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

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Abstract

Extensive b ooms of ~~the~~ the toxic epiphytic/benthic dinof age ate *Ostreopsis* cf *ovata* are being reported with increasing frequency and spatia distribution in temperate coasta regions inc uding the Mediterranean These b ooms are of human and environmenta hea th concern due to the production of isobaric pa ytoxin and a wide range of ovatoxins by *Ostreopsis* cf *ovata* Bacteria microa ga interactions are important regu ators in a ga b oom dynamics and potentia y toxin dynamics This study investigated the bacteria assemb ages co occurring with *O* cf *ovata* (OA) and from ambient seawaters (SW) during the ear y and peak phases of b oom deve opment in NW Adriatic Sea Fractions of the bacteria assemb ages co occurring with *O* cf *ovata* (OA) and more c ose y associated to the muci age ayer (LA) embedding *O* cf *ovata* ce s were a so reported

n tota 14 bacteria phy a were detected by targeted 454 pyrosequencing of the 16S rRNA gene The dominant bacteria phy a in the OA assemb ages were *Proteobacteria* and *Bacteroidetes*; whi e at the c ass eve *Alphaproteobacteria* were the most abundant (83 and 66% re ative abundance ear y and peak b oom phases) fo owed by *Flavobacteria* (7 and 19% ear y and peak phases) *Actinobacteria* and *Cyanobacteria* were of minor importance (<5% of the re ative bacteria abundance each) *Gammaproteobacteria* showed a notab y presence in OA assemb age on y at the ear y phase of the b oom (genus *Haliea* 13%) The *Alphaproteobacteria* were predominate y composed by the genera *Ruegeria* *Jannaschia* and *Erythrobacter* which represented about ha f of the tota phy otypes' contribution of OA at both ear y and peak phases of the *O* cf *ovata* b oom suggesting interactions between this consortium and the microa ga Moreover the highest contribution of *Ruegeria* (30% of the tota phy otypes) was observed at the ear y phase of the b oom in LA assemb age

Microbia assemb ages associated with the ambient seawaters whi e being a so dominated by *Alphaproteobacteria* and *Flavobacteria* were partia y distinct from those associated with *O* cf *ovata* due to the presence of genera a most not retrieved in the atter assemb ages

Keywords: *Ostreopsis*; ~~t~~Toxic dinof age ate; HAB associated bacteria; ~~Pyrosequencing~~ ~~Bacterial diversity~~ *Ruegeria*; *Haliea*; *Jannaschia*

1 Introduction

Extensive b ooms of toxic epiphytic/benthic dinof age ate *Ostreopsis* cf *ovata* are current y reported with increasing frequency and area distribution in the Mediterranean Sea (Vi a et a 2001; A igizaki and Niko aidis 2006; Mangia ajo et a 2011; Funari et a 2015) The dinof age ate grows epiphytica y forming brownish muci age mats on macroa gae but a so on other biotic and abiotic substrata in sha ow and she tered waters (Vi a et a 2001; A igizaki and Niko aidis 2006; Totti et a 2010; Accoroni et a 2011) Moreover *O* cf *ovata* is often found in p ankton samp es due to resuspension from the benthic substrata (Vi a et a 2001; A igizaki and Niko aidis 2006; Se ina and Or ova 2010; Totti et a 2010) Mediterranean *O* cf *ovata* produces pa ytoxin ike compounds name y isobaric pa ytoxin and a wide range of ovatoxins (OVTX a to k; ~~Garci~~Garc a A tares et a 2014; Brissard et a 2015; Tartag ione et a 2016) under both fie d and cu ture conditions (e g Accoroni et a 2011; Ciminie o et a 2011 ~~Ciminiello et al~~ 2012a b; Pezzolesi et al 2012 2014 Sca co et a 2012; ~~Pezzolesi et al 2012 Pezzolesi et al 2014~~ Vanucci et a 2012a) The b ooms can have a severe impact on human hea th causing intoxications through marine aeroso inha ation and contact (Ga ite i et a 2005; Kermarec et a 2008; Tichadou et a 2010; Funari et a 2015) They a so strong y affect invertebrate benthic communities causing

massive morta ities (Accoroni et a 2011; Faima i et a 2012; Gorbi et a 2012 2013; Care a et a 2015) Moreover bioaccumu ation of pa ytoxin ike compounds has been reported for biva ve mo usks and herbivorous echinoderms (e g A igizaki et a 2011; Amzi et a 2012; Fur an et a 2013; Brissard et a 2014) Driven by the negative impacts of *O* cf *ovata* Mediterranean outbreaks research efforts have been aimed at identifying the environmenta conditions and factors that trigger or regu ate the microa ga b oom dynamics (A igizaki and Niko aidis 2006; Totti et a 2010; Grané i et a 2011; ~~Pistocchi et al 2011~~ Mangia ajo et a 2011; Pistocchi et al 2011 Accoroni et a 2015a b) One of the major factors affecting *Ostreopsis* b ooms is hydrodynamic regime as consistent y higher abundances are found under ow hydrodynamism and in she tered sites compared with exposed ones (Chang et a 2000; Shears and Ross 2009; Mabrouk et a 2011; Se ina et a 2014)

n para e to the physicochemica factors a greater appreciation about the significance of the bidirectiona interactions between microa gae and bacteria in terms of regu ating harmfu a ga b ooms (HABs) has deve oped (Kodama et a 2006; Loureiro et a 2011) Cu ture based studies on harmfu p anktonic dinof age ates have shed ight on severa effects of bacteria on a ga ce u ar physio ogy which wi u timate y inf uence ce growth dynamics production degradation and/or modification of a ga toxins (e g Ho d et a 2001; Long et a 2003; Su et a 2005; Azanza et a 2006; Donovan et a 2009; Green et a 2010; Wang et a 2010~~Uncited references~~ Bo ch and Subramanian 2011~~ Bolch et al 2011~~ Santos and Azanza 2012) Patterns of association between cu tured harmfu dinof age ates and specific bacteria groups such as *Alpha* and *Gammaproteobacteria* *Cytophaga Flavobacteria Bacteroides* and some sub groups primari y the *Roseobacter* c ade and the *Alteromonas* have been observed (Amaro et a 2005; Jasti et a 2005; reviewed by Garcés et a 2007; Green et a 2010 and references therein; Onda et a 2015) Moreover severa members of the *Roseobacter* c ade (e g phy otypes of *Phaeobacter* and *Ruegeria* genera) associated with marine a gae and/or iso ated from non toxic and toxic dinof age ate cu tures are known as some of the most effective co onizers of surfaces in the coasta environments (e g S ightom and Buchan 2009 and references therein; E ifantz et a 2013) Furthermore *Roseobacters* have the capabi ity to produce potent antibacteria compounds main y affecting non *Roseobacter* phy otypes giving se ective advantage to this c ade (Long and Azam 2001; Brinkhoff et a 2004; Bruhn et a 2007) n addition it has been recent y demonstrated that the interaction between dinof age ates and these bacteria can be mutua istic antagonistic or switch between both (Wagner Döb er et a 2010; Wang et a 2014 2015) depending on a ga physio ogica status as aging a gae wi induce the production of a gicida compounds by bacteria (e g *Phaeobacter gallaeciensis* Seyedsayamdost et a 2011 2014; *Ruegeria* sp TM1040 Ric ea et a 2012) which cou d have an important ro e in the dec ining stages of a ga b ooms (Ric ea et a 2012)

Fie d impact of these associations or how e ements of natura bacteria assemb ages interact with the HAB popu ation however is sti poor y known (Maya i and Azam 2004; Maya i et a 2008) out ining the need for ~~in situ~~in situ studies assessing phy ogenetic diversity and its tempora changes of the natura co occurring bacteria popu ations during HABs The imited studies on harmfu p anktonic dinof age ates' microbiota genera y converge on a broad feature for the dominance of the two bacteria groups the *Rhodobacterales* (*Alphaproteobacteria*) and *Cytophaga Flavobacteria Bacteroides* (*Bacteroidetes*) during b ooms (Garcés et a 2007; Jones et a 2010; Park et a 2015)

The eco ogica ro e of bacteria assemb ages associated with epiphytic/benthic toxic dinof age ates has received considerab y ess attention than for their p anktonic counterparts Previous studies re ied upon bacteria p ate iso ation from cu tured dinof age ates (e g *Ostreopsis lenticularis* and *Gambierdiscus toxicus* Tosteson et a 1989; *Prorocentrum lima* Lafay et a 1995; Prokic et a 1998; *G* *toxicus* Sakami et a 1999) More recent y Pérez Guzmán et a (2008) showed that about ha f of tota bacteria associated with *O lenticularis* cu tures was made up by a sing e species be onging to *Cytophaga Flavobacteria Bacteroides* comp ex n contrast Ruh et a (2009) found *Alphaproteobacteria* to be the argest group in monoc ona cu tures of *Coolia monotis* and *Ostreopsis ovata* A aboratory study provided evidence that bacteria interfere indirect y with *Ostreopsis* cf *ovata* growth toxin production and ike y on toxin degradation (Vanucci et a 2012b) Current y there is no phy ogenetic data on the natura bacteria assemb ages associated with *O* cf~~ ~~ *ovata* b ooms and more genera y with b ooms of benthic dinof age ates

The present study focuses on the phy ogenetic characterization of the bacteria assemb ages co occurring over the ear y and the peak phases of a benthic *O* cf *ovata* b oom at Passetto station The Passetto station (Conero Riviera) has been c assified as a hot spot area for *Ostreopsis* cf *ovata* b ooms by the ta ian Agency for the Protection and Environmenta Research (SPRA 2012) n this region *O* cf *ovata* summer b ooms occur regu ar y and ce abundances are among the highest recorded a ong Mediterranean coasts (Mangia ajo et a 2011; Accoroni et a 2012 2015a) This site is a semi enc osed bay she tered by a natura reef and characterized by a most y rocky bottom and sha ow depth (up to 2 m) The shore is subjected to a moderate anthropogenic impact during the summer season (Marini et a 2002) being a popu ar area for summer ho idays when it is a so subjected to tramp ing by swimmers This site is a so characterized by the presence of sma caves derived from human boring of the natura c iffs with some wastewater discharge faci ities A c ear and important ro e of the hydrodynamic conditions on *Ostreopsis* b ooms' trend has been recognized in this area (Totti et a 2010; Accoroni et a 2011 2012) Recent y Accoroni et a (2015a) have proposed a conceptua mode for annua *Ostreopsis* cf *ovata* b ooms in the Northern Adriatic Sea based on the synergic effects of hydrodynamics temperature and the N:P ratio of water co umn nutrients pointing out that ca m conditions appeared to be a prerequisite for b ooms deve opment ndeed ow hydrodynamism wou d favor *O* cf *ovata* pro iferation by faci itating macroa ga co onization juxtaposing of the microa ga ce s and forming muci age mats (Vi a et a 2001; Totti et a 2010) which are known to be hotspots for microorganisms interactions (e g Co e et a 2014a~~b~~; Carreira et a 2015)

The aims of the present study were: (i) assessing the phy ogenetic composition of bacteria assemb ages co occurring with *O* cf *ovata* at the ear y and the peak phases of the b oom in order to high ight most prominent bacteria a ga associations; (ii) comparing bacteria assemb ages associated with *O* cf *ovata* with those from ambient seawaters for eva uating possib e contribution of a ochthonous bacteria to the former ones The bacteria assemb ages were assessed by high throughput para e tag sequencing (454 pyrosequencing)

2 Materials and methods

2.1 Study area and samples collection

Bacterial assemblages associated with *Ostreopsis* cf *ovata* aggregates (OA) (i e *O* cf *ovata* colonizing macrophytes) and those associated with the 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 taly 43°36 38 ~~N and 13°32 20~~ N and 13°32 20 E)

Surface temperature (approximately 0 5 m depth) and salinity were recorded ~~in situ~~ in situ by a YS Pro Plus probe Samples from six to eight dinoflagellate colonized macrophytes for microorganisms (i e *C streopsis* 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

2.2 ~~*O. cf. ovata*~~ *O.* cf. *ovata* and bacterial enumeration

For determination of *C streopsis* 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 320× magnification under bright field and phase contrast illumination

Bacterial abundances were assessed for OA and SW subsamples and for OA subsamples pre filtered onto sterilized 11 µm pore size filters (Millipore) All subsamples were fixed with 0 2 µm prefiltered formaldehyde (2%) and bacterial enumerations were carried out 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 1000×) under blue light excitation counting at least twenty fields per sample and a minimum of 300 cells Abundances of bacteria more closely associated with the mucilage layer embedding *C streopsis* cf *ovata* cells here defined as the bacterial fraction retained onto 11 µm pore size filters (i e mucilage layer bacterial assemblage LA) were estimated by subtracting bacterial counts obtained for 11 µm prefiltered OA subsamples to counts obtained for OA subsamples

2.3 DNA samples processing and extraction

For harvesting bacterial assemblages associated with *C streopsis* cf *ovata* (i e 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 collected under low vacuum onto 0 2 µm pore size Supor 200 PES filters (Pall Corporation/Pall Life Sciences) n order to assess the bacterial phylotypes more closely associated with the mucilage layer embedding *O* cf *ovata* cells (LA) aliquots of the same suspension were collected onto sterilized 11 µm pore size filters (Millipore) Hence LA assemblages represent fractions of the entire bacterial assemblages (OA) collected onto 0 2 µm pore size filters Samples for 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)

2.4.~~3.1~~ 16S rDNA pyrosequencing

For pyrosequencing analysis extracted DNA samples from three replicates were pooled together and diluted to 10 ng µL[1] nitial 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~~ Valencia CA) forward primer composed of the Roche Titanium Fusion Primer A (5 CCATCTCATCCCTGCGTGTCTCCGACTCAG 3) a 10 bp Multiplex dentifier (M D) sequence (Roche ndianapolis N) unique to each of the samples and the universal bacteria primer 8F (5 AGAGTTTGATCCTGGCTCAG 3) The reverse primer was composed of the Roche Titanium Primer B (5 CCTATCCCCTGTGTGCCTTGGCAGTCTCAG 3) the identical 10 bp M D sequence as the forward primer and the reverse bacteria primer 338R (5 ~~GCTGCCTCCCGTAGGAGT~~ GCTGCCTCCCGTAGGAGT 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 s~~ec~~ annealing at 50 °C for 30 s~~ec~~ and extension at 72 °C for 90 s~~ec~~ 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) nitial data analysis and base pair calling were performed by Research Computing at UNC (Chapel Hill NC)

2.5~~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 (10 349 reads) by randomly subsampling the datasets Sequences were aligned with the nfernal 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 2014b) 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

2.6 Statistical analysis

All statistical analysis except for Metastats analysis were performed with the PAST software package for Windows (Hammer et al 2001) Differences in *C streopsis* 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 (ANOS M) and Similarity Percentage analysis (S MPER) 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 *C streopsis* 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 (D N 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 *C streopsis* cf *ovata* cells colonizing macrophytes (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 approximately two fold higher in both OA and LA assemblages at the peak compared to the early phase of the bloom (OA mean values 6 62 10^{6} cells mL ^{1} vs 3 06 10^{6} cell mL ^{1} LA mean values 1 49 10^{6} cells mL ^{1} vs 0 68 10^{6} cell mL ^{1} $p < 0\ 01$ all 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 OA than in SW samples ($p < 0\ 01$ Student's *t* test)

3.2 Diversity of microbial assemblages

A total of 73 641 high quality reads spanning the 16S rDNA V1–V2 hypervariable region were used in the final analysis (average length 287 bp) 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 (SW Fig 1) curves reached higher number of OTUs per reads at the peak than at the early bloom phase for all the assemblages 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 assemblage at the peak phase of the bloom (OA) whereas the lowest values were found for bacteria more closely associated with the mucilage layer embedding *C streopsis* cf *ovata* cells at the early phase (i e LA) 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 assemblages while unique OTUs (31 6% of the total OTUs in OA and 30 9% in SW assemblages) were mainly represented by singletons (~60%)

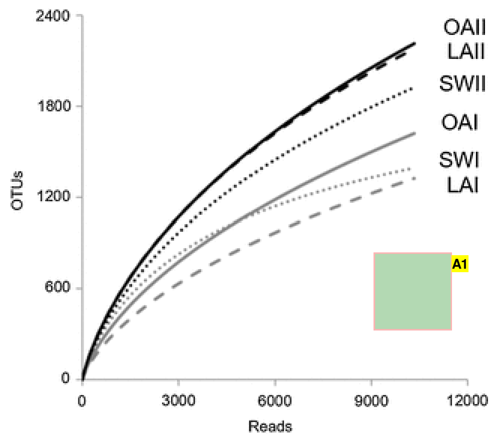

Fig. 1 Diversi y of bac erial assemblages associa ed wi h *O* cf *ovata* (OA) mucilage layer bac erial assemblages (A) and seawa er bac erial assemblages (SW) a he early and peak phase of he bloom (and respec ively) Rarefac ion curves were compu ed on bac erial OTUs a a dissimilari y level of 3%

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Table 1 Bacteria diversity parameters during *O.* cf *ovata* b oom Summary of tota high qua ity sequences after norma ization to the sma est ibrary (Reads) number of bacteria operationa taxonomic units detected at 97% identity (OTUs) estimated species richness (Chao 1) Shannon diversity index (H~~) and evenness (J~~) and evenness (J) obtained from pyrosequencing of bacteria assemb ages associated with *O.* cf *ovata* (OA) muci age ayer bacteria assemb ages (LA) and seawater bacteria assemb ages (SW) at the ear y and peak phase of the b oom (and respective y)

a t text: Tab e 1

Samp e	Reads	OTUs	Chao 1	H~~J OA 10~~	J
OA	10 349	1621	3229	5 28	0 715
OA	10 349	2214	4078	6 36	0 826
LA	10 349	1322	2582	4 63	0 644
LA	10 349	2177	3886	6 31	0 821
SW	10 349	1394	1798	5 79	0 800
SW	10 349	1921	3337	6 18	0 817

As expected higher numbers of OTUs were shared between OA and LA compared to SW assemblages and higher percentages of shared OTUs were observed at the peak than at the early phase of the bloom (data not shown) These findings were supported by the NMDS plot displaying Bray–Curtis similarities between samples (Fig 2) 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 at the early bloom phase (60 and 46% similarity) Nevertheless ANOS M test did not underline significant differences between assemblages or bloom phases ($p > 0$ 05 for both comparisons) S MPER 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 2 Table S1)

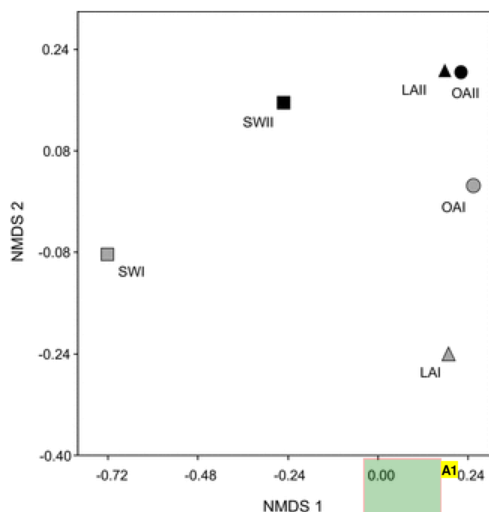

Fig. 2 NMDS ordina ion plo of bac erial assemblages s ruc ure using Bray-Curtis distances. -Curtis distances. Circles riangles and squares indica ed bac erial assemblages associa ed wi h *O* cf *ovata* (OA) mucilage layer bac erial assemblages (A) and seawa er bac erial assemblages (SW) respec ively Greay and black symbols represen he early ( ) and peak ( ) phase of he bloom respec ively The plo was cons ruc ed on he basis of bac erial OTUs re rieved from pyrosequencing da a

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Table 2 Major bacteria OTUs eading to dissimi arity Simi arity percentage ana ysis (S MPER) showing contribution of the seven OTUs exp aining 20% of the overa dissimi arity between bacteria assemb ages associated with *O.* cf *ovata* (OA) muci age ayer bacteria assemb ages (LA) and seawater bacteria assemb ages (SW) and between the ear y and peak phase of the b oom (and respective y) The number of sequences per OTU for each of the samp es a eis a so reported

a t text: Tab e 2

OTU	C osest re ative RDP c assifier	No of sequences per OTU						Contribution to dissimi arity (%)	
		OA	OA	LA	LA	SW	SW	Assemb ages	B oom 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

Table 3Supplementary Table S1 related to this article can be found in the online version at doi 10 1016/j hal 2016 04 003

TThe number of specific phylotypes (i e OTUs detected in only one kind of assemblage at both bloom phases) wereas 295 for OA and 82 for SW assemblage and all the phylotypes 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)

3.3 Microbial assemblages composition and comparisons

Overall 14 different bacterial phyla 20 classes and 165 genera were recovered supplementary Table S2 S3 and Supplementary Tables S2–S4 respectively) All microbial assemblages either related to *C streopsis* cf *ovata* (i e both OA and LA assemblages) or with the ambient seawaters (SW) 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) Phyla *Fusobacteria Firmicutes* and *Planctomycetes* were present at abundances just above 1% only in SW while *Parcubacteria* reached values ~1 5% in both SW and *O* cf *ovata*-related assemblages (OA and LA) at the bloom peak phase The remaining phyla *Acidobacteria Chloroflexi Chlorobi Spirochaetes TM7* and *Deinococcus Thermus* were rare representatives (i e <1% Table S2)

[SS]Appendix ASupplementary dataSupplementary data associated withSupplementary Tables S2–S4 related to this article can be found in the online version at doi 10 1016/j hal 2016 04 003

Assemblages related to *Ostreopsis* cf *ovata* (OA and LA) showed predominance of *Alphaproteobacteria* at both phases of the bloom (66 0 to 84 4% OA and LA respectively Fig 3) Genera *Ruegeria* and *Jannaschia* (*Roseobacter* clade) along with *Erythrobacter* were the most abundant ones with the highest values for *Ruegeria* and *Erythrobacter* at the early phase (30 2% in LA and 23 8% in OA respectively) and for *Jannaschia* at the peak phase (21 1% in OA Fig 4) primarily due to contribution of the three OTUs #6 #164 and #516 respectively (Table 2) Moreover the OTU #40 assigned to *Rhodobacteraceae* had also a consistent contribution in OA and LA assemblages particularly at the early bloom phase (Table 2) Other *Rhodobacteraceae* genera such as *Litoreibacter* Loktanella *Loktanella Paracoccus* showed contributions around 3% (Fig 4) Overall contribution of OTUs assigned to *Alphaproteobacteria* wereas significantly higher in *O* cf *ovata*-related assemblages (OA and LA) compared to SW assemblages (*p* < 0 05 Metastats analysis) n 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) Class *Gammaproteobacteria* was mainly represented by OTUs belonging to the genus *Haliea* at the early phase (>10% primarily OTU #35 Table 2) and then shifting to *Granulosicoccus* at the peak phase (~3%) The remaining three *Proteobacteria* classes (*Delta* Epsilon *Epsilon* and *Betaproteobacteria* Table S3) were rare Other notable groups showed an inverse trend with respect to *Alphaproteobacteria* (Fig 3) Members of *Flavobacteria* (7 2 and 22 2% OA and LA) followed by *Sphingobacteria* showed the highest contributions at the peak phase as well as *Ilumatobacter* (6% *Actinobacteria*) (Table 3)

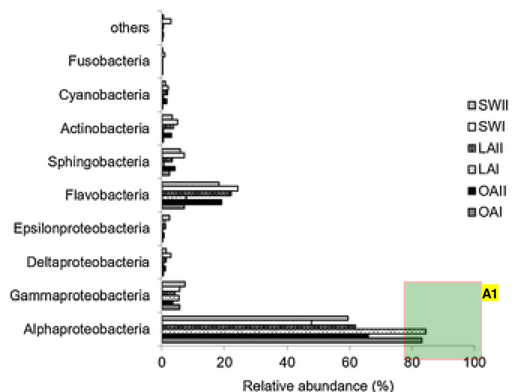

Fig. 3 Percen dis ribu ion of he dominan classes (≥1% in a leas one of he samples) in bac erial assemblages associa ed wi h *O* cf *ovata* (OA) mucilage layer bac erial assemblages (A) and in seawa er bac erial assemblages (SW) a he early and peak phase of he bloom (and respec ively) as revealed from pyrosequencing da a normalized for number of ribosomal operons per genome oO hers represen he classes wi h less han 1% of rela ive abundance individually Abbrevia ions Alpha *Alphaproteobacteria* Gamma *Gammaproteobacteria* Del a *Deltaproteobacteria* Epsilon *Epsilonproteobacteria* Flavo *Flavobacteria* Spingo *Sphingobacteria* Actino, Actinobacteria Actino, *Actinobacteria* Cyano *Cyanobacteria* uso, usobacteria. uso, *Fusobacteria.*

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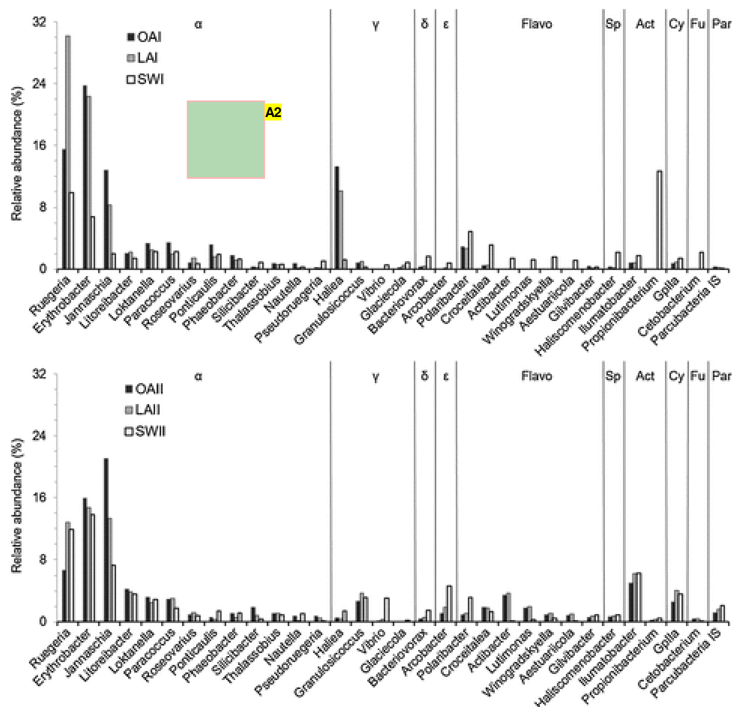

Fig. 4 Rela ive con ribu ion of he major bac erial genera (≥1% in a leas one of he samples) in bac erial assemblages associa ed wi h *O* cf *ovata* (OA) mucilage layer bac erial assemblages (A) and seawa er bac erial assemblages (SW) a he early phase (A) compared o he peak phase (B) of he bloom as revealed from pyrosequencing da a normalized for number of ribosomal operons per genome Ver ical lines separa e groups of differen phyla or classes Abbrevia ions α *Alphaproteobacteria* γ *Gammaproteobacteria* δ *Deltaproteobacteria* ε *Epsilonproteobacteria* Flavo *Flavobacteria* SpSphingobacteria Act, , *Sphingobacteria* Act, Actinobacteria Cy *Cyanobacteria* Fu *Fusobacteria* Par *Parcubacteria* S *Incertae Sedis*

~~**Table 3** Major bacteria OTUs eading to dissimi arity Simi arity percentage ana ysis (S M ER) showing contribution of the seven OTUs exp aining 20% of the dissimi arity between assemb ages and b oom phases The number of sequences per OTU for each of the samp es are~~is ~~a so reported~~ (Tab e 3 does not exist in the manuscript P ease remove comp ete y Tab e 3 and the re ative egend from the artic e)

a t text: ~~Tab e 3~~

~~OTU~~ (Table 3 does not exist in the manuscript Please remove completely Table 3 and the relative legend from the article)	~~C osest re ative RD c assifier~~	~~No of sequences per OTU~~						~~Contribution to dissimi arity (%)~~	
		OA	OA	LA	LA	SW	SW	Assemb ages	B oom 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

Ambient seawater bacterial assemblages were also dominated by *Alphaproteobacteria* (48–60%) although with a significant lower contribution with respect to *O* cf *ovata*-related assemblages (OA and LA) This class showed the highest contribution at

the peak phase of the bloom primarily with *Erythrobacte* _*Jannaschia* and *Ruegeria* (Fig 4) While OTU #40 (*Rhodobacteraceae*) overlapped with the *O* cf *ovata* related assemblages (OA and LA) OTU #33 (also a *Rhodobacteraceae*) had a large contribution only in SW (Table 2) 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 nterestingly the contribution of members affiliated to *Vibrio* w~~ere~~as significantly higher in SW than in OA and LA assemblages (e g ~~OTU #76 p <~~ 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 *O* cf *ovata*-_related assemblages (OA and LA) (OTU #335 p < 0 05 all Metastats analysis) Whereas *Flavobacteria*-_affiliated (24 and 18% SW and SW) did not differ significantly between assemblages (p > 0 05 all Metastats analysis) Notably *Propionibacterium* (*Actinobacteria*) was found at high relative abundance only in SW (12 7% primarily OTU #7 Table 2) whereas its contribution was negligible in OA and LA assemblages A similar pattern was also observed for *Cetobacterium* (*Fusobacteria* Fig 4)

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* (*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*) GpIIa (*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) Fig 5 summarizes bacterial assemblages' main shifts in relative abundance at genus level between early and peak phase of the bloom

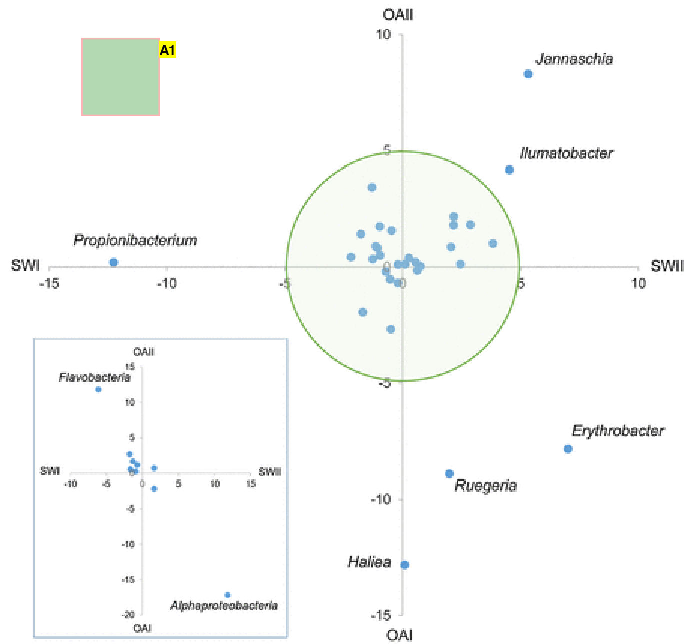

Fig. 5 Major changes in rela ive abundance a genus level and class level (inse) be ween peak phase ( ) and early phase ( ) of he bloom in bac erial assemblages associa ed wi h *O* cf *ovata* (OA) and seawa er bac erial assemblages (SW) Differences were calcula ed by sub rac ing percen ages recorded a he peak from values a he early phase of he bloom The posi ive values deno e higher con ribu ion a he peak as opposed o he early phase

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4 Discussion

4.1 General

Abundances of *Ostreopsis* cf *ovata* cells recorded during this study in both *O* cf *ovata* (OA) and ambient seawater (SW) assemblages 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 to infer on relationship dynamics between inorganic nutrients and *O* cf *ovata* bloom The physicochemical conditions the onset bloom N P ratio and nutrient concentration changes observed in this study

however 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* (OA) 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 Liu et al 2015~~ Piccini et al 2015~~ Piccini and García Alonso 2015) the latter being considered among the most diverse environments (Lozupone and Knight 2007) While the rarefaction curves indicated that species richness was not fully sampled 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

The comparison between *O*~~ ~~*streopsis* cf *ovata* (OA) and ambient seawater (SW) 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) n 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 Cells of *O* cf *ovata* and its mucilage 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*~~*O.* cf. *ovata*

n this study *Alphaproteobacteria* was the dominant class of the bacterial assemblages associated with *O*~~ ~~*streopsis* cf *ovata* (OA) during both bloom phases (83 and 66% relative abundance early and peak) followed by *Flavobacteria* that showed the highest contribution at the peak phase (up to 19%) The same trend was also observed for the bacterial fraction more closely associated to the mucilage layer embedding the *O* cf *ovata* cells (LA) These main bacterial composition feature and trend are consistent with previous ones reported for non toxic phytoplankton blooms (Fandino et al 2001 2005 Brussaard et al 2005 Alderkamp et al 2006 Lamy et al 2009) and also for the few available planktonic HABs (Garcés et al 2007 Hasegawa 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~~García Altares et al 2014~~ Gomez Pereira et al 2010~~ 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 Members of *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) n ~~our~~this context this genus might be directly related to the degradation of *O*~~ ~~*streopsis* 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* assemblages (OA) 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 2009 Lucena et al 2010) n 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*~~ ~~*kashiwo 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 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 data reported here This investigation however 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* assemblages (OA) 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

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* assemblages (OA) 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) Genus *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) Phylotypes belonging to *Erythrobacter* 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 (Mart nez Garc a 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 this 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 Elifantz et al 2013 Hahnke et al 2013 ~~Elifantz et al 2013~~) 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) n this study *Rhodobacteraceae* may have also taken advantage of the high D N 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 (Wagner Döbler and Biebl 2006 Wagner Döbler et al 2010 Riedel et al 2015) Diel anoxia may be expected in *C* ~~-~~ *streopsis* 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 *Pfiesteria piscicida* Alavi et al 2001 *Alexandrium catenella* Amaro et al 2005 *Pyrodinium bahamense* var *compressum* Onda et al 2015) Representatives of *Ruegeria* synthesize TDA prominently under static conditions (Bruhn et al 2007 Porsby et al 2008 Geng and Belas 2010 2011 D'Alvise et al 2014) and mainly inhibiting non *Roseobacter* phylotypes (e g members of *Vibrio* among *Gammaproteobacteria* Bruhn et al 2005 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 Seyedsayamdost et al 2011 and references therein) n this study the high contribution of *Ruegeria* in LA (30% of the total phylotypes) would worth further investigation to assess bacterial biofilm formation during *O* cf *ovata* bloom development High relative abundances 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 and Garc a Alonso 2015 ~~Piccini et al 2015~~) in association with algal cultures (Green et al 2015) but also as endosymbionts (Apprill et al 2009) Previous studies have shown that members of genera *Ruegeria* and *Jannaschia* are attracted by DMSP (e g *Ruegeria* sp TM1040 Miller and Belas 2004 2006 Geng and Belas 2010 *Jannaschia* sp Apprill et al 2009) and catabolize it (Moran et al 2007 ~~Todd 2012~~ Todd et al 2012 and references therein Reisch et al 2013 Dickschat et al 2015 and references therein) as many *Roseobacters* (Gonz~~a~~ález et al 2000 Newton et al 2010 Wagner Döbler et al 2010 Riedel et al 2015) Dimethylsulfoniopropionate is produced by several toxic dinoflagellates (reviewed by Caruana and Malin 2014) including *O* cf *ovata* (Vanucci et al in press)

At the peak of the bloom *Jannaschia* overcame *Ruegeria* while this study does not allow 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 *C* ~~-~~ *streopsis* 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 (Gonz~~a~~ález 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 ag~~e~~ing cells (Wagner Döbler et al 2010 Wang et al 2014 2015) by producing algicidal compounds induced by breakdown products 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 Bacterial assemblages in ambient seawaters

Ambient seawater bacterial assemblages (SW) were partially distinct from those associated with *C* ~~-~~ *streopsis* cf *ovata* (OA) 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 OA assemblages Seawater 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 (He et al 2014 Singh and Reddy 2014 ~~He et al 2014~~ Cleary et al 2015) Moreover some bacterial taxa possess many surface colonization traits (e g *Rhodobacteraceae*) 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 and Reddy 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

Dinof age ates bacteria re ationships are known to range from mutua istic to antagonistic interactions however we are just starting to appreciate the more ephemera and subt e aspects (Kodama et a 2006; Geng and Be as 2010;

Wagner Döb er et a 2010; Wang et a 2014 2015) n this study it has been found a strong association of a core bacteria genera with ~~C~~ streopsis cf *ovata* during the two investigated b oom phases Under the correct physicochemica conditions noted ear ier a positive interaction between the consortium of *Alphaproteobacteria* and *O* cf *ovata* favoring the dinof age ate pro iferation and the b oom deve opment phase it is hypothesized Low hydrodynamism is definite y considered a prerequisite for *Ostreopsis* b oom deve opment (A igizaki and Niko aidis 2006; Mangia ajo et a 2008; Totti et a 2010; Pistocchi et a 2011; Accoroni et a 2015a) Stab e weather conditions cou d a so favor *Ruegeria* co onization and biofi m formation sustaining this mutua istic/beneficia phase a though further investigations are needed to assess this hypothesis Moreover a focus on the ro e of both *Ruegeria* and *Jannaschia* in the b oom termination processes warrants future research considering the biva ent interaction ro e p ayed by *Roseobacters* in re ation to a ga age (Seyedsayamdost et a 2011 2014) The era of high throughput sequencing wi a ow further detai ed investigations on bacteria co occurring with epiphytic/benthic harmfu a ga b ooms at the consortia and ce ce eve s and their interactions

~~and Todd et al., 2012.~~Acknowledgements

This research was supported by ta ian PR N2009 project by ~~M UR and the~~ M UR and the FAMU Co ege of Science and Techno ogy We are gratefu to Dr Mauro Marini SMAR CNR Ancona ta y for providing nutrient data and to Natasha Butz Microbiome Core Faci ity UNC Chape Hi N~~orth Caro ina~~C USA for technica assistance in sequencing ~~Appendix ASupp ementary dataThe fo owing are the supp ementary data to this artic e:~~[SS]

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Multimedia Component 1

Table S1 Closes ma ches from he NCB GenBank da abase based on sequence similari y of he seven OTUs explaining 20% of he dissimilari y be ween assemblages and bloom phases as revealed by S MPER analysis

Multimedia Component 2

Table S2 Rela ive abundance (in %) of all he phyla de ec ed by pyrosequencing of bac erial assemblages associa ed wi h *O cf ovata* (OA) mucilage layer bac erial assemblages (A) and seawa er bac erial assemblages (SW) a he early and peak phase of he bloom (and respec ively) Rare phyla accoun ed for less han 1% in percen age

Multimedia Component 3

Table S3 Rela ive abundance (in %) of all he classes de ec ed by pyrosequencing analysis Rare classes accoun ed for less han 1% in percen age

Multimedia Component 4

Table S4 Rela ive abundance (in %) of all he genera de ec ed by pyrosequencing analysis Rare genera accoun ed for less han 1% in percen age

Queries and Answers

Query: The au hor names have been agged as g ven names and surnames (surnames are h gh gh ed n ea co or) P ease conf rm f hey have been den f ed correc y

Answer: Yes

Query: The number of keywords prov ded exceeds he max mum of 6 a owed by h s journa P ease prov de he f na s of 6 keywords for he ar c e

Answer: *Ostreopsis*; Tox c d nof age a e; HAB assoc a ed bac er a; *Ruegeria; Haliea; Jannaschia*

Query: "Your ar c e s reg s ered as a regu ar em and s be ng processed for nc us on n a regu ar ssue of he journa If h s s NOT correc and your ar c e be ongs o a Spec a Issue/Co ec on p ease con ac r sk nner@e sev er com mmed a e y pr or o re urn ng your correc ons "

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Answer: Yes

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Answer: No up ade s ava ab e, s s n press