Effect of Sulfide on Growth of Marine Bacteria

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Abstract

Severe hypoxia leads to excess production of hydrogen sulfide in marine environments. In this study we examine the effect of sulfide on growth of four facultative anaerobic marine bacteria in minimal media under anaerobic conditions. The Gram-negative chemolithoautotrophic *Marinobacter* sp. tolerated sulfide concentrations up to 0.60 mM, with doubling and lag times increasing as a function of increasing sulfide concentration but with no change in maximum culture yields; growth did not occur at 1.2 mM sulfide. Similar results were obtained for the metabolically diverse Gram-negative denitrifying *Pseudomonas stutzeri*, except that growth occurred at 1.2 mM and culture yields at 0.60 and 1.2 mM sulfide were approx. 10-fold lower than at sulfide concentrations between 0 and 0.30 mM. Increases in doubling and lag times accompanied by an overall 10-fold decrease in maximum culture yields were found for the Gram-negative chemoheterotrophic *Vibrio* sp. at all sulfide concentrations tested. In contrast, growth of a Gram-positive chemoheterotrophic *Bacillus* sp. was resistant to all sulfide concentrations tested (0.15 to 1.2 mM). Our results highlight the variable responses of marine bacteria to sulfide and provide some insight into shifts that may occur in microbial community structure and diversity as a consequence of changes in sulfide levels that are the result of hypoxia.

Keywords: Doubling times, growth yields, environmental stress, growth curves

Introduction

Dissolved oxygen concentrations in coastal, marine, and estuarine environments have changed drastically over the past decades as a result of both human induced eutrophication and global climate change (Diaz and Rosenberg 2008; Vaquer-Sunyer and Duarte 2008; Lavik et al. 2009; Levin et al. 2009; Vaquer-Sunyer and Duarte 2010) with hypoxia increasing exponentially at a rate of 5.54% per year (Vaquer-Sunyer and Duarte 2008). As a consequence of naturally occurring abiotic—e.g., volcanic, underground aquifer, mineral and geothermal spring activities (Babich and Stotzky 1978; Kalciene and Cetkauskaite 2006; Lloyd 2006; Ghosh and Dam 2009; Luther et al. 2011)—and biotic sulfide production, severe hypoxia and total lack of oxygen (anoxia) can lead to excess production of hydrogen sulfide (Lavik et al. 2009; Levin et al. 2009; Grote et al. 2012). Biotic production of sulfide includes the reduction of inorganic sulfate by sulfate-reducing bacteria (e.g. *Desulfovibrio* and *Desulfobacter* spp.), the reduction of S^0 (*Desulfuromonas* spp. and many hyperthermophilic *Archaea*) and the conversion of thiosulfate to sulfide and sulfate (disproportionation) (e.g. some *Desulfovibrio* spp.) (Jorgensen and Bak 1991; Lloyd 2006). Additionally, microbial decay of S-containing amino acids (cysteine and homocysteine), sulfolipids, and sulfated polysaccharides by various microbial groups (Cooper 1983; Lloyd 2006) contributes to biological sulfide production.

Excess sulfide levels promote the growth of sulfide utilizing microorganisms, mostly forming black mats in hypoxic and anoxic environments (Levin et al. 2009; Grote et al. 2012). As a result of sulfide oxidation by microaerophilic or anaerobic (often denitrifying)

chemolithoautotrophic bacteria (e.g., *Beggiatoa*, *Thioploca*, *Thioalkalivibrio, Thiohalomonas, Sulfurimonas,* and *Thiobacillus*) and Archaea (e.g. *Sulfolobales*), and anaerobic photolithoautotrophic bacteria (*Chlorobi*, *Chromatiaceae*, *Rhodospirillum*, *Rhodovulum*, and *Rhodopseudomonas*), which use a variety of enzymes, pathways, and mechanisms for sulfide oxidation (Lloyd 2006; Ghosh and Dam 2009; Luther et al. 2011), sulfide levels decrease in these ecosystems. On the other hand, the net balance is shifted towards sulfide accumulation in anaerobic aquatic ecosystems (Guidotti 1996; Kuster et al. 2005; Lloyd 2006; Ghosh and Dam 2009), where it exists mostly in the water-soluble form and can exceed 10 mM (Lloyd 2006).

In anaerobic environments sulfide is the most toxic form among all reduced sulfur compounds (Brouwer and Murphy 1995) and its toxicity has been demonstrated on higher organisms such as crustaceans (Powell et al. 1986; Kuster et al. 2005), plants (Koch et al. 1990; Heijs et al. 2000), algae (Castenholz 1976; Kuster et al. 2005), fish (Reiffenstein et al. 1992; Brouwer and Murphy 1995), and other vertebrates (Reiffenstein et al. 1992; Brouwer and Murphy 1995; Lloyd 2006). Understanding the effect of sulfide on growth of microorganisms, however, has been limited to examining its influence on the growth inhibition of methanogenic, anammox, and sulfate-reducing bacteria (Mountfort and Asher 1979; Ronnow and Gunnarsson 1981; Cohen et al. 1986; Mathrani et al. 1988; Reis et al. 1992; Brouwer and Murphy 1995; Kuster et al. 2005; Kalciene and Cetkauskaite 2006; Al-Zuhair et al. 2008; Jin et al. 2012). On the other hand, the response of non-sulfide utilizing marine microorganisms to elevated sulfide levels, which may be considered to be among the first indicators of environmental changes and contamination, is poorly understood. A few studies have examined the toxicity of sulfide on

79 microorganisms exclusively used in ecotoxicological biotests (e.g., *Vibrio fischeri*) (Brouwer and
80 Murphy 1995; Kuster et al. 2005; Kalciene and Cetkauskaite 2006). Median effective
81 concentration (EC_{50}) values for total sulfide (at pH 7.5) were found to vary between 62 μM (Van
82 Leeuwen et al. 1985; Postma et al. 2002) to 276 μM (Kuster et al. 2005).

83 Sulfides occur naturally in one of three states—H_2S, HS^- and S^{2-}—with H_2S and HS^- the
84 predominant species at physiological pH (Kuster et al. 2005; Al-Zuhair et al. 2008). Therefore,
85 total soluble sulfide concentrations are determined in order to evaluate the effect of sulfide on
86 different organisms (Castenholz 1976; Kuster et al. 2005). As a consequence of its volatility,
87 sulfide concentrations will decrease with time and studies that examine its effect on microbial
88 growth demand that they are done under conditions where contact with oxygen is restricted and
89 sulfide oxidation is minimized.

90 The goal of the current study was to examine the influence of soluble sulfide on growth
91 of four different facultative anaerobic marine bacteria from the families *Vibrionaceae*,
92 *Bacillaceae*, *Alteromonadaceae*, and *Pseudomonadaceae* to determine its influence on their
93 growth. Representatives of all four are found throughout the water column in the marine
94 environment (Dash et al. 2013) and we use them as examples for studying the effect of sulfide on
95 growth under anaerobic conditions. Sulfide concentrations in the absence of bacteria were also
96 measured to account for assumed losses through volatilization and oxidation that were the result
97 of reactivity with components of the microbial growth media formulations.
98

Materials and methods

Bacterial strains

Vibrio sp. strain OY15 *(Bacteria; Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae)* and *Bacillus* sp. strain S1 *(Bacteria; Firmicutes; Bacilli; Bacillales;* Bacillaceae) isolated from seawater shellfish were a gift from Dr. Gary Wikfors, NOAA Northeast Fisheries Science Center, Milford Laboratory, Milford, CT. DNA sequence analysis of OY15 and S1 16S rRNA gene sequences indicated these isolates to be related to *Vibrio sp.* EX25 and *Bacillus cereus*, respectively (Schreier, unpublished). *Marinobacter sp. (Bacteria; Proteobacteria; Gammaproteobacteria; Alteromonadales; Alteromonadacea)* is a laboratory strain isolated from a marine recirculating aquaculture system denitrification filter with a 16S rRNA gene sequence that shares 100% identity with *Marinobacter aquaeolei* (Schreier, unpublished). *Pseudomonas stutzeri (Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae)* was a gift from Dr. Russell Hill, University of Maryland Center for Environmental Sciences. Laboratory cultures of all bacteria were maintained on marine agar 2216 (Difco) plates.

Media and growth

Vibrio sp. OY15 and *Marinobacter* sp. were grown at 25°C in a medium (Medium 1) containing 400 mM NaCl, 10 mM KCl, 50 mM $MgSO_4 \cdot 7H_2O$, 9 mM NH_4Cl, 12.6 mM $CaCl_2 \cdot 6H_2O$, 0.1% Casamino acids (Difco, Detroit, MI, USA), 40 mM glucose, 4 mM $NaNO_3$ and 50 mM Tris-Cl (pH 8.0) (Proctor and Gunsalus 2000). *P. stutzeri* was grown at 25°C in a

120 medium (Medium 2) containing 1 g yeast extract (Difco), 0.5 g $(NH_4)_2PO_4$, 1.14 g KH_2PO_4, 1.45
121 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.001g MoO_3, 0.001g $FeSO_4$, 7.3 g KNO_3 and 5.0 g glucose per
122 1 L of deionized water (Spangler and Gilmour 1966). *Bacillus* sp. S1 was grown at 37°C in a
123 medium (Medium 3) containing 1.0 g NH_4C1, 0.45 g KH_2PO_4, 0.68g $NaNO_3$ and 5 g yeast
124 extract (Difco Laboratories) per L of deionized water plus 10 ml of Wolfe salts (Wolin et al.
125 1963) amended with 0.4 g of $Na_2MoO_4 \cdot 2H_2O$ in 1 L as described previously (Gocke et al. 1989).
126 The modified Hungate technique (Miller and Wolin 1974) was used to prepare media for
127 the growth of cultures under anaerobic conditions. Media was sparged with 99.998% (zero
128 grade) N_2 gas and dispensed anaerobically into 20 ml septum-stoppered Hungate tubes while
129 simultaneously flushing the headspace with N_2. Resazurin (1 mg L^{-1}) was added to visually
130 estimate anaerobic conditions. The tubes were then sealed with crimp-sealed aluminum caps and
131 autoclaved. Sodium sulfide (1000 mg L^{-1}) stock solution was made anaerobically and sterilized
132 separately.
133 Triplicate samples were inoculated using 0.8 ml of anaerobically grown mid-exponential
134 phase cultures and supplemented with sodium sulfide at concentrations of 0.15 mM, 0.30 mM,
135 0.60 mM, and 1.2 mM in a final volume of 15 ml. Cultures and sodium sulfide were added
136 aseptically through the septum of each tube using an N_2-flushed syringe.
137 To control for the loss of sulfide from sealed Hungate tubes, duplicates of abiotic controls
138 were prepared in the same manner as inoculated samples except that microorganisms were
139 omitted. In this manner, the loss/oxidation rate of sulfide over time was estimated.

140 Microbial growth was evaluated spectrophotometrically at a wavelength of 600 nm using
141 a Spectronic 20 Colorimeter. Periodically, 0.5 ml of sample was removed for each sample for
142 the determination of pH and sulfide concentrations.
143
144 Analytical methods
145 Microbial growth yields were determined by plating 0.1 mL of appropriate dilutions onto
146 solid medium (either Minimal Salt Media 2216 or Luria-Bertani agar) for colony counting.
147 Viable cell numbers per mL of culture were calculated as a function of sample OD_{600}. Indicator
148 paper was used to estimate culture pH, and total soluble sulfide concentrations were determined
149 using an LS-146AGSCM micro sulfide ion electrode (Lazar research Laboratories, Inc., Los
150 Angeles, CA) calibrated with sulfide standards ranging from 0.15 mM to 1.2 mM prepared under
151 anaerobic conditions.
152

153 ## Results

154 Effect of growth medium on sulfide concentration under anaerobic conditions

155 To determine the effect of sulfide on bacterial growth, sulfide concentrations were first
156 measured in each growth medium as a function of time in the absence of bacteria to assess
157 abiotic oxidation due to media components. Na_2S was added to each medium in sealed
158 anaerobic culture tubes through septa at concentrations of 0.15, 0.30, 0.60 and 1.2 mM and total
159 soluble sulfide was monitored as described in Materials and Methods. Sulfide concentrations
160 remaining as a function of time for each medium are shown graphically in Figure 1. While pH
161 changes will influence the HS^-/H_2S equilibrium (Kuster et al. 2005; Luther et al. 2011), no
162 significant change in pH was detected throughout the duration of the experiments, which
163 remained at 8.0 for Medium 1 and 7.0 for Medium 2 and Medium 3 (data not shown).

164 In general, a reduction in sulfide concentration was observed in all media regardless of
165 initial levels, although the rate of decrease varied for each medium. For Medium 1, sulfide loss
166 occurred gradually over time with 50% of the initial amount remaining after approximately 120 h
167 for the 0.15 mM sulfide sample and between 30 to 60 h for the other samples; from 1 to 20%
168 sulfide remained after almost 230 h. In Medium 2, on the other hand, sulfide concentrations
169 dropped dramatically after addition with 70 to 90% depletion observed within 16 h at all sulfide
170 concentrations; sulfide was barely detectable (<1.0%) after 34 h in the 0.15 and 0.30 mM
171 samples and were below 6% after 169 h in the 0.60 and 1.20 mM samples. While sulfide levels
172 were unchanged during the first 34 h of incubation in Medium 3, they subsequently decreased in

a manner similar to those observed for Medium 2, with 50% of the initial concentration remaining after 60 to 70 h. The variation in sulfide oxidation rates for the three media—estimated sulfide half-lives for Medium 2 ranged from 2 to 9 h, depending on initial concentrations, and between 40 and 97 h for Medium 1 and 3—likely reflect differences in oxidizing metal [e.g. iron and manganese (Yao and Millero 1995)] concentrations and are consistent with those reported for anoxic sulfide oxidation (Almgren and Hagström 1974; Luther et al. 2011). While we cannot rule out contributions due to the presence of trace amounts of O_2, sulfide oxidation rates under all conditions were significantly slower than those reported for O_2-facilitated processes (Almgren and Hagström 1974; Luther et al. 2011).

Effect of sulfide on bacterial growth under anaerobic conditions

Mid-exponential cultures of each marine bacterium grown in the absence of sulfide were used to inoculate media containing different concentrations of sulfide under anaerobic conditions and growth was monitored as described in Materials and Methods. Results are shown in Figure 2. In general, sulfide's influence on lag phase, doubling time, and maximum growth yields (as measured by maximum cell numbers) (Table 1) ranged from negligible to severe depending on the bacterium.

Marine gammaproteobacterial *Marinobacter* sp. was capable of tolerating initial sulfide concentrations up to 0.60 mM, although some perturbations in growth characteristics were detected. Doubling times in the presence of 0.15 mM, 0.30 mM, and 0.60 mM sulfide increased

193 from 18 to 25 h (Table 1, Fig. 2A) and lag phase times increased more than two-fold compared
194 to the absence of sulfide (Table 1, Fig. 2A). Growth yields for 0.15, 0.30 and 0.60 mM sulfide
195 concentrations (between 2.4 x 10^5 and 4.2 x 10^5 CFU mL^{-1}) were not significantly different from
196 those obtained in the absence of sulfide (Table 1). Growth was not observed in the presence of
197 1.2 mM sulfide.

198 Growth yields for the denitrifying marine bacterium, *P. stutzeri*, in the presence of 0.15
199 and 0.30 mM sulfide (1.1 x 10^{10} CFU mL^{-1}) were similar to those observed in the absence of
200 sulfide, although doubling times increased 2.6- to 3.4-fold at the 0.15 and 0.30 mM sulfide
201 concentrations (Table 1). While an appreciable lag was not observed for the 0.15 mM sulfide
202 culture, sulfide levels greater than 0.30 mM displayed a lag phase time of approx. 84 min. At
203 sulfide concentrations greater than 0.60 mM, cultures ceased growing after reaching a maximal
204 yield that was 10-fold lower than growth in the 0, 0.15, or 0.30 mM sulfide cultures (Table 1,
205 Fig. 2 B), although doubling times were not significantly different from the lower sulfide
206 treatments.

207 Both doubling times and growth yields were affected for *Vibrio* sp. OY15 at all sulfide
208 concentrations (Table 1 and Fig. 2C). In the absence of sulfide, OY15 grew with a doubling time
209 of approx. 5 h; doubling times increased with increasing sulfide concentrations to almost 12-fold
210 in the presence of 0.60 mM sulfide. Growth yield, on the other hand, decreased as a function of
211 sulfide concentration, with more than a 10-fold reduction at the 1.2 mM sulfide concentration.
212 At 1.2 mM sulfide, growth ceased prior to one doubling, at approx. 6 h after inoculation.

213 Growth of *Bacillus* sp. S1 was least affected by sulfide compared to the other marine
214 bacteria, exhibiting a negligible lag phase and achieving stationary phase between 8 to 14 h
215 (Table 1 and Fig. 2D) after inoculation in all treatments. In the presence of all sulfide
216 concentrations, doubling times were not significantly different compared to control cultures,
217 ranging between 6 to 10 h (Table 1). Cultures grown with sulfide concentrations of 0.15, 0.30,
218 and 0.60 mM yielded between 7.0-7.2 x 10^7 CFU mL^{-1}, which was similar to the growth yield
219 obtained in the absence of sulfide (7.8 x 10^7 CFU mL^{-1}). In the presence of 1.2 mM sulfide, the
220 maximal growth yield increased approx. 1.5-fold compared to the control culture (Table 1).
221

Discussion

Naturally and anthropologically driven sulfide concentrations measured in various marine habitats range from barely detectable in deep coastal basins, to several hundred μM at hydrothermal vents and several mM in sediment pore water in salt marshes and sewage outfalls (Bagarinao 1992). Gradients in sulfide concentrations change with depth into the sediment and the water column, or with the distance from the contamination point (Bagarinao 1992), which determines the composition and function of the local ecosystem. The expansion of anoxic/hypoxic environments, caused by the combined effects of eutrophication and climate-change (Vaquer-Sunyer and Duarte 2008; Lavik et al. 2009) is an additional source of abnormally high sulfide levels in marine, costal, and estuarine settings. This increase in natural sulfide levels magnifies the potential risk of toxic sulfide on native flora and fauna, and sulfide-induced animal mass mortalities are well documented in several environments (Grieshaber and Völkel 1998). Elevated sulfide levels were also shown to severely affect the biodiversity of higher organisms (Knezovich et al. 1996; Høgslund et al. 2008).

Effects of sulfide on microorganisms, however, have been explored predominantly for those whose metabolism involves some aspect of sulfur cycling, such as sulfate-reducing and sulfate-oxidizing microorganisms, methanogens that participate in interspecies hydrogen transfer with sulfate-reducing microbiota (Al-Zuhair et al. 2008; Luther et al. 2011), as well as communities associated with the anammox process (Jin et al. 2012). A consortium of sulfate-reducing microorganisms was completely inhibited by a sulfide concentration of 16.1 mM (Reis et al. 1992), while pure cultures exhibited IC_{50} (concentrations yielding 50% inhibition) for

sulfide ranging between 250 mg L^{-1} (~8 mM) to 926 mg L^{-1} (~29 mM) (Okabe et al. 1995; O'Flaherty et al. 1998). On the other hand, methanogens were found to be more sensitive to sulfide, with an IC_{50} varying between 175 mg L^{-1} (~5 mM) and 363 mg L^{-1} (~11 mM), depending on species (O'Flaherty et al. 1998). While anammox is functional below sulfide levels of 8 mg L^{-1} (0.23 mM), the IC_{50} of sulfide was found to be 264 mg L^{-1} (~8 mM) (Jin et al. 2012); 1.2 mM sulfide—the upper range for our study—showed only a 17% decrease in nitrogen removal rates (Jin et al. 2012). Aside from the anammox studies, we believe that our study is the first to examine the effect of sulfide on growth of marine bacteria that are not characteristically sulfide utilizers.

Microorganisms possess versatile and distinct metabolic strategies for coping with different environmental conditions and stressors (Storz and Hengge 2011), which is consistent with the varied response to sulfide levels observed for the four marine bacteria that we examined. For *Marinobacter* sp., *P. stutzeri*, and *Vibrio* sp. OY15, elevated sulfide concentrations were found to delay growth, influence doubling times, and decrease maximal growth yields. Growth of *Marinobacter* sp. occurred under all conditions except in the presence of 1.2 mM sulfide, which was toxic to the bacterium. At concentrations up to 0.6 mM, however, doubling times and growth yields, for the most part, were comparable to those observed in the absence of sulfide, with greater than 0.60 mM sulfide resulting in decreased growth yields. At 0.15, 0.30, and 0.60 mM sulfide, *Marinobacter* exhibited extended lag times (~two-fold) when compared to growth in the absence of sulfide, which likely represented the time required to produce protective cellular components necessary for adapting to sulfide (see below). The ability for *Marinobacter*

to commence growth after 15 to 20 h under these conditions cannot be explained by the disappearance of sulfide due to chemical oxidation since even at 34 h, 90%, 53%, and 52% sulfide remained in the 0.15, 0.30, and 0.60 mM sulfide-containing cultures, respectively (Fig. 1A).

Like *Marinobacter* sp., *P. stutzeri* growth yields for the 0.15 and 0.30 mM sulfide-containing cultures were comparable to cultures grown in the absence of sulfide. Doubling times, on the other hand, increased under both conditions and the 0.30 mM culture exhibited a nearly 100 h lag period, which was also observed for the 0.60 and 1.2 mM cultures. While sulfide levels in the *P. stutzeri* medium after 34 h were <5% of the initial dose at all concentrations (Fig. 1B), it is likely that the long adjustment period for 0.30, 0.60, and 1.20 mM cultures reflect direct inhibition of the *P. stutzeri* denitrification system by the original dose of sulfide. Partial and full inhibition of NO and N_2O reduction, respectively, by 0.30 mM sulfide has been reported (Sørensen et al. 1980) with a consequence of decreased energy yields and increased lag times (Miyahara et al. 2010). An accumulation of nitrite due to incomplete denitrification as well as sulfite generated by abiotic sulfide oxidation (Luther III et al. 1991) may also explain the behavior of the 0.60 and 1.2 mM cultures after ~110 h, which ceased growth after two to three doublings, resulting in a 6- to 10-fold decrease in maximum cell numbers compared to the control (no sulfide) culture. Nitrite and sulfite have been suggested as playing a role in restricting *P. stutzeri* growth in a synergistic manner (Mahmood et al. 2009). Whether the growth characteristics of *P. stutzeri* at 0.6 and 1.2 mM are a result of nitrite and sulfite toxicity is yet to be determined.

285 Unlike *Marinobacter* sp. and *P. stutzeri*, *Vibrio* sp. OY15 appeared to be least capable of
286 adapting to sulfide, with delays in growth occurring for the 0.60 and 1.2 mM cultures along with
287 increased doubling times and decreased growth yields. The effect of sulfide on OY15 growth
288 was detected within 10 to 30 h after inoculation, before any significant decreases in sulfide
289 concentrations due to abiotic activities of Medium 1 components (Fig. 1A). The response of
290 OY15 to sulfide is similar to that reported for *Vibrio fischeri*, which was found to display sulfide
291 toxicity that varied between 62 μM total sulfide (Van Leeuwen et al. 1985; Postma et al. 2002)
292 and 276 μM of total sulfide (Kuster et al. 2005).

293 In contrast to the other bacteria, *Bacillus* sp. S1 was resistant to as much as 1.2 mM
294 sulfide, with cell yields and doubling times under all conditions similar to growth without sulfide
295 addition; the minimum concentration of sulfide that could perturb growth was not determined.
296 Reduction of sulfide in the *Bacillus* growth medium (Medium 3) due to abiotic activities were
297 negligible during the 8 to 10 h period of growth as no losses were measurable after 34 h (Fig.
298 1C). Sulfide tolerance by *Bacillus* sp. S1 is likely due to the presence of a hydrogen sulfide
299 oxidase activity found in other *Bacillus* sp. (Nakada and Ohta 1999), which is similar to
300 activities identified in sulfide autotrophs like *Thiobacillus ferroxidans.*

301 In general, sulfide readily permeates the cell membrane and denatures proteins by
302 disrupting disulfide cross-links between polypeptide chains (Koster et al. 1986; Percheron et al.
303 1997). The main effect of sulfide on bacteria, however, has been attributed to its inhibitory
304 effects on cytochrome oxidase and other metalloenzymes (Guidotti 1996) by competitive
305 inhibition and subsequent reduction or elimination of redox activity. This could explain the

306 inhibition observed for *Vibrio* and *Marinobacter spp.*, which utilize cytochrome oxidases as part
307 of the electron transport chain necessary for dissimilatory nitrate reduction (Rehr and Klemme
308 1986; Singer et al. 2011). Similarly, inhibition of cytochrome oxidase and other metalloenzyme
309 activities would interfere with the ability for *P. stutzeri* denitrification (Lalucat et al. 2006).

310 A bacterial response to any damage caused by sulfide may include induction of general
311 systems that counteract oxidative stress such as *soxRS* and *oxyR* regulons (Lushchak 2011).
312 While *soxRS* responds to stress induced by superoxide anion and *oxyR* is activated by hydrogen
313 peroxide, both rely on sensors that act via sulfur chemistry and involve specific cysteine proteins
314 of key regulators (Lushchak 2011). Furthermore, sulfide has been shown to stimulate activities
315 of both catalase and superoxide dismutase (Shatalin et al. 2011), which are both controlled by the
316 oxidative stress regulons. Thus, the growth after an extended lag period that was observed in the
317 presence of sulfide for *Marinobacter* sp., *P. stutzeri*, and *Vibrio* sp. OY15 may be explained, in
318 part, by the induction of these or other stress-related systems.

319 The strain of *Marinobacter* used in this study is closely related to *M. aquaeolei*, a marine
320 chemolithoautotroph that can be found throughout the water column but was originally isolated
321 from offshore oil wells, which often contain small, but ubiquitous, amounts of sulfides due to
322 activities of sulfate-reducing bacteria (Nemati et al. 2001). As a consequence, these bacteria
323 have likely evolved mechanisms for coping with sulfides, which would explain its tolerance to
324 sulfide concentrations between 0.15 and 0.60 mM. While *Marinobacter* sp. has not been shown
325 to be capable of oxidizing sulfide (Handley et al. 2009), it has been observed to grow in
326 association with sulfate-reducing bacteria (Sigalevich and Cohen 2000) at sulfide concentrations

327 approaching 0.30 mM, which is consistent with our results. Furthermore, *Marinobacter* sp. has
328 been shown to possess *soxB* (Perreault et al. 2008), a gene involved in thiosulfate oxidation in
329 many Proteobacteria. Whether *soxB* is involved in protecting *Marinobacter* from sulfide toxicity
330 remains to be determined.

331 Our results demonstrate that there are several strategies utilized by marine bacteria for
332 coping with wide sulfide ranges in anoxic environments. On a global scale, the non-linearity and
333 unpredictability of the microbial growth patterns observed by our study suggests a potential shift
334 in microbial community structure and diversity in aquatic environments correlated with sulfide
335 concentration (in temporal and spatial gradients). At the lowest sulfide levels (less than 0.30
336 mM), microbial ecosystem stability may not be compromised and very few changes in microbial
337 community structure and function over time would be detected. However, while inducing
338 smaller changes in the short-term, non-lethal high sulfide levels (between 0.60 and 1.2 mM),
339 may, after longer periods of time, result in large community deviations that are driven by
340 selective pressure of sulfide and could eventually lead to a sulfide-tolerant or sulfide–resistant
341 population—a phenomenon characteristic of microbial communities associated with increasing
342 inputs of numerous contaminants (Baath et al. 1998; Konopka et al. 1999; Hunter et al. 2006). In
343 addition to the dominance of sulfide-oxidizing microorganisms (Lavik et al. 2009) and
344 disappearance of sulfide-sensitive species, highly sulfidic conditions will promote the growth of
345 species, e.g. *Bacillus* sp. S1, which can tolerate sulfide levels up to 1.2 mM. Thus, sulfide levels
346 that are inhibitory to some bacteria but not others will lead to disappearance of important
347 ecosystem links and the likely rearrangement of entire microbial community structures.

348 Predicting the effect of sulfide on an entire ecosystem on a large scale is a challenging
349 task and must be approached with caution. Besides direct involvement of microbial communities
350 in sulfide removal, the consequences of excess sulfide production in the water column can be
351 further complicated as a result of abiotic sulfide oxidation by oxidized metals such as Fe(III) and
352 Mn(III, IV) (Diaz and Rosenberg 2008; Luther et al. 2011) as co-reactants or in a catalytic
353 capacity, as noted above. Moreover, each of the numerous sulfide oxidation products (both
354 biotic and abiotic), e.g. sulfite, thiosulfate, tetrathionate, and polysulfides, may also support or
355 suppress both the metabolism of different microbial species and the rates of abiotic processes,
356 contributing to the spider-web like network of interactions and connections (Sievert et al. 2007;
357 Lavik et al. 2009).
358 Our results highlight the variable responses of marine bacteria to sulfide and may be
359 useful in providing some insight into shifts that may occur in microbial community structure and
360 diversity as a consequence of changes in sulfide levels that are the result of hypoxia.
361

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528

529 **Figure Legends**

530

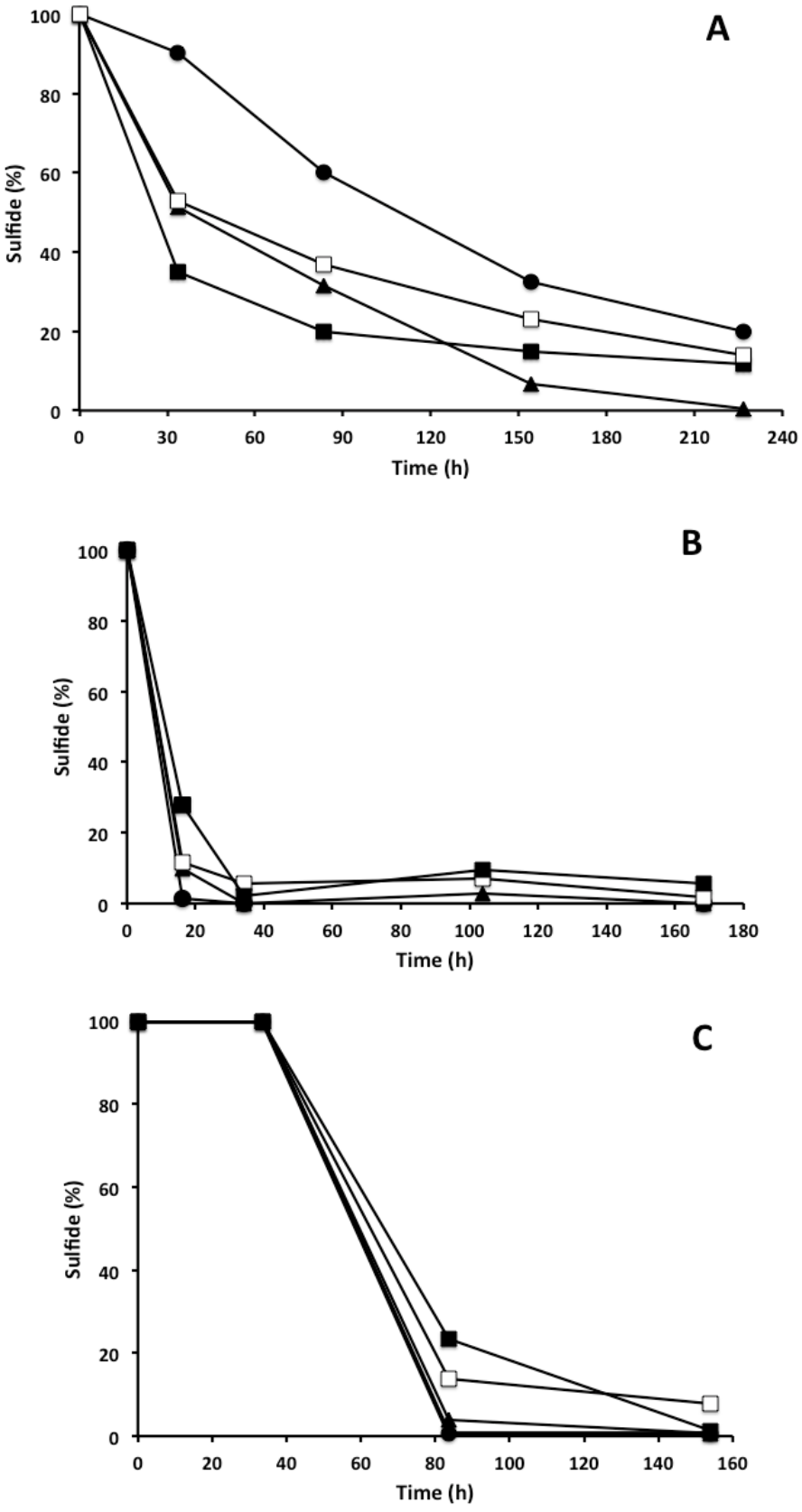
531 **Fig. 1.** Effect of medium composition on total sulfide concentration. Percent sulfide (mean ±
532 standard deviation of duplicates) remaining in (A) Medium 1, (B) Medium 2, and (C) Medium 3,
533 was calculated based on an initial Na_2S concentration of 0.15 mM (●), 0.30 mM (▲), 0.60 mM
534 (□) and 1.2 mM (■).

535 **Fig. 2.** Growth in the presence of varying concentrations of sulfide. (A) *Marinobacter sp.*, (B)
536 *P. stutzeri*, (C) *Vibrio* sp. OY15, and (D) *Bacillus* sp. S1. Sulfide was added to freshly diluted
537 cultures at 0 (◆), 0.15 mM (●), 0.30 mM (▼), 0.60 mM (□) and 1.2 mM (■) and growth was
538 monitored as described in Materials and Methods. Data represent mean ± standard deviation.

539

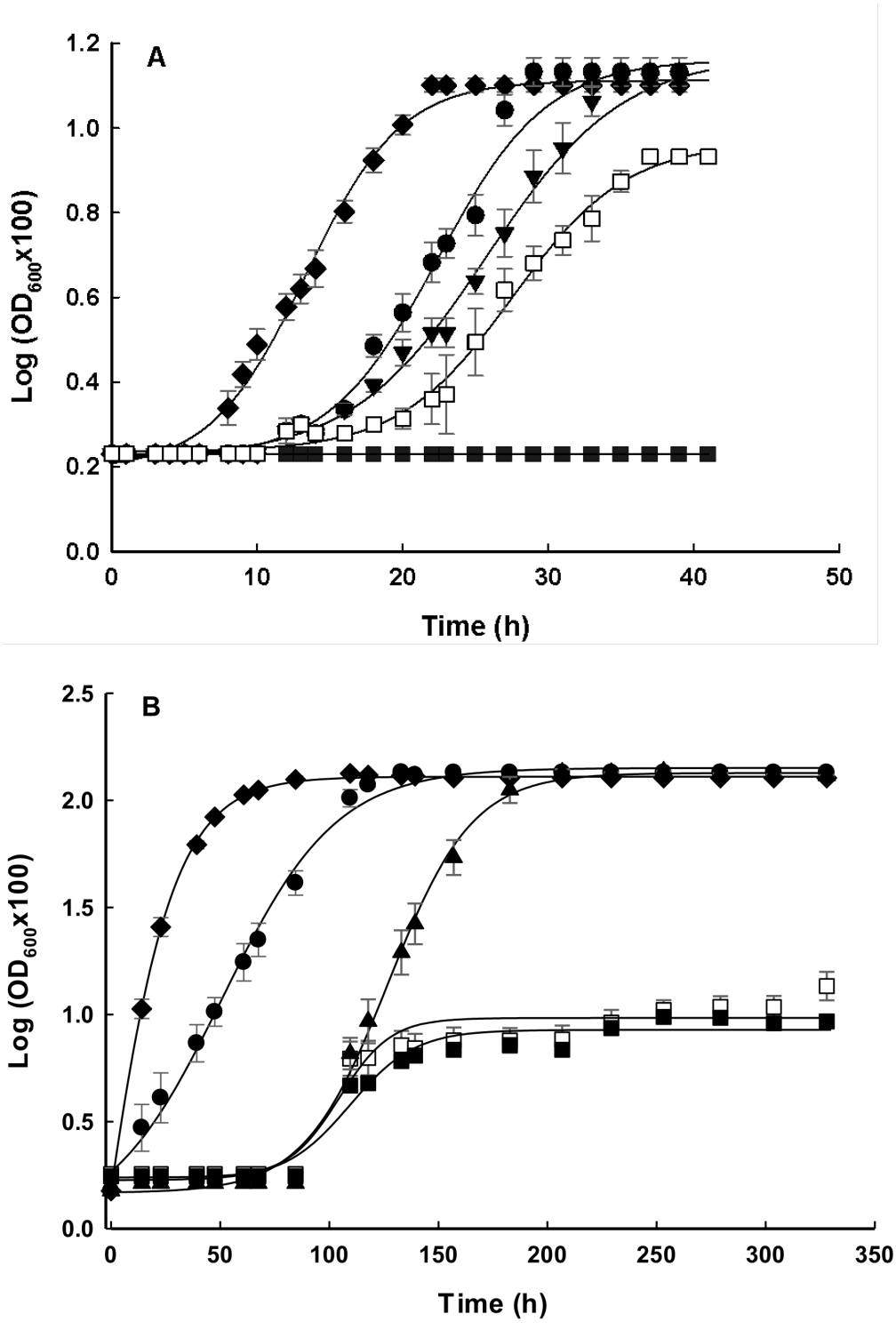
540

Fig. 1



541 Fig. 2

542



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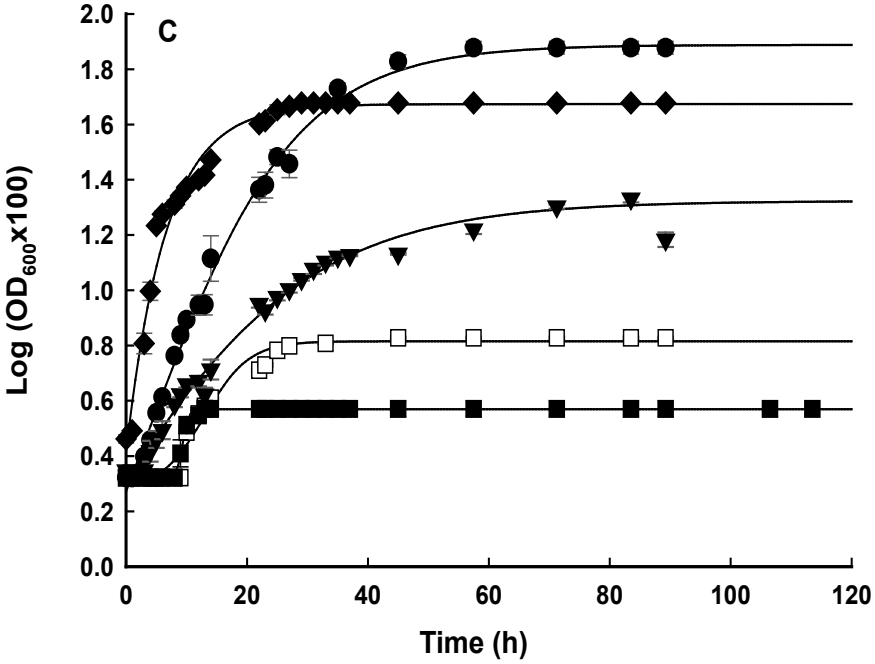
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60
80
100
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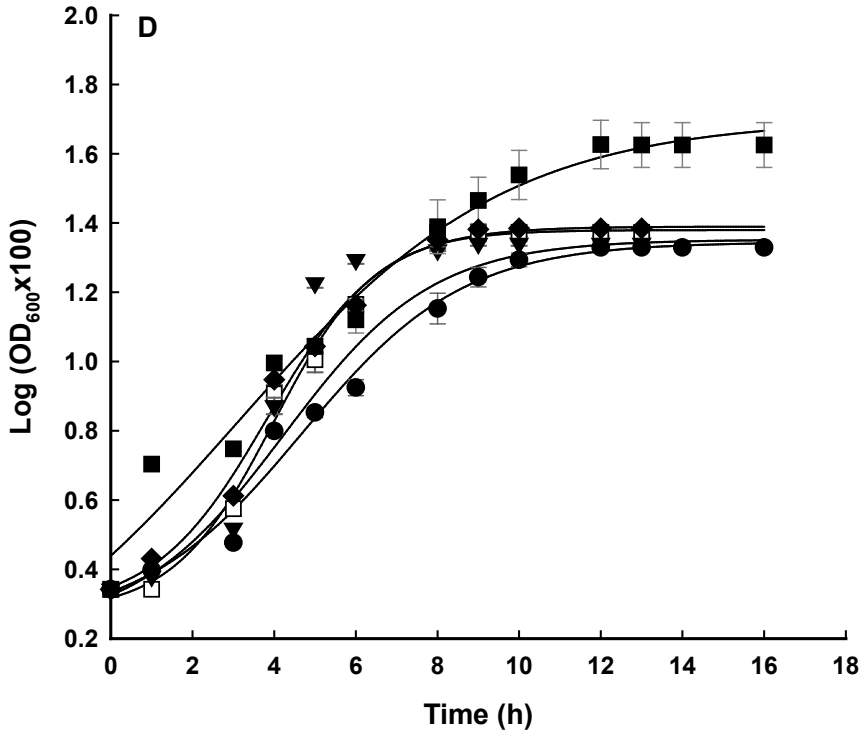
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