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Variability of planktonic and epiphytic vibrios in a coastal environment affected by *Ostreopsis* blooms

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Summary: Vibrios include several pathogenic bacteria that occur in aquatic environments. The presence of *Vibrio* has been assessed in many ecosystems by culture-based techniques. However, little is known on the contribution of vibrios in the sea, especially in areas subject to harmful algal blooms. A preliminary study in Sant Andreu de Llavaneres beach (NW Mediterranean) showed the presence of some *Vibrio* species during a recurrent bloom of the harmful benthic dinoflagellate *Ostreopsis* cf. *ovata*. In order to establish the importance of vibrios in a coastal area of the NW Mediterranean and to study the association with the dinoflagellate, we conducted a sampling monitoring for one year to quantify the concentration of vibrios both in the water (free-living and attached to particles) and in the epiphytic community of macroalgae. The aims were 1) to evaluate the relative abundance of *Vibrio* in the epiphytic and in the planktonic bacterial community, 2) to assess the percentage of free-living and attached vibrios in the planktonic community, and 3) to determine whether the presence of vibrios is associated with the blooms of the toxic dinoflagellate *Ostreopsis o*r with other environmental parameters. For this purpose, a CARD-FISH molecular probe was applied for the specific detection of bacteria belonging to the genus *Vibrio*. Cells were quantified and the abundance of both particles and bacteria attached to particles were assessed. The maximum *Vibrio* concentration ($1.3x10^4$ cells ml^{-1} and $1.4x10^6$ cells g^{-1} FW, for planktonic and epiphytic samples, respectively) was detected in September. Free-living vibrios contributed 0.38±0.24% to the total free-living planktonic community and 1.12±0.28% to the epiphytic bacterial community. However, their contribution was particularly high in the planktonic community attached to particles (17.37±20.49%). Although in the planktonic community *Vibrio* was found preferentially free-living (82.63±20.01%), particles are a niche for vibrios, since in particles vibrios may represent up to 72% of the total attached bacterial community. Abundance of planktonic *Vibrio* was correlated with *Ostreopsis* concentration and it is likely that they play a role in the wound infections suffered by beach users during the bloom.

Keywords: *Vibrio*; bacteria; particles; Mediterranean; HAB; dinoflagellates.

Variabilidad de vibrios planctónicos y epifíticos en un ambiente costero afectado por proliferaciones de *Ostreopsis*

Resumen: El género *Vibrio* incluye a varias bacterias patogénicas que se encuentran en ecosistemas acuáticos. La presencia de *Vibrio* se ha estimado en muchos ecosistemas mediante técnicas basadas en cultivos. Sin embargo, se conoce poco sobre la contribución de vibrios en el mar, especialmente en áreas afectadas por proliferaciones algales nocivas. Un estudio preliminar en la playa de Sant Andreu de Llavaneres (Mediterráneo NO) mostró la presencia de algunas especies de Vibrio durante una proliferación recurrente del dinoflagelado béntico nocivo *Ostreopsis* cf. *ovata*. Para poder establecer la relevancia de los vibrios en un área costera del Mediterráneo NO y estudiar su asociación con el dinoflagelado, realizamos un muestreo de monitoreo durante un año para cuantificar la concentración de vibrios tanto en el agua (de vida libre y adheridos a partículas) y en la comunidad epifítica de macroalgas con los objetivos de 1) evaluar la abundancia relativa de *Vibrio* en la comunidad bacteriana tanto planctónica como epifítica, 2) estimar el porcentaje de vibrios de vida libre y adheridos a partículas en la comunidad bacteriana planctónica y 3) determinar si la presencia de vibrios está relacionada con las proliferaciones del dinoflagelado *Ostreopsis* o con otros parámetros ambientales. Para este propósito, se aplicó una sonda molecular de CARD-FISH para la detección específica de bacterias pertenecientes al género *Vibrio*. Se cuantificaron las células y también la abundancia de partículas y de las bacterias adheridas a estas partículas. La máxima concentración de *Vibrio* ($1.3x10^4$ cels ml^{-1} y $1.4x10^6$ cels g^{-1} PF, para muestras planctónicas y epifíticas, respectivamente) fue detectada en Septiembre. Los vibrios de vida libre contribuyeron un 0.38±0.24% al total de la comunidad bacteriana de vida libre y un 1.12±0.28% a la comunidad bacteriana epifítica. Sin embargo, su contribución fue especialmente elevada en la comunidad bacteriana adherida a partículas (17.37±20.49%). Aunque en la comunidad planctónica *Vibrio* se encontraba preferentemente no adheridos a partículas (82.63±20.01%), las partículas constituyen un nicho para vibrios, ya que pueden llegar a representar hasta un 72% de la comunidad bacteriana adherida a partículas. La abundancia de *Vibrio* en el plancton se correlacionó con la concentración de *Ostreopsis*, y es posible que éstos jueguen un papel en las infecciones de heridas que sufren los bañistas durante las proliferaciones algales.

Palabras clave: *Vibrio*; bacterias; partículas; HAB; dinoflagelados.

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INTRODUCTION

Vibrio is a genus of heterotrophic bacteria that is widely spread in the ocean. It includes several potential pathogens such as *V. cholerae*, and bacteria associated with food-borne diseases such as *V. alginolyticus*, *V. parahaemolyticus* and *V. vulnificus* (Thompson et al. 2004), and with other infective syndromes such as otitis, pharyngitis and wound infections (De Paola et al. 1990, Mizunoe et al. 2000). Therefore, there has been considerable economic and health interest in determining their presence in coastal ecosystems.

Several studies have associated the presence of *Vibrio* with dinoflagellates (Mourino-Perez et al. 2003, Eiler et al. 2006). *Ostreopsis* is a genus of epiphytic and planktonic dinoflagellates that is found mainly attached to macroalgae but also free-living in seawater (Vila et al. 2001). It attaches to substrates by means of a rusty-brown mucilage that it secretes (Honsell et al. 2013). The genus is known for its ability to produce potent biotoxins known as palytoxins and analogues (Lenoir et al. 2004) and is widespread in the Mediterranean (Mangialajo et al. 2011 and references therein). At Sant Andreu de Llavaneres beach, NW Mediterranean, a recurrent massive bloom is found during summer, formed basically by *Ostreopsis* cf. *ovata* but also accompanied on some occasions by *Ostreopsis* cf. *siamensis* (Penna et al. 2005, Battocchi et al. 2010, Vila et al. 2012b). These blooms have often been related to respiratory symptoms in beach users or persons taking a nearshore walk in Mediterranean countries since the late 1990s (see Vila et al. 2016) and are thought to have been the cause of a massive mortality of benthic invertebrates in Llavaneres in August 1998 (Vila et al. 2008). The mortality of benthic invertebrates and fishes (Simoni et al. 2004, Shears and Ross 2009) has been documented at other locations, as have other noxious effects, such as human food-borne intoxications (Tubaro et al. 2011).

The bacterial community associated with harmful algal blooms (HABs) has been studied due to its possible role in the toxicity of dinoflagellate blooms (Gallacher et al. 1997, Lu et al. 2000, Sala et al. 2005, Kodama et al. 2006, Barlaan et al. 2007). This contribution might be due to bacterial lysis of algal cells or the active release of bacterial toxins (Lenes et al. 2013).

Studies of the microbial community associated with *Ostreopsis s*tarted some decades ago (Tosteson et al. 1989, Ashton et al. 2003, among others) and were conducted with cultures. They highlighted the need of symbiotic bacteria for the growth of *Ostreopsis* (Ashton et al. 2003); the bacterial community in *Ostreopsis* cultures was dominated by genus *Vibrio* or *Alteromonas,* in the class of γ-proteobacteria and by the complex Cytophaga-Flavobacter-Bacteroidetes (Tosteson et al. 1989, Ashton et al. 2003, Pérez-Guzman et al. 2008).

A preliminary study aimed at characterizing the bacterial community associated with field *Ostreopsis* blooms in Llavaneres beach (Borrull 2011) detected the presence of three OTUS of the genus *Vibrio* in epiphytic samples during the bloom of summer 2010: *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Vibrio tubiashi*. However, the technique used (DGGE) at that time could not provide information on their abundance. Therefore, the present study aims at determining the factors that drive the abundance of vibrios in the planktonic and epiphytic community and their potential contribution to the toxicity of the bloom.

Most studies on the occurrence of *Vibrio* in natural environments have been based on culture-dependent techniques, but some *Vibrio* may be unable to grow on conventional media, so molecular techniques are more appropriate. Although several studies have focused on the detection of the genus or certain species, data on the abundance of *Vibrio* in seawater are scarce and restricted to the Baltic and Skagerrak Seas (Eiler et al. 2006), the Arabian Sea (Gallacher et al. 1997) and the North Sea (Oberbeckmann et al. 2012). Although *Vibrio* seems to be an important pathogenic agent in the NW Mediterranean (e.g. Canigral et al. 2010, Lopez-Joven et al. 2015), to the best of our knowledge no information on abundance of planktonic *Vibrio* (using culture independent methods) is available for Mediterranean waters.

In the present study we carried out a sampling of both the planktonic and the epiphytic bacterial community in Sant Andreu de Llavaneres beach (NW Mediterranean), which is regularly affected by blooms of *Ostreopsis* in the summer months. Borrull (2011) reported on the importance of epiphytic *Vibrio* in the summer months, but did not assess its abundance. We therefore hypothesize a higher relevance of *Vibrio* in the epiphytic than in the planktonic communities, and also that *Vibrio* abundance is associated with *Ostreopsis* blooms.

Traditionally, vibrios have been generally thought to be associated with animals, probably because they were investigated only in intoxicated tissues (Thompson et al. 2004), and they have been found attached to several marine organisms (see Takemura et al. 2014 for a review) and also phytoplankton (e.g. Tamplin et al. 1990, Neogi et al. 2012). However, in some recent studies both free-living lifestyles or communities associated with aggregates have been reported for *Vibrio* (Lyons et al. 2007, Froelich et al. 2013, Szabo et al. 2013). A second hypothesis of our study is therefore that *Vibrio* is found more in particles than free-living in plankton.

Due to the direct and indirect effects on human health, *Vibrio* can impact the local economy by affect-

ing tourism, fisheries and aquaculture. Therefore, the prevalence of *Vibrio* spp. in coastal environments is of concern and the factors that regulate its dynamics need to be elucidated.

MATERIALS AND METHODS

Study area and sample collection

The study was carried out in Sant Andreu de Llavaneres beach (41°33.130'N, 2°29.540'E) in the NW Mediterranean from January to December 2010. The area is a fossil rocky beach that is highly colonized by different genera of macroalgae of the genera *Corallina*, *Jania*, *Halopteris*, *Dyctyota* and *Padina,* among others. Sampling was done monthly in winter and spring and the frequency was increased during the summer and autumn months, when *Ostreopsis* was detected. Both seawater and macrophyte fragments were collected in each sampling.

Environmental parameters in the water

Temperature and salinity were measured with a WTW Model LF 197 microprocessor conductivity meter. Chlorophyll-*a* determination followed the method in Yentsch and Menzel (1963). Briefly, 60 ml of surface water samples were filtered through GF/F glass fibre filters and frozen at –20°C until analysis. Samples were extracted in 6 ml of 90% acetone for 24 h at 4°C, and chlorophyll-*a* was measured with a Turner Designs fluorimeter. For inorganic nutrient analyses, 60 ml water samples were taken and frozen (–20°C). Analyses of dissolved inorganic nutrients (NO_3, NO_2, NH_4, PO_4 and SiO_4) were performed as described by Grasshoff et al. (1983) with a Seal Analytical AA3 continuous flow analyser (Bran+Luebbe).

Bacterial and particle abundance

Samples from both seawater and macroalgae were collected. Fragments of macroalgae (generally *Corallina*) of 2-7 g were placed in plastic tubes and filled up to 50 ml with in situ seawater filtered through 0.2 μm pore size filters. Water samples were collected using polyethylene acid-rinsed bottles. All samples were carried to the lab in the dark for further processing within two hours of collection. Once in the lab, the macroalgae solution was diluted with in situ filtered (0.2 μm) seawater up to 200 ml. The bottle was also vigorously shaken with a vortex mixer to detach organisms from the mucilage. The dilution was finally filtered through a mesh (140-200 μm) and this final solution was used for further processing. Both macroalgae and seawater samples were processed to assess particle size and density as well as abundance of both total bacteria and specific groups.

Bacterial and particle counts were assessed microscopically after staining with DAPI (4',6-diamino-2-fenilindol) according to Porter and Feig (1980). Briefly, after fixing samples with glutaraldehyde (3.6% final concentration), samples were filtered through a black Millipore filter with 0.2 μm pore size. The filters were stained with DAPI (final conc. 1 mg ml^{-1}) and observed under an epifluorescence microscope Olympus BX61. Discrete fields were counted for bacterial abundance, whereas for particles the filters were scanned with several transects in which the length and width of the particles were also measured. Particle size was calculated by assuming a square size.

Catalysed reporter deposition-fluorescence in situ hybridization (CARD-FISH) was used for the analysis of the abundance of single bacterial groups. We followed the protocol of Pernthaler et al. 2002), which is similar to that in Alonso-Sáez et al. (2007) and Ruiz-González et al. (2012). We used two horseradish peroxidase-labelled probes to identify bacterial groups in the samples: GAM42, which targets most of the γ-proteobacteria (55% formamide; Manz et al. 1992), and VIB572a, which targets most of *Vibrio* (50% formamide; Huggett et al. 2008). The probe VIB572a covers 15 different Vibrio strains that include *V. alginolyticus, V. parahemolyticus, V. vulnificus, V. cholera*, and also four *Photobacterium* strains (Huggett et al. 2008, Fig. 1). Briefly, 4.5 ml of seawater or of the epiphyte solution were fixed overnight with paraformaldehyde (1%) at 4°C. Samples were gently filtered on 0.2-μm Millipore polycarbonate filters. Filters were permeabilized with lysozyme (37°C, 1 h) and achromopeptidase (37°C, 30 min) before hybridization. Hybridizations were carried out overnight at 35°C with a percentage of formamide of 50% and 55% for GAM42a and VIB572a, respectively. The Beta42a (Manz et al. 1992) antisense probe was used as a negative control. For amplification, we used tyramide labelled with Alexa 488. Counterstaining of CARD-FISH preparations was done with DAPI (final concentration 1 mg ml^{-1}). DAPI and FISH-stained cells were counted. For γ-proteobacteria, between 500 and 1,000 positive cells were counted manually in a minimum of 30 fields. For *Vibrio*, due to the lower concentration, between 1 and 4 transects of the filters were scanned.

Phytoplankton abundance

Seawater samples for enumeration of planktonic *Ostreopsis* were fixed with lugol. An aliquot of 10-50 ml was placed in a counting chamber for 24 h, and for enumeration of the phytoplankton cells an area of the sample was scanned at 63-400× depending on cell density using a Leika-Leitz DM-II inverted microscope. For the abundance of epiphytic *Ostreopsis,* fragments of macroalgae (generally *Corallina*) of 10-20 g were placed in plastic bottles and filled up to 120 ml with in situ GF/F filtered seawater. They were shaken vigorously for 1 min to detach organisms from the macroalgae, and the solution was filtered through a mesh (140 μm) in order to separate the macroalgae and the bigger organisms. The samples were then fixed with Lugol's solution. An aliquot of 1-10 ml was placed in a Sedgwick-Rafter or Utermöhl chamber and *Ostreopsis* cells were counted as indicated above. The abundance of epiphytic phytoplankton was expressed as cell per gram of fresh weight of macroalgae.

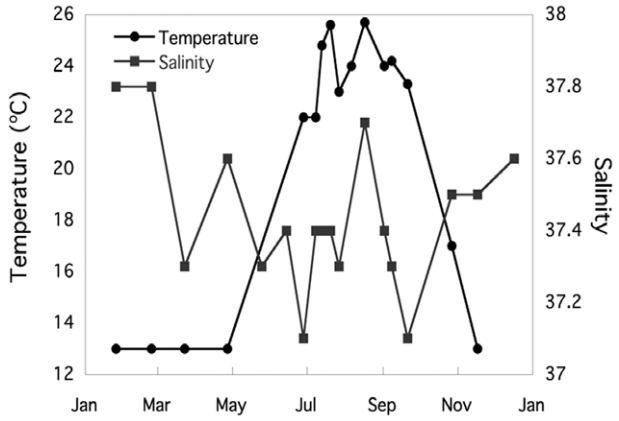

Fig. 1. – Temperature and salinity in the water of Sant Andreu de Llavaneres Beach in 2010.

Statistical analysis

Pearson's correlations were performed with STATISTICA software, version 8.0 (StaSoft).

RESULTS

Environmental parameters of the water

The thermohaline characteristics of the waters at Sant Andreu de Llavaneres in 2010 differed over the year (Fig. 1), with minimum temperatures in winter (13.0°C) and maximum temperatures in summer (25.7°C). Salinity ranged between 37.1 and 37.8, with no clear seasonal pattern. Chlorophyll-*a* concentrations ranged from 0.3 μg l^{-1} in July to 8.1 μg l^{-1} in November (data not shown).

Abundance of *Ostreopsis*

Abundance of *Ostreopsis* cells (both epiphytic and planktonic) was below the detection limit during the first part of the year (Fig. 2) and started increasing in June-July to achieve a first peak in August and a second peak in September-October. A huge peak of *Ostreopsis* that turned the water brown-red was found in September (9.9×10^6 cells l^{-1}), when epiphytic *Vibrio* also achieved a peak of 1.4×10^6 cells g^{-1} FW.

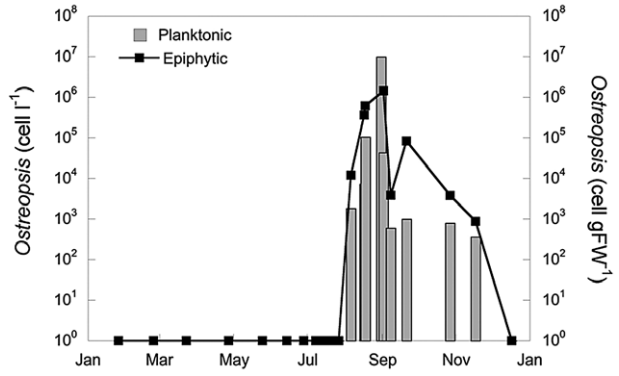

Fig. 2. – *Ostreopsis* sp. abundance in the plankton and in the epiphytic community of the macrophytes in 2010.

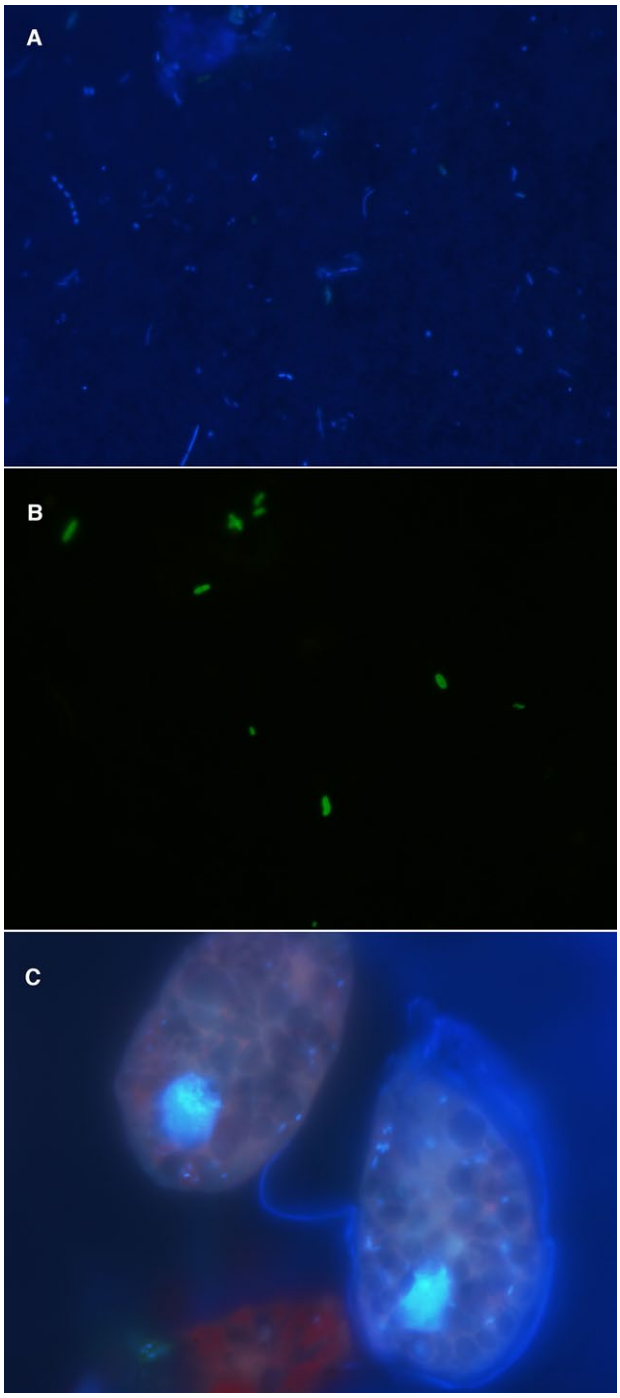

Fig. 3. – Epifluorescence microscopy of planktonic bacteria stained with DAPI (A), hybridized with the Vibrio CARD-FISH probe after amplification with tyramide-Alexa488 (B) and stained with DAPI and attached to an *Ostreopsis* cell (C).

Total bacterial and *Vibrio* abundance

Both bacterial and *Vibrio* abundance were assessed by epifluorescence microscopy (see photos in Fig. 3). Planktonic bacterial concentrations achieved their highest values in summer and autumn, with a maximum in September (1.5×10^6 cells ml^{-1}; Fig. 4A). Epiphytic bacterial concentrations also showed higher values between June and October and peaked in August (5.3×10^8 cells g^{-1} FW), but showed higher variability (Fig. 4B).

Planktonic vibrios showed a similar trend to that of total planktonic bacterial concentration, with higher concentrations in late summer and autumn, and a peak in September of 1.3×10^4 cells ml^{-1}. However, the range of percentage contribution to total bacterial concentration was low and varied between 0.11 and 0.86% (mean 0.38%), with the highest values in summer and winter (Fig. 4A).

The trend of epiphytic vibrios was similar to that of the total epiphytic bacteria. Concentrations varied between 7.1×10^4 and 1.1×10^7 cells g^{-1} FW, and the highest peaks were observed in July and August. The percentage contribution of *Vibrio* to total epiphytic bacteria was also low but higher than in the plankton (mean of 1.13%), with peaks in July of 4.3% (Fig. 4B).

Bacteria attached to particles

Seawater contained between 11 and 417 particles ml^{-1}, with higher concentrations between August and September (Fig. 5). A similar pattern was observed for the number of bacteria attached to particles, which varied between 380 and 8285 cells ml^{-1}, which corresponded to between 0.14 and 4.0% of total bacteria, with a mean of 0.8% (data not shown).

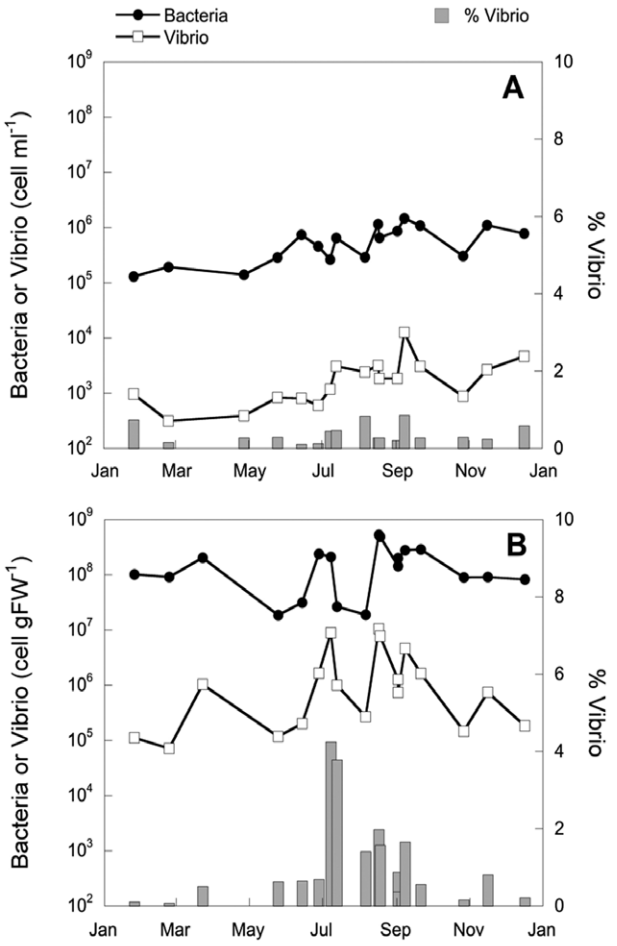

Fig. 4. – Total bacterial and *Vibrio* abundance, and *Vibrio* percentage of total cells in the plankton (free-living + attached) (A) and epiphytic community (B) in 2010.

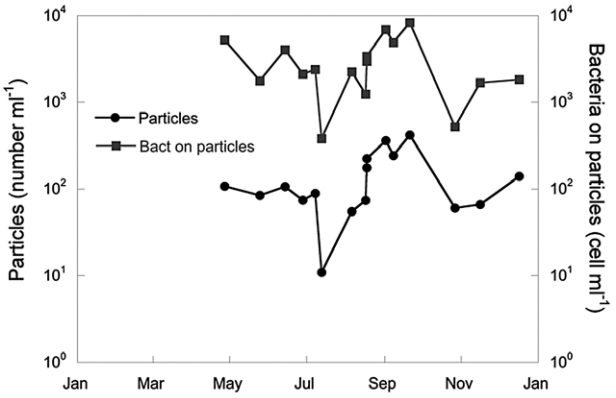

Fig. 5. – Particle abundance and bacteria attached to particles in 2010.

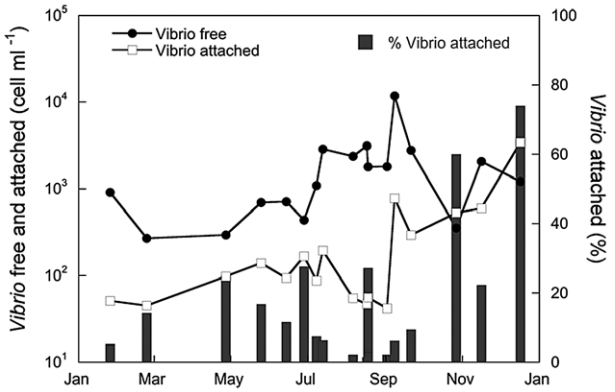

Fig. 6. – Concentration of planktonic free-living and attached *Vibrio*, and percentage contribution of vibrios to the attached bacterial community.

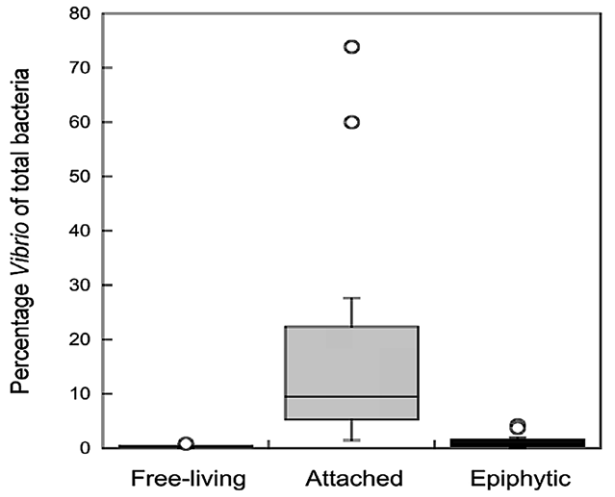

Fig. 7. – Percentage contribution of *Vibrio* to total planktonic (free-living or attached to particles) and epiphytic bacterial communities.

Vibrio cells appeared to be mostly free-living (353-11851 cells ml^{-1}), with higher values in summer and autumn (Fig. 6) representing a mean of 82.10±20.01% of the total concentration of planktonic vibrios. Concentration of vibrios attached to particles was low (42-3451 cells ml^{-1}), and the highest values were achieved

Table 1. – Pearson correlation coefficients between epiphytic (E) and planktonic (P) concentration of bacteria, vibrios and particles and selected abiotic and biotic variables at Sant Andreu de Llavaneres in 2010; n=13-17. Significant correlations ($p<0.05$) are indicated in bold. TIN, total inorganic nitrogen

		Temp	Salinity	Chl *a*	NO_3+NO_2	NH_4	TIN	Phosphate	Silicate	*Ostreopsis*
Bacteria	E	0.09	–0.10	0.23	0.14	0.12	0.10	0.08	–0.10	0.38
	P	**0.64**	–0,43	0.11	–0.43	–0.24	**–0.48**	0.11	–0.37	**0.57**
Vibrio total	E	**0.62**	–0.37	–0.17	–0.34	0.15	–0.20	–0.18	–0.38	0.36
	P	**0.66**	0.23	0.15	–0.25	–0.21	–0.33	0.02	–0.03	**0.47**
Vibrio free	P	**0.64**	–0.24	–0.00	–0.32	–0.13	–0.33	–0.55	–0.09	**0.52**
Vibrio attached	P	–0.03	–0.24	0.42	–0.09	–0.18	–0.07	0.21	0.01	–0.06
% γ-Proteobacteria	E	0.29	–0.25	–0.50	–0.04	–0.17	–0.07	–0.19	0.08	–0.28
	P	0.09	–0.12	–0.20	0.05	**0.57**	0.14	0.14	–0.31	–0.25
%Vibrio	E	**0.70**	–0.44	–0.39	**–0.55**	0.11	–0.34	–0.33	–0.44	0.22
	P	0.52	–0.06	0.15	–0.11	–0.17	–0.18	0.01	0.19	0.32
%Vibrio attached	P	–0.45	–0.18	0.35	0.24	–0.06	0.13	0.22	0.15	–0.37
Particles ml^{-1}	P	0.29	–0.19	0.18	–0.10	0.06	–0.09	0.06	–0.33	**0.54**

towards the end of the year, in autumn. In fact, from October to December the abundance of free-living vibrios and vibrios attached to particles was quite similar. These differences in the period of dominance of the free vs attached *Vibrio* population deliver large differences in the percentage contribution of *Vibrio* to the attached bacterial community, which varied from 1.5% in the summer months to around 12%-25% in spring, and up to 74% in December.

Mean percentage contribution of vibrios to the total bacterial community varied among the communities (Fig. 7). For the planktonic community, free-living vibrios accounted for a mean of 0.38% (range 0.11-0.86%) of total free-living bacteria. The epiphytic community on the macrophyte contained a larger percentage of vibrios (mean 1.13%; range: 0.07-4.25%). The highest contribution of *Vibrio* was found in the bacterial community attached to particles in the plankton (mean 17.32%; range:1.47-73.90%).

Correlations among parameters

Pearson's correlation coefficients between bacterial and particle parameters and several physico-chemical and biological parameters are shown in Table 1. Abundance of both total and free-living planktonic vibrios correlated positively with *Ostreopsis* concentration. *Ostreopsis* also correlated positively with the number of particles in the water. Abundance of total epyphitic and planktonic vibrios, and percentage of epiphytic vibrios of the total bacterial community showed a significant positive correlation with temperature. Percentage of γ-proteobacteria, the taxonomical group to which *Vibrio* belongs, showed a significant positive correlation with ammonia. Abundance of planktonic bacteria showed a significant positive correlation with both temperature and *Ostreopsis* concentration, and a significant negative correlation with total inorganic nutrient concentration.

DISCUSSION

Epiphytic and planktonic blooms of the dinoflagellate *Ostreopsis* cf. *ovata* occur recurrently at several localities of the Mediterranean Sea (Mangialajo et al. 2011). The relationship of these blooms with bacteria has rarely been analysed. Surprisingly, bacterial concentrations in Llavaneres were very similar to those found during the year in Blanes Bay, a coastal location on the Catalan coast (Alonso-Sáez et al. 2008), despite the fact that the sampling station at Llavaneres is shallower (50 cm depth) and close to anthropogenic influences, and has higher chlorophyll concentrations and occasional mucilage loads coinciding with the *Ostreopsis* blooms.

Is the epiphytic community more enriched in vibrios than the planktonic bacterial community?

Our study shows a temporal variation in the abundance of *Vibrio* spp. in a coastal marine area of the NW Mediterranean in both the epiphytic and planktonic communities, with higher concentrations in the warm period. With the specific CARD-FISH probe, we have provided the first values of the concentration of vibrios in Mediterranean coastal waters. However, we are aware that the coverage of our probe may be incomplete (see details in Huggett et al. 2008) and is not specific enough to monitor the different *Vibrio* populations that may appear at different stages of the year cycle (Thompson et al. 2005) The percentages of total *Vibrio* in the plankton detected at Sant Andreu de Llavaneres (mean 0.38%, maximum 0.9% in August) are slightly lower than the values detected recently in the North Sea with CARD-FISH (2%; Oberbeckmann et al. 2012), higher than those in the Baltic Sea detected with qPCR (0.002%-0.015%; Eiler et al. 2006), but in the same range as those in the Arabian Sea (0.2%-1.3%; Asplund et al. 2011).

The mean contribution of epiphytic vibrios was three times that of planktonic bacteria (mean 1.1%, with a peak of 4.2% of total epiphytic bacterial community). Presence of *Vibrio* on marine macroalgae has been well documented and *Vibrio* is the dominant culturable bacteria in several red algae (Takemura et al. 2014 and references therein). Apparently, macroalgae can serve as a refuge for vibrios, especially non-native macroalgal species (Gonzalez et al. 2014). Together with temperature, trophic resource availability is one of the most important controls of *Vibrio* abundance (Oberbeckman et al. 2012, Cavallo and Stabili 2004). Increased nutrient loads at this coastal station might have enhanced the growth of γ-proteobacteria and also of *Vibrio*, although a correlation between the latter and inorganic nutrients could not be found.

This study contributes to the knowledge that vibrios have a small representation in the planktonic bacterial community. However, their contribution in the epiphytic community is three times higher. Although numbers of planktonic vibrios are low, their biomass can be up to 100 times higher than that of SAR11, and they are known to play an important role in the ecosystem through biodegradation, nutrient regeneration and biogeochemical cycling (for example, as chitin degraders) (Takemura et al. 2014).

Are vibrios found preferentially attached to particles?

Vibrios have been detected on a large variety of biological surfaces, especially animals (Thompson et al. 2004, Baffone et al. 2006, Main et al. 2015), and are also associated with various types of organic particles of non-animal origin (Lyons et al. 2007, Froelich et al. 2013). Recently, there has been evidence that vibrios can remain free-living (Mourino-Perez et al. 2003, Worden et al. 2006, Eiler et al. 2006), although little is known on the factors determining whether they remain free-living versus particle-attached (Takemura et al. 2014). Whereas attachment to biofilms may provide a refuge from protozoan predation, attachment to particles may increase their susceptibility to being grazed by macrofauna but also increase their dispersal.

In the summer months, abundance of particles in the water showed a four-fold increase. In this study we frequently observed fragments of *Ostreopsis* thecae, and also mucilage associated with the dinoflagellates, which contributed to the pool of particles. Indeed, concentration of particles correlated with abundance of *Ostreopsis* in the water. The mucilage is rich in carbohydrates (Mestre pers. comm.) and the thecae in cellulose, both good sources of organic carbon for bacterial growth, which may favour particle colonization.

The contribution of plankton-attached vibrios to the total attached bacterial community was much higher (mean 4.0%, maximum 73.9%) than that of free-living vibrios to total free-living bacteria (mean 0.8%, maximum 17.37%). Particles concentrate more than half of the *Vibrio* population in the water, especially in autumn coinciding with the decaying *Ostreopsis* bloom. It is noteworthy that the lowest percentages of attached *Vibrio* are found in summer, when the concentration of attached bacteria is higher. Our data on the high percentage of free-living planktonic *Vibrio* (mean 83.8%, range 26.1%-99.5%) contribute to recent knowledge that, contrary to early studies, places *Vibrio* as a predominantly free-living bacteria, with comparable percentages (73-89%) to those in the Baltic Sea (Eiler et al. 2006).

Is abundance of vibrios linked to *Ostreopsis* concentration?

Although studies have shown a correlation between vibrios and chlorophyll-*a* concentration (e.g. Asplund et al. 2011), we found no relation between them in our study. Quantitative and qualitative differences in phytoplankton species composition may lead to pronounced differences in bacterioplankton species composition (Pinhassi et al. 2004). In particular, the relationship between vibrios and specific groups of phytoplankton is controversial, and some authors suggest a preferential association with dinoflagellates (Eiler et al. 2006), while others suggest a minor link between the two groups (Main et al. 2015). Our data show a positive relation between the abundances of vibrios and of the dinoflagellate *Ostreopsis* at Llavaneres. Such a relationship between the two genera was already established in early studies, when *Vibrio* sp. was isolated as an important bacteria in *Ostreopsis* cultures (Tosteson et al. 1989).

Bacterial composition during HABs is subject to study due to the possible contribution to the toxicity of the blooms (Groben et al. 2000); for example, toxigenic bacteria may contribute half the algal-associated PSP toxin levels in *Alexandrium* cultures, provided that there is physical contact with the alga (Doucette et al. 1998). At Sant Andreu de Llavaneres, coinciding with *Ostreopsis* blooms, beach users often suffer from skin irritation and wound infections. These symptoms have often been attributed to some *Vibrio* species, with severe cases of *V. vulnificus* in the Baltic Sea (Ruppert et al. 2004) and of *V. alginolyticus* in the North Atlantic (Shets et al. 2006, Reilly et al. 2011). In order to establish a connection between wound infections and the presence of pathogenic vibrios during *Ostreopsis* blooms, further research is being conducted to assess toxin profiles and identify the *Vibrio* species present at Llavaneres.

Relationships between *Vibrio* and environmental factors

Temperature is an important factor for the growth of *Vibrio* (Thompson et al. 2004, Vezzulli et al. 2013) and it was the most important environmental parameter correlating positively with the abundance of both epiphytic and planktonic vibrios, both total and free-living concentrations, and the percentage contribution of vibrios to the community. Indeed, long-term studies have provided evidence of a significant positive relationship between sea surface temperature and *Vibrio* occurrence (Vezzulli et al. 2012). In the NW Mediterranean, *Vibrio* infection together with temperature might have contributed to mass mortality events of benthic invertebrates (Vezzulli et al. 2010). On the Adriatic Sea coast, *Vibrio* expressing pathogenicity-associated properties were found mainly in the warmer months (Baffone et al. 2006).

Llavaneres is a very shallow beach and might be influenced by occasional seepage water from surrounding land. Salinity varies without a clear pattern probably due to these terrestrial influences of seepage water. Some studies have found positive correlations between vibrios and low salinities (Oberbeckmann et al. 2012), but this was not the case for our area of study. Vibrios have a high plasticity in their genome and seem to be adaptable to changes in salinity (Cavallo and Stabili 2004). The significant positive corre-

lations for % γ-proteobacteria and ammonia are also noteworthy. Ammonia has been adopted as a sewage water indicator since it is the result of urine decay. It is plausible that γ-proteobacteria, a group of generally fast-growing opportunistic bacteria, might have responded to these inputs of inorganic nitrogen or, alternatively, they might have been brought with the ammonia-rich terrestrial inputs.

In this study we have shown that vibrios may be associated with *Ostreopsis* blooms. As some *Vibrio* species are directly responsible for wound infections in marine waters (Ruppert et al. 2004, Shets et al. 2006, Reilly et al. 2011), it is plausible that part of the negative effects attributed to *Ostreopsis* blooms (e.g. Vila et al. 2012a) might be related not only to palytoxin analogues but also to specific bacteria such us *Vibrio* sp.

CONCLUSION

We have shown a positive relationship between the abundances of vibrios and of the benthic dinoflagellate *Ostreopsis* sp. at a coastal site of the NW Mediterranean. Abundance of bacteria of the genus *Vibrio* contributed up to 0.8% of the total planktonic bacterial community and up to 4.2% of the total epiphytic bacterial community, with a higher contribution in summer. Although most planktonic vibrios (mean of 83%) had a free-living lifestyle, particles in water constitute a niche for the *Vibrio* populations since they can occasionally represent up to 72% of the total bacterial community attached to particles.

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