CENTRO DE INVESTIGACIÓN CIENTÍFICA Y DE EDUCACIÓN SUPERIOR DE ENSENADA, BAJA CALIFORNIA

PROGRAMA DE POSGRADO EN CIENCIAS EN ECOLOGÍA MARINA

Bacteria, vitamins and *Lingulodinium polyedrum* – a bloom forming dinoflagellate

Tesis

para cubrir parcialmente los requisitos necesarios para obtener el grado de Doctor en Ciencias

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Ensenada, Baja California, México
2014

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Septiembre, 2014

Abstract of the thesis presented by Ricardo Cruz López as a partial requirement to obtain the Doctor of Science degree in Marine Ecology.

Bacteria, vitamins and *Lingulodinium polyedrum* – a bloom forming dinoflagellate

Abstract by:

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Ricardo Cruz López

We examined growth rates of the marine dinoflagellate *Lingulodinium polyedrum (Lp)* in culture in relation to the availability of vitamin B_1, B_7 and B_{12}. The results demonstrate independent threshold type growth limitation for vitamins B_1 and B_{12}. Using a factorial design based on different concentrations of B_1 and B_{12} we found that growth was inhibit at the lowest concentrations tested, 0.033 pM B_1 and 0.053 pM B_{12}. When the concentration of only one of the vitamins was increased then growth rate increased significantly suggesting that a limitation was multiplicative for the combination B_1 and B_{12} at these low concentrations. Dinoflagellates and bacteria form complex trophic interactions in nature and cultures. During growth of *Lp* the presence of bacteria could overcome vitamin limitation. The interaction will depend on the spatial arrangement between dinoflagellates and bacteria but the architecture of these arrangements has been scarcely investigated. Modifying a fluorescence *in situ* hybridization (FISH) protocol we identified and localized multiple bacterial taxa attached to dinoflagellate cells under different conditions of vitamin-limited growth. An average of six bacteria were attached to each dinoflagellate cell in both vitamin replete and deplete cultures but free suspended bacteria showed significantly higher concentrations in vitamin-limited cultures. Measured vitamin B_{12} concentrations produced by bacteria in vitamin-limited cultures (34 ± 5 nM) were lower but close to vitamin B_{12} in replete cultures (41 ± 3 nM). The culture medium was prepared without labile dissolved organics that could serve as a substrate for bacteria suggesting that bacterial growth was supported by organics released from the dinoflagellate host while the bacterial consortia provided vitamins B_1 and B_{12} in return.

Keywords: Dinoflagellates; nutrient limitation; vitamins; dinoflagellate-bacteria interactions; Fluorescence *in situ* hybridization.

Resumen de la tesis que presenta Ricardo Cruz López como requisito parcial para la obtención del grado de Doctor en Ciencias en Ecología Marina.

Bacteria, vitamins and *Lingulodinium polyedrum* – a bloom forming dinoflagellate

Resumen por:

Ricardo Cruz López

Usando cultivos del dinoflagelado marino *Lingulodinium polyedrum* (*Lp*) examinamos como la disponibilidad de vitaminas B_1, B_7 y B_{12} limitan la tasa de crecimiento. Los resultados muestran limitación independiente para B_1 y B_{12}. Usando un diseño factorial basado en diferentes concentraciones de B_1 y B_{12}, encontramos que a concentraciones menores de 0.033 pM para B_1 y 0.053 pM para B_{12}, el crecimiento es inhibido. Al aumentar la concentración de al menos una de las vitaminas el crecimiento se incrementó significativamente lo cual sugiere una limitación multiplicativa para B_1 y B_{12}. Los dinoflagelados y bacterias pueden llegar a formar complejas interacciones tróficas en condiciones naturales y en cultivo. Su interacción dependerá del arreglo espacial entre ambas partes aunque éste ha sido escasamente estudiado. Modificando un protocolo de hibridación de fluorescencia *in situ* (FISH) pudimos localizar e identificar múltiples grupos bacterianos adheridos a dinoflagelados los cuales fueron sometidos a diferentes concentraciones de vitaminas durante su crecimiento. Durante el crecimiento, la presencia de bacterias puede sostener la falta de vitaminas agregadas. Se documentó un promedio de 6 bacterias adheridas por célula de dinoflagelados en cultivos con y sin limitación de vitaminas. Las bacterias individualmente en suspensión, de vida libre, bajo condiciones de limitación por vitaminas presentaron el doble número en comparación de cultivos no limitados. Las concentraciones de B_{12} en cultivos limitados (34±5 nM) resultaron menores pero cercanas a las concentraciones de cultivos no limitados (41±3 nM). El medio de cultivo fue preparado sin orgánicos disueltos los cuales podrían servir como sustrato para las bacterias presentes, lo cual sugiere que el crecimiento bacteriano estuvo mantenido por la materia orgánica liberada por el dinoflagelado, mientras que el consorcio bacteriano proporcionó B_1 y B_{12} en respuesta.

Palabras clave: Dinoflagelados; limitación de nutrientes; vitaminas; interacción dinoflagelado bacteria; Hibridación de fluorescencia *in situ*.

Dedicatoria

A mi madre…

Agradecimientos

Al Consejo Nacional de Ciencia y Tecnología (CONACYT) por el financiamiento de la beca doctoral y del proyecto CB-2008-01 106003 otorgado al Dr. Helmut Maske.

Al comité conformado por los doctores Helmut Maske, Rosa Reyna Mouriño Pérez, Ramón Cajal Medrano, Axayacatl Rocha Olivares y Curtis Suttle, por la retroalimentación. Especial agradecimiento al Dr. Helmut Maske por su dedicación y compromiso, y al Dr. Curtis Suttle por recibirme en su laboratorio y facilitarme el entrenamiento en diferentes aspectos de ecología microbiana marina.

A miembros presentes y pasados del laboratorio de Ecología de Microbios Marinos CICESE/UABC. Helmut Maske, Ramón Cajal, César Almeda, Josué Villegas, Catalina Gutiérrez, Mary Carmen Ruiz, Leonardo de la Rosa. Agradecimiento especial para César Almeda y Helmut Maske, por todo el apoyo técnico.

Al personal administrativo. Elizabeth Farías, Elvia Serrano, Dolores Sarracino, Citlali Romero, Norma Fuentes e Ivonne Best.

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1 Introduction

Abstract

Dinoflagellates are typically associated with stratified surface layers following blooms of diatoms. In coastal waters dinoflagellates can form dense surface blooms (red tides) a phenomenon of increasing global frequency. The mechanisms controlling the formation of these red tides are not known, but interspecies interactions between dinoflagellates and bacteria may play a role, with bacteria taking the part of pathogens, competitors or symbionts. It has been shown that bacteria can positively influence dinoflagellate dynamics through the production of vitamins. Although stimulation of dinoflagellate growth from vitamins produced by bacteria has been shown, the simultaneous limitation by several vitamins has not been studied experimentally, nor has the mutualistic relationship between vitamin-producing bacterial consortia and dinoflagellates. Here I review the literature with respect to interactions among bacterial and dinoflagellates under conditions of high dinoflagellate concentrations such as occur during red tides.

Keywords: Dinoflagellates; vitamins; nutrient limitation; heterotrophic bacteria.

1.1 Dinoflagellates - an important group of eukaryotes in the marine environment

Dinoflagellates (Phylum Alveolata, Supergroup Chromoalveolata) are unicellular protists mostly 10-100 μm in size. After diatoms, this group is the most abundant eukaryotic phytoplankton in fresh water and coastal systems (Moustafa *et al.* 2010).

Approximately 50% of all nuisance bloom forming species and 75% of harmful algal bloom species are dinoflagellates. Some dinoflagellate species form massive toxic or non-toxic blooms in coastal areas leading to negative impacts on human health, the fisheries industry and recreational values of coastal zones (Moustafa *et al.*, 2010). Such blooms produce patches of higher cell concentrations often near the surface, by upward migration during the day (e.g. red tides, or thin layers at intermediate depths) probably as a result of the interaction of cellular movement and water-column stratification. The cells at the surface are further concentrated by processes responsible for the formation of red tides, such as convergent surface-water currents or daily wind driven transport towards shore (Ruiz *et al.*, 2014). When phytoplankton growth is limited by low nutrient concentrations in the euphotic layer, and stratification impedes renewal of surface nutrients by turbulent mixing, then dinoflagellates have a competitive advantage by being able to migrate below the pycnocline to take up nutrients during the night and migrate to the euphotic layer to photosynthesize during the day. Surface dinoflagellate blooms characteristically produce dense cell aggregates that are easily observed, and produce km-scale size patches with chlorophyll concentrations that are 100-fold above common oceanic concentrations (Ault, 2000; Doblin *et al.*, 2006; Ryan *et al.*, 2008). These exceptionally high cell concentrations within these surface blooms create a particular environment where dinoflagellates are potentially exposed to strong inorganic and organic nutrient limitation including vitamin limitation.

1.2 Role of vitamins in algal physiology

Although the importance of organic growth factors such as vitamins received early attention (Provasoli and Pintner, 1953; Droop, 1954, 1955), recently there has been renewed interest in the role of vitamins in regulating phytoplankton community growth and structure. Novel developments in analytical techniques such as high performance liquid chromatography (HPLC), and culture-based surveys of vitamin requirements have identified B_{12} (cobalamin) and B_1 (thiamine) as highly important growth factors for eukaryotic phytoplankton and suggest that these micronutrients have the potential to influence marine productivity and species composition (Tang *et al.*, 2010; Bertrand and Allen, 2012). B_{12} and B_1 play numerous essential roles in cellular biochemistry, and therefore starvation for these nutrients can affect phytoplankton cellular metabolism through a range of mechanisms (Bertrand and Allen, 2012). Vitamin B_{12} (cobalamin) is essential for the synthesis of amino acids, deoxyriboses, and the reduction and transfer of single carbon fragments in many biochemical pathways, vitamin B_1 (thiamine) plays a pivotal role in intermediary carbon metabolism and is a cofactor for several enzymes involved in primary carbohydrate and branched-chain amino acid metabolism; whereas, vitamin B_7 (biotin) is a cofactor for several essential carboxylase enzymes, including acetyl coenzyme A (CoA) carboxylase, which is involved in fatty acid synthesis, and so is universally required (Croft *et al.*, 2006; Tang *et al.*, 2010).

Culture-based studies on the requirement of vitamins (Droop, 2007; Tang *et al.*, 2010) were mostly focused on single-vitamin limitation; whereas, most HABs-vitamin related field studies have inversely correlated dissolved B vitamins and phytoplankton cell abundances (Carlucci, 1970; Gobler *et al*., 2007; Koch *et al*., 2014), or focused on the role of inorganic elements and B_{12} vitamin (Droop, 1974; Bertrand *et al.*, 2007; Gobler *et al.*, 2007; Panzeca *et al.*, 2009), but these studies did not contemplate colimitation with other vitamins.

Trophic modes of dinoflagellates are complex; about 50% of them are photoautotrophic or mixotrophic, and the other 50% lack plastids and live heterotrophically. The

mixotrophic species have food vacuoles and plastids and represent the majority of toxin-producing species (Burkholder *et al.*, 2008). Independent of their trophic lifestyle, almost all are auxotrophs for B_{12} (cobalamin), 78% for B_1 (thiamine) and 32% for B_7 (biotin) (Croft *et al.*, 2005; Tang *et al.*, 2010) acquiring these vitamins from the environment.

1.3 Dinoflagellates interact with Bacteria

Dinoflagellates and bacteria have co-occurred in the oceans, presumably since dinoflagellates diverged from apicomplexans at least 800-900 million years ago. The relationship has been reinforced through phylogenomic reconstruction of horizontal gene transfer events from bacteria to dinoflagellates of nuclear and plastid genes (Bhattacharya and Nosenko, 2008; Wisecaver and Hackett, 2011; Moszczyński *et al.*, 2012).

Thus, dinoflagellates represent a potential ecological niche for bacteria that may involve several different spatial relationships. These include (a) both partners being independently suspended, (b) bacteria being attached to the outside of dinoflagellates (both live and dead), or (c) bacteria being within the dinoflagellate cells. During the course of a dinoflagellate bloom, bacterial abundances typically increase substantially when dinoflagellate concentrations are ~10^6 cell l^{-1} and dissolved organic matter (DOM) concentrations are high (Mayali and Azam, 2004;, Pinhassi *et al.*, 2005; Gasol *et al.*, 2005; Jasti *et al.*, 2005). The higher abundances of dinoflagellates and bacteria also implies that the interactions and contact rate between dinoflagellates and bacteria intensifies. Also, the trophic interaction betweens dinoflagellates and bacteria can take different forms, and may include (a) phagocytosis or grazing of bacteria by dinoflagellates, (b) a diel cycle of organic substrates for bacteria controlled by dinoflagellate photosynthesis, (c) competition for inorganic nutrients caused by the daily vertical migration of dinoflagellates, or (d) the degradation of dead algal cells by saprophytic bacteria (Pinhassi *et al.*, 2005; Gasol *et al.*, 2005).

Since these interactions are partially driven by organic matter provided by dinoflagellates, the chemical characteristics of this matter may affect bacterial communitiy succession and lead to a phylogenetic composition that differs from that in the surrounding water (Pinhassi *et al.*, 2005). Environmental and culture-based studies have provided insight into the complex network of dinoflagellate-bacterial associations, where the physiological profile of attached bacteria likely differs from that of bacteria in suspension, suggesting host specificity (Riemman and Winding, 2001; Fandino *et al.,* 2001; Sapp *et al.,* 2007). These inter-species networks indicate a number of specific interactions, including negative feedback such as parasitism (Palacios and Marín, 2008; Maki and Imai, 2001a, 2001b), growth inhibition (Hare *et al.,* 2005) and algicidal activity (Doucette *et al.,* 1999). Other interactions include defense or protection of the host, such as factors affecting toxin induction (Green *et al.,* 2004, 2006) and cyst induction (Adachi *et al.,* 2003, 2004; Mayali *et al.,* 2007). Positive feedback has also been observed such as growth stimulation (Ferrier *et al.,* 2002) and chemotaxis towards photosynthates (Miller *et al.*, 2004); whereas, mutualism by exchange of vitamins and dissolved organic matter have been suggested by Croft *et al.* (2005) and Kazamia *et al.* (2012).

The cell wall and exudate biochemistry is characteristically different among dinoflagellates; hence, each dinoflagellate cell can be considered to be a unique microenvironment for its associated bacteria and may explain specific bacterial associations among dinoflagellate hosts (Jasti *et al.,* 2005). These substrates produced and released by dinoflagellates, including structural polysaccharide components of algal cell walls, are important in allowing cell-surface colonization by bacteria, but also for the dinoflagellate cell by supporting the growth of bacteria that can provide essential nutrients for dinoflagellate (Wagner-Döbler *et al.*, 2010); this is probably an important factor shaping specific dinoflagellate-bacteria interactions.

Bacteria can colonize dinoflagellates, but healthy phytoplankton cells generally can prevent bacterial colonization. The mechanisms that keep phytoplankton cells free of attached bacteria have not been clearly identified but may include the production of antibiotics, mucus low encounter rates with bacteria that can adhere, or the probability

that attached bacteria are diluted by relatively rapid dinoflagellate growth rates (Jasti *et al.,* 2005; Mayali *et al.,* 2007). Phylogenetic studies have provided insight into the complex attached and suspended bacterial communities associated with dinoflagellates (Green *et al.,* 2004; Jasti *et al.,* 2005; Mayali *et al.,* 2011) in the field and in culture. Although comprehensive assessments of whole bacterial communities on dinoflagellate surfaces are relatively scarce (Alavi *et al.,* 2001; Alverca *et al.,* 2002; Biegala *et al.,* 2002; Simon *et al.,* 2002; Mayali *et al.,* 2011), the available data suggest that bacterial communities associated with dinoflagellates show some degree of specificity for their host . These results are based mostly on 16S rRNA gene sequences of clone libraries, or molecular fingerprints (DGGE, RFLP), which have shown clear differences between the bacterial communities attached to dinoflagellates and those in the surrounding water. In addition, differences in bacterial composition have been observed among dinoflagellate species (Adachi *et al.,* 2003; 2004; Mayali *et al.,* 2007). Different dinoflagellate species in the same habitat showed different bacterial communities (Hold *et al.,* 2001; López-Pérez *et al.,* 2012), while the same algal species, even from different locations, had microbial communities that were very similar (Su *et al.,* 2007; Imai and Kimura, 2008; Lee *et al.,* 2008; Wang *et al.,* 2010).

Because only some members of Bacteria, Archaea and cyanobacteria can synthetize vitamins (Sañudo-Wilhelmy *et al.,* 2014) they must be the ultimate source for auxotrophic eukaryotic phytoplankton, either through cycling of the microbial loop and release of vitamins into the medium or through direct symbiotic interaction (Wagner-Döbler *et al.,* 2010; Bertrand *et al.,* 2011; Kazamia *et al.,* 2012). Croft *et al.,* 2005 showed that the B_{12}-producing bacterium *Halomonas* sp. provided most of the B_{12} requirements of several marine algae in culture. They concluded that the mutualism existed because the bacterium presumably used algal photosynthates to grow while providing vitamins to their hosts. On the other hand, Droop (2007) hypothesized that the extremely low requirements for cobalamin of many algal species could be fulfilled by scavenging the dissolved vitamins at concentrations typically found in seawater rather than requiring a symbiotic relationship based on spatial proximity. This is in contrast to recent studies suggesting that the ambient dissolved concentration of vitamins may be

insufficient to support maximum productivity in coastal areas during bloom events (Gobler *et al.,* 2007). This would conform with results by Tang *et al.* (2010) that showed the vitamin needs of harmful algal bloom populations are higher than non-bloom forming eukaryotic phytoplankton species and results of Wagner-Döbler *et al.,* (2010) showing that the aerobic anoxygenic phototrophic bacterium *Dinoroseobacter shibae* could supply both vitamin B_1 and B_{12} to its dinoflagellate host *Prorocentrum lima*.

At present, relatively little is known about how natural bacterial assemblages interact with harmful algal bloom (HAB) populations (Garcés *et al.,* 2007). Chemicals released by bacteria dramatically decrease in concentration at a distance of 10 μm from the bacterial cell due to diffusion and advection (Amin *et al.,* 2012; Stocker and Seymour, 2012); however if the bacterium is attached to a dinoflagellate cell, the algal cell would be exposed persistently to high concentrations of the chemical. The importance of bacterial attachment to the phytoplankton cell surface in mediating these interactions has not been fully evaluated. In the relatively dilute world of seawater, bacterial attachment to phytoplankton may be essential for these interactions to be effective (Mayali *et al.,* 2007).

Identification, localization and quantification of specific bacterial taxa, closely associated with phytoplankton, are thus of prime importance for a better understanding of the occurrence of blooms and more generally to assess bacteria-phytoplankton association in marine pelagic ecosystems. The physical association between bacteria and dinoflagellates has been studied by different technical approaches including: scanning-electron and epifluorescence microscopy for localization and quantification; whereas, identification of bacteria involved destructive techniques such as DNA extraction followed by sequencing or dot-blot hybridization. However, these techniques cannot simultaneously provide the identity and spatial localization of bacteria physically associated with phytoplankton. Both types of information could be obtained by *in situ* hybridization using oligonucleotide probes targetting 16S rRNA, associated with a precise method of detection such as confocal microscopy (Biegala *et al.,* 2002).

1.4 Thesis objectives

Based on studies which have shown that dinoflagellates require exogenous source of vitamins, mainly B_1 and B_{12}, the goals of this dissertation were to investigate vitamin auxotrophy and threshold-type limitation for the dinoflagellate *Lingulodinium polyedrum*, and to develop a microscope-base tool to evaluate the co-occurrence of distinct bacterial groups attached to dinoflagellates under different vitamin conditions, since the source of vitamins are at least for dinoflagellates, bacterial groups,
This dissertation addressed the following questions:

1. Is the dinoflagellate *Lingulodinium polyedrum* (*Lp*) auxotroph for vitamins?
2. Are the associated bacteria the primary source of vitamins?

To address these questions, the dissertation is structure as follows:

Chapter 2 analyzes the vitamins requirements and dual limitation of the dinoflagellate model *Lp*.

Chapter 3 describes a modified *in situ* method to observe and quantify with specific molecular probes the bacterial attachment on the dinoflagellate *Lp*.

Chapter 4 describes the vitamin production from a natural bacterial consortia and subsequent utilization from the dinoflagellate *Lp*.

Chapter 5 summarizes the results.

Chapter 6 gives a general conclusion on this study.

2 The marine dinoflagellate *Lingulodinium polyedrum* exhibits auxotrophy and threshold-type dual limitation for B_1 and B_{12} vitamins with possible multiplicative limitation at very low concentrations

Abstract

Using the marine dinoflagellate *Lingulodinium polyedrum (Lp)* in culture we examined the influence of vitamin B_1, B_7 and B_{12} availability on growth rate. Our approach involved the use of small volume bioassays of this dinoflagellate in batch cultures, and monitoring growth by *in vivo* chlorophyll-*a* fluorescence. The results demonstrate independent threshold type limitation for vitamins B_1 and B_{12}. Using a factorial design based on different concentrations of B_1 and B_{12} we found that at the lowest concentrations tested, 0.033 pM B_1 and 0.053 pM B_{12} growth was limited. When the concentration of only one of the vitamins was increased then growth rate increased significantly suggesting multiplicative B_1 and B_{12} limitation at these low concentrations.

Keywords: Dinoflagellates; vitamin auxotrophy; vitamin limitation.

2.1 Introduction

A major portion of eukaryotic phytoplankton need an external source of one or more B vitamins (auxotrophs) for growth. This implies that vitamin availability can be a limiting resource and can have an impact on marine phytoplankton growth and community composition (Peperzak *et al*., 2000; Bertrand *et al*., 2007; Gobler *et al*., 2007; Koch *et al*., 2011, 2012; Tang *et al*., 2010).

Over half of all species of eukaryotic phytoplankton require an exogenous supply of cobalamin (hereafter B_{12}), over 20% require thiamine (hereafter B_1) and over 5% need biotin (hereafter B_7) (Tang *et al*., 2010). B_{12} is essential for the synthesis of amino acids, deoxyriboses, and the reduction and transfer of single carbon fragments in many biochemical pathways. B_1 plays a pivotal role in intermediary carbon metabolism and is a cofactor for a number of enzymes involved in primary carbohydrate and branched-chain amino acid metabolism. B_7 is a cofactor for several essential carboxylases, including acetyl coenzyme A (CoA) carboxylase, which is involved in fatty acid synthesis (Croft *et al*., 2006, Tang *et al*., 2010). Tang *et al*., (2010) concluded that vitamin auxotrophy of harmful algal bloom (HAB) forming species is more common than for non-bloom forming species. Among bloom forming dinoflagellate species, 100% require B_{12}, 78% B_1 and 32% B_7.

There is little experimental information available on dinoflagellate growth because they are easily damaged by turbulence, making quantitative growth experiments difficult (Van de Waal *et al*., 2014).

The dinoflagellate *Lp* is a mixotrophic dinoflagellate (Jeong *et al*., 2005) that has been implicated in recurrent blooms along the coast of southern California and northern Baja California (Holmes *et al*., 1967; Kudela and Cochlan, 2000; Peña-Manjarréz *et al*., 2005). Although its physiology has been extensively studied (reviewed in Hastings 2007; Beauchemin *et al*., 2012), little is known about its vitamin auxotrophy. The only previous vitamin-related studies dates from Carlucci (1970) and Gobler *et al.* (2007) which

suggested that *Lp* utilizes B_1, B_7 and B_{12} vitamins. This conclusion was not based on culture experiments but on correlations of vitamin concentrations and cell abundances in coastal waters.

In vitamin experimental physiology most efforts have focused on single-vitamin limitation, for example B_{12} at different concentrations (reviewed in Droop, 2007; Tang *et al*., 2010), or the potential interaction of these with inorganic nutrients such nitrogen (Droop, 1974; Gobler *et al*., 2007; Bertrand and Allen, 2012), trace elements such as iron (Bertrand *et al*., 2007, 2011) or CO_2 (King *et al*., 2011). Previous studies did not investigate the potential for co-limitation of B_{12} with another essential vitamin. Here we report on the growth of *Lp* under conditions of single and multiple low vitamin concentrations. The data suggest that *Lp* is auxotrophic for both B_1 and B_{12}, where high concentration of one cannot overcome the lack of the other. At low, ecologically relevant concentrations multiple limitations allow increased growth when the concentration of only one vitamin is increased.

2.2 Materials and Methods

2.2.1 Strain and growth conditions

Natural oceanic seawater was collected off the coast of Ensenada, México (31.671° N, 116.693° W), treated with activated charcoal, filtered through GF/F, and 0.22-μm pore-size cartridge (Pall corporation) filters and aged for two months to remove residual organic matter. Aged seawater was sparged with CO_2 (5 min per 1 L of seawater), autoclaved for 15 min and then equilibrated with air. Non-axenic *Lp* strain HJ (Latz Laboratory, UCSD-SIO) was grown in L1 medium (NCMA-Bigelow, see Appendix A) prepared with aged oceanic water under 12:12 h light:dark cycle at an irradiance level of 100 μmol m^2s^{-1} and a temperature of 20°C.

2.2.2 Assessment of B vitamin auxotrophy

Bacteria are a potential source of B_1, B_7 and $B_{12,}$ making it necessary to establish axenic cultures for auxotrophy studies. *Lp* cultures were incubated three times with antibiotic solution (Sigma-Aldrich, P4083-100ML). Bacterial presence in the *Lp* culture was checked by staining filtered samples with the nucleic acid-specific stain 4',6-diamino-2-phenylindole (DAPI) (1μg ml^{-1}) and visual observation with epifluorescence microscopy (Axioskope II plus, Carl Zeiss, Oberkochen, Germany) connected by a liquid-light guide to a 175W xenon arc lamp (Lambda LS, Sutter) under X100 objective lens (Plan-Apochromat, Carl Zeiss).

To test the vitamin auxotrophic status of *Lp*, cultures were grown semi-continuously in 15 ml glass test tubes and silicon caps; before sampling they were carefully mixed by an inclined rotating test tube holder (10 rpm) before fluorescence measurements for growth determination. Semi-continuous cultures were used during the acclimation period of five consecutive transfers. Cultures were grown semi-continuously to maintain cells in the exponential growth phase to provide similar physiological conditions during sampling in different experimental treatments. The medium for axenic cultures of *Lp.* was

supplemented with the L1 vitamin mix (B_1, 2.96 x 10^{-7} M; B_7, 2.05 x10^{-9} M; B_{12}, 3.69 x 10^{-10} M final concentration), or with separate individually added vitamins at the same concentration (B_1, Sigma-Aldrich; B_7, Sigma-Aldrich; B_{12}, Sigma-Aldrich). These individually added vitamins were used in different combinations: B_1+B_{12}, B_1+B_7, B_7+B_{12}. Auxotrophy for a vitamin was declared when a culture ceased to grow in the absence of vitamins while growth persisted in parallel control treatments with added vitamin.

2.2.3 Assessment of vitamin B_1, B_7 and B_{12} auxotrophy

To assess the dinoflagellate B vitamin requirements, axenic semi-continuous cultures were grown for six weeks in cell culture flasks (BD Falcon™) containing 30 ml L1 medium, with an factorial experimental design combining 1) two vitamins (B_1 + B_{12}) and five vitamin concentrations ranging from 3.33 x 10^{-2} to 3.33 x 10^2 pM B_1 and 5.25 x 10^{-2} to 5.25 x 10^2 pM B_{12} (Table 1). In our semi-continuous culture design, weekly dilutions were used to prevent the cultures from entering stationary phase. The single culture tubes were placed in at random in the transparent test tube holders to minimize bias in the results due to a heterogeneous light field in the incubator or shading by other cultures tubes.

Table 1. Experimental design for testing vitamin limitation using triplicate sub-cultures for each condition, therefore the experimental set comprised 75 sub-cultures.

		factor 1 (pM B_{12})					
	level	1	2	3	4	5	
factor 2 (pM B_1)	level	0.053	0.526	5.26	52.5	525	
	1	0.033	x 3	x 3	x 3	x 3	x 3
	2	0.33	x 3	x 3	x 3	x 3	x 3
	3	3.33	x 3	x 3	x 3	x 3	x 3
	4	33.3	x 3	x 3	x 3	x 3	x 3
	5	333	x 3	x 3	x 3	x 3	x 3

In all experiments growth rate was measured by monitoring changes over time of *in vivo* chlorophyll fluorescence (IVF), (Turner Designs 10-000) as a proxy for cell abundances and is reported as relative fluorescence units. Comparison of cell counts and *in vivo* fluorescence were correlated (see Appendix B). Growth rates (hereafter μ (d-1)) were calculated from $\mu = \ln(\Delta F)/\Delta day$, using the exponential portion of the growth curve, specifically day 2 and day 10 for the growth rate estimate.

2.3 Results

2.3.1 Effect of B_1, B_7 and B_{12} requirements on specific growth rates

In order to assess the vitamin auxotrophy for the marine dinoflagellate *Lp*, our first approach was to determine its dependence on B_1, B_7 and B_{12} vitamins. Our initial experiment included axenic cultures amended with L1 medium. The positive control contained L1 medium plus vitamins; whereas, the negative control excluded the vitamin mixture from the medium. This test was designed to explore the ability of the dinoflagellate to thrive under vitamin depletion and determine the auxotrophy status. Dinoflagellate cultures were transferred to fresh medium within seven to nine days depending on the μ. Up to five subcultures were carried out, or until the algae ceased growth. Three culture replicates were carried out for each condition.

The initial experiments set a baseline for the vitamin auxotrophy of the dinoflagellate. The μ in the controls showed no significant differences between consecutive transfers ($p>0.05$). In contrast, after the second sub-culture, μ in the treatment started to decreased compared with the controls ($p<0.05$), and ceased to grow after the fourth sub-culture (Figure 1). This first experiment probed the auxotrophic status of the dinoflagellate *Lp*, and demonstrated for other HAB-producing species (Tang *et al*., 2010), vitamins probed to be required for growth.

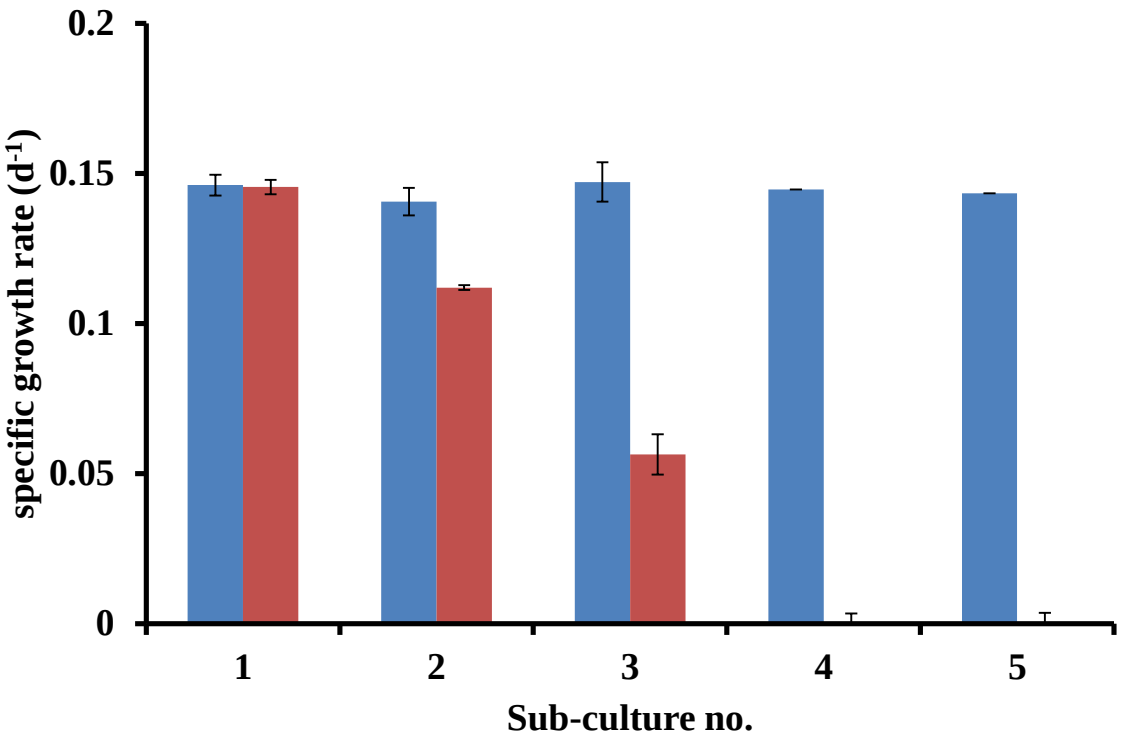

Figure 1. Specific growth rate for axenic *Lp* grown in B_1+B_7+B_{12}-replete (■) and B_1+B_7+B_{12}-limited (■) cultures. Error bars represent one s.d. (*n*=3).

The effect of individual vitamins on μ was examined by supplementing *Lp* cultures with all three vitamins (control), or with only one vitamin added, as follows: 2.96 x 10^5 pM B_1, 2.05 x 10^3 pM B_7 or 2.69 x 10^2 pM B_{12}. Cultures containing the three vitamins continued growing after five subcultures (p>0.05), while cultures to which medium containing only one of the three vitamins was added showed reduced growth after one transfer. After the third transfer, the cultures ceased growth, indicating that one vitamin in non-limiting concentration was not sufficient to support growth (Figure 2). The combinations of vitamin pairs tested in axenic cultures (B_1+B_{12}, B_1+B_7 and B_7+B_{12}), at the concentrations shown in Figure 2, showed that after five sequential subcultures the combination B_1+B_{12} showed the same μ as when the full complement of vitamins was added; whereas, the

other two combinations ceased to grow (Figure 3). In the experiments, all the cultures that eventually stopped growing due to vitamin limitation behaved similarly during the first three subcultures, with decreasing but positive growth before growth ceased completely. Growth in the vitamin-limited cultures could be initially sustained by residual vitamins carried over with the inoculum, or by luxury cell quota (i.e. excess intracellularly stored vitamins).

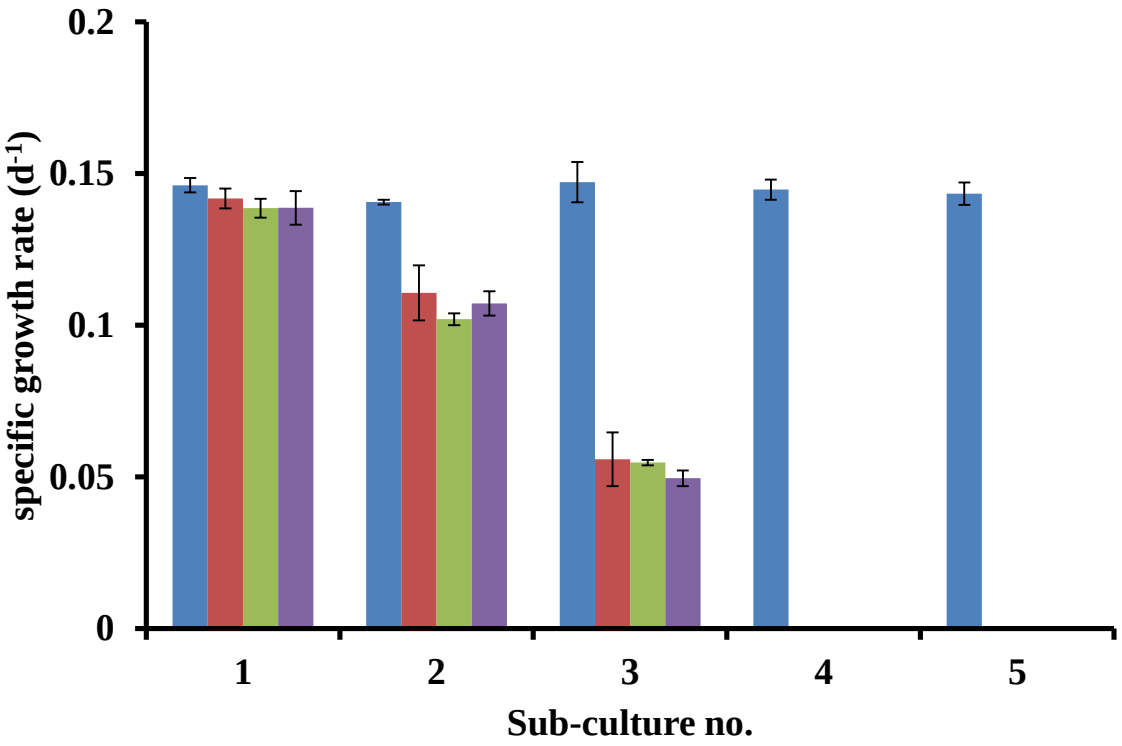

Figure 2. Specific growth rate for axenic *Lp* grown in B_1+B_7+B_{12}-replete (■), B_1-replete (■), B_7-replete (■) and B_{12}-replete (■) cultures. Error bars represent one s.d. (*n*=3).

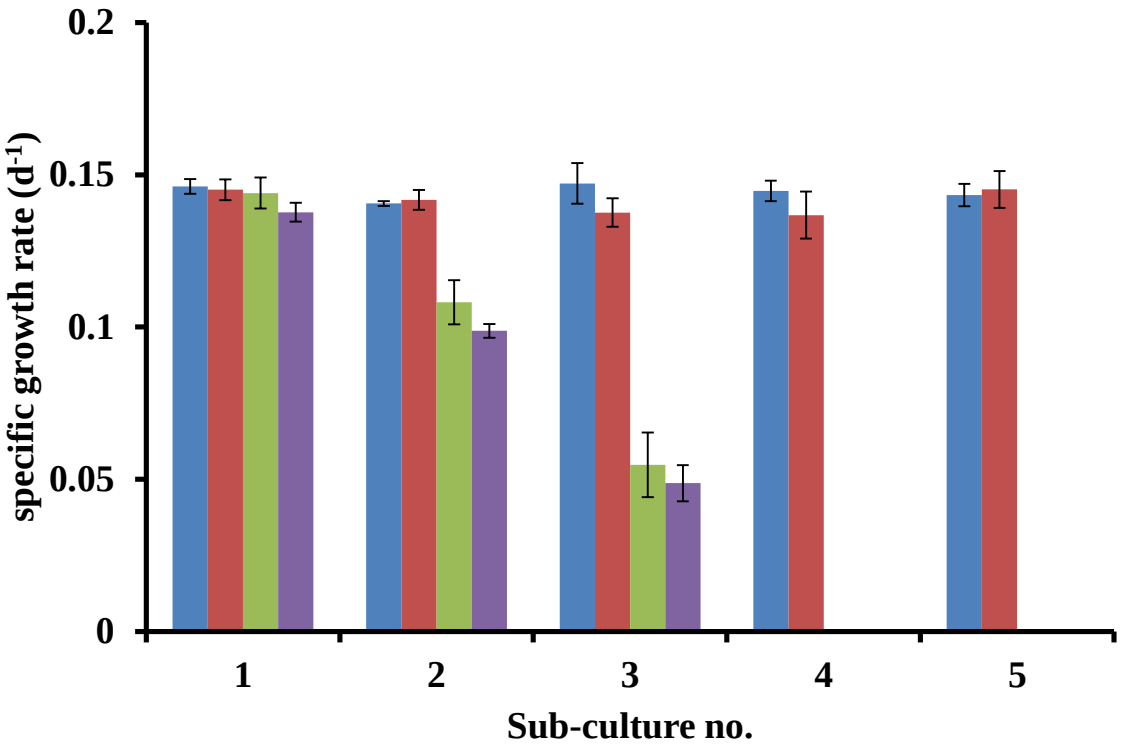

Figure 3. Specific growth rate for axenic *Lp* grown in B_1+B_7+B_{12}-replete (■), B_1+B_{12}-replete (■), B_1+B_7-replete (■) and B_7+B_{12}-replete (■) cultures. Error bars represent one s.d. (*n*=3).

2.3.2 Threshold-type vitamin limitation

In previous sections we clearly observed a classical Liebig´s limitation, biomass formation was prevented by the lack of one of the auxotrophic vitamins (Figure 2 and 3) similar to the threshold-type limitation established for vitamin and phosphorus for *Monochrysis lutheri* cultures (Droop, 1974).

We probed further the possible interactive growth control of the two auxotrophic vitamins with an experimental design where five different concentrations each of B_1 and B_{12} were combined ranging from B_1, $3.33x10^{-2}$ - $3.33x10^{2}$ pM and B_{12}, $5.52x10^{-2}$ – $5.52x10^{2}$ pM (Table 1). Three-dimensional representation of factorial experiment shows a clear threshold for B_1 at 0.333 pM and for B_{12} at 0.526 pM (Figure 4). The data could be modeled with a 3 x 3 polynomial that indicated that the maximum μ was found in the combination of 0.052 pM B_{12} and 3.33 pM B_1 (Figure 4). The polynomial model

parameters are specified in Table 2.2. The growth rate residuals calculated as data minus polynomial showed no bias in either the B_1 or B_{12} dimension (Figure 5).

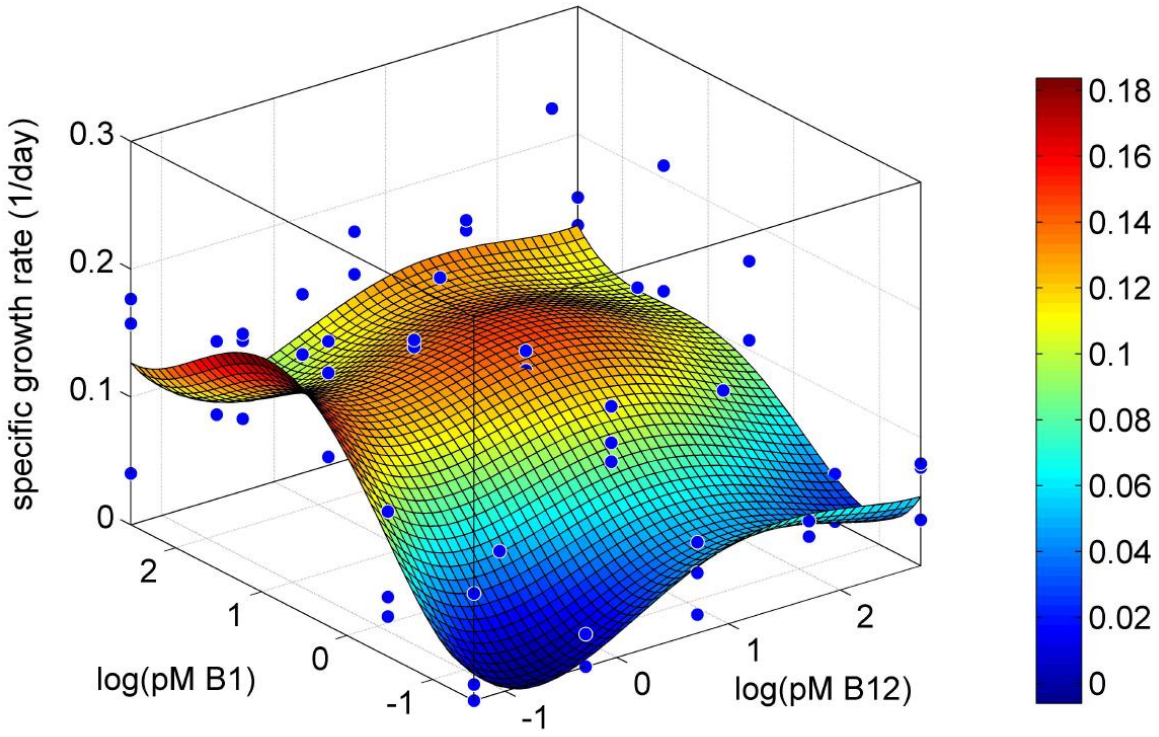

Figure 4. Three-dimensional representation of limitation with two vitamins (Table 1). Specific growth rate for axenic *Lp*. Colors represent specific growth rate (μ d^{-1}).

Table 2. The factors defining the polynomial calculating specific growth rate (d^{-1}) as a function of B_1 and B_{12} concentrations (pM), resulting in the response surface in Figure 4.

Polynomial: f(x,y) = p00 + p10*x + p01*y + p20*x^2 + p11*x*y + p02*y^2 + p30*x^3 + p21*x^2*y + p12*x*y^2 + p03*y^3.

p00=	0.08855	(0.0572, 0.1199)
p10=	0.000863	(-0.0224, 0.02413)
p01=	0.04867	(0.02308, 0.07427)
p20=	0.01635	(-0.002083, 0.03478)
p11=	-0.0097	(-0.02137, 0.00197)
p02=	-0.00673	(-0.02139, 0.00794)
p30=	-0.00773	(-0.01533, -0.0001249)
p21=	0.00099	(-0.004372, 0.006352)
p12=	0.006243	(0.0007606, 0.01172)
p03=	-0.00445	(-0.01209, 0.003187)

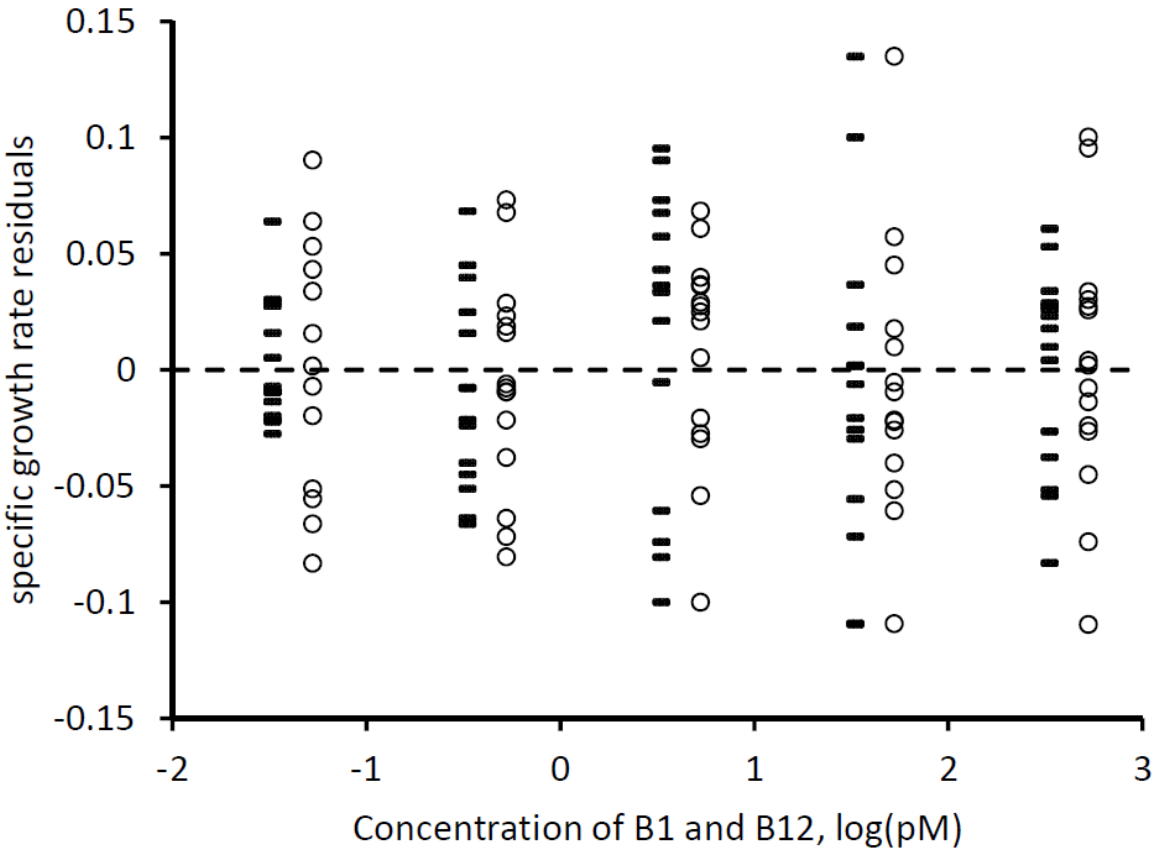

Figure 5. Residuals from polynomial model of specific growth rate as a function of vitamin concentrations, pM ○ log B_{12}, - log B_1.

2.4 Discussion

2.4.1 Vitamin auxotrophy

Lp is a notorious dinoflagellate specie in the California Current System because of its capacity to form extensive algal blooms. Despite the observations and physiological research on the genus *Lingulodinium* (Hastings, 2007; Beauchemin *et al*., 2012), its vitamin requirements have not been published. Previous work by Carlucci (1970) found a strong correlation between the increase in *Lp* cells and the decline of B_1, B_7 and B_{12} or for B_{12} (Panzeca *et al.,* 2009) off the California coast. Apart from this work no information on vitamin limitation in *Lp* has been published. Our initial experiments with axenic *Lp* cultures established a clear auxotrophy for B_1 and B_{12} but not for B_7 (Figures 2 and 3). There are no culture-based studies to compare B_1 and B_{12} growth rate limitation for any dinoflagellate. The lack of culture studies using dinoflagellates may stem from the experimental difficulty to work quantitatively with axenic cultures of this group, specifically because they are easily damaged by turbulence which makes mixing difficult (Van de Waal *et al*., 2014).

In a second experiment, a bioassay was developed to establish the pair of vitamins required for growth. Cells where grown under three pairs of vitamin combinations, B_1+B_{12}, B_1+B_7 and B_7+B_{12}, but only the first combination supported continuous growth, and showed no statistical difference with control cultures amended with the vitamin mix of the L1 medium that included B_1, B_7 and B_{12} (Figure 3). The dual vitamin dependence was expected because previous studies argued that phytoplankton species that have arisen as a result of secondary and tertiary endosymbiosis require a suit of B vitamins (Croft *et al.,* 2006). *Lp* follows the general pattern of B_1 and B_{12} auxotrophy but not B_7, consistent with reports that fewer HAB species were auxotrophic for B_7 than for B_1 and B_{12}.

For two substrates at near limiting concentrations, Droop (1974) defined ‘threshold-type’ limitation as both substrates limiting growth independently leading to a Liebig-type of response in biomass, and ‘multiplicative limitation’ as both substrates metabolically

interacting in such a way that an increase in the concentration of either substrate will increase growth and biomass formation. We conducted a growth experiment with triplicates in a 5 x 5 array of different concentrations of B_1 and B_{12}. The results showed a response surface that could be modeled as a 3 x 3 polynomial (Figure 4) and suggests that at low concentrations the two vitamins interact, and an increase in the concentration of either will increase growth rate, even when the other vitamin is at a growth-limiting concentration.

2.4.2 Ecological implications

A threshold-type of limitation for Fe/B_{12} and N/B_{12} has been documented for phytoplankton communities in HNLC areas of the Southern Ocean (Bertrand *et al.,* 2007; Bertrand and Allen, 2011; Koch *et al.,* 2011) and the Gulf of Alaska (Panzeca *et al.,* 2006); it took the addition of both limiting components to induce an increase in metabolic rate. These bioassays applied to ocean samples would be similar to our experiments reported in Figure 3, except in a community with different taxa, which may be limited by another substrate, and the community response might encompass a range of dissimilar responses from different fractions of the community. The uptake of B_1 and B_{12} uptake has been documented for phytoplankton communities in coastal areas (Gobler *et al.,* 2007; Koch *et al.,* 2011, 2012, 2013, 2014), but the potential for threshold-type limitation of B_1 and B_{12} has not been reported. Given the slow μ of dinoflagellates it is difficult to imagine that traditional bioassay experiments with natural populations probing vitamin limitation would yield conclusive results for dinoflagellates.

2.5 Conclusion

Here we show that *Lp* is auxotrophic for B_1 and B_{12}. From the factorial design experiment we can estimate the *in situ* concentrations of B_1 and B_{12} necessary to support maximum growth rates for *Lp* as 3.3 pM and 5.3 pM respectively. These concentrations can be compared with measured *in situ* concentrations. In coastal systems B_{12} ranged from undetectable to 87 pM (Panzeca *et al.,* 2009; Sañudo-Wilhelmy *et al.,* 2006, 2012) and for B_1 from undetectable to 200 pM (Gobler *et al.,*

2007; Koch *et al.,* 2012, 2013; Sañudo-Wilhelmy *et al.,* 2012). The comparison suggests that B_1 or B_{12} might limit the growth rate of *Lp* in parts of the ocean. The interpretation of *in situ* concentrations is complicated by the apparent multiplicative-type response of both vitamins at concentrations limiting to growth (Figure 4). Koch *et al.* (2014) measured B_1 and B_{12} concentrations inside and outside of dinoflagellate blooms and found concentrations higher than the limiting concentrations reported in Figure 3. They also reported that vitamin concentrations inside dinoflagellate blooms were lower than outside bloom waters which pointed to active uptake and the possibility of vitamin limitation. Further field data will have to show if coastal waters that are less eutrophic than their study area can effectively limit the development of dinoflagellate blooms.

3 A non-amplified FISH protocol to identify simultaneously different bacterial groups attached to eukaryotic phytoplankton

Abstract

Eukaryotic phytoplankton and bacteria form complex trophic interactions in nature and in culture. The nature of the interaction will depend on their spatial position of the bacteria and phytoplankton, but the architecture of these arrangements has been scarcely investigated. Here we modified a protocol in order to identify and localize multiple bacterial taxa attached to phytoplankton in culture and in natural samples, including dinoflagellates. Samples were embedded in agarose and hybridized simultaneously with different probes with distinct fluorescence properties. Embedding avoided losses and damage to host cells and attached bacteria during hybridization and washing, while allowing for efficient hybridization and identification of intact host-cells. After fluorescence *in situ* hybridization of the bacteria, the phytoplankton host cells, including dinoflagellates were still intact. Digital image stacks were taken with a wide-field epifluorescence microscope using different excitation-emission wavelength combinations allowed to locate the bacterial groups and their spatial position on the host-cell surface.

Keywords: Fluorescence *in situ* hybridization; epiphytic bacteria; phytoplankton cells.

3.1 Introduction

Eukaryotic phytoplankton represent a potential ecological niche for symbiotic or parasitic heterotrophic bacteria. The physiological profile of attached bacteria likely differs from those in suspension, suggesting host specificity (Riemann and Winding, 2001; Sapp *et al*., 2007). Environmental and culture-based studies of these inter-species networks have indicated a number of specific interactions among different microbes and eukaryotic phytoplankton, including intracellular interaction (Palacios and Marín, 2008; Maki and Imai, 2001a, 2001b), trade-offs of soluble factors such as vitamins (Wagner-Döbler *et al*., 2010), iron siderophores (Amin *et al*., 2009), growth stimulators (Ferrier *et al*., 2002), toxin inducers (Green *et al*., 2004, 2006), cyst inducers (Adachi *et al*., 2003, 2004; Mayali *et al*., 2007), growth inhibitors (Hare *et al*., 2005), algicidals (Doucette *et al*., 1999) and chemosensors (Miller *et al*., 2004). Some of these interactions are of practical importance for the success of commercial algal cultures or the development of harmful algal blooms. Despite these studies (reviewed in Amin *et al*., 2012; Goecke *et al*., 2013), little research has considered the physical association of epiphytic bacteria with eukaryotic phytoplankton.

Over the last 12 years a few studies have used fluorescence *in situ* hybridization (FISH) for the phylogenetic identification and enumeration of bacteria physically associated with phytoplankton, including dinoflagellates (Alavi *et al.*, 2001; Alverca *et al*., 2002; Biegala *et al.,* 2002; Simon *et al.,* 2002; Palacios and Marín, 2008; Wagner-Döbler *et al.*, 2010; Mayali *et al.*, 2011), chlorophytes (de-Bashan *et al.*, 2011), and diatoms (Bennke *et al.*, 2013) using either by widefield epifluorescence (EFM) or confocal laser scanning microscopy (CLSM).

Some technical problems of using standard FISH combined with widefield epifluorescence microscopy for studying epiphytic bacteria on phytoplankton, have been weak monolabel probe emission, optical interference of host chlorophyll autofluorescence and the filter background (Biegala *et al*., 2002; Palacios and Marin, 2008). Consequently, catalyzed reporter deposition-FISH (CARD-FISH) is a preferred

option, mainly because the stronger fluorescence signal provided by CARD, facilitates the observation of bacteria on phytoplankton. On the other hand, the amplified fluorescence signal of bacteria attached to phytoplankton produced by CARD-FISH is restricted to one pair of probes in a single assay (Palacios and Marín, 2008). FISH and CARD-FISH are often used with confocal microscopy where the use of different fluorochromes is limited by the number of lasers installed (Valm *et al*., 2011).

A general limitation of FISH is the low number of different target organisms that can be simultaneously detected using probes with different fluorochromes due to the limited optical discrimination; optical crosstalk generally restricts to three the number of different simultaneously used fluorochromes in each sample. This limitation has recently been overcome by Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH) (Valm *et al*., 2011). The CLASI-FISH method has lower sensitivity because different labeled probes compete for the same target site. To solve this problem a double-labeled probe (DOPE-FISH) with different fluorochromes was developed (Behnam *et al*., 2012), resulting in higher sensitivity and less bias. These modifications to the initial FISH protocol have been used to study bacterial communities, but have not been used to examine their association with eukaryotic phytoplankton. The CLASI-FISH and DOPE-FISH methods are effective but expensive in instrumental investment and consumables.

We developed a cost-effective method for FISH using three simultaneous probes labeled with three different single fluorochromes combined with widefield epifluorescence microscopy. The probes were specific for the following three major bacterial groups associated with phytoplankton: α-proteobacteria subclass, γ-proteobacteria subclass (*Alteromonas* clade) and the phylum *Bacteroidetes*. The method was developed to document the concurrent presence of different bacterial taxa on intact phytoplankton cells. The method was tested on natural samples and cultures of dinoflagellates and diatoms. We were interested in dinoflagellates because of the socioeconomic impact of their coastal blooms, but also because of the potentially symbiotic relationship between vitamin auxotrophic phytoplankton and epiphytic vitamin-producing bacteria.

3.2 Materials and methods

Seawater was collected off the coast of Ensenada, México (31.671° N, 116.693° W) treated with activated charcoal, filtered through a glass-fiber (GF/F) membrane filter, and a 0.22-μm pore-size cartridge filter (Pall corporation) and stored in the dark at room temperature to age for at least two months. Aged seawater was sparged with CO_2 (5 min per 1 L of seawater), autoclaved for 15 min and then equilibrated with air. *Lp* HJ (Latz laboratory, UCSD-SIO) was maintained in 250-mL flasks containing 100 mL L1 enriched (NCMA) aged seawater medium. Light was provided by cool white fluorescence tubes at an irradiance of 100 μmol m^2s^{-1} on a 12:12 h light:dark cycle and a temperature of 20°C. *Grammatophora angulosa* (Microalgae-Biology and Culture lab, CICESE) was maintained in a 200-ml flask containing 100 ml of f medium (Guillard and Ryther, 1962) and filtered seawater. Light was provided by cool-white fluorescence tubes at an irradiance of 100 μmol m^2s^{-1} on a 24h light cycle and a temperature of 20°C. Field samples were collected onshore and in the port of Ensenada (Ensenada, Baja California, México), gently centrifuged (700 rpm) for 5 min and fixed with 1% paraformaldehyde - phosphate-buffered saline (PFA-PBS).

Ten ml samples were fixed at a final concentration of 1% PFA-PBS in 15 ml clear polypropylene (PP) centrifuge tubes (Corning®). Fixed samples were gravity-settled for 4h at 4°C and washed in 1X PBS (0.1 M NaCl, 2mM KCl, 4 mM Na_2HPO_4, pH 8.1). Washed samples were immobilized onto an 8.0 μm pore size, 25 mm-diameter Nuclepore filter (Whatman International, Ltd., Maidstone, England) using a pressure difference of <3.5 kPa to avoid cell disintegration. To avoid cell losses in further processing, 15 μl of low-melting point agarose (0.05%, LMA) (BioRad, 161-3111) at 55°C were pipetted onto the center of the filter and dried for 15 min at 46°C. Embedded cells were dehydrated for 5 min in each step of 50%, 80% and 96% ethanol series at -20°C, and a final step of 70% at -20°C for 12h to reduce chlorophyll autofluorescence.

All in situ hybridizations were performed as described in Glöckner *et al.* (1999) with small modifications as in Pavlekovic *et al*., (2009). Before hybridization, bacterial

cells were partially digested with 400,000 U ml^{-1} lysozyme (Sigma, L6876) dissolved in buffer containing 100mM Tris-HCl, 50 mM EDTA, pH 8.0 for 1h at 37°C. The enzyme reaction was stopped by rinsing the filter three times with 5 ml sterile water for 1 min at 4°C. Embedded samples were hybridized with a buffer containing 900 mM NaCl, 20 mM Tris-HCl and 0.02% SDS at pH 8.0. When probes with different hybridization stringency optima were applied to the same sample, several hybridizations were performed, beginning with the probes requiring the most stringent conditions (Almstrand *et al.,* 2013). The probe sequences, hybridization conditions and spectral characteristics are listed in Table 3. Hybridizations containing 1μl of probe for every 20 μl of buffer (final probe concentration =25 ng μl^{-1}) were performed at 46°C for 2 h. After this, filters were washed with pre-warmed (48°C) buffer (900 mM NaCl, 20 mM Tris-HCl, 0.02% SDS, 5 mM EDTA) for 15 min and rinsed for 5 min in distilled H_2O. To localize the theca and bacterial cells, we used Calcofluor white (5 μg ml^{-1}) (Sigma-Aldrich, México City, México) to stain the theca of dinoflagellates and 4´,6´-diamino-2-phenylindole (DAPI; 1.5 μg ml^{-1})(Invitrogen, Eugene, OR) to stain the DNA in the host nucleus. This low DAPI concentration was chosen to limit the DNA fluorescence of the nucleus and avoid interference with the fluorescence from the FISH probe in the composite image. The stained sample was mounted with antifade reagent (Patel *et al.,* 2007) and a covered with cover slip.

For epifluorescence microscopy, we used an Axioskope II plus (Carl Zeiss, Oberkochen, Germany) microscope, with a 100X oil-immersion objective (Plan-Apochromat, Carl Zeiss), and 175W xenon-arc lamp (Lambda LS, Sutter) connected through a liquid light guide. Excitation and emission spectra were controlled by filter wheels (Lambda 10-3, Sutter); for FISH a triple Sedat filter configuration (Erdogan, 2006) with one dichroic filter with three transmission bands was used (Semrock FF444/521/608-Di01) (Table 4). Optical stacks with a 2.0 μm focal distance between images, were acquired with a computer controlled focusing stage (Focus Drive, Ludl Electronic Products, Hawthorne, NY, USA) and Micro-Manager (version 1.3.40, Vale Lab, UCSF) that controlled filter selection and the focusing stage. Images were captured with a cooled CCD camera (Clara E, Andor) with 100 or 500 ms integration time

depending on the fluorochrome signal intensity. For the processing of the image stacks, ImageJ software (Schneider *et al*., 2012) was used. Image stacks of up to 50 images were obtained for each color channel separately and stored in TIFF format for subsequent digital analysis. Focal depth steps of 2 μm assured the detection of all attached bacteria and fully covered the z-axis for phytoplankton cells. Images were converted from 16-bit to 8-bit for further processing because the additional resolution did not carry significant information but made image processing more cumbersome. For each spectral channel a summary image was composed by selecting the pixels of maximum intensity within the stack, and reducing the background noise by subtracting approximately 10% of the average values in the pixel group representing the bacteria. For the hybridized samples the resulting gray images were false colored to match the emission wavelengths of the fluorochromes and subsequently merged to compose one image of a single phytoplankton cell and attached bacteria.

One-way ANOVA was used to compare the number of retained dinoflagellate cells after the embedding and hybridization process. Since the distribution of the number of attached bacteria per dinoflagellate cell was not normal, a Kruskal-Wallis test was used to assess the significance (α=0.05) in the number of bacterial detached during the hybridization process.

Table 3. Oligonucleotide probes used in this study.

Probe	Target group	Sequence (5'-3')	Target site[a]	Formamide[b]	Reference
EUB338	*Bacteria*	GCTGCCTCCCGTAGGAGT	16S (338-355)[c]	0-50	Amann *et al.* (1990)
GAM42a	γ-*proteobacteria* class	GCCTTCCCACATCGTTT	23S (1027-1043)[c]	35	Glöckner *et al.* (1999)
CF319a	phylum *Bacteroidetes*	TGGTCCGTGTCTCAGTAC	16S (319-336)[d]	35	Manz *et al.* (1996)
ALF968	α-*proteobacteria* class	GGTAAGGTTCTGCGCGTT	16S (968-986)[e]	35	Glöckner *et al.* (1999)
AMAC137R	*Alteromonas* clade	TGTTATCCCCCTCGCAAA	16S (137-154)[f]	10	Brinkmeyer *et al.* (2000)

[a]*E. coli* numbering; [b]Percent in ISH buffer for *in situ* hybridization; labeled probes, [c]Cy3 (Ex 488/Em 570 nm)

[d]ATTO425 (Ex 436/Em 484 nm); [e]Alexa594 (Ex 590/Em 617 nm); [f]Oregon Green (Ex 495/Em 525 nm)

Table 4. Summary of spectral channels used in this study.

Channel	Fluorochrome	Excitation [nm]	Dichroic [nm]	Emission [nm]	Filter Company
1	DAPI	360	395	>397	Semrock & Zeiss
2	Cy3	532	562	593	Omega
3	ATTO 425	422-432	445	457-487	Semrock
4	Oregon Green 488X	498-510	520	529-556	Semrock
5	Alexa Fluor 594	582-597	605	621-643	Semrock

3.3 Results

3.3.1 Embedding

The FISH protocol involves a number of steps which can produce host and bacterial cell losses during the procedure. To minimize cell losses we initially worked with published cell-mounting protocols (Biegala *et al.,* 2002; Palacios and Marín, 2008; Mayali *et al.,* 2011) using non-axenic dinoflagellate cultures. Using dinoflagellate cultures and the mounting protocol as in Biegala *et al*., (2002) and Palacios and Marín (2008) we recovered no dinoflagellate cells after the lysozyme step which led us to adapt the embedding protocol applied in Mayali *et al*., (2011) but lowering the percentage of agarose from 0.1% to 0.05% to avoid partially blurred images unsuitable for FISH and image analysis. In previous studies the cell immobilization steps were not explained in detail, and the percentage of recovery or cell losses during FISH preparations was not quantified. We found that embedding dinoflagellate cells in agarose was critical for maintaining cell integrity during the FISH procedure. With our protocol, 100% of the host cells were retained after embedding but before hybridization using normal filtered samples as a reference; after FISH the recovery of host-cells was 81% (Figure 6). The cell loss of 19% during hybridization could be traced to detachment of part of the agarose together with the embedded cells. Some host-cell losses were expected as a result of sequential washing steps and staining. We examined the loss of attached bacteria from their hosts by staining samples with SYBR Green I (Patel *et al.,* 2007) before and after embedding and a treatment following the hybridization protocol without probes. After the procedure the number of attached bacteria per cell was not significantly different from before ($p>0.05$) (Figure 7).

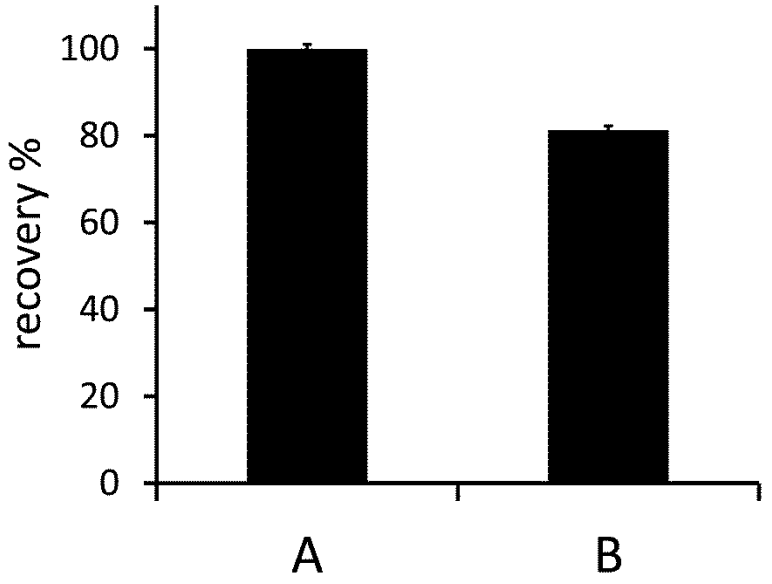

Figure 6. Dinoflagellate cells recovery using the embedding protocol using a *Lp* culture. A) Before hybridization; B) After hybridization (n=3, *p*<0.05).

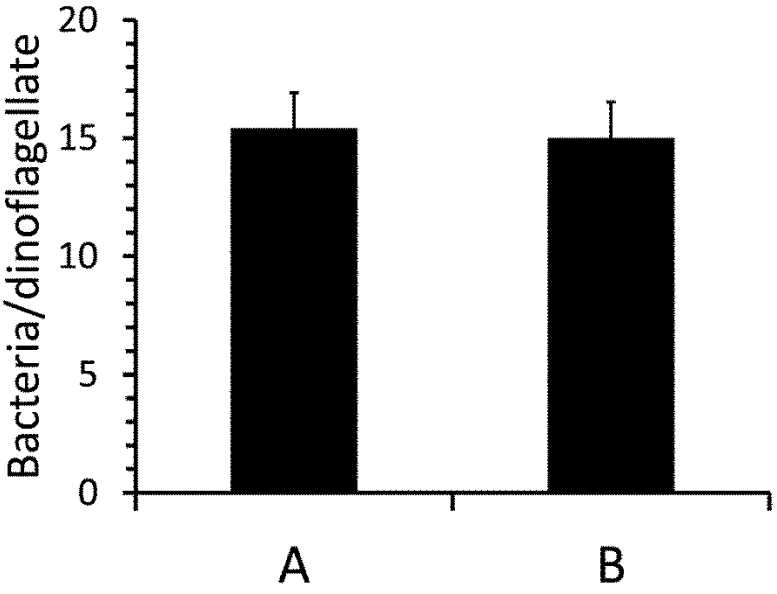

Figure 7. SYBR Green I stained attached bacteria per *Lp* cell in culture. A) Without hybridization protocol; B) After hybridization (n=3, *p*>0.05).

3.3.2 Fluorescence *in situ* hybridization

We tested our method with cultures of the dinoflagellate *Lp* which have been reported to harbor attached bacteria (Mayali *et al.,* 2011). Applying the probes ALF968, Gam42a and CF319a (Table 3), about 90% of the cells in the culture were free from attached bacteria, but those that were colonized by bacteria typically had more than one probe type (Figure 8). It is not surprising that most host-cells were free from attached bacteria, a pattern reported for phytoplankton in natural samples (Graff *et al.,* 2011). We applied FISH to a natural sample from Ensenada port that had more phytoplankton with attached bacteria than in oceanic samples; for example, the dinoflagellate cell with six attached α-proteobacteria and one attached γ-proteobacteria shown in Figure 9. In Figure 8 and 9 the DAPI-stained nucleus can be seen in the intact cells.

Double hybridization was tested on the cultured diatom *Grammatophora angulosa* using the general 16S eubacterial EUB338 probe and combined with the α-proteobacterial ALF968 probe (Table 3). Diatom cell walls were not stained; hence, Figure 10A shows the light-transmission image in order to orient the fluorescence images. The dark rings are the 8-μm filter pores. Both probes hybridized with the bacterial consortia associated with the diatoms (Figure 10B and 10C). The bacteria occurred on the diatom, even between closely spaced host cells. The *G. angulosa* cells were sampled in stationary phase, which explains the greater number of attached bacteria; in fast-growing cultures there were fewer attached bacteria. To demonstrate multiple-probe hybridization we choose a diatom from a natural sample, and applied probes for α-proteobacteria, *Alteromonas* clade, and the *phylum* Bacteroidetes.

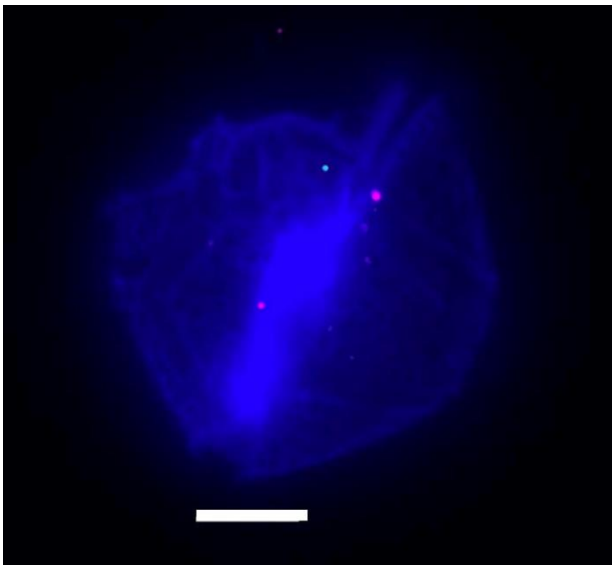

Figure 8. Composite image of the cultured dinoflagellate *Lp* cell and associated bacteria. Bacteria hybridized with probe AMAC137R-Oregon Green (magenta) and CF319a-ATTO425 (cyan) with standard FISH protocol; blue (center) DAPI-labeled nucleus surrounded by the calcofluor-labeled cellulose (blue) of dinoflagellate theca. Bar: 10 μm.

The transmitted light image (Figure 11A) shows an unidentified diatom with some extracellular protoplasma at 5 o clock; the dark rings are the filter pores (8 μm). Figure 11B shows a multitude of α-proteobacteria, γ-proteobacteria and *Bacteroidetes* attached to the lysed cell. The sample was stained with DAPI but the diatom nucleus was not visible, which is typical for a lysed cell. Hybridization with this combination of three probes allowed the bacteria covering the unidentified lysed diatom to be visualized.

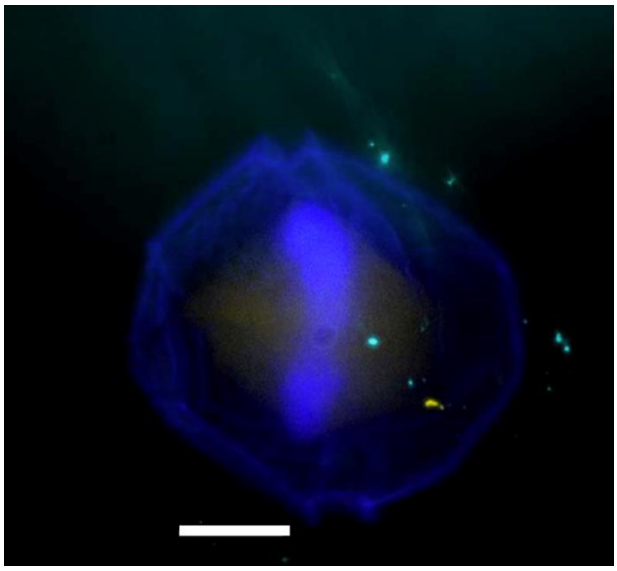

Figure 9. Composite image of the dinoflagellate *Lp* cell from a natural sample and its associated bacteria. Bacteria hybridized with probe CF319a-ATTO425 (cyan) and Gam42a-Cy3 (yellow) with standard FISH protocol; blue (center) DAPI-labeled nucleus surrounded by the calcofluor-labeled cellulose (blue) of dinoflagellate theca. Bar: 10 μm.

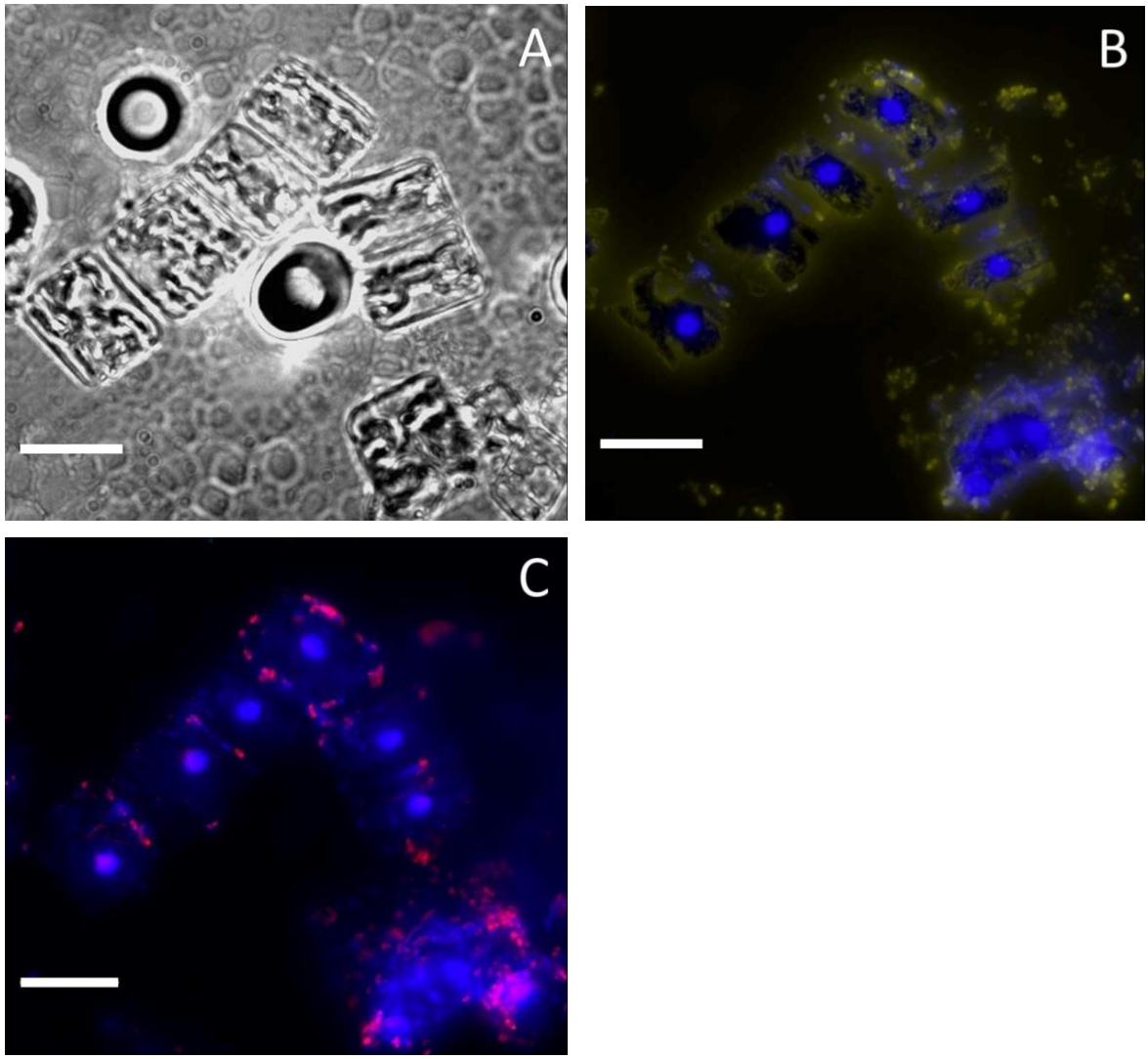

Figure 10. Images of the cultured diatom *Grammatophora angulosa* and associated bacteria. A) Composite image of light transmission. Dark circles correspond to 8 μm filter pores. B) Bacteria hybridized with probe EUB338- Cy3 (yellow) and DAPI-labeled nucleus (blue). C) Bacteria hybridized with probe ALF968-Alexa594 (red) and DAPI-labeled nucleus (blue). Bar: 10 μm.

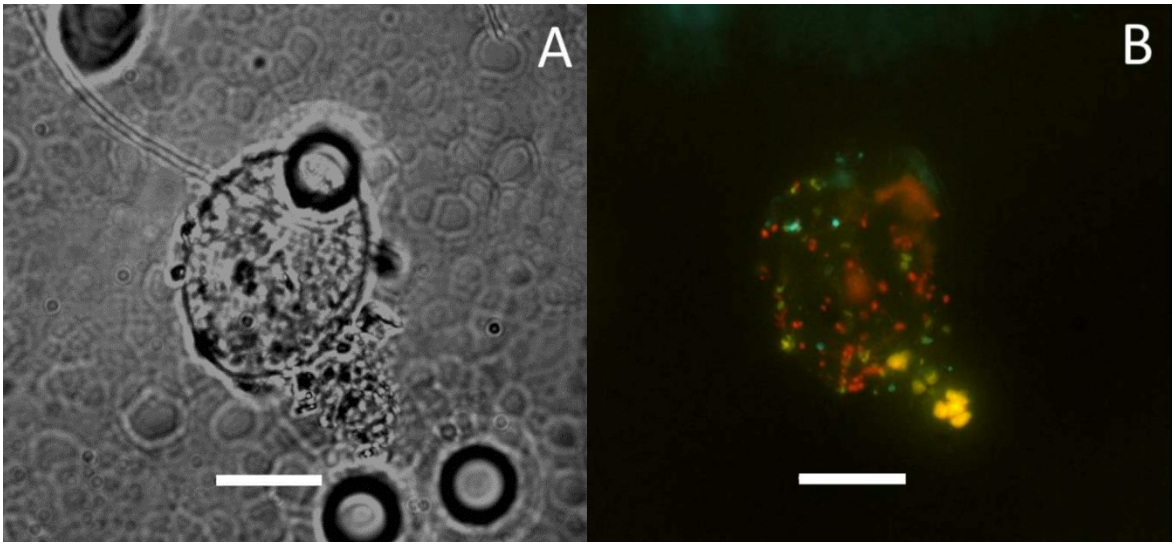

Figure 11. Image of an unidentified lysed diatom from a natural sample. A) Composite image of light transmission. Dark circles correspond to 8 μm pores in filter. B) Bacteria hybridized with probes Gam42a-Cy3 (yellow), Alf968-Alexa594 (red) and CF319a- ATTO425 (cyan). The sample was labeled with DAPI which is not observed in this cell. Bar: 10 μm.

3.4 Discussion

Previous studies used CARD-FISH for *in situ* hybridization of bacteria attached to phytoplankton. Biegala *et al*., (2002) concluded that standard epifluorescence microscopy did not allow all labeled bacteria to be observed due to interference by chlorophyll fluorescence from the phytoplankton host. The problem could partly have resulted from insufficient spectral discrimination by the emission filters, but despite having an optical filter with optical density $<10^{-4}$ out-of-band transmittance there was still a significant chlorophyll *a* emission signal with the Cy3-labeled probe. We used ethanol to wash samples and reduce chlorophyll autofluorescence, but could not eliminate it completely. However, chlorophyll autofluorescence could be eliminated numerically from the probe images of Cy3 by subtracting the autofluorescence images from the FISH image.

Probe specificity is critical for the FISH method because in natural samples there are many different bacterial species; hence, we selected probes sequences that had

been successfully applied (Knoll *et al.,* 2001; Biegala *et al.,* 2002). Fluorochromes were selected to maximize spectral discrimination by minimizing excitation and emission overlap. One of the advantages of wide-field epifluorescence microscopy over confocal laser scanning microscopy is the availability of a wide range of excitation spectra in comparison to the limited number of excitation lasers used in confocal microscopy (Manz *et al.,* 2000). The Xenon lamp in combination with the liquid light guide helped provide a more even lamp power spectrum to excite the different fluorochromes and a more homogeneous excitation light field. Our use of a Sedat triple transmission band dichroic filter eliminated the need to change the dichroic filter between the different probe images; this helped maintain the exact image geometry between the different probes. The image geometry is important to distinguish between bacteria in close proximity and marked by different probes. For each probe the filter wheels selected the excitation and emission filters, but these filter changes typically have little impact on image geometry.

Biegala *et al*., (2002) reported that blue fluorescence of Calcofluor (100 μg ml^{-1}) and DAPI (5 μl ml^{-1}) masked the FISH emission. They also reported that due to the strong chlorophyll autofluorescence, *in situ* identification of bacteria attached to dinoflagellate cells was not possible using the standard FISH protocol (Biegala *et al*., 2002). We applied DAPI or SYBR Green I to define the state of the host cell nucleus, and help identify intact host cells. Calcofluor helped to visualize the dinoflagellate cell outline by staining the theca (Figure 8).

The fluorescence from the condensed nuclei of the dinoflagellates is very bright when stained with DAPI and SYBR Green I; therefore, we diluted the typical concentrations of the stains to 0.1. DAPI and Calcofluor have similar excitation and emission wavelengths (Palacios and Marín, 2008), suggesting the use of SYBR Green I instead of DAPI, but SYBR Green I interfered with the Alexa594 fluorochrome label of the ALF968 probe. This limited the use of SYBR Green I to samples without the Alexa594 fluorochrome.

Embedding of the sensitive dinoflagellate cells in agarose is critical for maintaining cell integrity during the FISH procedure. Previous studies used FISH for bacteria associated with dinoflagellates, but the methodological details were not fully explained; thus, there are no data in the literature to compare with our cell losses during FISH preparations. Previous reports used 12 μm pore-size Isopore filters without embedding (Biegala *et al.,* 2002), but for our samples this approach was not successful and most dinoflagellate cells were lost or disintegrated. Mayali *et al.,* (2011) proposed a combination of Teflon-coated 10-well slides and 0.1% low melting point agarose as an embedding solution for FISH of attached bacteria on dinoflagellates. We tried a similar approach as Biegala *et al.* (2002), but with a 8.0 μm pore size polycarbonate filters, and adding 0.1% low melting point agarose (LMA) as used by Mayali *et al.* (2011); however, there were problems the penetration of the FISH probe resulting in blurry images (data not shown). In order to solve this issue, we modified the embedding protocol by changing to 0.05% LMA at 55^{o}C, and placing 13 μl on the 8.0 μm pore-size polycarbonate filters with the sample facing up. The embedded sample was then dried at 46°C for 15 min. Previous works used standard FISH and epifluorescence microscopy to report on the epiphytic bacteria associated with diatoms, but the physical context was not reported (Knoll *et al.,* 2001), and only one CARD-FISH probe was applied to each sample and visualized with confocal microscopy (Bennke *et al.,* 2013). Our study is the first to reports epyphitic bacteria associated with diatoms using simultaneous different probes on one sample. In Figure 10 all bacteria closely associated with the diatom cells belonged to the α-*Proteobacteria* group; whereas, different groups of bacteria were attached to the dead diatom (Figure 11) or to live dinoflagellates (Figures 8 and 9).

3.5 Conclusion

The method presented here allows the simultaneous detection of distinct bacterial groups physically associated with phytoplankton cells using non-amplified probes combined with epifluorescence microscopy. Using a combination of a modified protocol for cell immobilization we solved previously unreported problems, such as cell integrity and cell losses during the hybridization process. Using an extended ethanol series we reduced the background from chlorophyll fluorescence, facilitating the identification of bacteria attached to phytoplankton. The advantage of our method compared to previously published ones is that it allows the simultaneous observation of intact phytoplankton cells and the cell-surface localization of up to three different taxonomic groups of attached bacteria. This method can be easily adapted to other FISH-probe sequences targeting more specific taxonomic groups.

4 The vitamin B_1 and B_{12} requirement of the marine dinoflagellate *Lingulodinium polyedrum* can be provided by its associated bacterial community in culture

Abstract

The presence of bacteria could overcome vitamin limitation during growth of the dinoflagellate *Lingulodinium polyedrum* (*Lp*). Averages of six bacteria were attached to each dinoflagellate cell in both vitamin replete and deplete cultures, but suspended bacteria were in significantly higher concentrations in vitamin-limited cultures. Measured vitamin B_{12} concentrations produced by bacteria in vitamin-depleted cultures (34 ± 5 nM) were lower but not significantly different to vitamin B_{12} in replete cultures (41 ± 3 nM). The culture medium was prepared without labile dissolved organics that could serve as a substrate for bacteria suggesting that bacterial growth was supported by organics released from the dinoflagellate host, while the bacterial consortia provided vitamins B_1 and B_{12} in return. A sequential fluorescence *in situ* hybridization and digital imaging approach allowed quantification of the three major bacterial groups attached to *Lp* and in suspension in the vitamin-depleted and replete cultures.

Keywords: B vitamin auxotrophy; Dinoflagellate-bacteria interactions; Fluorescence *in situ* hybridization (FISH).

4.1 Introduction

Dinoflagellates are among the most abundant eukaryotic phytoplankton in freshwater and coastal systems (Moustafa *et al.,* 2010). Of the examined dinoflagellate species, 100% require vitamin B_{12}, 78% require vitamin B_1 and 32% require vitamin B_7 (Tang *et al*., 2010). B vitamin concentrations in coastal waters are generally higher than in the open ocean (Panzeca *et al.,* 2009; Sañudo-Wilhelmy *et al.,* 2012), and available genomic data indicate that some members of Bacteria and Archaea, as well marine cyanobacteria are vitamin producers (Bonnet *et al.,* 2010; Sañudo-Wilhelmy *et al.,* 2014), but so far the genetic potential for B_1 or B_{12} synthesis in aquatic environments is based only on a single study (Bertrand *et al.,* 2011). Dinoflagellates exhibit a diversity of trophic states including mixotrophy (Burkholder *et al*., 2008), thus they could acquire B-type vitamins from the environment either through osmotrophy of vitamins that are exuded (Droop, 2007; Kazamia *et al.,* 2012; Kuo and Lin, 2013; Xie *et al.,* 2013), through phagotrophy, for example of specific cyanobacterial groups that produce B_{12} (Jeong *et al*., 2005; Bonnet *et al*., 2010) or direct symbiosis with bacteria contacting the vitamin auxotroph (Croft *et al*., 2005; Wagner-Döbler *et al*., 2010). The relative contribution of these mechanisms to vitamin acquisition in dinoflagellates is not known; knowledge of these mechanisms would help in the understanding of dinoflagellate ecology and the possible role of vitamins in bloom development.

As stated in previous chapters, *Lp* is a dinoflagellate with a mixotrophic lifestyle (Jeong *et al.,* 2005) that forms recurrent blooms along the coast of southern California and northern Baja California (Holmes *et al.,* 1967; Kudela and Cochlan, 2000; Peña-Manjarrez *et al.,* 2005). Although its microbial ecology (Mayali *et al.,* 2008, 2011) has been previously studied, vitamin production by its associated bacteria has not been experimentally established. Here we investigate the role of vitamins and bacteria in the autecology of *Lp*, using non-axenic cultures of *Lp* under vitamin B_1, B_7 and B_{12} limitation. To document the association of natural bacterial consortia in *Lp* cultures under B-type vitamin-depleted and replete conditions, we employed a modified FISH method (see Chapter 3) to quantify suspended and attached bacteria and their taxonomic affiliation.

We found that of the attached bacterial community 80% were α-proteobacteria, 5% were *Alteromonas* and 5% were *Bacteroidetes*; this composition was not significantly different from the bacterial community suspended in the culture medium. Also, we quantified the contribution of soluble B_{12} vitamin by the bacterial consortium that was available to its dinoflagellate host.

4.2 Materials and Methods

4.2.1 Strain and growth conditions

See Chapter 2, Material and methods section. In brief, the medium was nutrient-amended and autoclaved. The culture growth of *Lp* was monitored by *in vivo* chlorophyll fluorescence.

4.2.2 Qualitative assessment of B_1 and B_{12} synthesis from the bacterial consortia

The axenic culture of *Lp* was inoculated with bacteria taken from natural seawater that was filtered through a 0.7 μm pore-size polycarbonate filter. *Lp* culture were divided into vitamin-replete and limited, and acclimated by culture transfer for several months to ensure depletion of the initial vitamins, and to make certain that the remaining microbial populations had the potential to synthesize vitamins.

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4.2.3 Cell fixation, immobilization and embedding

Dinoflagellate cells were harvested at lag, log and stationary phases and fixed with PFA-PBS at a final concentration of 1% for 12h at 4°C. For attached bacteria, fixed cells were immobilized onto an 8.0 μm pore-size, 25 mm-diameter Nuclepore filter (Whatman International, Ltd., Maidstone, England) using a pressure difference of <3.3 kPa to avoid cell disintegration, and rinsed with phosphate-buffered saline (PBS,0.1 M NaCl, 2mM KCl, 4 mM Na_2HPO_4, pH 8.1, Palacios and Marín 2008). For suspended bacteria, the fraction which passed through a 8.0 μm pore-size filter was collected on a 0.2 μm pore-size, 25 mm-diameter Nuclepore filter (Whatman International, Ltd., Maidstone, England) and rinsed with PBS. The cells collected on the 8.0 μm filter were covered with 13 μl of low-melting point agarose (0.05%, LMA) (BioRad, 161-3111) at 55 °C, dried for 15 min at 37 °C, before LMA was added again and the filter dried as previously described.

4.2.4 Fluorescence *in situ* hybridization

See Chapter 3, Material and methods section

4.2.5 Visualization

See Chapter 3, Material and methods section

4.2.6 Quantification of dissolved B_{12}

Dissolved B_{12} vitamin from the medium was quantified as follows: Dinoflagellate cells were harvested at log phase, and filtered through 8.0 μm and 0.45 μm pore-size, followed by a filtration step with 0.4 μm pore size 47 mm-diameter Nuclepore filters (Whatman International, Ltd., Maidstone, England) using a pressure difference of <3.3 kPa to avoid cell disintegration. Soluble B_{12} vitamin was pre-concentrated using a C_{18} resin (RP-C18) according to Okbamichael and Sañudo-Wilhelmy (2004), eluted with 5 ml methanol, concentrated at 60°C with vacuum, and quantified by ELISA (Immunolab GmbH, B12-E01. Kassel, Germany) according to Zhu *et al*., (2011).

4.3 Results

4.3.1 Co-culture of *Lp* with a natural bacterial consortia

Non axenic *Lp* cultures in vitamin free medium could maintain growth through more than five culture transfers at growth rates similar to the non-axenic and the axenic, vitamin replete cultures (Figure 12). These results suggest that the bacteria in the non-axenic culture could provide sufficient vitamins to sustain *Lp* growth. There was no statistical differences between culture treatments (p>0.05) which suggests that the bacterial consortia could meet the demand of B_1 and B_{12} vitamins during vitamin- depleted growth. In the non-axenic cultures the concentration of suspended bacteria was significantly higher without added vitamins than with vitamins added (Figure 13A). In the non-axenic culture the probability of *Lp* cells having bacteria attached or the average number of bacteria attached to *Lp* cells were not significantly different (Figures 13B and 13C).

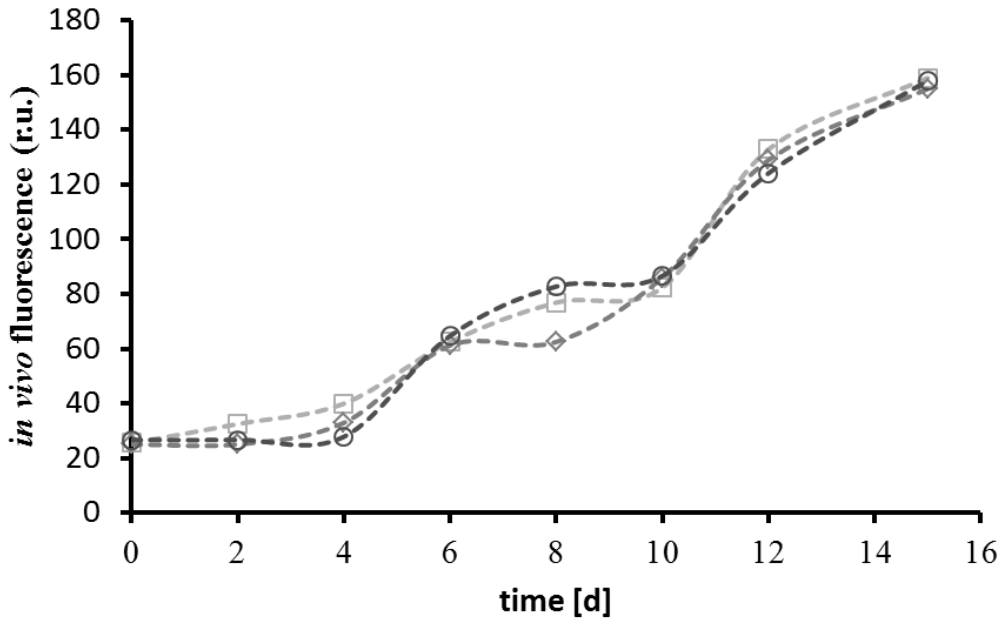

Figure 12. Growth of *Lp*, axenic or in co-culture with a natural marine heterotrophic bacterial consortia, after 8 culture transfers. *Lp* culture; axenic with vitamins (□), non-axenic without vitamins (○), non-axenic with B_1, B_7, B_{12} vitamins (◊).

The data in figure 13 show for the culture with vitamins added the percentage of dinoflagellate cells colonized by at least 1 bacterium that ranged from 38 to 98% between start and day 18. In the culture without added vitamin the frequency ranged from 46 to 100% (Figure 13A). The mean number of attached bacteria ranged from 1 to 6 in vitamin-replete conditions and from 1 to 12 in vitamin-depleted conditions, although in early stages of the culture in vitamin-depleted conditions some single dinoflagellate cells were colonized by up to 12 bacteria (Figure 13C).

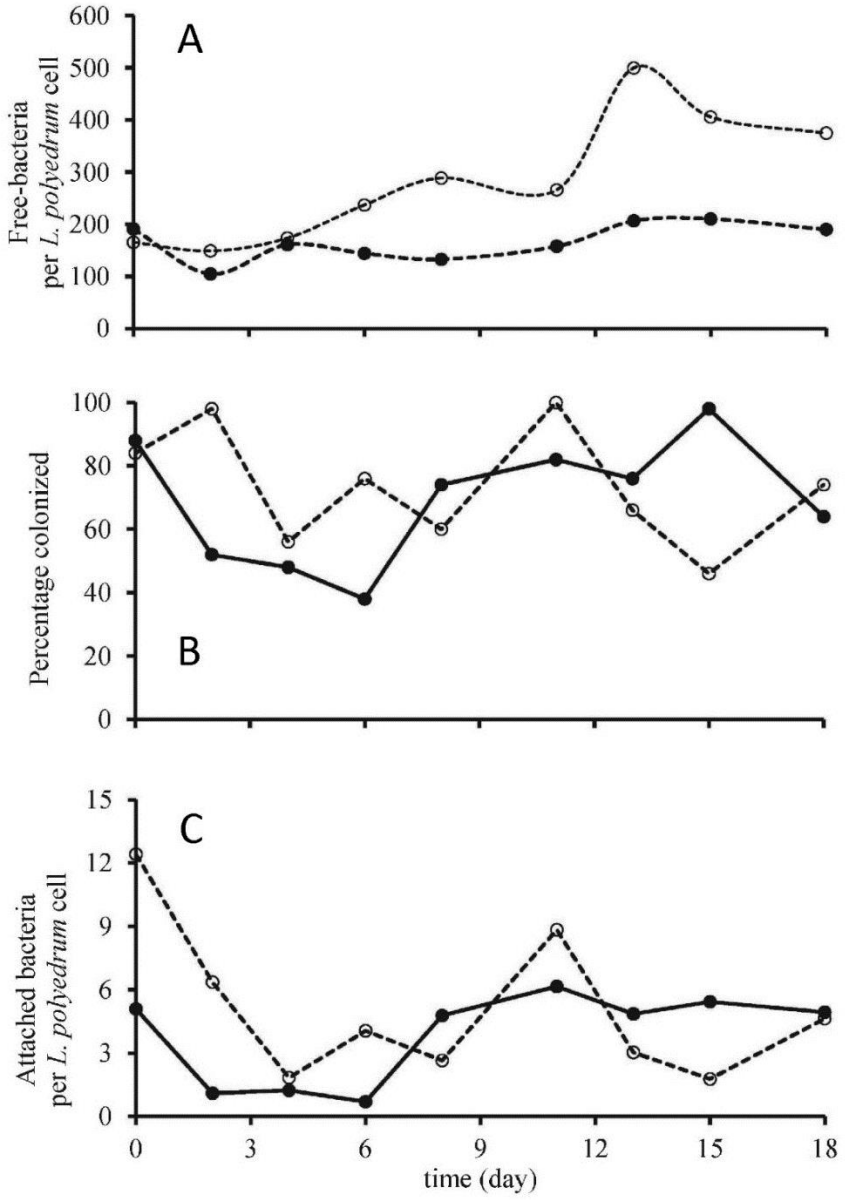

Figure 13. Growth of *Lp* and associated bacteria culture under vitamin replete (solid line) and deplete (dashed lines) conditions. A) Ratio of freely suspended bacteria to *Lp* cells. B) Percentage of *Lp* cells with 1 or more bacteria attached. C) Average number of bacteria attached to *Lp* cells having at least one bacteria attached. n= 50 dinoflagellate cells.

4.3.2 Abundance of free and attached bacterial groups in L. polyedrum cultures

To determine the phylogenetic identity of the major bacterial groups associated with *Lp*, a method using non-amplifying multiple probe fluorescence *in situ* hybridization and image analysis was developed (see Chapter 3). The method enables the simultaneous quantification of 4 bacterial taxa without probe signal amplification (see Table 3). Image analysis compensated for background fluorescence from the dinoflagellate, to avoid optical crosstalk and increase the detection of the non-amplified probes. Three of the major bacterial groups associated with dinoflagellates were detected either as suspended cells or attached to the cell surface. Changes in the composition of the bacteria associated with lag, log and stationary growth of *Lp* showed that 80% of the bacteria associated with both vitamin treatments were α-proteobacteria, while members of *Alteromonas* and *Bacteroidetes* were scarcely detected (Figures 14A and 14B). As stated in Biegala *et al*., (2002) when working with phytoplankton it is critical to use group-specific probes to discriminate the false positives coming from the plastids. Using image analysis we discriminated the eubacterial probe (EUB338) from the group-specific probes and were able to localize and quantify the bacteria attached to cells grown under the different vitamin treatments (Figures 15A and 15B). This is the first report of using FISH without catalyzed reported deposition (CARD) and epifluorescence microscopy to visualize bacteria attached to phytoplankton.

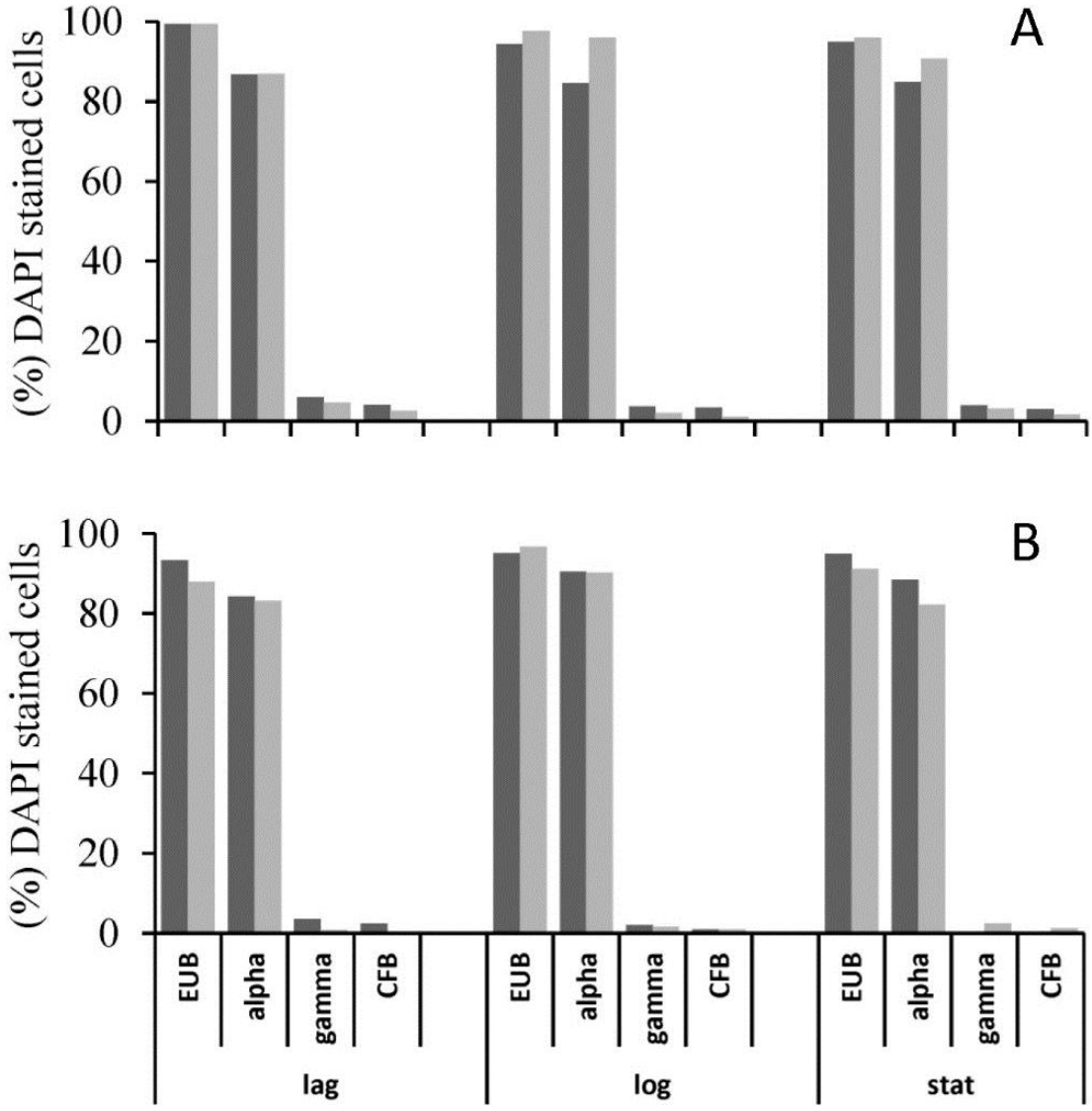

Figure 14. Suspended (A) and attached (B) bacteria associated with *Lp* in vitamin depleted (■) and replete (■) cultures during different growth phases (lag, log and stationary phases) given in percentages of the total number of DAPI-stained cells. Specific bacterial groups were quantified by FISH using the four probes listed in Table 3.

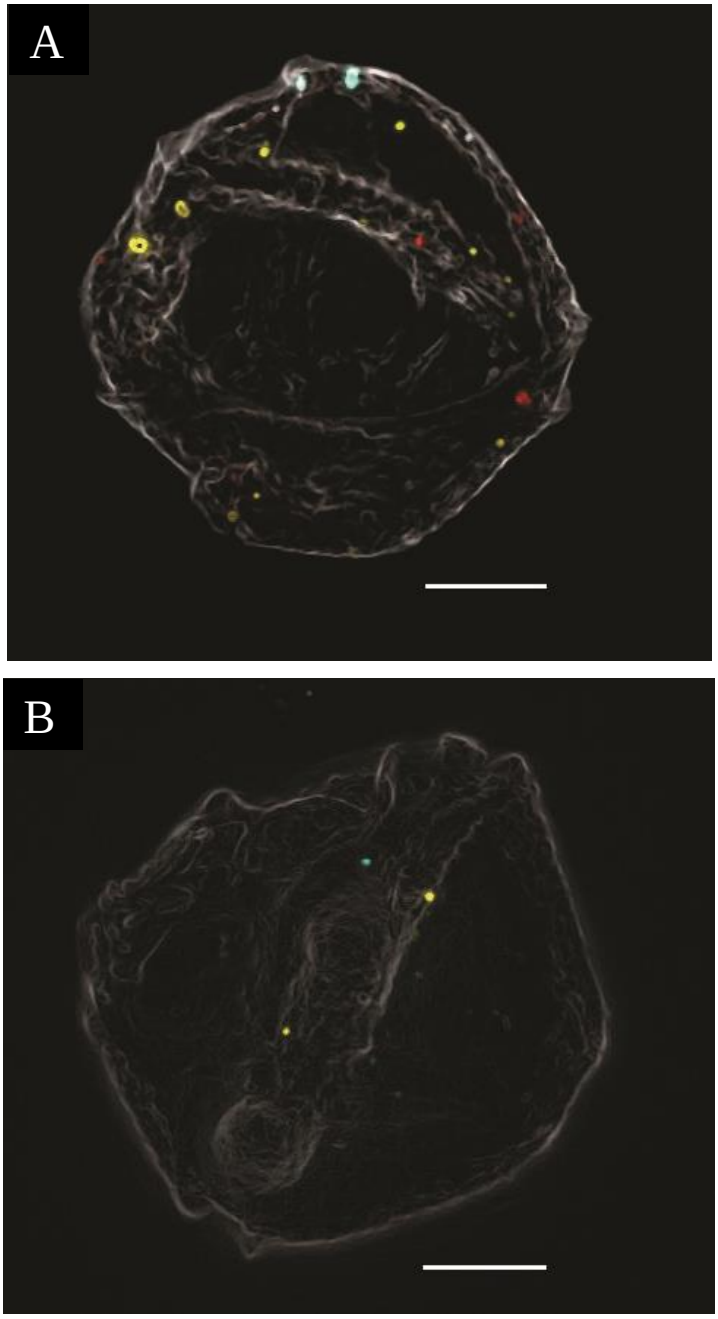

Figure 15. A) *Lp* cells without vitamins added. Composite image: 3 *Bacteroidetes* (CF319a, cyan); 11 *Alteromona*s (AMAC137R, yellow), 5 α-proteobacteria (ALF968, red). B) Vitamin replete *Lp* cell. Composite image: 3 *Bacteroidetes* (CF319a, cyan) and 2 *Alteromonas* (AMAC, yellow). *Lp* outline stained with Calcofluor. Bar: 5 μm.

4.3.3 Dissolved vitamin B_{12}

Vitamin B_{12} concentrations for both vitamin treatments were measured in exponentially growing non-axenic cultures of *Lp*. In replete cultures vitamin B_{12} was 41. ± 3 nM (n=3), and in cultures without vitamin B_{12} added 34 ± 5 nM (n=3), the concentration difference is not significant (p>0.05). The measured concentration of vitamin B_{12} in replete cultures was the sum of the dissolved B_{12} from the L1 medium and that synthesized by the microbial consortia; for limited conditions, the measured B_{12} came only from the microbial consortium.

4.4 Discussion

4.4.1 *Lp* and B_{12} production from the bacterial consortia

Phytoplankton vitamin B auxotrophy has been previously observed in culture (reviewed by Droop, 2007) and in natural phytoplankton assemblages in coastal areas composed mainly of diatoms and dinoflagellates (Sañudo-Wilhelmy *et al.* 2006; Gobler *et al*., 2007; Koch *et al*., 2012). *Lp* was chosen for our study because it forms coastal red tides and because previous studies had demonstrated high bacterial abundances and diversity of attached bacteria (Biegala *et al*., 2002; Mayali *et al*., 2007, 2011). Carlucci (1970) interpreted phytoplankton and B_{12} data from coastal waters of S. California suggesting that *Lp* was a B_{12} auxotroph but *Lp* vitamin B auxotrophy had not been tested in culture.

As a dinoflagellate, *Lp* is expected to be mixotrophic allowing for different modes of vitamin uptake through osmotrophy, phagotrophy (Jeong *et al*., 2005) or episymbiosis with heterotrophic bacteria (Croft *et al*., 2005; Wagner-Döbler *et al*., 2010). The latter two modes of uptake may be more efficient for acquiring B_{12} than osmotrophy, and hence may be preferred by dinoflagellates, given that dinoflagellates are more frequently vitamin auxotrophs than other phytoplankton (Tang *et al*., 2010). The ratio of suspended bacteria to *Lp* cells did significantly increase in vitamin limited cultures. This would

suggest that B_{12} produced by bacteria is being taken up by *Lp* through osmotrophy. The measured concentrations of B_{12} in replete and limited, non-axenic cultures was 20% lower in the latter, but much higher than concentrations in the ocean (Sañudo-Wilhelmy *et al*., 2006), suggesting that B_{12} production exceeded consumption and was not low enough to limit the growth rate of *Lp*.

Because the vitamin concentrations in cultures without added vitamins were measured during exponential growth, they probably represented equilibrium between the continuous supply from the bacterial consortia and uptake by the dinoflagellates.

Although we found no increases in bacteria attached to *Lp* the interactions between the bacterial consortia and *Lp* can still constitute a form of symbiosis between vitamin producing bacteria in suspension and *Lp* where the latter provides labile organics to the medium to sustain the growth of the suspended bacteria. Our data do not exclude the possibility of vitamin acquisition by either episymbiosis or phagocytosis, but we found no microscopic evidence for phagocytosis. We considered episymbiosis to be unlikely because the phylogenetic composition between attached and suspended bacteria were similar between vitamin treatments. On the other hand, suspended bacterial cells in vitamin-depleted treatment represents twice the numbers compared with vitamin-replete treatment, which apparently seems to be linked in the vitamin concentration in the medium; but still, attached epibionts could contribute to the B_{12} supply (Wagner-Döbler *et al*., 2010).

4.4.2 Fluorescence *in situ* hybridization of attached and suspended bacteria

The probes selected for FISH were based on those available for bacteria associated with dinoflagellates. This method identified α-proteobacteria as the dominant bacterial group in the attached and suspended bacterial community (Figure 14). It is difficult to relate the dominance of α-*proteobacteria* observed in this study to a particular functional phenotype, because members of α-proteobacteria are morphologically and metabolically extremely diverse. However, recent evidence indicates that α-

proteobacteria could contribute B_1 and B_{12} to their dinoflagellate hosts (Wagner-Döbler *et al.* 2010). *Roseobacter* spp. is an important clade of marine α-*proteobacteria* associated with dinoflagellates (Fandino *et al*., 2001; Hasegawa *et al*., 2007; Mayali *et al*., 2008, 2011) and includes species that produce B_1 and B_{12} vitamins (Wagner-Döbler *et al*., 2010). Bacteria within this group are known to be epibionts of dinoflagellates, particularly *Lp* (Mayali *et al*., 2011) and can represent the most abundant group within bacterial assemblages associated with phytoplankton cultures and during bloom conditions (Fandino *et al*., 2001; Hasegawa *et al*., 2007).

We also identified members of *Alteromonas* and *Bacteroidetes* as less frequent epibionts. Members of the taxa *Alteromonas* and *Bacteroidetes* have been found in samples during bloom conditions (Fandino *et al*., 2001; Garcés *et al*., 2007; Mayali *et al*., 2011); their low frequency of attachment in our cultures agrees with their low abundances reported in culture and field samples (Garcés *et al*., 2007). Recent genomic data about these two groups confirm that γ-*proteobacteria* have the metabolic potential to produce B_1 and B_{12}; whereas, members of the *Bacteroidetes* are known to include species that produce B_1 but so far there is no evidence that they can produce B_{12} (Sañudo-Wilhelmy *et al*., 2014).

The stable bacterial community observed in the culture suggests that α-proteobacteria and possibly *Roseobacter* species are an integral part of the epiphytic community of *Lp.* The similarity in community composition of attached and suspended bacteria in both vitamin treatments, suggests that bacteria in the different phylogenetic groups can move between attached and free-living lifestyle; thus, the bacterial community composition seemed more influenced by host-specificity rather than the capacity to produce vitamins. While free-living bacteria generally have higher growth rates, attached bacteria serve as a reservoir for suspended progeny (Riemman and Winding, 2001); hence, in a scenario with a dinoflagellate population relatively free of attached bacteria, it may be an exchange of bacterial cells between attached and suspended fraction, which results in the overlapping phylogenetic composition observed in this study.

4.5 Conclusion

Here we show that *Lp* is able to acquire vitamins B_1 and B_{12} from a consortium of bacteria. We used vitamin-replete and unamended cultures of *Lp* to produce contrasting conditions in which it was hypothesized that vitamin-limited cultures would promote the attachment of bacteria. Because the culture medium had no added dissolved organic carbon that could be used by organotrophic bacteria, our results suggest that the bacteria were able to use dinoflagellate photosynthates as a carbon source in return for supplying the host cell with vitamin B_1 and B_{12}.

The concept of algal-bacterial symbiosis is not novel, and has been recently shown with mono-specific algae and bacteria cultures (Croft *et al*., 2005; Wagner-Döbler *et al*., 2010; Kazamia *et al*., 2012), but the present study provides the first experimental evidence that natural marine bacterial consortia can supply the vitamins necessary to support dinoflagellate growth without the addition of organic substrates. The bacteria were probably being sustained by substrates supplied by the dinoflagellate host. Although the stable co-occurrence of bacteria in *Lp* cultures in medium without added vitamins suggests mutualism it leaves the question open how *Lp* acquires the vitamins from the bacterial consortia.

5 Discussion

5.1 Dinoflagellate vitamin auxotrophy

Lp is a notorious dinoflagellate species that forms extensive blooms in the California Current System. Despite observations and physiological research on the genus *Lingulodinium* (Hastings, 2007; Beauchemin *et al*., 2012), its vitamin requirements have not been published. However Carlucci (1970) and Panzeca *et al*., (2009) found strong correlations between the increases in *Lp* cells and declines of B_1, B_7 and B_{12} concentrations along the California coast. Other information on vitamin limitation of *Lp* has not been published.

Our initial experiments with axenic *Lp* cultures established a clear auxotrophy for B_1 and B_{12} but not for B_7. The dual vitamin dependence could be expected because previous studies argued that phytoplankton species that have arisen as a result of secondary and tertiary endosymbiosis require a suite of B vitamins (Croft *et al.,* 2006). Results from triplicate growth experiments in a 5 x 5 array of different concentrations of B_1 and B_{12} suggests that at low concentrations the two vitamins interact and that the increase in one concentration will increase growth rate, even though the other vitamin is at a growth limiting concentration. The B_1 and B_{12} uptake has been documented for phytoplankton communities in coastal areas (Gobler *et al.,* 2007; Koch *et al.,* 2012, 2013, 2014) but the potential for multiplicative limitation of B_1 and B_{12} has not been reported before. Given the slow μ of dinoflagellates it is difficult to imagine that traditional bioassay experiments that probe vitamin limitation would yield conclusive results for natural populations of dinoflagellates.

5.2 Fluorescence *in situ* hybridization

Previous studies used CARD-FISH for *in situ* hybridization of bacteria attached to phytoplankton. Biegala *et al*., (2002) concluded that standard epifluorescence microscopy did not allow the observation of all labeled bacteria due to interference by chlorophyll fluorescence from the phytoplankton host. We used ethanol to wash samples and reduce chlorophyll autofluorescence but we could not eliminate it completely. The chlorophyll autofluorescence could be eliminated numerically from the probe images of Cy3 by subtracting images of autofluorescence from the FISH images.

Fluorochromes were selected to maximize spectral discrimination by minimizing excitation and emission overlap. The use of a Sedat triple transmission band dichroic filter eliminated the need to change the dichroic filter between the different probe images; this helped in maintaining the exact image geometry among the different probes. The image geometry is important to distinguish between bacteria in close proximity and marked by different probes.

The condensed nucleus of dinoflagellates produces very strong fluorescence when stained with DAPI and SYBR Green I at normal concentrations; therefore, we diluted the stains to 0.1 of the normal concentration. DAPI and Calcofluor have similar excitation and emission wavelengths (Palacios and Marín, 2008), suggesting the use of SYBR Green I instead of DAPI but the latter interfered with the Alexa594 fluorochrome label of the ALF968 probe. This limited the use of SYBR Green I to samples without the Alexa594 fluorochrome.

Embedding dinoflagellate cells in agarose is critical for maintaining cell integrity during the FISH procedure. Previous reports used 12 μm pore size Isopore filters without embedding (Biegala *et al.,* 2002) or a combination of Teflon-coated 10-well slides and 0.1% low melting point agarose (Mayali *et al*., 2011), but without embedding. However, most cells were lost when 0.1% low melting point agarose was added, and

there were additional problems with the penetration of the FISH probes and blurry images. Modifying the embedding protocol changing to 0.05% LMA at 55^{o}C, and placing 13 μl on the 8.0 μm pore-size polycarbonate filters solved these problems.

5.3 *Lp* and B_{12} production from the bacterial consortia

Lp was chosen because previous studies had demonstrated high bacterial abundance and diversity of attached bacteria (Biegala *et al*., 2002; Mayali *et al*., 2007, 2011) in this species. In our cultures the probability of bacterial attachment and the number of attached bacteria per cell was not significantly different in vitamin limited and replete cultures of *Lp* which argues against episymbiosis. The ratio of suspended bacteria to *Lp* cells increased significantly in vitamin limited cultures. This suggests that B_{12} produced by bacteria is being taken up by *Lp* through osmotrophy. The measured concentrations of B_{12} in replete and limited, non-axenic cultures was 20% lower in the latter, but much higher than concentrations in the ocean (Sañudo-Wilhelmy *et al*., 2006), suggesting that B_{12} production exceeded consumption and was not low enough to limit the *Lp* growth rate.

From the factorial design experiment we can estimate the in situ concentrations of B_1 and B_{12} necessary to support maximum growth of *Lp* was 3.3 pM B_1 and 5.3 pM B_{12}. The comparison with published in situ concentrations suggests that typically the ocean may not be B_1 or B_{12} limiting for *Lp* under non-bloom conditions. These results are in accordance with Droop (2007) who argued that vitamins are typically not limiting in the ocean because phytoplankton can grow a very low vitamin concentrations. But during dinoflagellate blooms vitamins may become limiting because of the very high cell concentrations of these vitamin auxotrophs.

Because the concentrations in the culture without added vitamin were measured during exponential phase they probably represent equilibrium between the continuous supply from the bacterial consortia and uptake by the dinoflagellate. Although we found no increase in bacteria attached to *Lp*, the interaction between the bacterial consortia and

Lp can still constitute a form of symbiosis between vitamin producing-bacteria in suspension and *Lp* where the latter provides labile organics to the medium to sustain the growth of the suspended bacteria.

Members of the α-proteobacteria subclass were the dominant bacterial group in the attached and suspended bacterial communities, while members of *Alteromonas* and *Bacteroidetes* were much less frequent (Figure 14). It is difficult to relate the community composition observed in this study to particular functional phenotype, because α-proteobacteria are morphological and metabolically diverse. However, recent evidence indicates that α-proteobacteria could contribute B_1 and B_{12} to their dinoflagellate host (Wagner-Döbler *et al.* 2010). Members of *Roseobacter* spp. are an important marine α-proteobacterial lineage associated with dinoflagellates (Fandino *et al*., 2001; Hasegawa *et al*., 2007; Mayali *et al*., 2008, 2011) and include species that produces B_1 and B_{12} vitamins (Wagner-Döbler *et al*., 2010). Bacteria within this group are known to be epibionts of dinoflagellates, particularly *Lp* (Mayali *et al*., 2011) and can represent the most abundant group within bacterial assemblages associated with phytoplankton cultures and during bloom (Fandino *et al*., 2001; Hasegawa *et al*., 2007). The stable populations observed in the cultures suggests that α-proteobacteria and possibly *Roseobacter* species are integral parts of the epiphytic community of *Lp.*

6 Conclusion

Using a combination of bioassays, analytic and microscopic techniques we show:

- *Lp* is auxotrophic for vitamins B_1 and B_{12}.
- The *in situ* concentrations of vitamins B_1 and B_{12} necessary to support maximum growth of *Lp* is as follows: B_1 = 3.3 pM and B_{12} = 5.3 pM.
- Part of the ocean may be B_1 or B_{12} limiting for *Lp.*
- *Lp* is able to acquire B-type vitamins from a B_1 and B_{12}-synthesizing bacterial consortia.
- Bacteria were able to use dinoflagellate photosynthates as a carbon source.
- The present study provides the first culture-based evidence that natural marine bacterial consortia can supply the necessary vitamins without addition of organic substrates, which are probably supplied by the dinoflagellate host.
- The stable co-occurrence of bacteria in *Lp* cultures in medium without added vitamins suggests mutualism, but it leaves the question open how *Lp* acquires the vitamins from the bacterial consortia.

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8 Appendices

8.1 Appendix A. L1 medium.

$NaNO_3$ (75.0 g/L dH2O)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ (5.0 g/L dH2O)	1.0 ml
$Na_2SiO_3 \cdot 9H_2O$ (30.0 g/L dH2O)	1.0 ml
L1 Trace Metal Solution	1.0 ml
f/2 Vitamin Solution	0.5 ml
Filtered seawater to	1.0 L

L1 Trace Metal Solution:	
$FeCl_3 \cdot 6H2O$	3.15 g
$Na_2EDTA \cdot 2H_2O$	4.36 g
$CuSO_4 \cdot 5H_2O$ (9.8 g/L dH_2O)	0.25 ml
$Na_2MoO_4 \cdot 2H_2O$ (6.3 g/L dH_2O)	3.0 ml
$ZnSO_4 \cdot 7H_2O$ (22.0 g/L dH_2O)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (10.0 g/L dH_2O)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (180.0 g/L dH_2O)	1.0 ml
H_2SeO_3 (1.3 g/L dH_2O)	1.0 ml
$NiSO_4 \cdot 6H_2O$ (2.7 g/L dH_2O)	1.0 ml
Na_3VO_4 (1.84 g/L dH_2O)	1.0 ml
K_2CrO_4 (1.94 g/L dH_2O)	1.0 ml
Filtered seawater to	1.0 L

f/2 Vitamin Solution:	
Vitamin B12 (1.0 g/L dH2O)	1.0 ml
Biotin (0.1 g/L dH2O)	10.0 ml
Thiamine HCl	200 mg
Distilled water to	1.0 L

8.2 Appendix B. Comparison of *Lp* cell counts and *in vivo* chlorophyll *a* fluorescence.