Several coral species	Global distribution	Consortium of microorganism (including *Vibrio* spp.)	Arotsker *et al.*, 2009
Aspergillosis	Octocorals (Gorgonians)	Caribbean Sea	*Aspergillus sydowii*	Geise *et al.*, 1998
Skeletal eroding band	Several coral species	Global distribution	*Halofolliculina corallasia (protozoan)*	Antonius and Lipscomb, 2000

1.3.4. The role of *Vibrio* spp. in coral diseases and the effect of temperature

The genus *Vibrio* includes Gram-negative heterotrophic bacteria that occur naturally in marine, estuarine and freshwater environments around the world, being one of the most diverse marine bacterial genera (Gomez-Gil *et al.*, 2014), which occupies habitats ranging from the deep sea to shallow aquatic environments; being associated with phyto and zooplankton and other marine animals or as free-living inhabitants in the water column and sediments (Thompson *et al.*, 2004).

The distribution and dynamics of *Vibrio* spp. as well as the environment conditions that favor their proliferation have been widely studied, largely because many species are potential human and animal pathogens, such as corals, shellfish, shrimp and fish (reviewed by Pruzzo *et al.* 2005). It is known that temperature and salinity are the strongest environmental variable correlates to *Vibrio* abundance in the water column (see Takemura *et al.* 2014 for a review); explaining the greatest seasonal variations in temperate seas. Furthermore, Thompson *et al.* (2004) observed that temperature also influences the overall structure of *Vibrio* community, identifying distinct warm-water and year-round populations of *Vibrio*s. In addition, other environmental parameters can also influence *Vibrio* abundance; this is the case of chlorophyll *a* (which is an index of phytoplankton´s biomass), since phytoplankton blooms can serve as substrates by providing attachment surfaces and can also sustain bacterial communities (Karl, 2007).

Therefore, the increase of seawater temperature as a consequence of global warming promotes the proliferation of *Vibrio* spp. particularly in temperate aquatic regions, accordingly a rise of *Vibrio*-associated diseases could be occurred (Harvell *et al.*, 2002, Baker-Austin *et al.*, 2012, Vezzulli *et al.*, 2013a). Recent studies have shown that temperature can also affect *Vibrio* pathogenicity; Kimes *et al.* (2012) demonstrated that temperature has a direct effect over several virulence factors involved in motility, host degradation, secretion, and antimicrobial resistance of *Vibrio coralliilyticus*, which was linked to the disease of the purple gorgonian *Paramuricea clavata* (Vezzulli *et al.*, 2010).

1.4. BLEACHING OF *Oculina patagonica*

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 or not a role in the bleaching process. This process was first observed along the Israeli coastline in the summer of 1993 and Koch's postulates were applied to demonstrate that *Vibrio shilonii* (originally spelled *shiloni*), a later synonym of *Vibrio mediteranei* (Thompson *et al.*, 2001), was the causative agent of bleaching (Kushmaro *et al.*, 1996, 1997); being the increasing of seawater temperature the environmental factor that triggered the disease (Kushmaro *et al.*, 1998).

Subsequent studies were published outlining the sequential steps in the infection of *O. patagonica* by *V. mediterranei* and how temperature affects various virulence factors. The first step in the infectious process is adhesion of the pathogen to receptors on the coral mucus layer (Banin *et al.*, 2000a), being the temperature of bacterial growth critical for the adhesion (up to 20ºC) (Toren *et al.*, 1998). Then, the pathogen penetrates and multiplies into the coral (Banin *et al.*, 2000b), where it produces a toxin that binds to zooxanthellae membranes, forming a channel that allows ammonia to pass rapidly, thereby destroying the pH gradient across the membrane and blocking photosynthesis (Banin *et al.*, 2001b); this toxin is produced at much higher levels at 28ºC than at 16ºC. Furthermore, *V. mediterranei* produces an extracellular superoxide dismutase, at 30°C, but not at 16°C, which protects it from oxidative stress caused by the high concentration of oxygen produced by intracellular zooxanthellae photosynthesis (Banin *et al.*, 2003). Accordingly, the infection occurred during the summer (water temperatures from 25–30ºC), but not in winter (16–20ºC) (Israely *et al.*, 2001). Sussman *et al.* (2003) demonstrated that the marine fireworm *Hermodice carunculata* was a winter reservoir and a spring–summer vector for *V. mediteranei*.

Although *O. patagonica* bleaching has continuously occurring in the Mediterranean Sea during the summer months, *V. mediterranei* has not been detected in either bleached or healthy colonies of this coral since 2004 and laboratory stocks of *V. mediteranei* did not cause bleaching by experimental

infection in aquaria. According to these results Reshef *et al.* (2006) proposed the Coral Probiotic Hypothesis, which posits the existence of a dynamic relationship between symbiotic microorganisms, environmental conditions and corals; and how corals could acquire beneficial bacteria, which allow them to adapt to changing environmental conditions more rapidly and even to develop resistance to diseases. Some bacteria, isolated from *O. patagonica*, presumptively identified as *Pseudoalteromonas* sp. and *Roseobacter* sp, showed antibiotic activity towards *V. shiloi* (Nissimov *et al.*, 2009); supporting the concept that coral associated microorganism play an important role showing a probiotic effect on this coral.

Later studies suggested that V. *mediterranei* was not involved or was not the primary cause of the annual bleaching of *O. patagonica* in the Eastern Mediterranean (Ainsworth *et al.*, 2008). These authors stated that bacteria do not have a primary but rather a secondary role during coral bleaching because of the increase in susceptibility to microbial attack experienced by corals during environmental stress. However, a recent study (Mills *et al.*, 2013), showed that antibiotics inhibit temperature-induced bleaching of *O. patagonica* fragments and made the corals sensitive to *V. mediterranei* infection, providing support for the microbial hypothesis of coral bleaching. Furthermore, they suggest that even though *O. patagonica* might have developed resistance to infection by *V. mediteranei* in natural conditions, bacteria are responsible for bleaching, being *V. coralliilyticus* one possible causative agent.

1.5. OBJETIVES

The main goal of this PhD Thesis is to study the response of the coral *O. patagonica* and its microbial community to different environmental conditions, using this coral as a model to elucidate the role of *Vibrio* spp. and environmental factors in bleaching events, particularly in a global warming scenario.

To achieve this goal, this thesis has been structured in two parts and five chapters, each addressing a specific objective:

Part I. Biological and ecological characteristic of *Oculina patagonica*

Chapter 3.1 aims to describe the *O. patagonica* spatial distribution and its "invasive level" along the Valencian Region and to contribute to the understanding of the main factors that have allowed its spread. To achieve this goal its presence was mapped along the rocky coast, noting different factors such as algal community, coast morphology and distance from the nearest harbour.

Chapter 3.2 investigates the response, in terms of growth and bleaching, of this coral under different environmental conditions. For this, its growth rates were calculated in two contrasted environments, one eutrophic and turbid (Alicante Harbour) and one oligotrophic (Marine Protected Area of Tabarca); and the temporal growth and bleaching variations were also investigated in response to seawater temperature, as well as the role of light attenuation in coral bleaching.

Part II. The response of the coral associated microbial community with different environmental conditions.

Chapter 3.3 intends to know how zooxanthellae and bacterial communities respond to natural changes in environmental conditions. To carry out this chapter, *O. patagonica* associated bacterial communities were monitored under two contrasted environments every three months during a year by molecular approaches. In addition, changes in zooxanthellae community were also assesssed during summer, in two different locations of the Mediterranean Sea.

Chapter 3.4 tries to shed some light about the relationship between *Vibrio* spp., seawater temperature and *O. patagonica* bleaching. For this, seasonal patterns of abundance and diversity in *Vibrio* spp. communities associated with healthy and diseased *O. patagonica* colonies were analysed at a local and regional scale. Furthermore, *Vibrio* communities in the endemic coral *C. Caespitose* were compared with Vibriocommunities of *O.patagonica* in two different regions of the Mediterranean Sea.

Chapter 3.5 investigates the pathogenic potential of *Vibrio* spp. as a result of warmer seawater conditions. To mimic these conditions a laboratory experiment in aquaria was performed, testing two potential pathogens (*V. mediterranei* and *V. coralliilyticus*) and three temperatures (20, 24 and 28ºC).
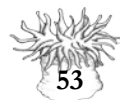
PUBLICATION STATUS OF THE CHAPTERS

This PhD has been conceived as a whole; however each study has been submitted as a separate paper for publication in scientific journals (four are already published while the other two are in preparation).

Distribution patterns of alien coral *Oculina patagonica* De Angelis D'Ossat, 1908 in western Mediterranean Sea (Chapter 3.1)

Rubio-Portillo, E., Vázquez-Luis, M., Izquierdo-Muñoz, A., Ramos Esplá, A. A.

Journal of Sea Research (2014), vol. 85: 372-378. Impact factor : 1.86; Q2 Marine and Freshwater Biology

Growth and bleaching of the coral *Oculina patagonica* under different environmental conditions in the western Mediterranean Sea (Chapter 3.2)

Rubio-Portillo, E., Vázquez-Luis, M., Valle, C., Izquierdo-Muñoz, A., Ramos-Esplá, A.A.

Marine Biology (2014), vol. 161: 2333-2343. Impact factor : 2.39; Q1 Marine and Freshwater Biology

Eukarya associated with the stony coral *Oculina patagonica* from the Mediterranean Sea (Chapter 3.3)

Rubio-Portillo, E., Souza-Egipsy, V., Ascaso, C., de los Rios Murillo, A.,. Ramos-Esplá, A. A., Antón, J.

Marine genomics (2014), vol 17: 17-23. Impact factor: 1.97; Q3 Genetics and Heredity

New insights into *Oculina patagonica* coral diseases and their associated *Vibrio* spp. communities (Chapter 3.4 and 3.5)

Rubio-Portillo, E., Yarza, P., Peñalver, C., Ramos-Esplá, A. A., Antón, J.

The ISME Journal (2014), vol. 8: 1794-1807. Impact factor: 9.27; Q1 Ecology

Coral associated *Vibrio* communities differ with geographic location and health status in scleractinian corals of the Mediterranean Sea (Chapter 3.4). In preparation

Bleaching effect on Bacteria community associated to *Oculina patagonica* (Chapater 3.3). In preparation**.**

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Universidad de Alicante

Photo/Figure: Scuba-diving sampling process (by Maite Vazquez Luis)

Numerous methodologies have been used within the framework of this thesis (see Fig. 2.1). From the samples collection and the live corals monitoring in field experiments in order to study the biological and ecological characteristic of *Oculina patagonica*, to the use of culture and molecular techniques to get a better insight into the temporal dynamics and spatial variation of *O. patagonica* microbial communities, including eukarya, bacteria and *Vibrio* spp.
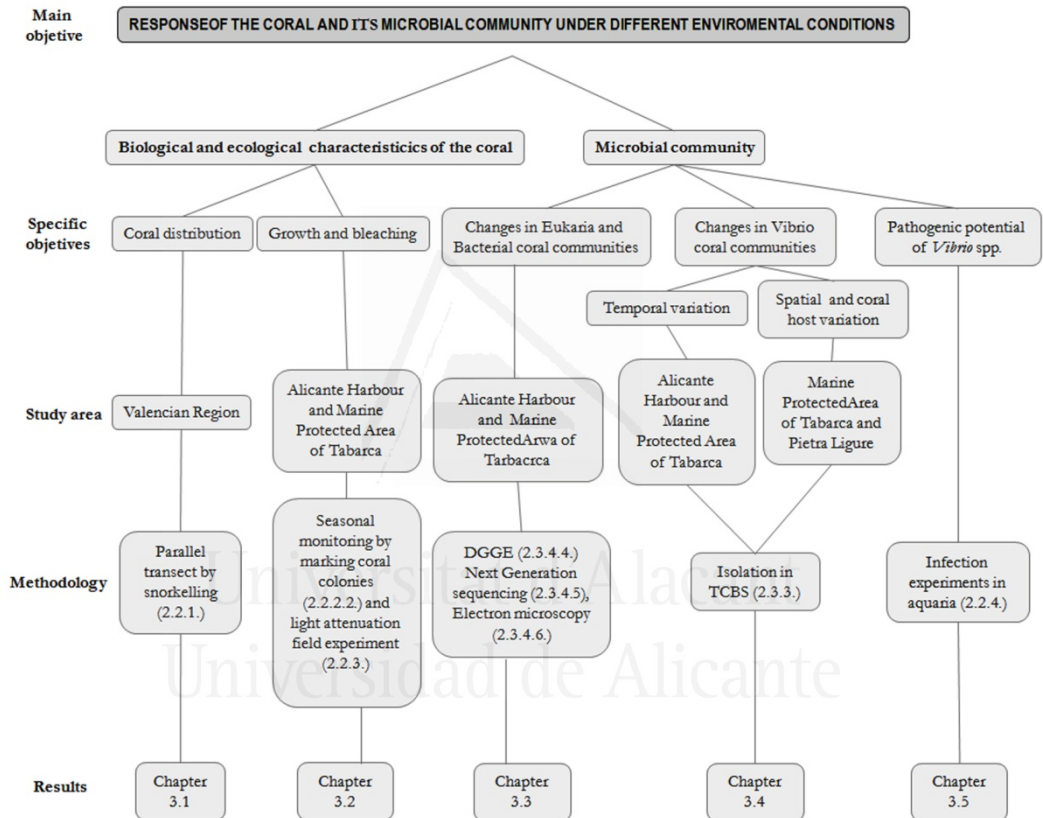
Figure 2.1. Summary methodology carried out in order to assesss each specific objective considered in this thesis.

2. 1. STUDY AREA

The two study areas, where most of the work conducted in this thesis has been carried out, are in the littoral coast of the Valencian Region (South East of Spain, South Western Mediterranean Sea). A third study area in the coast of Ligurian Region (North of Italy, North Western Mediterranean Sea) was selected to carry out the chapter 3.4 (Figure 2.2). From the oceanographic point of view, the Valencian Region corresponds to the central sector of the Western Mediterranean Sea, and shows subtropical characteristics, while the Ligurian Region situated in the North Western Mediterranean Sea, is one of the coldest areas of the Mediterranean Sea (Astraldi *et al.*, 1995)
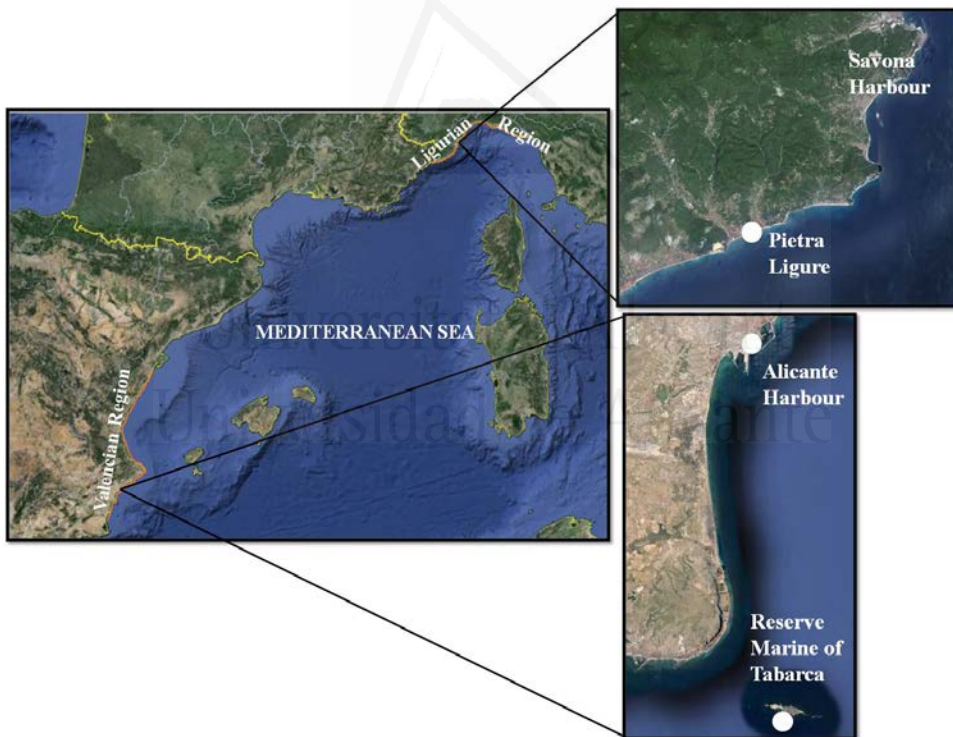
Figure 2.2. Map of the studied areas.

The main aim of Chapter 3.1 is to assess spatial distribution and the "invasive level" of *O. patagonica* through a field study along the rocky littoral of the Valencian Region, from Pilar de la Horadada (37°52'07"N; 00°45'15"W) to Vinaroz (40°31'09"N; 00°30'53"E). As result of the distribution of the coral, described in the chapter 3.1, two localities were chosen to carry out an in-depth study about the relationship among environmental parameters, *O. patagonica* ecology and its microbial community; these two sampling localitions were: the Alicante Harbour (38°20´11´´N, 00°29´11´´W) and the Marine Protected Area (MPA) of Tabarca in Alicante Bay (38°09´59´´N, 00°28´56´´W) (Figure 2.2).

The Alicante Harbour started to be built in 1476 and has around 15 m depth. The study site was located in the "Fishing Dock", an enclosed 9 m depth area with a low rate of water renewal. The MPA of Tabarca, which was established in 1986, is located 11 miles away from Alicante Harbour. This MPA consists of a main island called Isla Plana or Nueva Tabarca and some islets, two of which were chosen as sampling sites: La Galera (38°09´41´´N, 00°28´30´´) and El Scull Negre (38°09´39´´N, 00°28´35").

Pietra Ligure beach (44°08´50"N, 08°17´04"W), located in the North Mediterranean sea (Liguria, Italy) (Figure 2.2), is an artificial hard beach about 430m long with submerged breakwaters. It is 12 miles away from Savona Harbour, where *O. patagonica* was recorded for the first time in the Mediterranean Sea in 1966.

2.2. SAMPLING DESIGN AND SAMPLE COLLECTION

2.2.1. Spatial distribution of *O. patagonica* in the Valencian Region (Chapter3.1)

The study area was sub-divided into sub-areas A and B separated by San Antonio Cape (Figure 2.3). Sub-area A (Valencia Gulf) is located on the north of the San Antonio Cape and Sub-area B (Alicante Gulf) on the South. In the sub-area A the coastline is regular and almost rectilinear, dominated by sand deposits and with moderate influence of freshwater input; indeed, particles originating from the Ebro River and run-off from irrigation channels are easily found in this area (Díez, 1996; Serra, 1986). In contrast, sub-area B, with high and low cliffs present a limited continental influence (Serra, 2002).
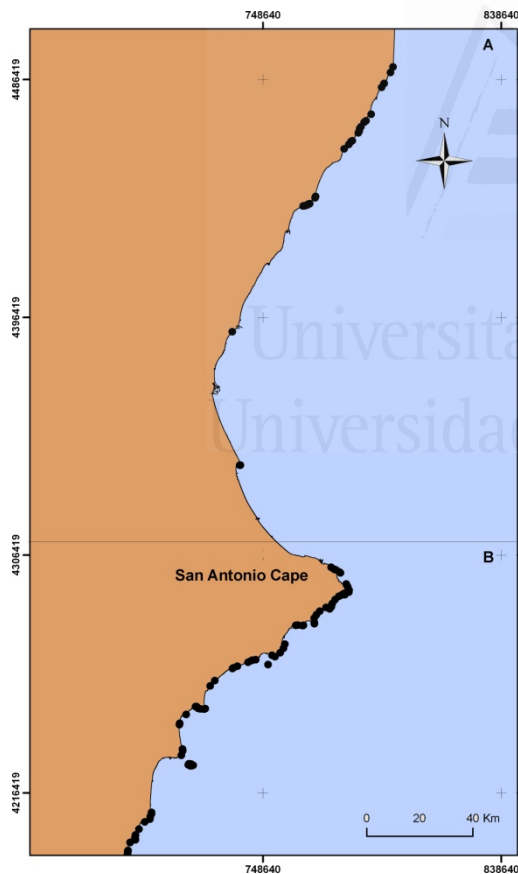
Figure 2.3. Study area (chapter 3.1).

All rocky substrata along Valencian Region were sampled by snorkelling during summer 2010 and 2011. A total of 122 parallel transects to the coastline of 100 m length were carried out, 34 in the sub-area A, due to the dominance of sandy shore, and 88 in the sub-area B (Figure 2.3), where the most coastline is rocky.

In each of these transects the "invasive level" of *O. patagonica* (developmental level of the species based on the size and aggregation of the colonies) was assesssed by taking into account the following categories: 1) small isolated colonies (<10 cm), 2) large isolated colonies (>10 cm), 3) colonies aggregated in the breaker zone, 4) continuous belt in the breaker zone and 5) colonies aggregated at

different depths. Each transect was also characterised according to environmental variables (algal community and cover, substrata and coast morphology) and spatial variables (geographical coordinates, sub-area and distance from the nearest harbour).

The associated algal community was classified into the following functional form groups of macroalgae (Diaz-Pulido *et al.*, 2010): Algal turfs (in our case mainly Ceramiales), "upright" fleshy macroalgae and "upright" calcareous geniculate macroalgae; each transect was assigned to only one macroalgae type, whereas in transects with the simultaneous presence of more than one macroalgae type, only the most abundant group was recorded. The coast morphology was divided into five categories: artificial substrate, natural blocks, abrasion platform, cliffs and islets. The distance from the nearest harbour to each transect was estimated and classified into three categories: near (0–5 km), medium (5–10 km) and far (>10 km).

2.2.2. Temporal trends of growth, bleaching (Chapter 3.2) and microbial community (Chapter 3.3 and 3.4)

2.2.2.1. Environmental parameters

In order to characterise the two sampling locations in Spanish coast (Alicante Harbour and Tabarca), the following environmental parameters were recorded during the study period (June 2010-December 2011): seawater temperature, sedimentation rates, and chlorophyll *a* (Chl *a*) concentration in seawater (which is an index of phytoplankton's biomass and an indicator of water trophic status).

Rates of sedimentation were assesssed using three sediment traps, consisting of five 0.5 l containers. Sediment traps were placed seasonally (every three months), at each location (Harbour and MPA) for a period of 24 hours. The sediment within the traps were separated by grain size using 2.0 and 0.063 mm sieves, and the remaining solution with smaller particulates was used to obtain the mud fraction by Whatman GF/F glass filters. The three sediment fractions obtained (gravel, sand and mud) were dried for 48 h at 110°C to obtain the total suspended solids and then were calcined at 550ºC for 1h to get the organic matter (ESS method, 1993). Values were expressed as g dry weight m^{-2} d^{-1}. Chlorophyll *a* concentration was also

determined, seasonally at each sampling location (Harbour and MPA), by filtering three seawater samples (5 l each one) onto Whatman GF/F glass fibre filters; pigment extraction was performed with 10 ml of 90% acetone, at 4Cº for 24h in the dark, and Chl *a* concentrations were determined according to Jeffrey and Humphrey (1975). Seawater temperatures were recorded *in situ*, at 5 m depth, with Madgetech Temperature Loggers (resolution of 0.1Cº), with data points taken automatically every 1 h.

During the study period, a cargo ship (73 meters in length) was located in the Alicante Harbour for 9 months (from August 2010 to April 2011) covering a part of our sampling location, so the shade effect was considered as a new environmental parameter. Therefore, shade effect was measured as present or absent.

2.2.2.2. Growth rates and bleaching

Nine colonies (~ 10 cm diameter) of *O. patagonica* were marked *in situ* with a screw nailed onto the rock at 3-5 m depth, in February 2010, in each sampling location (Harbour and Tabarca). Marks were located a few centimetres above the colonies to avoid damaging the corals. Underwater photographs of each marked colony were taken every three months, using a (20x20 cm) quadrate as a reference. Then, growth rates and bleaching extension were calculated by image analysis using Image-J software (Rasband, 2012) from June 2010 to August 2011.

Bleaching extension for each marked colony was categorised into the following six categories: (1) unbleached (normal coloration); (2) pale (lighter colour than usual for the season); (3) 0–20% of the surface bleached; (4) >20–50% bleached; (5) >50-80% bleached; and (6) >80% bleached. Furthermore, tissue necrosis was also calculated as the percentage of tissue lost for each marked colony. In addition, at each location, a bleaching index (BI) was calculated from ten randomly photographs (20x20 cm) made along three transects, every three months from June 2010 to December 2011 (McClanahan, 2004):

$$BI = (0c_1 + 1c_2 + 2c_3 + 3c_4 + 4c_5 + 5c_6) / 5$$

Where c_i is the percentage of colonies in each of the above six bleaching categories.

2.2.2.3. Microbial community analyses

In order to charterise microbial community associated to *O. patagonica* nine coral fragments and three samples of surrounding water were randomly collected from the Harbour and Tabarca, every 3 months from September 2010 to December 2011. Furthermore, to assess *Vibrio* community differences among coral host and geographic location, four coral fragments of *O. patagonica* and *C. caespitosa* and three samples of surrounding water were also removed from Tabarca and Pietra Ligure in June and September 2012 (see Table 2.1.). Coral colonies were removed at 3 m depth by SCUBA diving using a hammer and chisel, placed into plastic bags and transported to the laboratory in a cooler within the next 2h. Bleaching extension for each coral colony was visually estimated as described above (see 2.2.2.2.)

2.2.3. Light attenuation experiment (Chaper 3.2)

The influence of light over *O. patagonica* bleaching was studied by a field experiment that was carried out for a period of 8 months (February 2011 to September 2011) in the Alicante Harbour. To test the light attenuation effect, five experimental treatments were set: 1) opaque (colonies covered with opaque plates during all experiment); 2) opaque spring (colonies covered only in spring, March to May); 3) opaque summer (colonies covered only during the summer, June to September); 4) transparent control (colonies covered with transparent plates during all experiment); and 5) control (colonies not covered with plates). Incident light was attenuated using 20x20 cm methacrylate plates that were attached by two screws nailed onto the rock, and cleaned every month in order to avoid fouling. Three different sites were randomly selected along the study area and five colonies (~10 cm of diameter) were marked per treatment in each site. Thus, a total of 75 colonies were used during the experiment. Photosynthetic Active Radiation (PAR) was recorded by submersible dataloggers (MDS-MkV/L; ALEC ELECTRONICS) under opaque and transparent plates (Fig. 2.4), and control colonies (no plates), to check light availability for the corals. At the end of the experiment, bleaching extension, as a percentage of coral surface, was estimated using underwater

photography, as mentioned above. At the same time, coral samples (around 12 cm^2) were collected from each marked colony to determine Chl *a* concentration in the tissue. Pigment concentration, from crushed tissue homogenate, was measured as described above for the seawater samples. Additionally, seawater temperature was also recorded during the experiment with temperature loggers.
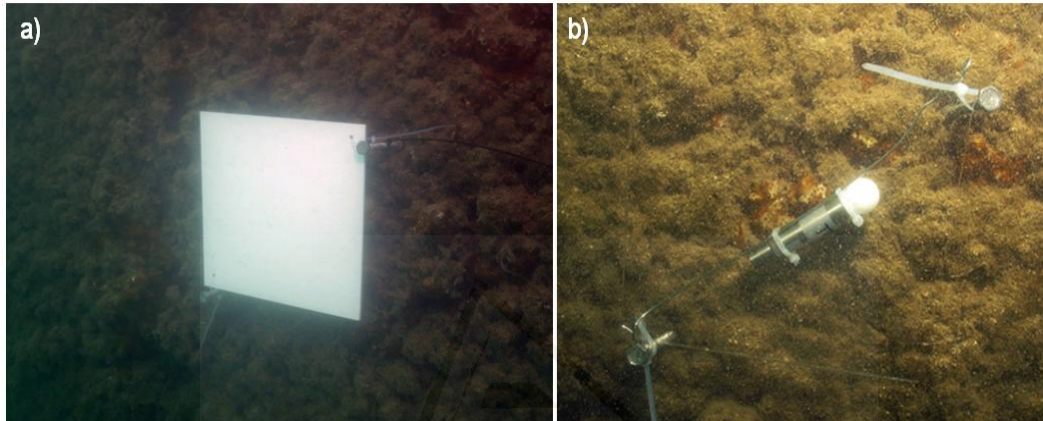
Figure 2.4. Plates used in the light attenuation experiment: an opaque plate (a) and a transparent plate with the light sensor (b). (Photos: M. Vázquez-Luis).

2.2.4. *Vibrio* infection experiment (Chapter 3.5)

Fragments (about 5 cm of diameter) of healthy *O. patagonica* were collected from Tabarca in June 2013. Each fragment was transferred to the laboratory and acclimated at 18ºC (seawater temperature at the sampling moment) for 3 days in aquaria (20 l) before being placed in separate aquaria (500 ml) for inoculation experiments (Fig. 2.5). Then, fragments were slowly acclimated to the experimental temperature by increasing the temperature by 0.5 °C per day. Fragments showing disease signs were immediately removed from the experiment. Prior to inoculation, the corals were maintained at the experimental temperature for 3 days in sterile filtered seawater (SFSW). Water was replaced every 3 days during the infection experiment.
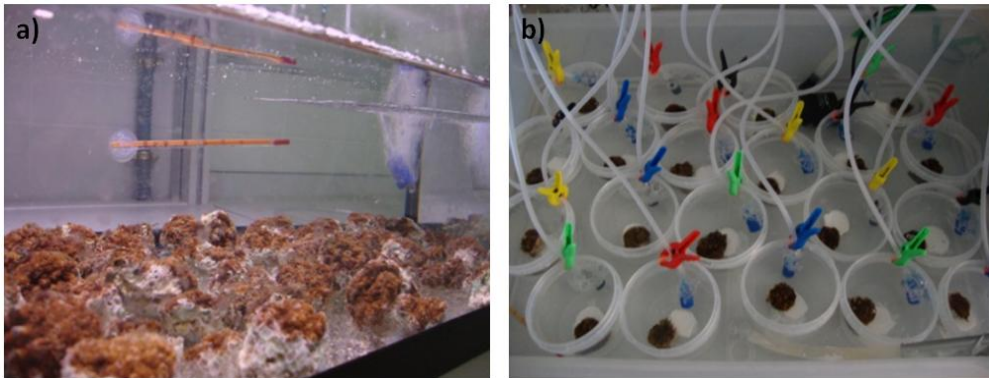
Figure 2.5. Aquaria used in the infection experiment: acclimation aquarium (a) individual aquaria (b).

Corals were infected with two representative *Vibrio* strains isolated from diseased *O. patagonica* colonies collected in June 2011 (see chapter 3.4): 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 *Vibrio gigantis* (*Vibrio splendidus* clade) was used as a control.

These bacteria were grown at 30 °C in Luria Bertani broth with 3% NaCl for 4 hours under agitation (130 rpm), harvested by centrifugation (4000 xg, 10 min) and washed twice with SFSW. Infections were carried out at three different temperatures: 20 ºC, 24 ºC and 28 ºC. 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 inoculum. 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 in coral tissues were measured as described above (2.2.2 and 2.2.2.1, respectively).

2.3. LABORATORY TECHNIQUES

2.3.1. Samples

Coral and water samples collected from the three study locations at different sampling times from September 2010 to June 2013 were analysed by diverse techniques in order to study the Eukarya, Bacteria and *Vibrio* community (see table 2.1), and the results obtained are shown in the chapters 3.3 and 3.4. Some of these techniques were also used to assesss changes produced in *Vibrio* community as consequence of the infection experiment and these results are displayed in the chapter 3.5.

2.3.2. Mucus, tissue and skeletal matrix separation

Corals were gently washed three times with 50 ml of SFSW to remove non-associated bacteria, broken into ca. 2×2 cm pieces, placed in 50 ml centrifuge tubes, and centrifuged for 5 min at 2,900 xg (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, the supernatant (i.e. coral tissue) was removed according to Koren and Rosenberg (2006) and the skeleton was washed with SFSW. In order to study their associate microbial communities, the different fractions were analysed using different laboratory techniques (Figure 2.6).

Table 2.1. Summary of samples collected in this thesis and the laboratory techniques used in different results sections. (C) *Cladocora caespitosa*; (O) *Oculina patagonica*; (VI) *Vibrio* isolation; (DGGE) Denaturing Gradient Gel Electroforesis; (EM) Electron Microscopy; (NGS) Next Generation Sequencing.

Sampling Location	Sampling Time	Techniques	Number of samples	Results Sections
Alicante Harbour	September 2010	VI	9	3.4
	December 2010	VI/DGGE 16S/ EM	9/5/4	3.3, 3.4
	February 2011	VI/DGGE 16S/EM	9/5/4	3.3, 3.4
	June 2011	VI/DGGE 16S, 18S, ITS/EM	9/5,4,4/4	3.3, 3.4
	September 2011	VI/DGGE 16S, 18S, ITS/ NGS/EM	9/5,4,4/2/4	3.3, 3.4
	December 2011	VI/DGGE 16S, 18S, ITS/EM	9/5,4,4/4	3.3, 3.4
MPA of Tabarca	September 2010	VI	9	3.3, 3.4
	December 2010	VI/DGGE 16S/ EM	9/5/4	3.3, 3.4
	February 2011	VI/DGGE 16S/EM	9/5/4	3.3, 3.4
	June 2011	VI/DGGE 16S, 18S, ITS/EM	9/5,4,4/4	3.3, 3.4
	September 2011	VI/DGGE 16S, 18S, ITS/ NGS/EM	9/5,4,4/2/4	3.3, 3.4
	December 2011	VI/DGGE 16S, 18S, ITS/EM	9/5,4,4/4	3.3, 3.4
	June 2012	VI/DGGE ITS	4C,4O/4O	3.4
	September 2012	VI/DGGE ITS	4C,4O/4O	3.4
Pietra Ligure	June 2012	VI/DGGE ITS	4C,4O/4O	3.4
	September 2012	VI/DGGE ITS	4C,4O/4O	3.4
Infection experiment	June 2013	VI/NGS/EM	4/1/1 per treatment	3.5
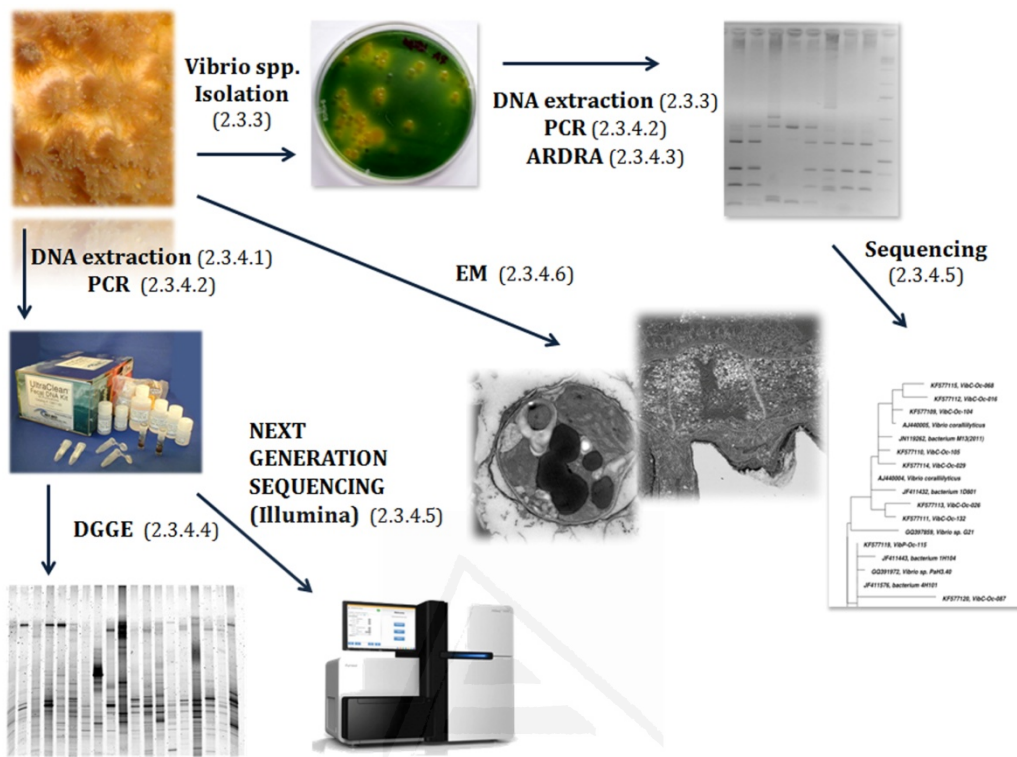
Figure 2.6. Laboratory techniques used to know the coral microbial community. (ARDRA) Amplified Ribosomal DNA Restriction Analysis, (DGGE) Denaturing Gradient Gel Electrophoresis, (EM) Electron microscopy

2.3.3. Isolation of *Vibrio* spp.

Vibrio spp. were isolated using Thiosulfate Citrate Bile Sucrose (TCBS) agar (Pronadisa, Spain) and Marine Agar (MA) (Pronadisa, Spain). Tenfold serial dilutions of sea water, coral mucus and crushed tissue were prepared in SFSW, plated and 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 or MA, incubated for further 48 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 analyze only the isolates which could belong to *Vibrio* genus.

2.3.4. Molecular approaches

2.3.4.1. DNA extraction

Total community DNA was extracted and purified with the Ultra Clean Soil DNA Kit (MoBio; Carlsbad, CA) following the manufacturer's instructions for maximum yield. Concentration of yielded DNA was determined by NanoDrop spectrophotometry (NanoDrop, Wilmington, DE).

For DNA extraction from *Vibrio* isolates, one colony of each 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 xg for 10 min (Biofuge-pico, Heraeus instruments). Supernatants were transferred to new tubes and used as template DNAs.

2.3.4.2. PCR amplification

Total DNA extracted from corals was used for PCR amplifications of bacterial 16S rRNA genes, eukaryal 18S rRNA, and the variable internal transcribed spacer region II (ITS 2) by using specific primers (Table 2.2.). Each PCR mixture contained 5 µl of 10x PCR reaction buffer (Invitrogen), 1.5 µl of 50 mM $MgCl_2$, 1 µl 10 mM dNTP mixture, 1.25 µl of 10 µM of each primer, 1 units of Taq polymerase, 3 µl of Bovine Serum Albumin (BSA, New England BioLabs), sterile MilliQ water up to 50 µl and 30 ng of l DNA. Furthermore, to eliminate heteroduplexes, 5 µl of PCR products were used as templates for a 5 cycle reamplification (56ºC, 55ºC and 52ºC of annealing temperature for Bacteria, Eukarya and ITS respectively) using fresh reaction mixture, as described by Thompson *et al.* (2002).

PCR primers specific for the V3-V4 region (Bakt-341f and Bakt-805r) of the 16S rRNA gene containing Illumina-specific adapter sequences (Table 2.2) were used to analyse coral bacterial communities. Each PCR mixture contained 5 µl of 10x PCR reaction buffer (Invitrogen), 1.5 µl of 50 mM $MgCl_2$, 1 µl 10 mM dNTP mixture, 1 µl of 100 µM of each primer, 1 units of Taq polymerase, 3 µl of BSA (New England BioLabs), sterile MilliQ water up to 50 µl and 10 ng of DNA.

DNA from isolated *Vibrio* colonies was used for PCR amplifications of bacterial 16S rRNA genes with the primers S and Ant1 (Table 2.2). The reaction mixtures contained 5 µl of 10x PCR reaction buffer (Invitrogen), 1.5 µl of 50 mM MgCl$_2$,1 µl 10 mM dNTP mixture, 1 µl of 10 µM of each primer, 1 units of Taq polymerase, sterile MilliQ water up to 50 µl and 2 µl of DNA.

Table 2.2. Oligonucleotide primers used in this study.

Primer	Sequence (5´to 3´)	Specificity	Reference
Euk1A	ACCAGACTTGCCCTCC	Eukarya	Sogin and Gunderson, 1987
Euk516r-GCa	CTGGTTGATCCTGCCAG	Eukarya	Amann et al., 1990
907r	CCGTCAATTCCTTTRAGTTT	Universal	Muyzer *et al.*, 1993
341f-GCb	CCTACGGGAGGCAGCAG	Bacteria	Muyzer *et al.*, 1993
ITSintfor2	GAATTGCAGAACTCCGTG	Symbiodinium (ITS 2)	LaJeunesse and Trench, 2000
ITS2CLAMPc	GGGATCCATATGCTTAAGTTCAGCGGGT	Symbiodinium (ITS 2)	LaJeunesse and Trench, 2000
S (1492r)	GGTTACCTTGTTACGACTT	Bacteria	Lane, 1991
Ant1 (27f)	AGAGTTTGATCATGGCTCAG	Universal	Lane, 1991
Bakt-341fd	CCTACGGGNGGCWGCAG	Bacteria	Herlemann *et al.*, 2011
Bakt-805re	GACTACHVGGGTATCTAATCC	Bacteria	Herlemann *et al.*, 2011

[a]GC clamp: CGCCCGGGGGCGCGCCCCGGGCGGGGGCGGGGGCACGGGGGG

[b]GC clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG

[c]GC clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCGCCC

[d]Illumina adapter: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

[e]Illumina adapter: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

PCR reactions were performed in a 2720 thermal cycler (Applied Biosystems) with the conditions specified in Table 2.3. Negative controls with no template DNA were always included. PCR products were checked on 1% agarose gels (LE; SeaKem) in 1X Tris-acetic acid-EDTA (TAE) buffer and visualized under UV light after ethidium bromide staining.

Table 2.3. PCR conditions

DGGE Bacteria	DGGE Eukarya	DGGE *Symbiodinium*	16SrRNA Bacteria	Illumina Bacteria
1. 94ºC 5´	1. 94ºC 5´	1. 94ºC 5´	1. 94ºC 3´	1. 94ºC 3´
2. 94ºC 1´	2. 94ºC 1´	2. 94ºC 1´	2. 94ºC 1´	2. 94ºC 45´´
3. 65ºC 1´	3. 56ºC 1´	3. 65ºC 1´	3. 55ºC 1´	3. 50ºC 1´
4. touchdown 1ºC/cycle	4. 72ºC 3´	4. 72ºC 3´	4. 72ºC 2´	4. 72ºC 2´
5. 72ºC 3´	5. Go to 2	5. 94ºC 1´	6. Go to 2	6. Go to 2
6. Go to 2	29 cycles	6. 62ºC 1´	34 cycles	34 cycles
9 cycles	6. 72ºC 30´	7. touchdown 1ºC/cycle	7. 72ºC 10´	7. 72ºC 10´
7. 94ºC 1´		8.72ºC 3´		
8. 55ºC 1´		9. Go to 5		
11. 72ºC 3´		9 cycles		
12. Go to 7		7. 94ºC 1´		
19 cycles		8. 52ºC 1´		
13. 72ºC 30´		11. 72ºC 3´		
		12. Go to 7		
		20 cycles		
		13. 72ºC 30´´		

2.3.4.3. Analysis of *Vibrio* isolates by Amplified Ribosomal DNA Restriction Anlysis (ARDRA)

PCR-amplified 16S rRNA genes of *Vibrio* isolates were compared by amplified rDNA restriction analysis (ARDRA) (Vaneechoutte *et al.*, 1992) with enzymes *Hinf*I and *Mbo*I (New England Biolabs). Enzymatic digestion was carried out by incubating (37ºC, 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 0.5X Tris-boric acid-EDTA buffer (TBE), stained and visualized as described above. At least two isolates were selected from each restriction profile for sequencing (see 2.3.4.5)

2.3.4.4. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using a DCode System (Bio-Rad, Hercules, CA). ITS region PCR products (500 ng) were separated by electrophoresis at 100 V during 16 h in a linear gradient from 40% to 65% (where 100% of denaturant consists of 7 M urea and 40% formamide) in a 8% (w/v) polyacrylamide gel (acrylamide-bisacrylamide gel stock solution 37.5:1; Bio-Rad), in 1x TAE buffer (40 mM Tris, pH 8.0; 20 mM acetic acid; 1 mM EDTA). Bacteria and Eukarya PCR products (500 ng) were loaded onto 6% (w/v) polyacrylamid with 40-60% and 30-50% denaturant gradient, respectively. DGGE gels were stained for 30 min with SYBR Green, visualized under UV light and photographed with a Typhoon 9410 (Amersham Biosciences) system.

DGGE gel images from Bacteria PCR products were analysed using the FPQuest Software Version 5.10 (Bio-Rad). In order to compensate for gel-to-gel differences and external distortion to electrophoresis, the DGGE patterns were aligned and normalized using an external reference ladder, containing PCR amplicons from seawater. The bands of interest were excised using sterile scalpel blades from DGGE gels and soaked overnight into 20 µl of MilliQ water. Two µl of supernatant were then reamplified with the same primer set and the PCR products were checked again by DGGE to make sure that they were single bands of the expected size.

2.3.4.5. Sequencing and sequence analyses

PCR products from *Vibrio* isolates 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). Anomaly tests, clustering and rarefactions from *Vibrio* isolates sequences retrieved from temporal variation study 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 from temporal and environmental study (chapter 3.3.1) 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. The sequences with similarity ≥97.8% (Stackerbrandt and Ebers, 2006) were grouped into phylotypes and the average relative abundances for each phylotypes were calculated for each kind of samples. All sequences were preliminary classified using BLASTn (Basic Local Alignment Search Tool, 30) and the reference NCBI database (http://www.ncbi.nlm. nih.gov). *Vibrio* isolates sequences retrieved from spatial and coral host study (chapter 3.3.2) were automatically also aligned using the SINA software but the phylogenetic tree was built using the Neighbor-Joining method (Jukes Cantor model) with the software Geneious version 7.1.5 (Biomatters), with bootstrap resampling using 100 replicates.

PCR products from DGGE bands were purified using the GeneJET PCR purification kit (Fermentas, EU) and 50 ng were sequenced with primers Euk1A (Eukarya), ITSintfor2 (ITS region) and 907r (Bacteria) using an ABI 3730xl sequencer (Applied Biosystems). Sequences were compared with reference sequences using the BLAST (Basic Local Alignment Search Tool) software at the National Centre of Biotechnology Information website (http://www.ncbi. nlm.nih.gov/).

Paired-end reads, obtained by next generation sequencing (Illumina), were assembled by aligning the 3′ ends of forward and reverse reads, to provide additional quality control in the lower-quality 3′ region of each read paired-end reads and paired-end reads that did not assemble as contigs were removed because they possessed sequencing errors (Bartram *et al.*, 2011). The assembling and subsequent screened of sequences according to quality (minimum of 27) and size (between 100-500 bp) were carried out using RDP Pyrosequencing Pipeline (Cole *et al.*, 2009). All sequences were assigned taxonomic affiliations based on Bayesian classification (RDP classifier) (Wang *et al.*, 2007) with an assignment cutoff of 0.5. Sequences of *Vibrio* pathogens isolated from coral samples were searched in Illumina libraries using Basic Local Alignment Search Tool (BLAST 2.2.22+) with the following command "blastn –query <input> –perc_identity 95 –evalue 0.00001 –num_alignments 0 –maz_target_seqs 300000 –db Illuminalibrary.fasta –outfmt 6 – out <output>".

2.3.4.6. Electron microscopy (EM) analyses

To determine zooxantellae and bacteria location in the coral tissue and their changes during bleaching, some coral fragments from Harbour and Tabarca were analysed by EM. These analyses were carried out by the group of Biogeochemical and Microbial Ecology of Natural Sciences Museum in Madrid. For this purpose, samples were fixed with 4% formaldehyde in sterile seawater and washed with 1mM cacodylate buffer (pH 7.4). Selected pieces were fixed with 3% glutaraldehyde in cacodylate buffer (pH 7.4) and postfixed with 1% osmium tetroxide, dehydrated in ethanol and embedded in LR-White resin hard grade (London Resin Company, England). The samples were polished (Ascaso and Wierzchos 1994; Wierzchos and

Ascaso 1994) and carbon coated for observation in a SEM (Zeiss DSM 960) equipped with a backscattered electron detector (KE Developments Cambridge, England). Observations were performed at 15kV and 10-15 mm working distances. Pieces of selected areas from SEM studied samples were removed and included in LR-White resin for ultrathin sectioning (Ultracut Reichert-Jung, Austria). Eighty nm ultrathin sections were collected on formvar coated copper grids and contrasted with lead citrate (Venable and Coggeshall 1965). Ultrathin sections were observed at 80kV with a Leo 910 TEM, and images were captured using a Gatan BioScan Camera model 792, and processed for scanning electron microscopy in backscattered electron mode (SEM-BSE) according to the method developed by Wierzchos and Ascaso (1994).

2.4. DATA ANALYSIS

Data obtained in this PhD thesis were analysed by means of both univariate and multivariate statistical analysis (Figure 2.6.) using three statistical packages: SPSS 20.0, PRIMER (Clarke and Gorley, 2001) and CANOCO 4.5 (ter Braak and Smilauer, 2002).
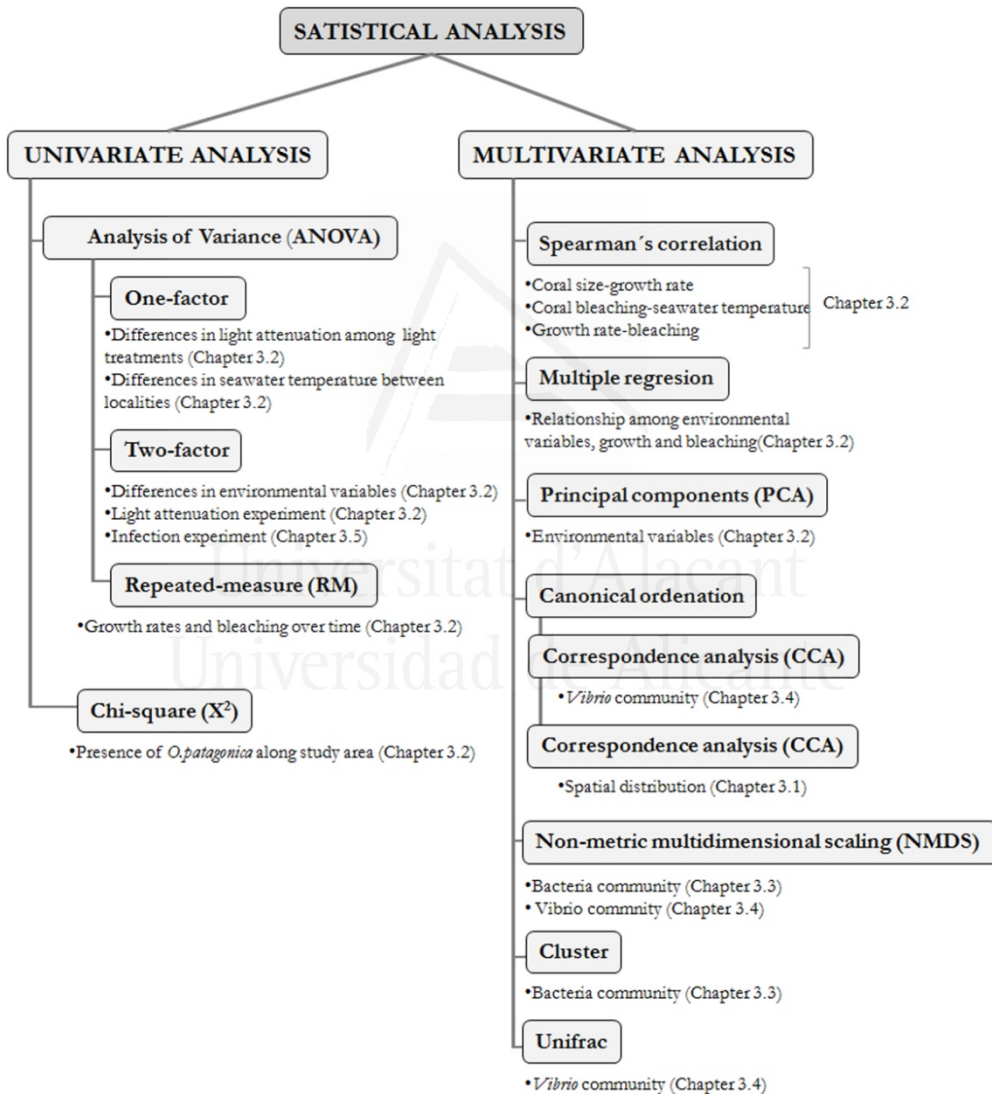
Figure 2.6. Summary of statistical analysis carried out in each chapter of this thesis.

2.4.1. Spatial distribution (Chapter 3.1)

The geographical position of each transect was entered in the data-base together with geographical information using Arc View 9.0 software, and later the spatial distribution of *O. patagonica* along the coast was mapped according to their "invasive level" (see 2.2.1.). The relationship between the presence/absence of the coral and the different variables (algal community, coast morphology and distance from the nearest harbour) was analysed using 2x2 chi-square contingency tables. Relationships between "invasive level" of *O. patagonica* and environmental and spatial variables were analysed using canonical ordination methods. Data were first analysed using detrended correspondence analyses (DCA), to explore the gradient length of the dataset and to decide an approach for subsequent direct gradient analyses. Since the length of the extracted gradients was relatively short (Standard Deviation units; SD = 2.08) a linear model was assumed and a redundancy analysis (RDA) (ter Braak and Smilauer, 2002) was generated for the "invasive level". The categories matrix was constrained by environmental and spatial variables, in order to identify the factors that better explain the variation in the "invasive level" of the species. Statistical significance of the generated ordination axes was tested using Monte Carlo permutation tests, and the statistical significance of the environmental and spatial variables in the RDA was determined using a Monte Carlo test with 999 permutations.

2.4.2. Environmental parameters (Chapter 3.2 and 3.4)

To test differences among environmental parameters, two-factors Analysis of Variance (ANOVA) was carried out with the sedimentation rate in absolute term as well as sediment composition (mud fraction and organic matter) as percentages on each location (see chapter 3.2). The experimental design comprised two factors: "Sampling location", fixed with two levels (Harbour and Tabarca); and "Sampling time", fixed with six levels (Summer 2010, Autumn 2010, Winter 2010, Spring 2011, Summer 2011 and Autumn 2011). For each combination of factors, 15 replicates were recorded (five replicates corresponding to each sediment trap), with a total of 180 replicates. The three variables obtained from the sediment analysis were tested separately using this design. Another two-factor ANOVA, with the same design, was carried out to test differences in Chl *a* concentration, with a total of 36

replicates. One-factor ANOVA was performed to test the differences in seawater temperatures between the two sampling locations. Prior to ANOVA, heterogeneity of variance was tested with Cochran's test and data were $\sqrt{x}+1$ transformed if the variances were significantly different at $p = 0.05$. When the ANOVA F-test was significant, post-hoc analyses were conducted using Student–Newman–Keuls (SNK) multiple comparisons (Underwood, 1981).

In order to characterise the two sampling locations (Harbour and Tabarca) environmental parameters (sea water temperature, sedimentation rates, chlorophyll *a* concentration in seawater) were analysed by a principal component analysis (PCA) (using correlation) to determine which environmental variables were important in driving differences between samples taken from Alicante Harbour and Tabarca (see chapter 3.2).

2.4.2. Growth and bleaching (Chapter 3.2)

Growth rates (GR) of the nine marked colonies, were seasonally calculated by the formula: GR= $((P_T - P_{T-1}) / P_{T-1})$ x 100; where P_T is the perimeter calculated at sampling time and P_{T-1} is the perimeter calculated at the previous sampling time (modified from Turon *et al.*, 1998), so this growth rate is the seasonal change in the aperimeter relative to the aperimeter at the beginning of the time interval. The annual growth rate was calculated from June 2010 to June 2011, from two sampling locations. By monitoring changes in growth rates and bleaching, the same coral colonies were retested over time, so data were not independent and a simple ANOVA cannot be applied to this method of data collection (Kingsford and Battershill, 1998). Therefore, repeated-measures (RM) ANOVA analyses were performed. The design comprised two factors: "Sampling location" as between-subject fixed factor, with two levels (Harbour and Tabarca) and "Sampling time" was the within-subject fixed factor, with six levels (Summer10, Autumn10, Winter10, Spring11, Summer11 and Autumn11). For each season, 9 replicates were recorded that represent a total of 108 replicates. Assumptions of compound symmetry were checked, examining the more conservative Greenhouse–Geiser and Huynh–Feldt correct F tests, and their results were not markedly different from the uncorrected ones, so only the standard F tests are presented here. When the RM

ANOVA F-test was significant, post-hoc analyses were conducted using the Bonferroni test for multiple comparisons (Winer *et al.*, 1991).

Spearman rank correlation was used to assesss relationships between: (i) size and growth rates (in the period in which the highest mean growth rate was recorded at both locations, Summer 10); (ii) bleaching and seawater temperatures; and (iii) growth rates and bleaching in marked colonies. To explore the relationship among growth rate, bleaching and environmental parameters, multiple regression analyses were performed. Quadratic and cubic terms were used to explore the possible nonlinear relationship. A stepwise forward selection of variables was run, with the aim of maximising the deviance reduction, followed by a stepwise backward elimination to prevent the loss of statistical significance of some variables due to the latter incorporation of new variables into the model. Before accepting any model, an analysis of residuals was carried out to detect outliers high influence on the models. The leverage and the Cook statistic of each sampling unit were measured (McCullagh and Nelder, 1989), so those with high values of leverage and influence were removed and the model refitted to ensure consistency.

2.4.3. Light attenuation experiment (Chapter 3.2)

To test the differences in light attenuation between opaque and transparent plates used to cover the corals, a one-factor ANOVA was carried out with PAR values measured in each kind of plates and compared with controls (without plates). To test if light differences affected the bleaching of *O. patagonica* (measured as a percentage of surface of bleached coral as well as Chl *a* concentration in the coral tissue) two-factors ANOVA was conducted. The experimental design comprised two factors: "light treatment", fix with five levels (opaque, opaque spring, opaque summer, transparent and control) and "site", random and nested in light treatment with three levels, with a total of 75 replicates. Prior analysis Cochran's test and SNK test were carried out as mentioned above.

2.4.4. Microbial community (Chapter 3.3 and 3.4)

2.4.4.1. Bacteria community (Chapter 3.3)

Denaturing Gradient gel Electrophoresis (DGGE) gel images were analysed using the FPQuest Software Version 5.1 (Bio-Rad). The presence/absence of individual DGGE bands in each sample was used to construct a binary matrix that represented the banding patterns, and multivariate analyses were performed, for exploring differences in the Bacteria assemblage composition, with Primer 5 software package (Clarke and Gorley, 2001). A distance matrix was constructed using Bray–Curtis similarity, and hierarchical clustering analysis (CLUSTER) (similarity dendrogram) and non-metric multidimensional scaling (NMDS) were used to explore groupings of the samples. To determine if coral microhabitat (mucus, tissue and skeleton), sampling time (cold and warm months), coral health status (healthy and unhealthy) or sampling location (Harbour and Tabarca) had an effect on the bacterial community, analyses of similarity (ANOSIM) and similarity percentage (SIMPER) was used to identify OTUs, using their relative abundances, that are responsible of the differences among samples.

2.4.4.2. Vibrio community (Chapet 3.4)

Two different studies were carried out in order to assesss changes in *Vibrio* community. Firstly we tried to elucidate temporal variation and differences related to environmental conditions. Environmental parameters (temperature, 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. 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 each 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).

Secondly, to compare the composition of *Vibrio* spp. communities and to determine which phylotypes were shared among localities (MPA of Tabarca in Spain and Pietra Ligure in Italy) or different coral species (*O. patagonica* and *C. caespitosa*), a distance matrix was constructed using Bray–Curtis similarity, and hierarchical clustering analysis (CLUSTER) (similarity dendrogram) and non-metric multidimensional scaling (NMDS) were used to explore groupings of the samples, using PRIMER v5 software package (Clarke and Gorley, 2001)). Analysis of Similarity (ANOSIM) was then performed on the Bray–Curtis resemblance matrix to determine the statistical significance ($\alpha < 0.05$) of compositional differences among *Vibrio* communities associated with coral samples from two sampling localities. Exploration of the phylotype composition in clusters generated by NMDS was conducted via the similarity percentages (SIMPER) routine (PRIMER v5). This software was also used to calculate Shannon index and estimate *Vibrio* diversity.

Furthermore, differences among *Vibrio* assemblages, in two *Vibrio* studies, were assesssed 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 with the UniFrac-based principal coordinate analysis (PCoA) To further identify environmental factors explaining differences among *Vibrio* assemblages, each sample was classified into different categories based on sampling location, time, coral species 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.

Photo/Figure: 3D figure of a DGGE gel and *Vibrio* spp. phylogenetic tree

Part I. BIOLOGICAL AND ECOLOGIAL CHARACTERISTICS OF *Oculina patagonica*

3.1. DISTRIBUTION

3.1.1. Spatial and bathymetrical distribution

According to our data *Oculina patagonica* is widely distributed in the Spanish Mediterranean coast, particularly in the Valencian Region, since it was found in 60.65% of transects (n=122). The coral was significantly more abundant at the north of the San Antonio Cape (sub-area A, Fig. 3.1a), where it was found in 76.47% of the sampled transects; while in the Southern part it was found in 54.54% of transects ($X^2 = 4.940$; p = 0.026). Nevertheless, in sub-area A small (<15 cm of diameter) isolates colonies were more abundant than in the sub-area B, whereas the biggest colonies were more concentrated. In fact, in the Marine Protected Area of Tabarca one of the biggest colony with 50 cm of diameter was detected. Hot spots (identified as the sampling units that displayed the highest aggregation of colonies) of *O. patagonica* were concentrated in the south of the study area (sub-area B; Fig. 3.1b). Three hot spots were detected in this sub-area: ¨La Zenia¨ (abrasion platform surrounded by sand banks), the MPA of Tabarca and Alicante Harbour, which represented the sampling point with the highest values of abundance within the entire study area (Fig. 3.1b). Only one hot spot was identified in the sub-area A (Peníscola), where big colonies were aggregated at different depths (Fig. 3.1b).With respect to the bathymetrical distribution, *O. patagonica* was found at depths from 0 (pools on abrasion platforms) to 12 m, being the highest abundances at the breaker zone, decreasing its below 4-5 meters depth.
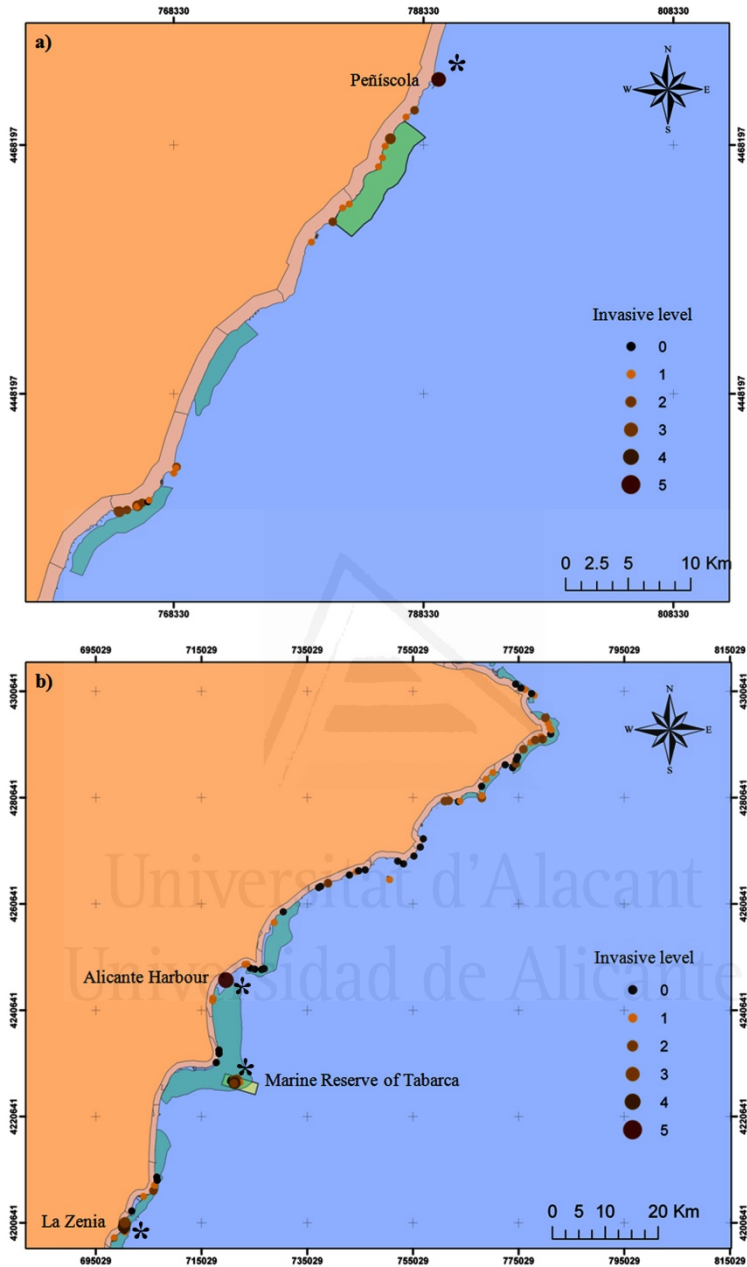
Figure 3.1. (**a**) Spatial distribution of *O. patagonica* categories in the sub-area A and (**b**) sub-area B. The invasive levels were definied as follows: 0) no presence, 1) small isolated colonies (<10 cm), 2) large isolated colonies (>10 cm), 3) colonies aggregated in the breaker zone, 4) continuous belt in the breaker zone and 5) colonies aggregated at different depths. Black stars indicate hot spots of species abundance.

3.1.2. Relationship with environmental variables

The most abundant algal functional groups were the calcareous macroalgae (50.83%), followed by fleshy macroalgae (37.70%) and algal turfs (11.47%). Calcareous macroalgae like *Corallina elongata* or *Jania rubens* were the most abundant group in sub- area B, particularly on the cliffs at northern coast. Fleshy macroalgae like *Dilophus fasciola* were present mainly on abrasion platforms with similar percentage of occurrence in both areas. Algal turfs with mixed filamentous species were concentrated in sub-area A, while in sub-area B they were only located on jetties or inside harbours. *O. patagonica* showed significantly high percentages of occurrence in areas dominated by algal turfs (85.71%), followed by calcareous macroalgae (65.57%) and fleshy macroalgae (47.82%) ($X^2 = 6.143$; $p = 0.046$) (Fig. 3.2a).The first two axes of the RDA explained 69% of the species data variance and the first axis explained 32.8%, with seven environmental variables statistically significant according to the Monte Carlo permutation test ($P<0.05$) (Fig. 3.3a). The distance from the nearest harbour and the sub-area were strongly significant in determining the presence of *O. patagonica* as well as the substrate type, while its "invasive level" were significatively determined by algae community. Most colonies were detected in artificial substrata and in some natural like islets and cliffs (Fig. 3.3b), which were less than 5 km away from the nearest harbour (Fig. 3.2b) while the hot spots (category 5) were mainly related to algal turf.
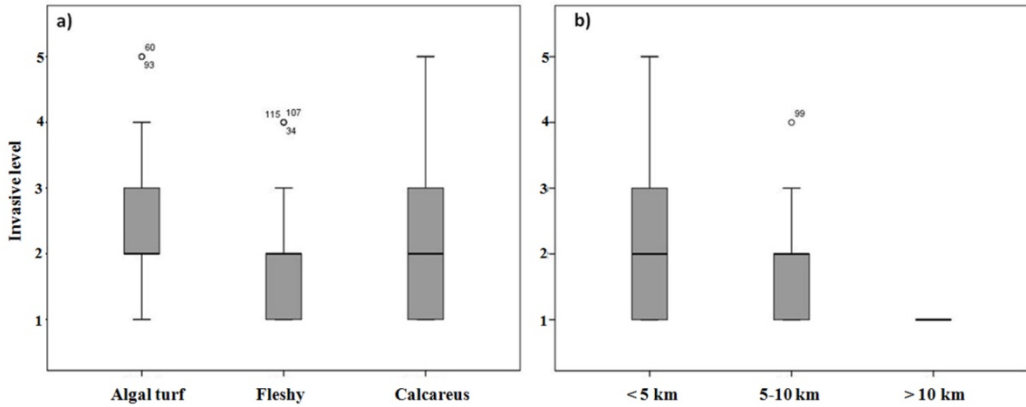
Figure 3.2. **(a)** Invasive level of *Oculina patagonica* with respect to algal community and **(b)** the distance from the nearest harbour. Bars show mean values ± standard deviation.
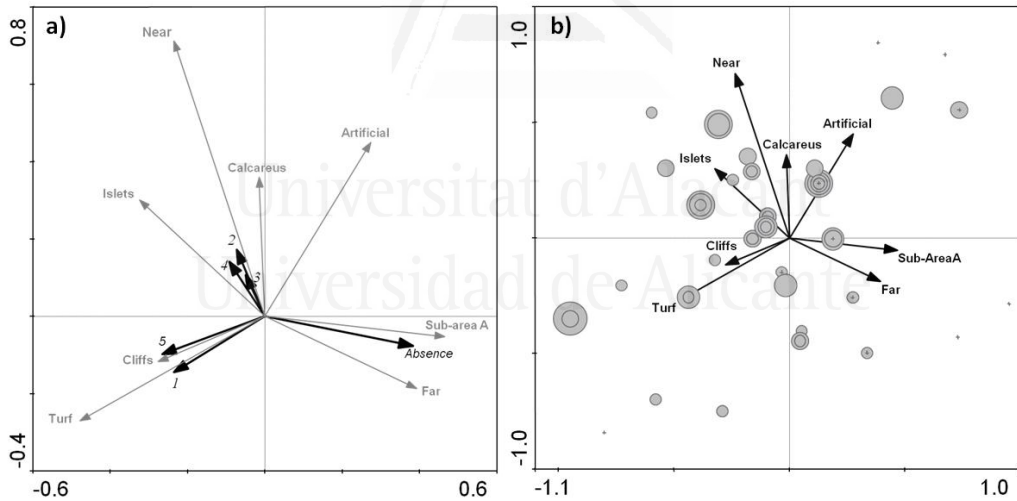
Figure 3.3. Redundancy analysis (RDA): **(a)** biplot showing the relationship between "invasion levels" categories and the significant environmental variables; **(b)** bubble attribute plots of the invasive level in the two-dimensional space determined by the RDA. (+) Empty sampling units. (Grey circle) Units in which the species is present, the circle diameter is proportional to the invasive level from the minimum up to the maximum value

3.2. GROWTH AND BLEACHING UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

3.2.1. Environmental parameters

Sedimentation rates at Alicante Harbour were 2.3 times higher than in the MPA of Tabarca (35.42 ± 0.51 and 15.43 ± 0.87 g dry weight m^{-2} d^{-1}; respectively). Regarding seasonality, higher sedimentation rates were detected in winter and autumn in Tabarca, while in the Harbour, the rates remained constant among seasons (Fig. 3.4a). Although mud and organic matter proportions were significantly higher in the Harbour; both localities presented a similar seasonal trend, showing the highest percentages during summer (Table 3.1 and Fig.3.4b, c).

During the study period, Chl a concentration was significantly higher in the Harbour than in the MPA (Table 3.1), ranging from 0.61 to 2.65 µg l^{-1} and from 0.14 to 0.31 µg l^{-1}, respectively. Highest Chl a concentrations were measured in both localities in February 2010 (winter), while the lowest concentrations were found during the warm period (June and September) (Table 3.1 and Fig. 3.4d).

Table 3.1. Summary of one-way ANOVA for each environmental variable: total sedimentation rate, mud fraction, organic matter and chlorophyll a in seawater. (df) degree of freedom; (MS) mean square; (F) F ratio. All factors were significant at $p<0.001$.

Source	df	Sedimentation rate		%Mud		%OM		Chl a	
		MS	F	MS	F	MS	F	MS	F
Locality	1	200.42	579,67	6667.72	168.04	233.95	598.72	12.51	10569.08
Time	5	6.43	18,59	462.93	11.67	15.71	40.20	1.38	1167.74
LocalityxTime	5	3.92	11,34	269.45	6.79	2.63	6.74	1.02	869.37
Error	168	0.34		39.67		0.39		.001	
Transformation		$\sqrt{x} + 1$		No		$\sqrt{x} + 1$		No	

■ Su10　⧄ Au10　□ Wn10　⧅ Sp11　■ Su11　⧄ Au11
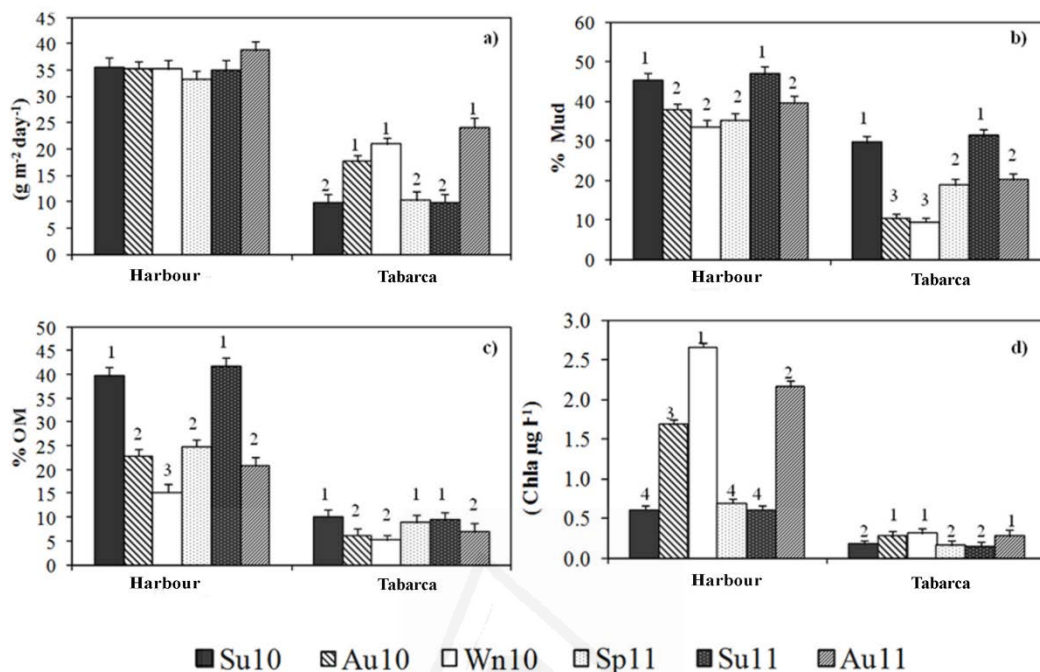
Figure 3.4. (**a**) Total sedimentation rates, (**b**) mud fraction, (**c**) organic matter, and (**d**) chlorophyll *a* concentration, for the two sampling localities throughout sampling time (Summer 2010, Autumn 2010, Winter 2010, Spring 2011, Summer 2011 and Autumn 2011). Groups defined by post-hoc SNK test after ANOVA were done within each locality and are indicated by numbers.

The mean seawater temperatures during the studied period in both localities weas not significantly different (one-way ANOVA, p= 0.133). The highest annual seawater temperature was recorded at the Harbour (28.8°C) in August 2010, and in Tabarca in July 2011 (28.17°C) (Table 3.2).

Table 3.2. Mean seawater temperature in 2010 and 2011, the minimum and maximum temperature recorded for each year and number of consecutive days that temperature was over 26°C, at the two sampling locations (Harbour and Marine Protected Area of Tabarca).

Locality	Year	SST (°C) Summer mean	Maximum SWT (°C)	Minimum SWT (°C)	Days up 26 °C
Harbour	2010	26.50 ± 1.55	28.80	15.84	49
	2011	26.48 ± 0.83	28.08	12.92	35
Tabarca	2010	26.27 ± 0.71	27.43	13.47	37
	2011	26.17 ± 1.56	28.17	12.89	54

The physicochemical characteristics of the two sampling locations were studied by PCA of the environmental data. Two components were needed to explain 89.1% of the total variance among environments. The first component, C1, had a very strong contribution of sedimentation rate and mud fraction and, to lesser extent, of organic matter and Chl *a* concentration, while C2 was mainly related with temperature. According to these two components, both Harbour and MPA samples were organized according to C1 which could be an indicator of trophic status, whereas C2 (i.e. the temperature) determined the segregation of summer, spring-autumn and winter samples (Fig. 3.5).

Variable	C1	C2
Tª	0,09	0,92
SR	0,90	-0,21
MF	0,90	0,20
OM	0,85	0,34
Chl *a*	0,79	-0,47
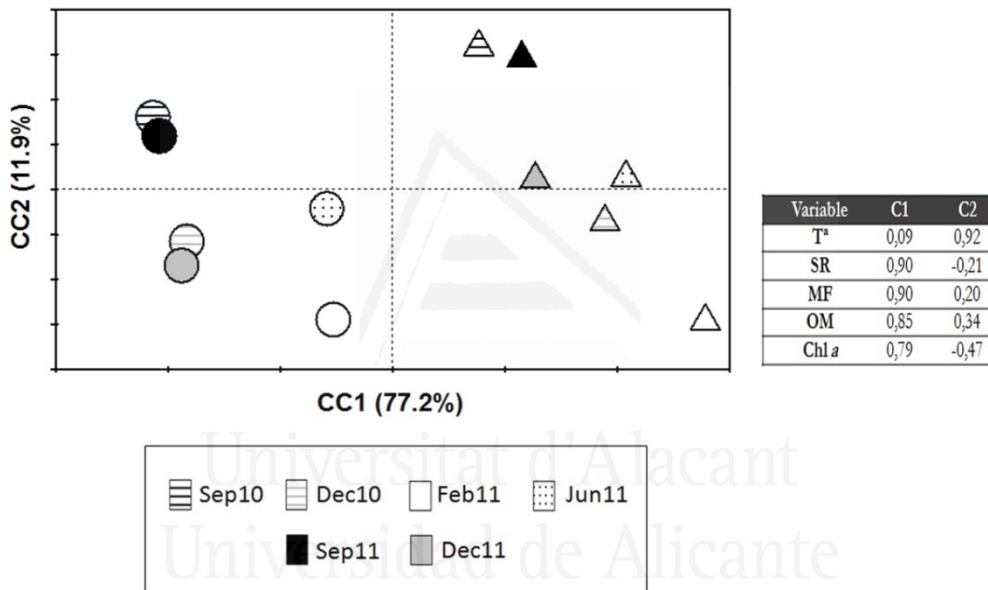
Figure 3.5. PCA ordination diagram of *O. patagonica* samples from Harbour (circles) and Tabarca (squares) collected at the different sampling times. Enviromental variables used in PCA analysis and their contribution to canonical axes are shown in the table. Variables: (Tª) Temperature, (SR) Sedimentation rate (g m^{-2} per day), (MF) Mud fraction (%), (OM) Organic matter (%) and(Chl *a*) Chlorophyll *a* (µg l^{-1}).

3.2.2. Bleaching and growth rates of *O. patagonica*

The health status of *O. patagonica* colonies (Fig. 3.6) in the Alicante Harbour and MPA of Tabarca was monitored every three months from June 2010 to December 2011 and maximum bleaching index (BI) values were similar in both localities; although they were recorded in different seasons (Fig. 3.7). In the Harbour, the highest BI (45.67) was detected in autumn 2010, while in Tabarca, it was recorded in summer 2011 (49.38), where a positive correlation between bleaching and seawater temperature was detected (rs= 0.707, N=48, p<0.01). Marked colonies also showed different bleaching dynamics between localities; detecting significant differences among the interaction time and locality. In the Harbour, marked colonies were most affected by bleaching in autumn 2010; while in the MPA, the highest values were recorded in summer and autumn 2011 (RM ANOVA, Table 3.3). Remarkably, most colonies (77%) affected by bleaching in the Harbour recovered their normal pigmentation; nevertheless, this was not the case for Tabarca corals, where some bleached colonies developed tissue necrosis (34.9±4.5% coral surface) at the end of the summer 2011, and three of them died as a result of algae overlaying.
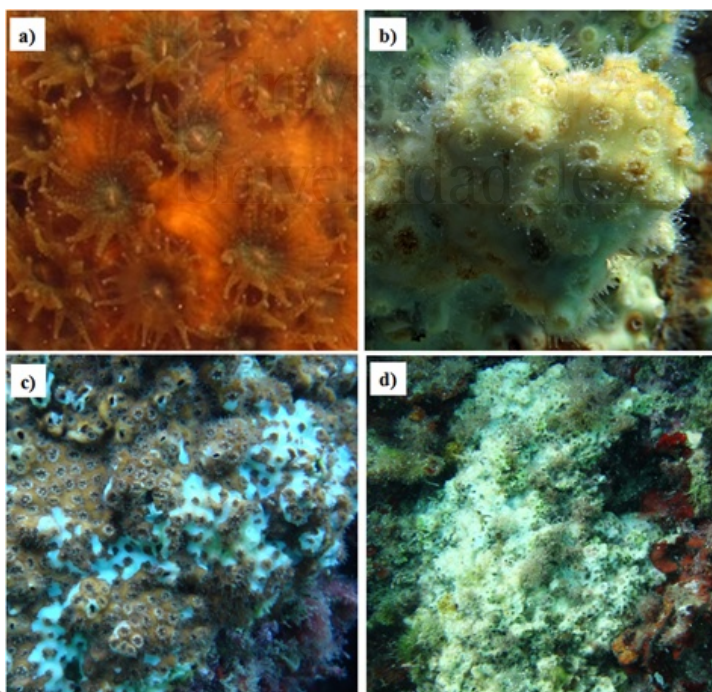
Figure 3.6. Different *O. patagonica* health status: Under water photographs of (**a**) a healthy coral, (**b**) a bleached coral in Alicante Harbour, (**c**) a coral displaying tissue necrosis in the Marine Protected Area of Tabarca and (**d**) a dead coral with overlaid algae.
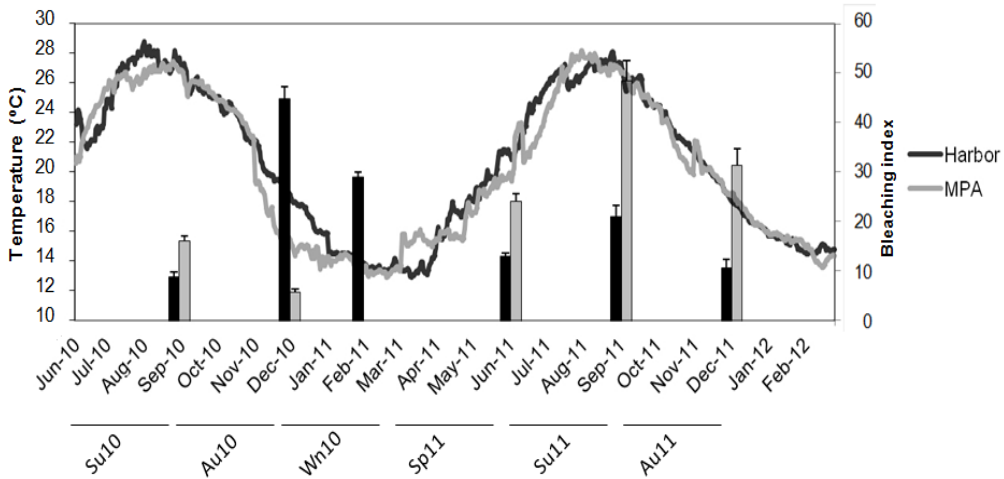
Figure 3.7. Seawater temperature for MPA of Tabarca (grey line) and Alicante Harbour (black line) and values of mean Bleaching Index (BI) of O*culina patagonica* recorded seasonally from MPA (grey bars) and Alicante Harbour (black bars) ±SE.

The annual growth rates calculated from the Harbour and Tabarca (39±3% and 37±4% increase in perimeter, respectively) did not show significant differences (one-way ANOVA, p= 0.680). The growth rates showed different time courses between environments, detecting significant differences among the interaction between time and locality. Thus, in both localities, higher growth rates were detected in summer 2010, being fewer in winter with decreasing of sea temperature, more remarkably in the Harbour. However, during summer 2011, the coral responses were different in both environments: in the Harbour, growth rates were increasing; while in the MPA, the lowest growth rates were recorded (Fig. 3.8, Table 3.3). Interestingly, a negative correlation between bleaching and growth rates was found, being a higher bleaching extension related to the lowest growth rates of *O. patagonica* (rs= -0.5267, N=29, p<0.01). In fact, corals in bleaching categories higher than 3 (> 20% of the coral´s surface bleached) showed growth rates negative or close to 0 (Fig. 3.9).
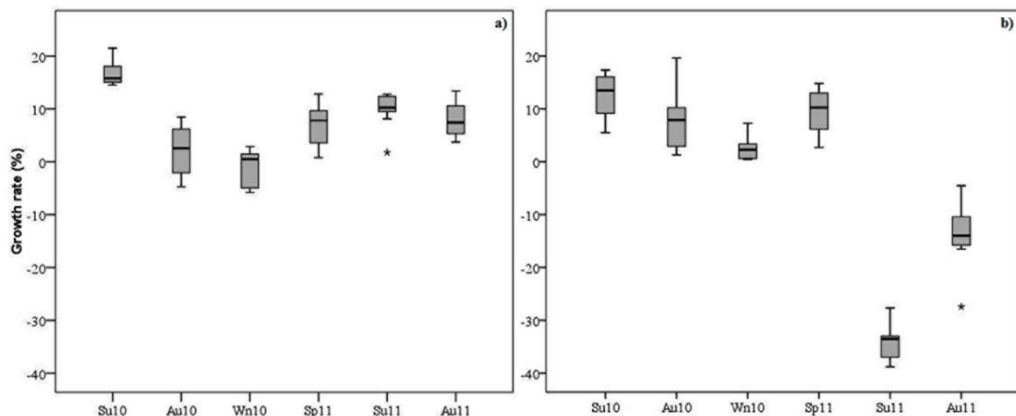
Figure 3.8. Boxplot representing seasonal growth rates of *Oculina patagonica* for (**a**) Alicante Harbour and (**b**) MPA of Tabarca. Bars show mean values ± standard deviation and asterisk represent extreme outliers.

Table 3.3. Summary of results of repeated measures growth and bleaching analysis. (df) degree of freedom; (MS) mean square; (F) F ratio; (p) p value; ns: no significant; (***) significant (p<0.001).

Source	ddf	Bleaching (%)		Growth rate	
		MS	F	MS	F
Between effects					
Locality	1	0.750	0.39ns	0.241	86.249***
Error	16	1.896		0.003	
Within effects					
Time	5	4.506	5.50***	0.145	82.296***
Time x Locality	5	13.572	16.591***	0.164	92.741***
Error	80	0.818		0.002	

In both localities, the highest growth rates were detected in summer 2010. During this season a negative correlation between coral perimeter and growth was found (Fig. 3.10), with smaller colonies showing higher growth rates (rs= -0.721, N=18, p<0.05).
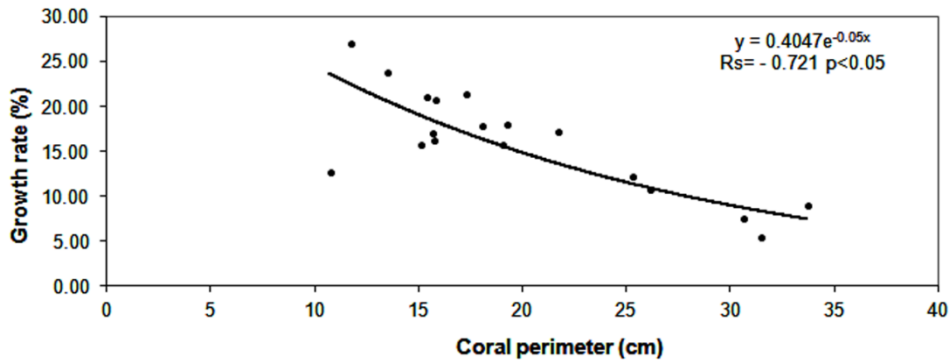
Figure 3.9. Correlation between coral size (cm²) and the annual growth rate. Rs: Spearman correlation coefficient; p: associated P value.
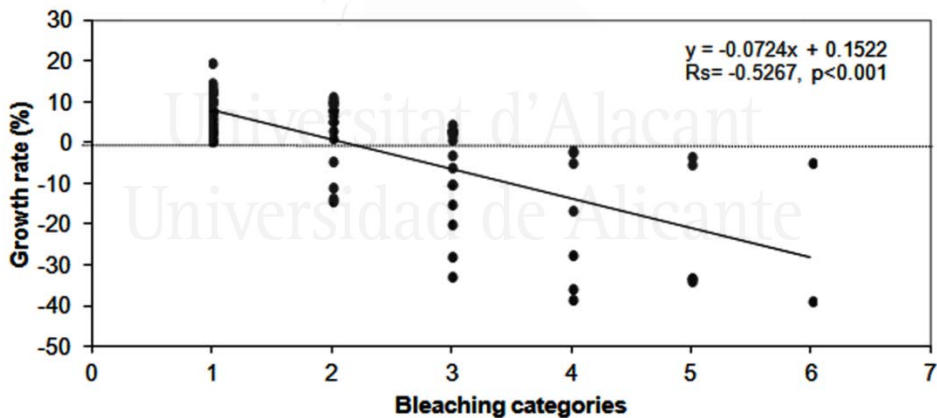
Figure 3.10. Relationship between seasonal growth rate of *Oculina patagonica* and bleaching. Bleaching categories: (1) unbleached (normal coloration), (2) pale (lighter colour than usual for the time of year), (3) 0–20% of the surface bleached, (4) >20–50% bleached, (5) >50-80% bleached, (6)>80% and (7) dead. Rs: Spearman correlation coefficient; p: associated P value

3.2.3. Influence of environmental parameters on corals' growth rates and bleaching

Multiple regression models explained a high proportion of variance mainly for bleaching (50.8%) and less for growth rates (27.8%) (Table 3.4). The regression model constructed with the observed values of *O. patagonica* bleaching incorporated four environmental variables: percentage of mud, percentage of organic matter, Chl *a* concentration in the seawater and light (shade effect). This model varied depending on the environment; in the Harbour, the only variable incorporated was light explaining 58.8% of the variation, while in the MPA, organic matter, mud fraction and seawater temperature were included explaining 64.4% of the variation.

Similarly, the regression model constructed with growth rate values was different for each environment. Organic matter, temperature and light were the environmental variables that explained the variation in general terms; nevertheless, when two environments were analysed separately, the percentage of the variation explained was higher reaching, i.e., 71.5% in the Harbour and 91.3% in Tabarca (Table 3.4). The environmental variables incorporated into the model were light and Chl *a* concentration in the seawater in the Harbour, and mud, organic matter and Chl *a* concentration in Tabarca.

3.2.4. Effect of light attenuation on *O. patagonica*

Reduction of Photosynthetic Active Radiation (PAR) values was detected in both kinds of methacrylate plates (one-way ANOVA; $p < 0.001$). Transparent plates produced a lower reduction than opaque plates (15.34% and 71.71%, respectively) compared with controls (without plates). There was a significant negative correlation between the percentage of bleaching and Chl *a* concentration in the coral tissue, with the highest bleaching percentages related to the lowest Chl *a* concentration in coral tissue (rs= -0.5488, N=75, $p < 0.001$).

ANOVA analysis did not showed significant differences in bleaching extension between control and transparent treatments (Table 3.5, Fig 3.11a). In other words, the placement of plates does not seem to produce damage in corals. Conversely, a slight increase of Chl *a* concentration in tissue was detected in transparent treatment compared with controls (Table 3.5, Fig 3.11b). An increase of bleaching percentage in corals with the opaque plates (suffering a drastic reduction of the light intensity), caused by a reduction in Chl *a* concentration in tissues, was detected. This effect was more evident in corals covered with opaque plates during summer, according to the rise of seawater temperature (Table 3.5, Fig 3.11a, b).
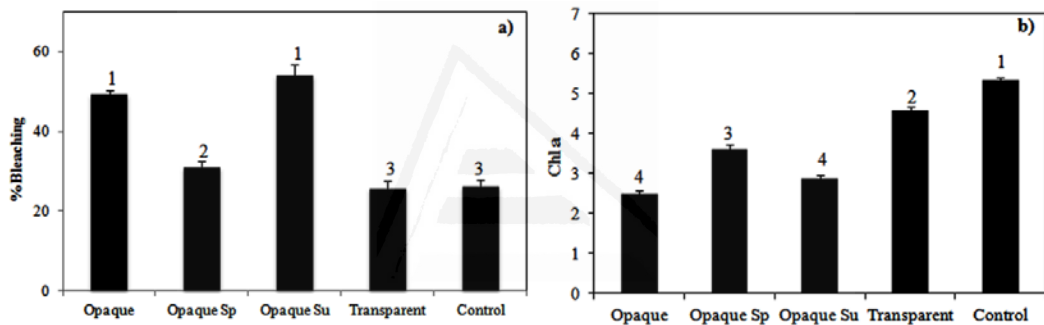
Figure. 3.11. (**a**) Mean values (±SE) of percentage of bleaching (%) and (**b**) chlorophyll a concentration in coral tissues recorded for each light treatment. Groups defined by post-hoc SNK test after ANOVA were done for each parameter and are shown by numbers.

Table 3.4. Results of multiple linear regression analysis of growth rates and against environmental variables.

	n	Adj R²	F	Cons	Sr	Mud	Mud³	OM	OM²	OM³	Chla	Chla²	Tᵃ³	Shade
GR	108	0.278	14.730***	0.075	-	-	-	-	1.576E⁻⁴	-	-	-	-.063E⁻⁵	-0.08
Harbor	54	**0.715**	67.646***	0.154	-	-	-	-	-	-	**-.042**	-	-	-0.089
Tabarca	54	**0.913**	140.310***	-0.948	-	0.016	-4.65E⁻⁵	-	-	0.001	**2.546**	-	-	-
Bleaching	108	0.508	23.055***	1.301	-	0.182		-.396	0.005	-	-	-.226	-	2.428
Harbor	54	**0.588**	76.512***	1.359	-	-	-	-	-	-	-	-	-	**2.118**
Tabarca	54	**0.644**	32.983***	24.750	-.522	0.520	-	-.598	-	-	-	-	**-0.233**	-

Table 3.5. Summary of one-way ANOVA for percentage of bleaching and Chl a concentration recorded from coral colonies at the end of light attenuation experiment. (df) degree of freedom; (MS) mean square; (F) F ratio; (ns) no significant; (***) significant at p<0.001.

Source	df	Bleaching (%)		Chl a concentration	
		MS	F	MS	F
Treatment	4	17.2378	60.44***	20.9759	260.31***
Si (Tr)	10	0.2852	0.82 ns	0.0806	0.89 ns
Error	60	0.3476		0.0905	
Transformation		$\sqrt{x+1}$			

Part II. RESPONSE OF THE CORAL ASSOCIATED MICROBIAL COMMUNTIES TO DIFFERENT ENVIRONMENTAL CONDITIONS

3.3. MICROBIAL COMMUNITIES ASSOCIATED TO *Oculina patagonica*

3.3.1. Eukaryotic community

A total of 46 *O. patagonica* colonies were used to assess changes in Eukarya community related to environmental conditions (Alicante Harbour/MPA of Tabarca), geographic location (MPA/Pietra Ligure) and coral health status (Bleached/Healthy). As discussed above, bleached corals were observed in the Spanish coast, both in Alicante Harbour and in MPA of Tabarca, mainly in September 2011 (see Fig. 3.7), although a few colonies taken in September 2012 displayed a color lighter than usual. Bleached corals were not observed in the Italian sampling locality (Pietra Ligure) (see Table 2.1, p. 67).

The presence of zooxanthellae located in the tissues of *O. patagonica* was verified by electron microscopy examination and DGGE. In all samples *Symbiodinium* sp. was detected, and was related (100% sequence identity) to clade B (AF427448), based on eukaryotic partial 18SrRNA genes (see Annexes, Fig. A1) and to type B2 (JN558062.1) based on molecular ITS2 analyses (see Annexes, Fig. A2). Electron microscopy showed that *Symbiodinium* was located in the gastrodermal layer of the *O. patagonica* (Fig. 3.12), where also nematocysts were observed. In healthy corals, zooxanthellae, displaying a round section with a continuous cell wall, were regularly distributed in the gastrodermis, which appeared as a consistent tissue (Fig. 3.12A, B, C and G). However, in bleached corals, the gastrodermal layer appeared damaged showing intercellular spaces and damaged cell remains both from gastroderm and zooxanthellae (Fig. 3.12D, E and F). In these damaged zooxanthellae, the chloroplasts were disorganized and starch and lipid globules were not observed (Fig. 3.12F).
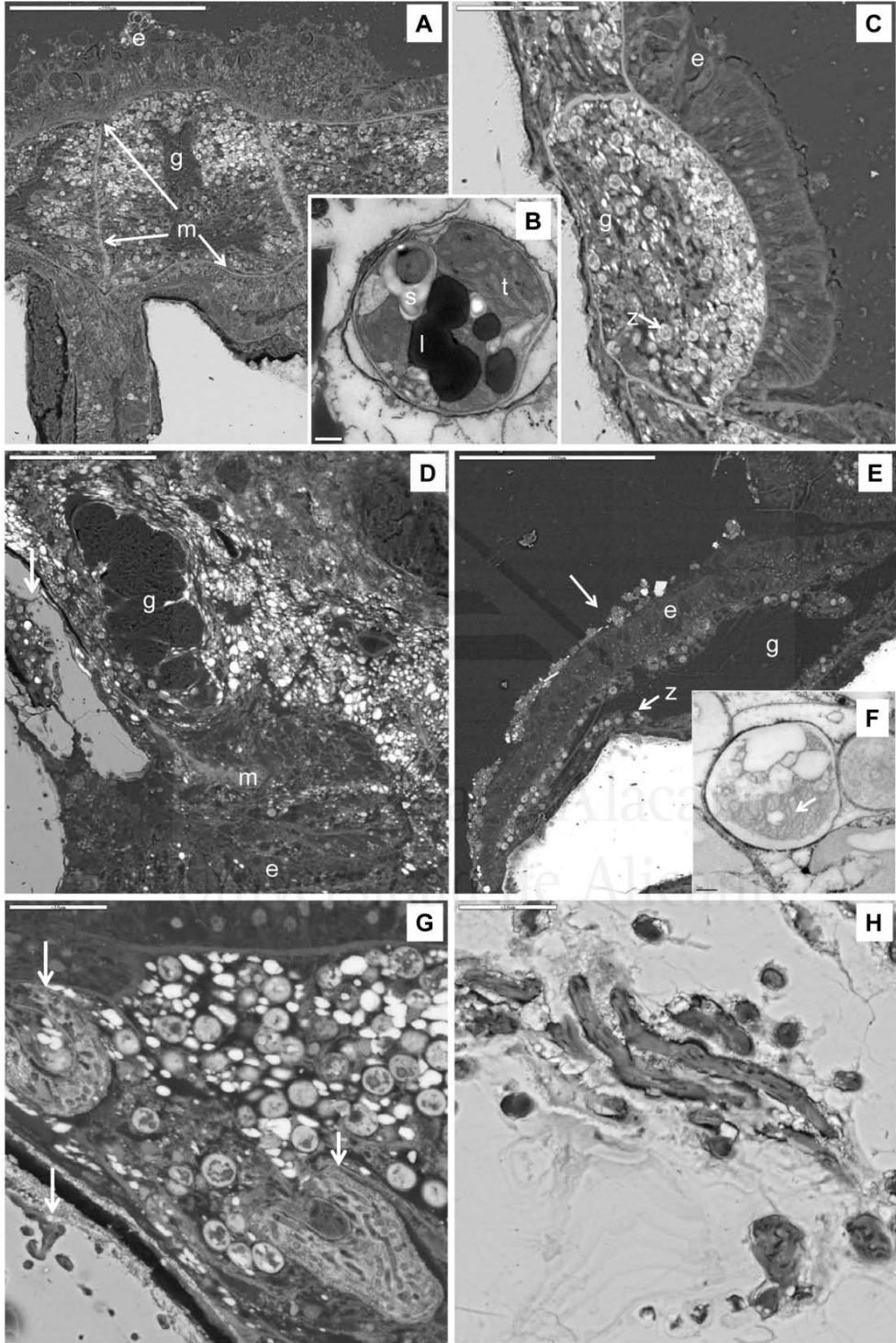
Figure 3.12 (previous page). (**A**) Healthy coral showing consistent gastroderm (g) with abundant zooxanthellae, surrounded by mesoglea (m) and well-defined epidermis (e) (bar=200 μm). (**B**) Healthy zooxanthella ultrathin section showing compact chloroplast with aligned thylakoids (t), abundant lipids globules (l) and starch (s) around the pyrenoid (bar=1 μm). (**C**) Healthy coral epidermis (e) with abundant zooxanthellae (z) and brilliant inclusion of mucus material in the gastroderm area (g) (bar=50 μm). (**D**) Unhealthy coral showing mesoglea and epidermis disorganization and inconsistent gastroderm (g), the presence of filamentous microorganism was detected into the skeleton part (arrows) (bar=100 μm). (**E**) Unhealthy coral showing inconsistent gastroderm (g) with empty spaces and few zooxanthellae (z), and mineral particles sedimented on the epidermis (arrows) (bar=200 μm). (**F**) Damaged zooxanthellae, showing disorganization of chloroplast thylakoids (arrow) (bar=1 μm). (**G**) Healthy gastroderm affected by the presence of colonization of the skeleton part (arrows) (bar=20 μm). (**H**) Detail image of the cyanobacteria filaments colonizing the coral skeleton (bar=20 μm). A,C,D,G and H correspond to SEM-BSE images; B and F to TEM images.

Eukarya sequences other than zooxanthellae and corals, which matched 100% with the 18S rRNA gene from *Phyllangia mouchezii* (see Annexes, Fig. A1), were detected in *O. patagonica* samples by 18S rRNA gene DGGE, in both tissue and skeletal matrix samples. Furthermore, SEM-BSE study permitted to detect the presence of endolithic microorganisms (Fig.3.11H) in both bleached (arrows in Fig. 3.11D) and healthy parts of the coral (arrows in Figure 3.11G). Most of the colonies from Tabarca and some from the Alicante Harbour showed barnacles, which, although readily visible, are difficult to identify by direct visualization, and were identified by DGGE, being closely related (99%) to *Megatrema anglicum* (see Annexes, Fig. A1). In addition to barnacles, two boring sponges were detected in skeletal samples, which showed a 99% sequence identity to *Cliona* sp. and *Siphonodictyon (=Aka) coralliphagum*. While *Cliona* sp. was associated mainly with samples from Tabarca after the bleaching event, *S. coralliphagum* was detected in both bleached and healthy corals collected from the Harbour (see Annexes, Fig. A1).

Eukaryotic plastids were also detected in *O. patagonica* samples by bacterial 16S rRNA gene DGGE (carried out to study bacterial community) using DNA extracted from the skeletal matrix (see Annexes, Fig. A5 and Table. A1). The proportion of plastids was higher in bleached (57.5% of the samples) than in healthy (11.3%) corals. These phototrophs included a photosynthetic cocolithophorid that showed a 99% identity in the 16S rRNA fragment analysed sequence with *Ochrosphaera* sp. (X99077), and was detected along the whole year in samples collected from Tabarca in both healthy and bleached corals. Two additional uncultured phototrophic eukaryotes were detected mainly in bleached samples. One of them, with no matches in databases, was more frequently retrieved from Harbour samples where it was detected in 33.7% of bleached corals. The other sequence, distantly related (91%) to a marine diatom, was found in 41.7% of bleached colonies from Tabarca.

3.3.2. Bacterial community

A total of 40 *O. patagonica* colonies were used to characterise changes in their bacterial community over a year including the bleaching event recorded on September 2011 (see Table 2.1, p. 67). DGGE profiles generated showed 40 different bands with distinct mobilities (see Annexes, Fig. A3, A4, A5 and Table A1). The different DGGE band patterns were analysed using Bray-Curtis similarities between samples, by the two-dimensional NMDS plot (Fig. 3.13; stress value= 0.21) and cluster analysis (Fig. 3.14), showing segregation of samples mainly related to coral microhabitats.

Further, an analysis of similarities (ANOSIM) test revealed that the composition of bacterial communities associated to the three microhabitats within the coral (mucus, tissue and skeleton) were significantly (R= 0.514; p= 0.001). The highest bacterial diversity was found in mucus (H´= 3.379), followed by tissue (H´= 3.305) and skeletal matrix (H´= 2.986). There was no clustering of the samples by sampling location and ANOSIM test confirmed that bacterial communities were not significantly different between environments (Harbour and Tabarca, R= 0.019; p= 0.710). Slightly seasonal differences between bacterial communities associated to coral in warm or cold months were only detected in tissue samples (ANOSIM; p=

0.03) and not in mucus or skeletal matrix (P-values of 0.128 and 0.111, respectively). Differences between bacterial communities associated with healthy and bleached *O. patagonica* colonies were detected in tissue and mucus (ANOSIM; P-values of 0.01 and 0.04, respectively), but not in the skeletal matrix (ANOSIM; p= 0.279).
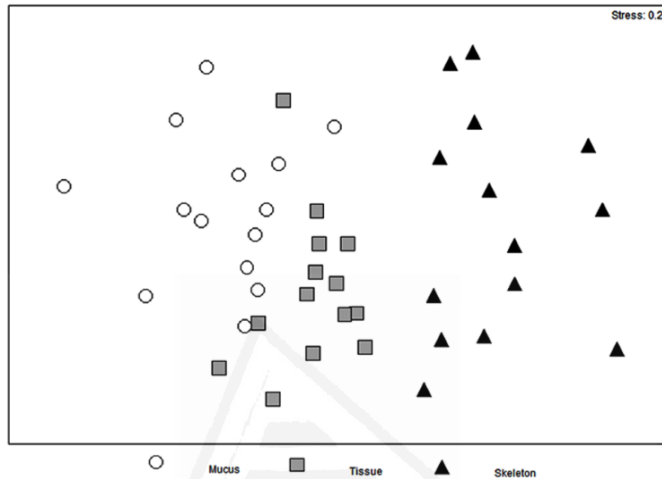
Figure 3.13. Non-metric Multimensional Scaling plots of the first two dimensions based on Bray-Curtis dissimilarities for mucus (white circles), tissue (grey squares) and skeletal matrix (black triangles).

3.3.2.1. Comparison between coral compartments

Most of the Operational Taxonomic Units (OTUs) retrieved from coral tissue (61.11%) and mucus layer (61.50%) of *O. patagonica* corresponded to *Proteobacteria* (Fig. 3.14), with *Alphaproteobacteria* as the dominant class of the microbial community in both microhabitats (84.89% and 64.8% of *Proteobacteria*, respectively), *Gammaproteobacteria* were mainly detected in tissue compartment (22.52% of tissue *Proteobacteria*) and *Deltaproteobacteria* in skeletal matrix (33.91% of skeletal *Proteobacteria*). In addition OTUs of *Chlorobi* and *Bacteroidetes* were mainly detected in mucus (12.25%) and skeletal matrix (22.51%), respectively. The presence of bacteria in *O. patagonica* tissue was also confirm by electron microscopy observation, bacteria was in the gastrodermis layer were among cnidocyst and zooxanthellae (Fig 3.15 a, b).
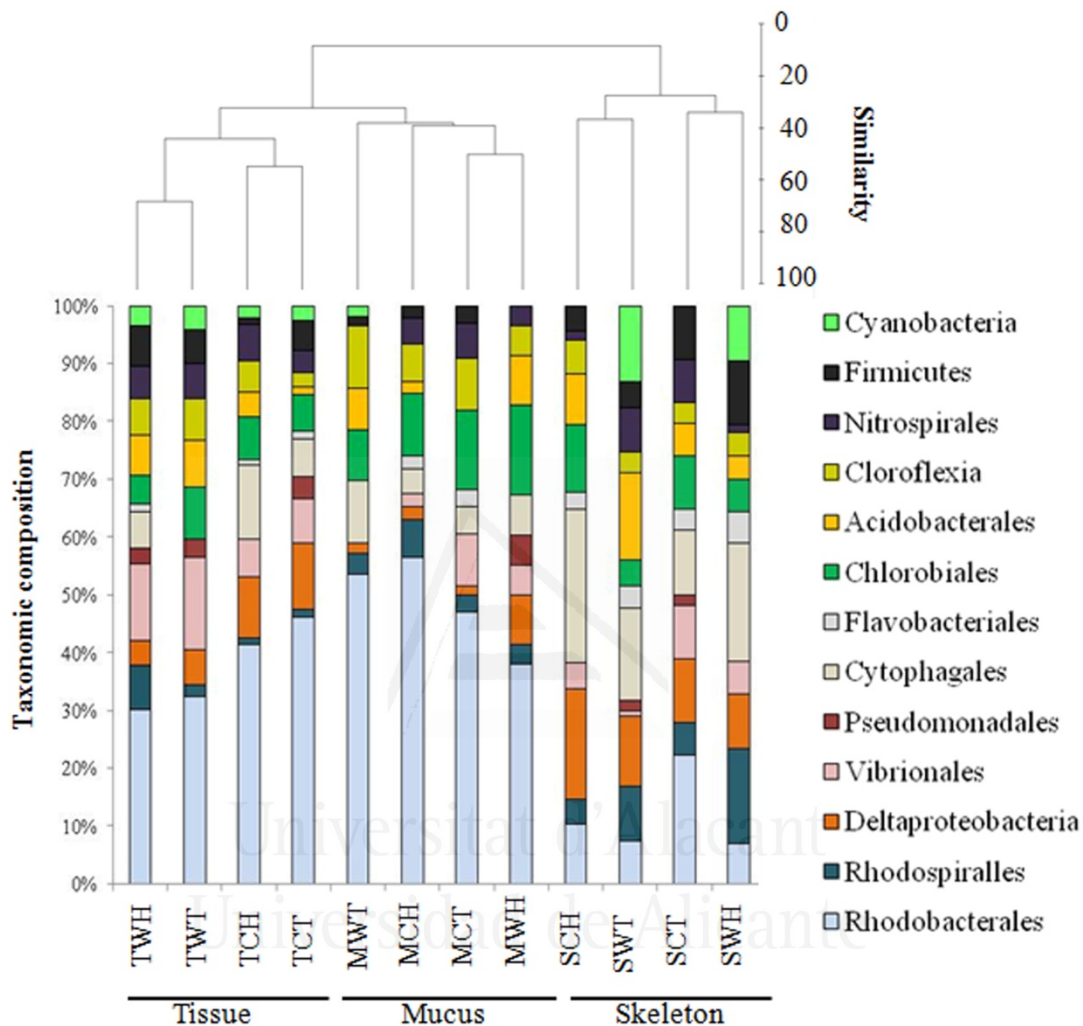
Figure 3.14. Cluster analysis and taxonomic composition (dominant bacterial sequence affiliations grouped into dominant ribotypes at the phylum and class level from RDP clasification) of mucus (M), tissue (T) and skeletal (S) collected in cold (C) or warm (W) months from Alicante Harbour (H) or Marine Reserve of Tabarca (T).

OTUs primarily responsible for the observed differences among the microhabitats studied (Table 3.6a) were determined by SIMPER. AP1 and AP3, classified as *Pseudovibrio* sp. and *Ruegeria* sp., were the most important OTUs for similarity of the mucus layer and coral tissue microbial community; especially in mucus layer where they were detected in 95% and 80% of samples, respectively. The OUT AP2, related to the genus *Oceanicola* , and GP2, which was classified in family *Vibrionaceae*, were mainly detected in tissue samples; being GP2 a tissue-specific OTU. In the skeletal samples a member of *Cytophaga* (BC1) detected in the 68% of the skeletal samples, was identified as the most important for the distinction of this microbial community.

Differences among cold and warm months microbiotas were only detected in the coral tissue. While in cold months *Alphaproteobacteria* OTUs (mainly AP1, and AP2, see Table 3.6b) occurred in more than 60% of colonies examined, in warm months this class was substituted by *Gammaproteobacteria* (GP2), *Chlorobia* (CB1) and *Acidobacteria* (AB) (Fig. 3.14).

Table 3.6. Percentage of contribution of each OUT to the Bacteria community structure occurring (a) between coral microhabitats (M: Mucus; T: Tissue and S: Skeleton); (b) between time (C) cold months (W) warm months, in tissue; and (c) between coral health status (H) healthy and (U) unhealthy samples in mucus and tissue. Based on SIMPER analysis, indicating the average contribution of each phylotypes to the similarity (S) within each grouping factor and differences (D) among them.

a) Microhabitat

Phylotype name	Phylogenetic group	Contribution %					
		Mucus S=49.63	Tissue S=56.33	Skeleton S=43.10	M-T D=55.18	M-S D=69.34	T-S D=60.50
AP3	Rhodobacterales	17.87	13.22	-	6.20	8.23	9.60
AP1	Rhodobacterales	17.46	6.02	-	-	6.93	4.40
CB1	Chlorobia	11.03	-	-	4.07	-	-
OTU11	Unidentified	9.93	-	-	4.03	-	-
AP2	Rhodobacterales	8.74	11.61	8.63	6.57	3.84	5.06
OTU7	Unidentified	-	7.16	-	4.18	-	3.72
GP2	Vibrionales	-	6.28	-	6.48	4.90	5.34
BC1	Cytophaga	-	-	9.61	-	-	3.75
OTU12	Unidentified	-	-	7.12	-	3.69	-
OTU10	Unidentified	-	-	6.67	-	-	-
AB	Acidobacteria	-	-	6.62	-	4.10	-

b) Microhabitat x time (Tissue)

Phylotype name	Phylogenetic group	Contribution %		
		Cold S=57.76	Warm S=58.38	C-W D=45.68
AP1	Rhodobacterales	20.09	9.88	5.85
AP2	Rhodobacterales	11.28	9.54	5.43
OTU7	Unidentified	7.66		
AP3	Rhodobacterales	5.70	6.27	
GP2	Vibrionales		9.31	6.22
DP1	Deltaproteobacteria	4.86		
CB1	Chlorobia		4.85	4.31
AB	Acidobacteria		4.41	4.44

c) Microhabitat x Coral health status

Phylotype name	Phylogenetic group	Mucus			Tissue		
		H S=52.11	U S=53.06	H-U D=52.90	H S=55.59	U S=63.28	H-U D=46.57
AP3	Rhodobacterales	8.06	18.75	8.25	11.09	13.96	5.08
AP1	Rhodobacterales	20.41	15.98	5.25	5.10	6.60	-
CB1	Chlorobia	12.52	-	5.15	-	9.61	6.05
OTU11	Unidentified	12.35	-	5.45	-	-	-
AP2	Rhodobacterales	7.53	9.18	4.90	14.82	-	6.27
BC1	Cytophaga	-	10.49	4.72	-	-	-
AB	Acidobacteria	-	8.31	4.19	-	6.69	5.10
OTU7	Unidentified	-	-	-	8.52	-	4.62
GP1	Vibrionales	-	-	-	3.02	5.07	3.32
NI	Nitrospirae	-	-	-	-	7.51	4.03

3.3.2.2. Comparison between healthy and bleached corals

Changes in the dominant bacterial groups in bleached compared with healthy samples were detected in the mucus layer (Fig 3.15a). Although *Rhodobacterales* were predominant in both healthy and diseased corals, *Pseudovibrio* spp. (AP1) were significantly associated with healthy assemblages, while *Ruegeria* sp. (AP3) was more important in diseased ones. In fact, these were the OTUs with the highest contribution to the differences found between healthy and unhealthy samples (Table 3.6c). Furthermore, the proportion of *Cytophaga* and *Acidobacteria* sequences became higher in mucus layer of unhealthy corals, contributing also to the differences found between healthy and unhealthy bacterial communities associated to mucus layer.

Bacterial communities associated to coral tissue were more similar for unhealthy than for healthy corals (Table 3.6c). The decrease of *Rhodobacterales* was more evident in unhealthy tissue than in mucus layer samples (Fig.3.15b), being *Oceanicola* sp. (AP2) together with *Pseudovibrio* spp. (AP1) the species that showed the highest more contribution to the differences between healthy and unhealthy tissue samples. OTUs related to the genus *Vibrio* (GP1) and to the phylum *Nitrospirae* also contributed to the differences detected in bleached tissues (Table 3.6c), increasing their proportion compared with healthy ones.

Although ANOSIM analysis did not show significant differences between healthy and unhealthy samples in the skeletal matrix, in unhealthy samples a trend to decrease *Alphaproteobacteria* and to increase *Acidobacteria* and *Cyanobacteria* was observed (Fig. 3.16C). The increase of *Cyanobacteria* in the skeletal matrix was also confirmed by electron microscopy (Fig 3.16C, D, E).
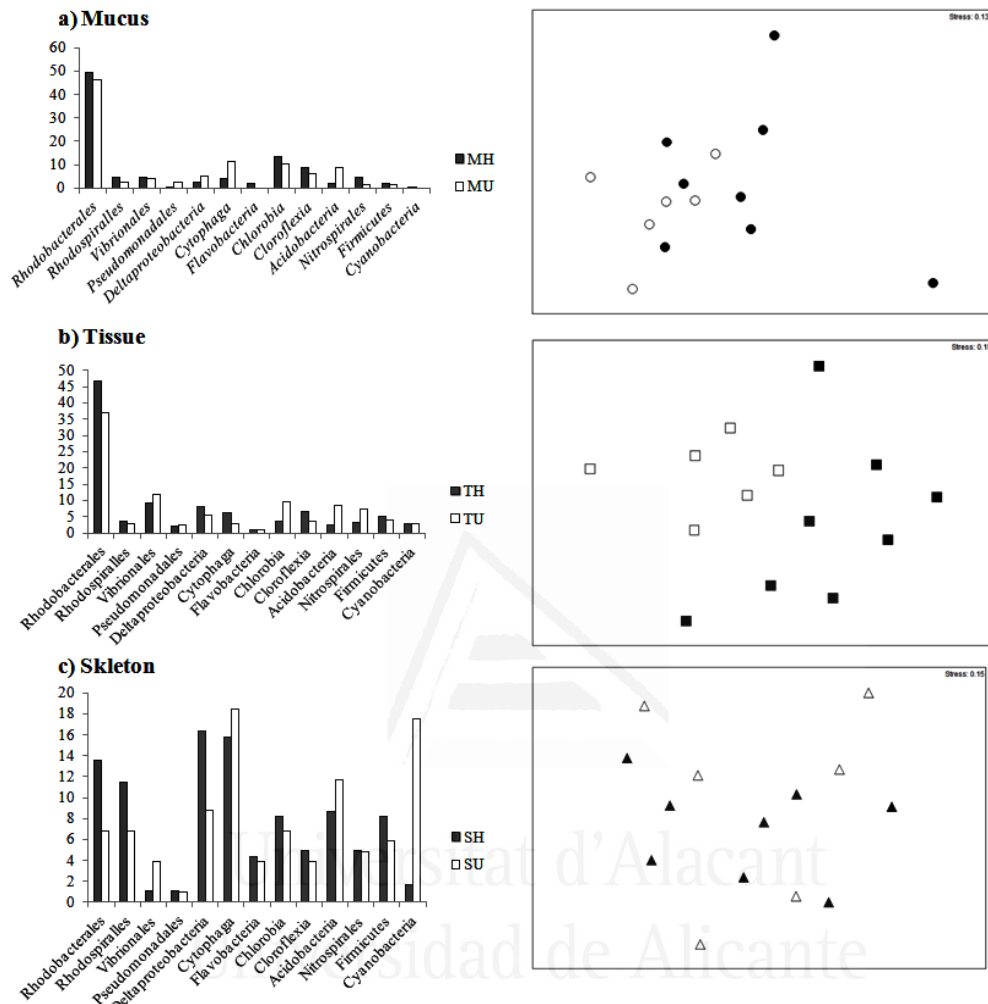
Figure 3.15. . Non-metric Multimensional Scaling plots of the first two dimensions based on Bray-Curtis dissimilarities and the percentage of bacterial classess from healthy (white) unhealthy (black): (a) samples of mucus(circles), (b) tissue (squares) and (c) skeletal matrix (triangles).
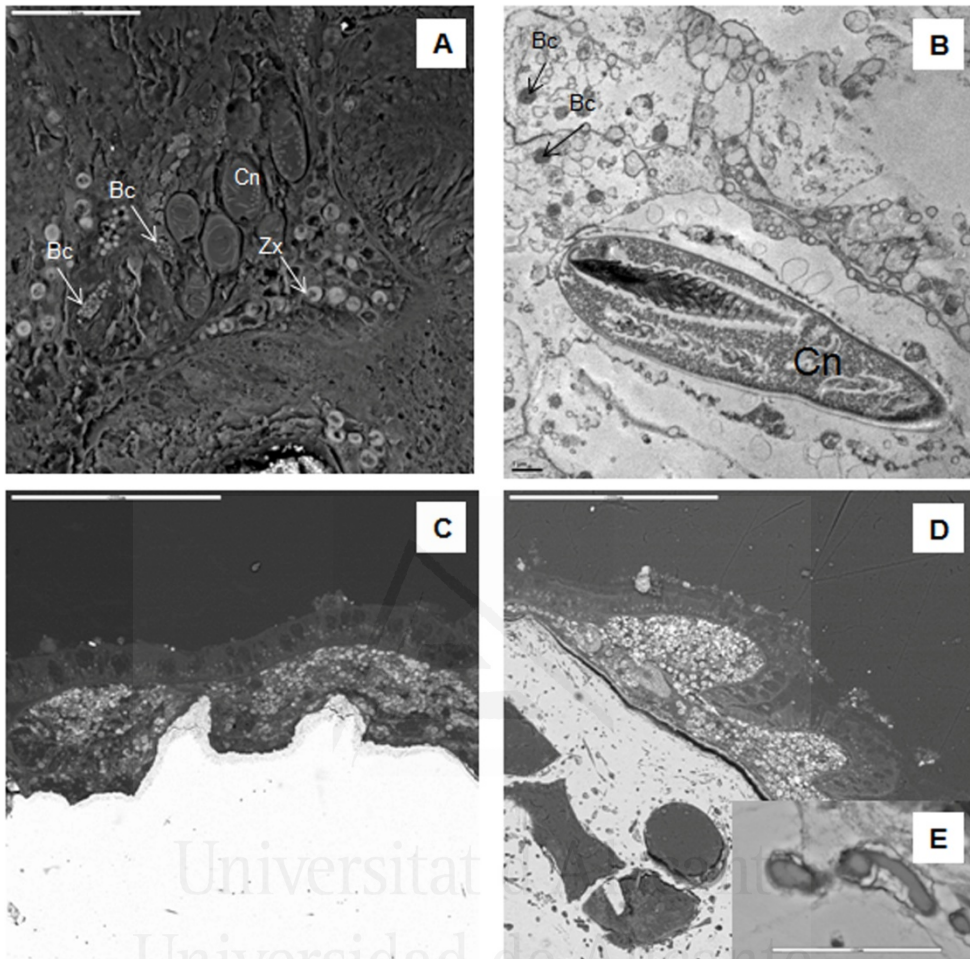
Figure 3.16. Electron microscopy images of *O. patagonica* holobiont. **(A)** Coral tissue showing consistent gastroderm with abundant cnidocyst (Cn) and zooxanthellae (Zx) and possible Bacteria (Bc) (bar=50μm). **(B)** Detail image of coral tissue of a cnidocyst and Bacteria around it (bar=1μm). **(C)** Healthy coral with a consistent skeletal matrix (bar=200μm). **(D)** Unhealthy coral with skeletal matrix affected by the presence a colonization of cyanobacteria (bar=200μm). **(E)** Detail image of the cyanobacteria filaments colonizing the coral skeleton (bar=20μm). A, C, D and E correspond to SEM-BSE images; and B to TEM images.

3.3.3.3. Illumina high-throughput 16S rRNA gene sequencing

A total of 648,384 quality filtered reads were recovered from the seven tissue samples sequenced using the Illumina platform. The corresponding number of OTUs at a 3% dissimilarity level and Shannon diversity indices are listed in Table 3.7.

Table 3.7. Counts of paired-end rRNA gene sequences obtained from Illumina (preassembly) and following assembly and screened (postassembly) for the libraries included in this study. Samples labels: location, (H) Harbour; (T) Tabarca; season, (C)Cold; (W) Warm; and coral status (H) Healthy and (U) Unhealthy.

Samples	Number of sequences		Remaining sequences (%)	Number of OTUs	Shanon index (H´)
	Preassembly	Postassembly and filtered			
HCH	3661044	295250	8.06	14778	6.23
HWH	126618	12426	9.81	2176	5.08
HWU	120202	14460	12.03	4831	7.05
TCH	146276	8125	5.55	18085	5.36
TWH	109296	22873	20.93	2127	6.00
TWU	1396170	147784	10.58	11159	4.28

RDP classifier revealed 27 phyla in cold samples from both sampling locations, decreasing until 15 and 18 in unhealthy samples from the Harbour and Tabarca, respectively. Not surprisingly, the diversity obtained by Illumina sequencing was higher than the diversity shown by DGGE, with slightly differences between sampling location, not detected by DGGE, unveiled by the massive sequencing approach. In both sampling locations the bacterial community was dominated by the phylum *Proteobacteria*, followed by the *Firmicutes* that was more frequently (18.5%) retrieved from the Harbour samples and only barely detected in Tabarca (0.35%). The second phylum most frequently retrieved from Tabarca was *Bacteroidetes*, which showed similar percentages in both localities (10.8% and 8.2% in the Harbour and Tabarca, respectively).

Although the proportions of 16S rRNA gene sequences corresponding to the main bacterial groups that were recovered by the Illumina and DGGE were different; they both indicated the same trends in the shifts experienced by tissue bacteria assemblages when comparing healthy and unhealthy corals. The phylum *Proteobacteria* was always the most dominant and constituted from 58 to 96% of the qualified bacterial Illumina reads in coral tissues (Fig. 3.17a). Within *Proteobacteria*, the class *Alphaproteobacteria* was dominant in cold samples (44-83 % in the Harbour and in Tabarca, respectively), with most sequences corresponding to the order *Rhodobacterales* (Fig. 3.17b), which decreased in samples collected during warm months and mostly in unhealthy samples. In good agreement with DGGE results, *Pseudovibrio* and *Ruegeria* were the genera most represented in healthy samples from the Harbour and Tabarca, respectively. The class *Betaproteobacteria*, not detected by DGGE, was retrieved mainly from healthy corals, being the order *Burkolderiales* the most frequently retrieved. Although *Gammaproteobacteria* were present both in healthy and unhealthy corals, their proportions were considerably higher in warm months (69-91% of total proteobacteria sequences).Within this class, a large number of sequences corresponded to the order *Vibrionale*s (Fig. 3.17b), and more specifically, to the genus *Vibrio* that accounted for up to 53% (Harbour) and 81% (Tabarca) of the sequences in unhealthy corals.
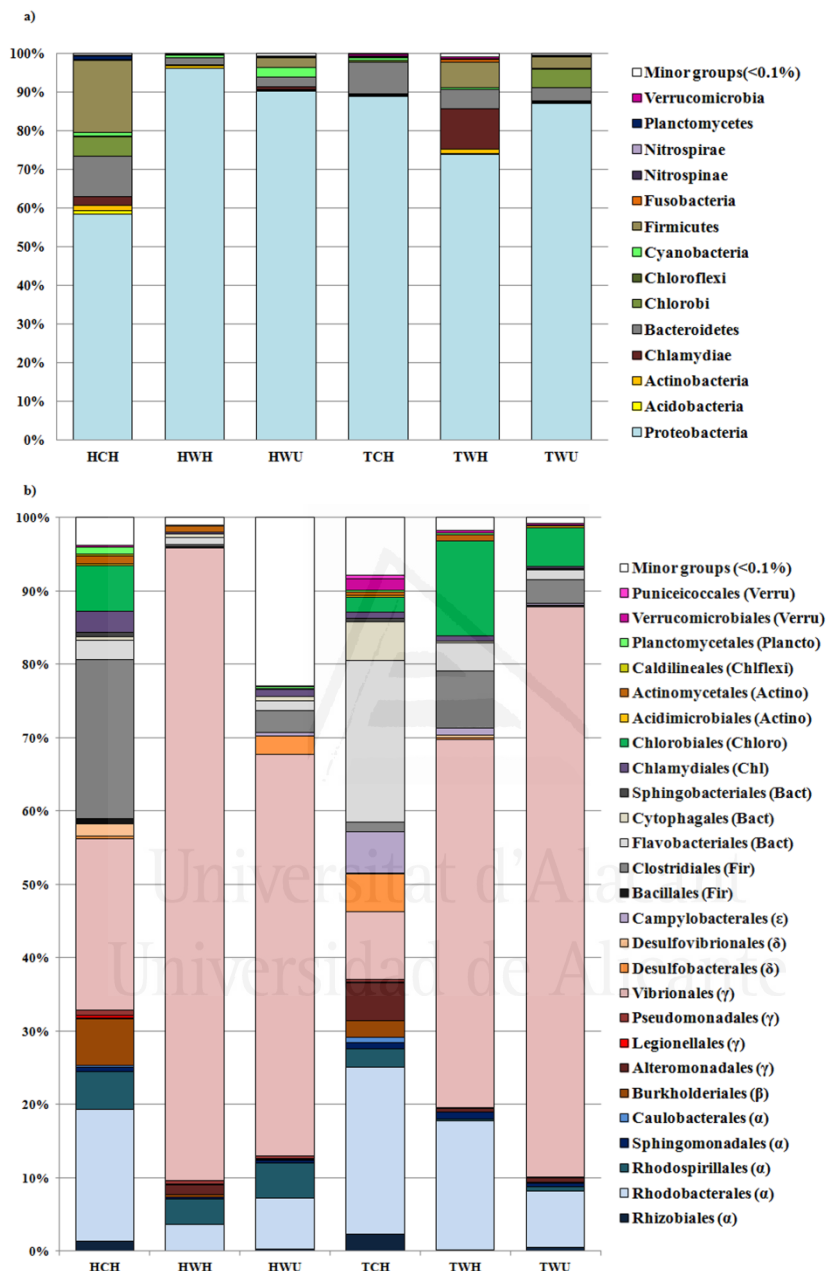
Figure3.17. Taxonomic classification of bacterial reads retrieved from Illumina high-throughput 16S rRNA gene sequencing from *Oculina patagonica* samples, collected in Alicante Harbour and Reserve Marine of Tabarca, into (a) phylum and (b) order levels using the RDP classifier. See Table 3.7 (p. 110) for sample identifiers.

3.4. *VIBRIO* spp. COMMUNITY

3.4.1. Temporal changes and relationship with environmental conditions

3.4.1.1. Coral health status and seasonal variations of *Vibrio* spp.

A total of 109 coral samples were taken from Alicante Harbour and MPA of Tabarca in order to characterise their associated communities of culturable *Vibrio* spp., and to ascertain how they change with environmental conditions and time, samples were collected seasonality from September 2010 to December 2011. The health status of *O. patagonica* colonies in the sampling locations was monitored every three months from September 2010 to December 2011 and the highest bleaching indexes were recorded in September 2011 in MPA of Tabarca and in December 2010 in the Harbour (see Fig.3.7). The status of coral colonies collected for this study is shown in Figure 3.18a.

The temporal variation of culturable *Vibrio* counts in seawater, mucus and coral tissue during the study period is shown in Figure 3.18. *Vibrio* spp. ranged from 1×10^0 to 1.8×10^3 CFU/l in seawater and from 9.8×10^1 to 2×10^5 and from 3×10^1 to 7.6×10^5 CFU/g in the mucus and coral tissue respectively. In seawater (Fig. 3.18b), culturable *Vibrio* spp. were only detected from June to December, while they could be detected in all the coral samples taken along the year (Fig. 3.18c), 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).
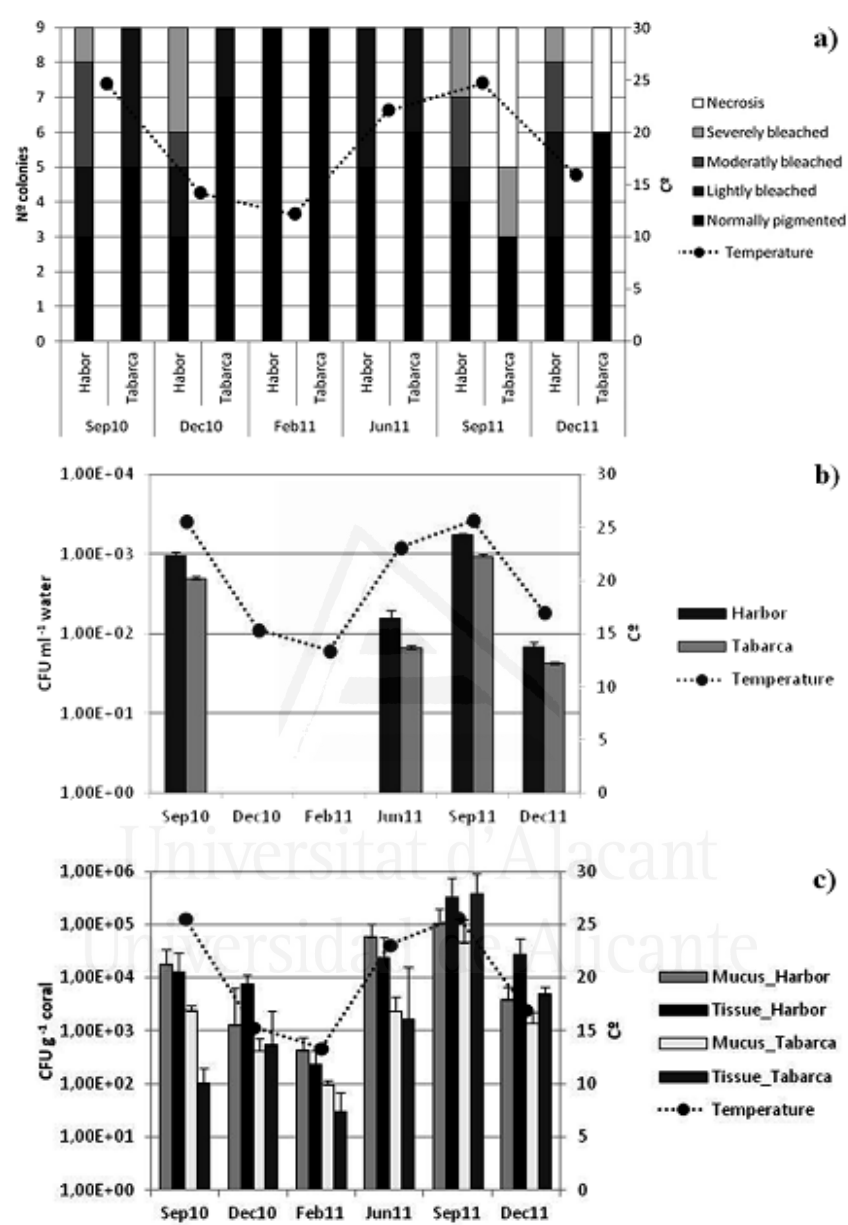
Figure 3.18. (a) Health status of *O. patagonica* samples and *Vibrio* spp. counts (b) in seawater and (c) in mucus and coral tissue (c) in both sampling locations. Temperature changes (averages calculated for each sampling time) are displayed in the three panels. (CFU) Colony Forming Units.

3.4.1.2. Diversity and dynamics of *Vibrio* community

The screening by ARDRA of the 296 *Vibrio* isolates obtained yielded a total of 53 different patterns. At least one isolate per restriction pattern was selected for complete sequencing, producing in total 133 sequences of adequate quality (1458 bp and 0% ambiguities as median values). The OTU (Operational Taxonomic Unit) saturation observed in the rarefaction curves (Fig. 3.19) 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* sp. 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, that were named according to the known taxa found within the clades (see Annexes, Fig. A4). *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 Harbor and MPA. 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 *Vibrio splendidus –V. gigantis – V. atlanticus – V. pomeroyi* (50%), *V. harvey 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. On the other hand, some phylotypes like the known pathogens *V. mediteranei* and *V. coralliilyticus*, were only present in warm months (Table 3.9 and see Annexes, Fig. A4).
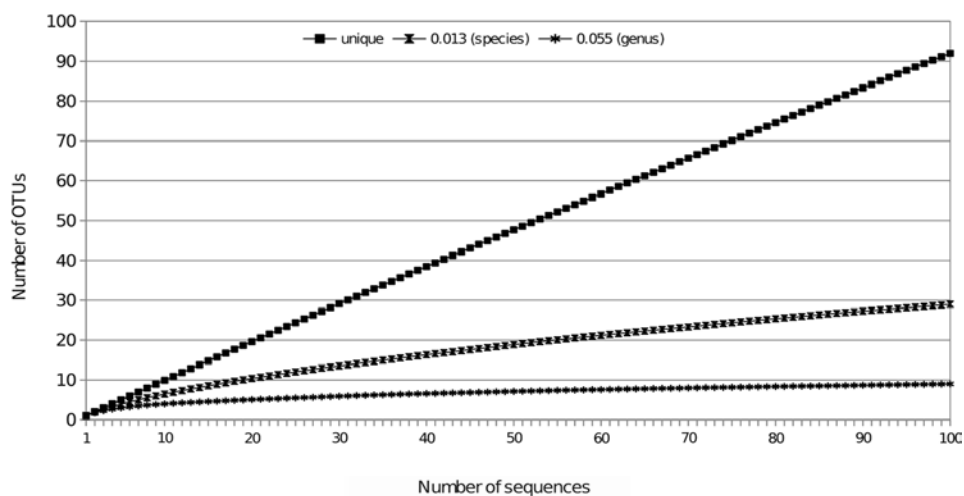
Figure 3.19. Rarefaction curves describing the deendence of discovering novel OTUs as function of the number of full-length 16S rRNA gene sequences. The clustering into OTUs was based on a distance matrix generated from a manually curated alignment (with gaps) compatible with SILVA. Three distance cutoffs have been modeled for identical sequences (0.000), distinct species (0.013) and distinct genera (0.055)

Table 3.8. Phylotypes detected in *O. patagonica* colonies (C), Healthy (H) and Unhealthy (U) and seawater samples (W) collected from Alicante Harbour and the Marine Protected Area of Tabarca. From September 2010 to December 2011.

Phylotipe Number (Nº)	Phylotype name	Location	Sep 10			Dec 10			Feb10			Jun 11			Sep 11			Dec 11		
			W	C H	C U	W	C H	C U	W	C H	C U	W	C H	C U	W	C H	C U	W	C H	C U
1	*Vibrio splendidus – Vibrio gigantis – Vibrio atlanticus – Vibrio pomeroyi*	Harbour	0	0	0	-	4	2	-	9	-	0	5	4	0	0	0	0	3	0
		Tabarca	0	2	0	-	4	0	-	9	-	2	6	3	0	2	0	0	3	0
2	*Vibrio mediterranei*	Harbour	0	0	3	-	0	0	-	0	-	0	0	4	0	0	5	0	0	4
		Tabarca	0	0	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	2
3	*Vibrio fortis*	Habour	0	0	0	-	0	0	-	0	-	0	0	2	0	0	0	0	0	0
4	*Vibrio coralliilyticus*	Habour	0	0	0	-	0	3	-	0	-	1	0	2	0	0	1	0	0	0
		Tabarca	0	0	0	-	0	2	-	0	-	0	0	3	0	0	5	0	0	3
5	*Vibrio hepatarius – Vibrio tubiashii*	Tabarca	0	0	0	-	0	0	-	2	-	0	2	2	0	0	0	0	0	0
6	*Vibrio xuii*	Harbour	0	0	0	-	0	0	-	0	-	0	2	0	0	0	0	0	0	0
		Tabarca	0	0	0	-	0	0	-	0	-	0	0	0	0	0	2	0	0	0
7	*Vibrio maritimus*	Harbour	0	0	0	-	0	0	-	0	-	0	0	3	0	0	0	0	0	0
		Tabarca	0	0	0	-	0	0	-	0	-	0	0	2	0	0	0	0	0	0
8	*Vibrio Sp3*	Tabarca	0	0	0	-	0	0	-	2	-	0	0	0	0	0	0	0	0	0
9	*Vibrio ponticus*	Habour	0	0	0	-	0	0	-	0	-	0	0	0	0	0	3	0	0	0
10	*Vibrio harveyi –Vibrio rotiferianus*	Harbour	1	0	0	-	0	0	-	0	-	0	2	3	0	0	0	1	0	8
		Tabarca	0	0	0	-	0	0	-	3	-	0	0	0	2	3	3	0	0	3
11	*Vibrio natriegens*	Harbour	0	0	0	-	0	0	-	0	-	0	0	0	0	0	3	0	0	0
		Tabarca	0	0	0	-	0	0	-	0	-	0	0	0	0	0	1	0	0	0
12	*Vibrio communis – Vibro owensii*	Harbour	3	0	3	-	0	0	-	0	-	1	0	0	1	0	1	0	0	1
		Tabarca	1	0	0	-	0	0	-	0	-	2	0	2	0	0	0	0	0	0
13	*Vibrio agarivorans*	Tabarca	0	0	0	-	0	0	-	0	-	0	0	1	1	0	6	0	0	0

Table 3.8. (Continued)

Phylotipe Number (Nº)	Phylotype name	Location	Sep 10 W	Sep 10 C-H	Sep 10 C-U	Dec 10 W	Dec 10 C-H	Dec 10 C-U	Feb10 W	Feb10 C-H	Feb10 C-U	Jun 11 W	Jun 11 C-H	Jun 11 C-U	Sep 11 W	Sep 11 C-H	Sep 11 C-U	Dec 11 W	Dec 11 C-H	Dec 11 C-U
14	*Vibrio comitans – Vibrio rarus – Virbio breoganii*	Harbour	0	2	0	-	0	0	-	0	-	0	0	2	0	0	0	0	0	2
		Tabarca	0	0	0	-	6	0	-	5	-	0	0	0	0	0	2	0	0	0
15	*Photobacterium rosenbergii*	Harbour	1	0	0	-	0	0	-	0	-	0	0	0	0	0	0	0	0	0
		Tabarca	0	0	0	-	0	3	-	0	-	0	0	0	0	0	0	0	0	0
16	*Photobacterium lutimaris*	Harbour	0	0	0	-	0	0	-	0	-	2	0	0	0	0	0	0	0	0
17	*Photobacterium swingsii*	Tabarca	0	0	0	-	0	2	-	0	-	0	0	0	0	0	0	0	0	0
18	*Providencia vermicola*	Habour	0	0	0	-	0	0	-	0	-	0	0	0	1	0	0	0	0	0
19	*Agarivorans albus*	Habour	0	0	0	-	0	0	-	4	-	0	0	0	0	0	0	0	0	0
		Tabarca	0	0	0	-	0	0	-	4	-	0	0	0	0	0	0	0	0	0
20	*Shewanella fidelis*	Habour	0	0	0	-	0	0	-	0	-	0	0	0	0	0	0	0	2	1
21	*Shewanella waksmanii*	Habour	0	0	0	-	0	0	-	0	-	0	0	0	0	0	0	0	1	2
22	*Sp2*	Habour	0	0	0	-	0	0	-	0	-	0	0	0	0	0	0	1	0	0

Table 3.9. Summary of phylotypes retrieved from coral samples. (*) In brackets, number of healthy and unhealthy samples. (**) Labeled as in Table 3.8; in brackets, number of subsamples in which each phylotype was retrieved and in bold (2) *V. medierranei* and (4) *V.coralliilyticus*. (***) Number of different phylotypes retrieved.

Sample	Subsample*	Retrieved phylotypes**	Diversity***
Harbour Sep10	Healthy (3)	14 (2)	1
	Unhealthy (6)	**2 (3)**, 12 (3)	2
Harbour Dec 10	Healthy (3)	1 (2)	1
	Unhealthy (6)	1 (4), **4 (3)**	2
Harbour Feb 11	Healthy (9)	1 (9), 19 (4)	2
	Unhealthy (0)	-	NA
Harbour Jun 11	Healthy (5)	1 (5), 6 (2), 10 (2)	3
	Unhealthy (4)	1 (4), **2 (4)**, 3 (2), **4 (2)**, 7 (3), 10 (3), 14 (3)	7
Harbour Sep 11	Healthy (4)	-	NA
	Unhealthy (5)	**2 (5)**, **4 (1)**, 9 (3), 11 (3), 12 (1)	5
Harbour Dec 11	Healthy (3)	1 (3), 20 (2)	2
	Unhealthy (6)	**2 (4)**, 10 (6), 12 (1), 14 (2), 20 (1), 21 (2)	6
Tabarca Sep10	Healthy (5)	1 (2)	1
	Unhealthy (4)	-	0
Tabarca Dec 10	Healthy (7)	1 (4), 14 (6), 15 (3)	3
	Unhealthy (2)	**4 (2)**, 17 (2)	2
Tabarca Feb 11	Healthy (9)	1 (9), 5 (2), 8 (2), 10 (3), 14 (5), 19 (4)	6
	Unhealthy (0)	-	NA
Tabarca Jun 11	Healthy (6)	1 (6), 5 (2)	2
	Unhealthy (3)	1 (3), **4 (3)**, 5 (2), 7 (2), 12 (2), 13 (1)	6
Tabarca Sep 11	Healthy (3)	1 (2), 10 (3)	2
	Unhealthy (6)	**4 (5)**, 6 (2), 10 (3), 11 (1), 13 (6), 14 (2), 22 (1)	7
Tabarca Dec 11	Healthy (6)	1 (3)	1
	Unhealthy (3)	**2 (2)**, **4 (3)**, 10 (3)	3

As shown in Figure 3.20, there was a succession of phylotypes along the organic matter gradient (from the up-left square to down-right) that was accompanied by a decrease in diversity. More *Vibrio* phylotypes were retrieved from Tabarca (16 phylotypes, 5 unique of this site) than from Harbour samples (14 phylotypes).
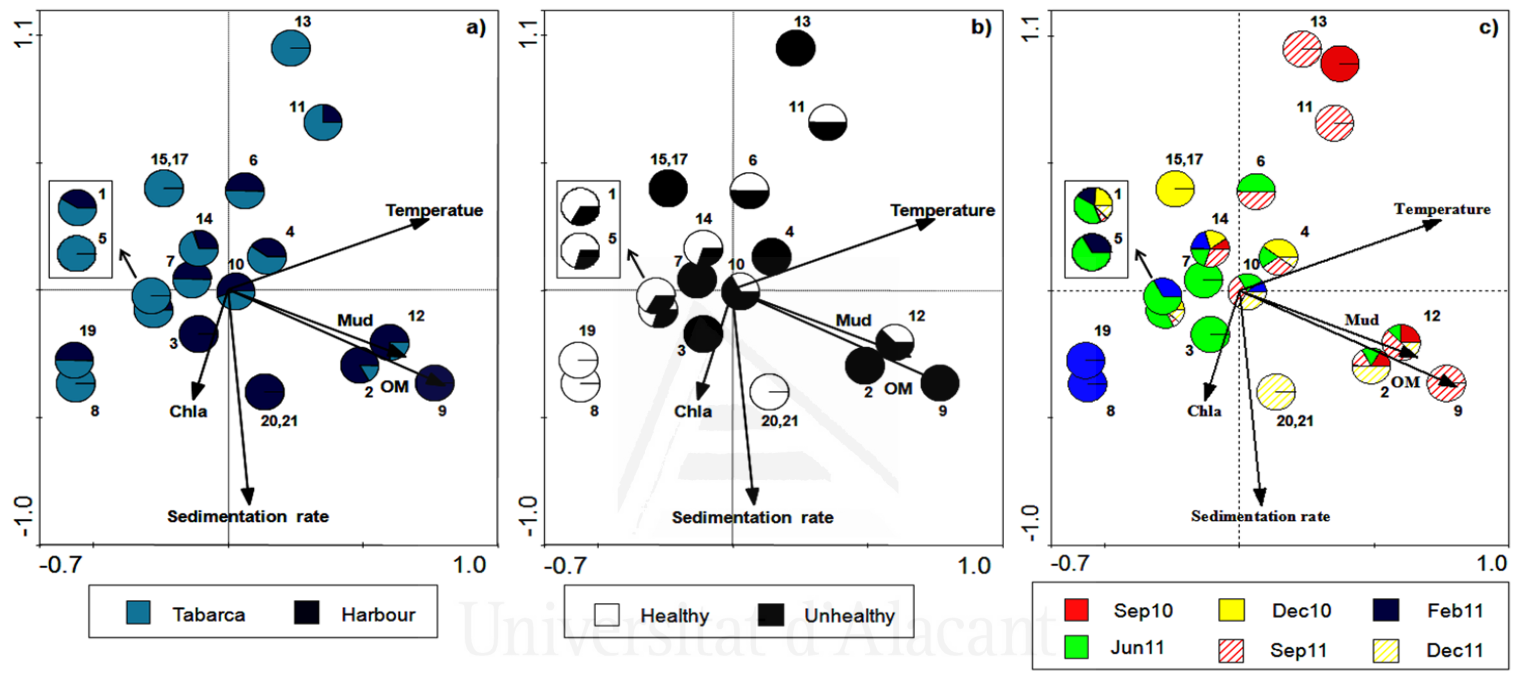
Figure 3.20. CCA biplot of the axis 1 and 2 for the phylotypes and environmental parameters. Every phylotype is represented by a circle that is divided into different sections corresponding to the proportions in sampling locations **(a)**, coral health status **(b)** and sampling time **(c)**.

Three phylotypes, *V. splendidus*-like (n° 1 in the figure), *V. harveyi*-like (11) and *V. comitans* (15), 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 17 and 19 in MPA, and 21 and 22 in the Harbour) displayed the same dependence of physicochemical parameters (Fig. 3.20b), 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 3.20a, *V. mediterranei* was more frequently retrieved from Harbor corals and V. *coralliilyticus*, from Tabarca's.

The *Vibrio* community was different in healthy and unhealthy corals as was also indicated by UniFrac-based principal coordinates analysis (PCoA) (Fig. 3.21) , where a small distance among samples implies that their communities are similar and consist of lineages sharing a common evolutionary history. *Vibrio* communities in healthy corals were similar between the two locations, both dominated by *V. splendidus*-like (55.56% of the isolates from healthy corals in Harbour and 42.86% in Tabarca). Bleaching events seemed to disrupt this equilibrium leading to a decrease in *V. splendidus*-like (13% in Harbour and 12% in Tabarca) and an increase of potential pathogens. Thus, *V. mediterranei* was isolated from 59.25% of unhealthy corals in the Harbour, while *V. coralliilyticus* was detected in 81.25% in Tabarca. The different distribution of these pathogens, and their different locations in the CCA space (Fig. 3.20), suggest that they may have different growth requirements in natural conditions.

Figure 3.21. Communities clustered using PCoA of the unweighted UniFrac distance matrix. (TH) healthy colonies from Tabarca; (HH) healthy colonies from the Harbour; (TU) unhealthy colonies from Tabarca; and (HU) unhealthy colonies from the Harbour.

3.4.1.3. Detection of *Vibrio* pathogens by Illumina high-throughput 16S rRNA gene sequencing

The sequences libraries obtained from tissue samples using the Illumina platform in chapter 3.3 (Table 3.7, p. 110) were used to search sequences related the possible *Vibrio* pathogens, *V. mediterranei* and *V.coralliilyticus*, retrieved in this study from unhealthy samples. Surprisingly, sequences related to (with a 98.7% of identity) phylotypes *V. mediterranei* and *V. coralliilyticus* were detected not only in diseased samples, but also in healthy samples, including samples collected in cold months, in which neither of the two pathogens were retrieved by cultivation. 16S rRNA genes sequences with 99.78% identity with isolates retrieved from the unhealthy samples collected during the bleaching event in September 2011were detected by massive sequencing approach in healthy corals (Fig. 3.22).
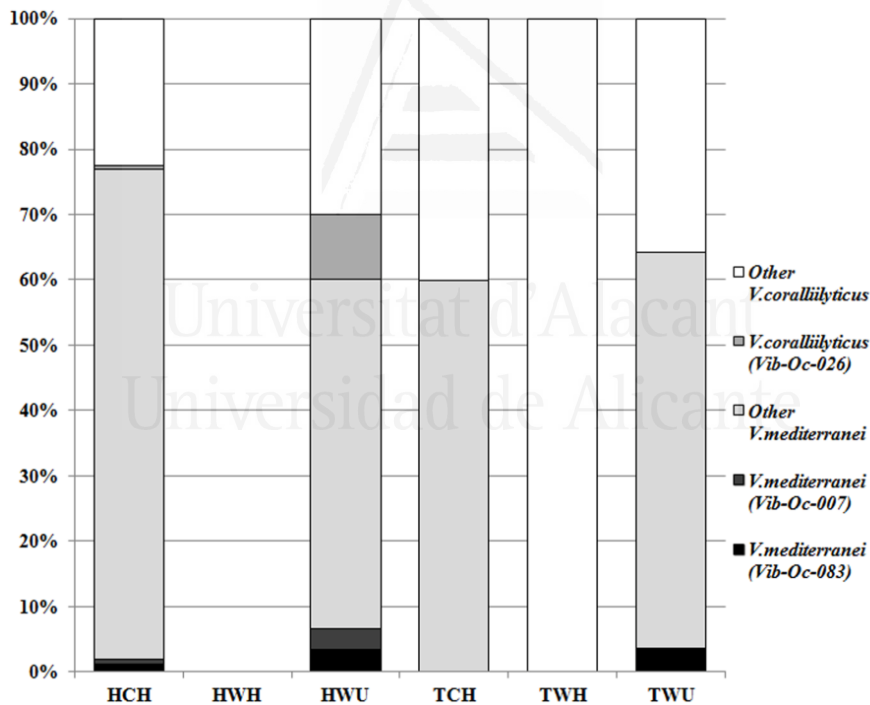
Figure 3.22. Percentages of Illumina reads that belong to phylotypes *Vibrio mediterranei* and *Vibrio coralliilyticus* and to strains isolated in this study from diseased samples of *O. patagonica.* (Vib-Oc-026, Vib-Oc-007, Vib-Oc-083). See Table 3.7, p 110, for sample identifiers.

3.4.2. Spatial variation and differences among coral host

3.4.2.1. *Vibrio* community analysis

Eight samples of *O. patagonica* and *C. caespitosa* and six of seawater were taken from Pietra Ligure (Italy) and MPA of Tabarca (Spain) (see Table 2.1, p. 67) in order to characterise their associated communities of culturable *Vibrio* spp., to ascertain how they change with geographic location and coral host. A total of 222 isolates were retrieved, from seawater and corals, and classified into 19 distinct phylotypes (defined at ≥98.7% sequence identity) (Table 3.10), which were named according to the known taxa found within the different clades observed in the phyogenetic tree (see Annexes, Fig. A5). Although bootstrap values were low for some branches, both neighbor-joining and maximum likelihood analyses rendered similar tree topologies. *Vibrio* assemblage was more diverse (according to H' values) in corals than in water samples (t-test, $p<0.001$), with H' values increasing at the end of summer (September) (t-test, $p<0.05$) regardless of the samples (Table 3.11). Differences among cultivable *Vibrio* counts in seawater and corals were also detected (t-test, $p<0.05$). For each location, *Vibrio* spp. counts per ml were always higher in corals than in seawater and always higher in September than in June. Among the samples taken in September, diseased corals yielded the highest number of cultivable counts (Table 3.11).

Differences in *Vibrio* community composition were assesssed using the phylogeny-based metric UniFrac. PCoA revealed strong primary clustering by habitat (water/coral) and locality, rather than by coral species or time; differences due to coral health status were also detected (Fig. 3.23). Additionally, NMDS analysis (Fig. 3.24) showed grouping of the samples first by habitat (water/coral) and then by coral health status, without differences among coral species. The analysis of similarities (ANOSIM) indicated that the *Vibrio* composition was significantly different between localities ($R=0.215$, $p=0.02$), habitat (water and corals; $R=0.439$, $p=0.001$) and coral health status ($R=0.344$, $p=0.03$), whereas no differences were detected between time (June and September; $R=0.088$, $p=0.193$) and coral species ($R=0.079$, $p=0.223$). However, a 2-way crossed ANOSIM test (Locality x coral host) indicated differences in *Vibrio* community between coral

species at a given locality, being these differences more noticeable in Spain (R=0.524, p=0.02 and R=0.456, p=0.04 for Spain and Italy respectively).

Table 3.10. *Vibrio* counts and Shannon diversity indices for the seawater and corals samples used in this study. (CFU) colony-forming units. (Loc) Sampling location; (Tª): Temperature (Cº).

Habitat	Loc.	Time	Health status	Tª	*Vibrio*s (CFU)	*Vibrio* Diversity (H´)
Water	Italy	June		24.2	$8.07 \times 10 \pm 5.81 \times 10$	1.60
		September		26.8	$8.43 \times 10^2 \pm 3.48 \times 10$	1.33
	Spain	June		23.9	$1.33 \times 10^2 \pm 5.77 \times 10^1$	1.38
		September		27.3	$1.39 \times 10^3 \pm 2.65 \times 10^2$	1.04
Oculina patagonica	Italy	June	Healthy	24.2	$6.32 \times 10^4 \pm 3.73 \times 10^3$	1.73
		September	Healthy	26.8	$8.87 \times 10^4 \pm 4.08 \times 10^3$	1.85
			Unhealthy		$1.14 \times 10^5 \pm 6.35 \times 10^4$	2.13
	Spain	June	Healthy	24.2	$5.32 \times 10^4 \pm 4.64 \times 10^3$	1.61
		September	Healthy	27.3	$1.30 \times 10^5 \pm 2.08 \times 10^3$	1.75
			Unhealthy		$5.08 \times 10^5 \pm 5.74 \times 10^4$	1.98
Cladocora caespitosa	Italy	June	Healthy	24.2	$1.38 \times 10^4 \pm 2.14 \times 10^3$	1.74
		September	Healthy	26.8	$1.14 \times 10^5 \pm 1.70 \times 10^4$	1.60
			Unhealthy		$2.36 \times 10^5 \pm 8.73 \times 10^4$	2.33
	Spain	June	Healthy	24.2	$3.76 \times 10^4 \pm 3.61 \times 10^3$	1.96
		September	Healthy	27.3	$5.32 \times 10^4 \pm 2.80 \times 10^3$	1.35
			Unhealthy		$2.08 \times 10^5 \pm 3.92 \times 10^4$	2.17

Table 3.11. Phylotypes detected in corals *Oculina patagonica* and *Cladocora caespitosa* (H: Healhty; U: Unhealthy) and seawater samples collected in two locations (Loc.): Italy and Spain, in June (J) and September (S).

Phylotype number	Phylotype name	Loc.	Water		Oculina patagonica			Cladocora caespitosa			TOTAL
			J	S	J	S-H	S-U	J	S-H	S-U	
1	*Photobacterium rosenbergii*	Italy	-	-	1	2	-	1	-	-	4
		Spain	-	-	-	1	-	1	-	-	2
2	*Vibrio splendidus* super clade	Spain	1	1	3	1	1	3	2	1	13
3	*Vibrio comitans- Virbio rarus- Vibro breoganii*	Italy	-	-	2	-	-	5	2	-	9
		Spain	-	1	1	2	-	7	8	1	20
4	*Vibrio ponticus*	Italy	-	-	-	-	-	1	-	1	2
5	*Vibrio mediterranei*	Italy	-	-	-	-	1	-	1	3	5
		Spain	-	-	-	-	2	-	-	1	3
6	*Vibrio tubiasii- Vibrio hepatarius*	Italy	-	-	-	-	2	-	-	2	4
		Spain	-	-	-	-	-	-	-	1	1
7	*Vibrio xuii*	Italy	-	-	-	1	-	2	1	-	4
8	*Vibrio maritimus*	Italy	-	-	-	1	1	-	-	-	2
		Spain	-	-	-	-	1	-	-	-	1
9	*Vibrio Sp1*	Italy	-	-	1	1	-	1	1	1	5
		Spain	-	-	1	-	2	-	-	1	4
10	*Vibrio coralliilyticus*	Italy	-	1	-	-	2	1	-	1	6
		Spain	-	-	-	-	4	3	-	2	9
11	*Vibrio Sp2*	Italy	-	-	-	1	1	-	1	1	4
		Spain	1	-	-	-	1	1	1	1	7
12	*Vibrio Sp3*	Spain	-	-	-	-	-	4	1	-	6
13	*Vibrio diazotrophicus- Vibrio hispanicus*	Italy	1	-	-	-	1	-	1	-	3
		Spain	-	-	-	-	-	-	-	1	1
14	*Vibrio agarivorans*	Italy	-	-	2	1	1	1	-	1	6
		Spain	-	-	1	-	-	-	1	-	2
15	*Vibrio Sp4*	Italy	-	-	-	1	-	-	-	-	1
		Spain	-	-	-	3	1	-	-	1	5
16	*Vibrio Sp5*	Italy	1	1	-	1	1	-	-	2	6
		Spain	1	-	1	1	-	-	-	-	3
17	*Virbio fortis*	Italy	1	1	2	3	1	1	1	-	10
		Spain	-	-	-	1	1	-	1	1	4
18	*Vibrio natriegens*	Italy	1	-	1	-	1	1	-	-	4
		Spain	-	1	4	1	-	3	-	-	8
19	*Vibrio harveyi-like* (*Vibrio* core group)	Italy	1	2	8	5	5	4	4	7	36
		Spain	1	2	4	7	5	1	-	4	24
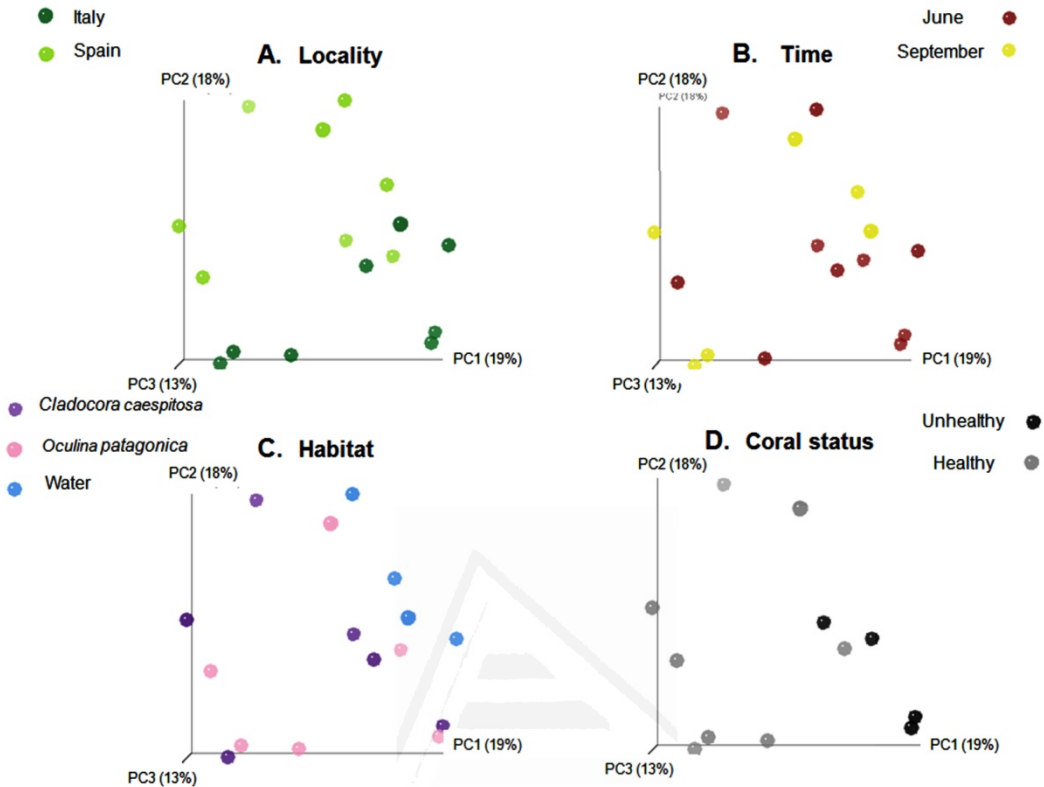
Figure 3.23. *Vibrio* communities clustered using principal coordinate analysis of the unweighted UniFrac distance matrix. Each point corresponds to a coral sample colored by (a) Locality, (b) Time (c) Habitat, or (d) Coral health status. The same plot is shown in each panel. The percentage of variation explained by the plotted principal coordinates is indicated on the axes.

3.4.2.2. Differences among water and corals

Coral *Vibrio* communities differed significantly from those in the surrounding water column (Fig. 3.23 and 3.24). *V. harveyi-like* (present in 60% of analysed water samples) and *Vibrio* Sp5 (30.4%) were the phylotypes most frequently retrieved from seawater, appearing both in Spain and Italy. The phylotypes most frequently retrieved from *O. patagonica* samples were *V. harveyi*-like (100% of samples) and *V. splendidus* super clade (56.25%). For *C. caespitosa, V. harveyi-like* was isolated only in 63% of the analysed samples, while *V. comitans-V. rarus-V. breoganii* super clade was detected in all samples.
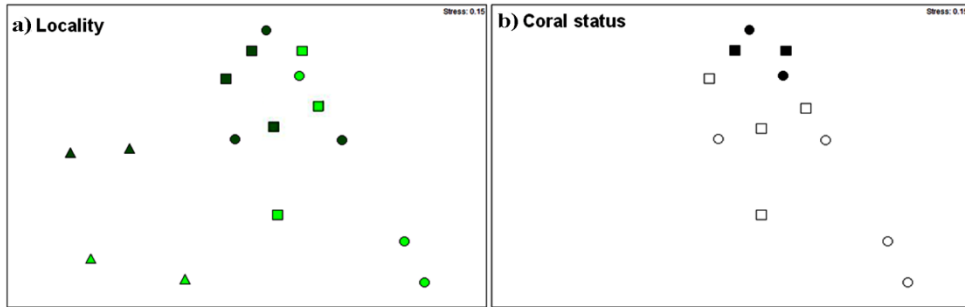
Figure 3.24. Non-metric Multimensional Scaling plots of the first two dimensions based on Bray-Curtis dissimilarities for water (triangles), *O. patagonica* (circles) and *C. caespitosa* (quadrats). (a) Locality: samples from Italy (dark green) and Spain (light green); and (b) Coral health status: healthy samples (white) and unhealthy ones (black).

3.4.2.3. Site-specificity of coral-*Vibrio* associations

SIMPER analysis was used to ascertain the differences between the *Vibrio* communities harbored by the two coral hosts in each locality. Results indicated that the *Vibrio* assemblages isolated from *C. caespitosa* and *O.patagonica* samples were more dissimilar for Spanish than for Italian corals (Table 3.14a). In Spanish samples, these differences were due to phylotypes 3 (*V. comitans- V. rarus- V. breoganii*) and 12 (*Vibrio sp.*3) most frequently retrieved from *C. caespitosa*, and phylotype 19 (*V. harveyi-like*) that was mainly present in *O. patagonica*. In Italy, besides 19 and 3, there were other phylotypes that contributed to the differences between coral hosts, such as *Vibrio xuii*, mainly present in *C. caespitosa*, and *Photobacterium rosenbergii*, *Vibrio agarivorans* and *Vibrio fortis*, mostly detected in *O. patagonica*. The endemic coral *C. caespitosa* showed slightly higher H' values (Table 3.10), with two phylotypes that could be considered species-specific: *Vibrio ponticus* (12.5% of samples) in Italy and *Vibrio sp.3* (31.25%) in Spain; accordingly, all phylotypes detected in *O. patagonica* were also present in *C. caespitosa*.

3.4.2.4. Differences among healthy and unhealthy corals

The composition of *Vibrio* communities present in unhealthy and healthy colonies weas significantly different; in both coral species. No differences between coral hosts were detected, but some differences between localities were found. The SIMPER analysis that highlighted the phylotypes primarily responsible for the observed differences within each locality is shown in Table 3.12 (b).

According to this analysis (Table 3.12b), in Spain, healthy samples were dominated by phylotypes 19 (100% of samples), 2 and 3 in *O. patagonica*, while in *C. caespitosa,* phylotypes 3 (100% of samples) and 2 were predominant. In diseased corals, *Vibrio*s closely related to *V. mediterranei* and *V. coralliilyticus* were retrieved from both corals, while *Vibrio tubiasii-Vibrio hepatarius* was only detected in *C. caespitosa*. Healthy samples collected from Italy were also dominated by phylotype 19 in *O. patagonica* and by phylotypes 3 in *C. caespitosa*. Whilst the phylotypes detected in diseased samples were the same as in Spain, the detection frequency was different, being *V. tubiashii-V.hetararius* retrieved from 100% and *V. coralliilyticus* from 50% of diseased samples in both species, the proportion of *V. mediterranei* was higher in *O. patagonica* than in *C. caespitosa*. Unexpectedly, the pathogen *V. coralliilyticus* was also detected in two healthy samples of *C. caespitosa* collected from Italy in June.

Table 3.12. Percentage of contribution of each phylotype to *Vibrio* community structure (based on SIMPER analysis), indicating the average contribution to the similarity (S) within each grouping factor and the dissimilarity (D) among them: (**a**) between corals host (O) *Oculina patagonica* and (C) *Cladocora caespitosa* and (**b**) between (H) healthy and (U) unhealthy corals, in each locality. Phylotypes with contribution higher than 50% are in bold

a) Coral hosts						
	Italy			Spain		
Phylotype name	**O** S=42.12	**C** S=38.03	**O/C** D=63.81	**O** S=51.18	**C** S=53.66	**O/C** D=45.80
V. harveyi-like	**57.57**	**53.66**	12.89	14.30	-	16.51
V.comitans	2.57	8.98	13.67	5.11	41.68	20.59
V. splendidus	-	-	-	14.30	18.62	1.22
Vibrio Sp3	-	-	-	-	14.00	8.33
V. natriegens	-	-	-	5.11	-	8.17
V. coralliilyticus	-	-	-	2.39	8.15	8.10
V. fortis	15.46	4.49	9.32	-	-	-
P. rosenbergii	3.95	-	6.42	-	-	-
V. xuii	-	4.49	5.76	-	-	-
V. agarivorans	11.51	-	4.92	-	-	-

b) Health status						
	Italy			Spain		
Phylotype name	**H** S=43.09	**U** S=54.71	**H/U** D=62.80	**H** S=27.84	**U** S=50.39	**H/U** D=76.10
V. harveyi-like	**75.69**	46.62	10.14	14.09	43.68	14.29
V. comitans	7.74	-	6.03	**63.40**	-	19.57
V. coralliilyticus	-	4.35	8.12	-	21.84	11.07
V. mediterranei	-	9.82	7.77	-	9.14	7.58
V. tubiashii	-	21.78	11.17	-	-	-
V. xuii	5.53	-	5.30	-	-	-
V. splendidus	-	-	-	3.01	-	5.89

3.5. ANALYSIS OF TWO POTENTIAL *VIBRIO* PATHOGENS BY EXPERIMENTAL INFECTIONS

3.5.1. Tissue damage, Chlorophyll *a* concentration and *Vibrio* counts

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. splendidus*, and a mixture of the three of them. The final concentration of *Vibrio*s in water was similar to that found in the environmental samples when seawater temperatures were between 15 and $20^{\circ}C$ (around 10^2 UFC/ml). Tissue damage on *O. patagonica* increased with temperature in all infection treatments (Figure 3.25), including controls, while concentrations of photosynthetic pigments decreased significantly (ANOVA, $p<0.01$; Table 3.13 and Figure 3.26a). In addition, significant differences were detected in the number of culturable *Vibrio*s from corals, with samples maintained at 28 °C showing higher abundances (ANOVA, $p<0.01$; Table 3.13 and Figure 3.26b).

At 20 °C, corals co-inoculated 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 assesssment (Figure 3.25a), together with reduced Chl *a* concentrations compared to the controls treatments (ANOVA, $p<0.01$; Table 3.13 and Figure 3.26a). Corals inoculated with either pathogen did not show significant differences in tissue damage at the end of the experiment compared to the controls Table 3.13 and Figure 3.26a).

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 $80 \pm 7.6\%$ and $36.6 \pm 5.8\%$ of tissue damaged respectively. Accordingly, significant differences in Chl *a* concentration were also detected

between the two distinct infections (ANOVA, p<0.01; Table 3.13 and Figure 3.26a). At this temperature *V. mediteranei* seemed thus to be more virulent than *V. coralliilyticus* (Figure 3.25).

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. splendidus*, respectively. On the other hand, while no significant differences were detected between single infections with either pathogen at this temperature, their mixture was more deleterious for the corals (Figure3.25).
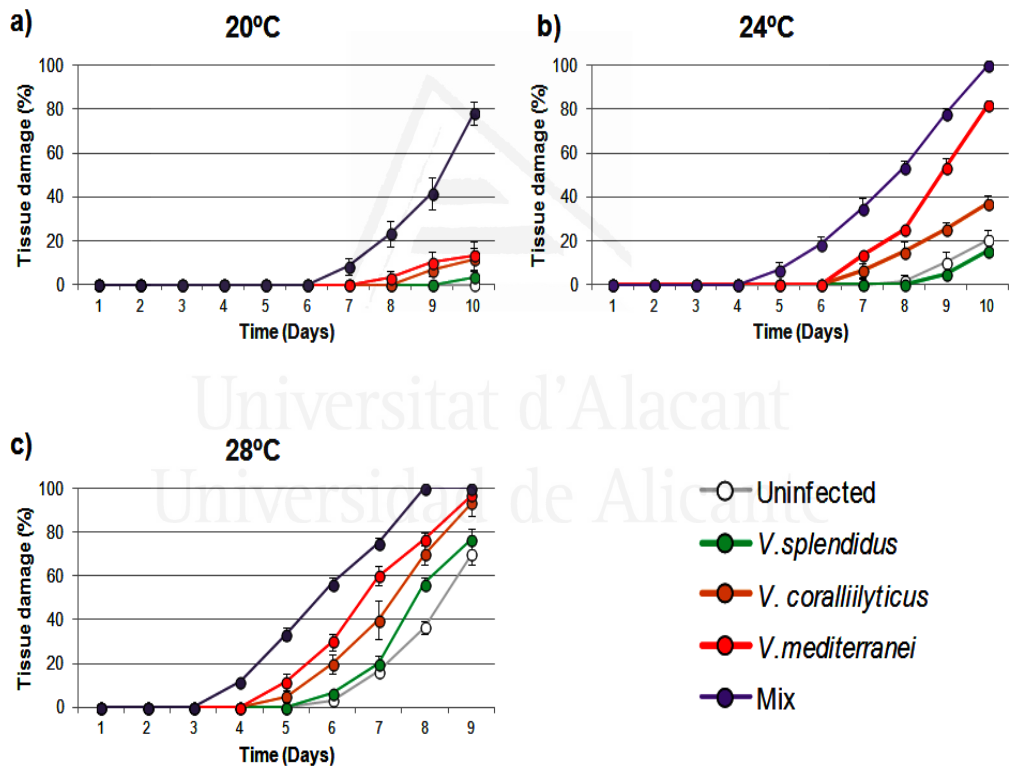
Figure 3.25. Development of tissue damage of *O. patagonica* colonies (average ± SD from three replicate colonies) following experimental infections in aquaria with the indicated inocula.

Table 3.13. Results of the two-factor ANOVA for: (CFUs) Colony-Forming Units and Chlorophyll a concentration. (Tª) temperature; (IT) Infection treatment; (Trans) Transformaton; (MS) mean square; (p) level of significance; (df) degrees of freedom. Infection treatments: (1) uninfected aquaria; (2) *Vibrio splendidus* (3) *V. coralliilyticus*; (4) *V. mediterranei*; (5) mixture.

Source	df	CFUs gr⁻¹		Chla a gr⁻¹	
		MS	p	MS	p
Tª	2	$3{,}476 \times 10^{14}$	0,001	1,370	0,001
IT	4	$2{,}328 \times 10^{14}$	0,001	0,386	0,001
Tª x IT	8	$2{,}011 \times 10^{14}$	0,001	0,057	0,001
Residual	3	$3{,}182 \times 10^{12}$		0,009	
Trans.		None		$\sqrt{x+1}$	
SNK		Tª: 28>24=20 IT: 5>4=3=2=1 TªxIT: 20: 5>1=2=3=4 24: 5>4>1=2=3 28: 5>4>3>1=2		Tª: 20>24>28 IT: 1=3>2>4>5 TªxIT: 20: 1=2=3=4>5 24: 1=2=3>4>5 28: 1=2>3=4>5	
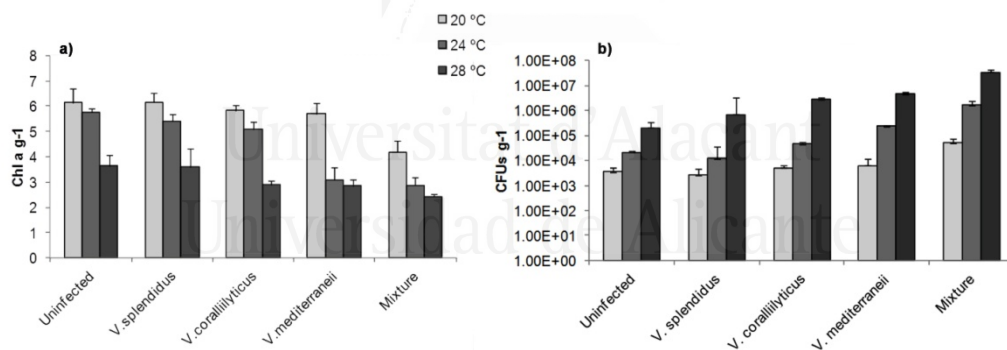
Figure 3.26. **(a)** Chlorophyll *a* and **(b)** concentration of culturable *Vibrios* (right) in *O. patagonica* samples after ten days of experimental infection with the indicated inocula at different temperatures (CFU: Colony-Forming Units).

3.5.2. Transmision electron microscopy

Electron microscopy observations were carried out from samples at the end of the experiment, confirming the presence of different bacteria morphologies. The untreated control maintained at 20ºC showed healthy zooxanthellae with possible active bacteria, with their content not condensed (Fig. 3.27a); while at 28ºC coral samples showed unhealthy zooxanthella with possible bacteria inside them (Fig. 3.26b) and many *Cyanobacteria* was alsoobserved in the tissue layer in touch with the skeletal matrix (Fig. 3.27c). The infected corals at 28ºC showed unhealthy zooxanthellae surrounded by diverse bacterial morphotypes (Fig. 3.27 d, e), distinguishing bacteria with bacillus morphology (Fig. 3.27f). Surprisingly, in some coral samples maintained at 28ºC we also observed what may be viral-like particles grouped inside vacuoles (Fig. 3.27e).
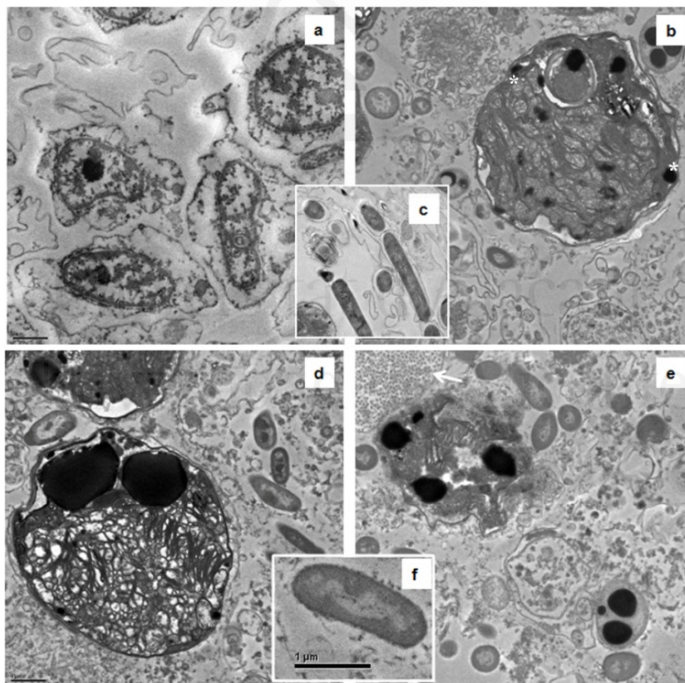
Figure 3.27. Representative transmission electron microscopy images of *O. patagonica* at the end of experiment (bar=1μm). (**a**) Bacteria in tissue of control coral at 20ºC.(**b**) Possible bacteria inside of unhealthy zooxanthela. (**c**) Detail image of Cyanobacteria. (**d**) Infected coral with unhealth zooxanthellae surrounded by different bacteria. (**e**) Coral at 28ºC with virus-like particles (arrow). (**f**) Detail of a bacillus in the gastrodermis of a infected coral.

3.5.3. Analysis of bacterial Community by Illumina high-throughput 16S rRNA gene sequencing

One sample of each infection treatment maintained at the three experimental temperatures, with the exception of infection with *V. splendidus,* was randomly chosen to be sequenced using Illumina platform in order to assesss changes in bacterial tissue communities and to detect *Vibrio* pathogens. A total of 1877643 quality filtering reads were recovered from twelve samples, the number of OTUs at a 3% dissimilarity level and Shannon diversity index are listed in Table 3.14, showing the highest value of diversity in the control samples maintained at 20ºC and the lowest in the samples infected with mixture of three *Vibrio*s at 28ºC.

Table 3.14. Counts of paired-end rRNA gene sequences obtained from Illumina (preassembly) and following assembly and screened (postassembly) for the libraries included in this study. (Tª) Temperature (ºC); (IT) Infection treatment; (Vm) *Vibrio mediterranei*; (Vc) *Vibrio corallilyticus*.

Tª	IT	Number of sequences		Remaining sequences (%)	Number of OTUs	Shannon index (H´)
		Preassembly	Postassembly and filtered			
	Uninfected	121872	3531	2.9	1093	5.71
20	Vm	96332	3189	3.31	809	4.21
	Vc	1271496	112280	8.83	1041	5.46
	MIX	337858	16429	4.86	4796	4.1
	Uninfected	2257916	355354	15.74	5514	4.71
24	Vm	1028308	92520	9	4389	4.48
	Vc	1545078	294205	19.04	5000	4.5
	MIX	2744288	332653	12.12	5501	4.51
	Uninfected	371834	2629	0.71	283	4.08
28	Vm	2300498	367346	15.97	1379	4.61
	Vc	2443642	281915	11.54	3055	4.68
	MIX	111724	15592	13.96	4643	3.33

Just as in tissue samples analysed in chapter 3.3. the phylum *Proteobacteria* was the most dominant and constituted 56 to 92% of the qualified bacterial Illumina reads in coral tissues (Fig. 3.28a). Within *Proteobacteria* the class *Alphaproteobacteria*, with the most of sequences belonging to the order *Rhodobacterales,* was the dominant in samples at 20ºC (20-29%), with the exception of the sample inoculated with the mixed culture in which the percentage of *Alphaproteobacteria* (8.6%) is more similar to samples maintained at 24 or 28ºC (Fig. 3.28b). In contrast, the class *Gammaproteobacteria,* with the order *Vibrionales* as the most abundant within this class, increased their percentages at higher temperatures, reaching more than 70% of the sequences retrieved in samples maintained at 28ºC. Remarkably, the coral sample inoculated with the mixed culture at 20ºC showed a high percentage of *Gammaproteobacteria* (47%) more similar to samples maintained at higher temperatures (53-74%) than the other samples maintained at 20ºC (3-10%).

Focus the attention on *Vibrio* genus and specifically on *Vibrio* strains used to infect the corals, we found that *V. splendidus* was the dominant in samples maintained at 20ºC, while the presence of our pathogens strains (*V. mediterranei* and *V. coralliilyticus*), together with other sequences belonging to these phylotypes (with a 98.7% of identity), were mainly detected in samples at 28ºC. In uninfected coral maintained at 20ºC no sequences related to pathogens were detected, and at 28ºC not one *Vibrio* sequences was retrieved; by contrast in the sample maintained at 24ºC sequences related with *V. mediterranei* and *V. coralliilyticus* were retrieved, including sequences with a 99.7% of identity with the *V. coralliilyticus* strain used in the experiment. Noticeably, the two samples without sequences related with our strains showed the lowest percentage of high quality sequences (Table 3.16). On the other hand, in all the samples inoculated with the mixed culture both pathogens were detected but *V. mediterranei* showed more number of sequences than *V. coralliilyticus* (Fig 3.28c).
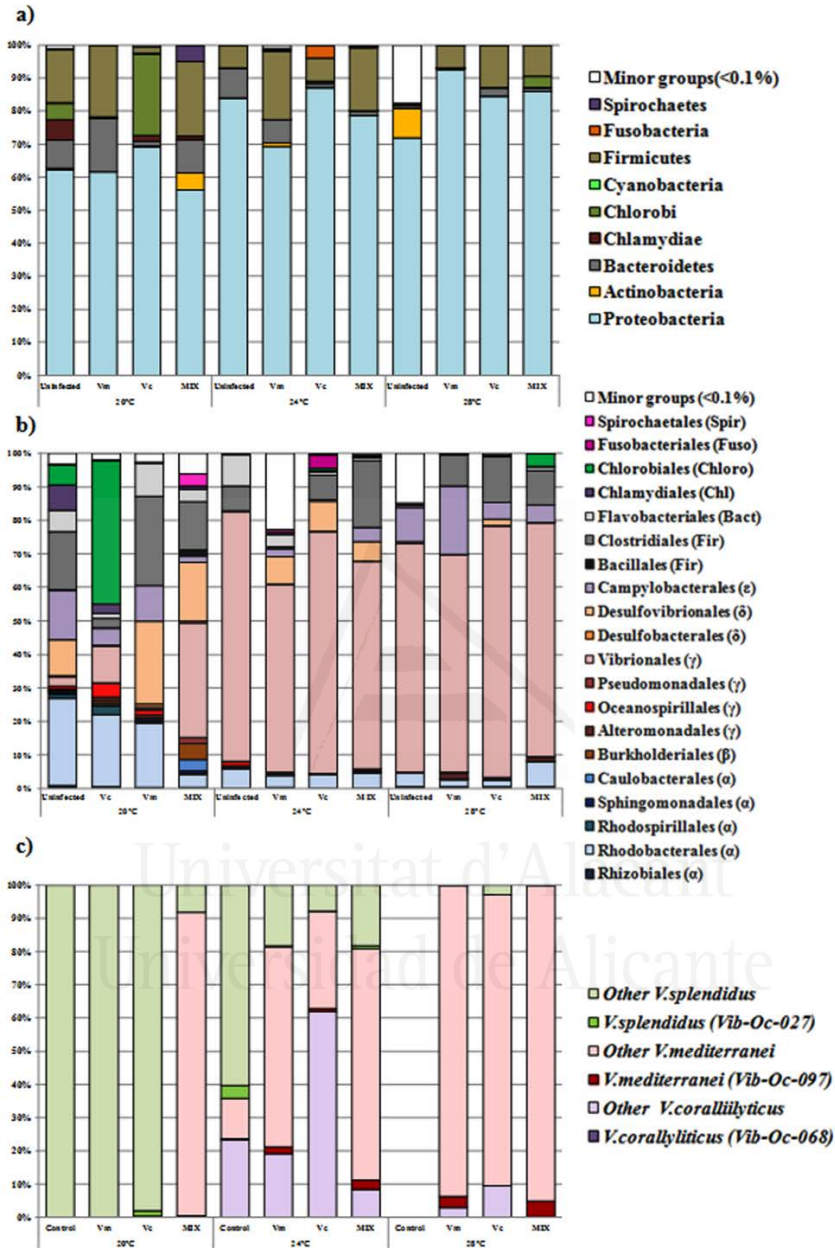
Figure 3.28. Taxonomic classification of bacterial reads retrieved from Illumina high-throughput 16S rRNA gene sequencing from infection experiment, into (**a**) phylum and (**b**) order levels using the RDP classifier. Percentages of reads belong to strains of *V. mediterranei*, *V. coralliilyticus* and *V. splendidus* used in the experiment (c). See Table 3.14 (p.134) for sample identifiers.

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Photo/Figure: *Oculina patagonica* corallites (from AINS) and electron microscopy tissue image (by Virgina Souza-Egipsy)

Part I. Biological and ecological characteristics of *Oculina patagonica*

4.1. DISTRIBUTION

Oculina patagonica was first observed in the Mediterranean Sea only in areas highly affected by humans (Zibrowius and Ramos, 1983), but later studies have discovered large populations in natural habitats (Ramos-Esplá, 1985; García-Raso *et al.*, 1992; Fine *et al.*, 2001; Ballesteros *et al.*, 2008; Coma *et al.*, 2011). Our studies (chapter 3.1) of the distribution patterns of *O. patagonica* in the Valencian Region indicate that this species is widely distributed along the infralittoral rocky bottoms. Although the coral was found between 0 and 12 m depths, the highest abundances were identified at the breaker zone and therefore it can be considered as a shallow water species. This coral was found under very different environmental conditions, such as a wide range of water salinities (areas exposed to brine discharge and areas with freshwater inputs), ultraviolet radiation (from shallow pools on abrasion platforms until 15 m depth), turbidity (areas with sand depositions), hydrodynamism (cliffs of the northern area of Alicante) and anthropogenic impact (areas exposed to domestic and industrial pollution and pristine areas such MPA of Tabarca).

Results obtained in chapter 3.1 suggest that the distance to the harbours seems to determine of *O. patagonica* distribution, since this coral has never been found at distances larger than 10 km from the nearest harbour. Therefore, harbours could be considered a focal point of dispersion of this species, confirming that *O. patagonica* spreads via the intense intra-Mediterranean maritime traffic (Fine *et al.*, 2001). Recent studies (Salomidi *et al.*, 2013; Serrano *et al.*, 2013) have also suggested that artificial coastal structures provide new substrata that promote the spread of this species.

Anthropogenic disturbances such pollution, eutrophication, physical destruction of habitats and increasing number of novel man-made structures, have the potential to change physical and biological environments, thus affecting native

species distribution and consequently favoring the introduction and spreading of invasive species (Bax *et al.*, 2003). According to Moyle and Light (1996), in aquatic systems with high levels of human disturbance, the range of invasive species that can develop is much higher than in systems with low levels of human disturbance. In the Mediterranean Sea, similar observations have been reported by Galil (2000). A recent study on the distribution of *O. patagonica* at the Saronikos Gulf (south Aegean Sea) suggested that the continuing degradation of the coastal zone, due to coastal contamination (Salomidi *et al.*, 2013) and the high tolerance of this species to pollution (Armoza-Zvuloni *et al.*, 2012), may contribute to its ability to successfully invade new areas in the Mediterranean Sea. However, this species is successfully established in the pristine Marine Protected Area of Tabarca, that can be considered as *O. patagonica* hot spot (see Fig. 3.1, p. 86), probably due to the intensity of recreational boating or because trophic interactions could be contributing to the success of this species. For example, sea urchins grazing activity can indirectly facilitate the expansion of the coral by creating open spaces that enhanced coral settlement and survival (Serrano *et al.*, 2013).

In shallow natural habitats of temperate ecosystems, the pattern of dominance of macroalgae is especially evident in the rocky infralittoral zone, and the presence of these benthic algae plays an important role in the scleractinian corals recruitment (McCook *et al.*, 2001, Birrell *et al.*, 2008). Previous studies have shown that different types of algae have very different effects on corals recruitment and that the variability in the interactions and effects can be largely explained in terms of the functional-form groups of algae (McCook *et al.*, 2001). Certainly, the spatial distribution of *O. patagonica*, in the Valencian Region is mainly driven by the presence of benthic algae, since the highest occurrences of this species were concentrated in the north of the San Antonio Cape (Fig. 3.1a; p. 86), in an area exposed to sand deposits and with moderate influence of freshwater input (Díez, 1996; Serra, 1986), where the rocky shallow water habitats were dominated by filamentous algal turf, which are more resistant to high sedimentation than erect and encrusting forms (Gorgula and Connell, 2004). Interestingly, Diaz-Pulido *et al.* (2010) showed that algal turfs and crustose coralline algae enhanced the settlement of coral larvae while all upright fleshy and calcareous macroalgae generally inhibited

it, likely due to both physical (shading) and chemical effects (chemical cues). Although the height and density of the turf are likely to have different effects on settling coral larvae, filamentous algal turfs seem to be relatively innocuous for coral settlement in comparison to thicker, corticated algal turfs (Birrell *et al.*, 2005).

Understanding the impacts of invasive species in natural ecosystems is an important component for the development of management strategies, because the alien species are considered as a biological pressure. However, it is nearly impossible to take measures to remove or reduce the impact on the natural system once an invasive species has established itself. This study shows that *O. patagonica* is really established in our study area, bearing a wide range of environmental conditions. Therefore, the knowledge acquired in this thesis about its baseline biota can not only determine its 'invasiveness' but also enhance the prediction of its spreading, which is determined by anthropogenic factors (e.g. man-made structures, pollution or physical destruction of habitats), while its proliferation depends on algae community that has influence over its recruitment.

4.2. GROWTH AND BLEACHING

The impact of temperature variations on coral growth rates and the correlation between coral calcification and extension rates with seawater temperatures have been widely reported before (see Lough and Cooper 2011). The results presented in this PhD thesis confirm that growth of *O. patagonica* is temperature dependent (Rodolfo-Metalpa *et al.*, 2008b). *Oculina patagonica* growth rates are smaller in cold (February, 13 °C) than in warm months (June to September, 18-28 °C), although it seems that there is temperature threshold (28 °C) over which growth rate starts again to decrease. Previous laboratory observations showed a significant decrease in *O. patagonica* growth rate when temperature was maintained over 24°C for prolonged periods, suggesting that this temperature is probably a breaking point in the coral growth (Rodolfo-Metalpa *et al.*, 2006). However, this coral is also spread along the Israeli coasts, where in summer, it has exposed to temperatures from 24 to 30 °C (Shenkar *et al.*, 2005), which are higher than temperature suggested by Rodolfo-Metalpa (2006) as a breaking point in its growth. Therefore, *O. patagonica* could have

some adaptive modifications (either symbiotics zooxanthellaes or bacteria, coral host or both) that allow withstanding different temperature ranges.

It is surprising that coral growth was similar in clear oligotrophic (Marine Protected Area of Tabarca) and in turbid eutrophic environments (Alicante Harbour), where high sedimentation rates cause a dramatic reduction in light penetration. Zooxanthellate corals depend on translocation of photosynthetic products from the algal symbionts, which are the principal source of energy for corals (Muscatine, 1992), and determine skeletal calcification rates, which are proportional to photosynthesis (Goreau and Goreau, 1959); consequently, light intensity may affect coral growth. However, previous studies have observed that high sediment loads had a strong positive effect on coral survival, due to available nutrition enhancement related to high turbidity (Anthony *et al.*, 2007). In fact, some hard coral species increase their heterotrophy rates to compensate light attenuation (Anthony and Fabricius, 2000). Therefore, the higher proportion of organic matter detected in the Harbour could be used by *O. patagonica* as food, increasing its growth rate in response to organic matter input contained in mud sediments. Thus, the presence of organic matter seems to compensate light attenuation in turbid environments, favoring *O. patagonica* growth. However, other authors (Tremblay and Peirano, 2011) observed that detrital particulate organic matter not seem to constitute a large input of carbon or nitrogen to *O. patagonica*.

Results showed in chapter 3.2 clearly indicate that temperature plays a key role in *O. patagonica* bleaching and growth. In fact, our results showed that a rapid increasing of seawater temperature and thermal stress impact negatively on coral growth. It seems that *O. patagonica* has different temperature thresholds of bleaching and growth depending on its location within the Mediterranean Sea. In the study area of this work (Western Mediterranean Sea), bleaching patterns were different between sampling locations with different environmental conditions. At the MPA of Tabarca; a seasonal pattern of bleaching was recorded with the highest percentages in summer 2011 (September); although the maximum temperature of this year was similar to the previous year (around 28 °C), temperatures over 26 °C were maintained for more consecutive days (see Table 3.2, p. 90). Therefore, for *O.*

patagonica bleaching the time of exposure to high temperatures seems to be more important than the maximum temperature raised. Nevertheless, temperature is not the only factor controlling bleaching, since at the Harbour the largest bleaching event was recorded in winter (December) when seawater temperature was low. In this case, bleaching was likely caused by light reduction, as was demonstrated with the light attenuation field experiment that showed how light reductions of 70% cause bleaching in this coral. Furtheremore, *O. patagonica* could be able to acclimatize to slight light reductions, which allow it to growth in turbid environments. This coral seems to promote an increase in Chl *a* concentration in its tissues in order to absorve more light, which agrees with previous studies carried out with other coral species (Dubinsky *et al.*, 1984; Anthony and Hoegh-Guldberg 2003).

Bleaching recovery has been previously described for *O. patagonica* in the Israeli coast (Kushmaro *et al.*, 1996; Shenkar *et al.*, 2005). This recovery might depend on food availability, which could modulate the effects of heat stress on zooxanthellae. An important recovery rate (77% of bleached colonies) was recorded in the Alicante Harbour, while most marked colonies in the showed tissue necrosis after bleaching, it might be due to the higher proportion of organic matter in the Habour. Several other studies have shown the importance of coral heterotrophy as a potential mechanism for colony survival through bleaching events (Borell *et al.*, 2008). Therefore coral responses to temperature stress and bleaching will likely be influenced by the availability of other kinds of nutrients for the coral host (Hoogenboom *et al.*, 2012).

Part II. Response of the coral associated microbial communities to different environmental conditions

4.3. MICROBIAL COMMUNITY

4.3.1. Eukarya

Molecular and microscopy techniques have unveailed (chapter 3.3) the presence of a complex *O. patagonica* associated Eukarya community constituted not only by zooxanthellae but also by other eukaryotic phototrophs, as well as coral-inhabiting barnacles and boring sponges. In spite of previous studies showing that, besides zooxanthellaes, Fungi are the group of Eukaryotes more abundant in corals (Sun *et al.*, 2014; Wegley *et al.*, 2007), we did not detect members of this group in our samples either by 18S rRNA gene or electron microscopy analyses. Although the lack of retrieval of fungal 18S rRNA gene sequences is often related to the low efficiency of DNA extraction protocols due to the extremely resistant fungal cell walls, this is most likely not the case in this work, since previous studies on coral diversity that retrieved fungal sequences used the same extraction protocol used in this work (Wegley *et al.*, 2007).

Coral-inhabiting crustaceans belonging to the Pyrgomatidae family, were identified by molecular methods as the species *Megatrema anglicum*, which was previously detected in *O. patagonica*, in samples collected in Portman (Spain) (Simon-Blecher *et al.*, 2007). These barnacles are considered obligatory symbionts of scleractinian and hydrozoans corals (Hiro, 1935 in Frank and Mokady, 2002). In addition to barnacles, the two genera of boring sponges (*Cliona* and *Aka*) identified in *O. patagonica* were also previously detected as part of the boring community in corals from the Mediterranean Sea, appearing as components of the red coral *Corallium rubrum* endobiotic sponge assemblage (Corriero *et al.*, 1997). Although both barnacles and sponges are metazoans that can be observed by the naked eye, they can be difficult to identify because they belong to morphologically cryptic but genetically divergent species (i.e., two or more distinct species classified as single species based on morphological similarities) (Blanquer and Uriz, 2007; Simon-

Blecher *et al.*, 2007). In such cases, the incorporation of molecular tools could help to solve the problem, as our data indicate.

The results showed in this thesis confirm the association of *O. patagonica* with clade B type 2 (i.e. clade B2) of *Symbiodinium*, which has been very recently reported in corals collected from the same sampling locations (Alicante and Ligurian Sea) at one time point in 2009, as well as in specimens from Israeli coast (Rodolfo-Metalpa *et al.*, 2014). *Symbiodinium* B2 was first isolated from the Caribbean jellyfish *Dichotomia* sp. (LaJeunesse, 2001). This clade has not been detected previously in other Mediterranean corals, which harbored clade temperate-A. However, clade B was found in *Bunodeopsis strumosa*, a sea anemone endemic of the Mediterranean but of ancient tropical origin (Visram *et al.*, 2006). Clade B is thus rare in the Mediterranean Sea, being more common in the tropical Western Atlantic (Baker, 2003); moreover this type 2 of clade B, has been previously detected in other corals of the genera *Oculina*, such as *Oculina diffusa* (LaJeunesse, 2001; Savage *et al.*, 2002), and *Oculina arbuscula* (Thornhill *et al.*, 2008).

Coral hosts can acquire *Symbiodinium*, either by the ocean environment (horizontal acquisition; Trench, 1987) or directly from the parent egg or brooded larvae (maternal or vertical acquisition; Trench, 1987). Karako-Lampert *et al.* (2004) demonstrated that coral species that transfer zooxanthellae by vertical transmission show homogeneity in their symbionts clades; nevertheless, when the transmission is horizontal the host generally forms associations with a broad range of symbiotic genotypes (Douglas, 1998). The results present in chapter 3.3 (see Annexes, Fig. A2) show homogeneity in *O. patagonica* symbionts (all ITS DGGE band were related with this clade), since the corals seem to bore the same clade regardless of geographic location (Spain or Italy), environmental differences within the littoral zone (Alicante Harbour or Marine Reserve of Tabarca), sampling time (June, September or December) or coral status (healthy or unhealthy colonies). This finding may be suggesting that larvae of *O. patagonica* acquire their symbionts via direct transmission from the parent colony. In addition, the finding of clade B2 supports the theory that *O. patagonica* is a exotic coral in the Mediterranean Sea, since this clade is more frequent in the Western Atlantic and has never been

observed in other Mediterranean corals. Until now, the description of *O. patagonica* as an alien coral in the Mediterranean was based on its morphological similarity with Pleistocene fossil samples collected in northern Argentina as well as on its simultaneous appearance in 1973 in the Alicante Harbour (Zibrowius and Ramos, 1983) with *Bostrycapulus odites* (Izquierdo *et al.*, 2007; Collin *et al.*, 2010). This gasteropod is an exotic species that phylogenetic studies have been demonstrated that it has as origin South America (Collin *et al.*, 2010). Therefore, *B. odites* together with *O. patagonica* could be introduced in the Mediterranean Sea by transoceanic transport from the temperate South Western Atlantic (Zibrowius, 1974; Zibrowius and Ramos, 1983).

Eukaryotic phototrophic plastids detected in *O. patagonica* increase their proportion in bleached corals, in good agreement with the previous studies that showed an increase in phototrophs other than zooxanthellae in bleached corals (Lins-de-Barros *et al.*, 2013). These phototrophs could partially compensate the lack of zooxanthellae by increasing the translocation of their photosynthetic products into the coral tissue (Fine and Loya, 2002; Fine *et al.*, 2002b, 2004). Plastids of *Ochrosphaera* sp., detected along the whole year in samples collected from Tabarca, have been previously detected in the coral *Montrastraea franksi* (Rohwer *et al.*, 2001), and *Siderastrea stellata* (Lins-de-Barros *et al.*, 2013), showing that plastid communities could play an important role during bleaching events. Previous studies also detected a shift in the endolithic community of *O. patagonica* (Ainsworth *et al.*, 2008). These authors observed that bleaching was accompanied by the appearance of an endolithic layer dominated by algae of the genus *Ostreobium*. This algal layer seems to take advantage of the fact that penetration of photosynthetically active radiation is deeper in bleached than in healthy corals, because light absortion is lower in transparent than in healthy tissues.

4.3.2 Bacterial community

As shown in chapter 3.3, the bacterial communities associated to *O. patagonica* have been characterised and monitored during a whole year, including a bleaching event recorded on summer 2011. Bacterial communities present in the three coral compartments (mucus, tissue and skeleton) are different. This compartmentalisation of bacterial communities within the holobiont has been previously described by Koren and Rosenberg (2006), who analysed the culturable and nonculturable bacteria present in the mucus and tissue of *O. patagonica*, and by Sweet *et al.* (2011) who carried out the first comparing the three compartments in the coral *Acropora palmata*.

We found that the OTUs most frequently retrieved from the mucus belonged to *Alphaproteobacteria* (see Fig. 3.14 and 3.17, p. 104 and 112), a class also associated with the tissue, where they were followed by *Gammaproteobacteria*; while in the skeletal matrix the class *Deltaproteobacteria* predominated together with the class *Cytophagales* of the Phylum *Bacteroidetes*. The microbiota inhabiting mucus and skeletal compartments were more stable than that of coral tissue, which showed different composition depending mainly on season and coral health status variations and to a lower extent on environmental conditions.

It is generally accepted that environmental factors, particularly temperature and salinity, may cause shifts in microbial communities, but in corals this question it is not clear. A previous study (Guppy and Bythell, 2006) carried out to investigate the bacterial community in the surface mucus layer of the reef coral *Montastraea faveolata*, using DGGE, did not find correlation between bacterial community structure and water quality parameters. These results are probably due to the lower sensitivity and resolution of this technique, in revealing microbial diversity and community structure, than newer approaches such as tag-pyrosequencing, which unveils differences among bacterial communities associated with corals from sites with different environmental conditions, including water nutrient content (Lee *et al.*, 2012). Similarly, not significant differences between bacterial communities due to environmental conditions were detected by DGGE, but Illumina sequencing indicated that the bacterial diversity associated to *O. patagonica* was lower in corals

collected in Tabarca than those from the Harbour. This is in good agreement with a recent study in which lower bacterial diversity values were detected in *Paramuricea clavata* from pristine areas compared with human impacted populations (Vezzulli *et al.*, 2013b). In addition, as shown in Figure 3.17b (p. 112), Illumina showed that corals from both locations harboured communities dominated by different taxa *Rhodospiralles*, *Burkolderiales*, *Clostridiales*, *Bacilliales* and *Chlamydiales* in the eutrophic Alicante Harbour and *Sphingomonadales*, *Alteromonadales* and *Flavobacterales* in pristine MPA of Tabarca. These results suggested that the occurrence of certain bacterial orders in *O. patagonica* tissues at different sites could be regulated by external environmental parameters.

Moreover, our results also showed changes in the coral tissue bacterial assemblages when comparing cold and warm months by DGGE and Illumina, suggesting that temperature caused microbiota shifts, which is consistent with the results obtained by Koren and Rosenberg (2006). A decrease of *Alphaproteobacteria* and an increase of class *Gammaproteobacteria* were detected in summer with the rise of seawater temperature. *Pseudovibrio*-related sequences accounted for between 17% and 35% of Illumina libraries, being the dominant genus within the class *Alphaproteobacteria* in healthy corals, as previously was reported by Koren and Rosenberg (2006). The presence of *Pseudovibrio* spp. mainly in healthy corals indicates that it could play a key role in the *O. patagonica* holobiont system and could be involved in the coral health status. Previous studies carried out with this genus have demonstrated that it does not only play a role in carbon and nitrogen cycles into the coral (Bondarev *et al.*, 2013), but also inhibits the growth of the coral pathogens, *V. mediterranei* and *V. coralliilyticus* (Nissimov *et al.*, 2009; Rypien *et al.*, 2010). The increase of *Gammaproteobacteria* in corals exposed to warmer temperatures, which is well documented in previous studies (Bourne *et al.*, 2008; Littman *et al.*, 2011), could probably be caused by the combined effect of increased of virulence (Rosenberg and Falkovitz, 2004; Kimes *et al.*, 2012), and antimicrobial resistance (Vizcaino *et al.*, 2010) exhibited by some *Gammaproteobacteria* during higher summer temperatures. Therefore, the increase of seawater temperatures and the consequent loss of antibiotic activity in the coral, caused by the loss of beneficial

bacteria (e.g. *Pseudovibrio* genus), could disrupt the holobiont equilibrium and make it more susceptible to pathogen domination (Mao-Jones *et al.*, 2010).

Many previous studies showed that microbial coral communities change during bleaching events (Bourne at al., 2008; Reis *et al.*, 2009; Lins-de-Barros *et al.*, 2013), also in *O. patagonica* (Koren and Rosenberg, 2008), being bacterial diversity higher in diseased corals (Bourne, 2005; Bourne *et al.*, 2008; Reis *et al.*, 2009; Sunagawa *et al.*, 2009). Both techniques used in this PhD thesis (DGGE and deep sequencing) confirm that bacterial communities associated to *O. patagonica* tissues are different depending on coral health status; however, bacterial diversity in unhealthy samples from Harbour was higher than in healthy samples, but in samples from the MPA was lower. Regardless of different trends in bacterial diversity, in both sampling location the genus *Vibrio* was dominant in colonies displaying disease signs, and *V. mediterranei* and *V.coralliilyticus* were retrieved in bleached samples by culture depending techniques and Illumina sequencing approach, even thought they were not detected by DGGE.

4.4. *VIBRIO* COMMUNITY

The analysis of the culturable *Vibrio* associated-community (chapter 3.4) as well as the direct Illumina sequencing of the coral microbiota (chapter 3.3) show a complex and dynamic assemblage of *Vibrio* species is present in *O. patagonica* along the whole year, under different environmental conditions and coral health status. Thus, these results support the view that corals harbour *Vibrio* spp. as autochthonous members of their microbial communities (Alves *et al.*, 2010). Moreover, the composition of the coral associated *Vibrio* assemblages was different between *O. patagonica* and *C. caespitosa* collected in the same geographic area, showing that coral-associated microbial communities could be coral species dependent (Rohwer *et al.*, 2001; Kvennefors *et al.*, 2010). Our results also confirmed the relevance of geographic location on the composition of the coral bacterial communities in a same coral host reported by Lee *et al.* (2012), at least for the culturable *Vibrio* fraction. Nevertheless, the shifts in *Vibro* composition were more

correlated with the coral health status than to geographical location, as previously shown for the gorgonian *P. clavata* (Vezzulli *et al.*, 2013b).

We identified two main dominant *Vibrio* phylotypes in *O. patagonica*, which could constitute their "core" microbiota: *V. harveyi-like* and *V. splendidus* super clade. The *V. harveyi* group, including six *Vibrio* species (i.e. *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio rotiferianus*, *V. harveyi*, *Vibrio campbellii,* and *Vibrio vulnificus*; Dorsch *et al.*, 1992), has been previously reported as one of the most dominant groups among *Vibrio*s found in the microbiota of Brazilian cnidarians (Chimetto *et al.*, 2009). *Vibrio* species belonging to this group were speculated to play an important ecological role for the coral host by fixing nitrogen (Chimetto *et al.*, 2008). On the other hand, members of this phylotype have also been previously reported as serious pathogens for a wide range of marine animals (Austin and Zhang, 2006) and hypothesized as possible pathogens for corals (Gomez-Gil *et al.*, 2004; Thompson *et al.*, 2004; Thompson *et al.*, 2005; Cervino *et al.*, 2008). Accordingly, in this study, bacteria belonging to *V. harveyi* clade were found in healthy and unhealthy corals, being its proportion higher in unhealthy ones. On the basis of these considerations, the role of this phylotype in the *O. patagonica* holobiont remains difficult to assesss. The other phylotype that could be part of the normal *O. patagonica* microbiota is *V. splendidus* super clade, which was mainly detected in healthy corals, decreasing its proportion in unhealthy ones. This clade had been previously detected in this species, being identified as one of the most abundant taxa present all year round in this coral in the Israeli coast (Koren and Rosenberg, 2006). However, this clade was only detected, during this study, in samples collected in Spanish coast, while it could not be detected in any of the coral samples collected in the Italian coast. Thus our results, at least in *Vibrio* culturable community, support the idea that coral microbial communities are determined by geographic location.

Increase of seawater temperature was not only accompanied by changes in coral health status (chapter 3.2) but also in the numbers and composition of their associated *Vibrio* spp. assemblages. Certainly, the occurrence of *Vibrio*s during the studied time frame was highly correlated with seawater temperatures, with a sharp increase in *Vibrio* abundances during summer months when the temperatures

reached around 28ºC. However, seawater temperature is not the only environmental variable that determines *Vibrio* abundance, that was also linked to organic matter concentration and Chl *a* (which is an index of phytoplankton biomass). This could be due to the fact that phytoplankton blooms may support the proliferation of *Vibrio* spp. (Asplund *et al.*, 2011). Together with the changes in *Vibrio* spp. numbers along the year, there was a succession of phylotypes with time (seasonal variation) as well as differences in the *Vibrio*s recovered from the Harbour and Tabarca, which is also related to the different environmental conditions at the two sampling locations (see Figure 3.20, p. 120). Therefore, *Vibrio* communities associated with *O. patagonica* are different at small-scale depending on the environmental conditions and at larger scale due to geographic location.

Most importantly, Unifrac analysis indicated that unhealthy and healthy samples of *O. patagonica* harboured distinct *Vibrio* spp. assemblages (see Fig. 3.21, p. 121), being the majority of isolates from healthy samples closely related to *V. splendidus* super clade, in Spain, while *Vibrio*s related to *V. mediterranei* and *V. coralliilyticus* were recovered mainly from unhealthy specimens, both in Spain and Italy. A clear spatial distribution of these two phylotypes, according to environmental conditions, was detected in Spain, being *V. mediterraei* mainly found in the Harbour and *V. coralliilyticus* in Tabarca, which was also confirmed by NGS. Accordingly, and considering the different disease signs experienced by corals in these two sampling locations, i.e. mainly bleaching in the Harbour and tissue necrosis in Tabarca (chapter 3.2), these two *Vibrio* spp. could be related with different *O. patagonica* diseases. The presence of isolates associated with *V. mediterranei* in diseased *O. patagonica* samples 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). 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, such as occurs between *Vibrio* spp. assemblages associated to healthy samples collected from Italy and Spain (see Fig. 3.23; p. 126)

In addition, the composition of *Vibrio* spp. assemblages changed with coral hosts. In this regard, the phylotypes within *V. splendidus* super clade and *V. harveyi-like* seemed to be part of the "core" microbiota in *O. patagonica* and *V. harveyi-like* clade and *V. comitans-V. rarus-V. breoganii*, in *C. caespitosa*. This last clade had been previously detected in other marine organisms such as zooplankton (Preheim *et al.*, 2011) or abalones (Sawabe *et al.*, 2007). However, *Vibrio* communities associated with unhealthy samples become more similar between the two coral species, even from samples collected in different geographic locations (see Fig. 3.23; p. 126). The pathogens *V. mediterranei* and *V. coralliilyticus* not seem coral specific since they were recovered not only from diseased *O. patagonica* but also from *C. caespitosa* samples with tissue necrosis. In fact, *V. corallilitycus* has been related with coral diseases worldwide (Indo-pacific, Sussman *et al.*, 2008; Mediterranean Sea, Bally and Garrabou, 2007; Red Sea, Ben-Haim *et al.,*, 2003; and Caribbean Sea, Vizcaino *et al.,* 2010). Besides these two coral pathogens, one major shellfish pathogen, *Vibrio tubiashii* (Estes *et al.*, 2004) was also detected in unhealthy samples of the two corals hosts. Although this *Vibrio* was previously detected in other coral species (Alves *et al.*, 2010; Vezzulli *et al.*, 2013b), it has never been related to coral diseases. *V. tubiashii* contains a zinc-metalloprotease which could affect photosynthesis in *Symbiodinium* (Sussman *et al.*, 2009). This, together with the phylogenetic proximity of this species to *V. coralliilyticus* and its likely missclassification (Ben-Haim *et al.*, 2003), provides some basis for its possible role in coral disease.

The presence of the two possible *Vibrio* pathogens (*V. mediterranei* and *V. coralliilyticus*) recovered by culture from diseased samples was also detected in healthy samples of *O. patagonica* by NGS. These pathogens have been only found in association to diseased corals but never found in healthy ones, considering them as non-resident pathogens. Actually, previous studies suggest that new infections are produced every summer by *V. mediterranei*, which is maintained in the viable-but-non-culturable (VBNC) state during winter in the marine fireworm *Hermodice carunculata* that acts as transmission vector when feeding on the coral (Sussman *et al.*, 2003). Interestingly, the detection of both *Vibrio* pathogens in healthy corals, even during cold months, suggests that these pathogens could be part of the normal coral microbiota, maintaining in VBNC inside of coral tissues, in good agreement with

the results of Sharon and Rosenberg (2010) that found *Vibrio* spp. in VBNC in mucus layer of *O. patagonica*. Accordingly, *Vibrio* pathogens could be inside the coral during cold months and with the increase of seawater temperatures and its consequent coral microbiota alteration, possibly trigger the occurrence of *O. patagonica* diseases.

The detection of *V. mediterranei* and *V. coralliilyticus* in diseased specimens of the endemic coral *C. caespitosa* together with the fact that these pathogens are part of the alien species *O. patagonica* microbiota, which is spreading along the Mediterranean Sea, raises the possibility of the secondary horizontal transmission of *Vibrio* spp. by physical contact between neighboring corals from the two species, as reported for the transmission of *V. coralliilyticus* among colonies of *Pocillopora damicornis* (Ben-Haim and Rosenberg, 2002). In this way, the dispersion of *O. patagonica* to areas where *C. caespitosa* is established could enhance the impact over the endemic coral of increasing seawater temperatures in the predicted global warming scenario.

4.5. ANALYSIS OF TWO POTENTIAL *VIBRIO* PATHOGENS BY EXPERIMENTAL INFECTIONS

The data on seasonal *Vibrio* spp. dynamics in the coral *O. patagonica* 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 also clearly correlated with an increase in water temperature (see chapter 3.2). The effect of temperature on coral disease and the increase of *Vibrio* numbers in marine environments have been widely reported before (Kushmaro *et al.*, 1998; Ben-Haim *et al.*, 2003; Bourne *et al.*, 2008; Vezzulli *et al.*, 2010), although there is still much controversy on the role of *Vibrio* spp. in causing *O. patagonica* bleaching. The last objective of this thesis was to get a new insight into this issue by analysing the response of *O. patagonica* to experimental infection, with the *Vibrio* phylotypes retrieved from unhealthy samples, under different conditions. Althought this experimental approach has well-known limitations (Ainsworth and Hoegh-Guldberg, 2009) it 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" *Vibrio*s than in untreated controls, with higher/faster disease signs in the corals inoculated with the mixed inocula (see Fig. 3.25; p. 131), in good agreement with the results 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 when they were inoculated individually, leading to the suggestion that these four *Vibrio*s were acting as a consortium. Subsequently, these authors confirmed that a consortium of different *Vibrio* species caused YBD in both Caribbean and Pacific Sea, being the infection initiated at 25ºC and increased with rising seawater temperature (29–30ºC) (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 *P. 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, even in corals not inoculated with *Vibrio*s. This fact suggests that corals used in the experiment probably had *Vibrio* pathogens in VBNC state in their tissues, as was detected in our field samples (see above). However, no *Vibrio* sequences were retrieved by NGS from control coral at 28ºC, probably due to the low quality of this sample sequences, because *Vibrio* spp. were detected by culture from the same samples. On the contrary, in the control sample maintained at 24ºC *V. mediterranei* and *V.coralliilyticus* were retrieved by NGS, including sequences with a 99.7% identity with the *V. coralliilyticus* strain used in the experiment. Therefore, the tissue damage experienced by the corals without *Vibrio* inocula could have been caused by VBNC *Vibrio*s inside the coral as was suggested by Sharon and Rosenberg (2010). However, for these authors, VBNC *Vibrio*s 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 (there is not a clear cut classification). Furthermore, these 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 etiologies. 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 carried out in this thesis is the development of disease signs at 20 °C when mixed cultures of *V. mediterranei*, *V. coralliilyticus* and *V. splendidus* were inoculated into healthy corals. The fact that only the mixture of *Vibrio*s was inducing disease signs at low temperature indicated that these bacteria could be harmful to coral under conditions in which the individual pathogens would not have any deleterious effect. In field studies carried out during this PhD thesis, *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.

4.6. COULD VIRUSES BE PLAYING ANY ROLE IN *O. patagonica* HEALTH STATUS?

At the end of infection experiment with *Vibrio* spp. we could observer, by transmission electron microscopy (TEM) (See Fig. 3.27; p. 133F), viral-like particles (VLPs) inside of vacuoles near *Symbiodium* cells in corals that were maintained at 28ºC. Since the focus of this study was on the role of *Vibrio* spp. in *O. patagonica* bleaching the relationship of coral thermal stress and the appearance of VLPs did not investigate. However, it can not rule out the possibillity that viruses could be playing any role in *O. patagonica* diseases during thermal stress. In fact, previous studies confirm that different kinds of stressess produce viral induction in cnidarians. For instance, Wilson *et al.* (2005) carried out the first characterisation of VLPs morphology in the scleractinian coral *Pavona danai* and observed by TEM a greater abundance of VLPs associated with the heat-shocked tissues, suggesting that a viral outbreak occurred in this coral during heat stress. These authors also found

that VLPs were associated with resident *Symbiodinium* cells, which could imply the active viral penetration and infection of zooxanthellae during their experiment. Furthermore, observations of VLPs in TEM images in different species of Indo-Pacific corals thermally stressed, as well as the cell lysis of non-stressed zooxanthellae with medium around heat-degraded zooxanthellae, support the infection of zooxanthelae within reef corals by viruses (Davy *et al.*, 2006).

Observations of VLPs communities in corals have generally been described as a latent pathogen reservoir, susceptible to induction by environmental stressors (Wilson *et al.*, 2005; Davy *et al.*, 2006; Vega Thurber *et al.*, 2008); in fact, the presence of eukaryote-specific viruses in corals has been also demonstrated (Marhaver *et al.*, 2008, Vega Thurber *et al.*, 2008). Thus, given the abundance and diversity of coral-associated viruses, it is expected that these virus communities will have potential roles on coral holobiont. More recent studies, using metagenomic approaches, have suggested that the presence of bacteriophages in coral tissues could help to regulate microbial communities on the coral (Wegley *et al.*, 2007). This would be the case, for instance, of *Vibrio*phages present in coral tissue that may affect the pathogenesis of coral-associated *Vibrio* spp. (Marhaver *et al.*, 2008). In fact, phage therapy has been proposed as a solution in some coral diseases such as white plague-like disease in *Favia favus* and bleaching and tissue lysis in *Pocillopora damicoranis*, which are produced by the coral pathogens *Thalassomonas loyana* and *V. coralliilyticus* respectively, both inhibited by specific phages isolated from seawater (Efrony *et al.*, 2006; Atad *et al.*, 2012; Cohen *et al.*, 2013).

4.7. GENERAL DISCUSION

The acquired knowledge throghtout this PhD Thesis about the biology and ecology of *O. patagonica* holobiont is summarized in Figure 4.1 and it helps us to better understand the complex interactions among human, environmental and biotic factors with the coral host *O. patogonica* and with its microbial community, as well as to increase our knowledge about bleaching dynamics.

Oculina patagonica is considered as an alien species in the Mediterranean Sea from South Western Atlantic. The only evidence found about its origin was its morphological similarity with Pleistocene fossil samples collected in Argentina. This researche presents new evidences that support this theory, such as its association with *Symbiodinium* clade B2, typically Atlantic, as well as the spreading of *O. patagonica* in the last years, mainly favored by human activities. Anthropogenic impacts can promote the spreading of *O. patagonica* directly by the construction of man-made structures, that act as focus of dispersion to this species, or indirectly by coastal pollution and physical destruction of habitats that simultaneously produce changes in composition of macroalgal community and the dominance of filamentous algae turf that seems to favour the recruitment of *O. patagonica* (see red arrows in Fig.4.1).

It is evident that the most significant risk to coral ecosystems is global warming, due to corals are thermally sensitive and a little increase as 1-2°C of seawater temperature can be harmful for them (Brown, 1997). This PhD thesis highlight the importance to study the changes produced over all organisms that form part of the coral holobiont in order to understand better and evaluate the potential effects of global warming on *O. patagonica*. The increase of seawater temperatures as consequence of global warming produces bleaching on *O. patagonica* and in this study we found that spatiotemporal variations exit in bleaching events. Field surveys and aquaria experiments allowed us to establish a clear link between elevated water temperature and bleaching progression. A bleaching event was recorded on summer 2011 when seawater was maintained over 26°C for a prolonged period of time and throughout aquaria experiments, confirming that tissue damage on *O. patagonica* was higher and occured faster with the increase of temperature. Not only were detected

external signs of tissue damage but degradation of coral gastrodermis was also observed by electron microscopy. Moreover, the consequences of the increase seawater temperature over zooxanthellae were also shown by electron microscopy. A decrease of zooxanthellae density as well as the thylacoids disruption was observed, probably resulting from changes in lipid characteristics of their membranes (Iglesias-Prieto *et al.*, 1992), suggesting the dysfunction of photosystem II (Warner *et al.*, 1999) and the potential consequent reduction of photosynthetic rates (see "consequences section" in Fig. 4.1).

The temporal variability of bleaching can partially be explained by changes in water temperature, though it is not the only environmental variable controlling this process (see blue arrows in Fig.4.1). In addition to seawater temperature, the light attenuation also has influence over *O. patagonica* bleaching process, being its effect more evident with the increase of seawater temperature. These observations are in good agreement with the work by Downs *et al.* (2013) who provided evidences that heat stress and low-light conditions act on the zooxanthellae via different mechanisms, resulting in physical disruption of photosynthetic electron transport.

Bleaching events showed spatial variation, probably related to other environmental variables such as organic matter in suspension and Chlorophyll *a* (as phytoplankton index), which might have influence over bleaching recovery in *O. patagonica*. It can be assumed that *O. patagonica* is able to increase its heterotrophy rates as a potential mechanism for coral survival through bleaching events (Borell *et al.*, 2008; Hoogenboom *et al.*, 2012), and feeding on organic matter in suspension (dashed blue line in Fig. 4.1) and/or zooplankton (dashed green line in Fig. 4.1), whose abundance depends on phytoplankton abundance. Nevertheless, in this work only a half of the bleaching variability could be explained by environmental variables, suggesting that other factors also play an important role in bleaching process (e.g. Biotic factors, green arrows in Fig. 4.1). In fact, many coral diseases are likely caused by intricate multi-factorial sources, with the pathogen(s) and the environment acting synergistically to cause disease (Work *et al.*, 2008; Sokolow, 2009).

This work shows that environmental variables not only have an effect over coral hosts and their zooxanthellae community but also over its bacterial assemblages, which show changes in their composition as consequence of the increase of seawater temperature. The bacteria composition of *O. patagonica* tissue associated microbiota depends on seawater temperature. The phylum *Proteobacteria* is always predominant. However, there is a seasonal variation within this phylum, where the class *Alphaproteobacteria* is dominant in cold months, while the proportion of *Gammaproteobacteria* increases considerably during warm months and the order *Vibrionales* contributes most to this increase. Some of *Alphaproteobacteria* detected belong to *Pseudovibrio* genus and could have beneficial roles in the *O. patagonica* holobiont such as biogeochemical cycling (Bondarev *et al.*, 2013; Bourne and Webster, 2013) or antimicrobial activity (see dashed black lines in the holobiont section in Fig. 4.1). It was previously demonstrated that this genus has a potential role on protecting corals against invading pathogens, inhibiting their growth (Nissimov *et al.*, 2009; Rypien *et al.*, 2010; Vizcaino *et al.*, 2010). Among the order *Vibrionales* detected in warm months, the two recognized coral pathogens *V. mediterranei* and *V. coralliilyticus* were only retrieved, by culture methods, in *O. patagonica* with diseased sings. Thus, the effect of these pathogens over *O. patagonica* was studied by experimental infections in aquaria, which confirmed that the two pathogens induce acute *O. patagonica* tissue damage, increasing their effect under heat-stress.

The two *Vibrio* pathogens (biotic factor in Fig.4.1) have been also detected in seawater during warm months and in healthy corals during winter (by molecular techniques). The fact that pathogens were not retrieved by culture methods but were detected by deep sequencing approachess could imply that *V. mediterranei* and *V. coralliilyticus* could survive in the VBNC state in *O. patagonica* tissues until the conditions were favorable (i.e. warmer temperatures) ("inside holobiont" section in Fig. 4.1). However, it is difficult to determine the accurate origin of these pathogens, which could be seawater, the coral itself (pathogens in VBNC state) or even other habitats not studied in this work such as benthic algae, which have been suggested as reservoir of coral pathogens (Sweet *et al.*, 2013).

According to our findings it can be suggested that there is a relationship between changes in normal coral microbiota, as a consequence of increase of seawater temperature, and the presence of *Vibrio* pathogens and *O. patagonica* bleaching. Hence, these results support that global warming could be leading to the increase of coral diseases as a consequence of the interactions between *Vibro* pathogens and corals (see Sokolow, 2009). However, the knowledge acquired in this PhD thesis does not seems to be enough to elucidate the action mechanism of *Vibrio* pathogens in *O. patagonica*, neither if they are the primary cause of bleaching nor if they act as opportunistic pathogens when the coral is weakened as consequence of thermal stress (dark green lines from potential pathogens to coral holobiont Fig. 4.1). It is clear that thermal stress has several effects over coral hosts (see "consequences" section in Fig. 4.1), such as the degradation of the gastrodermis. Thermal stress effect was also evident in zooxanthellas that showed thylacoid disruption. These responses together with changes in bacterial coral community, such as minor presence of bacteria that could have antimicrobial activities (possibly *Pseudovibrio* spp.), might lead to decrease the immune capacities of the coral, facilitating the infection by *V. mediterranei* and *V. coralliilyticus*. These complex relationships currently make difficult to determine if *Vibrio* pathogens are the direct cause of bleaching, as well as their interactions with the host and/or environment, and the overall understanding of coral *O. patagonica* bleaching dynamics.

Figure 4.1. Summary of the interactions between different factors, human (red lines), environmental (blue lines)and biotic (green lines), and *O. patagonica* holobiont and their implication on coral bleaching. Dashed lines and light grey letters represent results of other research studies that could be applied to this model; and continue lines and black letter are results from this thesis.
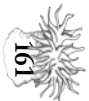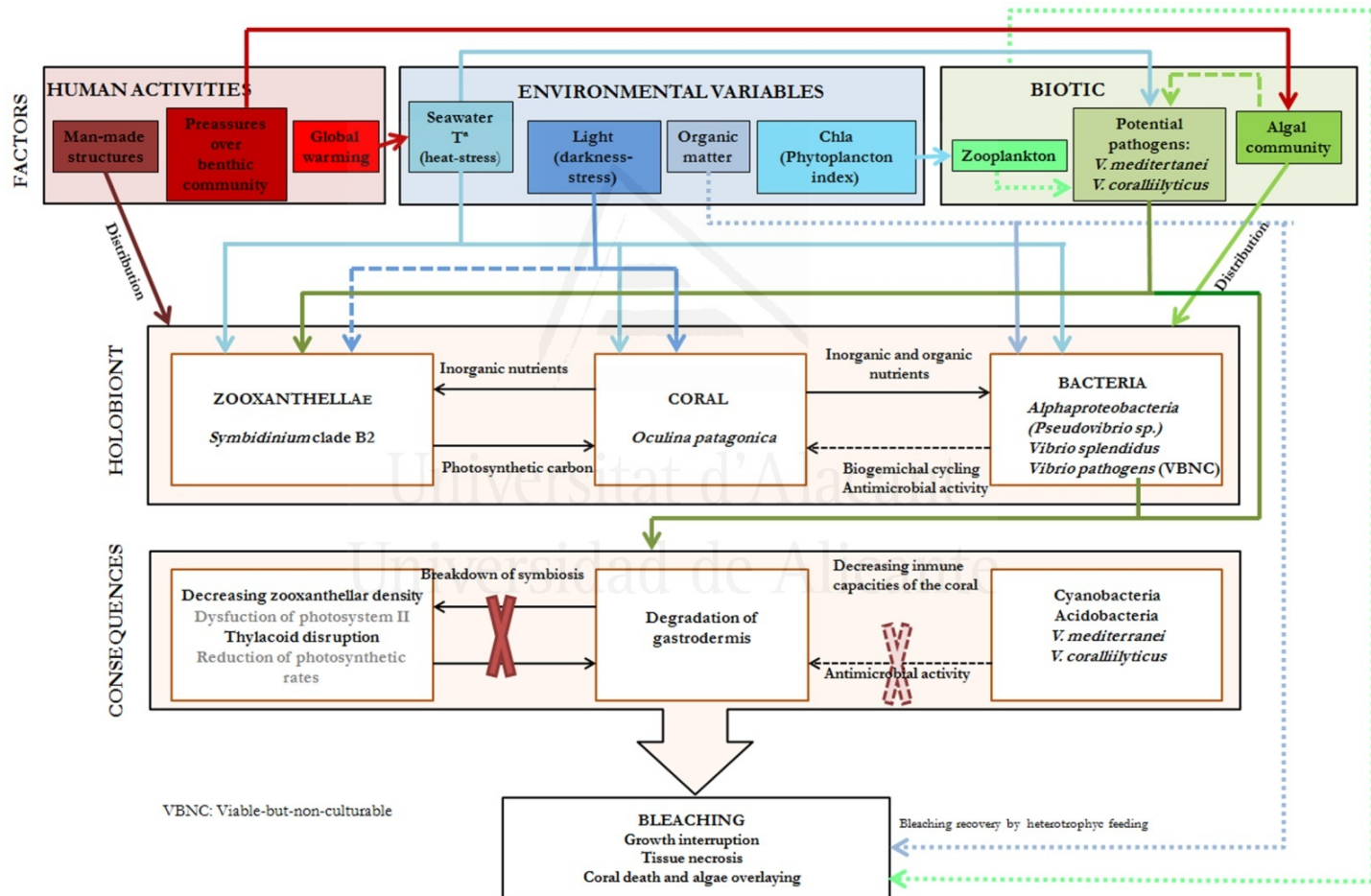
4.7. GAPS OF KNOWLEDGE AND FUTURE RESEARCH

The information on *O. patagonica* bleaching gathered in this thesis provides a baseline for future research on this disease. For example, this study provides new details insight into the bacteria community dynamics that occur in *O. patagonica* with temperature stress such as decreasing of *Alphaproteobacteria* class, some of which (e.g. *Pseudovibrio* spp.) theoretically could play key roles in the coral holobiont. However, more detail analysis are necessary to investigate their bacterial potential functions and metagenomic approach could be a useful tool for understanding the complexity of the *O. patagonica* microbiome (Wegley *et al.*, 2007; Vega-Thurber *et al.*, 2009; Littman *et al.*, 2011) and to detect not only taxonomic but also metabolic shifts in *O. patagonica* microbial communities during a bleaching event, in order to understand better the role of different microorganism in coral diseases. Furthermore, metagenomic approached would allow uncovering changes at other microbial levels, which are not studied in this thesis, such as viruses. There is a lack of knowledge about the potential role of viruses in *O. patagonica* holobiont and the full molecular characterisation of viruses associated with this coral would imply a great step on the way to understand the role of the viral community in *O. patagonica* health; considering that has been previously demonstrated that the virus abundance in corals increase during thermal stress (Vega-Thurber *et al.*, 2008; Littman *et al.*, 2011), as well their potential role in coral diseases (Davy *et al.*, 2006; Soffer *et al.*, 2013).

Another new line of research could derive from the results of infection experiment that suggest a possible synergic effect between *V. mediterranei* and *V. coralliilyticus* affecting coral tissues at temperatures lower than investigated until now. It has been demonstrated that communication between bacteria is based on chemical signal exchange of molecules defined as quorum sensing. Bacteria produce small molecules (autoinducers) that ordinarily regulate their gene expression in response to cell density or interspecies bacterial interactions. This communication can be implied for example in the resuscitation of dormant *Vibrio cholera* cells (Bari *et al.*, 2013) or in the control of its virulence factors (De Kievit and Iglewski, 2000). The production of acyl homoserine lactones, which normally act as autoinducter in Gramnegative bacteria, has been previously demonstrated in both *V. mediterranei*

and *V. coralliilyticus* (Tait *et al.*, 2010). This fact suggests that quorum sensing could have important roles in the interactions between these pathogens and the coral host and may be linked to their growth capability, virulence potential as well as their capacity to revival from the VBNC state inside the coral. Therefore, further researches are necessary in order to improve the knowledge about *in situ* roles of quorum sensing in these pathogens, as well as the possible mechanisms evolved to interfere with their cell-cell communication by inhibition or degradation of AHL signals to control *Vibrio* virulence. One of the mechanisms of cross-inhibition envolved to interfere bacterial comunication is termed quorum quenching, an enzymatic *in situ* degradation of AHL signals produced by other bacteria that could controll infection process, becaming in a novel target for antimicrobial therapy in *O. patagonica* bleaching.

Finally, the fact that *V. mediterranei* and *V. coralliilyticus* have been detected in the endemic coral *C. caespitosa* with necrosis signs open new lines of research in order to elucidate the role of these pathogens in this coral species, as well as the possibility of coral diseases related to *Vibrio* spp. can be transmited by physical contact between *O. patagonica* and *C. caespitosa*. A previous study has demonstrated that tissue lysis produced by *V. coralliilyticus* in *P. damicornis* is contagious by direct contact between an infected diseased coral and a healthy one (Ben-Haim and Rosenberg, 2002). Since *O. patagonica* is spreading along the Mediterranean Sea in the same habitats than the endemic coral and could be a vector of *Vibrio* pathogens transmission, enhancing the impact of increasing seawater temperatures in the predicted global warming scenario over the endemic coral that is an endangered species.
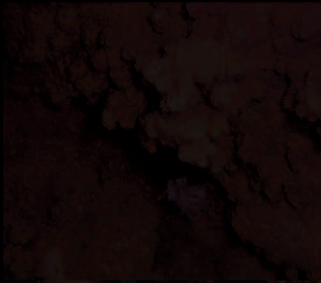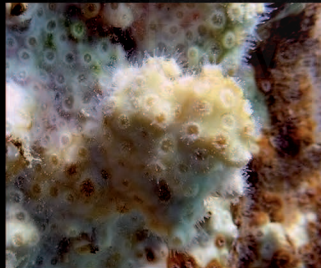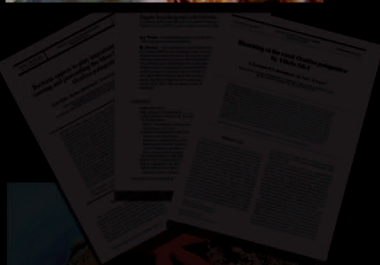
Photo/Figure: Bleached *Oculina patagonica* colony

Part I. Biological and ecological characteristic of *Oculina patagonica*

1. The spread of *Oculina patagonica* in shallow Mediterranean rocky habitats is mainly determined by anthropogenic factors, such as the maritime traffic and the existence of man-made structures like harbours.

2. The proliferation of *O. patagonica* is driven by its recruitment, which is determined by the algae community, and is favored by filamentous algae turf more than by fleshy and calcareous macroalgae. Therefore, the effect of sediment scouring also seems to facilitate the recruitment of *O. patagonica* on the rocky substrata.

3. The between-environments comparison (Harbour and Marine Protected Area) confirmed that *O. patagonica* has a broad tolerance to seawater irradiance and nutrient concentration in water, showing similar growth rates in eutrophic and turbid environments than in oligotrophic and clear ones.

4. Sea water temperature is the environmental variable that has more influence over coral growth, showing a

Parte I. Características biológicas y ecológicas de *Oculina patagonica*

1. La propagación de *Oculina patagonica* en los habitats rocosos someros del Mediterráneo está principalmente determinada por factores antropogénicos, como el tráfico marítimo o la existencia de estructuras construidas por el hombre.

2. La proliferación de esta especie está determinada por su reclutamiento, el cual se ve influenciado por la comunidad algal, viéndose más favorecido por el césped de algas filamentosas que por las algas erectas ya sean carnosas o calcáreas.Además, el efecto de la abrasión del sedimento sobre la roca parece facilitar el reclutamiento de *O. patagonica*.

3. La comparación entre ambientes (Puerto y Área Marina Protegida) confirmó que *O. patagonica* tiene un rango de tolerancia amplio frente a la irradiancia y laconcentración de nutrientes en el agua, mostrando tasas de crecimiento similares tanto en ambientes oligotróficos como eutróficos.

4. La temperatura del agua es la variable ambiental que mayor influencia tiene

clear seasonality with a minimum rate during cold months (February, 13 °C), and increasing during the warm months (June to September, 18-28 ºC).

5. The environmental variables that have more influence over bleaching are temperature, being more important the exposition time to high temperatures than the maximum temperature raised, and light whose attenuation produces a reduction of photosynthetic pigments in coral tissue.

6. *O. patagonica* is able to recovery from bleaching in eutrophic areas, showing the importance of coral heterotrophy as a potential mechanism for colony survival through bleaching events.

7. The fact that *O. patagonica* bores a single *Symbiodinium* clade (B2), not previously detected in other Mediterraenean corals, which is more frequent in the tropical Western Atlantic, represents a new evidence that this coral is an alien species in the Mediterranean Sea.

sobre el crecimiento de esta especie, que presenta un mínimo en invierno (febrero, 13ºC) aumentando en los meses cálidos (junio-septiembre,18-28ºC).

5. Las variables ambientales que mayor influencia tienen sobre el blanqueamiento son la temperatura, siendo más importante el tiempo de exposición a altas temperaturas que la máxima temperatura alcanzada, y la luz, cuya atenuación produce la disminución de pigmentos fotosintéticos en los tejidos del coral.

6. *O. patagonica* es capaz de recuperarse del blanqueamiento en áreas con eutrofización, lo que resalta la importancia de la nutrición heterótrofa en los corales, siendo posiblemente el mecanismo que permite a los corales sobrevivir durante eventos de blanqueamiento.

7. El hecho de que *O. patagonica* tenga un único clado de *Symbiodinium* (B2), no detectado previamente en otros corales del Mediterráneo, y que además es más frecuente en el Atlántico tropical Occidental, es una nueva evidencia de que es un coral introducido en el

Part II. Response of *O. patagonica* **associated microbial communities to different environmental conditions**

8. Phototrophic Eukarya increase their proportion in skeletal matrix of bleached *O. patagonica,* whose transparent tissues allow a deeper penetration of photosynthetically active radiation.

9. Bacterial communities hosted in the three coral compartments (mucus, tissue and skeletal matrix) are different among them, being the bacterial communities associated to tissue coral more sensitive to temporal, environmental and coral health status changes.

10. The occurrence of certain bacterial orders in *O. patagonica* tissues at different sites could be regulated by external environmental parameters.

11. Increase of seawater temperature exerted a strong influence over coral holobiont, producing changes in its tissue microbiota and decreasing putatively beneficial bacteria, such as *Pseudovibrio* genus, while increasing *Vibrio* representatives.

Mediterráneo.

Parte II. Respuesta de la comunidad microbiana asociada a *O. patagonica* **en diferentes condiciones ambientales**

8. La proporción de eucariotas fotótrofos aumenta en el esqueleto de *O. patagonica* con blanqueamiento, cuyos tejidos transparentes permiten una penetración de la radiación fotosintéticamente activa más profunda.

9. Las comunidades bacterianas presentes en los tres compartimentos del coral (mucus, tejido y esqueleto) son diferentes entre sí, siendo la del tejido la más sensible a cambios estacionales, ambientales o del estado del coral.

10. La presencia de ciertos órdenes de bacterias en los tejidos de *O. patagonica* depende de los parámetros ambientales externos.

11. El aumento de la temperatura tiene una gran influencia sobre el coral holobionte, causando cambios en la microbiota de sus tejidos, disminuyendo algunas especies posiblemente beneficiosas para el coral, como el género *Pseudovibrio*, y un aumento del

12. *O. patagonica* harbours *Vibrio* spp. as autochthonous members of their microbial communities along the whole year, although they are different between healthy and unhealthy corals.

13. Experimentally ii has been demonstrated that *Vibrio mediterranei* and *Vibrio coralliilyticus* are involved in *O. patagonica* bleaching, increasing their effect with rising of temperatures.

14. *Vibrio* community is coral species dependent, being different between *O. patagonica* and *Cladocora caespitosa*. However, unhealthy corals of both species harbor similar *Vibrio* assemblages.

15. *O. patagonica* could be considered as a *Vibrio* reservoir and an important vector of *Vibrio* diseases transmission to nearby endemic coral stocks, such as *C. caespitosa*, putatively enhancing the impact of increasing seawater temperatures in the predicted global warming scenario.

genero *Vibrio*.

12. *O. patagonica* alberga especies del género *Vibrio* como miembros autóctonos de sus comunidades microbianas durante todo el año, aunque son diferentes entre corales sanos y enfermos.

13. Se ha demostrado de forma experimental que *Vibrio mediterranei* y *Vibrio coralliilyticus* están involucrados en el blanqueamiento de *O. patagonica*, incrementando su efecto con el aumento de la temperatura.

14. La comunidad de *Vibrio*s depende de la especie del coral hospedador, siendo diferente entre *O. patagonica* y *Cladocora caespitosa*. Sin embargo, se vuelve similar entre corales enfermos de ambas especies.

15. *O. patagonica* podría ser considerada como un reservorio de *Vibrio* y por tanto como un importante vector de transmisión de enfermedades, relacionadas este género, a otras poblaciones de corales endémicos como *C. caespitos*a, aumentando así el impacto del incremento de la temperatura en el escenario de cambio de global.

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Photo/Figure: Peer-reviewed scientific publications about *Oculina patagonica*

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Photo/Figure: *O. patagonica* colonies in a Mediterranean ecosystem (by Juan Cuetos/OCEANA)

OTU	Blastn (identity)	Description (Geenbank)
E1	AF052887 (100%)	*Phyllangia mouchezii*
E2		
E3	AF427448 (100%)	*Symbiodinium* clade B
E4		
E5	KC902056 (99%)	*Cliona* sp. (Sponge)
E6	KC902084 (99%)	*Siphonodictyon coralliphagum* (Sponge)
E7	AM4978888 (99%)	*Megatrema anglicum* (Barnacle)
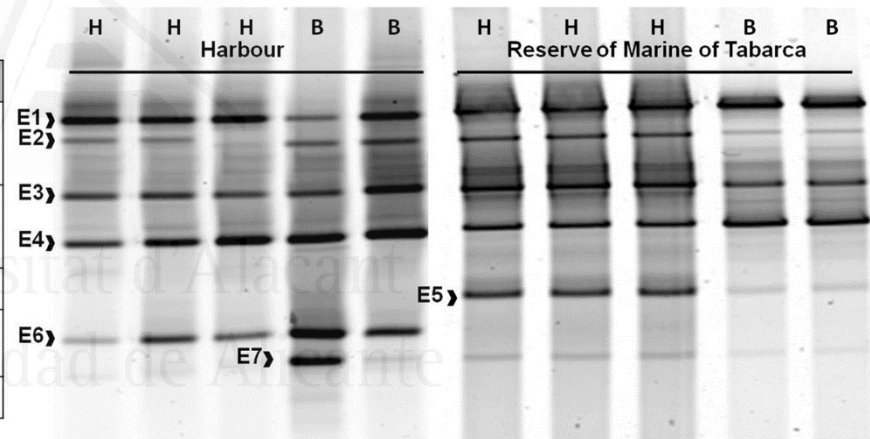
Figure A1. Example of DGGE profiles obtained with 18S primers (Eukarya), from tissue samples collected September from Alicante Harbour and Marine Protected Area of Tabarca, sequenced bands are marked with arrows

OTU	Blastn (identity)	Description (Geenbank)
I 1	JN558062 (100%)	*Symbiodinium sp. B2*
I 2		
I 3	JN558060 (100%)	
I 4	JN558061 (100%)	
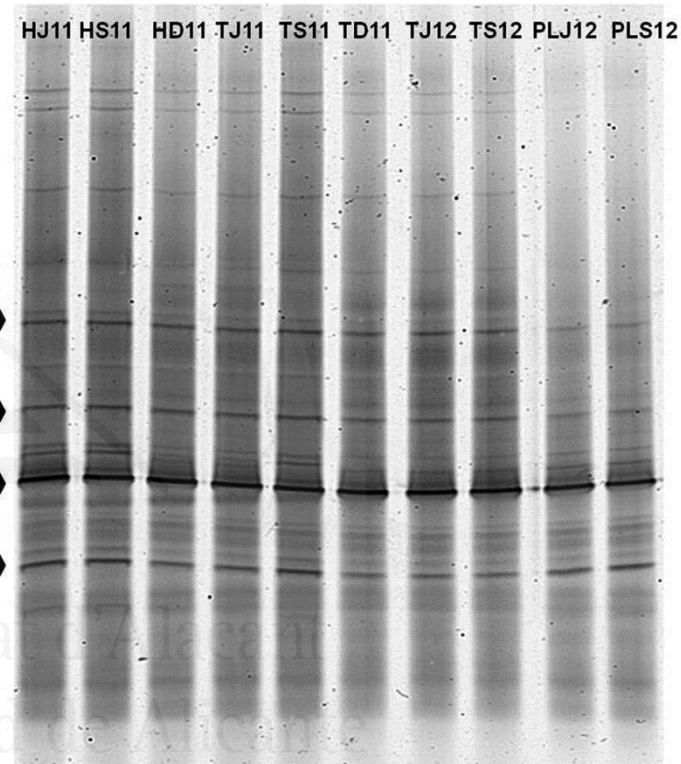
Figure A2. DGGE profiles obtained with ITS2 primers, from tissue samples collected in J11: June 2011, S11: September 2011, D11: December 2011, J12: June 2012 and S12: September 2012; from H: Alicante Harbour (Spain), T: Marine Protected Area of Tabarca (Spain), and PL: Pietra Ligure (Italy) sequenced bands are marked with arrows.
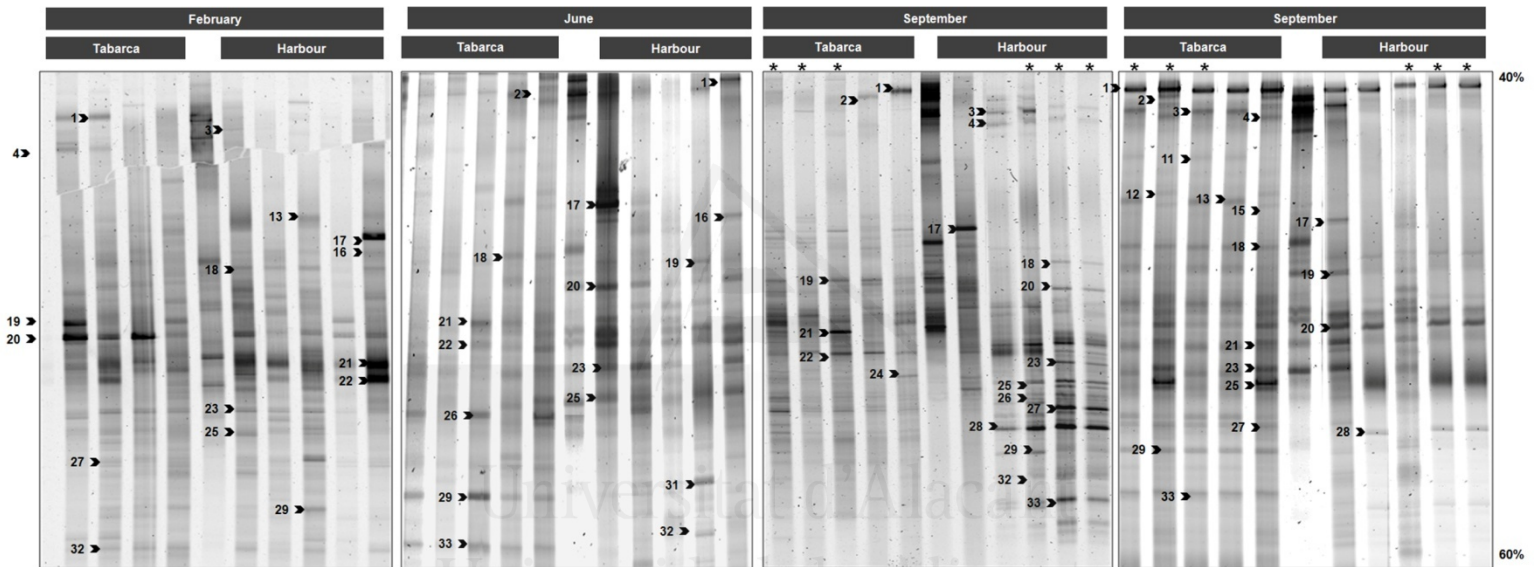
Figure A3. DGGE profiles obtained with 16S primers (Bacteria), from mucus samples. The arrows indicate bands that were excised and sequenced (See Table A1). (*) Samples with disease signs.

Figure A4. DGGE profiles obtained with 16S primers (Bacteria), from tissue samples. The arrows indicate bands that were excised and sequenced (See Table A1). (*) Samples with disease signs

Figure A5. DGGE profiles obtained with 16S primers (Bacteria), from skeletal matrix samples. The arrows indicate bands that were excised and sequenced (See Table A1). (*) Samples with disease signs

Table A.1. Bacterial 16S rRNA sequences of selected DGGE bands.

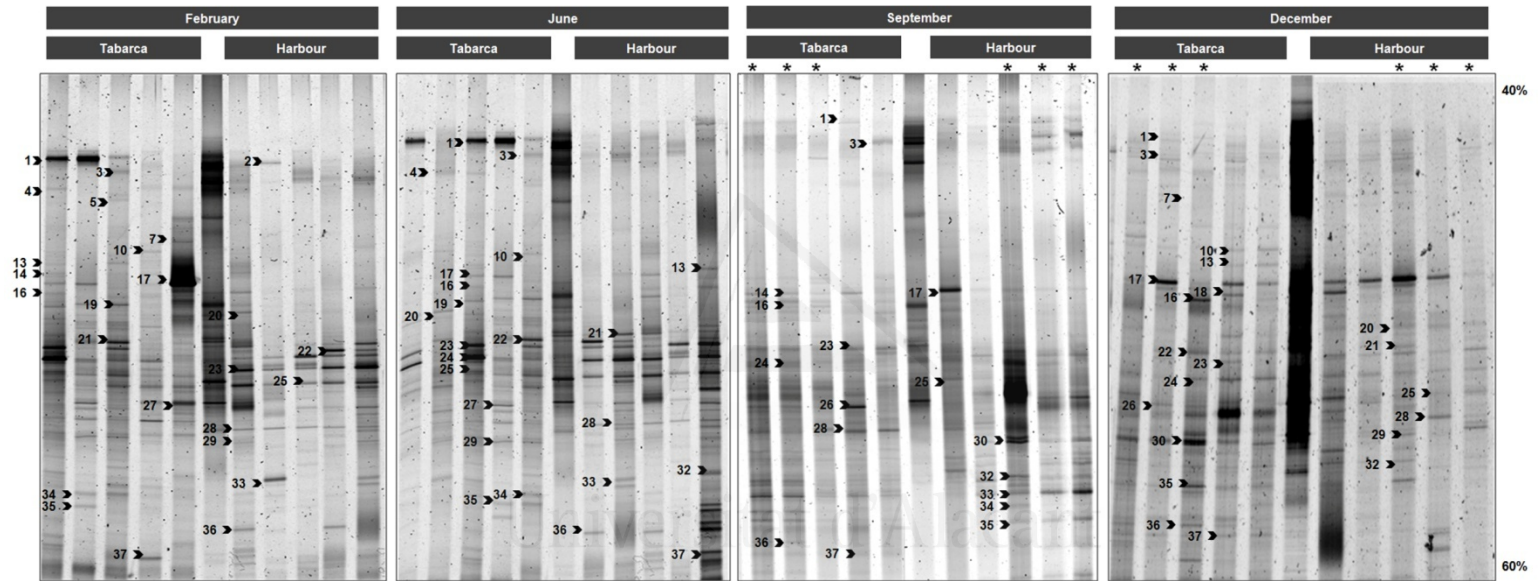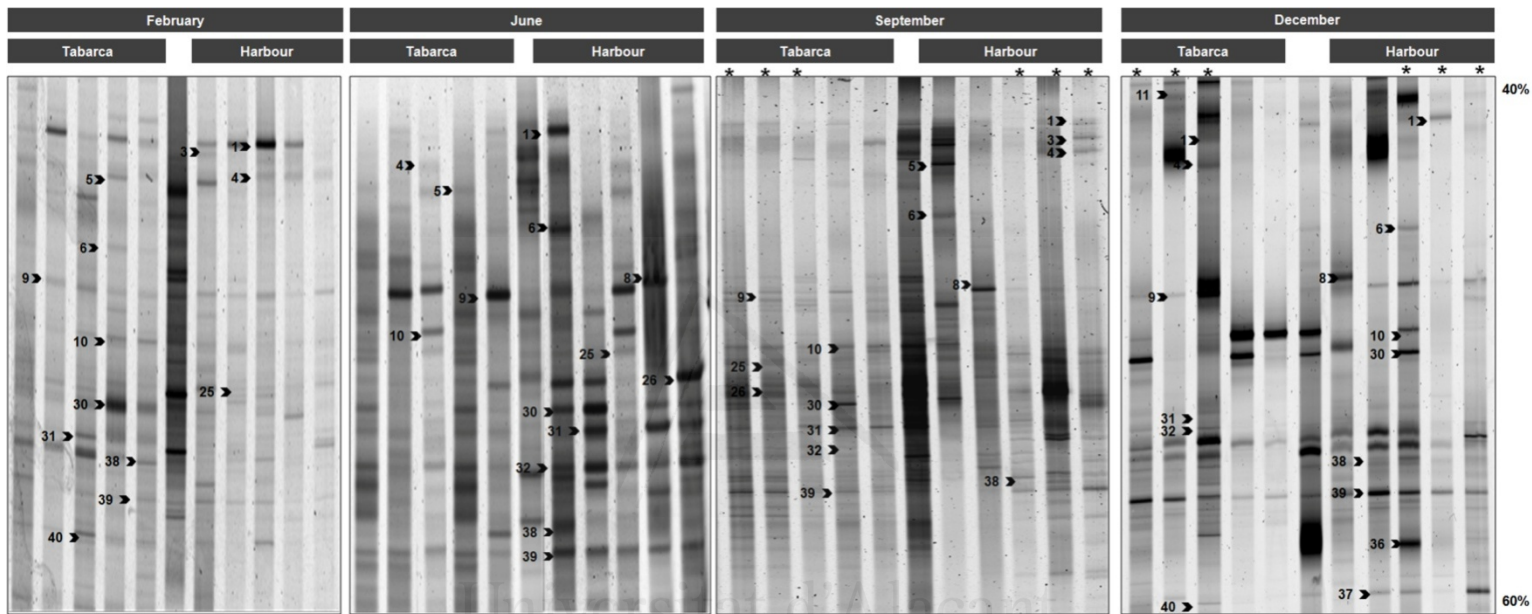| Band	OTUs	Phylogenetic group	BLASTn best hit	Source (% sequence identity, accesion no.)	Closest type strain(% sequence identity, accesion no.)
B1	BC1	Bacteroidetes (Cytophaga)	Uncultured Cytophaga	Marine sediments (88, AJ240979)	*Alkaliflexus imshenetskii Marinilabiliaceae* (86, AJ784993)
B2	BC2	Bacteroidetes (Flavobacteria)	Uncultured Bacteroidetes bacterium	Sponge-associated (99,AM259925)	*Vitellibacter aestuarii* (89, EU642844)
B3	BC3	Bacteroidetes (Cytophaga)	Uncultured Rhodothermaceae bacterium	Sponge-associated (100, JQ612356)	*Rhodothermus profundi* (92, FJ624399)
B4	BC1	Bacteroidetes (Cytophaga)	Uncultured Cytophaga	Marine sediments (89, AJ240979)	*Alkaliflexus imshenetskii Marinilabiliaceae* (86, AJ784993)
B5	BC4	Bacteroidetes (Cytophaga)	Uncultured Cytophaga	Hydrothermal vent chimney (97,FJ640814)	*Marivirga serice* (92, AB078081)
B6	BC1	Bacteroidetes (Cytophaga)	Uncultured Cytophaga	Marine sediments (88, AJ240979)	*Alkaliflexus imshenetskii Marinilabiliaceae* (86, AJ784993)
B7	OTU7				
B8	BC1	Bacteroidetes (Cytophaga)	Uncultured Cytophaga	Marine sediments (88, AJ240979)	*Alkaliflexus imshenetskii Marinilabiliaceae* (86, AJ784993)
B9	BC5	Bacteroidetes (Flavobacteria)	Coralibacter albidofladus	Hard coral (99, AB377124)	*Pseudozobellia thermophila* (93, AB084261)
B10	PL	Phototrophic plastid	Ochrosphaera sp.	Coral (99, X99077)	
B11	PL	Phototrophic plastid	Marine diatom	Coral (91, AY221721)	
B12	OTU10	Unidentified			
B13	CB1	Chlorobia	*Prosthecochloris Vibrioformis*	Marine aquaculture pond water (99, AM690798)	*Prosthecochloris Vibrioformis* (98, M62791)
B14	OTU11	Unidentified			
B15	OTU12	Unidentified			
B16	CX1	Chloroflexi	Uncultured bacterium	Sponge-associated (98,FJ900573)	*Bellilinea caldifistulae* (81,AB243672)
B17	CB1	Chlorobia	Uncultured Chlorobi bacterium	Marine aquaculture pond water (99, AJ428420)	*Chlorobium phaeobacteroides* (98, CP000492)
B18	CX1	Chloroflexi	Uncultured bacterium	Sponge-associated (99, JX06654)	

Table A.1. (Continued)

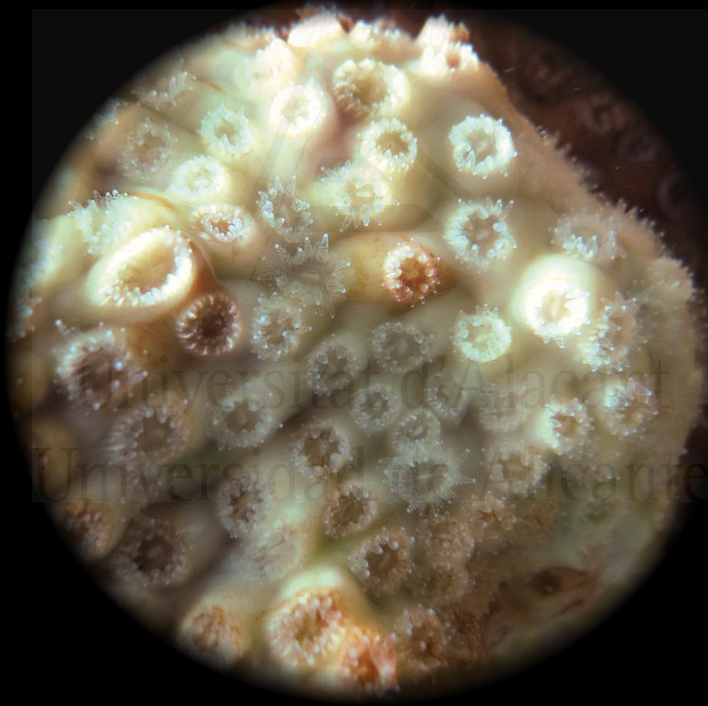| Band	OTU	Phylogenetic group	BLASTn best hit	Source (% sequence identity, accesion no.)	Closest type strain(% sequence identity, accesion no.)
B19	**AP1**	Alphaproteobacteria (Rhodobacterales)	*Pseudovibrio japonicus*	Seawater (100, AB246748)	*Pseudovibrio japonicus* (100, AB246748)
B20	**AB**	Acidobacteria	uncultured bacterium KM3-173-A5	Mediterranean Sea (93, EU686629)	
B21	**AP2**	Alphaproteobacteria (Rhodobacterales)	Uncultured bacterium	Seawater (89, HQ203925)	*Oceanicola batsensis* (99,AY424898)
B22	**AP1**	Alphaproteobacteria (Rhodobacterales)	Bacterium 1H215	Coral-associated (99, JF411476)	*Pseudovibrio denitrificans* (99, AY486423)
B23	**AP1**	Alphaproteobacteria (Rhodobacterales)	Rhodobacteraceae bacterium 1tb2	Coral associated (100,FJ952774)	*Pseudovibrio denitrificans* (99, AY486423)
B24	**AP3**	Alphaproteobacteria (Rhodobacterales)	Uncultured bacterium	Seawater (100,KC120680)	*Ruegeria atlantica* (99,D88526)
B25	**AP3**	Alphaproteobacteria (Rhodobacterales)	Ruegeria sp. JZ11ML32	Marine sponge (100,KC429919)	*Ruegeria conchae* (98,HQ171439)
B26	**AP3**	Alphaproteobacteria (Rhodobacterales)	Uncultured bacterium	Seawater (100,KC120680)	*Ruegeria atlantica* (99,D88526)
B27	**AP1**	Alphaproteobacteria (Rhodobacterales)	*Pseudovibrio denitrificans*	Abalone (99, HE584768)	*Pseudovibrio japonicus* (99, AB246748)
B28	**AP3**	Alphaproteobacteria (Rhodobacterales)	*Roseobacter sp.* 7m33	Soil (99,JQ66197)	*Ruegeria halocynthiae* (98, HQ852038)
B29	**DP1**	Deltaproteobacteria	Uncultured bacterium	Marine sediments (99,EU488075)	*Sandaracinus amylolyticus* (92, HQ540311)
B30	**NI1**	Nitrospirae	Uncultured bacterium	Sponge (99, EU035954)	*Nitrospira moscoviensis* (89,X822558)
B31	**FI1**	Firmicutes	*Clostridium sp.* AN-AS8	Sediments (97,FR872934)	*Defluviitalea saccharophila* (95,HQ020487)
B32	**AP4**	Alphaproteobacteria (Rhodospiralles)	Uncultured alpahaproteobacteria	Sponge-associated (98, JF824774)	*Nisaea nitritireducens* (94, DQ665839)
B33	**GP1**	Gammaproteobacteria (*Vibrio*nales)	*Photobacterium sp.* 1983	Phytoplankton culture (99, HF549205)	*Photobacterium frigidiphilum* (99,AY538749)

Table A.1. (Continued)

Band	OTU	Phylogenetic group	BLASTn best hit	Source (% sequence identity, accesion no.)	Closest type strain(% sequence identity, accesion no.)
B34	**GP1**	Gammaproteobacteria (*Vibrio*nales)	*Vibrio sp.* S3855	Sea weed (98, FJ457563)	*Vibrio orientalis* (97,X74719)
B35	**GP1**	Gammaproteobacteria (*Vibrio*nales)	*Vibrio campbellii*	(110, X56575)	*Vibrio rotiferianus* (100, 316187)
B36	**CY1**	Cyanobacteria	*Oscillatroia corallinae*	(99, X84812)	*Loriellopsis cavernicola* (93, HM748318)
B37	**CY2**	Cyanobacteria	Filamentous cyanobacterium	Coral (96, EU196366)	*Prochlorothrix hollandica* (89, AM709625)
B38	**DP2**	Deltaproteobacteria	Uncultured microorganism	Sponge-associated (100,JN002375)	*Desulfonatronum thiosulfatophilum* (85,FJ469578)
B39	**DP3**	Deltaproteobacteria	Uncultured microorganism	Sponge-associated (100,JN002375)	*Desulfonatronum thiosulfatophilum* (85,FJ469578)
B40	**GP2**	Gammaproteobacteria (Pseudomonadales)	*Psychrobacter glacincola*	Sea ice (99, U85879)	*Psychrobacter piscatorii* (99, AB453700)

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Figure A6. Consensus phylogenetic tree of 16S rRNA gene sequences. The final tree topology is based on three different reconstructions calculated using Neighbor Joining with the Jukes Cantor correction, Maximum Likelihood (RaxML, model GTRGAMMA) and Maximum Parsimony methods as implemented in the ARB software. Partial sequences were added a posteriori without affecting the underlying topology. Scale bar indicates estimated sequence divergence. Color labels indicate time of sampling (blue for Winter, red for Spring, green for Summer and yellow for Autumn), location (blue for Harbor and red for Tabarca), niche (blue for tissue, red for mucus and green for water) and bleaching status (blue for healthy and red for unhealthy corals).

Figure A7. Phylogenetic tree based on the neighbor-joining method, using the 16S rRNA gene, showing the different clades of vibrios. Bootstrap percentages after 100 simulations are shown. Color labels indicate locality (dark green for Italy and light green for Spain); sampling time (yellow for June and red for September); habitat (blue for water, pink for *Oculina patagonica* and purple for *Cladocora cæspitosa*); and coral status (white for healthy and black for unhealthy corals).