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The harmful benthic dinoflagellate *Ostreopsis* cf. *ovata*: biotic factors affecting its growth and toxicity

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Esame finale anno 2016

This thesis is dedicated to my mother and father, my inspiration

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Introduction

1. HABs: Harmful Algal Blooms

The acronym HABs (i.e. Harmful Algal Blooms) designate microalgae outbreaks directly or indirectly harmful for the ecosystem and the human health (Anderson, 1992; Hallegraeff, 1993). In the last years, HABs have been reported with increasing frequency and areal distribution, thus research efforts have been aimed at investigating the conditions and the factors that trigger or regulate these phenomena. Within the approximately 4000 identified marine microalgae, about 200 including diatoms, dinoflagellates, haptophytes, raphydophytes, cyanophytes and pelagophytes can generate negative events (mostly dinoflagellates), whereof about 80 synthetize toxins (Zingone and Enevoldsen, 2000; Smayda and Reynolds, 2003). In addition, the number of harmful algal species discovered is constantly growing.

A noxious phytoplanktonic bloom (also known as "red tide"; Fig. 1) is characterized by a sudden increase in microalgal cell abundances, which reach high values (e.g. 10^4-10^5 cells mL^{-1}) in a variable time span (1-3 weeks; Masò and Garcés, 2006). Blooms maybe harmful either due to the high biomass or to toxin production depending on the algal species involved; synergy of both the factors is sometimes reported. Algal species producing toxins can have severe impacts on human health (i.e. biointoxications) causing sometimes the death mainly through seafood consumption or aerosol inhalation and contact, while high biomasses can generate negative effects on the ecosystem (hypoxia/anoxia) and on the recreational activities (Garcés et al., 1999; Botana et al., 2014).

Fig. 1. A spectacular non-toxic "red tide" bloom of *Noctiluca scintillans* in New Zealand (A; photo by M. Godfrey), a toxic cyanobacterial bloom at Lake Erie (B; photo by Tom Archer/University of Michigan), and a non-toxic bloom of *Noctiluca miliaris* in Hong Kong (C; photo by Kin Cheung).

Toxins produced by algal species encompass a wide range of organic compounds with different molecular weight (from few hundreds to more than 1.000 Da) and solubility characteristics. These molecules are mainly produced by phytoplanktonic and phytobenthic organisms but also from heterotrophic bacteria, the latter being involved in modification processes as well. Bivalve mollusks are one of the most common vectors, as they can bioaccumulate large amount of toxins in tissues by filter-feeding. A first grouping within algal biotoxins can be done between water-soluble and fat-soluble toxins (Poletti et al., 2003). For long time biotoxins have been classified on a symptomatic base as: Paralytic shellfish poisoning (PSP); Diarrhoetic shellfish poisoning (DSP); Neurotoxic shellfish poisoning (NSP); Azaspiracid poisoning (AZP); Amnesic shellfish poisoning (ASP), related to diatoms; Ciguatera fish poisoning (CFP), provoked by dinoflagellates and spread to the humans by fishes. Nowadays this classification is rather debated, as some compounds has been wrongly grouped in a cluster only because of the concomitantly presence of other known toxins, although they showed different effects and chemical structure. Therefore, it

has been considered more adequate to distinguish toxin based on their chemical structure as follows (Toyofuku, 2006; Tubaro et al., 2010; Botana et al., 2014): Saxitoxin group (STX); Okadaic acid group (OA); Pectenotoxin group (PTX); Yessotoxin group (YTX); Domoic acid group (DA); Brevetoxin group (BTX); Azaspiracid group (AZA); Ciguatera group; Cyclic imine group and Palytoxin group (PLTX).

Toxins are secondary metabolites differing for structure, elemental composition and functional activity (Granéli and Flynn, 2006), and their synthesis rate depends on availability and utilization of primary metabolites which are precursors. In turn, primary metabolites synthesis vary in relation to nutrients availability, cell cycle and cell growth phase (Flynn and Flynn, 1995; Pistocchi et al., 2014). As general, changes in toxin cell content are related with physiological stress or with the shift from exponential to the stationary growth phase of the algae (Edvardsen et al., 1990, Anderson 1994; Flynn et al., 1994, 1996; Granéli et al., 1998; Johansson and Granéli 1999a,b; Vanucci et al., 2012a; Pezzolesi et al., 2014; Pistocchi et al., 2014). However, the abiotic and biotic factors that trigger or regulate toxin production in many algal species are still largely unknown (Katircioglu et al., 2004).

2. Target species: *Ostreopsis* cf. *ovata*

Phylum Dinoflagellata
Class Dinophyceae
Order Peridiniales
Family Ostreopsidaceae
Genus *Ostreopsis*
Species *Ostreopsis* cf. *ovata*

Dinoflagellates belonging to the Ostreopsidaceae family are found in the benthic microalgal assemblage both in temperate and tropical areas (Faust et al., 1996). As for their planktonic counterparts, it is hypothesized that benthic dinoflagellates are mixotrophic and auxotrophic for at least some vitamins. Only the two species *Ostreopsis siamensis* and *Ostreopsis* cf. *ovata* have

been retrieved in the Mediterranean in the 70's and the 90's, respectively (Taylor, 1979; Tognetto et al., 1995), whereof *O.* cf. *ovata* (Fig. 2) is the most common and abundant species (Vila et al., 2001; Penna et al., 2005; Aligizaki and Nikolaidis, 2006; Turki et al., 2006; Battocchi et al., 2010; Totti et al., 2010; Accoroni et al., 2011; Mangialajo et al., 2011). The lack of a monitoring program for the microphytobenthic communities along the Mediterranean coasts do not allow to establish when these species have been introduced in the basin (with ballast water for instance) or whether they were already present as autochthonous at low densities. However, the increasing in *Ostreopsis* spp. bloom event frequencies, intensities and distribution along the Mediterranean coasts in the last decade is evident, raising the attention of the scientific community and of the environmental management authorities. As a result, taxonomical, genetic, ecological and toxicological studies intensified in this period (e.g. Vila et al., 2001; Penna et al., 2005; Turki, 2005; Aligizaki and Nikolaidis, 2006; Ciminiello et al., 2006, 2008; Riobò et al., 2006; Aligizaki et al., 2008; Ledreux et al., 2009; Guerrini et al., 2010; Vanucci et al., 2012a; Pezzolesi et al., 2012, 2014; Accoroni et al., 2015a,b; Pinna et al., 2015; Tartaglione et al., 2016).

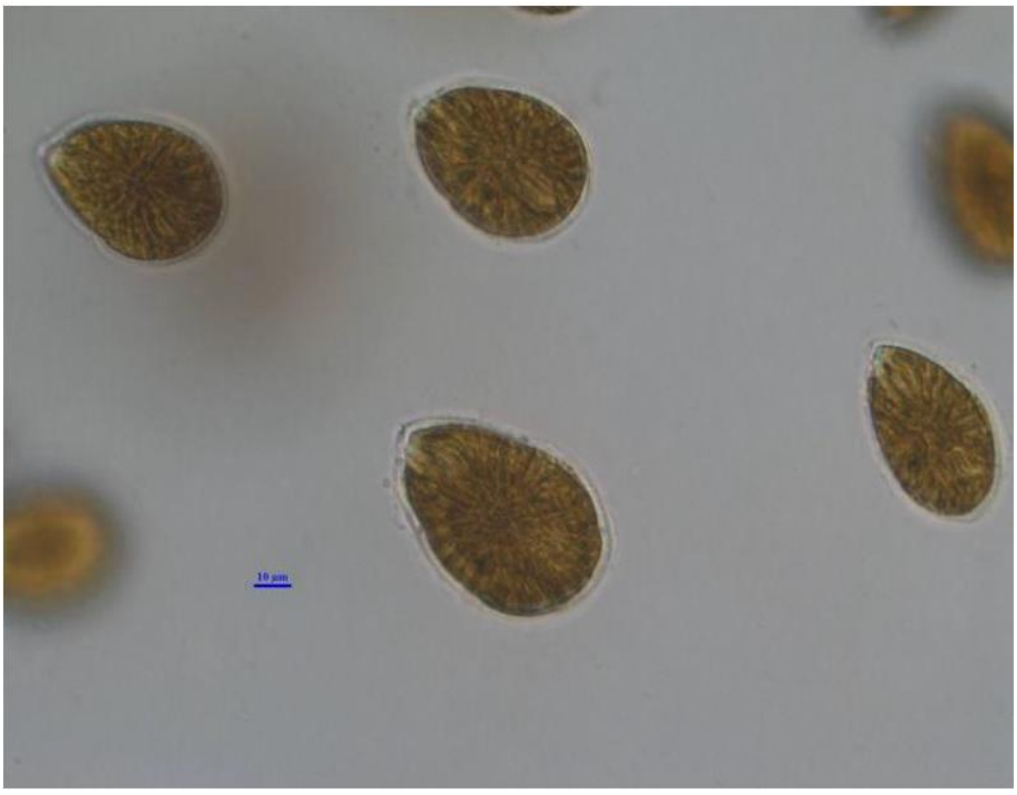

Fig. 2. Light microscopy micrograph of an *O.* cf. *ovata* culture.

The genus *Ostreopsis* was firstly designated with the species *O. siamensis* Schmidt (1901). Nevertheless, the genus was negligibly taken into consideration until taxonomical studies of Fukuyo allowed to describe two more species: *O. ovata* Fukuyo (1981) and *O. lenticularis* Fukuyo (1981). Six other species were successively added: *O. heptagona* Norris, Bomber and Balech (1985), *O. mascarenensis* Quod (1994), *O. labens* Faust and Morton (1995), *O. marinus* Faust (1999), *O. belizeanus* Faust (1999) and *O. caribbeanus* Faust (1999). Taxonomy of the genus *Ostreopsis* rely on morphological traits as thecal arrangement, morphology and shape (Penna et al., 2005), although it is still unclear (Rhodes et al., 2000; Vila et al., 2001).

It was showed that *Ostreopsis* spp. cell dimensions can vary over the different growth phases in both environmental and laboratory conditions (Aligizaki and Nikolaidis, 2006; Guerrini et al., 2010). However, despite the presence of different dimensional and morphological classes belonging to the genus *Ostreopsis*, (Pin et al., 2001; Penna et al., 2005; Aligizaki and Nikolaidis, 2006; Rossi et al., 2010), intraspecific morphological analyses were rarely conducted so far.

Species affiliated to this genus are important in the tropical and subtropical coral reef marine environment; however, they are also present in temperate areas, where they remarkably increased in abundance in the last decade (Shears and Ross, 2009; Laza-Martinez et al., 2011; Mangialajo et al., 2011; Rhodes, 2011; Penna et al., 2012; Funari et al., 2015). *Ostreopsis* cf. *ovata* is a benthic dinoflagellate living associated to a broad range of biotic and abiotic substrata (macrophytes, marine phanerogams, benthic invertebrates, sand and rocks), albeit it can be find in the water column due to resuspension. *O.* cf. *ovata* cells appear egg-shaped, thinned in the ventral part and flattened in the anteroposterior direction. Epitheca and hypotheca have the same dimensions, and shape of the plates is essential for identification (Fig. 3 and 4). Thecal arrangement is Po, 3', 7", 6c, 6s, 5''', 1p, 2'''' (Faust and Gulladge, 2002).

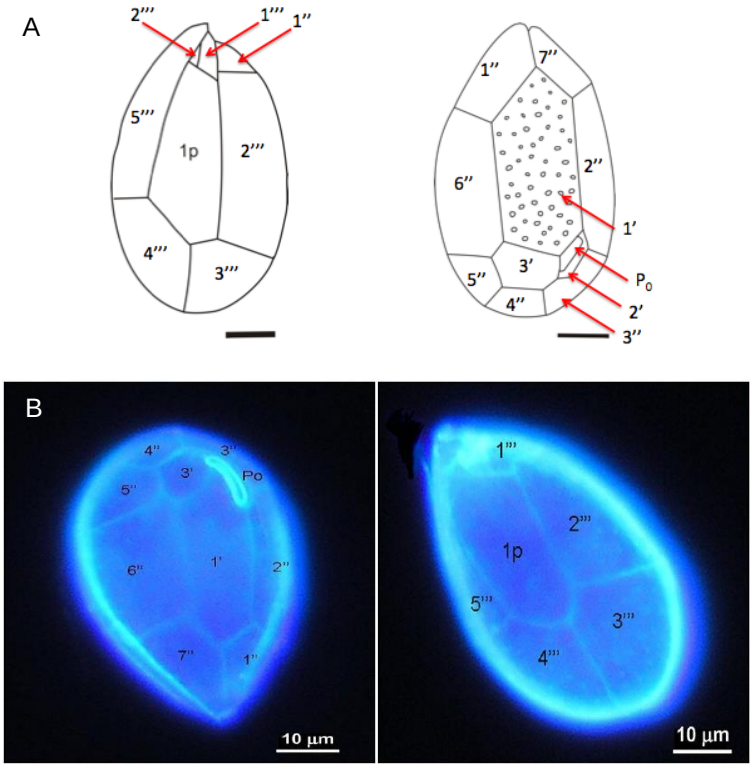

Fig. 3. Schematic diagram of *Ostreopsis ovata* hypotheca and epitheca, bar scale: 10 µm (A; from Tomas et al., 1997), and tecal arrangement of calcofluor stained *O. ovata* (B).

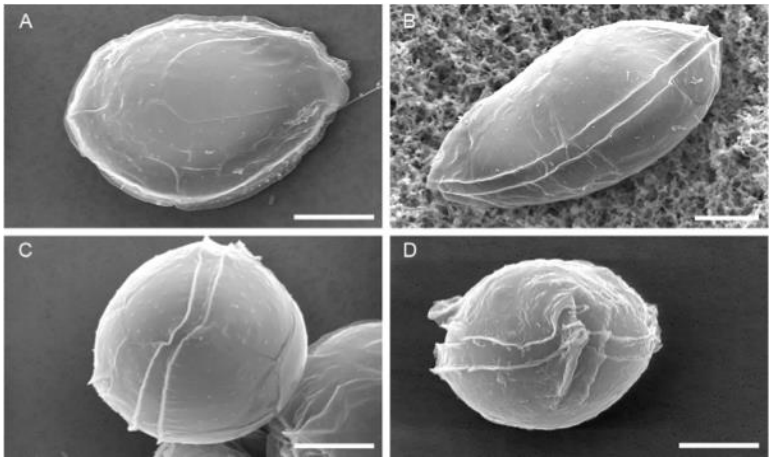

Fig. 4. *Ostreopsis* cf. *ovata* at the electron mycroscopy (SEM): (A) hypotheca, (B) lateral view, (C) dorsal view and (D) ventral view. Bar scale: 10 µm. (from Accoroni et al., 2012).

Besides the ability to perform photosynthesis, Faust et al. (1998) proposed the possibility of *O. ovata* to ingest microalgae, cyanobacteria and ciliates through the ventral pore that can stretch and contract, hypothesizing a mixotrophic lifestyle of this dinoflagellate; however, evidence for phagotrophy in *Ostreopsis* species is still elusive (Zingone, 2013). *O.* cf. *ovata* prefer shallow, calm waters (Pistocchi et al., 2011; Funari et al., 2015) and lives in benthic algal assemblages together with other dinoflagellates as *Amphidinium sp*., *Coolia monotis*, *Gambierdiscus toxicus*, *Prorocentrum lima* and the centric diatom *Coscinodiscus* sp. (Blanfunè et al., 2015; Carnicer et al., 2015). In low hydrodinamism conditions, *O.* cf. *ovata* release exopolymeric substances from the ventral pore forming mucilaginous networks that constitute a biofilm which trap small invertebrates (Barone and Prisinzano, 2006; Honsell et al., 2013). Since the end of 90's vast *O.* cf. *ovata* bloom event increased in frequency, intensity and areal distribution. High *O.* cf. *ovata* cell densities along the Italian coasts were firstly registered in the Ligury region (Abbate et al., 2007; Mangialajo et al., 2008), Sardinia (Lugliè, pers. comm.), Tyrrhenian sea (Sansoni et al., 2003; Simoni et al., 2004; Barone and Prisinzano, 2006; Congestri et al., 2006; Bianco et al., 2007) and in the Southern Adriatic area (Bottalico et al., 2002; Di Turi et al., 2003; Ungaro et al., 2005). Only since 2006 *O.* cf. *ovata* was retrieved along the Northern Adriatic rocky coastal areas (Monti et al., 2007; Totti et al., 2007).

O. cf. *ovata* outbreaks are usually related to human health effects ascribed to aerosols or cutaneous exposure to seawater, with symptoms mainly involving the upper respiratory tract but also eyes and skin (Gallitelli et al., 2005; Vivaldi et al., 2007; Funari et al., 2015). In the last years, severe human intoxications were reported in concomitance with *Ostreopsis* spp. blooms (Gallitelli et al., 2005; Kermarec et al., 2008; Tichadou et al., 2010; Honsell et al., 2011). The typical symptoms of intoxication (fever, dyspnoea, bronchospasm, conjunctivitis and skin irritation) and the benthic invertebrate deaths occasionally observed (Di Turi et al., 2003; Sansoni et al., 2003; Congestri et al., 2006; Shears and Ross, 2009, 2010; Accoroni et al., 2011; Carella et al., 2015) have been linked to palytoxin-like compounds (Ciminiello et al., 2011, 2012a,b), which can also bioaccumulate in tissues of filter-feeding bivalve mollusks (Rhodes et al., 2006; Aligizaki et al., 2008, 2011; Louzao et al., 2010; Gorbi et al., 2012, 2013; Brissard et al., 2014; Ciminiello et al., 2015).

3. Palytoxin group

In the last years, many bloom events originated by toxic or potentially toxic bloom-forming species (e.g. *Fibrocapsa japonica*, *Protoceratium reticulatum*, *Gonyaulax spinifera*, *Pseudo-nitzschia* spp.) were reported along the Italian coasts. However, the most serious cases in terms of sanitary implications were attributable to the benthic dinoflagellate *Ostreopsis* cf. *ovata*. The most known circumstances occurred during summer of 2005 along the Ligurian coasts, when a few hundred people staying in proximity of the beaches needed hospitalization due to the above-mentioned symptoms. *O.* cf. *ovata* outbreaks recorded in such areas during those days were considered the causative agent.

A putative palytoxin (pPLTX), later named also isobaric palytoxin (García-Altares et al., 2015), was identified through analysis of algal extracts from samples harvested during the 2005 outbreak (Ciminiello et al., 2008). Further analysis, performed during the course of following investigations in the environment and in *O.* cf. *ovata* laboratory cultures, showed the presence in the extracts of toxins with a pPLTX-like molecular structure, named ovatoxins (OVTX) -a to -k, whereof OVTX-a is the major component of the toxin profile (≥ 50%) both in natural blooms and algal cultures (Fig. 5 and 6) (Ciminiello et al., 2010, 2012a, 2015; Ciminiello and Penna, 2014; Garcìa-Altares et al., 2015; Brissard et al., 2015; Tartaglione et al., 2016). The palytoxin molecule is made up by a long, partially unsaturated aliphatic chain containing polycyclic ethers, 64 chiral centers, 40-42 hydroxylic groups and 2 amidic groups (formula: $C_{129}H_{233}N_3O_{54}$), being the longest carbon chain known in nature and one of the most powerful and lethal non-protein marine toxins.

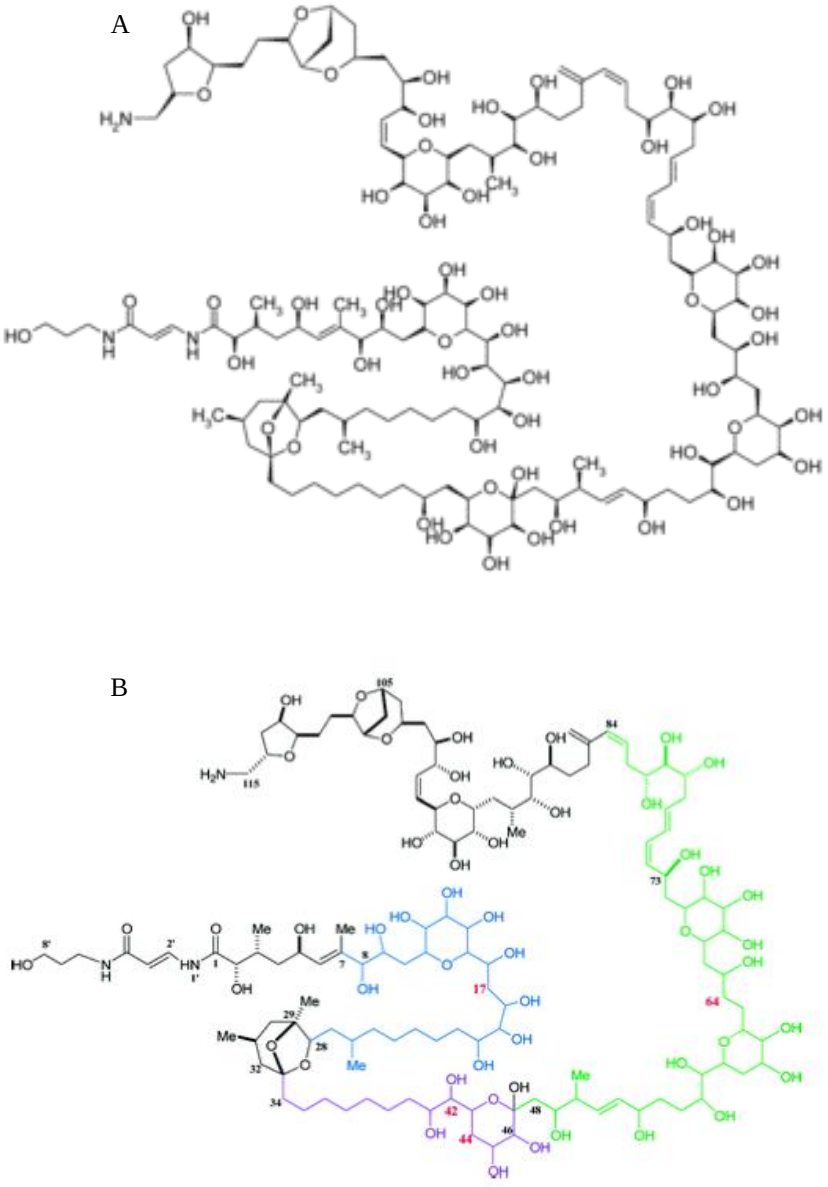

Fig. 5. Molecular structure of palytoxin (A) and ovatoxin-a (B) (from Ciminiello et al., 2012).

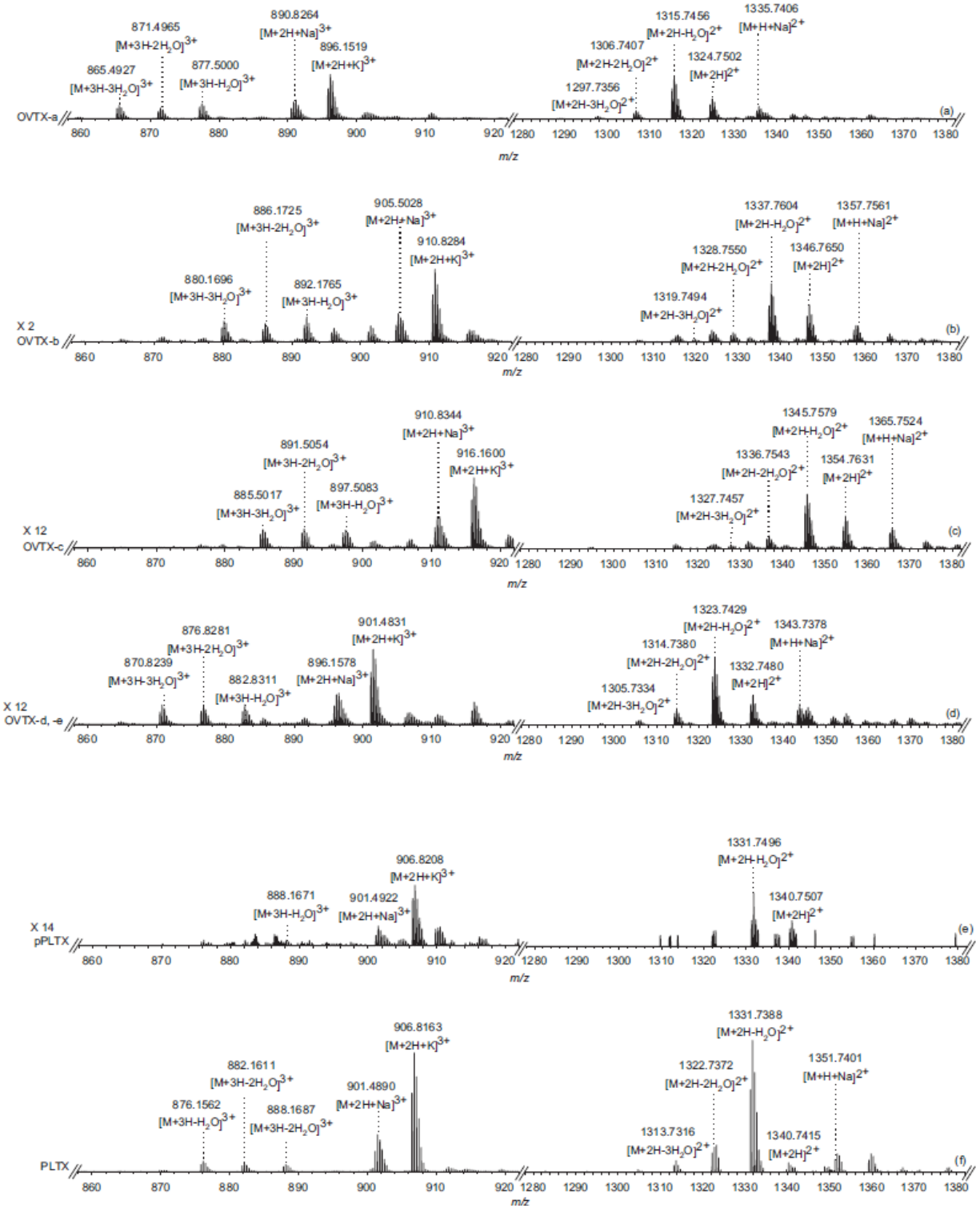

Fig. 6. HR Full MS spectra (mass range m/z 800–1400) of putative palytoxin (pPLTX), ovatoxin (OVTX)-a, -b, -c, and -d plus -e of *O.* cf. *ovata* cells *vs* palytoxin standard (PLTX). Magnification grade used to have spectra of the same intensity as that of OVTX-a is shown on the left side of each spectrum (from Vanucci et al., 2012a).

With regard to palytoxin's mechanism of action, the main character is the delayed haemolysis of mammalian erythrocytes based on the capacity of PLTX and analogues to convert the Na^+/K^+-ATPase pump into a non-specific channel, causing ion imbalance; such activity could be neutralized by ouabain. Binding to this pump, PLTX initiates a cell death cascade characteristic of Ca^{2+} overload (Schilling et al., 2006; reviewed in Funari et al., 2015). Although not being considered an emolysin, PLTX promotes a fast and complete loss of K^+ from erythrocytes, leading to a delayed haemolysis of the colloid-osmotic type and hemoglobin release (Malagoli, 2007; Pelin et al., 2015). PLTX causes a wide range of secondary pharmacological actions, including cardiovascular effects, hemolysis, intense contraction of skeletal, smooth and cardiac muscles, hormon release, thrombocyte aggregation, bone resorption and inhibition of sperm motility (Ramos and Vasconcelos, 2010 and references therein; reviewed in Funari et al., 2015).

4. Biotic factors influencing *O.* cf. *ovata* bloom dynamics with emphasis on microalgal-bacterial interactions

Water temperature and salinity, hydrodinamism, substratum, depth and nutrient availability are the abiotic factors mainly affecting *O.* cf. *ovata* bloom development (reviewed in Pistocchi et al., 2011; Carnicer et al., 2015). Summarizing, in the Mediterranean Sea higher *Ostreopsis* spp. abundances were commonly recorded during the periods of the year characterized by higher temperature and salinity waters, and a relative water column stability; in particular, as the algal cells are easily resuspended in the water column, the role of hydrodinamism in bloom triggering and decline has been largely pointed out (e.g. Totti et al., 2010; Accoroni et al., 2015a). Studies conducted on different *O.* cf. *ovata* strains isolated from different sites along the Italian coasts revealed different temperature optima according with the bloom period, and higher toxin concentrations produced concomitantly with the best growth conditions. As general, nitrogen and phosphorous limitation determined lower cell toxin content (Pistocchi et al., 2011; Vanucci et al., 2012a), in contrast with the pattern observed for many toxic dinoflagellates (Pistocchi et al., 2011).

On the other hand, little research is available with regard to the biotic factors influencing *O.* cf. *ovata* bloom dynamics. Pezzolesi et colleagues (2011) reported a negligent effect of the toxins produced by *O.* cf. *ovata* Adriatic strains in batch cultures towards the benthic dinoflagellate *Coolia monotis*, generally coexisting with *O.* cf. *ovata* in the benthic assemblage. These authors suggested that growth dynamics observed experimentally for the co-growing dinoflagellates are mainly influenced by competition for resources through different nutrient uptake patterns and/or different optimal N:P ratio, rather than negative allelopathic effects of the toxins released, as similarly proposed by Carnicer et al. (2015) on the basis of environmental data. Such findings are consistent with the results obtained from Monti and Cecchin (2012), who evidenced only a weak inhibitive effect of an *O. ovata* filtrate on the growth of *P. minimum* and *C. monotis* in laboratory cultures. Recently, evident negative allelopathic effects of three macroalgae towards *O.* cf. *ovata* have been highlighted in experimental conditions (Accoroni et al., 2015b), and have been hypothesized to be responsible for the significant lower *O.* cf. *ovata* cell abundances recorded on seaweeds compared with rocks in the environment (Totti et al., 2010; Accoroni et al., 2015b). As earlier mentioned, different nutrient uptake strategies and nutrition modes can play a relevant role

in competition for an ecological niche between microphytobenthic algal species. Mixotrophy, the combination of phototrophy (i.e. acquirement of energy through photosynthesis and inorganic carbon) and heterotrophy (i.e. the uptake of dissolved organic compounds and/or phagotrophy, that is feeding on particulate organic matter and/or live preys; Burkholder et al., 2008) has been recently suggested for an increasing number of dinoflagellate species (Flynn et al., 2012). While no literature is available so far for *O.* cf. *ovata* with regard to phagotrophy/prey ingestion, osmotrophy (Glibert and Legrand, 2006) of organic substrates such as urea and glycerol-3-phosphate has been observed in *O.* cf. *ovata* cultures, with growth rate comparable to those retrieved in mineral media (Guidi, unpublished data). Thus, myxotrophy can lend competitive advantages to *O.* cf. *ovata* over strict phototrophs and heterotrophs microorganisms.

In relation to biotic factors, bacteria associated to toxic microalgae and the different modes of microalgal-bacterial interactions have increasingly received attention in the last years, as they are considered between the most relevant ecological factors in triggering and/or regulation of HAB dynamics (e.g. Granéli and Turner, 2006; Kodama et al., 2006; Loureiro et al., 2011). Relationships between algal-associated bacteria, that are bacteria adhering to the algal surface (i.e. attached) or swimming in the water column (i.e. free-living) (Riemann and Winding, 2001), range from species-specific to general (Garcès et al., 2007; Saap et al., 2007; Teeling et al., 2012) and from positive to negative ones (Doucette et al., 1998; Seyedsayamdost et al., 2011a,b, 2014; Wang et al., 2014, 2015). The presence and the succession of specific and phylogenetically diverse bacterial groups with partially different metabolic activity observed during the development of several HABs suggests their possible role in modulate HABs' initiation, maintenance and termination processes (Doucette, 1995; Adachi et al., 1999; Doucette et al., 1999; Buchan et al., 2014), and also HABs' toxicity extent, acting directly and/or indirectly on production and degradation of microalgal toxins (e.g. Gallacher et al., 1997; Shetty et al., 2010; Albinsson et al., 2014). For instance, it is well known the primary role of bacteria in nutrient recycling and production of vitamins that stimulate algal growth, along with other more or less specific interactions that interfere with microalgal growth cycle (e.g. cysts formation; Granèli e Turner, 2006 and references therein).

More in detail, microalgal-bacterial relationships may be synergic (e.g. symbiosis, mutualism, commensalism) or competitive/antagonistic (e.g. competition for nutrients uptake, algicidal activity, parassitism) (Doucette et al., 1998; Amin et al., 2009, 2012, 2015; Jones et al., 2010;

Cooper and Smith, 2015; Ramanan et al., 2016). The majority of these interactions take part at the “phycosphere”, which is the region around the algal cell (or chain, or colony of cells) where bacteria feed on the extracellular products released from the alga (Bell and Mitchell, 1972), represented by a thin, fluid diffusive boundary layer surrounding algal cells, through which the transport occurs entirely for diffusion (Amin et al., 2012 and references therein). Bacteria reach phycosphere by sensing and directing movement in response to chemical gradients through negative and/or positive chemiotaxis (Seymour et al., 2009; Stocker and Seymour, 2012). Then, some bacteria can remain within the phycosphere by adhering to the algal surface (Fig. 7), likely via production of extracellular molecules as proteins or polysaccharides secreted by the alga (Fig. 8) or by the bacteria (Biegala et al., 2002; Kaczmarska et al., 2005; Jung et al., 2008; Mayali et al., 2008; Gardes et al., 2011).

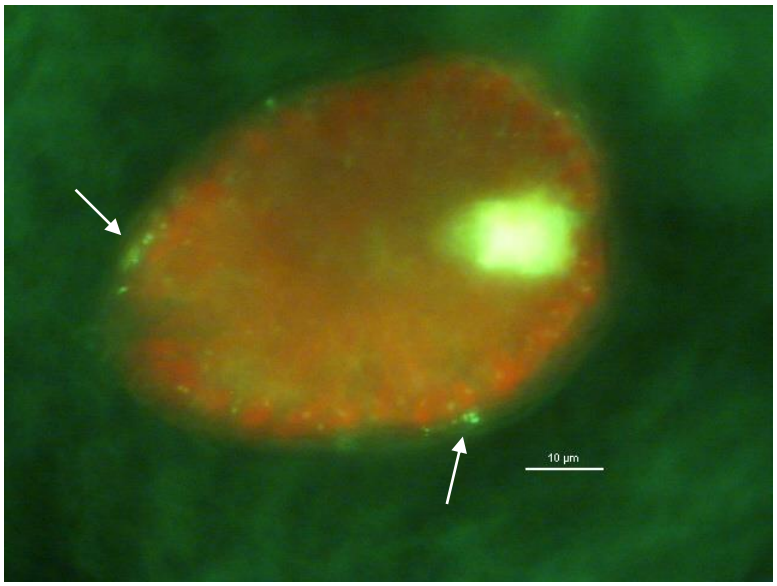

Fig. 7. *O.* cf. *ovata* at 1000x magnification, Sybr Gold staining. White arrows point bacteria attached to the algal surface. (Courtesy of Prof. Silvana Vanucci).

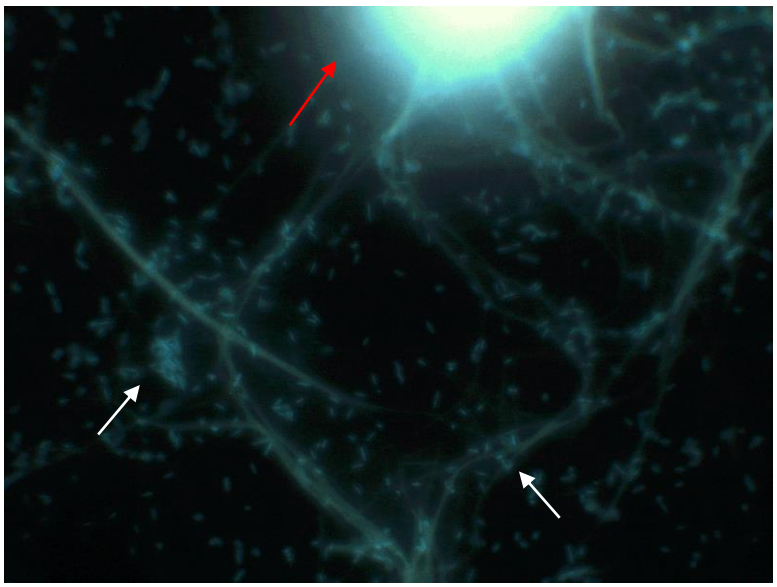

Fig. 8. *O.* cf. *ovata* at 1000x magnification, DAPI staining. Red arrow point the dinoflagellate cell in the upper side, white arrows point bacteria attached to the mucous strands. (Courtesy of Prof. Silvana Vanucci).

4.1. Microalgal-bacterial interactions

The consistent presence of specific bacterial species associated with toxic and non-toxic dinoflagellates both in cultures and in the environment suggests that such bacteria are able to efficiently use the products deriving from microalgal cells. Bacteria, in turn, may benefit the dinoflagellates by making available some nutrients or protecting the alga by harming from parasitic bacteria. On the other hand, the same or other bacterial groups may opportunistically take advantage of the microalga, causing cell stress or lysis (reviewed in Ramanan et al., 2016). A mutualistic, often symbiotic interaction commonly known between bacteria and dinoflagellates (and phytoplankton as general) rely upon the synthesis of essential vitamins for the dinoflagellate growth by bacteria (Fig. 9), and the utilization of algal exudates from the latter (Cooper and Smith, 2015; Ramanan et al., 2016). Cobalamin (vitamin B_{12}) is essential for the synthesis of amino acids, deoxyriboses, reduction and transfer of single carbon fragments in many biochemical pathways, whereas thiamine (vitamin B_1) plays a central role in intermediary carbon metabolism and is a cofactor for several enzymes involved in primary carbohydrate and branched-chain amino acid metabolism, while biotin (vitamin B_7) is a cofactor of several essential carboxylase enzymes, such as acetyl CoA (Tang et al., 2010 and references therein). All these three are hydrosoluble vitamins that need therefore to be quickly assimilated from the dinoflagellates before spreading out of the phycosphere. This type of interaction appears to be ecologically very relevant with regard to toxic bloom-forming dinoflagellates (Sañudo-Wilhelmy et al., 2014). So far, the vast majority of HAB researches focused on dynamics of macronutrients as nitrogen and phosphate, owing to the recurrent linkage between eutrophication and toxic microalgae outbreaks, whereas the importance of coenzymes and specific vitamins (e.g. vitamin B_{12}, B_1 and B_7) were rarely investigated. Many evidences suggest that vitamins could play a prominent role in the occurrence of dinoflagellate blooms, as dinoflagellate are well known being an algal group auxotrophic for vitamins (Croft et al., 2005; Tang et al., 2010). Recently has been pointed out that more than 90% of the surveyed species required vitamin B_{12}, about 50% vitamin B_1 and roughly 40% were auxotrophic for vitamin B_7 (Tang et al., 2010). The strong auxotrophy manifested from toxic dinoflagellates along with their known osmotrophic and mixotrophic ability imply a huge uptake of vitamins from the environment, especially during bloom development (Koch et al., 2014).

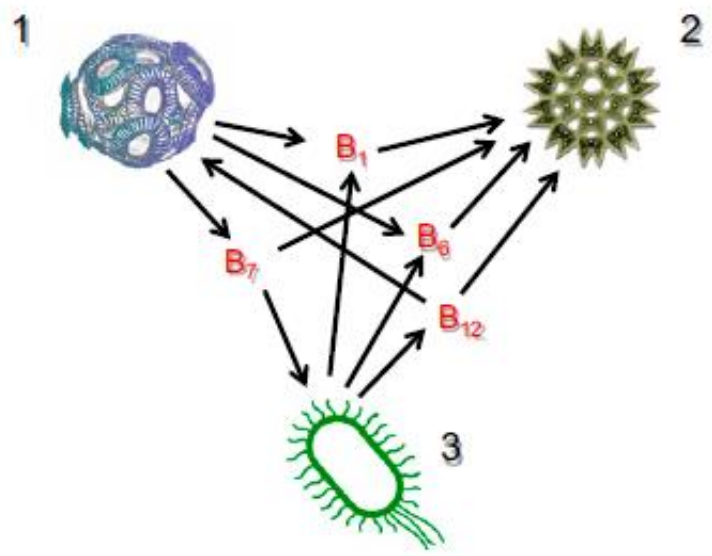

Fig. 9. Schematic model of vitamin exchange in a plankton community where algal species 1 synthesize three B vitamins, species 2 none, and bacterium species 3 produces three B vitamins. Note that none of the species in this example can grow without vitamins provided by at least one of the other community member, suggesting relevant metabolic-driven evolutionary implications. (from Giovannoni, 2012 modified).

The type of association (e.g. mutualism, commensalism, parasitism) that a bacterium or a group of bacteria can engage may vary in relation to environmental conditions and algal physiological status (Azam and Smith, 1991; Amaro et al., 2005; Seyedsayamdost et al., 2011a). Hence, it is reasonable that the bacterial community associated with a toxic microalga during a certain stage of the bloom would depend by availability and characteristics of organic substrata, nutrients (e.g. nitrogen, phosphate), micronutrients (e.g. vitamins, iron) and algal physiological conditions, that on the other hand are influenced by several environmental factors and also in turn from algal-bacterial interactions (see forward). Progression and dynamics of a microalgal bloom would be then associated with successional stages of the bacterial community co-occurring with, which would be characterized by different modes of predominant algal-bacterial interactions during the different phases (Teeling et al., 2012; Buchan et al., 2014; Wemheuer et al., 2014, 2015; Voget et al., 2015). For instance, analyzing the different colonization dynamics of diverse bacterial taxa during distinct bloom phases, Mayali et al. (2011) postulated that host-benefic bacteria would be present during high abundance, exponential growth phases of microalgal cells; antagonistic/parasitic bacteria would instead increase in abundance just before decline phases of the blooms (senescent phases), while saprophytic/scavenger bacteria specialized in death algal

cell degradation would dominate suddenly after bloom termination, in response to the availability of phytodetritus. Along with microalgal-bacterial relationships and environmental conditions, the recurring ecological succession that occurs within the microbial community is also driven by the different bacteria-bacteria interactions (e.g. Long and Azam, 2001; Rabe et al., 2014; Ziesche et al., 2015), which encompassed the same wide spectrum listed for algae and bacteria, ranging from symbiosis to competition. Similarly to superior organisms, bacteria may display two different growth strategy, being either r-strategist (e.g. *Alphaproteobacteria*, *Gammaproteobacteria*) or k-strategist (e.g. *Bacteroidetes*). The former are first-colonizer of new substrata, and are characterized by high exponential growth rates that subsist until nutrient availability and environmental conditions are favorable, whereas the latter usually respond as secondary colonizers showing a slower but rather steady growth (Jones et al., 2010 and references therein; Elifantz et al., 2013). r-strategist species are commonly considered opportunistic and pioneer bacteria, as they are able to thrive in favorable transitory conditions rapidly colonizing the environment. On the other hand, k-strategist species proliferate under stable environmental conditions, where high biodiversity levels and steady fluxes of energy and matter allow them to firmly colonizing substrata; for this reasons they usually dominate the bacterial community in steady-state, mature ecosystems (Garcès et al., 2007; Jones et al., 2010; Abby et al., 2014).

4.2. Main bacterial taxa found associated with toxic dinoflagellates

Culture-based studies on harmful planktonic dinoflagellates, using both biochemical and traditional molecular methods, revealed that mechanisms as nutrient competition, production of growth-stimulant or -inhibiting compounds, or the algicidal activity of some bacterial species may have different effects on algal cellular physiology, which will ultimately influence cell growth dynamics, production, degradation, and/or modification of algal toxins (e.g. Hold et al., 2001a,b; Amaro et al., 2005; Jasti et al., 2005; Su et al., 2005; Donovan et al., 2009; Green et al., 2010; Wang et al., 2010; Albinsson et al., 2014; Wang et al., 2014, 2015). A summary of the different approaches used to characterize algal–bacterial interactions has been depicted in Fig. 10.

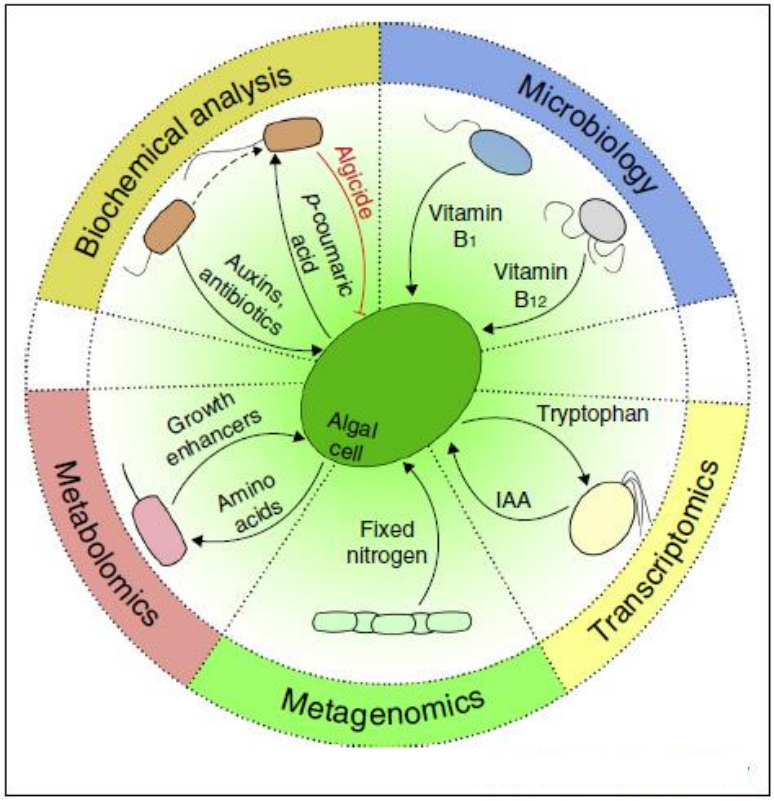

Fig. 10. Metodological approaches utilised to investigate algal–bacterial interactions. The phycosphere contains algal exudates accessible to the bacteria, which in turn can supply micronutrients such as vitamin B_1 or B_{12}, as demonstrated in laboratory co-cultures (Croft et al., 2005; Wagner and Dobler et al., 2010). Transcriptomics analysis of a co-culture between *P. multiseries* and *Sulfitobacter*-related bacteria revealed both up-regulation of algal genes involved in tryptophan biosynthesis and indole 3-acetic acid production in the bacterium, an auxin-family compound enhancing diatom growth (Amin et al., 2015). Pyrosequencing of 16S and 18S rRNA gene was performed to point out algal-bacterial symbioses relying on nitrogen provision in the Atlantic Ocean (Krupke et al., 2014). *T. pseudoniana* has been showed to increase synthesis of sugars and amino acids when in presence of *D. shibae* compared to an axenic control through metabolomic approaches (Paul et al., 2013). Lastly, Seyedsayamdost et al. (2011a) by using biochemical analysis revealed unique characters of the mutualistic/parasitic relationship between *Emiliana huxleyi* and *Phaeobacter gallaeciensis* in a dual model systems. (from Cooper and Smith, 2015 modified).

Recurrent associations between cultured harmful dinoflagellates and specific bacterial groups such as *Alpha-* and *Gammaproteobacteria*, *Cytophaga-Flavobacteria-Bacteroides* and some sub-groups, primarily affiliated with the *Roseobacter* clade and *Alteromonas*, have been observed (Amaro et al., 2005; Jasti et al., 2005; reviewed in Garcés et al., 2007; Green et al., 2010; Yang et al., 2012, 2015; Onda et al., 2015; Park et al., 2015), although no univocal associations have been reported for the same dinoflagellate species or strongly related (e.g. for *Alexandrium* spp.,

reviewed in Garcés et al., 2007). The ecological role of bacteria assemblages associated with ciguatera epiphytic dinoflagellates, such as those included in the genera *Gambierdiscus*, *Ostreopsis*, *Coolia*, *Amphidinium* and *Prorocentrum*, has received considerably less attention than for their planktonic counterparts. The previous available studies relied upon bacteria plate isolation from cultured dinoflagellates (Tosteson et al., 1989; Lafay et al., 1995; Prokic et al., 1998; Sakami et al., 1999). More recently, with regard to *Ostreopsidaceae* species, Ashton et al. (2003) have reported the presence of nonculturable bacterial strains (*Pseudomonas/Alteromonas* and *Acinetobacter*, class *Gammaproteobacteria*) in association with *Ostreopsis lenticularis* cultures, while Pérez-Guzmán et al. (2008) have shown that about 50% of bacteria associated with *O. lenticularis* clones belonged to the *Cytophaga-Flavobacteria-Bacteroides* complex. Differently, Ruh et al. (2009) found an important contribution of *Alphaproteobacteria* in two clonal cultures of *Coolia monotis* and *Ostreopsis ovata* (52% and 35% of the total retrieved sequences, respectively). So far, with regard to *Ostreopsis* cf. *ovata*, a recent study based on laboratory cultures provided evidence that bacteria interfere indirectly with algal growth and toxin production via their effects on algal physiology and likely on toxin removal (Vanucci et al., 2012b). Whereas, no information is available on natural bacterial assemblages co-occurring with *O.* cf. *ovata* blooms.

Although dinoflagellate-bacterial associations and interactions observed in laboratory cultures are the outcome of a strong selective pressure exerted by the experimental conditions (Garcés et al., 2007), and the variability of the bacterial response could be influenced, for instance, by nutrient concentrations in the culture medium, algal growth phase, or algal species (e.g. Skerratt et al., 2002; Amaro et al., 2005), culture-based studies are indispensable to elucidate several aspects related to this interactions (Loureiro et al., 2011; Flynn et al., 2012; Buchan et al., 2014).

The *Proteobacteria* phylum encompass the classes *Alpha-*, *Beta-*, *Gamma-*, *Delta-* and *Epsilonbacteria*. Within *Alphaproteobacteria* the clade *Roseobacter* is one of the most abundant and metabolically versatile group existing in the Ocean. Marine members of this clade are heterotrophic and photoheterotrophic bacteria most abundant in coastal waters and often able to assimilate organosulfur compounds like dimethylsulfoniopropionate (DMSP), a major osmolyte released in relevant amount during algal blooms (González et al., 1999, 2005; Yoch 2002; Miller and Belas 2004, 2006; Todd et al., 2012; Dickschat et al., 2015). Thus, bacteria belonging to the clade *Roseobacter* (hereafter *Roseobacters*) assume an important role in carbon and sulfur

biogeochemical cycles in the marine environment (Moran et al., 2004; Selje et al., 2004; Buchan et al., 2005; Howard et al., 2006; Wagner-Dobler and Biebl, 2006; Moran and Miller, 2007; Brinkhoff et al., 2008; Wagner-Dobler et al., 2010; Buchan et al., 2014; Durham et al., 2015).

Many *Roseobacters* species live as epibionts on marine macro- and microalgae and reach high cellular densities during phytoplanktonic blooms in general (Fig. 11; reviewed in Buchan et al., 2014) and during blooms of toxic dinoflagellates (Lafay et al., 1995; Prokic et al., 1998; Miller and Belas, 2004; Hasegawa et al., 2007; Mayali et al., 2008; Yang et al., 2012, 2015; Park et al., 2015). *Roseobacters* isolated from cultured dinoflagellate are able to grow on algal-derived compounds as the sole carbon source (Moran and Miller, 2007; Mayali et al., 2008; Newton et al., 2010), and to state what are the triggering factors that can induce a switch from synergic to competitive relationships is still an issue (Wagner-Dobler et al., 2010; Seyedsayamdost et al., 2011a,b; Wang et al., 2014, 2015).

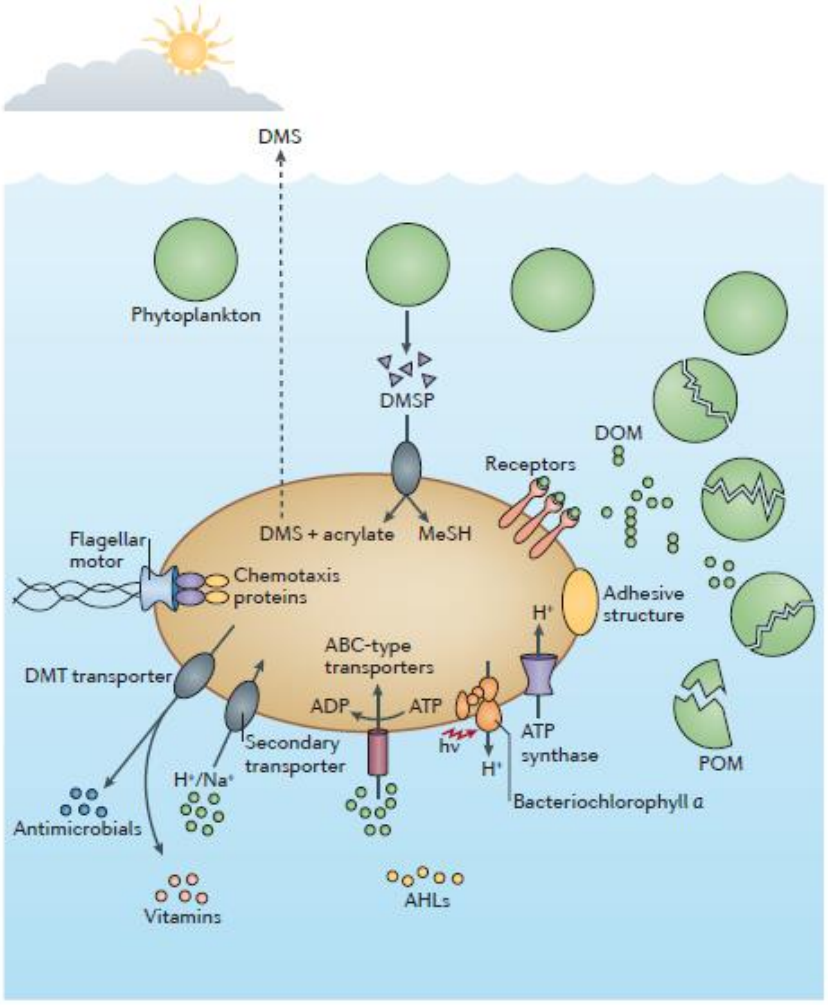

Fig. 11. Metabolic and physiological traits of *Roseobacters* favoring associations with phytoplankton. *Roseobacters* harbor several metabolic characteristics facilitating interactions with phytoplankton and phytodetritus. The algal osmolyte dimethylsulphoniopropionate (DMSP) is degraded via i) cleavage to form dimethyl sulphide (DMS) and acrylate, or ii) demethylation to form methanethiol (MeSH). While acrylate and MeSH are used as carbon and reduced sulphur source by the bacteria, DMS contributes to cloud formation fluxing to the atmosphere. Similarly, *Roseobacters* are specialized in utilization of a wide range of low molecular weight algal compounds. Chemotaxis towards such algal-derived moleules has been proven, and encoding by roseobacters of several transport systems involved in the uptake of small molecules (e.g. ABC, TRAP and DMT transporters) has been elucidated. TRAP transporters are predicted to import carboxylic acids, whereas DMT transporters seem to be involved in secondary metabolites' export, including phytoplankton growth-promoting factors like vitamins and auxins, and antimicrobial molecules. *N*-acyl homoserine lactones (AHLs), secreted by many roseobacter strains with a quorum sensing function, have been recently shown to regulate the synthesis of antimicrobial compounds depending on cell density. Along with organic matter oxidation, aerobic anoxygenic photosynthetic (AAP) *Roseobacters* can also count

on light-provided energy to generate ATP via ATP synthases. Adhesive structures such as pili or fimbriae for attachment to biotic or abiotic surfaces are also commonly detected in *Roseobacter* isolates. (from Buchan et al., 2014 modified).

With regard to *Gammaproteobacteria*, relatives of this class are commonly considered copiotrophic and opportunistic bacteria, and have been frequently retrieved from toxic dinoflagellate blooms and cultures (e.g. Brinkmeyer et al., 2000; Green et al., 2004; Amaro et al., 2005; Sala et al., 2005; Sapp et al., 2007; Bolch et al., 2011; Yang et al., 2012, 2015; Park et al., 2015). Particularly, members belonging to the group *Alteromonas-Pseudomonas-Vibrio* are described like r-strategist bacteria in marine ecosystems, as they fast grow in response to favorable environmental conditions rapidly colonizing organic substrates, hence showing large fluctuations in terms of abundance (Jones et al., 2010; Amin et al., 2012; Abby et al., 2014). In addition, some members exhibit a strong algicidal activity (Skerratt et al., 2002; Wang et al., 2005; Su et al., 2007).

The *Bacteroidetes* phylum, also known as *Cytophaga-Flavobacteria-Bacteroides* or more recently *Flavobacteria-Sphingobacteria* group (Grossart et al., 2005), is composed of the three large classes *Bacteroidia*, *Flavobacteria* and *Sphingobacteria* (Bernardet et al., 2002). Members of this phylum have been retrieved in a variety of marine habitats where they occupy a wide range of ecological niches, representing a prominent part either of the heterotrophic bacterioplankton in coastal surface and oceanic waters or of the microbial community associated with marine sediments (De Long et al., 1993; Bowman et al., 1997; Pinhassi et al., 1997; Glockner et al., 1999; O'Sullivan et al., 2002; Mann et al., 2013; Buchan et al., 2014); their conspicuous presence has also been observed during phytoplanktonic blooms and in cultures of toxic dinoflagellates (e.g. De Long et al., 1993; Gonzàles and Moran, 1997; Gasol and Duarte, 2000; Alverca et al., 2002; Biegala et al., 2002; Green et al., 2004, 2010; Garcés et al., 2007). *Bacteroidetes* can live free in the water column or attached to the substrate/host, and are specialists in hydrolysis of biopolymers like proteins, complex polysaccharides and glycoproteins (Kirchman 2002; Gòmez-Pereira et al., 2012; Riedel et al., 2013), along with being able to degrade complex plant polymers and macroalgal polysaccharides trough fermentative processes (Teske et al., 2011). Specifically, *Flavobacteria* relatives are considered among the main remineralizers of high-molecular weight organic substances, indeed they are often found abundantly associated with particulate matter/algal detritus but also colonizing the surface of

living organisms like plants, corals, macrophytes and microalgae (Fig. 12; Wagner-Dobler et al., 2010; Kolton et al., 2013; Mann et al., 2013). Recently, a strong algal host-dependent lifestyle has been observed for a member of this class highly specialized in degradation of monosaccharides and polysaccharides produced by green, red and brown algae (Mann et al., 2013). Furthermore, it has been pointed out that both *Flavobacteria* and *Sphingobacteria* quickly decompose phytodetritus of surface sediments (Gihring et al., 2009), and that *Sphingobacteria* are better competitors in more anoxic condition compared with *Flavobacteria* (Teske et al., 2011). Similarly to *Gammaproteobacteria*, algicidal activity of some bacterial strains affiliated with *Cytophaga-Flavobacteria-Bacteroides* has been reported (Mayali et al., 2004; Hare et al., 2005; Shi et al., 2013).

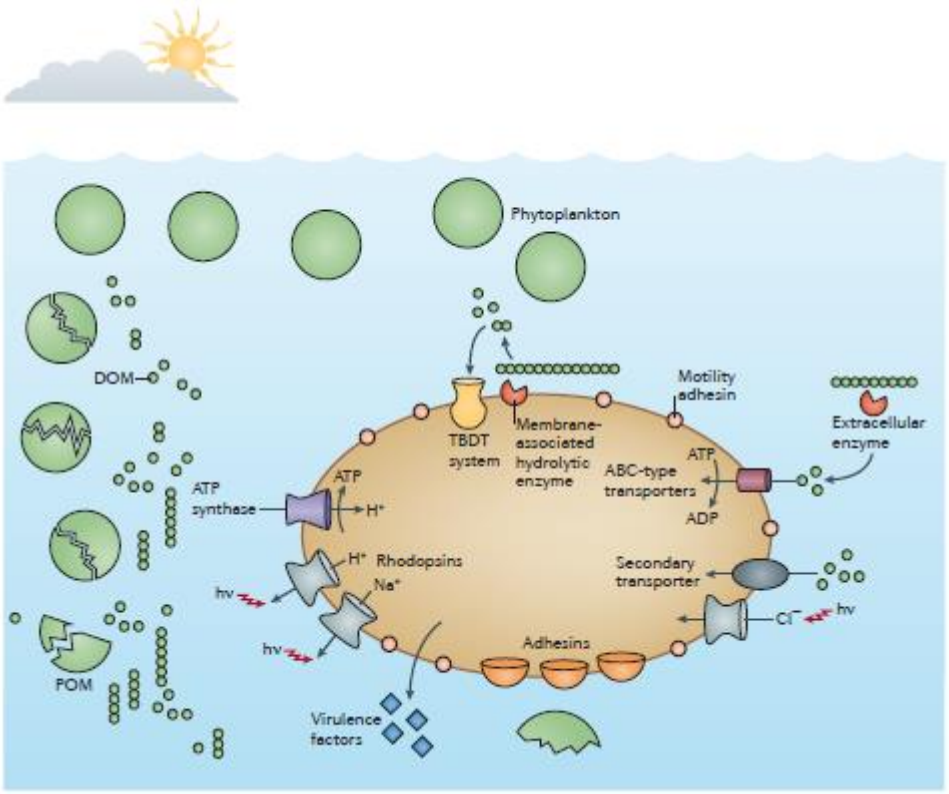

Fig. 12. Metabolic and physiological traits of *Flavobacteria* favoring associations with phytoplankton. *Flavobacteria* genomes show a vast inventory of genes involved in interactive processes with phytoplankton and organic matter of algal origin. They are efficiently specialized in degradation of high molecular weight compounds that occur through membrane-associated and extracellular hydrolytic enzymes, such as laminarinases and β-D-fucosidases, harbored in multiprotein extracellular systems. Degradation products are then incorporated through TonB-dependent transport (TBDT) systems. Additional transporters (ABC-type and secondary transporters) for the

uptake of low molecular weight phytoplanktonic components are present. In some strains, adhesins (e.g. SprB and RemA) allow gliding cell motility over surfaces. Other putative adhesins are thought to favor attachment to both living and dead surfaces. Proteases with supposed algicidal activity are transcribed in some flavobacterial genomes. Additionaly, many *Flavobacteria* possess rhodopsins, that are light-driven ion (H^+, Cl^-, or Na^+) pumps providing additional energy to the cell and guaranteeing an adequate ion balance during striking variations as occur throughout bloom development. (from Buchan et al., 2014 modified).

4.3. Some aspects on bacterial-viral relationships

Bacteria are tipically unicellular prokaryotic organisms whose dimensions normally range from 0.2 and 2 µm (Sieburth et al., 1978). Although their ubiquitous presence in the marine environment was already well known, their enumeration has been possible only after development of epifluorescence microscopy counting methods in the 70's, which revealed average abundances in the order of 10^6 - 10^9 cells L^{-1} (Ferguson and Rublee, 1976; Hobbie et al., 1977).

It is known that heterotrophic bacteria (along with archaea) in the marine environment degrade both dissolved and particulate organic matter (DOM and POM, respectively) of different origin, but most directly or indirectly deriving from the phytoplanktonic production (Azam et al., 1983; Ducklow and Carlson, 1992; Buchan et al., 2014). In this way, bacteria make available inorganic forms of the elements and participate significantly to the biogeochemical turnover of the matter in the oceans (Paerl 1975; Smith et al. 1992; Duhram et al., 2015).

Existence of viruses (biological entities with dimensions ranging from 0.2 and 2 µm; Sieburth et al., 1978) in the marine environment is recognized since several decades (Spencer, 1955; Valentine et al., 1966, Torrella and Morita, 1979). However, their great importance in marine microbial community functioning has been hypothesized only around the end of the last century, when new fluorochromes made possible visualization and counting of these infectious agents, disclosing viral densities in the order of 10^8 - 10^{10} VLPs L^{-1} (virus like particles; Fig. 13) (Bergh et al., 1989; Paul et al., 1993; Hara et al., 1996; Proctor, 1997; Wilhelm and Suttle, 1999).

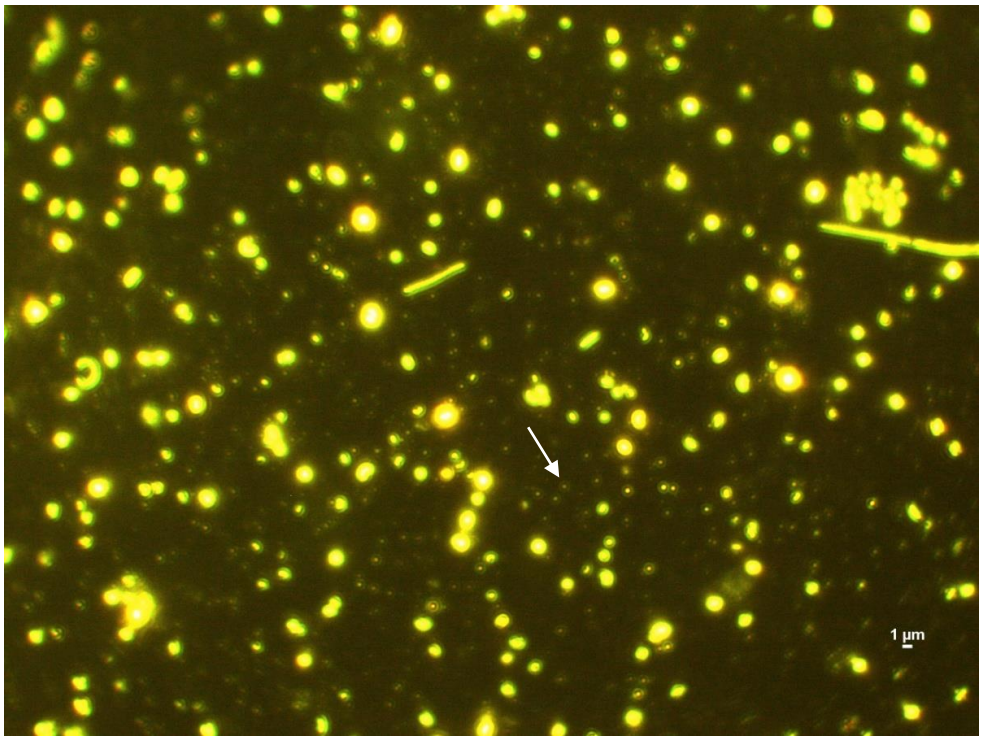

Fig. 13. Water sample from the brackish Lake of Ganzirri (Messina, Italy) at 1000x magnification, Sybr Gold staining. The tiny little points are virus like particles (e.g. white arrow), while larger spots are heterotrophic and/or phototrophic bacteria. (Courtesy of Prof. Silvana Vanucci).

The enormous abundances recorded for such metabolically inert bodies in aquatic habitats suggest a significant role of viruses in turnover of substances and cycling of nutrients in trophic webs. In addition, it has been emphasized the key impact of viruses on population and community dynamics of organisms involved in the microbial loop (Fenchel et al., 2008), as viruses are important mortality agents for bacteria and phytoplankton (Fuhrman, 1999; Wommack and Colwell, 2000). As a result, cell abundances and taxonomic structure of microbial populations (mostly bacterial communites) are modulated by viral infection extents, along with other drivers as resource availability (e.g. inorganic nutrients, organic matter) and grazing activity by protists (Wilhelm and Matteson, 2008 and references therein). Viral infection is species-specific, and viruses randomly come into contact with host cells. The modes through which viruses reproduce are mainly lytic and lysogenic infection. During lytic process the virus attach to the host cell, injects genetic material and induce the infected cell machinery to synthesize and assemble new viral nucleic acids and proteins. Then, the new viral progeny is released into the environment after host-cell lysis. During lysogenic infection, the viral genetic material integrates

within the bacterial chromosome by recombination becoming a prophage, which reproduce as genetic material in the host-cell line (Fig. 14; Breitbart et al., 2008).

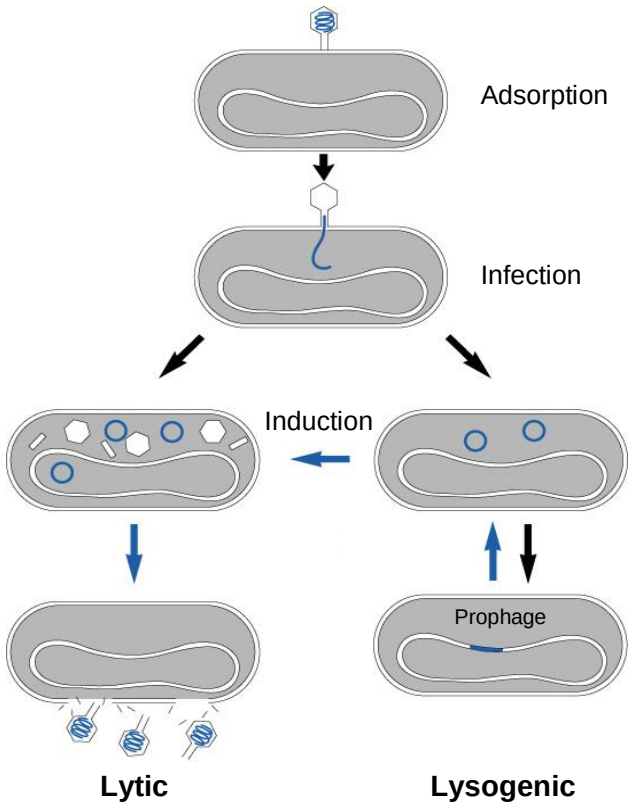

Fig. 14. Schematic diagram of lytic and lysogenic viral infection (from Ptashne et al., 2004 modified; Breitbart et al., 2008 modified).

Lysogenic infection represents a "survival" strategy that virus adopt when host cellular densities are low (thus the host-encounter rate is low), or more in general, when environmental condition are adverse. Lytic infection is induced by high density and/or metabolic activity of the host cells colonizing the environment (Fuhrman and Suttle, 1993; Breitbart et al., 2008, 2012). It is postulated that winners in the competition for resources have the highest abundances in planktonic (and sediment) bacterial communities, and that such bacterial groups are subjected to a striking decline after viral lysis events; such theory has been termed "Kill the Winner" (Thingstad and Lignell, 1997). Thus, owing to the viral pressure accounting for 20 to 50% of the bacterial mortality in surface coastal waters (Fuhrman and Noble, 1995), any bacterial species will reach considerable higher abundances than the others within a steady-state microbial

community, allowing a high bacterial diversity in the same habitat. In the majority of marine environments viral abundance overcame the bacterial abundance (Danovaro et al., 2011), and the average virus to bacteria ratio (VBR) is about 10 (Wommack and Colwell, 2000). Higher VBR values (40-50) are recorded in eutrophic environments while lower values are registered in oligotrophic habitats or in steady-state conditions (Needham et al., 2013), suggesting a greater infection rate and a higher number of viral attacks when environmental conditions promote high bacterial growth and productivity (e.g. Breitbart et al., 2012; Liu et al., 2015). When lysis occurs, the lysed cell become detritus, made up by dissolved molecules (monomers, oligomers, polymers), colloidal substances and cellular fragments (Shibata et al., 1997). Proctor and Fuhrman (1990) and Bratbak et al. (1993) showed that quite the whole DOM deriving from viral lysis is assimilated from bacteria. Hence, if the lysed cell is a bacterium, it will establish a kind of trophic ring known as "viral shunt" (Fig. 15), where bacteria themselves degrade the bacterial biomass (Fuhrman, 1999).

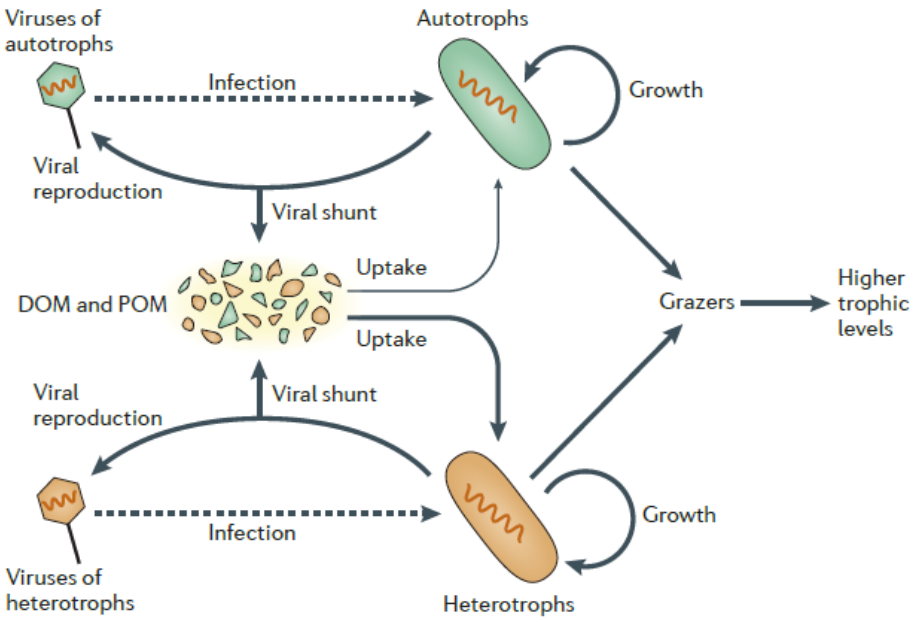

Fig. 15. Schematic of the viral shunt. (from Jover et al., 2014 modified).

4.3.1. Bottom-up and top-down control on bacterial communities

The size in terms of abundance of a bacterial population is firstly determined by the balance between growth and mortality rate; however, community composition and structure in relation to

dominant taxa is driven by nutrient availability and quality, and more generally by environmental resources. Such kind of regulation is defined "bottom-up", and take place through the competitive interactions engaged between different bacterial groups for utilization of the same resources. When resources are limiting, the bacterial community will be mainly characterized by the few dominant species (i.e. *core taxa*) best adapted to the occurring nutrient and environmental conditions, which therefore overcame rare taxa (*seed bank*; Pedròs-Aliò, 2006). Viral infection play a primary role in this mechanism, making available dissolved or particulate organic forms of nutrients through bacterial and/or phytoplankton cell lysis (viral shunt; Fuhrman, 1999). On the other hand, along with grazers, viruses can exert a relevant top-down regulation on bacterial communities via the "Kill the Winner" strategy, usually with a larger extent in eutrophic environments (Winter et al., 2010; Liu et al., 2015) (see also before, 4.3).

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Aims

Intense blooms of toxic epiphytic/benthic dinoflagellate *Ostreopsis* spp., once constrained to tropical and subtropical areas, have recently spread to more temperate regions, including the Mediterranean Sea, with increasing frequency and areal extension.

Ostreopsis' bloom events in the Mediterranean area are mainly due to the species *Ostreopsis* cf. *ovata*, which proliferates in shallow and sheltered waters under low hydrodinamism, growing epiphytically on a wide range of benthic substrata (e.g. macroalgae, invertebrates, rocks), producing brownish mucilage mats. Mediterranean *O.* cf. *ovata* produces palytoxin-like compounds (i.e. isobaric palytoxin and a wide range of ovatoxins) that cause both mortality of benthic marine organisms and noxious effects on human health through aerosol inhalation, skin contact, or ingestion of seafood that can bioaccumulate algal-derived toxins.

Several studies have been conducted to assess the role of abiotic factors (mainly hydrodynamics, water temperature and nutrients) on bloom dynamics, while biotic factors such as competition with other microalgal species and interaction with bacteria have only been barely addressed.

The aim of this thesis is to provide insights about the effect of the mentioned biotic agents in affecting *O.* cf. *ovata* growth and physiology with emphasis on dinoflagellate-bacterial interactions, and ultimately make some inferences on their role in *O.* cf. *ovata* blooms' development, maintenance and decline processes. Specifically, the main goals are: (i) to evaluate inorganic nutrients uptake and organic phosphorus utilization by *O.* cf. *ovata* in order to address the dinoflagellate competitivness in relation to other microalgal species co-occurring in the mycrophytobenthyc assemblage; (ii) to assess the phylogenetic composition of bacterial assemblage co-occurring with *O.* cf. *ovata* at the early and peak phases of a natural bloom in order to highlight most prominent bacterial-algal associations, and compare bacterial assemblages associated with *O.* cf. *ovata* with those from ambient seawaters for evaluating possible contribution of allochthonous bacteria to the former ones; (iii) to investigate the temporal dynamics of microbial (bacteria and viruses) abundances, bacterial taxonomic composition and algal toxin production in *O.* cf. *ovata* batch cultures throughout the algal growth cycle.

This thesis is composed of three chapters, whereof one of them has been published as conference proceedings and the other two have been submitted to ISI journals. Scientific and technical

supports have been provided by Prof. Silvana Vanucci (Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina), Prof. Rossella Pistocchi (Algal Biology Lab, University of Bologna), Prof. Richard A. Long (Acquatic Microbial Molecular Ecology Lab, University of South Carolina), Prof. Carmen Dell'Aversano and Dr. Luciana Tartaglione (Natural Chemical Substances Lab, University Federico II of Naples).
The chapters of this thesis address the following topics:

- Chapter 1 reports on a laboratory study performed in order to get better insights into nutrient utilization by *Ostreopsis* cf. *ovata*. Nutrient uptake kinetics for nitrate and phosphate were characterized, along with induction of alkaline phosphatase activity under different N:P ratios. Retrieved data were compared with available literature for other microalgae, hypothesizing potential advantages conferred to *O.* cf. *ovata* by its nutrient utilization strategy towards other species of the microphytobenthos competing for resources.

- Chapter 2 reports on an environmental survey of the bacterial assemblages co-occurring with *Ostreopsis* cf. *ovata* bloom. Phylogenetyc composition of the bacterial assemblages associated with *O.* cf. *ovata* mats and ambient seawater bacterial assemblages were assessed at two different phases of the bloom through 16S rDNA pyrosequencing, providing first phylogenetic data on bacteria associated with toxic benthic/epiphytic dinoflagellates in the environment.

- Chapter 3 reports on an investigation of microbial (bacteria and viruses) dynamics and phylogenetic composition of the bacterial community co-occurring with *Ostreopsis* cf. *ovata* in batch cultures during the different algal growth phases along with toxin production. *O.* cf. *ovata*, total bacteria, highly respiring bacteria (CTC^+) and viral cell abundance were monitored along with major nutrients removal from the medium, while bacterial phylogenetic composition and algal toxin production were determined through 16S rDNA ION torrent sequencing and LC–HRMS analysis, respectively. The study was undertaken in order to infer interactions that synergistically affect algal and bacterial physiology and metabolism with potential relevance on bloom dynamics. A brief comparison between the bacterial assemblages co-occurring with *O.* cf. *ovata* bloom with the microbiota selected under batch cultures of the *O.* cf. *ovata* strain isolated during the same bloom event has been also reported.

Chapter 1

Inorganic nutrients uptake and organic phosphorus utilization by *Ostreopsis* cf. *ovata*

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ABSTRACT

Although nutrient implication in bloom dynamics has not yet been clarified, in *Ostreopsis* cf. *ovata* cultures the importance of nutrient concentrations (N and P) for cell growth and toxicity has been remarked. Nutrient uptake pattern in cultured strains also evidenced the need for balanced N/P ratios for optimal growth of this species; moreover it appears that the intracellular nutrient ratios tend towards balanced conditions independently by concentrations (N:P ratio) in the external medium. The present study was therefore performed in order to get better insights into nutrient utilization by *O.* cf. *ovata*. The characterization of the nutrient uptake kinetics showed that this species has higher V_{max} and higher affinity values for P uptake than for N. In addition, a study performed with cultures grown at different N:P ratios, showed that alkaline phosphatase activity was induced when external P was depleted or present in low amounts. These findings attest to the high efficiency of *O.* cf. *ovata* in both inorganic and organic P acquisition, an aspect that could confer advantages towards competing species.

Keywords: alkaline phosphatase, N:P ratio, nutrient uptake, *Ostreopsis* cf. *ovata*

1. Introduction

Ostreopsis cf. *ovata* blooms along Italian coasts display different characteristics for intensity, toxic effects and blooming period; it is therefore difficult to relate them to specific environmental conditions. Among the different parameters involved, nutrient influence on blooms is one of the most difficult to ascertain due either to the complexity in obtaining a large dataset of measurements and to the difficult interpretation of the data. Culture studies can thus be of help in understanding the nutrient dynamics of a microalga species. Previous studies performed in order to correlate nutrient availability, growth and toxicity of this species have left some questions open. For example: 1) which is the main limiting nutrient? P uptake occurred slightly faster than N uptake, however when N was limiting P uptake significantly decreased while when P was limiting N uptake rate remained mostly unaffected (Vanucci et al., 2012); ii) is toxin production influenced by cell nutrient status? Nutrient depletion did not cause any increase in toxin content per cell both under unbalanced (Vanucci et al., 2012 and balanced conditions (Pezzolesi et al., in prep.), however, cell toxin production sharply increased as soon as external nutrients were depleted (Pezzolesi et al., 2014); iii) can this species obtain nutrients from organic matter? The cells were able to maintain a constant intracellular N/P ratio despite the fast P uptake resulted in an unbalanced N:P ratio (high) in the external medium (Pezzolesi et al., in prep.; Pinna et al., submitted).

The present study was aimed to integrate previous results with new data, obtained using *O.* cf. *ovata* cultures, in order to better understand: i) the nutrient utilization pattern, ii) the growth and cellular responses to different N:P ratios, iii) the relationship between nutrient utilization pattern and toxicity.

2. Material and Methods

Ostreopsis cf. *ovata* cultures were grown in 50 mL medium having nutrients of f/2 medium (Guillard, 1975) with the exception of N and P which were added in different concentrations in order to obtain five N:P ratios (16, 24, 30, 50,100); these N:P values were achieved by adding the two macronutrients both at a low (N=11,8 μM; P= 0,74-0,12 μM) and a high level (N=176,6 μM; P= 11,0-1,76 μM). Four replicates for each condition were performed. Low nutrient cultures were grown for 8 days and those at high nutrients for 11 days. On the last day the following parameters were measured: cell numbers (Utermöhl, 1958) (n=4), alkaline phosphatase activity (as described in Cucchiari et al., 2008) (n=2), dissolved N and P (kit analysis with DR/2010; Hach, Colorado, USA) (n=2), cellular N, C (through ThermoFisher organic elemental analyzer Flash 2000) and P (according to Menzel and Corwin, 1965) (n=2).
Nutrient uptake rates were measured as described in Kwon et al. (2013).

3. Results and Discussion

3.1. Inorganic nutrients uptake

N and P uptake kinetics were studied allowing the calculation of the semi-saturation constant (K_s) and the maximal transport rate (V_{max}) for *O.* cf. *ovata* (Fig. 1).
P uptake was characterized by higher V_{max} (mean value: 1.36 pmol cell^{-1} h^{-1}) and lower K_s (mean value: 4.82 μM) values than those measured for N (mean values: 1.02 pmol cell^{-1} h^{-1} and 8.4 μM, respectively) attesting that this species has a higher transport affinity for P than for N and that the former nutrient is taken up by cells at higher rates. This finding was in agreement with the pattern previously observed measuring the external nutrient consumption during growth (Vanucci et al., 2010; Pezzolesi et al., 2014) and gives consistency to those results. A comparison between the kinetic parameters of *O.* cf. *ovata* with those of different species evidenced that Ks values for N and P in *O.* cf. *ovata* were in the range of those observed in benthic diatoms (Kwon et al., 2013) but higher than those measured in planktonic toxic dinoflagellates (e.g. *Alexandrium tamarense* and *A. catenella*) (Yamamoto and Tarutani, 1999; Jauzein et al., 2010); conversely, the V_{max} values were higher for *O.* cf. *ovata* then for the other species and, in particular, P value was twice the average value.

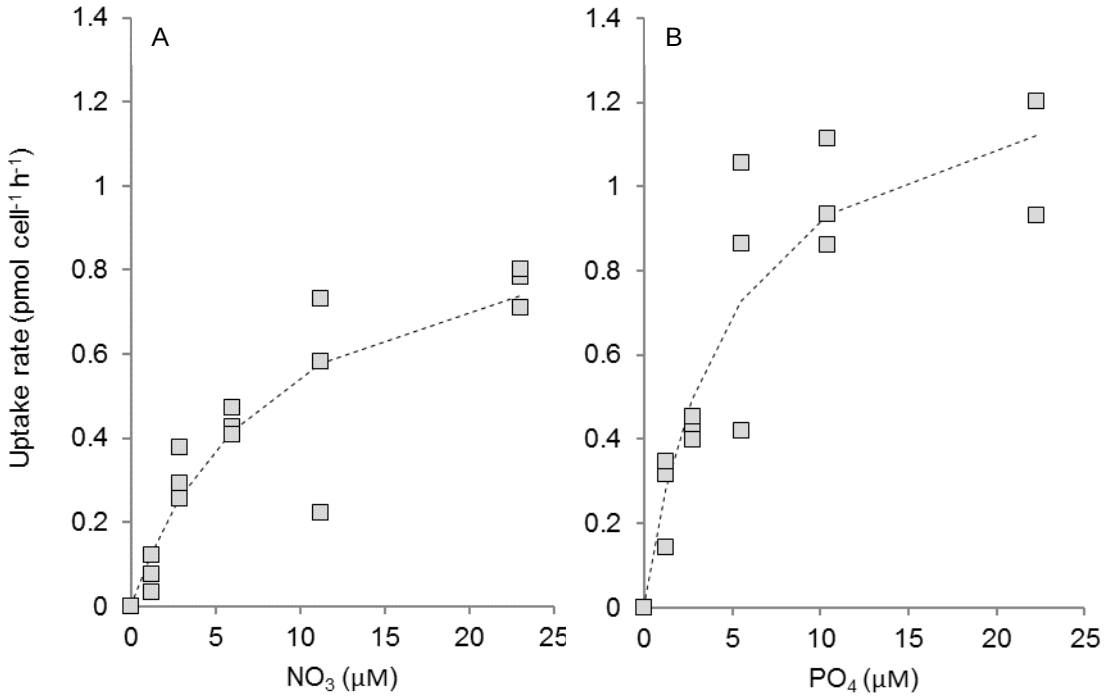


Fig. 1. Nitrate (A) and phosphate (B) uptake rates of *O.* cf. *ovata* as a function of the ambient nitrate and phosphate concentration. The curves were fitted to the observed values using a non-linear least square method.

3.2. Effect of N:P ratio on growth

By performing cultures under different initial N:P ratios it was observed that *O.* cf. *ovata* growth, evaluated as cell numbers (data not shown) or as carbon (Fig. 2), was affected mainly by the nutrient level rather than by the N:P ratio. Carbon concentrations were 4 to 5-fold higher in HN than in LN cultures but, within the nutrient level, values were similar among the different N:P conditions. Cultures in low nutrients (LN) reached a similar final cell concentration, not higher than 1,500 cell mL^{-1}, and stopped growing before depleting the external N and P (data not shown). Those in high nutrients (HN) reached the highest cell concentrations at N:P ratios of 24 and 30 (12,369 and 12,525 cell mL^{-1}, respectively) and only cultures with N:P ratios equal or higher than 30 depleted all the P, while N was not completely exhausted (data not shown).

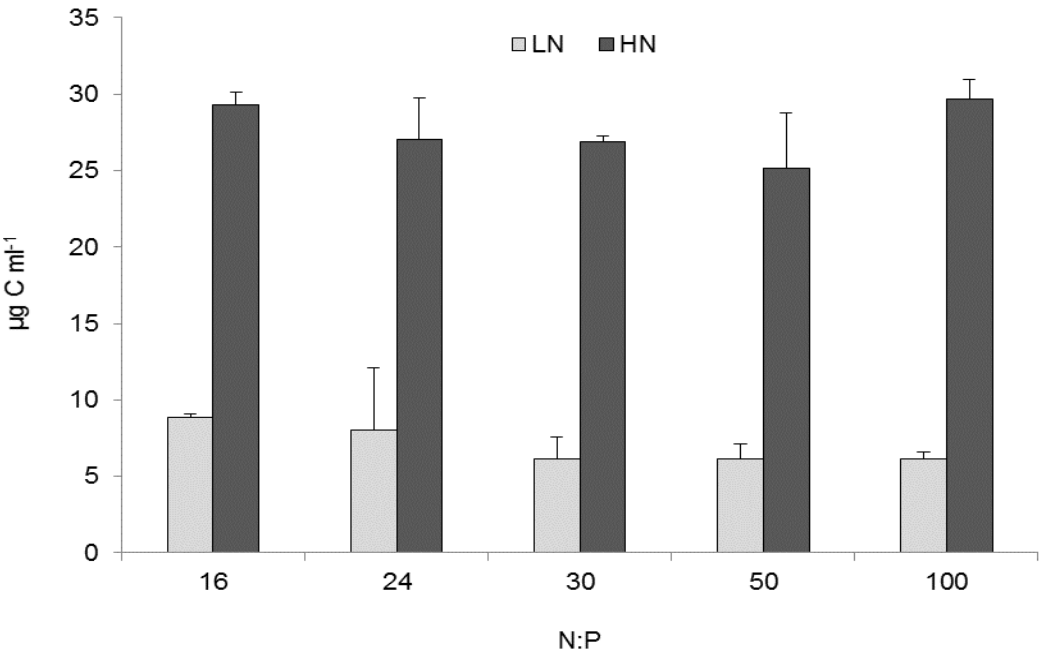
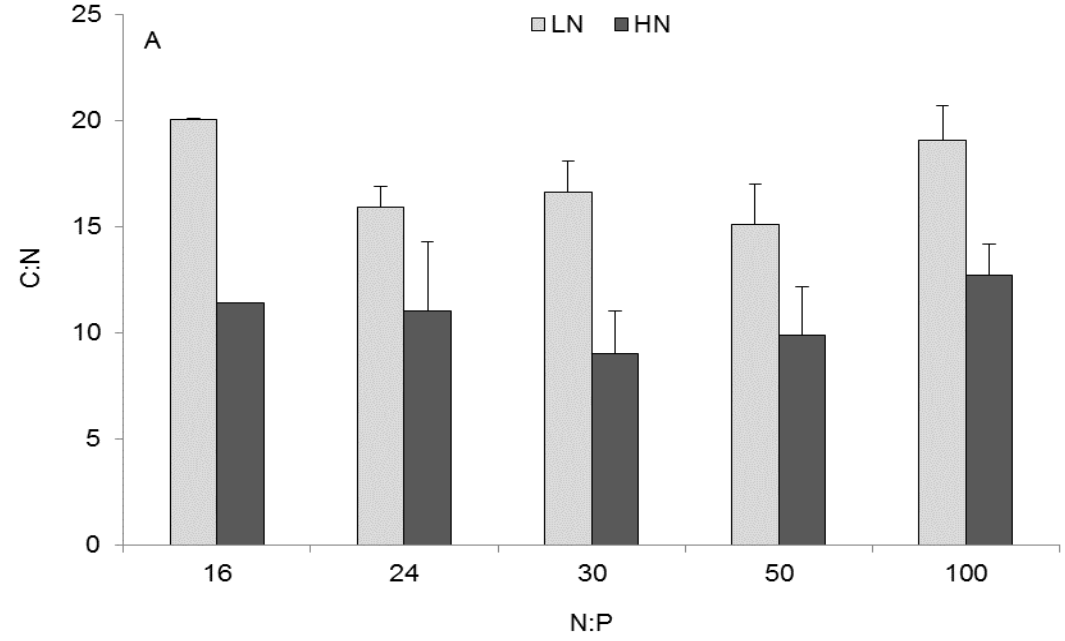


Fig. 2. Carbon concentrations measured in *O.* cf. *ovata* cultures grown in the presence of high (HN) or low (LN) nutrients levels: N and P were added in order to obtain different initial N:P ratios. The measurements were performed at day 8 (LN) or 11 (HN) of growth. Bars indicate standard deviation.

C, N and P contents in *O.* cf. *ovata* cells were measured in the last day of growth (day 8 and 11 for LN and HN, respectively), which represents the beginning of the stationary phase, and allowed the corresponding C:N and C:P values to be obtained. Cell C:N ratios (Fig. 3a) remained stable and displayed higher values in cultures grown with lower than those with higher nutrients content, presumably due to an increased polysaccharide production. Cell C:P (Fig. 3b) and N:P (Fig. 3c) ratios increased only in HN cultures which grew at higher levels and which depleted the added phosphate faster; both ratios increased in cultures having an external N:P $\geq$ 30 and increased with the increasing of P-depletion. These results attest that the best condition for *O.* cf. *ovata* growth is represented by the presence of high macronutrient levels in a balanced ratio, between 16 and 30. Nitrogen seems to be the most important element for high biomass accumulation while the P stored, thanks to high uptake capacity, can sustain growth when the environment becomes P-limited.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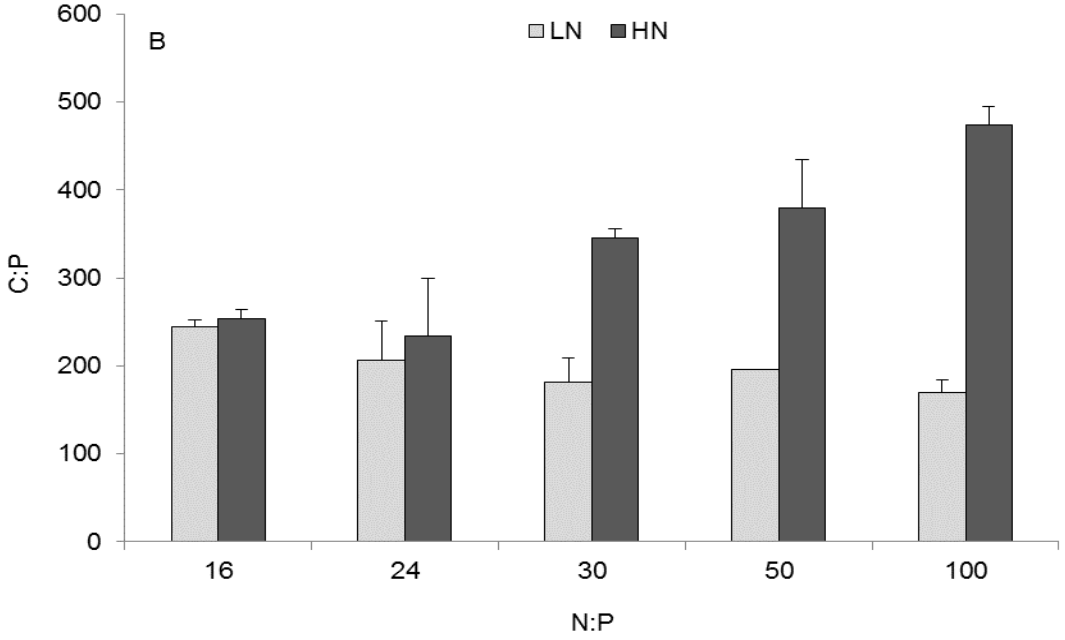
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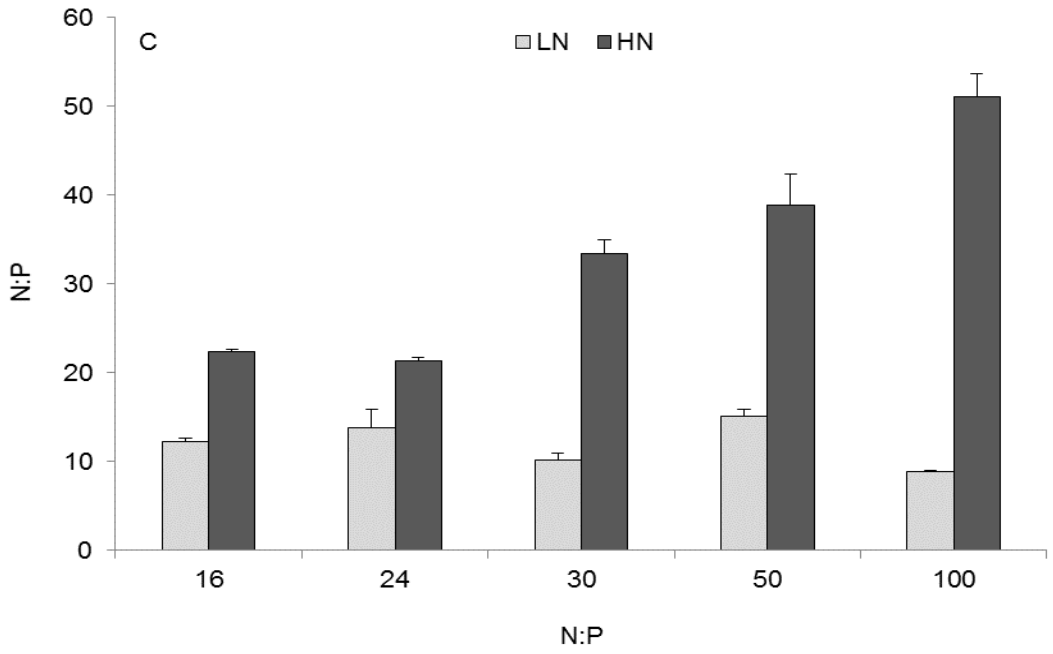

Fig. 3. Cell C:N (A), C:P (B) and N:P (C) ratios measured in *O.* cf. *ovata* cultures grown in the presence of high (HN) or low (LN) nutrients levels, at day 8 and 11, respectively. Bars indicate standard deviation.

3.3. Organic phosphate utilization

Alkaline phosphatase activity (APA) showed the lowest (and comparable) values in cultures with N/P = 16 and 24, both at low and high nutrient levels (Fig. 4). The enzyme activity was induced in cultures with a N:P ratio higher than 24 both in HN and LN conditions. The enzyme was induced also in LN cultures which did not deplete all the external P and where the intracellular C:P and N:P ratios were not as much affected as in HN cultures (Fig. 3b,c). This is consistent with the observation (Jauzein et al., 2013) that intracellular P concentrations are not as direct in APA regulation as are the extracellular concentrations.

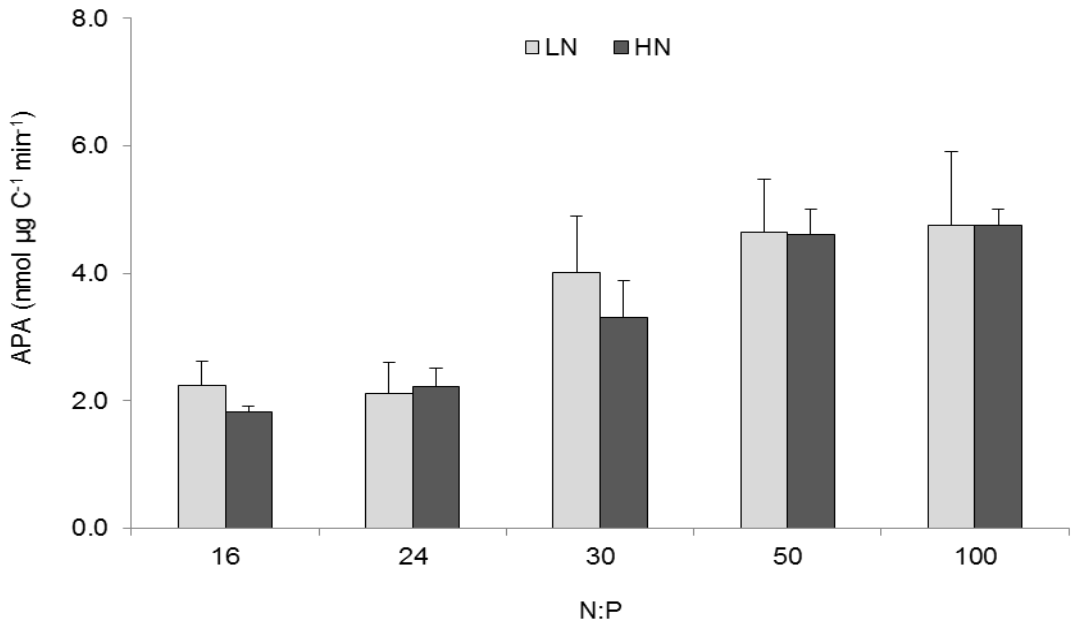

Fig. 4. Alkaline phosphatase activity measured in *O.* cf. *ovata* cultures grown in the presence of high (HN) or low (LN) nutrients levels; N and P were added in order to obtain different initial N/P ratios. The measurements were performed at day 8 (LN) or 11 (HN) of growth. Bars indicate standard deviation.

In addition, in LN cells a pgP pgC^{-1} value, very close to the threshold value of 0.016 reported as necessary for AP synthesis (Jauzein et al. 2013), was observed. In HN cultures, the enzyme was induced in the culture condition where the external P was firstly depleted. In a preliminary experiment, where APA was measured at day 8 also in HN cultures, any APA induction was observed (Pistocchi, unpublished). Thus the possibility to use organic P as soon as the inorganic form is depleted attests the high capacity of *O.* cf. *ovata* to scavenge this nutrient from the environment, an aspect that, in addition to highly efficient uptake parameters, could confer a competitive advantage towards other microalgae species.

In the attempt to establish a link between the present results and *O.* cf. *ovata* toxin production, it has been recently observed (Pinna et al., submitted) that an increase in cellular C:P and C:N ratios along the growth curve, due to an ongoing primary production coupled with poor nutrient conditions, corresponds to an increase in toxin production (increasing tox/C ratio), as it has usually been observed in the stationary phase. The present results allowed us to ascertain that the presence of high initial nutrient levels, characterized by N:P ratios higher than the optimal ones (e.g. for nitrogen inflow), can sustain high cells growth determining a high C:P ratio in

short time. On the other hand, in the presence of low nutrient levels, C:N values higher than those considered as physiological were observed. Both conditions could thus be linked to an increased relative cell toxin production.

Acknowledgements

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Chapter 2

Phylogenetic structure of bacterial assemblages co-occurring with *Ostreopsis* cf. *ovata* bloom

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ABSTRACT

Extensive blooms of the toxic epiphytic/benthic dinoflagellate *Ostreopsis* cf. *ovata* are being reported with increase frequency and spatial distribution in temperate coastal regions including the Mediterranean. These blooms are of human and environmental health concern due to the production of isobaric palytoxin and a wide range of ovatoxins by *Ostreopsis* cf. *ovata*. Bacterial-microalgal interactions are important regulators in algal bloom dynamics and potentially toxin dynamics. Our study investigated the bacterial assemblages co-occurring with *O*. cf. *ovata* biofilms (OA) and from ambient seawaters (SW) during the early and peak phases of bloom development in NW Adriatic Sea.

In total, 14 bacterial phyla were detected by targeted 454 pyrosequencing of the 16S rRNA gene. The dominant bacterial phyla in the OA assemblages were *Proteobacteria* and *Bacteroidetes*; while at the class level, *Alphaproteobacteria* were the most abundant (83 and 66%, relative abundance, early and peak bloom phases), followed by *Flavobacteria* (7 and 19 %, early and peak phases). *Actinobacteria* and *Cyanobacteria* were of minor importance (<5% of the relative bacterial abundance each). *Gammaproteobacteria* showed a notably presence in OA assemblage only at the early phase of the bloom (genus *Haliea*, 13%). The *Alphaproteobacteria* were predominately composed by the genera *Ruegeria*, *Jannaschia* and *Erythrobacter* which represented about half of the total phylotypes' contribution of OA at both early and peak phases of the *O.* cf. *ovata* bloom, suggesting interactions between this consortium and the microalga. Microbial assemblages associated with the ambient seawaters while being also dominated by *Alphaproteobacteria* and *Flavobacteria* were partially distinct from those associated with *O*. cf. *ovata* due to the presence of genera almost not retrieved in the latter assemblages.

Keywords: *Ostreopsis*, toxic dinoflagellate, HAB-associated bacteria, pyrosequencing, bacterial diversity, *Ruegeria*, *Haliea*

1. Introduction

Extensive blooms of toxic epiphytic/benthic dinoflagellate *Ostreopsis* spp., once limited to tropical and subtropical regions of the oceans and Caribbean Sea (Fukuyo, 1981; Besada et al., 1982; Chang et al., 2000; Hurbungs et al., 2001; Rhodes, 2011) are currently reported with increasing frequency and areal distribution in temperate regions including the Mediterranean Sea (Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011; Rhodes, 2011; Parsons et al., 2012; Furlan et al., 2013 and references therein). The *Ostreopsis* blooms along the Mediterranean coasts are mostly due to *Ostreopsis* cf. *ovata* (Battocchi et al., 2010; Perini et al., 2011) that usually grow epiphytically forming brownish mucilaginous biofilms on macroalgae; in addition, mats can form on reefs, rocks, soft sediments, seaweeds, marine angiosperms, and invertebrates in shallow and sheltered waters (Vila et al., 2001; Turki, 2005; Aligizaki and Nikolaidis, 2006; Totti et al., 2010; Accoroni et al., 2011). Moreover, *O.* cf. *ovata* is often found in plankton samples due to resuspension from the benthic substrates, sometimes showing high cell numbers in the overlying water column (Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Selina and Orlova, 2010; Totti et al., 2010).

Mediterranean *O.* cf. *ovata* produces palytoxin-like compounds, namely, isobaric palytoxin and a wide range of ovatoxins (OVTX-a to-k; Garcìa-Altares et al., 2014; Brissard et al., 2015; Tartaglione et al., 2016). Toxin production has been observed under both field and culture conditions (e.g. Accoroni et al., 2011; Ciminiello et al., 2011, 2012a,b; Scalco et al., 2012; Pezzolesi et al., 2012; Vanucci et al., 2012a; Pezzolesi et al., 2014). *O.* cf. *ovata* Mediterranean blooms can have a severe impact on human health causing intoxications through marine aerosol inhalation and contact (Gallitelli et al., 2005; Kermarec et al., 2008; Tichadou et al., 2010; Funari et al., 2015), and they also strongly affect invertebrate benthic communities causing massive mortalities due to severe cytotoxic effects and hypoxia/anoxia episodes (Shears and Ross, 2009; Louzao et al., 2010; Accoroni et al., 2011; Faimali et al., 2012; Gorbi et al., 2012, 2013; Pagliara and Caroppo, 2012; Carella et al., 2015; Funari et al., 2015). Moreover, palytoxin-like compounds produced by *Ostreopsis* spp. bioaccumulate in tissues of both filter-feeding bivalve mollusks and herbivorous echinoderms (Aligizaki et al., 2008, 2011; Ramos and Vasconcelos, 2010; Ciminiello et al., 2011, 2015; Amzil et al., 2012; Furlan et al., 2013; Brissard et al., 2014) representing a further possible threat for human health associated with seafood consumption. Driven by the negative ecological, economic and human health impacts of *O.* cf. *ovata*

Mediterranean outbreaks, research efforts have been aimed at identifying the environmental conditions and factors that trigger or regulate the microalgal bloom dynamics, including hydrodynamism, water temperature, nutrient concentrations, and substrata (Aligizaki and Nikolaidis, 2006; Totti et al., 2010; Granéli et al., 2011; Pistocchi et al., 2011; Mangialajo et al., 2011; Accoroni et al., 2015a,b). One of the major factors affecting *Ostreopsis* blooms is hydrodynamic regime, as consistently higher abundances are found under low hydrodynamism and in sheltered sites compared with exposed ones (Chang et al., 2000; Shears and Ross, 2009; Totti et al., 2010; Mabrouk et al., 2011; Selina et al., 2014). Storm events may disrupt bloom development leading to a sudden decrease of cell abundances on the benthic substrata (Totti et al., 2010; Accoroni et al., 2011), however, abundance can be rapidly restored under suitable conditions (Accoroni et al., 2015a).

In parallel to the physicochemical factors, a greater appreciation about the significance of the bidirectional interactions between microalgae and bacteria in terms of regulating harmful algal blooms (HABs) has developed (Kodama et al., 2006; Loureiro et al, 2011). The relationship between dinoflagellates and their associated bacteria is recognized to be a complex association that can range from being mutualistic to antagonistic (e.g. Azam and Ammerman 1984; Doucette et al., 1998; Fuchs et al., 2002; Kodama et al., 2006; Wang et al., 2014, 2015). Culture-based studies on harmful planktonic dinoflagellates, using both biochemical and traditional molecular methods, have shed light on several effects of bacteria on algal cellular physiology by mechanisms such as nutrient competition, production of stimulatory or inhibitory compounds, which will ultimately influence cell growth dynamics, production, degradation, and/or modification of algal toxins (e.g. Hold et al., 2001; Long et al., 2003; Su et al., 2005; Azanza et al., 2006; Donovan et al., 2009; Wang et al., 2010; Green et al., 2010; Bolch et al., 2011; Santos and Azanza, 2012). Patterns of association between cultured harmful dinoflagellates and specific bacterial groups such as *Alpha-* and *Gammaproteobacteria*, *Cytophaga-Flavobacteria-Bacteroides* and some sub-groups, primarily the *Roseobacter* clade and the *Alteromonas*, have been observed (Jasti et al., 2005; Amaro et al., 2005; reviewed by Garcés et al., 2007; Green et al., 2010 and references therein; Onda et al., 2015). Moreover, several members of the *Roseobacter* clade (e.g. phylotypes of *Phaeobacter* and *Ruegeria* genera) associated with marine algae and/or isolated from non-toxic and toxic dinoflagellate cultures are known as some of the most effective colonizers of surfaces in the coastal environments (e.g. Slightom and Buchan,

2009 and references therein; Elifantz et al., 2013). Furthermore, *Roseobacters* have the capability to produce potent antibacterial compounds, mainly affecting non-*Roseobacter* phylotypes, giving selective advantage to this clade (Long and Azam 2001; Brinkhoff et al., 2004; Bruhn et al., 2007). These characteristics serve as potential mechanisms explaining the dominance of this clade in the association with marine microalgae (reviewed in Geng and Belas, 2010; Teeling et al., 2012; Buchan et al., 2014). In addition, it has been recently demonstrated that the interaction between dinoflagellates and these bacteria can be mutualistic, antagonistic, or switch between both (Wagner-Döbler et al., 2010; Wang et al., 2014, 2015), depending on algal physiological status as aging algae will induce the production of algicidal compounds by bacteria (e.g. *Phaeobacter gallaeciensis,* Seyedsayamdost et al., 2011, 2014; *Ruegeria* sp. TM1040, Riclea et al., 2012) which could have an important role in the declining stages of algal blooms (Riclea et al., 2012).

However, field impact of these associations or how elements of natural bacterial assemblages interact with the HAB population is still poorly known (Mayali and Azam 2004; Mayali et al., 2008), outlining the need for *in situ* studies assessing phylogenetic diversity and its temporal changes of the natural co-occurring bacterial populations during HABs. The limited studies on harmful planktonic dinoflagellates' microbiota generally converge on a broad feature for the dominance of the two bacterial groups the *Rhodobacterales* (*Alphaproteobacteria*) and *Cytophaga-Flavobacteria-Bacteroides* (*Bacteroidetes*) during blooms, despite geographical locations (Garcés et al., 2007; Jones et al., 2010; Park et al., 2015). Differently, Yang et al. (2012, 2015) recorded the dominance of *Alphaproteobacteria* and *Gammaproteobacteria* during *Akashiwo sanguinea* blooms.

The ecological role of bacterial assemblages associated with epiphytic/benthic toxic dinoflagellates has received considerably less attention than for their planktonic counterparts. Previous studies relied upon bacteria plate isolation from cultured dinoflagellates (e.g. *Ostreopsis lenticularis* and *Gambierdiscus toxicus,* Tosteson et al., 1989; *Prorocentrum lima,* Lafay et al., 1995; Prokic et al., 1998; *G. toxicus,* Sakami et al., 1999; *O. lenticularis,* Ashton et al., 2003), thus retrieving only bacteria capable of growing under this selective conditions and artificial nutrient regimes. Pérez-Guzmán et al. (2008) showed that about half of total bacteria associated with *O. lenticularis* cultures was made up by a single species belonging to *Cytophaga-Flavobacteria-Bacteroides* complex, and it was hypothesized that this species could be

implicated in ciguatoxin production. In contrast, Ruh et al. (2009) found *Alphaproteobacteria* to be the largest group in monoclonal cultures of *Coolia monotis* and *Ostreopsis ovata* (52% and 35% of the total retrieved sequences, respectively). A laboratory culture-based study provided evidence that bacteria interfere indirectly with *Ostreopsis* cf. *ovata* growth, toxin production, and likely on toxin degradation (Vanucci et al., 2012b). Currently, there is no phylogenetic data on the natural bacterial assemblages associated with *O.* cf. *ovata* blooms, and more generally with blooms of benthic dinoflagellates. The present study focuses on the phylogenetic characterization of the bacterial assemblages co-occurring over the early and the peak phases of a benthic *O.* cf. *ovata* bloom at Passetto station, a coastal moderate anthropogenic impacted area of the North-western Adriatic Sea.

The Passetto station, (Conero Riviera) has been classified as a hot-spot area for *Ostreopsis* cf. *ovata* blooms by the Italian Agency for the Protection and Environmental Research (ISPRA, 2012). In this region, *O.* cf. *ovata* summer blooms occur regularly, and cell abundances are among the highest recorded along Mediterranean coasts (Mangialajo et al., 2011; Accoroni et al., 2012; Accoroni et al., 2015a). This site is a semi-enclosed bay, sheltered by a natural reef and characterized by a mostly rocky bottom, and shallow depth (up to 2m). The shore is subjected to a moderate anthropogenic impact during the summer season (Marini et al., 2002), being a popular area for summer holidays, when it is also subjected to trampling by swimmers. This site is also characterized by the presence of small caves derived from human boring of the natural cliffs, with some wastewater discharge facilities. A clear and important role of the hydrodynamic conditions on *Ostreopsis* blooms' trend has been recognized in this area (Totti et al., 2010; Accoroni et al., 2011, 2012). Recently, Accoroni et al. (2015a) have proposed a conceptual model for annual *Ostreopsis* cf. *ovata* blooms in the Northern Adriatic Sea based on the synergic effects of hydrodynamics, temperature, and the N:P ratio of water column nutrients, pointing out that calm conditions appeared to be a prerequisite for blooms development. Indeed, low hydrodynamism would favor *O.* cf. *ovata* proliferation by facilitating macroalgal colonization, juxtaposing of the microalgal cells, and forming mucilaginous mats (Vila et al., 2001; Totti et al., 2010) which are known to be hotspots for microorganisms interactions (e.g. Cole et al., 2014; Carreira et al., 2015).

The aims of the present study were: (i) assessing the phylogenetic composition of bacterial assemblage co-occurring with *O.* cf. *ovata* at the early and the peak phases of the bloom in order

to highlight most prominent bacterial-algal associations; (ii) comparing bacterial assemblages associated with *O*. cf. *ovata* with those from ambient seawaters for evaluating possible contribution of allochthonous bacteria to the former ones. The bacterial assemblages were assessed by high-throughput parallel tag sequencing (454 pyrosequencing).

2. Material and Methods

2.1. Study area and samples collection

Bacterial assemblages associated with *Ostreopsis* cf. *ovata* aggregates (i.e. *O.* cf. *ovata* colonizing macrophytes) and ambient seawaters (SW) were collected at the early and at the peak phases (19 September and 2 October 2012, respectively) of an *O.* cf. *ovata* bloom along the coast of North-western Adriatic Sea (Passetto, Italy; 43°36'38" N and 13°32'20" E, Fig. 1).

Surface temperature (approximately 0.5 m depth) and salinity were recorded *in situ* by a YSI Pro Plus probe. Samples from six to eight dinoflagellate-colonized macrophytes for microorganisms (i.e. *O*. cf. *ovata* and bacteria) cell counting and bacterial assemblages structure analysis were base-cut using a sterile blade and immediately collected in 700-mL aseptic re-closable polyethylene bags with minimal seawater. Ambient seawater samples for microbial assemblages were collected in acid-washed, autoclaved 1-L polypropylene bottles. Additional water samples for nutrient analysis were collected in polyethylene bottles close to the sampled macrophytes. All samples were stored on ice and in the dark for transport to the laboratory.

Nutrient samples were prepared by filtering through Whatman GF/F filters (nominal pore size 0.7 μm) and stored at -20 °C until analysis. Nitrate, nitrite, ammonium, and phosphate concentrations were determined according to the methods described by Strickland and Parsons (1972), using an Autoanalyzer QuAAtro Axflow.

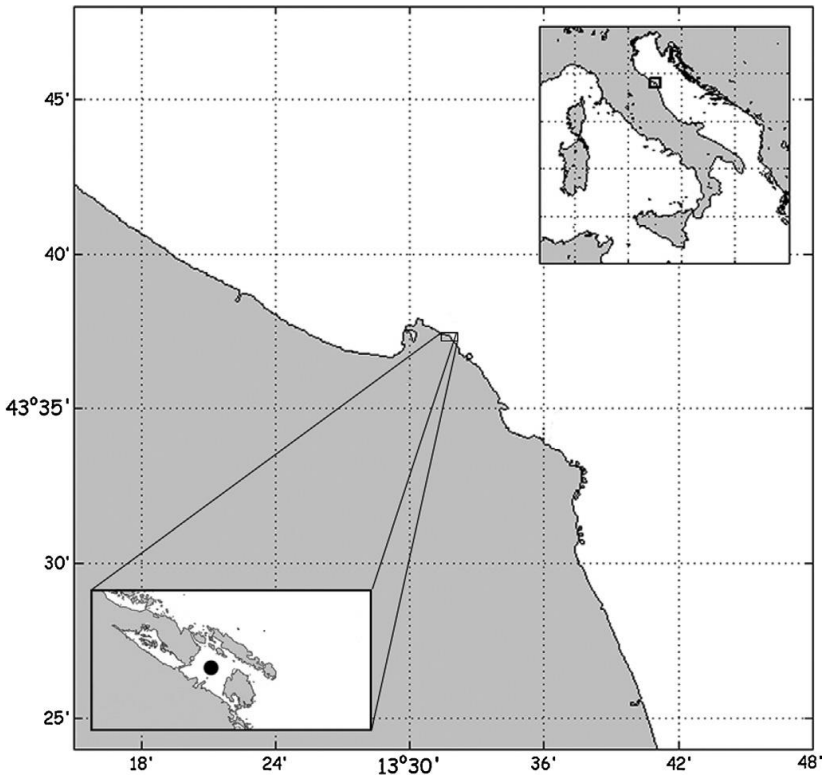

Fig. 1. Map of the study area, showing the location of the sampling site along the Conero Riviera (NW Adriatic Sea).

2.2. O. cf. ovata and bacterial enumeration

For determination of *O.* cf. *ovata* epiphytic abundances, dinoflagellate-colonized macrophyte samples were treated following the procedure described in Totti et al. (2010) and fixed with 1% Lugol solution (Throndsen, 1978). Seawater subsamples for *O*. cf. *ovata* planktonic abundances were also fixed with 1% Lugol solution. After fixation both epiphytic and planktonic *O.* cf. *ovata* cell counts were performed following Utermöhl method (Hasle, 1978) using a Zeiss Axioplan inverted microscope at 320X magnification under bright field and phase contrast illumination.

Total bacterial abundances were enumerated for subsamples fixed with 0.2 µm prefiltered formaldehyde (2%) following method described by Shibata et al. (2006). Briefly, aliquots were concentrated onto 0.2 µm pore size Anodisc filters (Whatman, 25 mm diameter), stained with 100 µL of 8X SYBR Gold (Life Technologies), mounted onto microscopic slides, and stored at -20°C. Enumeration was performed using epifluorescence microscopy (Nikon Eclipse 80i, magnification 1000X) under blue light excitation counting at least twenty fields per sample and a minimum of 300 cells.

2.3. DNA samples processing and extraction

For harvesting of total bacteria associated with the *O.* cf. *ovata* aggregates (OA), dinoflagellate-colonized macrophyte samples were shaken in the storage water (3 min) to allow the detachment of *O.* cf. *ovata* cells, then up to 100 ml of the suspension was filtered under low vacuum onto Supor 200 PES filters (Pall Corporation/Pall Life Sciences, pore size 0.2 µm). In order to assess the bacterial phylotypes more closely associated with *O.* cf. *ovata* and its mucilage layer (LA), aliquots of the same suspension were collected onto sterilized 11 µm pore size filters (Millipore). Samples for total DNA analysis of seawater bacterial assemblages (SW) were collected as previously described for storage water. All filters were stored at -80°C in sterile 2 mL microcentrifuge tubes until analysis. Filters were shredded under sterile conditions, and DNA from cells on the filters was extracted using the UltraClean Soil DNA isolation kit (MoBio Laboratories) according to the manufacturer's instructions. DNA concentrations and purity were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington DE).

2.3.16S rDNA pyrosequencing

For pyrosequencing analysis, extracted DNA samples from three replicates were pooled together and diluted to 10 ng μL^{-1}. Initial amplification of the hypervariable V1-V2 region of the bacterial 16S rDNA was performed on total DNA from samples. Master mixes for these reactions were prepared with Qiagen Hotstar Hi-Fidelity Polymerase Kit (Qiagen,Valencia CA), forward primer composed of the Roche Titanium Fusion Primer A (5'-CCATCTCATCCCTGCGTGTCTCCGA CTCAG -3'), a 10 bp Multiplex Identifier (MID) sequence (Roche, Indianapolis IN) unique to each of the samples and the universal bacteria primer 8F (5'- AGAGTTTGATCCTGG CTCAG-3'). The reverse primer was composed of the Roche Titanium Primer B (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG -3'), the identical 10 bp MID sequence as the forward primer and the reverse bacteria primer 338R (5'- GCTGCCTCCCGTA GGAGT-3'). Amplification in triplicate of each sample was performed under the following conditions: an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturing of 94°C for 45 sec, annealing at 50°C for 30 sec and extension at 72°C for 90 sec, then a final extension at 72°C for 10 min, and a final hold at 4°C. Samples with pooled replicates were gel purified individually using the Qiaquick Gel Extraction Kit (Qiagen, Valencia CA) and combined at equimolar ratios. The 16S rDNA amplicons from the pooled samples were sequenced on a

Roche 454 Genome Sequencer FLX Titanium instrument using the GS FLX Titanium XLR70 sequencing reagents and protocols (Microbiome Core Facility, Chapel Hill, NC). Initial data analysis and base pair calling were performed by Research Computing at UNC (Chapel Hill, NC).

2.4. Sequence processing and diversity analysis

The 16S rRNA gene amplicons data were processed through the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu). Pre-processing included screening and removing of short reads (<250 bp) and low quality reads. After sorting based on sequence tags and trimming of primer and tag sequences, derived high quality reads were checked for artificial chimeric formations using the Uchime algorithm. Community taxonomy information was obtained using the RDP classifier tool (Wang et al., 2007) and those sequences either related to chloroplasts or not belonging to the Domain Bacteria were discarded from further analysis, then samples were standardized to the size of the smallest library (10349 reads) by randomly subsampling the datasets. Sequences were aligned with the Infernal aligner and operational taxonomic units (OTUs) were clustered at a 97% similarity level by the furthest neighbor algorithm. The representative sequence for each cluster was assigned according to the minimum sum of the square of distances between sequences within a cluster (Cole et al., 2014). Taxa abundances were normalized at phylum, class and genus level based on the average 16S rRNA copy number values reported by rrnDB database (Stoddard et al., 2014). When the 16S copy number for a specific taxon was not available in the database, the average value for the upper taxonomical level was considered. Alpha diversity was analyzed through rarefaction curves, and diversity estimators (Chao 1, Shannon index and evenness) were calculated.

2.4. Statistical analysis

All statistical analysis except for Metastats analysis were performed with the PAST software package for Windows (Hammer et al., 2001). Differences in *O.* cf. *ovata* cell numbers, bacterial abundances and diversity estimators within and between sampling times were assessed through Student's *t*-test. Beta diversity was addressed by non-metric multidimensional scaling (NMDS) performed using a Bray-Curtis similarity matrix of OTUs abundance data; community level differences between groups were tested by analysis of similarities (ANOSIM) and Similarity

Percentage analysis (SIMPER) was utilized to identify the OTUs most contributing to the dissimilarity between bloom phases and assemblages. Finally, Metastats (White et al., 2009) was used for detection of differences in contribution of bacterial OTUs among samples. Statistical significance was set at $p < 0.05$ for all the analysis.

3. Results

3.1. Environmental conditions and cell abundances

Sampling was carried out at an early and at the peak phases of the *O.*cf. *ovata* bloom (1st and 2nd sampling times, respectively). Surface seawater temperature and salinity were 22.2 °C and 34.7, and 21.5 °C and 36.8, at early and peak, respectively. Dissolved inorganic nitrogen (DIN, i.e. NH_4^+ plus NO_2^- plus NO_3^-) was 9.40 µM at the early and 0.55 µM at the peak phase of the bloom. While, phosphate concentration (PO_4^{3-}) was 0.31 µM at both sampling times, leading to a N:P ratio of 30.2 and 1.8 (1st and 2nd sampling, respectively).

Abundances of *O.* cf. *ovata* cells colonizing macrophytes (i.e. *O.* cf. *ovata* aggregates; OA) were two fold higher at the peak phase than at the early phase of the bloom (mean value: 2.06×10^6 vs. 9.49×10^5 cells g^{-1} fw; $p < 0.05$, Student's *t*-test). Cell densities of *O.* cf. *ovata* in ambient seawater samples (SW) were on average 1.98 and 3.10×10^4 cells L^{-1} at the early and peak phase, respectively ($p > 0.05$, Student's *t*-test).

Bacterial abundances were also approximately two fold higher in OA samples at the peak compared to the early phase of the bloom (mean value: 6.62×10^6 cells mL^{-1} vs. 3.06×10^6 cell mL^{-1}, $p < 0.01$, Student's *t*-test), whereas abundances in SW samples did not change significantly between the two sampling periods (mean value: 7.94×10^5 cell mL^{-1} vs. 6.34×10^5 cell mL^{-1}; $p > 0.05$, Student's *t*-test). Overall, bacterial abundances were almost one order of magnitude higher in the OA than in SW samples ($p < 0.01$, Student's *t*-test).

3.2. Diversity of microbial assemblages

A total of 73641 high-quality reads spanning the 16S rDNA V1-V2 hypervariable region were used in the final analysis (average length = 287bp). This yielded 4765 different OTUs at 97% similarity from the whole dataset. Rarefaction analysis based on OTUs indicated that sampling did not achieve complete coverage except for SW sample at the early phase (SWI, Fig. 2); curves

reached higher number of OTUs per reads at the peak than at the early bloom phase for all the assemblages.

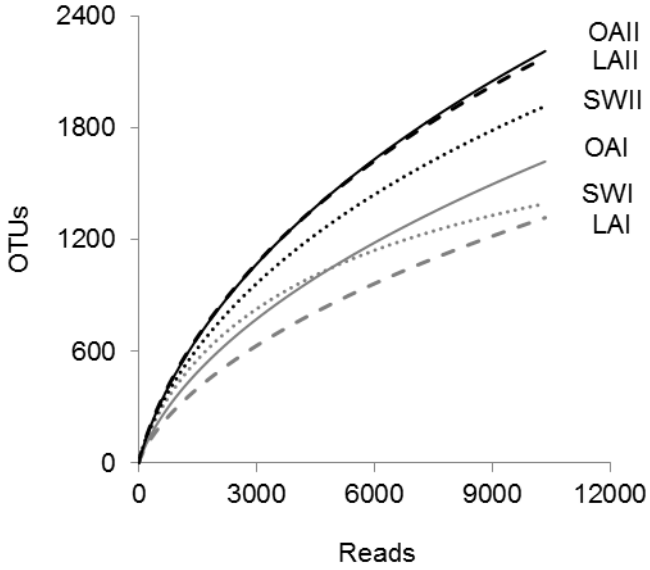

Fig. 2. Diversity of bacterial assemblages in *O.* cf. *ovata* aggregates (OA, continuous curves), *O.* cf. *ovata* and its mucilage layer (LA, dashed curves), and ambient seawater samples (SW, dotted curves) at the early and peak phase of the bloom (I and II, respectively). Rarefaction curves were computed on bacterial OTUs at a dissimilarity level of 3%.

The number of bacterial OTUs and estimated species richness (Chao 1), together with Shannon diversity (H') and evenness (J') reached the highest values in OA samples at the peak phase of the bloom (OAII), whereas the lowest values were found for bacteria more closely associated with *O.* cf. *ovata* and its mucilage layer at the early phase (i.e. LAI), except for Chao1 (Table 1). OTUs number and Shannon index were significantly higher at the peak than at the early phase ($p < 0.05$, all; Student's *t*-test), whereas the other indexes did not show significant differences between phases and assemblages ($p > 0.05$, all; Student's *t*-test). The majority of OTUs, including the most abundant ones, were shared among samples, while unique OTUs (31.6% of the total OTUs in OA and 30.9% in SW assemblages) were mainly represented by singletons (~60%).

In general, higher numbers of OTUs were shared between OA and LA compared to SW samples, and higher percentages of shared OTUs were observed at the peak than at the early phase of the bloom (Table 2). These findings were supported by the NMDS plot displaying Bray-Curtis

similarities between samples (Fig. 3), which revealed that OA temporal samples were more similar than SW temporal samples (54 and 39% similarity, respectively); while the assemblages were more similar at the peak than the early bloom phase (60 and 46% similarity). However, ANOSIM test did not underline significant differences between assemblages or bloom phases ($p > 0.05$ for both comparisons). SIMPER analysis showed that seven OTUs explained 20% of the dissimilarity either between assemblages or between bloom phases; four OTUs were related to *Rhodobacteraceae* and three to the genera *Erythrobacter* (*Alphaproteobacteria*), *Haliea* (*Gammaproteobacteria*) and *Propionibacterium* (*Actinobacteria*), respectively (Table 3; Table S1).

Specific phylotypes (i.e. OTUs detected in only one kind of assemblage at both bloom phases) were 295 for OA and 82 for SW assemblage and all of them were rare (i.e. <1% of the total reads in a sample), accounting for 6.2 and 1.7% of the total OTUs, respectively. While more than half of the OA-specific OTUs belonged to the *Rhodobacteraceae* family, SW-specific OTUs were more uniformly distributed between taxa (data not shown).

Table 1. Bacterial diversity parameters during an *O.*cf. *ovata* bloom. Summary of total high quality sequences after normalization to smallest library (Reads), number of bacterial operational taxonomic units detected at 97% identity (OTUs), estimated species richness (Chao 1), Shannon diversity index (H') and evenness (J') obtained from pyrosequencing of *O.* cf. *ovata* aggregates (OA), *O.* cf. *ovata* and its mucilage layer (LA), and ambient seawater samples (SW) at the early and peak phase of the bloom (I and II, respectively).

Sample	Reads	OTUs	Chao 1	H'	J'
OAI	10349	1621	3229	5.28	0.715
OAII	10349	2214	4078	6.36	0.826
LAI	10349	1322	2582	4.63	0.644
LAII	10349	2177	3886	6.31	0.821
SWI	10349	1394	1798	5.79	0.800
SWII	10349	1921	3337	6.18	0.817

Table 2. Bacterial community structure similarity matrix in a *O.*cf. *ovata* bloom. OTU overlap ratios (i.e. the percent of shared OTUs in total OTUs of two groups) between samples.

Shared OTUs (%)	OAII	LAI	LAII	SWI	SWII
OAI	35.3	50.3	29.0	19.1	26.9
OAII		25.7	62.1	19.2	29.4
LAI			25.1	17.0	27.0
LAII				17.0	30.3
SWI					19.1

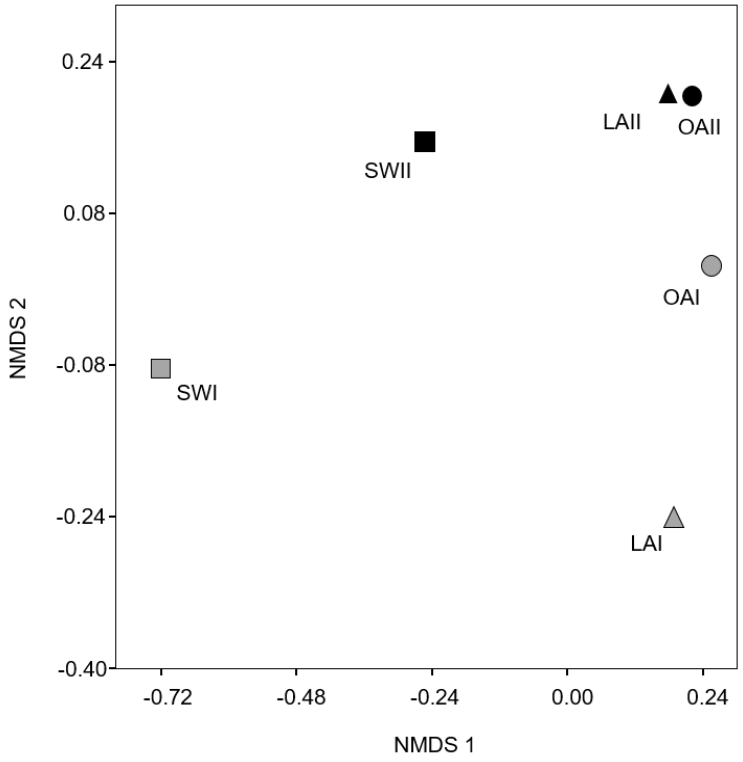

Fig. 3. NMDS ordination plot of bacterial assemblages' structure using Bray-Curtis distances. Circles, triangles and squares indicated *O.* cf. *ovata* aggregates (OA), *O.* cf. *ovata* and its mucilage layer (LA), and ambient seawater samples (SW), respectively. Black and grey symbols represent the early and peak phase of the bloom, respectively. The plot was constructed on the basis of bacterial OTUs retrieved from pyrosequencing data.

Table 3. Major bacterial OTUs leading to dissimilarity. Similarity percentage analysis (SIMPER) showing contribution of the seven OTUs explaining 20% of the dissimilarity between assemblages and bloom phases. The number of sequences per OTU for each of the samples are also reported.

OTU	Closest relative RDP classifier	No. of sequences per OTU						Contribution to dissimilarity (%)	
		OAI	OAII	LAI	LAII	SWI	SWII	Assemblages	Bloom phases
6	*Ruegeria*	1519	462	2268	644	579	647	7.09	4.97
40	*Rhodobacteraceae*	812	412	1184	583	437	330	3.51	2.64
35	*Haliea*	568	9	638	10	23	48	2.83	2.86
33	*Rhodobacteraceae*	13	20	13	32	692	49	2.22	3.37
7	*Propionibacterium*	0	15	1	14	418	14	1.32	2.27
516	*Jannaschia*	219	278	91	153	28	61	1.27	1.56
164	*Erythrobacter*	355	247	333	193	88	167	1.22	1.63

3.3. Microbial assemblages composition and comparisons

Overall, 14 different bacterial phyla, 20 classes and 165 genera were recovered (supplementary Table S2, S3 and S4, respectively). All microbial assemblages either associated with the *O.* cf. *ovata* aggregates or with the ambient seawaters were dominated by *Proteobacteria* (50.9-85.7%), followed by *Bacteroidetes* (12.7-36.0%) and *Actinobacteria* (0.6-6.3%). *Cyanobacteria* was the fourth most represented phylum, yet never exceeding 2.5% in relative abundance (Table S2). *Fusobacteria*, *Firmicutes*, and *Planctomycetes* were present at abundances just above 1% only in SWI, while *Parcubacteria* reached values ~1.5% in both SW and OA bacterial assemblages at the bloom peak phase. The remaining phyla *Acidobacteria*, *Chloroflexi*, *Chlorobi*, *Spirochaetes*, *TM7*, and *Deinococcus-Thermus* were rare representatives (i.e. <1%; Table S2).

O. cf. *ovata*-related assemblages (OA and LA samples) showed predominance of *Alphaproteobacteria* at both phases of the bloom (66.0 to 84.4%, OAII and LAI, respectively; Fig. 4). *Ruegeria* and *Jannaschia* (*Roseobacter* clade) along with *Erythrobacter* were the most abundant genera, with the highest values for *Ruegeria* and *Erythrobacter* at the early phase (30.2% in LAI and 23.8% in OAI, respectively), and for *Jannaschia* at the peak phase (21.1% in OAII, Fig. 5), primarily due to contribution of the three OTUs, #6, #164 and #516, respectively (Table 3). Moreover, the OTU #40 assigned to *Rhodobacteraceae* had also a consistent

contribution in OA and LA assemblages, particularly at the early bloom phase (Table 3). Other *Rhodobacteraceae* genera such as *Litoreibacter*, *Loktanella*, *Paracoccus* showed contributions around 3% (Fig. 5). Overall, contribution of OTUs assigned to *Alphaproteobacteria* was significantly higher in *O.* cf. *ovata*-related assemblages compared to SW assemblages ($p < 0.05$, Metastats analysis). In addition, OTUs significantly more abundant in OA and LA than in SW assemblages were all affiliated to the *Rhodobacteraceae* family, including one *Ruegeria* (OTU #1637) and several *Jannaschia*-related OTUs (i.e. OTUs #2431, #2072, #6042, #4675). Within the *O.* cf. *ovata*-related assemblages, OTU #2431 was significantly more present in OA than LA samples ($p < 0.05$, Metastats analysis). *Gammaproteobacteria* were mainly represented by OTUs belonging to the genus *Haliea* at the early phase (>10%, primarily OTU #35, Table3), and then shifting to *Granulosicoccus* at the peak phase (~3%). The remaining three *Proteobacteria* classes (*Delta-*, *Epsilon-* and *Betaproteobacteria*; Table S3) were rare.

Other notable groups showed an inverse trend with respect to *Alphaproteobacteria* (Fig. 4). *Flavobacteria* (7.2 and 22.2%, OAI and LAII) followed by *Sphingobacteria* showed the highest contributions at the peak phase, as well as *Ilumatobacter* (6%, *Actinobacteria*).

Ambient seawater bacterial assemblages were also dominated by *Alphaproteobacteria* (48-60%), although with a significant lower contribution with respect to OA assemblages. This class showed the highest contribution at the peak phase of the bloom primarily with *Erythrobacter*, *Jannaschia* and *Ruegeria* (Fig. 5). While OTU #40 (*Rhodobacteraceae*) overlapped with the *O.* cf. *ovata*-related samples, OTU #33 (also a *Rhodobacteraceae*) had a large contribution only in SWI (Table 3). Differently from *O.* cf. *ovata*-related assemblages, *Haliea* accounted only for 1.3% at both samplings, whereas *Granulosicoccus* was about 3% at the peak phase as in the former ones. Interestingly, the contribution of members affiliated to *Vibrio* were significantly higher in SW than in OA and LA assemblages (e.g. OTU #76; $p < 0.05$, Metastats analysis), where the genus was essentially absent. Similarly, members belonging to *Haliscomenobacter* (2%, early phase), the most abundant representative of *Sphingobacteria*, had a significantly higher contribution in SW with respect to OA assemblages (OTU #335; $p < 0.05$, Metastats analysis). Whereas, *Flavobacteria*-affiliated (24 and 18%, SWI and SWII) did not differ significantly between assemblages ($p > 0.05$, Metastats analysis). Notably, *Propionibacterium* (*Actinobacteria*) was found at high relative abundance only in SWI (12.7%, primarily OTU #7;

Table 3), whereas its contribution was negligible in OA and LA samples. A similar pattern was also observed for *Cetobacterium* (*Fusobacteria*; Fig. 5).

When comparing the two phases of the bloom, several *Flavobacteria* OTUs (e.g. OTUs #265, #678, #733, #1340) were significantly more abundant at the peak phase ($p < 0.05$, Metastats analysis). Contribution of OTU #208 related to *Granulosicoccus* genus (*Gammaproteobacteria*) as well as OTUs #150 and #366 affiliated with genus *Arcobacter* (*Epsilonproteobacteria*) were also differentially higher at the peak than at the early phase ($p < 0.05$, Metastats analysis). Lastly, *Ilumatobacter* (*Actinobacteria*), Gplla (*Cyanobacteria*) and *Parcubacteria Incertae Sedis* showed the highest relative abundances at the peak phase for all assemblages, and contributions of a large number of OTUs assigned to these genera were significantly higher at the peak than at the early phase of the bloom ($p < 0.05$, Metastats analysis). Figure 6 summarizes bacterial assemblages' main shifts in relative abundance at genus level between early and peak phase of the bloom.

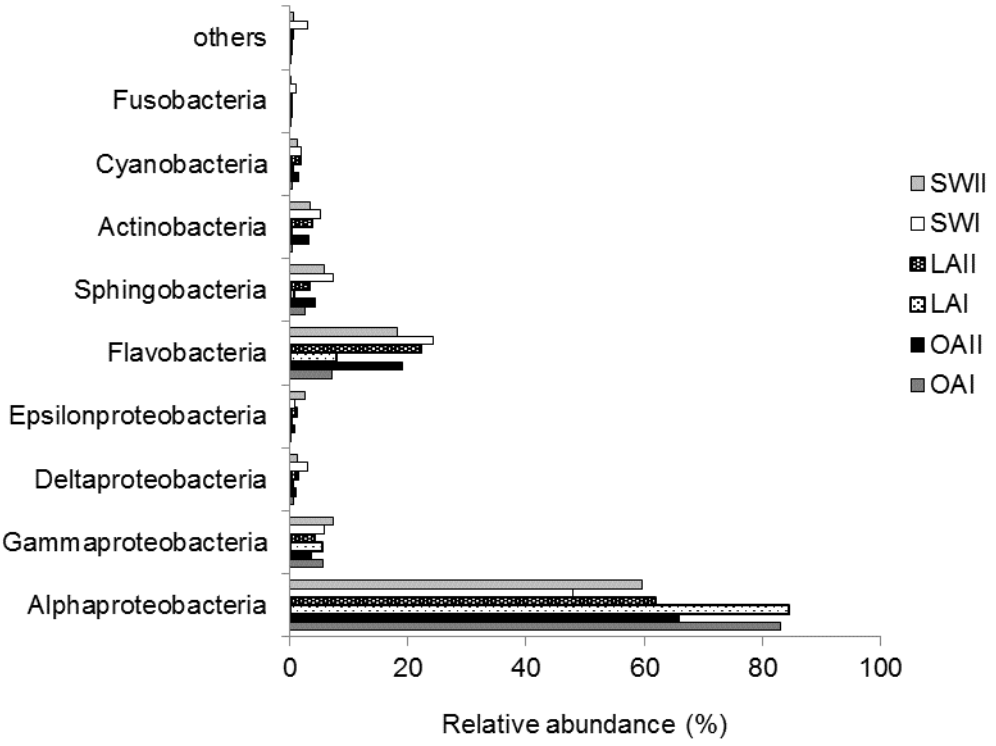

Fig. 4. Percent distribution of the dominant classes (≥1% in at least one of the samples) in *O.* cf. *ovata* aggregates (OA), *O.* cf. *ovata* and its mucilage layer (LA), and ambient seawater (SW) bacterial assemblages at the early and peak phase of the bloom (I and II, respectively), as revealed from pyrosequencing data normalized for number of ribosomal operons per genome. 'Others' represent the classes with less than 1% of relative abundance individually.

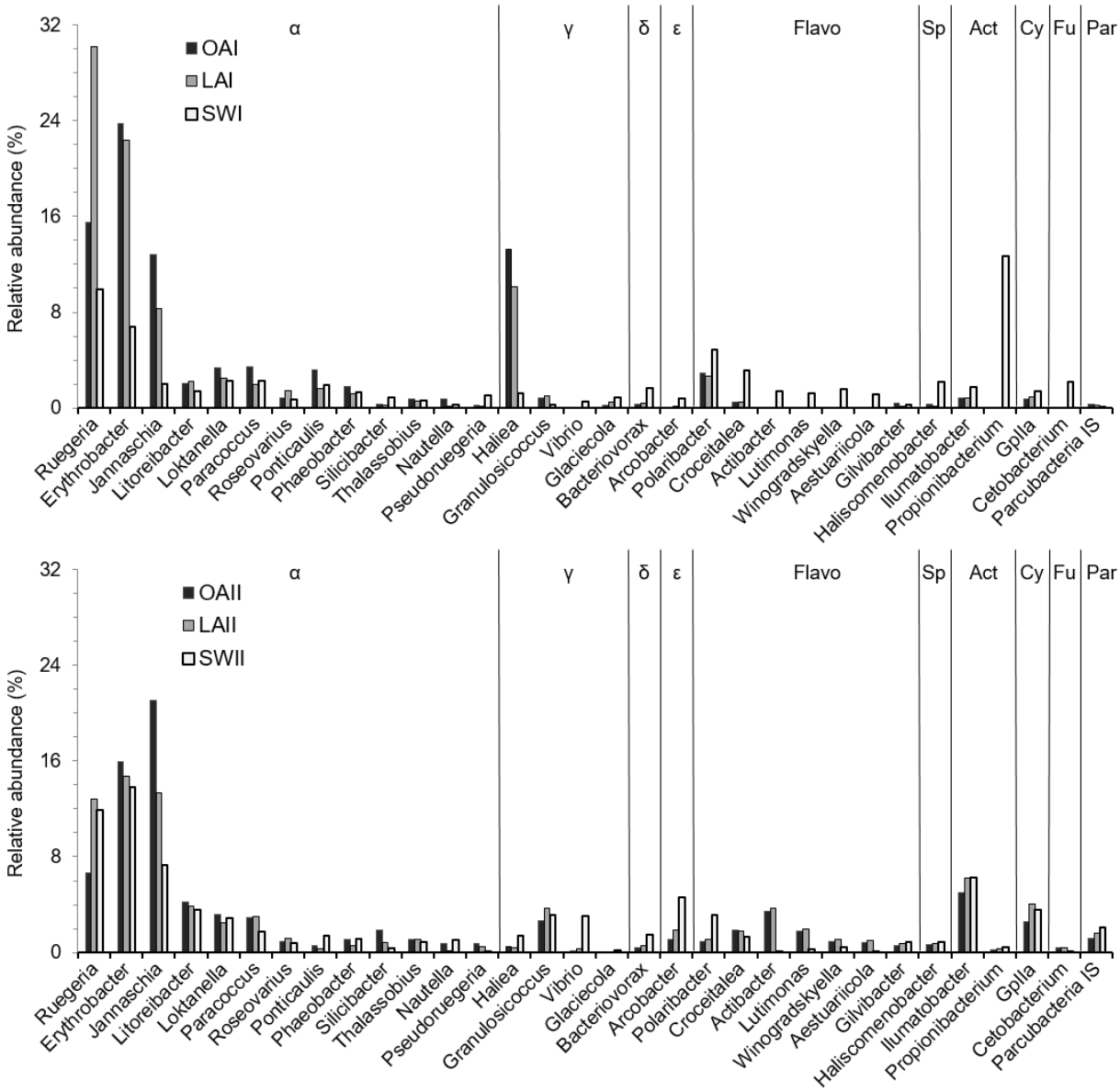

Fig. 5. Relative contribution of the major bacterial genera (≥0.5% of the total reads) in *O.* cf. *ovata* aggregates (OA), *O.* cf. *ovata* and its mucilage layer (LA), and ambient seawater bacterial assemblages (SW) at the early phase (I, A) compared to the peak phase (II, B) of the bloom, as revealed from pyrosequencing data normalized for number of ribosomal operons per genome. Vertical lines separate groups of different phyla or classes.

Abbreviations: α, *Alphaproteobacteria;* γ, *Gammaproteobacteria;* δ, *Deltaproteobacteria*; ε, *Epsilonproteobacteria; Flavo, Flavobacteria; Sp, Sphingobacteria; Act, Actinobacteria; Cy, Cyanobacteria; Fu, Fusobacteria; Par, Parcubacteria; IS, Incertae Sedis.*

3. Discussion

4.1. General

Ostreopsis cf. *ovata* cell abundances recorded during this study in both *O.* cf. *ovata* aggregates and ambient seawater samples were in the range of values reported previously for the same area and more generally for Mediterranean Sea (Totti et al., 2010; Mangialajo et al., 2011 and references therein; Accoroni et al., 2015a; Carnicer et al., 2015). Bloom development occurred under stable weather conditions, low hydrodynamism, and accompanied by a drop of nitrogen concentration at the peak phase. The limited sampling (two timepoints) does not allow us to infer on relationship dynamics between inorganic nutrients and *O.* cf. *ovata* bloom. However, the physicochemical conditions, the onset bloom N:P ratio, and nutrient concentration changes observed in this study do fulfill the recent conceptual model proposed for *O.* cf. *ovata* blooms in this region (Accoroni et al., 2015a). The model postulates that calm conditions are a prerequisite for blooms, and only when this state is established temperature and suitable N:P ratio will have a decisive effect (Accoroni et al., 2015a). Bacterial cell densities reported for the ambient seawaters were in the range of abundances found during non-toxic or harmful microalgal blooms (e.g. Lamy et al., 2009; Jones et al., 2010; Mayali et al., 2011; Park et al., 2015).

Diversity indexes of bacterial assemblages associated with *O.* cf. *ovata* were comparable with pyrosequencing derived values reported for a broad-range of bacterial-benthic eukaryote associations (Webster et al., 2010; Lee et al., 2011; Carlos et al. 2013; He et al., 2014) and for shallow-water sediments (Wang et al., 2013; Piccini et al., 2015; Liu et al., 2015), the latter being considered among the most diverse environments (Lozupone and Knight, 2007). While the rarefaction curves indicated that we did not fully sample the species richness, they do suggest a higher bacterial richness at the peak than at the early phase of the bloom in accordance with Chao1 and Shannon estimators. Basu et al. (2013) reported similar observations when comparing the declining with the active phase of a *Noctiluca miliaris* bloom. These findings support the hypothesis that enhanced algal substrate availability and dissolved organic matter release provides a series of ecological niches in which specialized populations could develop (Teeling et al., 2012).

While the molecular approach applied here precludes a direct comparison of bacterial diversity parameters with most previous HABs investigations (Jones et al., 2010; Yang et al., 2012; Basu et al., 2013) as the number of OTUs in the present work are at least one order of magnitude

greater, species richness and diversity temporal trends found in this study are partially consistent with those reported by Yang et al. (2015). The comparison between *O.* cf. *ovata* and ambient seawater assemblages did not indicate a clear distinction in terms of alpha and beta diversity, differently from other studies that compared benthic eukaryote-associated bacteria with the surrounding seawaters bacteria (Webster et al., 2010; Lee et al., 2011; Carlos et al. 2013; He et al., 2014). In fact, in this study, assemblages differed mostly in terms of the OTU relative contribution rather than in the presence or absence of specific phylotypes, as the latter were detected in very low abundances. *O.* cf. *ovata* cells and its mucilaginous layer adhere only loosely to the substrata and can be easily resuspended in the water column, particularly as blooms progress and mats become heavier (Totti et al. 2010; Mangialajo et al., 2011). This phenomenon may explain the higher Bray-Curtis similarities found among samples at the peak than at the early bloom phase, and a portion of the phylogenetic overlap between OA and SW assemblages, as revealed by the NMDS plot.

4.2. Bacterial assemblages associated with O. cf. ovata

In this study, *Alphaproteobacteria* was the dominant class associated with *O*. cf. *ovata* during both bloom phases (84 and 66%, relative abundance, early and peak), followed by *Flavobacteria* that showed the highest contribution at the peak phase (up to 19 %). These main bacterial composition feature and trend are consistent with previous ones reported for non-toxic phytoplankton blooms (Fandino et al., 2001; Fandino et al., 2005; Brussaard et al., 2005; Alderkamp et al., 2006; Hasegawa et al., 2007; Lamy et al., 2009) and also for the few available planktonic HABs (Garcés et al., 2007; Jones et al., 2010; Park et al., 2015) with the exception for Yang et al. (2012; 2015).

The highest contributions found in this study for several *Flavobacteria*-affiliated OTUs at the peak than at the early bloom phase are in accordance with the main metabolic traits ascribable to the members of this class, being recognized as specialists for degradation of particulate organic matter and high molecular weight compounds (e.g. cellulose, chitin and proteins; Kirchman 2002; Rink et al., 2007; Gómez-Pereira et al., 2010; Fernández-Gómez et al., 2013; Buchan et al. 2014). As such, these OTUs probably respond as secondary colonizers (Elifantz et al. 2013) to the wider pool of refractory organic matter such as phyto-detritus as the bloom proceeded.

Actinobacteria, the third most abundant phylum, generally recognized as organotrophic bacteria able to decompose recalcitrant and poorly accessible substrates at later stages of microbial succession (Holt et al., 1994; Zakharova et al., 2013; Bagatini et al., 2014), were present predominately at the peak phase with *Ilumatobacter* as the main representative. This genus has been recently found abundant during degradation processes of freshwater diatoms (Zakharova et al., 2013) and at the stationary growth phase of freshwater-cultured phytoplankton (Bagatini et al., 2014) and other algal cultures (Green et al., 2015). In our context, this genus might be directly related to the degradation of *O*. cf. *ovata* senescent cells but also probably responding to the epiphytic diatoms that colonize the macrophytes throughout the year, co-occurring in minor proportion with *O*. cf. *ovata*, and presumably undergoing to nutrient stress during the bloom (Totti et al., 2010; Carnicer et al., 2015).

At the early phase of the bloom, *Gammaproteobacteria* (~ 6%) associated with *O*. cf. *ovata* aggregates were mainly represented (13%) by the recently designated genus *Haliea* (Urios et al., 2008), primarily with OTU #35. Members and sequences of *Haliea* have been found in surface waters of the North-western Mediterranean Sea (Urios et al., 2008; Urios et al., 2009; Lucena et al., 2010). In addition, representatives have been reported in the presence of methane source in oxic water layers (Mau et al., 2013) and in association with an *A. sanguine* bloom event (Yang et al., 2015), suggesting that phylotypes affiliated to this genus may be involved in the dynamics of methane that is produced through not yet identified microorganisms under summer phytoplankton bloom by using dimethylsulfoniopropionate (DMSP) (Damm et al., 2008; Damm et al., 2010, Dickschat et al., 2015). Overall, the limited contribution of *Gammaproteobacteria* reported here is partially consistent with that one found for assemblages co-occurring with *Alexandrium* spp. blooms in which specific groups such as *Alteromonadaceae* could not be retrieved in significant proportions (Garcés et al., 2007). By contrast, Yang et al. (2012, 2015) found bacterial assemblages dominated by *Gammaproteobacteria* and *Alphaproteobacteria* during *A. sanguinea* blooms, with an increase of the former at the decline phase (Yang et al., 2012), suggesting an algicidal activity for this class, as also proposed for *Cochlodinium polykrikoides* (Park et al., 2015), that conversely it could not be inferred by our data. However, our investigation was limited to the early and peak phases of the bloom and did not sample its decline phase. The remaining phyla associated with the *O*. cf. *ovata* were rare (<1%). Representatives from *Acidobacteria*, *Chloroflexi*, *Chlorobi*, *Fusobacteria*, *Firmicutes*, *Spirochaetes*, *TM7*, and

Deinococcus-Thermus were observed. While their relative abundances were low, it cannot be precluded their importance in the nutrient dynamics and interactions within the assemblages.

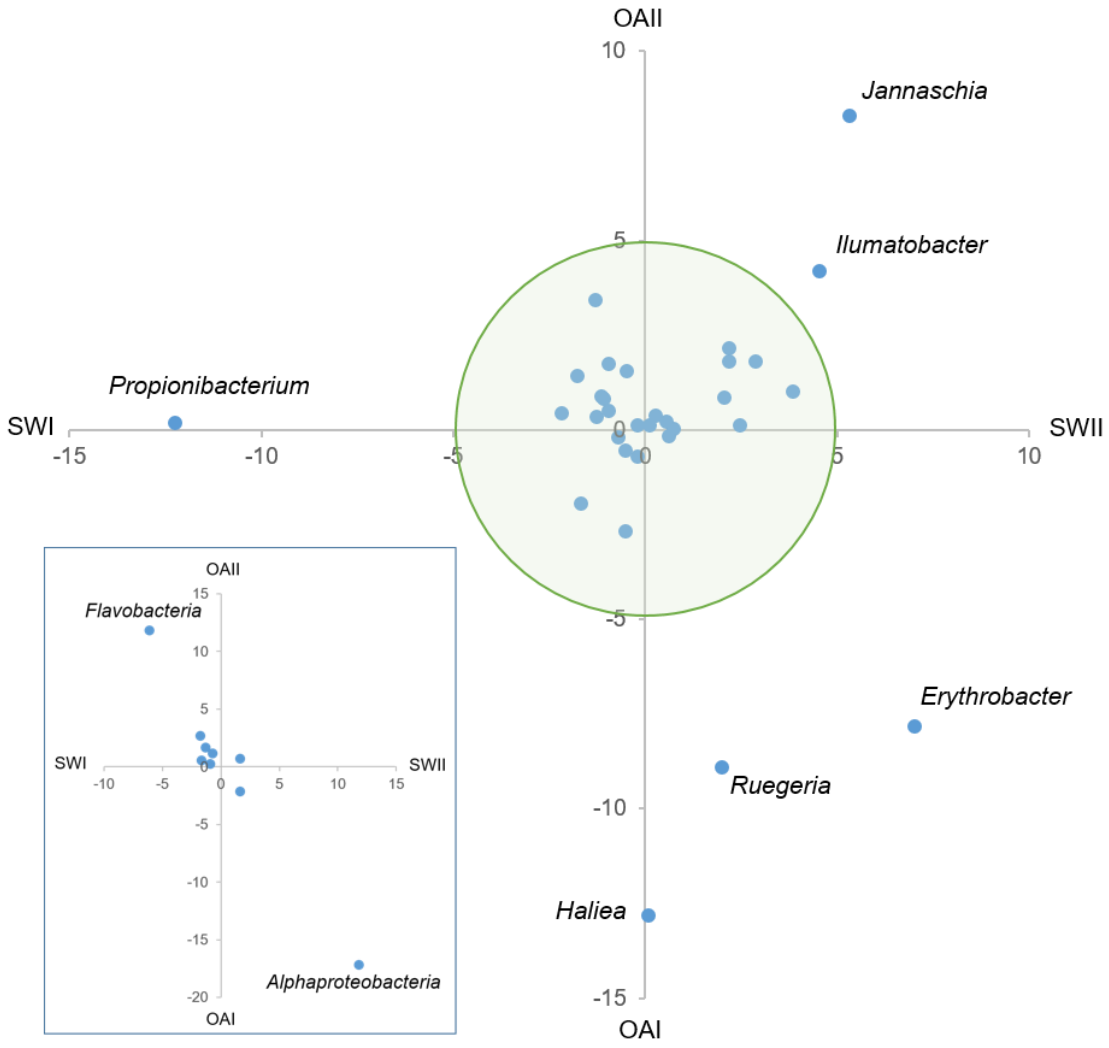

Fig. 6. Major changes in relative abundance at genus level and class level (inset) in *O.* cf. *ovata* aggregates (OA) and ambient seawater bacterial assemblages (SW) between peak phase (II) compared with early phase (I) of the bloom. Differences were calculated by subtracting percentages recorded at the peak from values at the early phase of the bloom. The positive values denote higher contribution at the peak as opposed to the early phase.

Focusing on *Alphaproteobacteria*, a *Rhodobacteraceae* consortium composed by *Ruegeria*, *Jannaschia* and the OTU #40 (closely related to *Roseovarius*, Table S1) together with *Erythrobacter* appears to be associated with the *O.* cf. *ovata* aggregates representing more than half of the total phylotypes' contribution at both phases of the bloom, with the highest values at

the early phase for OTU #40, *Ruegeria* and *Erythrobacter* and at the peak phase of the bloom for *Jannaschia*. Additionally, more than half of the *O.* cf. *ovata*-specific OTUs were affiliated to the *Rhodobacteraceae*, remarking the crucial role of this family in the bacterial-algal interactions (e.g. Buchan et al., 2014).

Erythrobacter is a very relevant component of the marine planktonic communities, becoming in some cases one of the most dominant groups in eutrophic coastal environments (Shiba and Simidu, 1982; Frette et al., 2004) adapted to grow on refractory carbon (Frette et al., 2004). *Erythrobacter* phylotypes have been found associated with cultured microalgae (e.g. *Skeletonema costatum*, Jasti et al., 2005; *Coolia monotis*, Ruh et al., 2009), macroalgae (Burke et al. 2011), colonial ascidians (Martínez-García et al., 2007), phytoplankton blooms (Borsodi et al., 2013; Yang et al., 2015), and also endosymbiont (e.g. in *Pyrodinium bahamense* var. *compressum*, Azanza et al., 2006). Moreover, as in our case, *Erythrobacter* has been found dominant together with *Jannaschia* in limestone biofilm (Berdoulay and Salvado, 2009), suggesting a potential relationship between these two aerobic anoxygenic phototrophic (AAnP) genera.

The *Rhodobacteraceae* are among the most abundant and ecologically relevant coastal marine bacteria, their ecological niches range from free-living plankton, to symbiont, to biofilm pioneers (reviewed by Geng and Belas, 2010; Hahnke et al., 2013; Elifantz et al. 2013; Yang et al., 2015). These bacteria, in fact, show high metabolic versatility, including aerobic anoxygenic photosynthesis, the degradation of the algal osmolyte dimethylsulfoniopropionate (DMSP), and the synthesis of bioactive metabolites such as tropodithietic acid (TDA) which has potent antibacterial properties (Geng and Belas 2010; Hahnke et al., 2013 and references therein). In this study, *Rhodobacteraceae* may have also taken advantage of the high DIN levels (mainly nitrate) detected at the early phase of the bloom, as they are able to use nitrate as a terminal electron acceptor to sustain an active energy metabolism also in the absence of oxygen (Dobler and Biebl, 2006; Wagner-Döbler et al., 2010; Riedel et al., 2015). Diel anoxia may be expected in *O.* cf. *ovata* mats, as regularly recorded in photosynthetic biofilms at night as a consequence of intense respiration, as often observed in microbial mats (Steunou et al., 2008). Follow up studies of diel and longitudinal gene expression in response to physicochemical fluctuations is necessary to further elucidate these microbial consortial interactions. The genus *Ruegeria* phylotypes have been retrieved from surface ocean waters of most climatic zones in both coastal zones and open oceans (e.g. Gram et al., 2010; Lai et al., 2010), in tidal flat sediments (Oh et al., 2011),

associated with marine invertebrates (e.g. Menezes et al., 2010; Lee et al., 2012; Kim et al., 2014), but also being reported in association with cultured toxic dinoflagellates (e.g. *Alexandrium catenella*, Amaro et. al. 2005; *Pyrodinium bahamense* var. *compressum*, Onda et al., 2015) including some DMSP-producing species (e.g. *Pfiesteria piscicida*, Alavi et al., 2001).

High relative abundance of *Jannaschia*-related clones have been reported in the Baltic Sea (Salka et al., 2008), in tidal flat sediments (Yoon et al., 2010; Fang et al., 2014; Piccini et al., 2015), in association with algal cultures (Green et al., 2015) but also as endosymbionts (Apprill et al., 2009).

Although 16S rDNA phylogenetic data do not directly elucidate functionality they still provide insights on how the different bacterial groups correlate within the assemblages and with the associated alga considering certain metabolic characteristics significant to the groups and to the associated organism (Amin et al., 2012; Newton et al., 2010; Gifford et al., 2014). The dominance of this consortium at both phases of the bloom suggests the establishment of interactions chemically mediated among the bacteria and between the bacteria and *O*. cf. *ovata* that would likely interfere on algal growth and perhaps decline. Members affiliated to *Ruegeria* and *Jannaschia* genera share several metabolic traits showing high versatility; however, comparative genome reveal differences in the presence of potential interaction genes (e.g. secondary metabolites production; Moran et al., 2007; Newton et al., 2010) that might favor their co-occurrence and outcompeting other marine bacteria. Indeed, a beneficial phase in the interaction between *Ruegeria* phylotypes or other *Roseobacters* such as *Dinoroseobacter shibae* (closely related to several *Jannaschia* phylotypes; Slightom and Buchan 2009; Newton et al., 2010) and healthy dinoflagellates have been reported where these bacteria protect the algae from other bacteria by producing TDA or other secondary metabolites, and the exchange of essential growth metabolites occur between the counterparts (Alavi et al., 2001; Brinkhoff et al., 2004; Geng and Belas, 2010; Wagner-Döbler et al., 2010; Seyedsayamdost et al., 2011; Wang et al., 2014). In addition DMSP appears also involved in these relationships (Miller and Belas, 2004; Geng and Belas, 2010; Seyedsayamdost et al., 2011; Todd et al., 2012; Wang et al., 2014). Although in the present study TDA was not measured, all previously tested *Ruegeria* representatives synthesize TDA (Alavi et al., 2001; Miller and Belas, 2006; Bruhn et al., 2007; Porsby et al., 2008; D'Alvise et al., 2014); TDA synthesis appears to be attached-life phase dependent, correlating with formation of rosettes and biofilms (Miller and Belas, 2006; Geng and

Belas, 2010), and most prominent under static conditions (Porsby et al., 2008; D'Alvise et al., 2014). TDA mainly inhibit non-*Roseobacter* phylotypes, primarily members of *Vibrio* among *Gammaproteobacteria* (Bruhn et al., 2005; Bruhn et al., 2007), *Flavobacteria*, and *Actinobacteria* (Brinkhoff et al., 2004; Rabe et al., 2014), thus enhancing *Roseobacters* symbiosis by limiting competition from other species, and then promoting algal growth (Miller and Belas, 2006; Geng and Belas, 2010; Geng and Belas, 2011; Seyedsayamdost et al., 2011 and references therein). In our study, attachment/biofilm formation by *Ruegeria* was also suggested by its largest contribution in the LAI sample (30.2%). Moreover, the nearly absence of *Vibrio* in *O.* cf. *ovata* aggregates as well as of *Propionibacterium* (*Actinobacteria*), while being abundantly present in the ambient seawaters, suggest unfavorable conditions for these genera within the mats. In addition, a previous study found that in marine larval rearing units under stagnant conditions, TDA-producing *Ruegeria* spp. inhibits *Vibrio anguillarum* (Porsby et al., 2008). Therefore our observations are consistent with the TDA inhibiting colonization.

In this study, both *Ruegeria* and *Jannaschia* were associated with *O.* cf. *ovata* aggregates. Previous studies have shown that members of these two genera are attracted by DMSP (e.g. *Ruegeria* sp. TM1040, Miller et al 2004; Miller and Belas, 2006; *Jannaschia* sp., Apprill et al. 2009) and catabolize it (Moran et al., 2007; Todd, 2012 and references therein; Reisch et al., 2013; Dickschat et al., 2015 and references therein), as many *Roseobacters* (Gonzàlez et al., 1999, 2000; Newton et al., 2010; Wagner-Dobler et al., 2010; Riedel et al., 2015). DMSP can be secreted by several toxic dinoflagellates (reviewed by Caruana and Malin, 2014), and recently its production under laboratory conditions has been also assessed for *O.* cf. *ovata* strains isolated from the same samples collected for this study (Vanucci et al., unpublished data). This finding might suggest an important role of DMSP in the interactions between *O.* cf. *ovata* and the *Rhodobacteraceae* consortium, especially during the development phase of the bloom when the bulk of the algal cells is expected to be mostly in an healthy status which is a requisite for engaging positive bacterial-algal relationships between *Roseobacters* and dinoflagellates (Wagner-Dobler et al., 2010; Buchan et al., 2014; Wang et al., 2014, 2015). In this study, the production of DMSP by *O.* cf. *ovata* could have been responsible for the high relative abundance of *Haliea* at the early phase of the bloom. However, further studies are necessary to elucidate the initiation and progression of these microbial interactions.

At the peak of the bloom, *Jannaschia* overcame *Ruegeria*; while our study does not allow us to infer on conditions and/or bacterial-algal interactions which would have favored the growth of one genus over the other and their individual effects on *O.* cf. *ovata* bloom dynamics, it is well known that *Roseobacters* are involved in algal bloom decline processes, and may exert significant control over phytoplankton biomass (Gonzalez et al., 2000; Mayali et al., 2008; Teeling et al., 2012; Buchan et al., 2014). Particle associated phylotypes of this clade have been also noted as being highly antagonistic to other bacteria (Long and Azam, 2001). Recently it has been demonstrated that *Roseobacters*-dinoflagellates relationship shifts from a mutualistic to a pathogenic phase in response to ageing cells (Wagner-Döbler et al., 2010; Wang et al., 2014, 2015) by producing algicidal compounds induced by breakdown product release in the case of aging algae (e.g. *Phaeobacter gallaeciensis*, *P. inhibens*, Seyedsayamdost et al., 2011, 2014; *R. pomeroyi*, Riclea et al., 2012). The presence of aging and potentially nutrient stressed *O.* cf. *ovata* cells would increase with bloom progression, likely inducing a shift from a mutualistic to an antagonistic interaction. Further studies focusing on the potential transition from mutualistic to antagonistic interactions between *O.* cf. *ovata* and the associated *Roseobacters* are needed.

4.3. Comparison with bacterial communities co-occurring with Ostreopsis spp. cultures

To our knowledge, no data are available on natural bacterial assemblages co-occurring with epiphytic/benthic HABs. Previous studies based on restriction fragment length polymorphism (RFLP) analysis of the bacterial community associated with *O. lenticularis* cultures revealed that most species were grouped within *Alpha-*, some closely related to *Roseobacters*, and *Gammaproteobacteria* (9 and 5 out of 16 species, respectively), while one of the two species belonging to *Cytophaga-Flavobacteria-Bacteroides* (CFB) represented almost approximately half of total bacteria identified (Pérez-Guzmán et al., 2008). A similar number of phylotypes were obtained from *Coolia monotis* and *Ostreopsis ovata* cultures (19 and 17, respectively), although most of the sequences from 16S rDNA libraries were assigned to *Alphaproteobacteria* (~ 44%) and *Gammaproteobacteria* (~36%), followed by CFB group (Ruh et al., 2009). Ruh et al. (2009) emphasized that the sequences similar to *Roseobacter* sp. found in both *C. monotis* and *O. ovata* cultures were previously reported in association with various dinoflagellates (e.g. *Alexandrium* spp., *Pfiesteria* spp., *Prorocentrum* spp.) and that *Roseobacter* sp. is responsible for DMSP degradation (Ruh et al., 2009 and references therein). Our deep sequence data reinforce the

aspects emphasized by Ruh et al. (2009) regarding *Roseobacter* phylotypes, on the other hand they provide a different bacterial composition associated with *O. cf. ovata*. This finding is not surprising considering the different depth of sequencing in the two studies, but it also points out that bacteria associated with algal cultures are subjected to selective factors that may differ significantly from field conditions (e.g. Amaro et al., 2005; Garcés et al., 2007).

4.4. Bacterial assemblages in ambient seawaters

Ambient seawater bacterial assemblages were partially distinct from those associated with *O*. cf. *ovata*, primarily containing taxa almost not retrieved in the latter and also by showing a significant lower contribution of OTUs assigned to *Alphaproteobacteria* compared to *O*. cf. *ovata* assemblages. Moreover, SW assemblages while sharing the major genera of this class with OA assemblages were also characterized by a large contribution of OTU #33 (closely related to *Salinihabitans*; Table S1) at early bloom, not observed in the same high proportion in *O*. cf. *ovata* aggregates. SW assemblages contained members of OTUs belonging to *Vibrio*, *Propionibacterium*, *Haliscomenobacter* and *Cetobacterium*, which were absent or in low abundance in the OA assemblages. These genera are commonly connected with host organisms or sewage pollution (Vorobjeva, 1999; Finegold et al., 2003; Mulder and Deinema, 2006; Ceccarelli and Colwell, 2014). This finding was not surprising as the sampling area is moderately affected by anthropogenic impact during summer season (Accoroni et al., 2011). Dynamics between benthic hosts' associated and the surrounding seawater bacteria can be tightly coupled, as the latter can serve as a major seeding source for epibiotic consortium, as well as the former may diffuse into the surrounding planktonic assemblages (Singh et al., 2014; He et al., 2014; Cleary et al., 2015). Moreover, some bacterial taxa possess many surface colonization traits (e.g. *Rhodobacteraceae*, see before) and can be cosmopolitans living in both habitats. Whereas, other taxa may be specialists of water column and lack the capacity to live in certain interfaces (e.g. He et al., 2014), or being selectively excluded in the presence of competitive bacteria associated to the benthic substrata (e.g. Singh et al., 2014), as it could be the case for *Vibrio* and *Propionibacterium*. Thus, it appears that the retrieved allochthonous bacteria (i.e. bacteria anthropogenic impact related) harbored in the ambient seawaters marginally affect bacterial assemblages associated with *O*. cf. *ovata* during bloom development; whereas dislodgment and resuspension from *O.* cf. *ovata* mats could be possibly responsible for some bacterial diffusion

into seawater assemblages, as also suggested by similarities pattern between samples in the NMDS plot.

5. Conclusions

Dinoflagellates-bacteria relationships are known to range from mutualistic to antagonistic interactions, however, we are just starting to appreciate the more ephemeral and subtle aspects (Kodama et al., 2006; Wagner-Dobler et al., 2010; Geng and Belas, 2010; Wang et al., 2014, 2015). In this study it has been found a strong association of a core bacterial genera with *O.* cf. *ovata* during the two investigated bloom phases. Under the correct physicochemical conditions noted earlier, a positive interaction between the consortium of *Alphaproteobacteria* and *O.* cf. *ovata* favoring the dinoflagellate proliferation and the bloom development phase it is hypothesized. Low hydrodynamism is definitely considered a prerequisite for *Ostreopsis* bloom development (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008; Totti et al., 2010; Pistocchi et al., 2011; Accoroni et al., 2015). Stable weather conditions could also favor *Ruegeria* colonization and biofilm formation, sustaining this mutualistic/beneficial phase, although further investigations are needed to assess this hypothesis. Moreover, a focus on the role of both *Ruegeria* and *Jannaschia* in the bloom termination processes warrants future research, considering the bivalent interaction role played by *Roseobacters* in relation to algal age (Seyedsayamdost et al., 2011, 2014). The era of high throughput sequencing will allow further detailed investigations on bacteria co-occurring with epiphytic/benthic harmful algal bloom at the consortia and cell-cell levels and their interactions.

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Supplementary material

Table S1. Closest matches from the NCBI GenBank database based on sequence similarity of the seven OTUs explaining 20% of the dissimilarity between assemblages and bloom phases as revealed by SIMPER analysis.

OTU	Closest relative RDP classifier	Closest matched sequence BLASTn	NCBI accession number	Similarity (%)	Source
6	*Ruegeria*	*Ruegeria marina**	NR116848.1	96	Marine sediment, East China Sea
		Uncultured *bacterium*	KJ002192.1	99	Coastal seawater, California
		Uncultured *bacterium*	JQ200136.1	99	Seawater next to dolphins, California
		Uncultured *bacterium*	KF373193.1	99	*Haliclona* sp., coral reef, India
		Uncultured *Rhodobacteraceae*	JQ515086.1	99	Filtered seawater surrounding *Montastra faveolata*, Puerto Rico
		Uncultured *bacterium*	KF036080.1	99	Seawater from a coral reef system, India
		Uncultured *bacterium*	KC873282.1	99	Surface seawater, China
		Uncultured *bacterium*	JX864693.1	99	Seawater, Northern Adriatic Sea
40	*Rhodobacteraceae*	*Roseovarius sediminilitoris**	NR109620.1	95	Seashore sediment, South Sea-Korea
		Uncultured *bacterium*	JQ432669.1	99	Seawater, Northern Adriatic Sea
		Uncultured *bacterium*	FN435443.1	99	Surface coastal seawater, Spain
35	*Haliea*	*Haliea mediterranea**	NR116976.1	94	Seawater, Spain
		Uncultured *bacterium*	AY568791.1	97	Intertidal flat, Ganghwa Island
		Uncultured *Gammaproteobacteria*	LC018948.1	97	Seawater, Northern Adriatic Sea
33	*Rhodobacteraceae*	*Salinihabitans flavidus**	NR044594.1	96	Marine solar saltern, Korea
		Uncultured *bacterium*	EU627932.1	99	Coastal seawater, free-living fraction, Philippines

7	*Propionibacterium*	*Propionibacterium acnes**	NR074675.1	100	Human skin
		Uncultured *Propionibacterium* sp.	KP967484.1	100	*Rutilus rutilus' s* gut
		Propionibacterium acnes	KF933807.1	100	Human oral cavity
516	*Jannaschia*	*Jannaschia seohaensis**	NR116203.1	98	Tidal flat sediment, Yellow Sea-Korea
		Uncultured *bacterium*	KC631555.1	99	Marine finfish aquaculture farm sediment
		Uncultured *bacterium*	JF272022.1	99	Natural marine biofilms, Hong Kong
		Uncultured *bacterium*	GU066488.1	99	Natural biofilm with silver nanoparticles treatment, Singapore
164	*Erythrobacter*	*Erythrobacter litoralis**	NR074349.1	100	Seawater, Sargasso Sea
		Uncultured *bacterium*	KR921225.1	100	Bulk soil associated with *Rhizophora mangle* root zones, Brasil
		Uncultured *bacterium*	KJ998483.1	100	Uppermost 2 mm of an intertidal microbial mat, Mexico
		Uncultured *bacterium*	KF179645.1	100	*Porites lutea*' s healthy tissue, Mayotte
		Uncultured *bacterium*	AB948566.1	100	Methane hydrate sediment, South Sea-Korea
		Uncultured *Erythrobacter sp.*	JQ917658.1	100	Marine carbonate sediment, Bahamas
		Uncultured *bacterium*	JX864532.1	100	Seawater, Northern Adriatic Sea
		Erythrobacter sp.	KC169807.1	100	Marine sediment, Taiwan

* Best match from the typestrains/prokaryotic 16S ribosomal RNA database. All the other best matches are from the nucleotide collection (nr/nt) database.

Table S2. Relative abundance (in %) of all the phyla detected by pyrosequencing analysis. Rare phyla accounted for less than 1% in percentage.

Phylum	% in at least one sample	OAI	OAII	LAI	LAII	SWI	SWII
Proteobacteria	≥ 1	85.0	64.6	85.7	60.8	50.9	64.4
Bacteroidetes	≥ 1	13.5	26.6	12.7	29.3	36.0	27.3
Actinobacteria	≥ 1	0.6	4.4	0.6	5.1	6.3	4.4
Cyanobacteria	≥ 1	0.4	2.1	0.6	2.3	2.4	1.6
Parcubacteria	≥ 1	0.3	1.2	0.2	1.4	0.0	1.7
Fusobacteria	≥ 1	0.0	0.4	0.0	0.4	1.2	0.1
Firmicutes	≥ 1	0.0	0.1	0.0	0.1	1.1	0.1
Planctomycetes	≥ 1	0.0	0.1	0.0	0.0	1.0	0.1
Acidobacteria	rare	0.0	0.3	0.0	0.3	0.5	0.1
Chloroflexi	rare	0.0	0.2	0.0	0.1	0.0	0.0
Chlorobi	rare	0.0	0.0	0.0	0.0	0.4	0.0
Spirochaetes	rare	0.1	0.0	0.1	0.1	0.2	0.1
TM7	rare	0.0	0.1	0.0	0.1	0.1	0.1
Deinococcus-Thermus	rare	0.0	0.1	0.0	0.1	0.0	0.1

Abbreviations: refer to Table 1 for sample ID abbreviations.

Table S3. Relative abundance (in %) of all the classes detected by pyrosequencing analysis. Rare classes accounted for less than 1% in percentage.

Class	% in at least one sample	OAI	OAII	LAI	LAII	SWI	SWII
Alphaproteobacteria	≥ 1	83.1	66.0	84.4	61.9	47.8	59.6
Gammaproteobacteria	≥ 1	5.7	3.6	5.4	4.2	5.8	7.4
Deltaproteobacteria	≥ 1	0.5	1.1	0.6	1.3	3.0	1.3
Epsilonproteobacteria	≥ 1	0.1	0.8	0.1	1.1	0.9	2.5
Flavobacteria	≥ 1	7.2	19.1	7.8	22.2	24.3	18.2
Sphingobacteria	≥ 1	2.5	4.2	0.7	3.2	7.2	5.9
Actinobacteria	≥ 1	0.4	3.1	0.4	3.7	5.1	3.3
Cyanobacteria	≥ 1	0.3	1.5	0.4	1.7	1.9	1.2
Fusobacteria	≥ 1	0.0	0.3	0.0	0.3	1.0	0.1
Betaproteobacteria	rare	0.1	0.1	0.0	0.1	0.6	0.2
Bacteroidia	rare	0.0	0.0	0.0	0.0	0.2	0.0
Bacilli	rare	0.0	0.0	0.0	0.0	0.5	0.0
Clostridia	rare	0.0	0.0	0.0	0.0	0.2	0.1
Acidobacteria Gp16	rare	0.0	0.1	0.0	0.1	0.2	0.0
Acidobacteria Gp4	rare	0.0	0.0	0.0	0.0	0.0	0.1
Caldilineae	rare	0.0	0.1	0.0	0.0	0.0	0.0
Ignavibacteria	rare	0.0	0.0	0.0	0.0	0.2	0.0
Deinococci	rare	0.0	0.0	0.0	0.0	0.0	0.0
Phycisphaerae	rare	0.0	0.0	0.0	0.0	0.8	0.1
Spirochaetes	rare	0.0	0.0	0.1	0.0	0.1	0.1

Abbreviations: refer to Table 1 for sample ID abbreviations.

Table S4. Relative abundance (in %) of all the genera detected by pyrosequencing analysis. Rare genera accounted for less than 0.5% of the total reads.

Genus	% of total reads	OAI	OAII	LAI	LAII	SWI	SWII
Ruegeria	≥ 0.5	15.5	6.6	30.2	12.8	9.9	11.9
Erythrobacter	≥ 0.5	23.8	16.0	22.4	14.7	6.8	13.8
Jannaschia	≥ 0.5	12.8	21.1	8.3	13.3	2.0	7.3
Litoreibacter	≥ 0.5	2.1	4.3	2.2	3.8	1.4	3.5
Loktanella	≥ 0.5	3.3	3.2	2.5	2.5	2.3	2.9
Paracoccus	≥ 0.5	3.4	2.9	1.9	3.0	2.3	1.7
Roseovarius	≥ 0.5	0.8	0.9	1.4	1.2	0.7	0.8
Ponticaulis	≥ 0.5	3.2	0.5	1.6	0.3	1.9	1.4
Phaeobacter	≥ 0.5	1.8	1.1	1.2	0.6	1.3	1.1
Silicibacter	≥ 0.5	0.3	1.9	0.2	0.8	0.8	0.4
Thalassobius	≥ 0.5	0.7	1.1	0.6	1.1	0.6	0.9
Nautella	≥ 0.5	0.8	0.8	0.1	0.2	0.3	1.1
Pseudoruegeria	≥ 0.5	0.3	0.8	0.1	0.5	1.1	0.1
Haliea	≥ 0.5	13.3	0.5	10.1	0.4	1.3	1.4
Granulosicoccus	≥ 0.5	0.9	2.7	1.0	3.7	0.3	3.1
Vibrio	≥ 0.5	0.1	0.2	0.1	0.3	0.6	3.0
Glaciecola	≥ 0.5	0.3	0.1	0.5	0.1	0.9	0.2
Bacteriovorax	≥ 0.5	0.3	0.4	0.4	0.6	1.7	1.5
Arcobacter	≥ 0.5	0.1	1.1	0.1	1.9	0.8	4.6
Polaribacter	≥ 0.5	2.9	1.0	2.7	1.1	4.9	3.2
Croceitalea	≥ 0.5	0.5	1.9	0.5	1.8	3.1	1.3
Actibacter	≥ 0.5	0.0	3.5	0.1	3.7	1.4	0.1
Lutimonas	≥ 0.5	0.1	1.8	0.1	1.9	1.3	0.3

Winogradskyella	≥ 0.5	0.1	1.0	0.1	1.1	1.6	0.4
Aestuariicola	≥ 0.5	0.1	0.9	0.1	1.0	1.1	0.1
Gilvibacter	≥ 0.5	0.4	0.6	0.1	0.8	0.3	0.8
Haliscomenobacter	≥ 0.5	0.3	0.7	0.2	0.8	2.2	0.9
Ilumatobacter	≥ 0.5	0.8	5.0	0.8	6.2	1.7	6.3
Propionibacterium	≥ 0.5	0.0	0.2	0.0	0.3	12.7	0.4
GpIIa	≥ 0.5	0.8	2.6	0.9	4.0	1.4	3.6
Cetobacterium	≥ 0.5	0.0	0.4	0.0	0.4	2.2	0.0
Parcubacteria Incertae Sedis	≥ 0.5	0.3	1.1	0.2	1.6	0.1	2.1
Hellea	rare	0.8	0.1	0.7	0.2	0.2	1.0
Acinetobacter	rare	0.0	0.2	0.0	0.0	0.0	0.2
Aestuariibacter	rare	0.1	0.1	0.0	0.0	0.0	0.0
Ahrensia	rare	0.0	0.0	0.0	0.0	0.1	0.0
Algibacter	rare	0.0	0.0	0.2	0.3	0.2	0.3
Algicola	rare	0.0	0.1	0.0	0.0	0.0	0.1
Altererythrobacter	rare	0.0	0.0	0.0	0.1	0.6	0.1
Alteromonas	rare	0.0	0.0	0.0	0.0	0.6	0.0
Amaricoccus	rare	0.0	0.0	0.0	0.1	0.3	0.0
Aquimarina	rare	0.0	0.1	0.1	0.2	0.4	0.1
Arenicella	rare	0.5	0.5	0.5	0.4	0.3	0.9
Aureispira	rare	0.0	0.1	0.0	0.1	0.1	0.1
Bacteroides	rare	0.0	0.0	0.0	0.0	0.1	0.0
Balneola	rare	0.0	0.0	0.0	0.0	0.1	0.0
Bizionia	rare	0.0	0.0	0.0	0.0	0.4	0.0
Bowmanella	rare	0.0	0.0	0.0	0.0	0.0	0.1
Brevundimonas	rare	0.0	0.0	0.0	0.0	0.2	0.0
Caldilinea	rare	0.0	0.1	0.0	0.0	0.1	0.1
Celeribacter	rare	0.0	0.1	0.1	0.0	0.6	0.1
Citreicella	rare	0.0	0.0	0.0	0.0	0.2	0.1

Clostridium sensu stricto	rare	0.0	0.1	0.0	0.0	0.2	0.0
Clostridium XI	rare	0.2	0.2	0.0	0.0	0.1	0.2
Cocleimonas	rare	0.0	0.0	0.0	0.0	0.1	0.0
Corynebacterium	rare	0.0	0.1	0.0	0.0	0.4	0.0
Coxiella	rare	0.0	0.2	0.1	0.5	0.1	0.3
Crocinitomix	rare	0.3	0.2	0.4	0.1	0.0	0.5
Cupriavidus	rare	0.0	0.0	0.0	0.0	0.9	0.0
Dasania	rare	0.0	0.1	0.0	0.0	0.1	0.0
Delftia	rare	0.0	0.0	0.0	0.0	0.2	0.0
Desulfopila	rare	0.1	0.0	0.0	0.1	0.3	0.1
Desulforhopalus	rare	0.0	0.0	0.0	0.1	0.0	0.1
Desulfotalea	rare	0.0	0.0	0.0	0.1	0.2	0.0
Ectothiorhodosinus	rare	0.0	0.0	0.0	0.1	0.1	0.0
Ekhidna	rare	0.0	0.0	0.0	0.0	0.2	0.0
Enterovibrio	rare	0.0	0.0	0.0	0.0	0.8	0.0
Eudoraea	rare	0.2	0.2	0.0	0.3	0.2	0.2
Fabibacter	rare	0.1	0.1	0.2	0.2	0.2	0.9
Fangia	rare	0.0	0.1	0.0	0.0	0.0	0.0
Ferrimonas	rare	0.0	0.0	0.0	0.0	0.2	0.0
Flavobacterium	rare	0.0	0.1	0.0	0.0	0.1	0.1
Gaetbulibacter	rare	0.6	0.2	0.1	0.4	0.0	0.3
Gp16	rare	0.0	0.1	0.0	0.1	0.0	0.1
Gp4	rare	0.0	0.0	0.0	0.0	0.0	0.0
GpI	rare	0.3	0.1	0.0	0.0	0.1	0.2
GpIV	rare	0.0	0.1	0.0	0.2	0.0	0.0
GpIX	rare	0.0	0.2	0.0	0.2	0.2	0.0
GpVIII	rare	0.0	0.3	0.0	0.2	0.1	0.2
GpX	rare	0.0	0.0	0.0	0.0	0.1	0.0
GpXII	rare	0.0	0.0	0.0	0.0	0.6	0.0

Gracilimonas	rare	0.2	0.6	0.1	0.1	0.2	0.3
Granulicatella	rare	0.0	0.0	0.0	0.0	0.1	0.0
Henriciella	rare	0.0	0.0	0.0	0.0	0.0	0.0
Hirschia	rare	0.0	0.0	0.2	0.0	0.3	0.0
Hyphomonas	rare	0.0	0.0	0.2	0.1	0.1	0.0
Ignavibacterium	rare	0.0	0.0	0.0	0.0	0.3	0.0
Joostella	rare	0.0	0.1	0.0	0.3	0.2	0.2
Kordiimonas	rare	0.2	0.1	0.0	0.0	0.0	0.1
Krokinobacter	rare	0.0	0.1	0.0	0.0	0.4	0.0
Labrenzia	rare	0.0	0.0	0.2	0.2	0.9	0.0
Legionella	rare	0.0	0.0	0.0	0.1	0.3	0.0
Leisingera	rare	0.2	0.2	0.1	0.5	0.2	0.4
Leucothrix	rare	0.6	0.1	0.1	0.1	0.0	0.0
Lewinella	rare	0.1	0.3	0.1	0.3	0.8	0.3
Listonella	rare	0.0	0.0	0.0	0.0	0.1	0.0
Litorimonas	rare	0.0	0.4	0.1	0.3	0.2	0.0
Lutibacter	rare	0.0	0.0	0.0	0.0	0.3	0.1
Mameliella	rare	0.0	0.0	0.0	0.0	0.0	0.0
Maribacter	rare	0.3	0.1	0.0	0.7	0.1	0.3
Maribius	rare	0.0	0.1	0.0	0.0	0.1	0.1
Maricaulis	rare	0.0	0.2	0.1	0.1	0.1	0.0
Marinicella	rare	0.0	0.2	0.1	0.1	0.0	0.2
Marinoscillum	rare	0.0	0.0	0.0	0.1	0.6	0.0
Marivita	rare	0.0	0.1	0.1	0.2	0.3	0.0
Microcella	rare	0.0	0.1	0.0	0.1	0.1	0.1
Muricauda	rare	0.1	0.1	0.0	0.1	0.0	0.1
Muriicola	rare	0.0	0.0	0.0	0.1	0.2	0.1
Mycobacterium	rare	0.0	0.1	0.0	0.0	0.1	0.1
Nereida	rare	0.1	0.4	0.2	0.2	0.4	0.4

Oceanicola	rare	0.5	0.4	0.4	0.2	0.4	0.4
Oceanospirillum	rare	0.0	0.0	0.0	0.0	0.1	0.0
Orientia	rare	0.0	0.0	0.0	0.3	0.1	0.2
Pacificibacter	rare	0.2	0.1	0.1	0.1	0.3	0.2
Parabacteroides	rare	0.0	0.0	0.0	0.0	0.1	0.1
Parvularcula	rare	0.2	0.4	0.1	0.5	0.9	0.5
Pelagibius	rare	0.0	0.0	0.0	0.0	0.0	0.0
Photobacterium	rare	0.1	0.0	0.0	0.0	0.3	0.3
Phycisphaera	rare	0.1	0.3	0.0	0.0	0.6	0.1
Pibocella	rare	0.0	0.0	0.0	0.2	0.3	0.0
Plesiocystis	rare	0.0	0.0	0.0	0.0	0.2	0.0
Prevotella	rare	0.0	0.1	0.0	0.0	0.0	0.0
Propionigenium	rare	0.5	0.0	0.0	0.0	0.0	0.2
Pseudoalteromonas	rare	0.1	0.4	0.0	0.2	0.4	0.1
Pseudomonas	rare	0.0	0.1	0.0	0.0	0.6	1.1
Psychrilyobacter	rare	0.0	0.0	0.0	0.0	0.1	0.0
Psychroserpens	rare	0.0	0.1	0.0	0.2	0.2	0.2
Psychrosphaera	rare	0.0	0.0	0.0	0.0	0.7	0.0
Reichenbachiella	rare	0.1	0.4	0.5	0.1	0.7	0.3
Rhodovulum	rare	0.0	0.3	0.4	0.3	1.0	0.5
Robiginitalea	rare	0.1	0.3	0.1	0.9	1.3	0.4
Robiginitomaculum	rare	0.0	0.0	0.1	0.0	0.2	0.1
Roseibacterium	rare	0.0	0.1	0.0	0.4	0.0	0.6
Roseivirga	rare	0.0	0.0	0.0	0.0	0.0	0.3
Roseobacter	rare	0.1	0.1	0.0	0.1	0.0	0.1
Saprospira	rare	0.0	0.0	0.0	0.0	0.1	0.0
Sediminitomix	rare	0.0	0.0	0.0	0.1	0.6	0.0
Shimia	rare	1.0	0.2	0.8	0.2	0.1	0.6
Sorangium	rare	0.0	0.0	0.0	0.0	0.1	0.0

Sphingobium	rare	0.0	0.0	0.0	0.0	0.3	0.1
Sphingomonas	rare	0.0	0.0	0.0	0.0	0.5	0.1
Sphingopyxis	rare	0.0	0.1	0.1	0.0	0.1	0.2
Spirochaeta	rare	0.0	0.1	0.0	0.0	0.0	0.0
SR1 incertae sedis	rare	0.0	0.0	0.0	0.0	0.0	0.0
Stakelama	rare	0.0	0.0	0.0	0.0	0.2	0.0
Staphylococcus	rare	0.5	0.3	0.0	0.0	0.1	0.3
Stenothermobacter	rare	0.0	0.0	0.0	0.0	0.2	0.0
Stenotrophomonas	rare	0.0	0.0	0.0	0.0	0.2	0.0
Streptococcus	rare	0.0	0.2	0.0	0.0	0.2	0.3
Sulfitobacter	rare	0.5	0.3	0.1	0.3	0.7	0.3
Sulfurimonas	rare	0.1	0.1	0.0	0.0	0.2	0.2
Sulfurovum	rare	0.5	0.1	0.0	0.3	0.1	1.3
Tateyamaria	rare	0.0	0.0	0.0	0.0	0.2	0.0
Tenacibaculum	rare	0.5	0.3	0.1	0.1	0.1	0.5
Terasakiella	rare	0.0	0.0	0.0	0.0	0.0	0.0
Thalassobacter	rare	0.0	0.0	0.0	0.0	0.0	0.0
Thalassomonas	rare	0.1	0.6	0.0	0.0	0.0	0.2
Thioprofundum	rare	0.0	0.0	0.0	0.0	0.1	0.0
TM7 incertae sedis	rare	0.0	0.1	0.0	0.0	0.1	0.0
Tropicimonas	rare	0.0	0.0	1.7	0.1	0.1	0.1
Truepera	rare	0.0	0.1	0.1	0.0	0.0	0.0
Ulvibacter	rare	0.1	0.4	0.0	0.0	0.9	0.6
Vampirovibrio	rare	0.0	0.0	0.0	0.2	0.1	0.1
Veillonella	rare	0.0	0.0	0.0	0.0	0.3	0.1
Wenxinia	rare	0.0	0.0	0.0	0.0	0.2	0.0

Abbreviations: refer to Table 1 for sample ID abbreviations

Chapter 3

Microbial dynamics along the toxic dinoflagellate *Ostreopsis* cf. *ovata* growth: bacterial phylogenetic succession and viruses top-down control

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ABSTRACT

The increasing occurrence of toxic epibenthic *Ostreopsis* cf. *ovata* blooms has been reported in the Mediterranean area since the last two decades. Microalgae and bacteria synergistically affect their physiology and metabolism, and microalgal-associated bacteria can either promote or inhibit algal growth in different phases, with potential relevant effects on harmful algal bloom dynamics. This study investigated the temporal dynamics of microbial (bacteria and viruses) abundances and bacterial phylogenetic succession pattern in *O.* cf. *ovata* batch cultures during different algal growth phases. Phylogenetic structure of the bacterial community was assessed by high-throughput parallel tag sequencing of the 16S rDNA gene, while algal toxin production was determined through LC–HRMS analysis. Total toxins (range: 7.4 to 18.0 pg cell^{-1}) showed an increasing trend from the exponential to the stationary algal growth phase. Bacterial and viral temporal dynamics suggest a more relevant top-down control by lytic activity on bacteria at the end of the algal exponential phase. *Alphaproteobacteria* (65-96%) followed by *Sphingobacteria* (2-34%) and *Actinobacteria* (0.1-2%) primarily composed the microbiota. The bacterial composition and successional trend showed dominance of vitamin- and antibacterial-synthesizing taxa promoting *O.* cf. *ovata* growth during exponential phase (i.e. *Oceanicaulis*, *Dinoroseobacter*, *Roseovarius*, *Sphyngopyxis*), followed by saprophytic/algicidal phylotypes as *Labrenzia* and *Dietzia* at later phases of the algal growth. The algal-bacterial functional association pattern retrieved paves the way for further investigations on the role of allochthonous vitamins in *O.* cf. *ovata* bloom dynamics.

Keywords: *Ostreopsis*, toxic dinoflagellate, algal-bacterial interactions, ION torrent, virus, vitamins, *Dinoroseobacter*

1. Introduction

The increasing occurrence of *Ostreopsis* cf. *ovata* blooms has been reported in the Mediterranean Sea since the last two decades (Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011; Funari et al., 2015). Human respiratory, skin problems (reviewed in Funari et al., 2015), and massive mortalities of invertebrate benthic communities (Accoroni et al., 2011; Faimali et al., 2012; Pagliara and Caroppo, 2012; Carella et al., 2015) reported during periodic outbreaks of the dinoflagellate, have been linked to the algal production of isobaric palytoxin and several ovatoxins (OVTX-a to -k; Garcìa-Altares et al., 2015; Brissard et al., 2015; Tartaglione et al., 2016). Toxin production occurs under both field (Accoroni et al., 2011; Ciminiello et al., 2011) and laboratory conditions, with an increasing trend over the growth phases (Vanucci et al., 2012a,b; Pezzolesi et al., 2014). The epiphytic/benthic dinoflagellate thrives better in shallow and sheltered waters with low hydrodynamism, where it grows onto a wide range of biotic and abiotic substrata forming thick, brownish mucilaginous biofilms that embed the algal cells inside a matrix (Totti et al., 2010; Honsell et al., 2013; Giussani et al., 2015). Concurrently with the environmental conditions and abiotic factors (reviewed in Pistocchi et al., 2011; Mangialajo et al., 2011; Accoroni et al., 2015; Carnicer et al., 2015), a larger appreciation about the importance of the interactions between microalgae and bacteria in terms of regulating harmful algal bloom (HAB) dynamics has established (Kodama et al., 2006; Loureiro et al, 2011). Microalgae and bacteria reciprocally affect their physiology and metabolism through complex and intimate relationships (Bolch et al., 2011; Santos and Azanza, 2012; Albinsson et al., 2014) ranging from mutualism to parasitism (reviewed in Amin et al., 2012; Cooper and Smith, 2015; Ramanan et al., 2016). The presence and the succession of specific and phylogenetically diverse bacterial groups with partially different metabolic activity observed during the lifetime of the blooms (Teeling et al., 2012; Buchan et al., 2014) suggest a fundamental role of algal-bacterial relationships in ecosystem functioning (Amin et al., 2012; Ramanan et al., 2016), synthesis/modification of algal toxins (Donovan et al., 2009; Green et al., 2010; Albinsson et al., 2014) and ultimately bloom dynamics (Wang et al., 2010; Riclea et al., 2012; Buchan et al., 2014). Indeed, specific bacterial taxa such as *Rhodobacteraceae* have been observed to switch from mutualistic symbionts to opportunistic pathogens of their algal hosts (Wagner-Dobler et al., 2010; Wang et al., 2014, 2015) in response to algal senescence signaling molecules (Seyedsayamdost et al., 2011a,b; 2014; Sule and Belas, 2013), implying a relevant role of these bimodal interactions in both bloom triggering and termination phases.

Moreover, changes in amount and composition of algal-released compounds depending on algal species, growth phase and physiological status may determine the type (Bennke et al., 2013; Christie-Oleza et al., 2015; Xing et al., 2015) and the successional pattern of the associated bacterial community (Schafer et al., 2002; Grossart et al., 2005; Saap et al., 2007; Teeling et al., 2012; Bagatini et al., 2014).

Studies on harmful planktonic and benthic dinoflagellates conducted both *in situ* and on laboratory cultures revealed recurrent associations between the toxic microalgae and specific bacterial groups such as *Alphaproteobacteria* mainly affiliated with the clade *Roseobacter*, *Gammaproteobacteria*, members of the *Flavobacteria-Sphingobacteria* cluster, and *Actinobacteria* to a lesser extent (reviewed in Garcés et al., 2007; Pérez-Guzmán et al., 2008; Ruh et al., 2009; Green et al., 2010; Jones et al., 2010; Yang et al., 2015; Onda et al., 2015; Park et al., 2015). *Roseobacters* are high versatile bacteria capable to establish intimate relationships with phytoplankton and utilize a wide-spectrum of dissolved algal-derived low-molecular-weight organic matter including the algal osmolyte dimethylsulfoniopropionate (DMSP) (Wagner-Döbler et al., 2010; Teeling et al., 2012; Buchan et al., 2014; Durham et al., 2015). Differently, members of *Flavobacteria-Sphingobacteria* generally include secondary colonizers bacteria specialized in ectohydrolitic breakdown of high-molecular weight particulate organic matter of algal origin (Teeling et al., 2012; Mann et al., 2013; Williams et al., 2013; Klindworth et al., 2014).

While most studies on HABs considered the importance of macronutrients, the influence of micronutrients such as vitamins in bloom stimulation and development has been barely addressed, although laboratory screenings elucidated the striking auxotrophy of dinoflagellates for B-vitamins (Croft et al., 2005, Tang et al., 2010). Furthermore, recurrent associations between microalgae and potential vitamin-synthesizing bacteria have been observed in cultures (Kuo and Lin, 2013; Abby et al., 2014; Ramanan et al., 2015), and the bacterial provision of essential vitamins to the algal host during mutualistic phases of the microbial succession has been demonstrated for the bacterium *Dinoroseobacter shibae* and the dinoflagellate *Prorocentrum minimum* in a dual-species co-cultivation system (Wagner-Döbler et al., 2010, Wang et al., 2014, 2015), suggesting a critical role of vitamin availability in triggering algal blooms and driving bacterial succession (Koch et al., 2013, 2014; Sãnudo-Wilhelmy et al., 2014).

Recently, viruses have been also suggested to be important elements in bloom dynamics of toxic planktonic dinoflagellate (*Karenia brevis*, Meyer et al., 2014), possibly inducing shifts in the bacterial community composition and activity rather than straightly impact the alga

(Paul et al., 2002; Meyer et al., 2014). In fact, viruses can significantly top-down regulate bacterial community composition infecting the most abundant and metabolically active taxa (reviewed in Del Giorgio and Gasol, 2008; Martiny et al., 2014). However, little is known about the specific roles that phages have in controlling bacterial composition and abundance, and consequently bloom mechanisms (Buchan et al., 2014).

Concerning *O.* cf. *ovata*, limited information is available on the potential relationships between the dinoflagellate and its associated microbial (bacteria and viruses) community (Vanucci et al., 2012b; Vanucci et al., in press-a). A deep knowledge on phylogenetic composition and successional dynamics of the bacterial communities associated with HAB populations, limited to date, will be a crucial step for understanding the complex algal-bacterial interactions underlying the different bloom phases (Mayali et al., 2011; Tada et al., 2012; Bagatini et al., 2014; Yang et al., 2015). Indeed, although 16S rDNA phylogenetic analysis do not directly decode bacterial functionality, they still provide insights on how the diverse bacterial groups correlate within the assemblages and with the microalgal partner, taking into account certain metabolic traits significant to the groups and to the associated organism (Newton et al., 2010; Amin et al., 2012; Langille et al., 2013; Gifford et al., 2014; Buchan et al., 2014). In general, most of the available studies describing bacterial communities associated with both planktonic and benthic bloom-forming dinoflagellates have been relied on traditional molecular methods (e.g. FISH, DGGE, 16S rDNA clone libraries) with few exceptions (*Akashiwo sanguine*, Yang et al. 2015; *Ostreopsis* cf. *ovata*, Vanucci et al., in press-a), and do not provide information on temporal dynamics. These previous surveys have been based on a little number of sequences, allowing a limited assessment and comparison of the bacterial communities' phylogenetic diversity co-occurring either with different toxic algal species, or with distinct algal bloom stages or experimental growth phases (Garcés et al., 2007; Jones et al., 2010; Mayali et al., 2011, Yang et al., 2012; Park et al., 2015). Next Generation Sequencing approaches typically allow a deep phylogenetic analysis, reducing considerably the gap of knowledge on taxonomic composition of microalgal-associated bacteria. Moreover, culture-based studies, although only partially simulate nature, still represents an essential tool for uncover overlooked interactions, providing the possibility to isolate individual species from their multifaceted natural background under defined experimental conditions (Jasti et al., 2005; Ruh et al., 2009; Buchan et al., 2014; Sison-Mangus et al., 2014).

The present study was undertaken to assess temporal dynamics of microbial (bacteria and viruses) abundances and bacterial phylogenetic succession pattern associated with *O.* cf. *ovata*

batch cultures during the different phases of the algal growth, in order to infer interactions that synergistically affect algal and bacterial physiology and metabolism with potential relevance on bloom dynamics. A brief comparison between the bacterial assemblages co-occurring with *O.* cf. *ovata* bloom with the microbiota selected under batch cultures of the *O.* cf. *ovata* strain isolated during the same bloom event is also reported. Bacterial phylogenetic composition was assessed by high-throughput parallel tag sequencing using ION torrent PGM platform, while algal toxin production was determined through LC–HRMS analysis. Additionally, the bulk active bacterial fraction was identified through the use of the fluorogenic redox dye 5-cyano-2,3-dytolyl tetrazolium chloride (CTC), in order to provide details on bacterial community's metabolic and physiological state during culture evolvement.

2. Materials and methods

2.1.Experimental setup and culture conditions

Ostreopsis cf. *ovata* strain OOAP0912 was isolated using capillary pipette method (Hoshaw and Rosowski, 1973) from macrophyte samples collected at the early phase of an *O.* cf. *ovata* bloom along the coast of North-western Adriatic Sea (Passetto, Italy, 43°36'38" N and 13°32'20" E; Vanucci et al., in press-a) and washed sequentially several times with sterile medium, in order to leave only those bacteria strongly associated with the single algal cell at the time of isolation. After initial growth in microplates, cells were maintained for 18 months (20 subculture generations) in sterile flasks sealed with cotton plugs at 20 ± 1 °C under a 16:8 h Light:Dark cycle in a growth chamber (photon flux density 110-120 µmol m^2 s^{-1} by cool white lamp); cultures were established in natural seawater adjusted to salinity value of 36, with macronutrients added at a five-fold diluted f/2 concentration (Guillard, 1975) plus selenium. Experimental cultures consisted in duplicate 3-L Erlenmeyer flasks, inoculated with *O.* cf. *ovata* collected from a culture at end exponential/early stationary phase and fresh medium to a final volume of 2650 mL, in order to have a concentration of about 300 cell mL^{-1} at the beginning of the experiment (day 0). All culture transfers and experimental manipulation was carried out in a laminar flow hood using sterile equipment.

2.2. O. cf. ovata enumeration

O. cf. *ovata* cell counts were performed at day 0, 3, 6, 9, 12, 18, 24, 32, and 42. As enumeration of *O.* cf. *ovata* cells in batch cultures is difficult due to the presence of mucilaginous cell aggregates, sampling for cell counts was performed by using method

developed by Guerrini et al. (2010) and slightly modified: aliquots of at least 50 mL were collected and treated with HCl to a 4mM final concentration, then aliquots were fixed with 1% Lugol solution (Throndsen, 1978). Before counting, cells were allowed to settle for about 5 min, then cell counts were performed following the Utermöhl method (Hasle, 1978) using a Zeiss Axiovert 100 inverted microscope at 320x magnification under bright field and phase contrast illumination. For each sample, *O.* cf. *ovata* cell concentration was estimated by averaging the cells enumerated in four transects of the chamber, at least in triplicates. Specific growth rate (μ, day^{-1}) was calculated using the following equation:

$$\mu = \frac{\ln N_1 - \ln N_2}{t_1 - t_0}$$

where N_0 and N_1 were cell density values at time t_0 and t_1.

All counted cells were measured using a digital monitor system (Nis Elements BR 2.20 software) with a Nikon Digital Sight DS-U1 camera connected to the inverted microscope.

2.3. Nutrient determination

Nitrate and phosphate analyses were performed on filtered culture medium aliquots (Whatman GF/F filters, pore size 0.7 µm) and analyzed spectrophotometrically (UV/VIS, JASCO 7800, Tokyo, Japan) according to Strickland and Parsons (1972). The nutrient (NO_3-N and PO_4-P) uptake (U) was calculated according to Pezzolesi et al. (2014) from the residual nutrient concentrations in the medium (C) and the difference in cell densities (Y) between days, when the depletion of nutrients was linear. The following equations were used:

$$U = -\frac{C_1 - C_0}{Y\Delta t}$$

$$\mu = \frac{N_1 - N_0}{\ln N_1 - \ln N_0}$$

where C_0 and C_1 were the nutrient concentrations (µM) at time t_0 and t_1, and N_0 and N_1 were the corresponding cell densities (cells mL^{-1}).

2.4. Toxin analysis

2.4.1. Sample extraction

Cell samples and growth medium of *O.* cf. *ovata* cultures were separately extracted. Cell samples were obtained by filtering a variable volume of culture (500–800 mL) using glass fiber filters (Whatman GF/F, pore size 0.7 μm), and the obtained filters were stored at -80 °C until the extraction. All organic solvents used for the toxin extraction and analysis were of distilled-in-glass grade (Carlo Erba, Milan, Italy). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). For the toxin extraction, 8 mL of a methanol/water (1:1, v/v) solution was added to each filter and then sonicated for 2 min in pulse mode, while cooling in an ice bath. The mixture was centrifuged at 3000 x *g* for 15 min, the supernatant was decanted and the pellet was washed twice with 4 mL of methanol/water (1:1, v/v). The extracts were combined and the volume adjusted to 16 mL with extracting solvent. The obtained mixture was analyzed directly by LC–HRMS. Recovery percentages of the above extraction procedures were estimated to be 98% (Ciminiello et al., 2008). Aliquots of 200 ml of the growth medium flowed-through during cell filtering were loaded onto SPE cartridges OASIS HLB LP 6 cc (500 mg) (Waters, USA) with the following conditions (Ciminiello et al., 2015):

1. Conditioning: MeOH–H_2O 8:2, 1:9, H_2O (20 ml each)
2. Load: Growth medium (200 ml)
3. Wash: H_2O, MeOH–H_2O 1:9 (3 ml each)
4. Elute: MeOH–H2O 8:2 with 0.1 % trifluoroacetic acid (E_1 – E_{10}, 10 ml)

The obtained elutes were analyzed directly by LC–HRMS. Recovery percentages of the above extraction procedures (50 ± 9%) were assessed by contaminating blank growth medium with PLTX standard at 5 μg l^{-1} and repeating the whole procedure over three replicates.

2.4.2. Liquid chromatography–high resolution mass spectrometry (LC–HRMS)

LC–HRMS experiments were carried out on an Agilent 1100 LC binary system (Palo Alto, CA, USA) coupled to a hybrid linear ion trap LTQ Orbitrap XL™ Fourier Transform mass spectrometer (FTMS) equipped with an ESI ION MAX™ source (Thermo-Fisher, San José, CA, USA). The following conditions were used: a column of 2.7 μm Poroshell 120 EC-C18, 100×2.10 mm (Agilent), was kept at room temperature and eluted at 0.2 mL min^{-1} with water (eluent A) and 95 % acetonitrile/water (eluent B), both containing 30 mM acetic acid (HPLC grade, Sigma-Aldrich). Injection volume for both algal extracts and growth medium elutes

was 5 µL. A slow gradient elution was used: 20–50% B over 20 min, 50–80% B over 10 min, 80–100% B in 1 min, and hold 5 min. This gradient system allowed a partial chromatographic separation of most palytoxin-like compounds. HR full-MS experiments (positive ions) were acquired in the range m/z 800–1400. The source settings were: spray voltage = 4.8 kV, capillary temperature = 290 °C, capillary voltage = 50 V, sheath gas = 38 and auxiliary gas = 2 (arbitrary units), tube lens voltage = 120 V. Quantitative determinations of putative palytoxin, ovatoxin-a,- b,-c,-d, and -e in the extracts were carried out using a calibration curve (triplicate injection) of palytoxin standards (Wako Chemicals GmbH, Neuss, Germany) at four levels of concentration (100, 25, 12.5, 6.25 ng mL^{-1}) and assuming that their molar responses were similar to that of palytoxin. Linearity of calibration curve was expressed by R^2 = 0.999. Extracted ion chromatograms (XIC) for palytoxin and each ovatoxins were obtained by selecting the most abundant ion peaks of both $[M+2H-H2O]^{2+}$ and $[M+H+Ca]^{3+}$ ion clusters. A mass tolerance of 5 ppm was used.

2.5. Bacterial and viral enumeration, bacterial physiological state

Total bacterial and virus like particle abundance (VLP) were determined in the same culture subsamples fixed with 0.02 µm prefiltered formaldehyde (2%), following method described by Shibata et al. (2006). Briefly, aliquots were concentrated onto 0.02 µm pore size Anodisc filters (Whatman, 25 mm diameter) and stained with 100 µL SYBR Gold (Life Technologies) at 8X final concentration, then mounted onto microscopic slides and stored at -20°C. Enumeration was performed in duplicate using epifluorescence microscopy (Nikon Eclipse 80i, magnification 1000X) under blue light excitation counting at least twenty fields and a minimum of 300 cells per sample. Viruses were discriminated from bacteria based on their dimensions (Noble and Fuhrman, 1998).

Measurement of the different bacterial physiological state was performed by determining highly respiring bacteria as those able to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Sigma-Aldrich), which turns into a red fluorescent formazan detectable by epifluorescence microscopy (Sherr et al., 1999). Sample aliquots (0.9 ml) were amended with 100 µL of a 50 mM CTC solution (final concentration 5 mM) immediately following collection and were incubated for 3 h in the dark at room temperature. After the incubation, samples were fixed with 0.2 µm prefiltered formaldehyde (2%) and then filtered onto 0.2 µm black-stained polycarbonate membrane filters (Millipore). Cells were counted manually using 1000× magnification (oil immersion) as described above for total bacteria and VLPs.

2.6. Bacterial DNA extraction and PCR amplification

O. cf. *ovata* cultures in aliquots of 30-100 ml volume were harvested at the time of the inoculum and at exponential, mid and late stationary algal growth phases (day 0, 6, 24, 42, respectively) by filtration under low vacuum onto Supor 200 PES filters (Pall Corporation/Pall Life Sciences, pore size 0.2 µm). All filters were stored at -80 °C in sterile 2-mL centrifuge tubes until analysis. For DNA extraction, filters were shredded under sterile conditions, and DNA from cells on the filters was extracted using the ZR Soil Microbe DNA MiniPrep (Zymo Research) according to the manufacturer's instructions. DNA concentrations and purity were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific), then extracted DNA samples from three replicates were pooled together at equimolar amounts.

Partial bacterial 16S rRNA genes (hypervariable V1-V2 region) were amplified using universal bacterial primers 8F (5'- AGAGTTTGATCCTGGCTCAG-3') and 338R (5'-GCTGCCTCCCGTAGGAGT-3') and master mixes prepared with Qiagen Hotstar Hi-Fidelity Polymerase Kit (Qiagen). Amplification in triplicate of each sample was performed with following conditions: an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturing of 94°C for 45 sec, annealing at 50°C for 30 sec and a 1 min 30 sec extension at 72°C, ending with a 10 min extension at 72°C and a final hold at 4°C. Each amplification was checked by electrophoresis on a 1% agarose gel. In order to remove primer dimers, the replicate PCR reactions were pooled and purified using Agencourt AMPure XP PCR purification kit (Beckman Coulter Inc.) according to manufacturer instructions. Purified amplicons were quantified using a Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies) and pooled at equimolar ratio. Barcoded amplicon libraries were realized using the Ion Plus Library Kit and Ion Xpress Barcode Adapters (Life Technologies) in preparation for clonal amplification. Emulsion PCR was performed using the Ion PGM Template OT2 400 Kit (Life Technologies) according to the manufacturer's instructions. Multiplexed sequencing of the amplicon libraries was carried out on a 316 v2 chip with the Ion Torrent PGM system using 850 flows and employing the Ion PGM Sequencing 400 Kit (Life Technologies) according to the supplier's instructions.

After sequencing, the individual sequence reads were filtered by the PGM software for low quality and polyclonal sequences removal. Sequences matching the PGM 3' adaptor were also

automatically trimmed. All PGM quality-approved, trimmed and filtered data were exported as fastq files.

2.7. Sequence processing and diversity analysis

The fastq files were processed using MOTHUR (Schloss et al., 2009). Quality control retained sequences with a length between 250 and 400 bp, average sequence quality score >25, with truncation of a sequence at the first base if a low quality rolling 10 bp window was found. Sequences with presence of homopolymers >6 bp, any ambiguous base call, mismatched primers and more than one error on barcode sequence were omitted. Community taxonomy information was obtained using a Ribosomal Database Project naive Bayesian rRNA classifier (Wang et al., 2007) and those sequences either related to chloroplasts and mitochondria or not belonging to the Domain Bacteria were discarded from the dataset. The remained unique sequences were aligned against the Silva bacteria database. After screening, filtering, preclustering, and chimera removal, samples were standardized to the size of the smallest library (19662 reads) by randomly subsampling datasets and the retained sequences were used to build a distance matrix. Bacterial sequences were grouped into operational taxonomic units (OTUs) by clustering at 97% similarity, then singleton OTUs were discarded from the analysis if they were not found in at least two different samples. The representative sequence for each OTU was picked and classified using the RDP classifier and the resulting matches for each set of sequence data were summarized at various levels of taxonomic hierarchal structure (i.e. phylum, class and genus). According to Baltar and colleagues (2015), OTUs were defined as *abundant* when representing ≥1% of the community in at least one of the samples, *common* when their relative abundance ranged between 0.1–1.0%, and as *rare* those OTUs with ≤0.1% in total contribution. Bacterial community diversity was addressed through three diversity indices (observed richness, Shannon index and Good's coverage) and a Bray-Curtis similarity matrix of square root transformed OTUs abundance data performed in PAST (Hammer et al., 2001).

2.8. Statistical analysis

All statistical analysis were performed with PAST. Differences in the investigated variables were tested by the analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$ for all the analysis.

3. Results and discussion

3.1. Nutrient concentrations and Ostreopsis cf. ovata cell growth

This study investigated the temporal dynamics of microbial (bacteria and viruses) abundance and bacterial taxonomic composition in *O.* cf. *ovata* batch cultures, along with removal of major nutrients from the medium. Inorganic nutrient (i.e. NO_3-N and PO_4-P) concentration changes in culture medium during *O.* cf. *ovata* cell growth are shown in Figure 1. Nutrients were rapidly taken up during the first days and almost depleted by day 12 when algal cells entered the stationary growth phase (Fig. 2, see forward). The maximum uptake rate of 19.9 pmol $cell^{-1}$ day^{-1} for NO_3-N was recorded between days 3 and 6 (ANOVA, $p < 0.05$), while the highest value of 0.75 pmol $cell^{-1}$ day^{-1} recorded for PO_4-P temporally preceded that one of NO_3-N (days 0-3; ANOVA, $p < 0.05$), as attested by the increase of the N:P ratio until day 9 (112; Fig. 1 inset). Thereafter, N:P ratio progressively decreased to values around 1-2 (day 24 and 42). Inorganic nutrient uptake's patterns reported here were similar to those previously observed in non-axenic batch cultures established under comparable experimental conditions (Vanucci et al., 2012a; Pezzolesi et al., 2014).

Growth curve of *O.* cf. *ovata* is shown in Figure 2. The algal growth trend was consistent with those previously described (Vanucci et al., 2012a; Pezzolesi et al., 2014). Cultures had initial cell densities of 372 ± 37 cells mL^{-1} and a nine-day exponential phase with a mean growth rate of 0.22 day^{-1}. At the end of the exponential phase cell yield was $2.63 \times 10^3 \pm 9.55 \times 10^1$ cells mL^{-1}, and at mid and late stationary phase an increase in mucilaginous cell aggregates was evident in *O.* cf. *ovata* cultures.

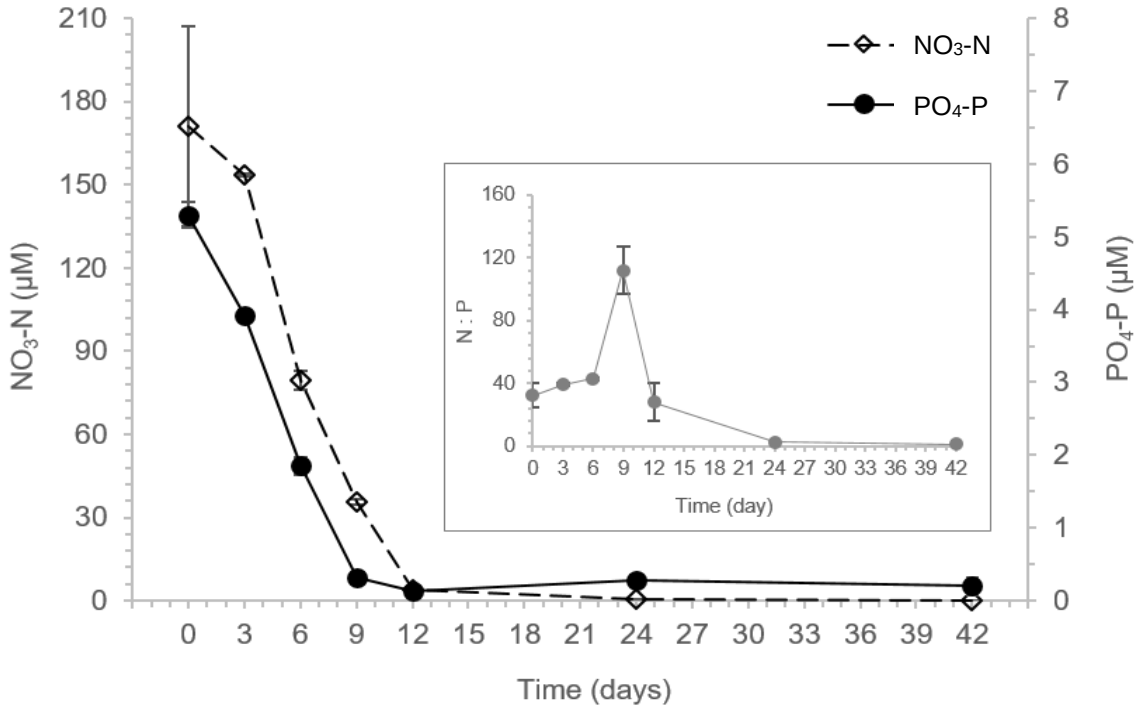

Fig. 1. Nitrogen (NO_3-N) and phosphorus (PO_4-P) concentrations measured in *O.* cf. *ovata* culture medium and corresponding N:P ratios (inset). Bars indicate standard deviation.

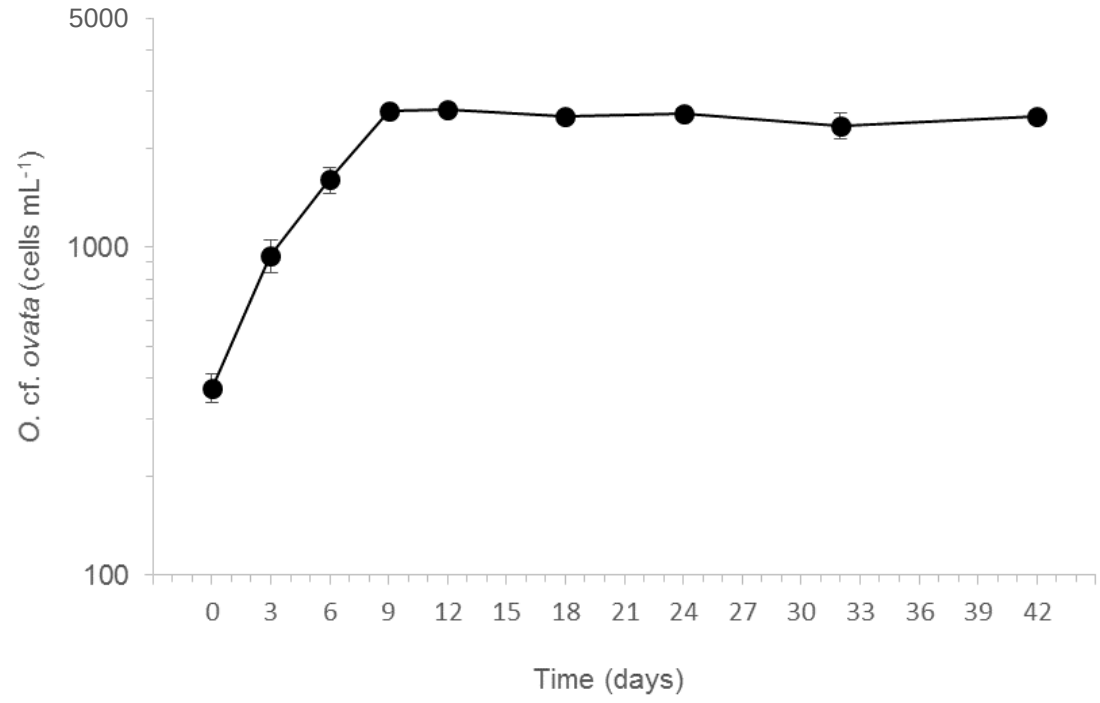

Fig. 2. Growth pattern of *O.* cf. *ovata* cells. Bars indicate standard deviation.

3.2 Bacterial abundance and physiological state

During the experiment, bacterial densities rise up of more than one order of magnitude (range: 7.24 x 10^5 to 2.01 x 10^7 cells mL^{-1}, day 0 and 24, respectively; mean value: 9.41 x 10^6 ± 6.77 x 10^6 cells mL^{-1}), consistently with the range of values previously reported for *O.* cf. *ovata* batch cultures (Vanucci et al., 2012b; Pezzolesi et al., in press). Bacterial community growth was characterized by a biphasic pattern, with a first exponential phase (first growth step) co-occurring with the algal exponential growth phase (days 0-9), and a second exponential phase (second growth step) coupled with the algal mid-stationary phase (days 12-24) and characterized by a lower growth rate than the first one (μ = 0.24 and 0.10 day^{-1}, days 0-9 and 12-24, respectively; ANOVA, $p < 0.01$) (Fig. 3). The first growth step suggests utilization of the inorganic nutrients available in the culture medium not only by *O.* cf. *ovata*, but also by bacteria (Bradley et al., 2010; Sebastian et al., 2012; Pepe-Ranney and Hall, 2015), along with low-molecular weight (LMW) photosynthetic products (e.g. organic acids, amino acids, peptides, oligosaccharides; Wagner-Dobler et al., 2010; Buchan et al., 2014). Differently, the second growth step suggests thriving of bacteria able to grow in a wide availability of LMW and high-molecular weight (HMW, e.g. polysaccharides, proteins, nucleic acids, lipids; Thornton et al., 2014; Buchan et al., 2014) algal-derived organic matter together with a low concentration of inorganic nutrients in the culture medium.

Abundance of highly respiring bacteria (CTC^+) showed a similar pattern as total bacterial abundance (data not shown), representing on average 45 ± 15% of the latter (corresponding to 3.75 x 10^6 ± 2.46 x 10^6 cells mL^{-1} , Fig. 3). Particularly, the highest relative abundances were found synoptically with the first and the beginning of the second bacterial exponential step (62.8 and 59.9%, day 6 and 12, respectively), in accordance with the recognized correlation between CTC-based estimates and bacterial production (Sherr et al., 1999; Paoli et al., 2006). Whereas, a significant drop in CTC^+ contribution, likely related to viral lytic activity (see forward), was recorded at day 9 (ANOVA, $p < 0.05$). A decreasing trend was observed during the algal stationary phase (days 12-42), with another drop at day 24 (ANOVA, $p < 0.01$), suggesting changes in bacterial taxa contribution within the community (see forward). The lowest CTC^+ percentage (13.1%) was recorded at the end of the experiment (day 42), indicating the presence of senescent or died cells. The high contributions of active bacteria reported here are rarely detectable in natural environments, where high metabolic bacterial cells are mainly grazed (Jürgens and Massana, 2008) besides being targeted by virus in order to ensure a successful propagation of their progeny (Del Giorgio and Gasol, 2008). Given the

premise that comparison between laboratory and natural events is not always straightforward, the pattern of CTC^+ found in *O.* cf. *ovata* cultures resembles the thymidine incorporation trend observed during a bloom of the toxic planktonic dinoflagellate *Karenia brevis*, where increasing and decreasing uptake rates were reported during bloom initiation and maintenance, respectively (Meyer et al., 2014).

3.3. Viral abundance

Abundance of virus like particles (VLPs) ranged within 1.29×10^7 and 5.50×10^7 VLPs mL^{-1} (day 0 and 12, respectively), showing a four-fold higher mean value ($3.86 \times 10^7 \pm 1.33 \times 10^7$ VLPs mL^{-1}) than average bacterial abundance. While during the first bacterial growth step (days 0-9) viral abundance exhibited a similar increasing pattern, during the second step viral densities were slightly decreasing, almost stationary (Fig. 3). This trend suggests a more tight relationship between viral abundance and bacterial growth rate rather than bacterial abundance, as previously proposed for several marine environments (e.g. Danovaro et al., 2008; Breitbart et al., 2012 and references therein). The second step of bacterial growth was likely characterized by higher abundance of slow-growing bacteria and/or prevalence of lysogeny (Lymer and Lindström, 2010; Meyer et al., 2014; Montanié et al., 2015) and/or partially absence of species-specific phages (Breitbart et al., 2008, Montanié et al., 2015 and references therein); indeed, differences in contribution of the dominant taxa were observed with respect to the first bacterial growth step (see forward). Accordingly, mean virus to bacteria ratios (VBR, Fig. S1) of 11 during the first bacterial growth step (days 0-9, concomitantly with *O.* cf. *ovata* exponential phase) and of 2.7 during the algal mid and late stationary phases (days 18-42), suggest a more relevant top-down control on the bacterial host populations responsible for the first step (Jacquet et al., 2005). This is also supported by the drop in CTC^+ contribution recorded at day 9, followed by steady-state conditions (Thingstad, 2000; Needham et al., 2013), often detected in cultures (Breitbart et al., 2008, 2012 and references therein). Besides, the low VBR observed at mid and late stationary phase may be partially due to high rates of viral decay due to bacterial enzymatic activities occurring in the mucilage network (see forward), as postulated for diverse marine habitats (Danovaro et al., 2011 and references therein; Meyer et al., 2014; Dell'Anno et al., 2015).

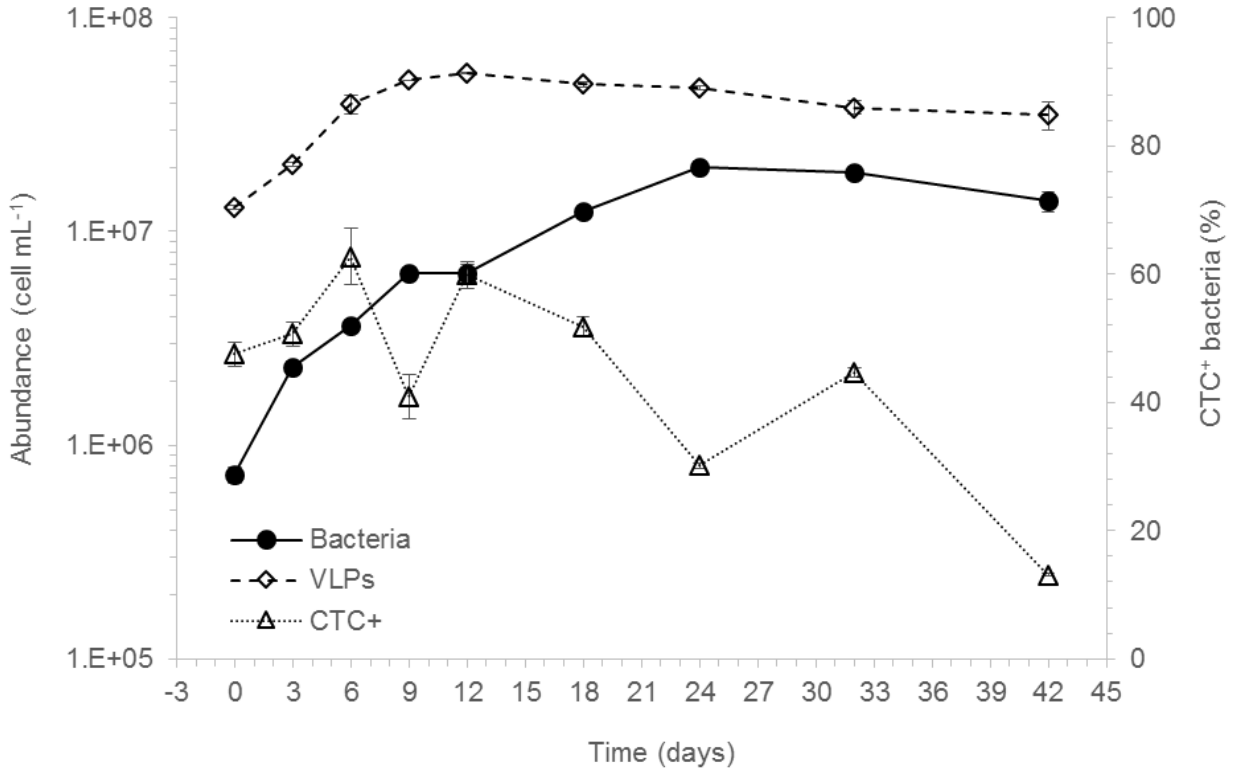

Fig. 3. Bacterial abundance, viral abundance (VLPs) and percentage contribution of highly respiring bacteria (CTC^+). Bars indicate standard deviation.

3.4. Toxins

The LC–HRMS analysis of algal and culture medium extracts showed the presence of the main algal toxins produced by *O.* cf. *ovata* (Fig. 4). The most abundant toxin was ovatoxin-a (55-56%), followed by ovatoxin-b (28-30%), d + e (11-12%), c (3-4%), and isobaric palytoxin (i.e. putative palytoxin, 0-1%), accordingly to the most common toxin profile observed for the dinoflagellate (Ciminiello and Penna, 2014). The relative percentages of the single toxins remained constant throughout the algal growth phases (ANOVA, $p > 0.05$ for all comparisons, Fig. 4a), whereas intracellular toxins on a per cell basis increased progressively over the growth phases (ANOVA, $p < 0.05$ for all comparisons, Fig. 4b) ranging from 7.4 ± 0.2 to 18.0 ± 0.7 pg $cell^{-1}$. Extracellular toxins showed the same qualitative profile (data not shown) and quantitative temporal trend described for intracellular ones, accounting for 0.9, 1.4 and 10.8% of the total toxins synthesized by *O.* cf. *ovata* at days 6, 24 and 42, respectively. Toxin temporal dynamics were generally consistent with those previously reported for non-axenic *O.* cf. *ovata* cultures (Vanucci et al., 2012a,b; Pezzolesi et al., 2014). However, in this study a lower relative extracellular release was observed in comparison with previous works (Pezzolesi et al., 2012; Vanucci et al., 2012b). These variations could be due

to physiological and genetic characteristics typical of the strains used in the investigations that have been isolated from different coastal regions of the Adriatic Sea or during different bloom events (Guerrini et al., 2010). Moreover, the two-fold higher bacterial densities coupled with a 20% lower algal cell yield observed here compared to Vanucci et al. (2012b) data may have accounted for a higher removal of *O.* cf. *ovata* extracellular toxins. In fact, despite being carbon-rich HMW refractory molecules (Pinna et al., 2015), *O.* cf. *ovata* toxins are still degraded by bacteria (Vanucci et al., 2012b), as observed for other algal toxins (Stewart, 2008; Shetty et al., 2010; Weissbach et al., 2012; Sison-Mangus et al., 2014).

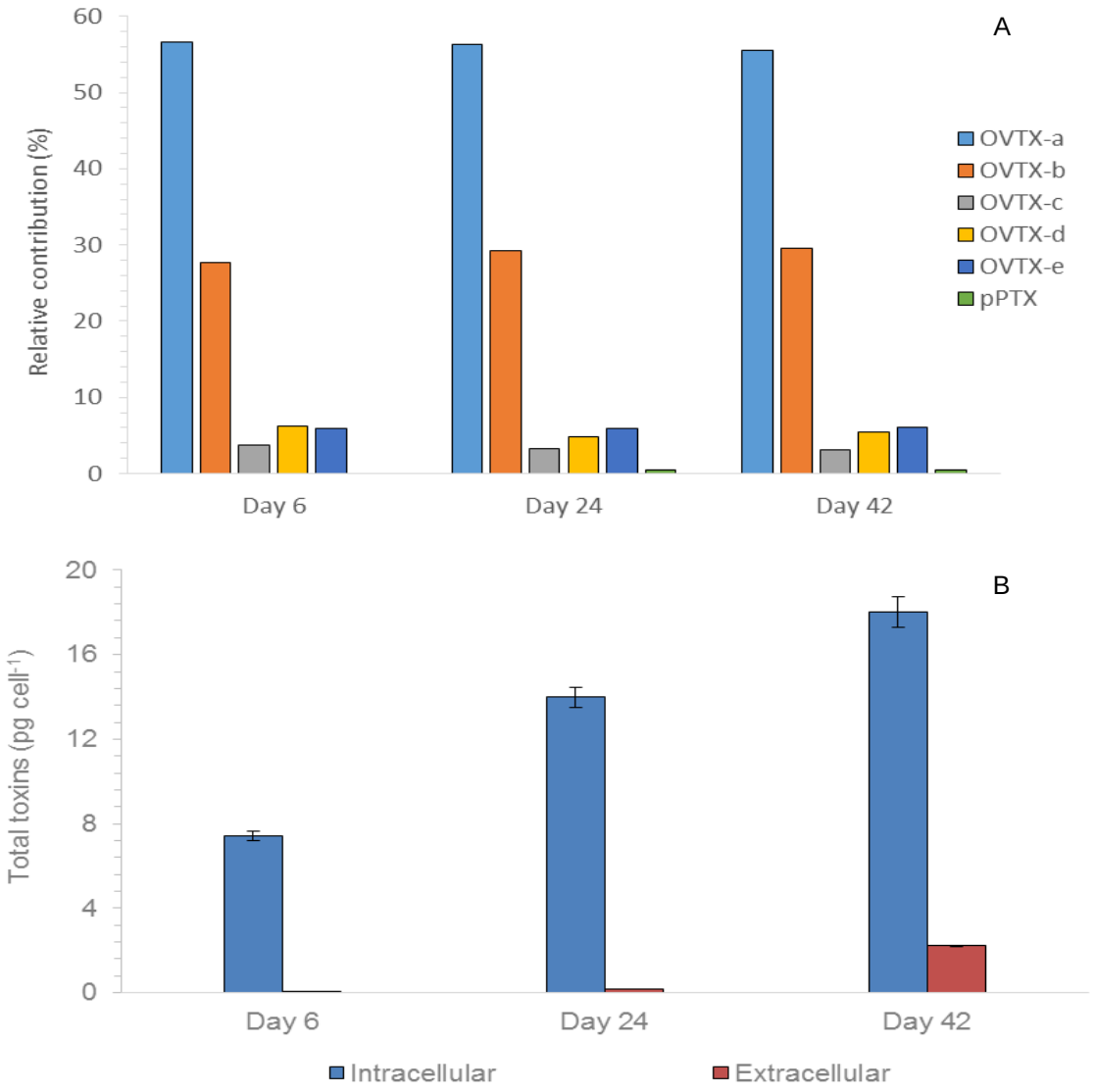

Fig. 4. Relative concentration (%) of the single toxins (A) and total intracellular and extracellular toxin content of *O.* cf. *ovata* cells expressed on a cell basis (B) during the different algal growth phases (day 6, 24 and 42). Bars indicate standard deviation.

3.5. Bacterial community diversity

Overall, 247426 high quality reads spanning the hypervariable regions V1-V2 of the 16S rDNA were obtained (average length = 290bp), yielding a total of 4441 different OTUs after normalization on the smallest sample size (19662 reads), singletons removal and chloroplast and mitochondrial sequences discharge. Rarefaction curves (Fig. S2) as well as coverage values (Table 1) revealed that most of the bacterial diversity was recovered by sequencing analysis. Diversity indexes of the bacterial community co-occurring with *O.* cf. *ovata* in batch cultures varied in a restricted range of values along the different growth phases (Table 1). The highest OTU richness (3031) was recorded during *O.* cf. *ovata* mid-stationary phase (i.e. at the end of the second bacterial growth step, day 24), implying the presence of other rare OTUs that were not detected in the analysis of the bacterial source community (day 0), as initially masked by more dominant phylotypes. This high bacterial diversity might have partially reduced virus-sensitive host-encounter rate (Filippini et al., 2006; Montanié et al., 2015), contributing to the lower VBRs observed at the stationary compared to the algal exponential growth phase (see before). As it was expected, ION torrent 16S rDNA sequencing revealed a higher diversity than previous traditional molecular methods used for assessing bacterial communities associated with cultured dinoflagellates (e.g. *Ostreopsis lenticularis*, Ashton et al., 2003; Pérez-Guzmán et al. 2008; *Alexandrium* spp., Sala et al., 2005; *O. ovata* and *Coolia monotis*, Ruh et al., 2009; *Gymnodinium catenatum*, Green et al., 2010; *Pyrodinium bahamense*, Onda et al., 2015), while being comparable with the non-dinoflagellate pyrosequencing data available to date (Sison-Mangus et al., 2014; Bagatini et al., 2014). In this study, the highest bacterial diversity was detected at mid and late stationary phase of the *O.* cf. *ovata* growth (i.e. day 24 and 42), as found in concomitance with the maintenance/decline phase of dinoflagellate natural blooms (*Noctiluca miliaris*, Basu et al. 2013; *O.* cf. *ovata*, Vanucci et al., in press-a), likely as a consequence of the increased array of specialized niches provided by the enhanced pool of available organic matter (Teeling et al., 2012).

The UPGMA dendrogram of Bray-Curtis pairwise distances between samples (Fig. 5) showed that samples collected at earlier (i.e. day 0 and 6) and later (i.e. day 24 and 42) algal growth phases formed two different clusters although differences were not significant (ANOSIM, $p > 0.05$), nevertheless suggesting a shift in bacterial OTU composition during algal growth proceeding, consistently with previous observations (Grossart et al., 2005; Bagatini et al., 2014).

Table 1. Bacterial diversity parameters during *O.* cf. *ovata* growth. Summary of total sequences after normalization (Reads), number of bacterial operational taxonomic units detected at 97% identity (OTUs), Shannon diversity and Good's sample coverage obtained from Ion torrent sequencing at the time of the inoculum (Day 0), and during different algal growth phases (Day 6, 24 and 42) in batch cultures.

Sample	Reads	OTUs	H' Shannon	Good's coverage
Day 0	19662	2855	4.53	0.91
Day 6	19662	2624	4.26	0.91
Day 24	19662	3031	4.62	0.89
Day 42	19662	2906	4.54	0.90

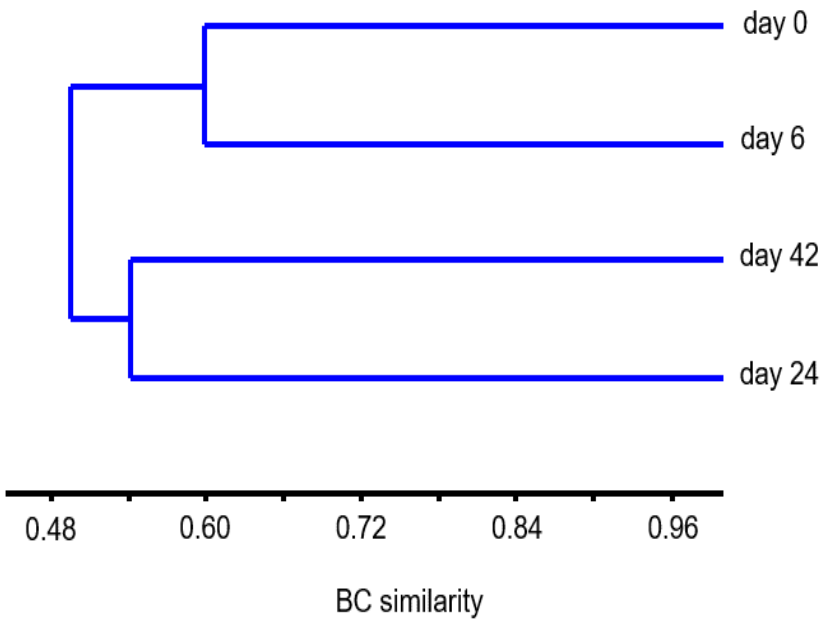

Fig. 5. UPGMA cluster of bacterial community 's structure using Bray-Curtis distances. Samples were collected at the time of the inoculum (day 0) and during different algal growth phases (day 6, 24 and 42) . The dendrogram was constructed on the basis of square root transformed bacterial OTU abundances retrieved from ION torrent sequencing data. Total similarity between samples is indicated by 1, and total dissimilarity is indicated by 0.

3.6. Bacterial community composition

Bacterial OTUs retrieved by ION torrent analysis along the different *O.* cf. *ovata* growth phases were affiliated with 11 different bacterial phyla, 16 classes and 127 genera (supplementary Table S1, S2 and S3). *Alphaproteobacteria* (range: 64.7 to 95.7%, day 24 and 6, respectively), *Sphingobacteria* (2.4-33.6%, day 6 and 24) and *Actinobacteria* (0.1-2.2%, day 42) dominated the community, together accounting for more than 96% in relative contribution in all samples (Fig. 6). *Alphaproteobacteria* were the most abundant class at all algal growth phases ($p < 0.05$; ANOVA), being mostly represented by the genus *Oceanicaulis* (fam. *Hyphomonadaceae*; range: 14.3 to 39.7%, day 24 and 0, respectively) and the three *Rhodobacteraceae*-affiliated genera *Dinoroseobacter* (21.7-34.6%, day 0 and 6), *Labrenzia* (5.7-24.9%, day 0 and 24) and *Roseovarius* (4.8-15.9%, day 42 and 6). *Sphingobacteria* and *Actinobacteria* were mainly represented by members belonging to the genera *Fabibacter* (fam. *Flammeovirgaceae*; 0.6-21.9%, day 6 and 24) and *Dietzia* (0.03-1.4%, day 42), respectively (Fig. 7). Changes of the main bacterial taxa during *O.* cf. *ovata* growth mostly relied upon the contribution of the sixteen abundant OTUs (i.e. ≥1% of the total reads in at least one of the samples; Table 2 and 3), together accounting for 64-70% of the total community in all samples. While at the time of the inoculum (day 0), OTUs #2, #9, #13, #18 and #20 affiliated to *Oceanicaulis* showed the highest contribution (15.4% for OTU #2, 1.7-4.5% for the others), during the algal exponential growth phase (i.e. first bacterial growth step) the overall most abundant *Dinoroseobacter*-related OTU #1 represented a quarter of the total community showing the highest percentages together with *Roseovarius* OTUs #7, #11 (5.0 and 9.4%, respectively) and OTU #9 (6.5%). At the *O.* cf. *ovata* mid-stationary phase (i.e. end of the second bacterial growth step, day 24), *Fabibacter*- and *Labrenzia*-related OTUs #6 and #4 (17.8 and 13.7%, respectively) together with OTU #1 (15.5%) made up around half of the total bacteria. Lastly, at the *O.* cf. *ovata* late stationary phase (day 42) *Dinoroseobacter* and *Oceanicaulis* restored the dominance observed at the exponential phase (day 6), while OTU #4 still contributed for ~14% to the total community (Table 2).

The dominance of *Alphaproteobacteria* (mostly *Roseobacters*), *Flavobacteria-Sphingobacteria* and *Actinobacteria* and the successional pattern of these groups observed in this study is consistent with the general bacterial composition feature and trend reported during phytoplankton blooms (Rooney-Varga et al., 2005; Garces et al., 2007; Jones et al., 2010; Mayali et al., 2011; Vanucci et al., in press-a) and also in mesocosms/batch cultures

(Riemann et al., 2000; Schafer et al., 2002; Grossart et al., 2005; Tada et al., 2012; Bagatini et al., 2014).

At a lower taxonomic level, composition and succession of bacteria co-growing with *O.* cf. *ovata* appeared to be determined by: i) the establishment of mutualistic associations between bacteria and healthy algal cells or saprophytic/algicidal interactions between bacteria and senescent cells; ii) changes of inorganic and organic resources available during the diverse algal growth phases with an overall bottom-up effect, affecting the succeeding bacterial populations to different extents; iii) the engagement of bacteria-bacteria enhancing and/or antagonistic interactions; iv) a viral top-down control and the ability of bacterial taxa to resist viral lysis through complex defense systems.

The bacterial successional trend suggests a more intense top-down regulation on the genus *Oceanicaulis,* although the lack of viral genetic identification did not allow verifying such hypothesis. The dominance of the genera *Oceanicaulis* e *Dinoroseobacter* at distinct algal growth phases deeply differing in terms of inorganic nutrient concentrations and organic matter characteristics and availability, is likely abscribable to their high metabolic plasticity.

Oceanicaulis representatives were firstly isolated from *Alexandrium tamarense* non-toxic cultures (*Oceanicaulis alexandrii,* Strömpl et al., 2003). An ubiquitous and consistent presence of this genus was also recently observed in marine algal cultures (*Emiliania huxleyi*, Zabeti et al., 2010; *Eutreptiella* sp., Kuo and Lin, 2013; *Ostreococcus tauri*, Abby et al., 2014), and it was suggested to complement the host for vitamins and additional growth-promoting factors (Helliwell et al., 2011; Kuo and Lin, 2013; Abby et al., 2014), as supported by detection of genes and regulons involved in biosynthesis pathways of cobalamin (vitamin B_{12}), thiamine (vitamin B_1) and biotin (vitamin B_7) in *Oceanicaulis* phylotypes (Oh et al., 2011), also retrieved in *Sphyngopyxis* relatives (Lauro et al., 2009; Oelschlägel et al., 2015). The high contributions of *Oceanicaulis* at both *O.* cf. *ovata* exponential and late stationary phase were likely due to a versatile chemoheterotrophic metabolism (Strömpl et al., 2003; Oh et al., 2011; Chen et al., 2012), together with an efficient phosphate uptake in carbon-limited medium and inorganic nutrient depleted conditions through high-affinity phosphate transporters located in the prosthecae (McAdams et al., 2006; Oh et al., 2011).

Similarly to *Oceanicaulis, Dinoroseobacter*-affiliated members were firstly retrieved in association with cultured toxic planktonic and benthic dinoflagellates (*Alexandrium ostenfeldii*, *Procentrum lima*, Biebl et al., 2005a; *Protoceratium reticulatum*, Wagner-Döbler et al., 2010) and other non-toxic algal species (reported in Wagner- Döbler et al., 2010). The abundance temporal trend of this genus along the *O.* cf. *ovata* growth is likely linked to a

mutualistic/antagonistic bimodal behavior in relation to changes in the algal physiological status over the different growth phases. In fact, it has been recently stated for *D. shibae* in co-culture with toxic dinoflagellates the ability to switch from a mutualistic phase, where the bacterium synthesize vitamin B_1, B_{12} and antibacterial compounds in exchange for algal-released dimethylsulfoniopropionate (DMSP) and other photosynthetic metabolites, to an opportunistic/pathogenic phase in response to algal senescence molecules (Wagner- Döbler et al., 2010; Wang et al., 2014, 2015), as previously suggested also for other *Roseobacters* (Seyedsayamdost et al., 2011a; Sule and Belas, 2013). While DMSP production has been assessed for *O.* cf. *ovata* (Vanucci et al., in press-b), its vitamin requirements are not known; however, consistently with all tested dinoflagellates to date (Tang et al., 2010), auxotrophy for some essential vitamins could be hypothesized. The amendment of exogenous vitamins (B_{12}, B_1, and B_7) to the media used for cultures onset likely only marginally affect bacterial-algal associations in cultures (Abby et al., 2014), which are the outcome of consolidated functional relationships coevolved in different environments (Ramanan et al., 2016). Moreover, a synergistic effect of algal-associated vitamin-producing bacteria and exogenous vitamins in enhancing algal growth has been recently pointed out (Kuo and Lin, 2013).

Ability of *Dinoroseobacter* as well as *Roseovarius* and *Labrenzia* to perform aerobic anoxygenic photosynthesis (Biebl et al., 2005a,b; Biebl et al., 2007) might have been helpful in outcompeting strictly chemoheterotrophs and becoming a highly active part of the microbial community when growing in a light-dark regime under carbon-limited condition (Wang et al., 2014, 2015; Koblížek et al., 2015; Soora et al., 2015). Moreover, *Dinoroseobacter* as well as *Oceanicaulis* and *Roseovarius* are able to grow anaerobically (Oh et al., 2011; Wagner-Dobler et al., 2010; Laass et al., 2014; Riedel et al., 2015), retaining a more active energy metabolism than strictly aerobic bacteria in *O.* cf. *ovata* mucilaginous aggregates more abundant at mid and late stationary phase, in which occurring of anoxic/suboxic niches was suggested by detection of ammonia (~3.0 µM at both days 24 and 42, data not showed). In addition, *Dinoroseobacter*'s high viral resistance (Wagner-Döbler et al., 2010) may have prevent viral top-down regulation on this genus compared with other taxa (e.g. *Oceanicaulis*). *Roseovarius*-affiliated phylotypes were retrieved from cultures of different marine algal species (Biebl et al., 2005b; Onda et al., 2015), also in co-occurrence with *Oceanicaulis* (Kuo and Lin, 2013; Abby et al., 2014) and *Fabibacter* relatives (Green et al., 2015) and during toxic dinoflagellate blooms (Yang et al., 2015; Vanucci et al., in press-a). Metagenomic and biochemical analysis highlighted the large metabolic portfolio of *Roseovarius* (e.g. Bruns et al., 2013; Riedel et al., 2015), including synthesis of dual nature

compounds (i.e. algal growth-promoting and algicidal ones; Ziesche et al., 2015). While *Roseovarius* and *Labrenzia* have been shown to require both vitamin B_1 and B_7 for the growth (Biebl et al., 2005b, 2007), *Dinoroseobacter* has been found to be auxotrophic only for vitamin B_7 (Biebl et al., 2005a). Therefore, the main bacterial taxa forming the alphaproteobacterial consortium in *O.* cf. *ovata* cultures appear synergistically suit the nutritional needs of the algal partner and functionally complement the auxotrophic bacteria for vitamins, an hypothesis that is worthy of further investigation.

According to chemoorganotrophic *Flammeovirgaceae*'s general metabolic and ecological features (Nedashkovskaya et al., 2011; Kim et al., 2013; Mohit et al., 2014), the high contribution of *Fabibacter* at *O.* cf. *ovata* mid-stationary phase indicated occurrence of HMW biopolymers less susceptible to *Roseobacters* attack (reviewed in Buchan et al., 2014) such as complex polysaccharides, phytodetritus and also carbon-rich macromolecules like *O.* cf. *ovata* toxins (see before). *Fabibacter*-relatives were retrieved in association with coral mucus (de Castro et al., 2010), and their metabolic features, in particular slow-growth and high exo-enzimatic activity, would presumably contribute to explain both the drop in CTC^+ bacterial fraction and the low VBRs recorded during the algal mid-stationary phase (see before).

Bacteroidetes-mediated conversion of HMW algal molecules into LMW compounds (Fernández-Gómez et al., 2013; Buchan et al., 2014) likely explain the high contribution of genera such as *Dinoroseobacter* and *Labrenzia* at *O.* cf. *ovata* mid and late stationary phase, as *Roseobacters* are more specialized in LMW organics uptake and may function synergistically with *Bacteroidetes* to remineralize algal-derived matter larger components (Teeling et al., 2012; Taylor et al., 2014). Moreover, *Labrenzia alexandrii* (OTU-4, 100% similarity; Biebl et al., 2007) warrants further investigation since killing-host activity also by R-bodies has been hypothesized for this species (Fiebig et al., 2013). Inhibitory/algicidal activity has been also strongly suggested for *Dietzia*-affiliated members (Kim et al., 2008; Le Chevanton et al., 2013). Relatives of this genus became abundant in the microbiota at the late stationary *O.* cf. *ovata* growth phase (OTU-16), consistently with the pattern generally observed for *Actinobacteria* in microbial succession both in monoclonal cultures and *in situ* algal blooms, where members of this class are predicted to decompose recalcitrant and poorly accessible substrates (Basu et al., 2013; Bagatini et al., 2014; Sakami et al., 2016; Vanucci et al., in press-a).

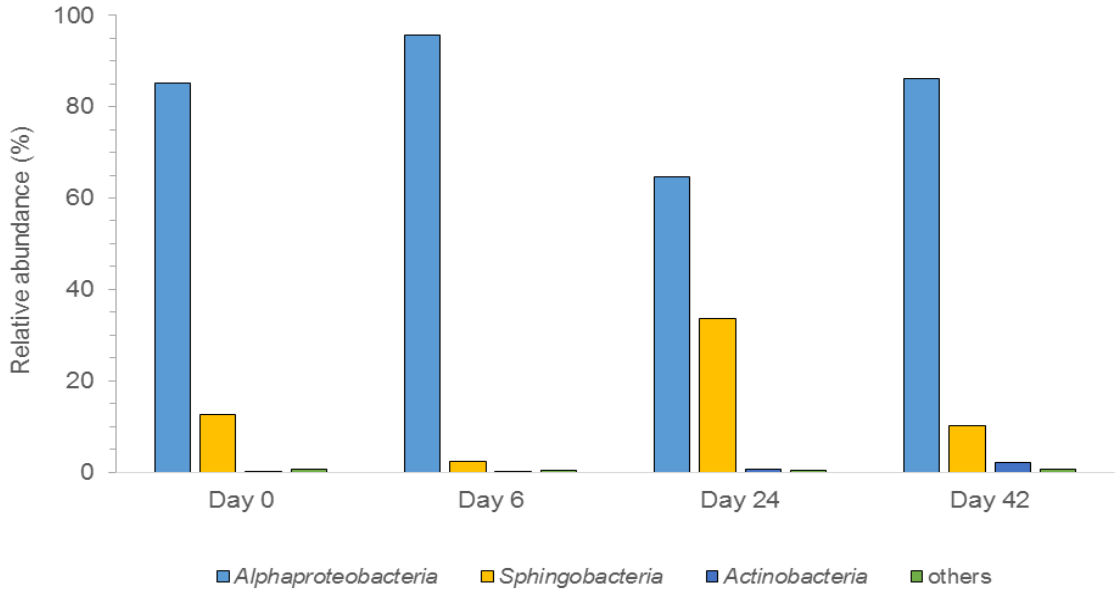

Fig. 6. Percent distribution of the dominant classes (≥1% in at least one of the samples) detected in *O.* cf. *ovata* batch cultures at the time of the inoculum (day 0), and during the different algal growth phases (day 6, 24 and 42), as revealed from ION torrent data. 'others' represent the classes with less than 1% of relative abundance individually.

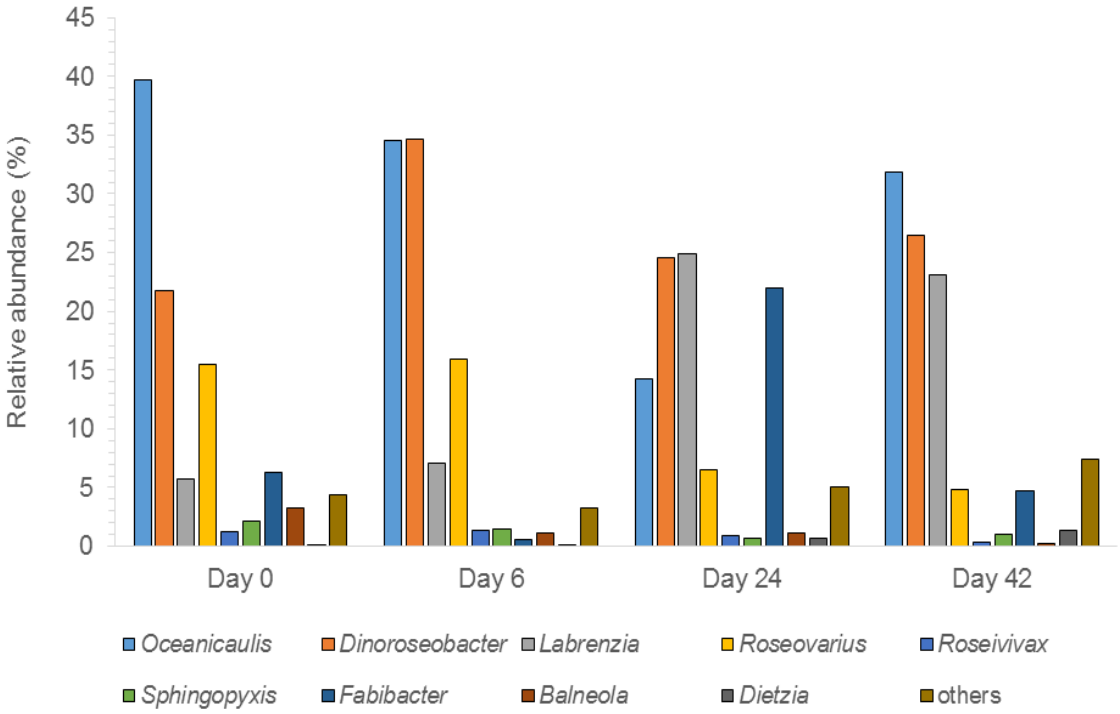

Fig. 7. Relative contribution of the major bacterial genera (≥1% in at least one of the samples) retrieved in O. cf. ovata batch cultures at the time of the inoculum (day 0) and during the different algal growth phases (day 6, 24 and 42), as revealed from ION torrent data. 'others' represent the genera with less than 1% of relative abundance individually.

Table 2. Relative contribution (%) of the most abundant OTUs (≥1% in at least one of the samples) retrieved at the time of the inoculum (day 0) and during the different algal growth phases (day 6, 24 and 42), as revealed from ION torrent data.

OTU	Closest relative RDP classifier	day 0	day 6	day 24	day 42
1	*Dinoroseobacter*	15.1	24.6	15.5	18.7
2	*Oceanicaulis*	15.4	12.1	3.5	11.5
4	*Labrenzia*	3.6	4.3	13.7	13.9
6	*Fabibacter*	5.7	0.5	17.8	4.0
7	*Roseovarius*	9.2	9.4	3.3	3.0
9	*Oceanicaulis*	4.5	6.5	2.8	5.4
11	*Roseovarius*	4.4	5.0	1.9	1.4
13	*Oceanicaulis*	2.6	1.8	0.8	1.8
16	*Dietzia*	0.0	0.0	0.6	1.3
18	*Oceanicaulis*	1.9	1.8	0.6	1.5
20	*Oceanicaulis*	1.7	1.2	0.4	1.2
21	*Flammeovirgaceae*	0.1	0.0	1.3	1.2
33	*Rhodobacteraceae*	1.1	1.3	0.7	0.3
35	*Balneola*	1.7	0.6	0.6	0.1
58	*Rhodobacteraceae*	0.6	0.5	0.2	0.2
67	*Sphingopyxis*	0.6	0.4	0.1	0.2

Table 3. Closest matches from the NCBI GenBank database based on sequence similarity of the 16 abundant OTUs (≥1% of the total reads in at least one of the samples) as revealed by Ion torrent sequencing.

OTU	Closest matched sequence (% similarity)	NCBI accession number	Closest cultured neighbor (% similarity)	NCBI accession number
1	*Dinoroseobacter shibae* (99)	NR074166.1	*Dinoroseobacter shibae* (99)	NR074166.1
2, 9, 13, 18, 20	Uncultured bacterium (99)	JQ337901.1	*Oceanicaulis alexandrii* (96)	NR025456.1
4	*Labrenzia alexandrii* (100)	NR042201.1	*Labrenzia alexandrii* (100)	NR042201.1
6	Uncultured bacterium (99)	JX016873.1	*Fabibacter pacificus* (92)	NR109732.1
7, 11	*Roseovarius* sp. (100)	AB114422.1	*Roseovarius tolerans* (99)	NR026405.1
16	Uncultured bacterium (98)	FJ594833.1	*Dietzia cinnamea* (81)	NR116686.1
21	*Reichenbachiella* sp. (94)	JX854345.1	*Reichenbachiella faecimaris* (93)	NR117445.1
33	*Thalassococcus lentus* (100)	NR109663.1	*Thalassococcus lentus* (100)	NR109663.1
35	Uncultured *Balneola* sp. (98)	JX529426.1	*Balneola alkaliphila* (95)	NR044367.1
58	*Loktanella* sp. (98)	AB917988.1	*Poseidonocella sedimentorum* (98)	NR113210.1
67	Uncultured organism (100)	DQ395425.1	*Sphingopyxis flavimaris* (97)	NR025814.1

3.7. Community composition comparisons between cultures and Ostreopsis cf. ovata bloom

The comparison between structure of the bacterial community in batch cultures with that one co-occurring with *O. cf. ovata* bloom, both investigated through high-throughput molecular approaches, revealed that bacterial richness in cultures was consistent with the values documented *in situ* (1621-2214; Vanucci et al., in press-a). Whereas, Shannon diversity was generally lower than that one observed during the bloom (4.26-4.62, this study *vs* 5.28-6.36, *O.* cf. *ovata* bloom), indicating a prominent dominance of few OTUs, as previously found in relation to several cultured algal strains (Schafer et al., 2002; Yang et al., 2013; Bagatini et al., 2014; Sison-Mangus et al., 2014). The limited number of abundant OTUs detected in *O.* cf. *ovata* cultures with respect to that one recorded during *O.* cf. *ovata* bloom suggests that culture conditions exerted a selective pressure on the bacterial communities, considering bacterial specific survivability in coltures (Jasti et al., 2005; Green et al., 2004, 2010) and also bacteria-bacteria mutualistic/antagonistic interactions (reviewed in Cooper and Smith, 2015; Ramanan et al., 2016). Nevertheless, the relative abundance and temporal trend of the major bacterial groups found in *O.* cf. *ovata* cultures resembled those reported during the bloom (Vanucci et al., in press-a). In fact, *Alphaproteobacteria* accounted for ~90% of the community at exponential growth phase in culture and during bloom development phase, and for ~65% at culture's mid-stationary/bloom maintenance phase, with an ubiquitous presence of *Roseobacters* (40-60% of the total bacteria). Whereas, *Flavobacteria-Sphingobacteria* and *Actinobacteria* showed the highest contributions (~20-30% and ~2-3%, respectively) at culture's stationary/ bloom maintenance phases. Interestingly, in both conditions an *Alphaproteobacteria* consortium composed by some phylogenetically closely related taxa was retrieved: the *Rhodobacteraceae*-affiliated *Dinoroseobacter* and *Roseovarius* together with *Oceanicaulis* composed the consortium in cultures, while the *Rhodobacteraceae Ruegeria, Jannaschia, Roseovarius* along with *Erythrobacter* dominated during the *O.* cf. *ovata* bloom. The members forming the two consortia altogether share comparable metabolic traits, including self-sustenance for vitamins B_1, B_7, and B_{12} production and the ability to synthesize both algal-promoting antibacterial metabolites and algicidal compounds (reviewed in Newton et al., 2010; Pujalte et al., 2014), suggesting to some extent similar functionality, and also redundancy in bacterial communities co-occurring with microalgae, similarly to macroalgae-associated bacteria (Burke et al., 2011; Dittami et al., 2016). These findings suggest that while culture conditions partially modify bacterial phylogenetic structure, the algal-bacterial functional association pattern is somewhat maintained, indicating laboratory cultures as a

valuable system for future investigations on algal-bacterial relationships. This consideration, while inconsistent with few assertions (e.g. Garcés et al., 2007), is in agreement with several previous statements underlying culture studies as a reliable and essential path to elucidate the functional significance of microalgal-associated bacteria (e.g. Jasti et al., 2005; Ruh et al., 2009; Kuo and Lin, 2013; Buchan et al., 2014; Sison-Mangus et al., 2014; Cooper and Smith, 2015).

4. Conclusions

Data in the present study showed that *Alphaproteobacteria* (mainly *Roseobacters*), followed by *Sphingobacteria* and *Actinobacteria*, mainly composed the *Ostreopsis* cf. *ovata* microbiota in batch cultures. Bacterial composition and the successional trend exhibited the dominance of vitamin- and antibacterial-synthesizing taxa (i.e. *Oceanicaulis*, *Dinoroseobacter*, *Roseovarius*, *Sphyngopyxis*) promoting *O.* cf. *ovata* growth during exponential phase, followed by abundant contributions of saprophytic/algicidal phylotypes as *Labrenzia* and *Dietzia* at later phases of the algal growth, encompassing taxa with an assessed bimodal activity (e.g. *Dinoroseobacter*). Moreover, the algal-bacterial functional association pattern in this study indicate laboratory cultures as a valuable system for future investigations on algal-bacterial relationships.

So far, the vast majority of studies on HABs have focused on dynamics of macronutrients as nitrogen and phosphate, owing to the recurrent linkage between eutrophication and toxic microalgae outbreaks. Whereas, the importance of coenzymes and specific vitamins has been rarely investigated although strongly suggested, specifically for blooms of toxic dinoflagellates, which are well known to be strikingly auxotrophic for vitamins. Data reported here pave the way for further investigations on the role of allochthonous vitamins in *O.* cf. *ovata* bloom development and maintenance, that would promote both algal growth directly but also likely thriving of the host-beneficial bacteria which biosynthesize vitamins at an high metabolic cost.

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Supplementary material

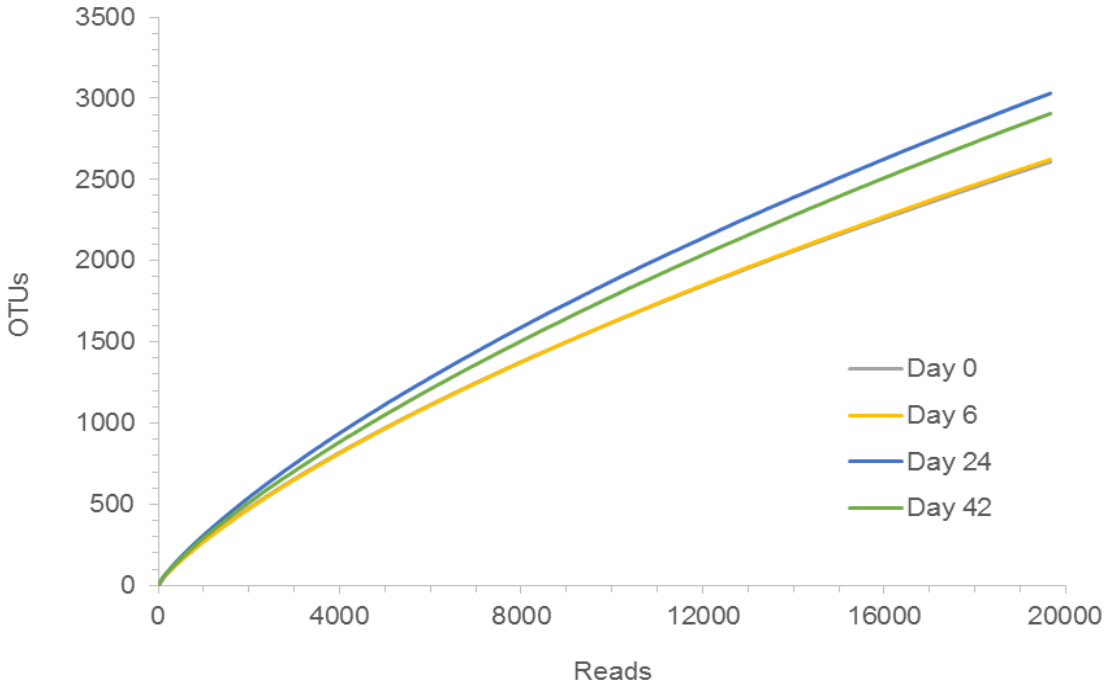

Fig. S1. Diversity of the bacterial community associated with *O.* cf. *ovata* at the time of the inoculum (Day 0), and during the different algal growth phases (Day 6, 24 and 42). Rarefaction curves were computed on bacterial OTUs at a dissimilarity level of 3%.

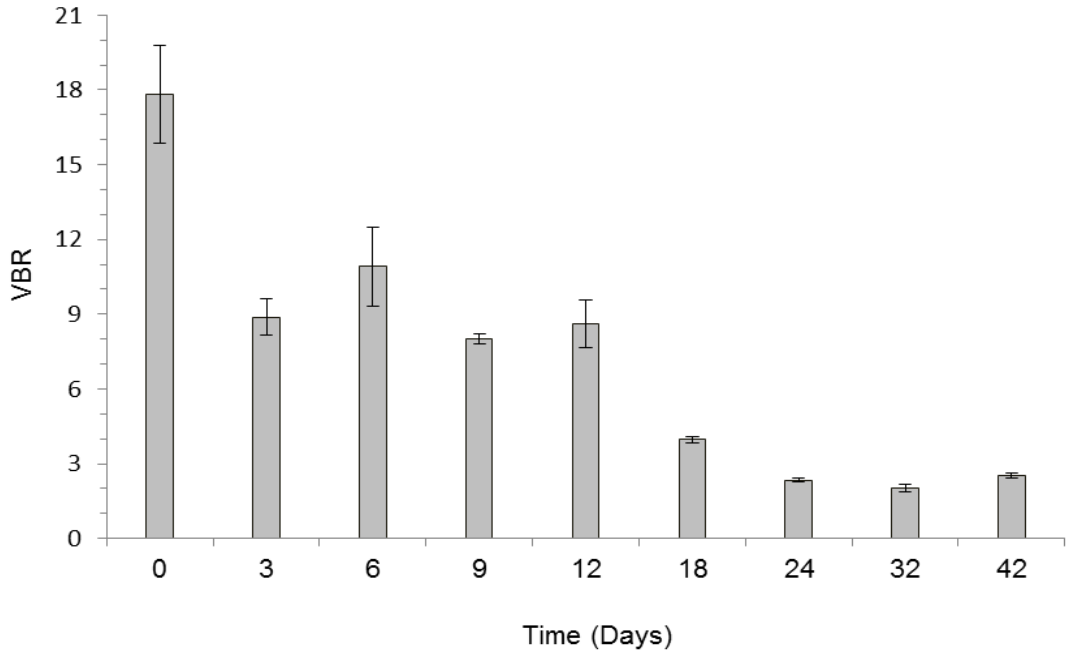

Fig. S2. Virus to bacteria ratio (VBR) throughout the *O.* cf. *ovata* growth. Bars indicate standard deviation.

Table S1. Relative abundance (in %) of all phyla detected by ION torrent sequencing analysis. Rare phyla accounted for less than 1% in percentage.

Phylum	% in at least one sample	Day 0	Day 6	Day 24	Day 42
Proteobacteria	≥ 1	86.6	97.0	65.3	86.9
Bacteroidetes	≥ 1	12.9	2.7	34.0	10.8
Actinobacteria	≥ 1	0.1	0.1	0.6	2.2
OD1	rare	0.0	0.0	0.0	0.0
Planctomycetes	rare	0.0	0.0	0.0	0.0
Fusobacteria	rare	0.0	0.0	0.0	0.0
Cyanobacteria	rare	0.0	0.0	0.0	0.0
Deinococcus-Thermus	rare	0.1	0.1	0.0	0.0
Firmicutes	rare	0.0	0.0	0.0	0.1
TM7	rare	0.1	0.1	0.0	0.0
Spirochaetes	rare	0.0	0.0	0.0	0.0

Abbreviations: refer to Table 1 for sample ID abbreviations.

Table S2. Relative abundance (in %) of all classes detected by ION torrent sequencing analysis. Rare classes accounted for less than 1% in percentage.

Class	% in at least one sample	Day 0	Day 6	Day 24	Day 42
Alphaproteobacteria	≥ 1	85.1	95.7	64.7	86.0
Sphingobacteria	≥ 1	12.6	2.4	33.6	10.3
Actinobacteria	≥ 1	0.1	0.1	0.6	2.2
Gammaproteobacteria	rare	0.9	0.9	0.3	0.5
Betaproteobacteria	rare	0.6	0.4	0.3	0.3
Flavobacteria	rare	0.3	0.2	0.4	0.5
Phycisphaerae	rare	0.0	0.0	0.0	0.0
Deltaproteobacteria	rare	0.0	0.0	0.0	0.0
Epsilonproteobacteria	rare	0.0	0.0	0.0	0.0
Fusobacteria	rare	0.0	0.0	0.0	0.0
Cyanobacteria	rare	0.0	0.0	0.0	0.0
Deinococci	rare	0.1	0.1	0.0	0.0
Clostridia	rare	0.0	0.0	0.0	0.0
Spirochaetes	rare	0.0	0.0	0.0	0.0
Bacilli	rare	0.0	0.0	0.0	0.0
Bacteroidia	rare	0.0	0.0	0.0	0.0

Abbreviations: refer to Table 1 for sample ID abbreviations.

Table S3. Relative abundance (in %) of all genera detected by ION torrent sequencing analysis. Rare genera accounted for less than 1% in percentage.

Genus	% in at least one sample	Day 0	Day 6	Day 24	Day 42
Oceanicaulis	≥ 1	39.7	34.6	14.3	31.8
Dinoroseobacter	≥ 1	21.7	34.6	24.6	26.5
Labrenzia	≥ 1	5.7	7.0	24.9	23.1
Roseovarius	≥ 1	15.5	15.9	6.5	4.8
Roseivivax	≥ 1	1.2	1.4	0.9	0.3
Sphingopyxis	≥ 1	2.2	1.5	0.7	1.0
Fabibacter	≥ 1	6.3	0.6	21.9	4.7
Balneola	≥ 1	3.3	1.1	1.2	0.3
Dietzia	≥ 1	0.0	0.0	0.7	1.4
Ponticaulis	rare	0.0	0.0	0.0	0.0
Ruegeria	rare	0.1	0.1	0.1	0.2
Nautella	rare	0.0	0.1	0.0	0.1
Phaeobacter	rare	0.0	0.0	0.0	0.1
Thalassococcus	rare	0.0	0.0	0.0	0.0
Jannaschia	rare	0.0	0.0	0.0	0.1
Sulfitobacter	rare	0.0	0.0	0.0	0.0
Planktomarina	rare	0.0	0.0	0.0	0.0
Leisingera	rare	0.0	0.0	0.0	0.0
Loktanella	rare	0.0	0.0	0.0	0.0
Pelagibacter	rare	0.5	0.5	0.5	0.7
Marivita	rare	0.0	0.0	0.0	0.0
Gaetbulicola	rare	0.0	0.0	0.0	0.0
Devosia	rare	0.1	0.1	0.1	0.1
Methylobacterium	rare	0.1	0.0	0.0	0.0
Parvularcula	rare	0.0	0.0	0.0	0.0
Glaciecola	rare	0.1	0.1	0.1	0.1
Maricurvus	rare	0.0	0.0	0.0	0.0
Alteromonas	rare	0.0	0.0	0.0	0.0
Vibrio	rare	0.0	0.0	0.0	0.0
Alcanivorax	rare	0.4	0.7	0.1	0.1
Polaribacter	rare	0.1	0.0	0.1	0.1
Hyunsoonleella	rare	0.0	0.0	0.0	0.0
Flavobacterium	rare	0.0	0.0	0.0	0.0
Bizionia	rare	0.0	0.0	0.0	0.0
Aquimarina	rare	0.1	0.0	1.6	1.3
Algibacter	rare	0.0	0.0	0.0	0.0
Croceitalea	rare	0.0	0.0	0.0	0.0
Formosa	rare	0.0	0.0	0.0	0.0
Maribacter	rare	0.0	0.0	0.0	0.0
Fluviicola	rare	0.0	0.0	0.1	0.1

Lishizhenia	rare	0.0	0.0	0.0	0.0
Gracilimonas	rare	0.0	0.0	0.0	0.0
Haliscomenobacter	rare	0.0	0.0	0.0	0.0
Phycisphaera	rare	0.0	0.0	0.0	0.0
GpIIa	rare	0.0	0.0	0.0	0.0
OD1 Incertae Sedis	rare	0.1	0.0	0.0	0.1
Acidovorax	rare	0.0	0.0	0.0	0.0
Acinetobacter	rare	0.0	0.0	0.0	0.0
Actibacter	rare	0.0	0.0	0.0	0.0
Amphritea	rare	0.0	0.0	0.0	0.0
Anaerococcus	rare	0.0	0.0	0.0	0.0
Antarctobacter	rare	0.0	0.0	0.0	0.0
Aquabacterium	rare	0.1	0.0	0.0	0.0
Arcobacter	rare	0.0	0.0	0.0	0.0
Aurantimonas	rare	0.0	0.0	0.0	0.0
Aureispira	rare	0.0	0.0	0.0	0.0
Azospirillum	rare	0.0	0.0	0.0	0.0
Blastococcus	rare	0.0	0.0	0.0	0.0
Brevundimonas	rare	0.1	0.1	0.0	0.0
Chryseobacterium	rare	0.0	0.0	0.0	0.0
Cloacibacterium	rare	0.0	0.0	0.0	0.0
Clostridium_XI	rare	0.0	0.0	0.0	0.0
Cohnella	rare	0.0	0.0	0.0	0.0
Comamonas	rare	0.0	0.0	0.0	0.0
Corynebacterium	rare	0.0	0.0	0.0	1.0
Deinococcus	rare	0.1	0.1	0.0	0.0
Desulfopila	rare	0.0	0.0	0.0	0.0
Desulfosarcina	rare	0.0	0.0	0.0	0.0
Donghicola	rare	0.0	0.0	0.1	0.1
Dyadobacter	rare	0.0	0.0	0.0	0.0
Enhydrobacter	rare	0.0	0.0	0.0	0.0
Enterovibrio	rare	0.0	0.0	0.0	0.0
Erythrobacter	rare	0.0	0.0	0.0	0.0
Finegoldia	rare	0.0	0.0	0.0	0.0
Flavisolibacter	rare	0.1	0.1	0.0	0.0
Flexithrix	rare	0.0	0.0	0.0	0.0
GpVIII	rare	0.0	0.0	0.0	0.0
Granulosicoccus	rare	0.0	0.0	0.0	0.0
Haliea	rare	0.0	0.0	0.0	0.0
Hydrogenophaga	rare	0.0	0.0	0.0	0.0
Ilumatobacter	rare	0.0	0.0	0.0	0.0
Ilyobacter	rare	0.0	0.0	0.0	0.0
Limnobacter	rare	0.2	0.1	0.3	0.3
Lysobacter	rare	0.0	0.0	0.0	0.0
Marinoscillum	rare	0.0	0.0	0.0	0.0
Massilia	rare	0.1	0.0	0.0	0.0
Meiothermus	rare	0.0	0.0	0.0	0.0

Methylobacillus	rare	0.0	0.0	0.0	0.0
Microbacterium	rare	0.0	0.0	0.0	0.0
Microvirga	rare	0.1	0.1	0.0	0.0
Nereida	rare	0.0	0.0	0.0	0.0
Nocardioides	rare	0.0	0.0	0.0	0.0
Novosphingobium	rare	0.0	0.0	0.0	0.0
Oceanicola	rare	0.0	0.0	0.0	0.0
Pannonibacter	rare	0.0	0.0	0.0	0.0
Paracoccus	rare	0.0	0.0	0.0	0.0
Parvibaculum	rare	0.0	0.0	0.0	0.0
Phenylobacterium	rare	0.1	0.0	0.0	0.0
Pontibacter	rare	0.0	0.0	0.0	0.0
Porphyrobacter	rare	0.0	0.0	0.0	0.0
Prevotella	rare	0.0	0.0	0.0	0.0
Propionibacterium	rare	0.0	0.0	0.0	0.0
Propionigenium	rare	0.0	0.0	0.0	0.0
Pseudomonas	rare	0.4	0.1	0.0	0.1
Reichenbachiella	rare	0.3	0.2	0.7	0.8
Rhizobium	rare	0.4	0.2	0.0	0.1
Rhodocista	rare	0.0	0.0	0.0	0.0
Robiginitomaculum	rare	0.0	0.0	0.0	0.0
Roseivirga	rare	0.0	0.0	0.0	0.0
Roseomonas	rare	0.0	0.0	0.0	0.0
Rubellimicrobium	rare	0.0	0.0	0.0	0.0
Salinarimonas	rare	0.0	0.0	0.0	0.0
Skermanella	rare	0.0	0.0	0.0	0.0
Sphingobium	rare	0.0	0.0	0.0	0.0
Sphingomonas	rare	0.0	0.0	0.0	0.0
Sphingosinicella	rare	0.0	0.0	0.0	0.0
Spirochaeta	rare	0.1	0.0	0.0	0.0
Staphylococcus	rare	0.0	0.0	0.0	0.0
Sulfurimonas	rare	0.0	0.0	0.0	0.0
Sulfurovum	rare	0.0	0.0	0.0	0.0
Tenacibaculum	rare	0.0	0.0	0.0	0.0
Terasakiella	rare	0.0	0.0	0.0	0.0
Thalassomonas	rare	0.0	0.0	0.0	0.0
Thermus	rare	0.0	0.0	0.0	0.0
TM7 Incertae Sedis	rare	0.1	0.1	0.0	0.0
Vasilyevaea	rare	0.0	0.0	0.0	0.0
Vogesella	rare	0.0	0.0	0.0	0.0

Abbreviations: refer to Table 1 for sample ID abbreviations.

Overall conclusions

Over the last two decades, *Ostreopsis* cf. *ovata* blooms have been reported with increased frequency, intensity and areal distribution in temperate coastal regions including the Mediterranean Sea. Previous studies indicate stable weather conditions and low hydrodynamism as a prerequisite for blooms' onset and maintenance.

Data reported in the first chapter of this thesis show the high efficiency of *O.* cf. *ovata* in both inorganic and organic phosphate acquisition, an aspect that could confer advantages towards competing species. As phosphorous has been pointed out to be the major nutrient affecting benthic dinoflagellates' growth, these findings may partly contribute to explain why differently from *Coolia monotis*, *Prorocentrum lima*, and *Amphidinium carterae*, *O.* cf. *ovata* is the only dinoflagellate mainly composing the microphytobenthic assemblage that reach high cell concentrations in the Mediterranean area.

Studies presented in the second and in the third chapters evidence intimate and recurrent associations between *O.* cf. *ovata* and specific bacterial taxa (mostly *Roseobacters*) both *in situ* and in laboratory cultures, the latter being demonstrated as a reliable tool for future investigations on *O.* cf. *ovata*-bacterial interactions. For some of these retrieved bacterial species or closely related ones, switching from mutualistic relationships with healthy algal cells to algicidal/scavenger activity towards senescent cells has been previously revealed in dual-system co-cultures, suggesting a primary role of dinoflagellate-bacterial interactions in triggering and demising of *O.* cf. *ovata* toxic algal blooms. Both the natural and the cultured *O.* cf. *ovata*-associated bacterial consortia include functionally similar members harboring the whole metabolic pathways for biosynthesis of several vitamins, supporting the hypothesis that *O.* cf. *ovata*-bacterial beneficial interactions mostly rely on the exchange of micronutrients often limiting in the marine environment. While the vast majority of studies on HABs to date have focused on dynamics of macronutrients as nitrogen and phosphate, owing to the recurrent linkage between eutrophication and toxic microalgae outbreaks, the importance of coenzymes and specific vitamins has been rarely investigated although strongly suggested, specifically for blooms of toxic dinoflagellates, which are well known to be strikingly auxotrophic for vitamins. Data in this thesis suggest a relevant role of vitamins in bloom dynamics, and they pave the way for further investigations on

the role of allochthonous vitamins in *O.* cf. *ovata* bloom development and maintenance. In addition, for several *Roseobacter* relatives retrieved in association with *O*. cf. *ovata* in these studies, synthesis of secondary metabolites with antibacterial action against other non-*Roseobacters* interacting with dinoflagellate phycosphere has been previously observed. Antibiotic compounds' synthesis occur to a larger extent during attached-life phase of the bacteria, and is most prominent under low hydrodinamism. As a result, by preventing algicidal bacterial species from harming the microalgal host, *Roseobacters* enhance their symbiosis with the dinoflagellate and could favor its proliferation during bloom development phase.

In conclusion, in the attempt to bring further information on mechanisms that might increase the occurrence of *O.* cf. *ovata* blooms in the Mediterranean area, a conceptual model could be postulated. Under prolonged periods of steady weather conditions and low hydrodinamism, expected also as a consequence of the global climate change in the Mediterranean area, *O.* cf. *ovata* recruits and likely engages beneficial relationships with alphaproteobacterial phylotypes (mostly *Roseobacters*), mainly based on algal-released DMSP and bacterial-synthesized vitamin exchange between the counterparts. These positive interactions would favor algal proliferation and mucilage mats development, which further tighten *O.* cf. *ovata-Roseobacters* symbiosis favoring production of antibiotic compounds that would limit colonization by non-*Roseobacters* phylotypes harming the dinoflagellate, further supporting bloom development and maintenance. The increased availability of allochthonous vitamins in the environment due to a moderate anthropogenic impact during the summer period would promote both algal growth directly but also likely thriving of the host-beneficial bacteria that biosynthesize vitamins at an high metabolic cost.

The era of high throughput sequencing will allow further detailed investigations on bacteria co-occurring with epiphytic/benthic harmful algal blooms at the consortia and cell-cell levels and their interactions.

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