Iron and Harmful Algae Blooms: Potential Algal-Bacterial Mutualism between *Lingulodinium polyedrum* and *Marinobacter algicola*

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22   **Abstract**

23   Phytoplankton blooms can cause acute effects on marine ecosystems either due to their

24   production of endogenous toxins or due to their enormous biomass leading to major impacts on

25   local economies and public health. Despite years of effort, the causes of harmful algal blooms

26   (HAB) are still not fully understood. Our hypothesis is that bacteria that produce photoactive

27   siderophores may provide a bioavailable form of iron to commensally associated phytoplankton,

28   which could in turn affect algal growth and bloom dynamics. Here we report a laboratory-based

29   study of binary cultures of the dinoflagellate *Lingulodinium polyedrum*, a major HAB species,

30   with *Marinobacter algicola* DG893, a phytoplankton-associated bacterium that produces the

31   photoactive siderophore vibrioferrin. Comparing binary cultures of *L. polyedrum* with both the

32   wild type and the vibrioferrin minus mutant of *M. algicola* shows that bacteria are necessary to

33   promote dinoflagellate growth and that this growth promotion effect is at least partially related to

34   the ability of the bacterium to supply bioavailable iron via the siderophore vibrioferrin. These

35   results support the notion of a carbon for iron mutualism in some bacterial-algal interactions.

36

Introduction

Phytoplankton blooms are a frequent phenomenon in the coastal regions of every continent in the world. Certain phytoplankton algae occurring in mass proliferations produce toxins. Such harmful algal blooms (HABs) can, directly or indirectly, impact marine ecosystems with repercussions on local economies and public health (Lewitus et al., 2012). Direct effects on human health are often related to the consumption of shellfish that have ingested toxic phytoplankton, resulting in the accumulation of toxins and, consequently upon human consumption, paralytic, diarrheic, and neurotoxic poisoning which can sometimes be fatal (Anderson 1994: Honner et al., 2012). HABs also have indirect impacts such as impairment of water quality leading to losses in the tourism and recreational sectors. Even phytoplankton blooms that do not produce toxins can be detrimental and lead to ecological impacts such as the displacement of indigenous species, habitat alteration, or oxygen depletion (Glibert et al., 2005). The economic effects caused by HABs in the U.S. alone were estimated at $82 million per year in 2006 (Hoagland and Scatasta, 2006). Current strategies to reduce health-related impacts due to HABs are based on frequent coastal monitoring and early detection of HAB species and toxin levels. Nevertheless, the factors leading to the origins of HABs are still not well understood. Consequently, there is no strategy for their ultimate prevention.

There is an ongoing debate whether the factors leading to HABs are natural or anthropogenic. Oceanographic factors and global climate change including and impacting upwelling, reversal and relaxation of winds may be the major drivers initiating phytoplankton blooms (Roegner et al., 2002: Tweddle et al., 2010). HABs have also been proposed to be the consequence of human activities including eutrophication, changes in land use and agriculture, overfishing, and ballast water discharge (Gilbert 2005). Others consider that better analytics

60   results in increased detection of HAB species (Burkholder, 1998). Nevertheless, HABs are a

61   global threat to human health, fisheries and aquaculture resources. While numerous studies

62   concentrate on the causes of HABs, few have considered that bacterial species coexisting with

63   microalgae could contribute to their development. However since Bell and Mitchell reported that

64   specific bacterial communities occur and microbial activity was altered in the so-called

65   phycosphere (Bell and Mitchell, 1972; Bell and Lang, 1974), more and more studies suggest that

66   such algal-bacterial interactions are very specific and important (Azam and Malfatti, 2007; Amin

67   et al., 2015; Bertrand et al., 2015; Ramanan et al., 2016; Seymour et al. 2017). A plausible

68   hypothesis suggests that the mutualistic association of some phytoplankton and bacteria is based

69   on nutrient exchange. Nitrogen and phosphorus are the best studied in this regard, although the

70   molecular nature of interactions involving these nutrients is not well understood (Hallegraeff and

71   Gollasch, 2006). Alternatively specific bacteria may affect algal growth and bloom dynamics by

72   their control of iron, a trace element which is often growth limiting to phytoplankton in the

73   marine environment (Miethke and Marahiel, 2007; Maldonado et al., 2005: Croot and Heller,

74   2012).

75   Iron is an essential element for all living organisms including phytoplankton and bacteria

76   due to its involvement in photosynthesis and respiration. Despite iron being the fourth most

77   abundant element on the Earth, its bioavailability in the marine environment is extremely low

78   due to its poor solubility under the mildly alkaline aerobic conditions present in the ocean

79   (Martin and Fitzwater, 1988; Wu and Luther III, 1994). To overcome this low bioavailability,

80   bacteria and fungi have evolved sophisticated systems to produce high-affinity iron-chelating

81   compounds called siderophores to acquire, transport, and process this essential metal ion (Sandy

82   and Butler, 2009). The major role of siderophores is to bind mineral phases of iron and to deliver

83 the iron siderophore complex to specific outer membrane receptors on microbial cells. Several

84 hundred siderophores have been isolated and extensively studied with respect to their synthesis,

85 structures and transport mechanisms over the last three decades (Yamamoto et al., 1994; Challis,

86 2005; Sandy and Butler, 2009: Raymond et al., 2015). While much research has been done on

87 terrestrial siderophores, the study of marine siderophores is less extensive and only a relatively

88 few have been fully elucidated (Vraspir and Butler, 2009). Nevertheless one of the two major

89 attributes that seem to distinguish marine siderophores from those of terrestrial origin is the

90 tendency of the former to contain a α- and β-hydroxy acid group in their iron-binding domain

91 (Barbeau et al., 2001, 2002; Küpper et al., 2006). The significance of the presence of these

92 functional groups in siderophores is in their ability to make the iron-siderophore complex

93 photoreactive. The chelated Fe(III) in such siderophores is reduced in the presence of sunlight

94 via an internal redox process to release Fe(II), a more soluble form of iron (Amin et al., 2009). It

95 has been proposed that sunlight-driven reduction of the Fe(III) would transiently produce Fe(II)

96 which might be utilized not only by the siderophore-producing bacteria themselves but also by

97 non-siderophore producing bacteria and other organisms such as phytoplankton (Maldonado et

98 al., 2005; Naito et al., 2008; Amin et al., 2009, 2012).

99 While iron acquisition by bacteria is well understood, that of phytoplankton remains less

100 so. There are data to support the possibility of a variety of iron uptake mechanisms being

101 operative simultaneously depending on the species involved. These include an iron-reductive

102 route via a cell surface reductase, a direct xenosiderophore-mediated mechanism and cell

103 surface-enhanced processes (Sutak et al., 2012; McQuaid et al., 2018). However as of yet there

104 are no well documented examples, with some exceptions among the cyanobacteria, where

105 phytoplankton have been shown to produce their own siderophores for iron acquisition (Raven,

106   2013). Thus how phytoplankton effectively acquire this metal from the low concentration iron

107   environment is still unclear.

108   Our hypothesis is that algal growth and bloom dynamics may be affected by the increased

109   bioavailability of iron engendered by the presence of photoreactive siderophores produced by

110   mutualistic bacteria. Previously, we studied a bloom of the dinoflagellate, *Lingulodinium*

111   *polyedrum*, at the Scripps Pier (San Diego, CA, USA) in 2011. *L. polyedrum* is one of the HAB

112   species known to produce Yessotoxins, a group of polyether toxins which can accumulate in

113   shellfish and show high toxicity to mice via intraperitoneal injection (Tubaro et al., 2004). As the

114   first step to search for an association of phytoplankton with specific bacterial photoreactive

115   siderophore producers (producing petrobactin, aerobactin, and vibrioferrin siderophores), we

116   monitored both the population of *L. polyedrum* and the bacterial siderophore producers before,

117   during, and after the 2011 bloom. The results showed that both *L. polyedrum* and the bacterial

118   siderophore producers simultaneously increased and decreased during the bloom period

119   (Yarimizu et al., 2014). At the *L. polyedrum* bloom maximum, the total number of bacterial

120   producers of photoreactive siderophore reached their maximum accounting for roughly 9% of the

121   total bacterial population suggesting that such high abundance of photoreactive siderophore

122   producers could potentially provide bioavailable iron to the phytoplankton in this environment.

123   Furthermore, when the PCR-derived amplicons were sequenced, a phylogenetic tree constructed

124   from the sequencing results showed that the community of siderophore producers in pre-bloom

125   was statistically significantly different than those found during and after the bloom by UniFrac

126   analysis suggesting that this particular bacterial community could be involved in bloom

127   initiation.

128   As a proof of concept, the present study was performed using laboratory culture data to

129   correlate the association of the vibrioferrin siderophore producing bacterium, *Marinobacter*

130   *algicola DG893* and the dinoflagellate *L. polyedrum* with iron as a nutrient. It is hoped that

131   finding an algal-bacterial mutualism based on iron availability may be useful for a more

132   thorough understanding of the mechanisms of HAB formation.

133

Materials and Methods

Trace iron cleaning procedures: The container cleaning technique was adopted from Bruland and Franks (1979). All plastic and glass containers were washed with pure water and soaked in 3N hydrochloric acid for at least two weeks at ambient temperature. The acid-washed containers were rinsed with Milli-Q water and dried in a laminar-flow air bench. For those experiments requiring sterile conditions, acid-washed containers were placed in pouches and autoclaved at 121°C for 30 minutes followed by a 45 minute dry cycle. Alternatively, purchased sterile containers Nunc™ Cell Culture Treated Flasks with Filter Caps were used.

Growth media: Of eight sources of natural waters screened, Pacific Ocean oligotrophic water was initially selected for our use due to its extremely low dissolved iron concentration (pM). This was replaced later by Scripps Pier seawater because of its easier accessibility and comparable *L. polyedrum* growth patterns observed in the two sources of seawater. Seawater from Scripps Pier (32.153°N, 117.115°W, San Diego, CA) was collected (pH 8.2-8.4, 980 mOsm/Kg H_2O, total dissolved iron 3-4 nM) and filtered immediately through 0.22 mm pore size membrane. The filtered seawater was mixed with approximately 0.005% metal free hydrochloric acid, autoclaved for 30 minutes, cooled at ambient temperature for a day, and stored in 5°C until use. The pH of the autoclaved seawater was ensured to be between 8.0 and 8.2 at ambient temperature. L1 nutrient was added to sterile seawater per manufacturer's instruction (L1 medium). For those cultures requiring controlled iron concentrations in media, L1 trace element solution without $FeCl_3$ and Na_2EDTA was prepared in house (Guillard and Hargraves, 1993) and added along with other L1 nutrients (nitrate, phosphate, silicate, vitamins) to sterile seawater (L1-Fe medium). Serial dilutions were made on L1 medium with L1-Fe medium to prepare growth medium with defined total iron concentrations, $[Fe]_T$.

157   Artificial Sea Water (ASW): The component solutions were prepared in three separate

158   containers, one with 15 g NaCl, 0.75 g KCl, 1 g NH_4Cl in approximately 500 mL water, one with

159   12.4 g $MgSO_4 \cdot 7H_2O$ in small volume of water, and one with 3.0 g of $CaCl_2 \cdot 2H_2O$ in a small

160   volume of water. The three solutions were mixed and 0.1 g disodium ß-glycerol phosphate was

161   added. The pH was adjusted to 8.0-8.2 and volume was adjusted to 1L. The solution was

162   autoclaved at 121°C for 30 minutes.

163   Algal maintenance and growth monitoring: The *L. polyedrum* strain used in this study was

164   isolated from Venice Beach, California, and kindly provided to us by Avery Tatters (University

165   of Southern California). *L. polyedrum* cells were maintained in sterile one liter Erlenmeyer flasks

166   containing L1 medium and capped with a ventilation sponge. Cultures were exposed to 100 μmol

167   photons m^{-2} s^{-1} on a 12-hour alternating light and dark cycle at a temperature of 20±2 °C

168   (standard growth condition). The upper portion of a culture containing healthy cells was diluted

169   to 1/5 with L1 medium every three weeks. Algal growth was monitored by direct cell count

170   using an inverted microscope (Nikon Eclipse TE2000-U, 4x objective). To prepare cells in

171   controlled iron medium, cultures containing 10^4-10^5 cells per mL was placed in a 15-mL Falcon

172   tubes and centrifuged for a few seconds to pellet cells which were gently washed three times and

173   re-suspended to an appropriate volume with controlled iron medium.

174   "Axenic" *L. polyedrum* preparation: Non-axenic *L. polyedrum* cells were grown to 10^5 cells/mL

175   in L1 medium. Antibiotics 0.1% (v/v) (Penicillin 5units/mL, Streptomycin 5 μg/mL, Neomycin

176   10 μg/mL) were added to a non-axenic culture and incubated for 24 hours under the standard

177   growth condition. Antibiotic-treated cultures were placed in 15 mL Falcon tubes and gently

178   centrifuged for a few seconds. The pelleted cells were washed three times and re-suspended with

179   L1 medium containing 0.05% (v/v) antibiotics in a sterile culture flask to appropriate volume.

180   Antibiotics 0.05% (v/v) was added every 3 days to maintain the culture "axenic". The absence of

181   cultureable bacteria in   the antibiotic treated *L. polyedrum* was tested by spreading a 10 μL

182   sample of the culture on a marine broth plate (5 g/L peptone, 1 g/L yeast extract, 15 g/L agar in

183   75% seawater) followed by incubation at 25°C for 2-3 days. The absence of any visible bacterial

184   colonies was considered indicative of an "axenic" culture of *L. polyedrum*.

185   <u>Bacteria strains and growth monitoring</u>: The model bacterium used was *Marinobacter algicola*

186   DG893 (GenBank code NZ_ABCP00000000.1) since it has been isolated by Green et al. (2004)

187   and well characterized as a producer of the photoactive siderophore vibrioferrin (Amin et al.,

188   2007). The mutant *ΔpvsAB*-DG893 was made as described by Amin et al. (2012) by 872 base

189   pair deletion from the wild type to knock out two siderophore biosynthesis genes, *pvsA* and *pvsB*.

190   To monitor bacterial growth, two methods were used: Optical density at 600 nm was applied to

191   apparently turbid samples which generally contained $\geq 10^7$/mL of bacterial cells. Enumeration of

192   bacteria by serial dilution was applied for samples containing lower numbers of bacteria cells

193   ($<10^7$/mL). For enumeration techniques, serial dilution was made on those samples with sterile

194   seawater, each diluted sample was spread on marine broth plates, and the number of colony

195   forming units (CFU) recorded from the lowest diluted sample plate. CFU in original sample per

196   mL was calculated by (CFU in diluted sample) / (volume plated in mL) x dilution factor.

197   <u>Stock bacteria solution</u>: One liter of marine broth was prepared (5 g/L peptone, 1 g/L yeast

198   extract, 15 g/L agar in 75% seawater, pH 8.2, sterile) and dispensed into sterile tubes with a 5

199   mL volume. A few colonies of bacteria picked from stock plates were transferred into the marine

200   broth tubes and shaken at 25°C for 1-2 days until they reached log phase. The cells were

201   collected by centrifugation, washed three times and re-suspended in sterile medium to an optical

202   density (OD600) of 0.3 (stock bacteria solution).

203 <u>CAS-dye assay</u>: The method was adopted from Alexander and Zuberer (1991) to detect

204 siderophore production by DG893. Using acid washed glassware, the following stock solutions

205 were prepared: 10 mM HDTMA in water, 2 mM CAS in water, 1 mM $FeCl_3.6H_2O$ in water, 50

206 mM piperazine anhydrous buffer (pH5.6), and 0.2 M 5-sulfosalicylic acid in water. CAS solution

207 was prepared by pouring a mixture of 15 mL of CAS and 3 mL of $FeCl_3.6H_2O$ slowly into 12 mL

208 of HDTMA followed by addition of 50 mL of piperazine. The final volume was adjusted to 200

209 mL with water. CAS shuttle solution was prepared by adding 20 µL of 5-sulfosalicylic acid to

210 every mL of CAS solution (used within one day of preparation). Samples to be tested were

211 centrifuged to remove particles and 0.5 mL supernatant was mixed with 0.5 mL of CAS shuttle

212 solution followed by incubation in the dark at ambient temperature for 10 minutes. A color

213 change from dark purple to clear red indicated siderophore production.

214 <u>Materials and reagents</u>: The following materials were purchased from Sigma-Aldrich: Chrome

215 azurol S (CAS, C-1018, MW605.28), antibiotics (Penicillin/Streptomycin/Neomycin, P4083-100

216 mL), peptone (P-1265), iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, 10025-77-1, MW270.30),

217 manganese(II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$, 13446-34-9, MW197.91),

218 hexadecyltrimethylammonium bromide (HDTMA, H6269-100G, MW364.45), 1,4-

219 diazacyclohexane, diethylenediamine (Piperazine, Sigma-Aldrich, P45907-100G, MW 86.14), 5-

220 sulfosalicylic acid (390275, MW218. 18), zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, 1088830500,

221 MW287.54), cobalt(II) chloride hexahydrate ($CoCl_2 \cdot 6H_2O$, 8025400010, MW129.83), copper(II)

222 sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 209198-5G, MW249.69), sodium molybdate dehydrate

223 ($Na_2MoO_4 \cdot 2H_2O$, 331058-5G, MW241.95), selenous acid (H_2SeO_3, 211176-10G, MW128.97),

224 nickel(II) sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$, 227676-100G, MW262.85), sodium orthovanadate

225 (Na_3VO_4, 450243-10G, MW183.91), potassium chromate (K_2CrO_4, 216615-100G, MW194.19),

226   salicylaldoxime (SA, 84172-100G). The following materials were purchased from Fisher

227   Scientific: trace metal-free hydrochloric acid (Optima, A466-250), yeast extract (BP9727-500),

228   agar (BP1423-500), Casamino Acids (BP1424-500), Nunc™ Cell Culture Treated Flasks with

229   Filter   Caps   (12-565-57),   ethylenediaminetetraaceticacid,   tetrasodium   salt   dihydrate

230   (Na$_4$EDTA$\cdot$2H$_2$O, BP121-500, MW416.2), boric acid (AAJ67202A1). Other materials were

231   purchased as follows: 0.22 μm filter membrane (Millipore, MillexGV, SLGV033RS), L1

232   medium kit (Bigelow, MKL150L), pure water (Barnstead water system, 18.2 mΩ). Vibrioferrin

233   (VF, MW434.35) was isolated and purified in house according to Amin et al. (2007).

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Results

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Bacterial growth: The growth of DG893 and mutant $\Delta pvsAB$-DG893 in L1 medium containing

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different total iron concentrations $[Fe]_T$ (1,000, 100, 10, and 0 nM) was monitored. All media

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were divided in two parts, one maintaining [EDTA] at 10,000 nM (L1 nutrient level) with

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variable $[Fe]_T$, and the other reducing [EDTA] along with $[Fe]_T$ to maintain molar ratio of Fe:

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EDTA of 1:1. All media were prepared with and without sterile Casamino Acids. In each

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medium, ca. 10^6 cells/mL of DG893 or mutant $\Delta pvsAB$-DG893 were added and shaken in the

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dark at 30°C. The bacterial growth in each medium was monitored for 5 days by optical density

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at 600 nm. Neither WT DG893 nor the mutant grew in simple seawater or L1-Fe medium

243

without a carbon source (Figure 1A). Addition of iron to seawater or L1-Fe medium also did not

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maintain growth (Figure 1B). However upon addition of Casamino Acids, both DG893 and the

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mutant grew and their growth increased with increasing $[Fe]_T$ in media (Figure 1C). There was

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no remarkable difference in growth pattern between DG893 and the mutant, consistent with the

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report by Amin et al. (2012). In summary, both DG893 and the mutant require a carbon source to

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grow to a detectable level in seawater medium.

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Siderophore production and total iron concentration $[Fe]_T$: The ability to produce the siderophore

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vibrioferrin (VF) by DG893 but not by the mutant was confirmed by the CAS-dye assay. Since

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siderophore biosynthesis is generally turned on under low iron concentrations and turned off

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under high concentrations (Braun, 1995 and 1998; Miethke and Marahiel, 2007), WT was grown

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in L1 medium containing various $[Fe]_T$ with 0.3% Casamino Acids at 30°C in dark for 24 hours

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to find the approximate $[Fe]_T$ under which DG893 produces VF. The WT did not produce VF in

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the media with $[Fe]_T \geq 10$ μM but did so with a $[Fe]_T$ of 1 μM or less . The mutant $\Delta pvsAB$-

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DG893 did not show signs of siderophore production under any of the conditions tested. The

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258   results suggest that under normal seawater conditions, where $[Fe]_T$ is estimated to be nM to pM,

259   that siderophore biosynthesis in DG893 is likely turned on.

260   <u>Axenic culture preparation</u>: The difficulty in preparing and maintaining axenic cultures of many

261   marine algae has been reported in the past (Green et al., 2004; Jauzein et al., 2015; Liu et al.,

262   2017). In fact, Lupette et al. (2016) noted that completely axenic cultures of a model green algal

263   species could not be maintained despite the use of antibiotic treatment protocols. We were

264   partially successful in our efforts to prepare axenic *L. polyedrum*. Initially, non-axenic *L.*

265   *polyedrum* cultures containing 10^5 cells/mL were treated with antibiotics (1% v/v Penicillin 50

266   units/mL, Streptomycin 50 μg/mL, Neomycin 100 μg/mL) for 24 hours under the normal growth

267   conditions. The treated cells were washed and re-suspended with sterile L1 media and while the

268   "axenicity" of the culture at this point was confirmed, bacterial colonies reappeared after only a

269   few days. An attempt was made to keep 1% antibiotics in the culture longer than 24 hours,

270   however, dinoflagellate cells began to disintegrate after 2 days suggesting that 1% antibiotics

271   must be removed from the culture within 24 hours. The fact that the negative control and sterile

272   L1 medium without phytoplankton cells remained bacteria-free for weeks ruled out a possibility

273   of culture contamination. We believe that the antibiotics are most likely eliminating only free-

274   living bacteria leaving attached bacteria unaffected. The next effort of preparing axenic cultures

275   was made by immediate dilution of antibiotic-treated cultures. The rationale of this attempt was

276   to minimize attached bacteria on *L. polyedrum* cells by dilution. The antibiotic treated *L.*

277   *polyedrum* culture was diluted 200-fold with sterile L1 medium and incubated under standard

278   growth conditions. The diluted culture remained bacteria-free for the first 10 days, however

279   microbial growth was observed on the 12th day as the number of *L. polyedrum* cells also

280   increased. Although this dilution method suggested that the culture stayed bacteria free for up to

281   10 days, the *L. polyedrum* cell count was too dilute to be useful for further study. The next

282   attempt at axenic culture preparation was made with three sequential antibiotic treatments. The

283   non-axenic culture was treated with 1% antibiotics for 24 hours, washed three times, and re-

284   suspended with sterile L1 medium. The cells were allowed to recover under normal growth

285   conditions for a day and then again treated with 1% antibiotics. This procedure was repeated

286   three times. This procedure also failed as the dinoflagellate cells began to disintegrate. Finally,

287   using a similar procedure but reducing the concentration of antibiotics to 0.1% for the first

288   treatment and adding 0.05% subsequently every 2-3 days resulted in *L. polyedrum* cultures

289   without visible cell damage that appeared to be free of free-living bacteria for more than a

290   month.

291   *L. polyedrum* growth screening: A matrix containing thirty cultures of *L. polyedrum* in L1

292   medium containing different numbers of DG893 cells in the initial inoculum (10^7, 10^6, 10^5, 10^4,

293   10^3, 0 CFU/mL) and different $[Fe]_T$ (10^4, 10^3, 10^2, 10^1, and 0 nM) was set up. The same matrix

294   was set up with the mutant *ΔpvsAB*-DG893. Bacterial growth was monitored by enumeration of

295   bacteria by serial dilution and phytoplankton growth was monitored by direct cell counts under

296   the microscope. Regardless of the starting bacterial cell number, both DG893 wild type and

297   mutant grew in all the binary culture matrices over 28 days to eventually reach a bacterial

298   population of ca. 10^5 CFU/mL. The group of media containing 1,000 nM $[Fe]_T$ showed the

299   greatest growth of *L. polyedrum* regardless of the starting bacterial inoculum (Figure 2A). The

300   group of media containing ≤10 nM $[Fe]_T$ showed very slow *L. polyedrum* growth regardless of

301   starting bacterial inoculum. No significant differences in *L. polyedrum* growth co-cultured with

302   DG893 or the mutant were observed, which left a question as to whether VF secreted by DG893

303   would have a significant effect on *L. polyedrum* growth. It was speculated that under these

304 growth conditions, the amount of VF being produced by DG893 was insufficient to see

305 detectable changes in *L. polyedrum* growth. This was later confirmed (*vide infra*) when excess

306 VF artificially added to the culture produced pronounced *L. polyedrum* growth. Finally, the

307 growth pattern of axenic *L. polyedrum* was similar to that of a non-axenic culture. This

308 observation left open another question as to whether DG893 and *L. polyedrum* were

309 commensally associated. However this also was confirmed by experiments described below

310 where after subsequent culturing the "axenic" *L. polyedrum* ceased to grow while binary *L.*

311 *polyedrum* DG893 kept growing exponentially (Figure 3).

312 Subsequent batch culture growth of *L. polyedrum* in DG893 binary culture: *L. polyedrum* grown

313 in L1 media containing various $[Fe]_T$ and DG893 (Figure 2A) for 28 days were diluted with

314 appropriate media to adjust *L. polyedrum* to 10^2 cells/mL in the 2nd subsequent batch culture. The

315 *L. polyedrum* growth in this 2nd subsequent culture was monitored for another 28 days under

316 each growth condition. The result showed that the 2nd subsequent batch culture *L. polyedrum* in

317 the higher two $[Fe]_T$ (10,000 and 1,000 nM) maintained their exponential growth while those in

318 the lower three $[Fe]_T$ did not grow (Figure 2B). Although the cells under the latter conditions did

319 not completely die out, the few cells that survived had their swimming activity under the

320 microscope clearly reduced.

321 Axenic *L. polyedrum* culture and rescue: The growth of "axenic" and binary cultures of *L.*

322 *polyedrum* with DG893 in L1 was compared over several subsequent batch cultures. No

323 significant difference in growth was observed in the 1st culture, however, a clear difference was

324 observed in the 2nd subsequent batch culture of *L. polyedrum* with and without DG893. While *L.*

325 *polyedrum* with DG893 grew fully in both the 1st and 2nd subsequent batch cultures, "axenic" *L.*

326 *polyedrum* ceased to grow (Figure 3). When DG893 was added to the 2nd subsequent "axenic"

327 batch culture, *L. polyedrum* cells began to regrow exponentially demonstrating that DG893 could

328 rescue "axenic" *L. polyedrum*.

329 <u>Non-axenic *L. polyedrum* growth and rescue</u>: The non-axenic *L. polyedrum* was maintained in

330 L1 medium and assumed to contain not only siderophore-producing bacteria but also other

331 unknown bacteria. The non-axenic culture was divided in two parts, one diluted to 1/5 with L1

332 medium and other diluted to 1/5 with L1-Fe medium (batch subculture 1). When the cell count

333 reached approximately 10^4 cells/mL, both cultures were further diluted with appropriate medium.

334 Therefore, one culture maintained $[Fe]_T$ at L1 level throughout the subsequent batch cultures

335 while the other contained successively reduced $[Fe]_T$. The *L. polyedrum* growth was monitored

336 for 28 days for each subculture. After the 6th subculture where $[Fe]_T$ in the medium was

337 estimated to be 2 nM, *L. polyedrum* stopped growing completely (Figure 4). On 29th day of the

338 6th subculture, an iron supplement to give a $[Fe]_T$ of 5850 nM was added to the culture and

339 growth was continued to be monitored for additional 28 days. The results indicate that the *L.*

340 *polyedrum* culture which stopped growing under 2 nM $[Fe]_T$, could be rescued by addition of

341 iron after 28 days (Figure 5).

342 *L. polyedrum* growth in DG893 supernatant: Earlier it was stated that when *L. polyedrum* was

343 co-cultured with either DG893 or the mutant *ΔpvsAB*-DG893, little difference was observed in

344 its growth pattern. We speculated that this may have been due to the small amount of VF

345 produced by the equilibrium population of DG893 in the binary culture. To test this idea,

346 samples of the supernatant of the media from the WT DG893 grown under ideal conditions and

347 containing detectable amounts of VF by CAS-dye assay, as well as the mutant *ΔpvsAB*-DG893

348 which did not, respectively, were added to *L. polyedrum* cultures and growth under these

349 different conditions was compared. It should be noted that while we traditionally grow DG893

350   and the mutant in marine broth containing Casamino Acids, these were found to be toxic to *L.*

351   *polyedrum* cells. In this experiment, we instead used ASW with succinic acid (at from 0.001% to

352   0.1%) as a carbon source (pH 8.2) to grow DG893 and the mutants prior to their supernatant

353   being added to *L. polyedrum* cultures. *L. polyedrum* growth was enhanced the most by the

354   addition of the DG893 supernatant. The mutant supernatant also showed a slightly positive effect

355   on *L. polyedrum* growth compared to media without bacterial supernatant, however the degree of

356   growth was not as large as that induced by the DG893 supernatant (Figure 6). These results

357   suggest that VF has potential influence on *L. polyedrum* growth, but that other extracts from

358   bacteria (vitamin B_{12}?) may also have a potential effect on their growth. Further experiments

359   were performed using purified VF itself. When purified VF at 57μM was directly added to a *L.*

360   *polyedrum* culture in L1 media containing 100 nM $[Fe]_T$, *L. polyedrum* reached cell counts

361   approximately 1.5 times as large as those in media without VF.

362   DG893 growth in *L. polyedrum* supernatant: "Axenic" *L. polyedrum* cells were resuspended in

363   L1 media containing 100 nM, 1,000 nM, 10,000 nM $[Fe]_T$ and incubated under the standard

364   growth conditions for two days to collect their organic extracts. The *L. polyedrum* cultures were

365   divided in two parts, one of which was filtered through a 0.22 μm membrane to collect only

366   organics and the other was kept without filtration to keep phytoplankton cell debris. The *L.*

367   *polyedrum* supernatant with and without cells was added to the stock DG893 containing 0.3%

368   Casamino Acids, and the mixture shaken at 30°C for several days. The control was prepared in

369   the same manner without *L. polyedrum* supernatant and cells. In the media containing 1,000 nM

370   and 10,000 nM $[Fe]_T$, DG893 growth was enhanced by the presence of *L. polyedrum* supernatant

371   and was even more pronounced with the supernatant containing cell debris (Figure 7). In media

372   with 100 nM [Fe]$_T$, only a subtle difference was observed in DG893 growth with and without *L.*

373   *polyedrum* supernatant.

374   **Discussion**

375   Before proceeding to a discussion of the results presented here it is important to

376   operationally define what we mean by [Fe]$_T$ and "axenic" cultures. Here we used the term total

377   iron concentrations [Fe]$_T$ to mean the total analytical amount of iron added to culture which

378   includes all phases of iron present in the solution. This is different from the total dissolved iron

379   concentration which we routinely measure for environmental seawater samples (and which is

380   approximately 2-6 nM for Scripps Pier water for example) as determined by cathodic stripping

381   voltammetry (CSV). Total dissolved iron in such environmental samples is processed by

382   filtration through a 0.22 μm membrane followed by acidification. As a result, total dissolved iron

383   in a sample is much lower than the total analytical iron concentration. We use this operational

384   definition as it is not possible to measure total dissolved iron in culture media using the CSV

385   method because equilibrium between the various iron oxo/hydroxo species potentially present in

386   seawater at pH 8.2 is reached only extremely slowly and thus concentrations of total dissolved

387   iron are constantly changing over the time course utilized in these experiments. Using a straight

388   forward expression of total analytical iron concentration is thus a potentially more reproducible

389   approach with which to compare the effects of iron on bacterial and algal growth in a laboratory

390   setting.

391   Secondly we use the term "axenic" in quotation marks for describing cultures of *L.*

392   *polyedrum*, to mean the absence of any observable *cultureable* bacteria based on the absence of

393   visual bacterial colonies when cultures were placed on solid media and incubated for several

394   days. We were only partially successful making the culture "axenic" by treating the culture with

395   low concentrations of antibiotics and sequential addition of the antibiotics to the culture over

396   extended times. Using this method the culture appeared to remain bacteria free for at least a

397   month. However when the sequential addition of antibiotics to the culture was stopped, bacteria

398   growth was observed to return within a few days. Thus clearly the cultures were not truly axenic.

399   Nevertheless our main concern was that the dominant species of bacteria growing in our binary

400   culture should be DG893 and that "axenic" cultures should contain insignificant numbers of

401   unknown bacteria. We confirmed the former by qPCR and the latter was further supported by

402   presence of few if any bacteria seen via DAPI staining. To our knowledge, there is at present no

403   general protocol for preparing a completely axenic culture *of L. polyedrum* hence our use of the

404   operational definition of "axenic" described here.

405   We initially investigated conditions under which the bacterium *Marinobacter* DG893 and

406   the dinoflagellate *L. polyedrum* could grow independently. As expected, in the absence of an

407   added carbon source DG893 could not grow in natural unamended seawater or in L1

408   supplemented ASW irrespective of the iron concentration. In the presence of an adequate

409   artificial source of carbon however bacterial growth was controlled by the available

410   concentration of iron. Under idealized conditions, both DG893 and the VF null mutant reached a

411   final concentration of around 10^5 CFU/mL independent of the initial inoculum. However even

412   *without any externally added carbon source* DG893 grew well in the presence of *L. polyedrum*

413   indicating that the dinoflagellate leaked enough dissolved organic carbon (DOC) to fully support

414   the growth of the bacterium. The notion that DG893 could utilize *L. polyedrum* as a carbon

415   source was further supported by the observation that growth of DG893 was stimulated when it

416   was incubated in the media containing the supernatant from a filtered *L. polyedrum* culture

417   which contains non-specific organics secreted from *L. polyedrum*. These may possibly include

418   dimethylsulfoniopropionate (DMSP) a labile carbon and sulfur source known to "leaked" from

419   dinoflagellates (Caruana and Malin, 2014).

420   In the case of both "axenic" and nonaxenic cultures of the photosynthetic dinoflagellate

421   *L. polyedrum*, iron proved to be a growth limiting nutrient. Thus maximum growth occurred at

422   total iron concentrations of 100-1000 nM while higher concentrations proved toxic and lower

423   concentrations (0-10 nM) were growth limiting. In the first subculture, both "axenic" and non-

424   axenic cultures grew equally well. However, their growth showed a remarkable difference in the

425   second batch subculture. Here the non-axenic *L. polyedrum* culture continued to grow

426   exponentially, although now only at the higher $[Fe]_T$. When the iron concentrations were kept

427   high, *L. polyedrum* cultures continued to thrive up to at least 6 subcultures. However, when the

428   iron concentrations were allowed to be reduced by dilution in subsequent subculturing, the

429   growth gradually ceased. Poorly growing subcultures in low iron media could however be

430   rescued by the addition of additional iron to the culture. Remarkably "axenic" *L. polyedrum*

431   subcultures ceased to grow regardless of $[Fe]_T$ but could be rescued by the addition of DG893.

432   These results emphasize the importance of both bacteria and iron to *L. polyedrum* growth.

433   Our results further suggest that the *L. polyedrum* growth promoting effects of the

434   bacterium *Marinobacter* DG893 were in fact not unspecific but at least partially related to its

435   production of the photoactive siderophore vibrioferrin. Thus only the supernatant of DG893 that

436   contained sufficient amounts of vibrioferrin facilitated *L. polyedrum* growth. While the addition

437   of the supernatant from a culture of the VF null mutant of DG893 also improved growth of *L.*

438   *polyedrum* the effect was much less than that from the WT. This observation was further

439   confirmed by direct addition of purified VF into the *L. polyedrum* culture which growth

440   increased 1.5 times more than those without VF. These results imply that the presence of the

441 siderophore vibrioferrin clearly influences *L. polyedrum* growth, but that other factors such as

442 vitamin B12 (Cruz-Lopez and Maske, 2015) provided by bacteria may also be important in

443 promoting this apparent bacterial-algal mutualism.

444   Here we have focused on a bacterium that produces the photoreactive siderophore,

445 vibrioferrin. Vibrioferrin was chosen for more detailed study for a number of reasons. First

446 vibrioferrin has been isolated from several different bacteria which are known to be closely

447 associated with HAB species (Amin et al., 2007) and we found that bacteria that had the ability

448 to synthesize vibrioferrin were by far the most numerous as compared to the producers of other

449 photoreactive siderophores which we followed during the bloom of *L. polyedrum* at Scripps Pier

450 in 2011. We initially supposed that vibrioferrin was a good candidate for a siderophore that could

451 provide bioavailable iron for both the bacterial producers and their algal partners as it is the most

452 rapidly photolyzed of the photoactive siderophores tested and that photolyzed vibrioferrin has no

453 further affinity for iron. This feature is unusual in that most other photoactive siderophores retain

454 the ability to strongly bind Fe(III) even after photolysis (Amin et al., 2009). However we have

455 recently showed that while vibrioferrin was the best source of iron for *L. polyedrum* of the ones

456 we tested (Yarimizu et al., 2017), it was not due to its photolysis since iron uptake from VF was

457 the same in the dark as in the light. We therefore attribute its ability to provide bioavailable iron

458 to the dinoflagellate to its relatively weak iron binding properties (with respect to other more

459 traditional siderophores) as it lacks the sixth donor group required to complete the octahedral

460 coordination geometry preferred by Fe(III) (Amin et al., 2009). This in turn leads to a less

461 negative reduction potential (it falls within the biological range) so that the iron can readily be

462 released from it by the cell surface reductases known to be present in *L. polyedrum* .

As a final note, while this laboratory study strongly supports a carbon for iron mutualism between the dinoflagellate *L. polyedrum* and *Marinobacter* DG893 as previously proposed (Amin et al, 2009), it is unclear at present how, or indeed if, such a mutualism will operate in the field. To this end we have collected data from several field studies involving both HAB and non-HAB bloom events and are currently searching for relationships between phytoplankton, vibrioferrin producing bacteria, and available iron. We hope that both these laboratory and field studies will complement each other and advance our knowledge of the potential role of bacterial-algal interactions in understanding the mechanisms of HAB formation.

Acknowledgements

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Figure 1. DG893 growth in media with and without Fe and carbon

A) DG893 growth in media without added Fe. The bold lines represent media containing 10 mg/mL Casamino Acids. The dotted lines represent media without Casamino Acids. The grey lines represent L1-Fe media. The black lines represent seawater. The VF null mutant of DG893 showed a similar pattern of growth (data not shown). B) DG893 incubated in L1 media containing different $[Fe]_T$ but without an added carbon source. The black bold line, grey bold line, grey wide dotted line, and grey narrow dotted line represent L1 media with 1,000 nM, 100 nM, 10 nM, 0 nM $[Fe]_T$, respectively. The mutant DG893 showed a similar pattern. C) DG893 incubated in L1 media containing 10 mg/mL Casamino Acids and different $[Fe]_T$. The black bold line, grey bold line, grey wide dotted line, and grey narrow dotted line represent L1 media with 1,000 nM, 100 nM, 10 nM, 0 nM $[Fe]_T$, respectively.

Figure 2. *L. polydrum* co-cultured with a starting inoculum of DG893 at 10^3 cells/mL in media with various $[Fe]_T$

A) 1st generation growth of *L. polyedrum* in the presence of DG893. The starting inoculum of DG893 was 10^3 cells/mL which reached 10^5 cells/mL after 28 days incubation under the standard growth conditions. A similar pattern was observed in all other groups: i.e. media containing a starting inoculum of DG893 of 10^7, 10^6, 10^5, 10^4, 10^3, 0 cells/mL and $[Fe]_T$ of 10^4, 10^3, 10^2, 10^1, or 0 nM. B) 2nd generation growth of *L. polyedrum* in the presence of DG893. After 28 days the cultures in panel A were diluted to a *L. polyedrum* concentration of 10^2 cells/mL with appropriate media and incubated under the standard growth condition for an additional 28 days.

Figure 3. Rescue of *L. polyedrum* by addition of DG893

A) 1st generation of *L. polyedrum* growth maintained in L1 media either "axenic" or co-cultured with DG893. B) Growth of *L. polyedrum* in the 2nd subculture either "axenic" or co-cultured with DG893. After the 2nd subculture of "axenic" *L. polyedrum* was incubated under the standard growth conditions for 21 days, an inoculum containing approximately 10^2 cells/mL of DG893 was added and incubation continued for an additional 14 days.

Figure 4. Non-Axenic *L. polyedrum* growth in L1 media and reduced $[Fe]_T$ over 6 subcultures

Solid line) cultures maintained with a $[Fe]_T$ at the L1 level throughout the subculturing Dotted line) cultures where the $[Fe]_T$ continually decreased over the subcultures due to dilution.

Figure 5. Iron starved non-axenic *L. polyedrum* growth rescued by iron addition

The iron sufficient culture (solid black line) contained 11700 nM $[Fe]_T$ while the iron deficient culture contained 2 nM (dotted black line). After 28 days 5850 nM $[Fe]_T$ was added to the iron deficient culture which rescued *L. polyedrum* growth.

Figure 6. *L. polyedrum* growth in media with DG893 supernatant, mutant supernatant, and without bacterial supernatant

640   The black lines, grey lines, and dotted lines represent *L. polyedrum* growth in media with added
641   DG893 supernatant, with added VF null mutant supernatant, and without any bacterial
642   supernatant, respectively.
643
644   **Figure 7. DG893 growth in media containing different [Fe]$_T$ with and without presence of**
645   ***L. polyedrum* supernatant and cell debris**

646   The solid black lines represent growth of DG893 in the presence of *L. polyedrum* supernatant
647   with their cell debris, the grey lines are DG893 growth in *L. polyedrum* supernatant without their
648   cell debris, and the dotted lines are DG893 without any *L. polyedrum supernatant*.
649