

The Pennsylvania State University

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**THE EFFECT OF ARCHEAN OCEANS ON CYANOBACTERIA, GREEN  
SULFUR BACTERIA AND THE RISE OF OXYGEN**

A Thesis in

Geosciences

by

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## Abstract

Growth of a common marine cyanobacterium, freshwater cyanobacterium, and a green sulfur bacterium was examined under different oxygen, sulfide, and ferrous iron concentrations. The goal of this study was to explore how early Archean oceans might have affected cyanobacterial proliferation and the response of major preexisting phototrophs to increasing oxygenation in order to better constrain the timing of the rise of oxygen. The results of this study illustrate that cyanobacteria are very negatively affected by reduced iron and to a lesser degree by sulfide. In addition, increasing oxygen provides no discernable advantage to cyanobacteria, suggesting it is unlikely cyanobacteria evolved and proliferated in  $\text{Fe}^{+2}$ -rich oceans. Archean oceans rich in  $\text{Fe}^{+2}$  would likely have confined cyanobacteria to local  $\text{Fe}^{+2}$ -poor environments, prevented their immediate global propagation, and led to a significant lag time between cyanobacterial evolution and the rise in  $\text{O}_2$ . Sulfur-rich Archean oceans are more likely to have allowed for rapid widespread proliferation of cyanobacteria and an earlier rise in atmospheric  $\text{O}_2$ . Furthermore, the negative response of green sulfur bacteria to slight decreases in reducing power supports early  $\text{O}_2$  accumulation in  $\text{H}_2\text{S}$ -rich Archean oceans.

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# Chapter 1

## Introduction

The rise of free oxygen (O<sub>2</sub>) on early earth following the evolution of cyanobacteria is debatably the most significant transition Earth has undergone<sup>1</sup>. Prior to this transition, Earth's Archean biosphere, or global ecosystem, was anoxic<sup>2,3</sup>. Carbon dioxide (CO<sub>2</sub>) and nitrogen (N<sub>2</sub>) were the major atmospheric constituents<sup>4,5,6</sup>. Reduced iron and sulfur compounds, including ferrous iron (Fe<sup>+2</sup>) and hydrogen sulfide (H<sub>2</sub>S), are theorized to have been abundant in Archean oceans<sup>7,8,9</sup>. Earth's early oceans were inhabited by anaerobic prokaryotic microorganisms, presumably poisoned by O<sub>2</sub>, and often metabolically reliant on reduced oceanic compounds.<sup>10,11</sup> Green sulfur bacteria, microorganisms that photosynthetically oxidize reduced sulfur compounds were likely abundant on early Earth.<sup>10</sup> By ~ 2.7 Ga and possibly as early as 3.5 Ga, oxygen-producing microorganisms, or cyanobacteria, evolved<sup>12,13,14</sup> and transformed the biosphere from an anoxic to an oxic world, rich in free oxygen and populated by more complex aerobic organisms that thrive on O<sub>2</sub>.<sup>15</sup>

While this transition was critical to the development of Earth and life, the timing and nature of this transition is poorly understood. Many researchers support a late rise in O<sub>2</sub> around 2.3 Ga, more than 400 million years after the appearance of cyanobacteria.<sup>8</sup> A competing theory refutes such a significant lag time and suggests O<sub>2</sub> accumulation occurred much earlier in the Archean, immediately following cyanobacterial evolution.<sup>4,16</sup>

Understanding the nature of Earth's transformation from an anoxic to oxic world requires understanding what oceanic conditions allowed for the development and



proliferation of cyanobacteria, yet the chemical makeup of Archean oceans are still debated. While investigators agree the Archean biosphere was anoxic and reducing, levels of major oceanic constituents are poorly constrained. Recent studies point towards ancient oceans rich in reduced iron, but some researchers argue instead for sulfur-rich Archean oceans.<sup>8,17,18</sup> Additionally, better confining the timing of the rise of oxygen necessitates understanding how key preexisting photosynthesizers like green sulfur bacteria responded to increasing oxygen and decreasing availability of electron donors.

This study focused on how increasing levels of  $\text{Fe}^{+2}$ ,  $\text{H}_2\text{S}$ , and  $\text{O}_2$  might affect viability and growth of cyanobacteria and green sulfur bacteria. The broad objective was to better constrain the chemical and biological makeup of Archean oceans prior to the evolution of cyanobacteria and understand how these conditions might influence the timing of the rise of  $\text{O}_2$ .

## Background

### Transition From an Anoxic to Oxic World

Photosynthetic organisms use sunlight to cycle electrons, provided by electron donors, and reduce  $\text{CO}_2$  to organic matter. During most of the Archean,  $\text{H}_2\text{S}$  served as a primary electron donor to early anoxygenic photosynthesis.<sup>10</sup> Recent discoveries of photosynthetic  $\text{Fe}^{+2}$ -oxidation suggest  $\text{Fe}^{+2}$  may also have been an important early electron donor.<sup>19, 20</sup> Green sulfur bacteria use a single photosystem to photosynthetically oxidize reduced sulfur compounds in complete absence of  $\text{O}_2$ .<sup>21</sup> This group of bacteria is composed of deeply branching lineages, eluding to their importance in early Archean oceans<sup>10</sup>. They have unique extramembranous antennae systems, called chlorosomes. These light-harvesting structures are densely-packed with different bacteriochlorophyll pigments, allowing for efficient absorption of sunlight. As a result, green sulfur bacteria can thrive within a wider variety of habitats, even in extremely low-light environments.<sup>21,</sup>

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At some point during the Archean, photosynthetic microbes acquired an additional photosystem (Photosystem II) that performs oxygenic photosynthesis. This new photosystem utilizes  $\text{H}_2\text{O}$  as an electron acceptor, splitting  $\text{H}_2\text{O}$  in the process and generating  $\text{O}_2$  as byproduct.<sup>1, 23</sup>

The development of oxygenic photosynthesis was significant for several reasons. First, it cut the dependency of primary producers on the availability of reduced chemical compounds.<sup>1</sup> Prior to cyanobacterial evolution, primary productivity was dominated by anaerobic photosynthetic microorganisms that used reduced sulfur and iron as their

electron donors.<sup>10,24</sup> Since H<sub>2</sub>O is ubiquitous, the ability of cyanobacteria to use it as an electron donor dramatically increased ocean productivity.<sup>25</sup> Furthermore, the byproduct of Photosystem II, O<sub>2</sub>, is a very electronegative electron acceptor; its covalent bonds are highly attractive to electrons. This means for microorganisms to use O<sub>2</sub> as an electron acceptor to breakdown organic matter requires minimal energy input and generates maximum energy output.<sup>1</sup>

Perhaps the most significant consequence of the evolution of oxygenic photosynthesis was oxidation of the biosphere. As cyanobacteria proliferated and free oxygen began to accumulate, the biosphere became increasingly oxidizing. Eventually, O<sub>2</sub> levels rose to modern levels, making up 21% of the atmosphere.<sup>26</sup> Increasing productivity and O<sub>2</sub> availability, in conjunction with oxygen's electronegative properties, led to increasing size and complexity of organisms.<sup>1</sup>

The exact timing of the evolution of oxygenic photosynthesis still engenders research and debate. Evidence exists supporting the existence of possible oxygenic photosynthesis by the late Archean, including stromatolites<sup>27</sup>, biomarker evidence<sup>28, 29</sup>, and carbon isotopes.<sup>30</sup> Stromatolites are lithified material, left by layers of bacteria, that resemble stratified mounds. Modern stromatolites are comprised largely of cyanobacteria and suggest ancient stromatolites may have been as well.<sup>27</sup> 2-alpha-methylhopanes are lipid biomarkers found in cyanobacteria, and their existence in ancient sediments points to oxygenic photosynthetic activity.<sup>28</sup> This process discriminates between <sup>12</sup>C and <sup>13</sup>C when CO<sub>2</sub> is reduced to organic matter and can leave isotopic fractionations in the rock record. The preponderance of highly <sup>13</sup>C-depleted organic matter in 2.7 Ga sediments is strong evidence of Rubisco activity, suggesting oxygenic photosynthesis.<sup>30</sup>

Increasing evidence suggests that oxygenic photosynthesis evolved earlier than 3.5 Ga. Carbon isotopic excursions in 3.7 Ga Archean sediments have been interpreted as evidence of early cyanobacterial activity.<sup>31</sup> Cyanobacteria-like microfossils in 3.45 Ga Apex Chert from Western Australia have also been attributed to the presence of oxygenic photosynthetic microorganisms.<sup>32</sup>

### **Timing of the Rise of Oxygen Based on Sulfur Isotopes**

In recent years, growing evidence supports the widely held view that free oxygen remained low throughout the Archean (< 0.1% PAL) and did not rise to significant levels until the Proterozoic, around 2.3 Ga.<sup>33,34</sup> The mass-independent fractionation of sulfur (MIF-S) is considered by many researchers as the smoking gun. Sulfur cycling by (bio)chemical processes separates, or fractionates, stable sulfur isotopes (<sup>32</sup>S, <sup>33</sup>S, <sup>34</sup>S, and <sup>36</sup>S) based on their mass differences. These fractionations fall along the terrestrial fractionation line (TFL) and are denoted by  $\delta^{34}\text{S}$  and  $\delta^{33}\text{S}$ . Sulfur isotopic fractionations that deviate from the TFL are considered mass-independent fractionations (MIF-S) and are denoted by  $\Delta^{33}\text{S}$ . Laboratory experiments show photolysis of sulfur dioxide (SO<sub>2</sub>) fractionates S isotopes independent of mass, forming sulfur molecules with this MIF signature. As these S molecules fall to the earth and are buried, their MIF-S signature is preserved in sediments. In the presence of ozone (O<sub>3</sub>), produced by photolysis of O<sub>2</sub> high in the atmosphere, SO<sub>2</sub> can no longer undergo photolysis and MIF-S would disappear. Researchers have demonstrated that the MIF-S signatures in ancient sediments cease at 2.3 Ga, indicating oxygen rose to appreciable levels around this time.<sup>35, 36, 37,</sup>

A few researchers have reexamined the MIF-S signature and uncovered discrepancies. First, Archean rocks containing no MIF-S signature exist, particularly in mid-Archean sediments, suggesting MIF-S signatures may not be solely linked to atmospheric O<sub>2</sub> levels.<sup>38</sup> MIF-S in Archean sediments may instead signify periods of intense volcanic activity. Violent eruptions would eject large amounts of SO<sub>2</sub> above the ozone layer, into the stratosphere, where it would be subject to photochemical reactions.<sup>9</sup> Ohmoto *et al* cites the presence of large MIF-S signatures in recent volcanic ashes originating from particularly violent eruptions compared to those ashes coming from more minor eruptions.<sup>39</sup> He concludes then the frequent occurrence of large MIF-S in Archean rocks and the decreasing degree of MIF-S in younger rocks only supports the universally held belief that early volcanic eruptions were more frequent and violent owing to a hotter early Earth.<sup>9, 40, 41</sup>

Mass-dependent fractionation of sulfur in Archean sediments also serves as a pO<sub>2</sub> proxy. Today, oceanic SO<sub>4</sub><sup>-2</sup> primarily originates from pyrite oxidation during oxidative weathering. Therefore, low oceanic sulfate suggests an anoxic atmosphere.<sup>42</sup> δ<sup>34</sup>S values ~ 0‰ of sedimentary sulfides in some Archean sediments reflect decreased activity of sulfate reducing bacteria and low SO<sub>4</sub><sup>-2</sup> levels.<sup>43, 44</sup> Moreover, increasing S isotopic excursions after 2.3 Ga points towards increasing oceanic sulfate and pO<sub>2</sub>.<sup>45</sup> However, Ohmoto *et al* analyzed the sulfur isotopic composition of individual pyrite grains from the 3.4 Ga Barberton Greenstone Belt. Their data showed pyrite grains were generated by bacterial sulfate reduction, indicating very active bacterial sulfate reduction in sulfate-rich oceans and generated by an oxygen-rich Archean atmosphere.<sup>17</sup>

## Timing of the Rise of Oxygen based on Paleosols and Banded-Iron Formations

Paleosols, ancient soils, develop at the interface between the lithosphere and atmosphere and have long been considered  $pO_2$  proxies.<sup>26</sup> Ferrous iron is soluble under anoxia but becomes insoluble and immobile when oxidized. Paleosols older than 2.2 Ga are depleted in iron, indicating mobilization of  $Fe^{+2}$  during weathering and anoxic conditions.<sup>26</sup> The youngest of these Fe-depleted paleosols is the 2.2 Ga Hekeport Formation. According to this view, Fe-leaching from rocks ceased around this time because oxygen had risen to significant levels.<sup>46</sup>

Questioning the significance of paleosols as  $pO_2$  indicators, Ohmoto *et al* pointed out the possibility of iron remobilization by reducing metasomatic alteration or organic acids.<sup>16</sup> Additionally, recent studies have called into question the interpretation of the Hekeport paleosol, showing it actually represents part of an ancient lateritic weathering profile with an iron-rich upper zone and an iron-depleted lower zone. Previous studies took place only in areas where the lower, iron-poor zone has been preserved.<sup>47</sup> Abundant free oxygen is among the requirements for laterite formation. Furthermore, paleosols in the 3.34 Ga Pilbara Craton were described as probable lateritic soils, suggesting an  $O_2$ -rich atmosphere by the early Archean.<sup>48</sup>

Banded-iron formations (BIFs) are geological structures often used to support a more than 400 million year delay between Earth's oxidation and the evolution of oxygenic photosynthesis. BIFs are massive sedimentary deposits made from alternating layers of iron, specifically magnetite ( $Fe_3O_4$ ) and hematite ( $Fe_2O_3$ ), and chert ( $SiO_2$ ). Oceans would have to have been anoxic for  $Fe^{+2}$  to accumulate and generate such large iron deposits. BIFs precipitated on continental shelves following oxidation of  $Fe^{+2}$  to

$\text{Fe}^{+3}$ , arguably by  $\text{O}_2$  produced by cyanobacteria at the water's surface. The presence of these iron-rich formations during the Archean and Paleoproterozoic has been argued to signify deep ocean anoxia and low atmospheric  $\text{O}_2$ .<sup>46, 49</sup> Problems with this interpretation begin when considering other possible mechanisms for BIF deposition, including photooxidation of ferrous iron<sup>50</sup>, the transition of deep oceans to euxinic conditions<sup>51</sup> and direct iron oxidation by phototrophic bacteria.<sup>20</sup> Moreover, by measuring oxygen isotopic ratios and rare earth elements (REE) of BIFs in Canada, Ohmoto *et al* demonstrated that these iron deposits formed by mixing of large amounts of anoxic hydrothermal water and oxic deep-ocean seawater.<sup>52</sup>

### **Timing of the Rise of Oxygen based on Redox Sensitive Elements**

Detrital minerals are minerals found in sediments which were transported to the depositing water body as a solid particle; complete mineral dissolution and later reprecipitation did not occur during the weathering process. Several minerals, including uraninite ( $\text{UO}_2$ ) and pyrite ( $\text{FeS}_2$ ) can only be deposited under anoxic conditions and are often considered  $\text{pO}_2$  proxies. Uraninite ( $\text{UO}_2$ ) is a mineral precipitated under anoxic conditions. This mineral exists in rocks today but is typically oxidized to the soluble state during weathering. Dissolved  $\text{U}^{+6}$  mobilizes and is transported to the oceans, settles into anoxic sediments, is reduced to  $\text{U}^{+4}$ , and reprecipitates uraninite.<sup>26</sup> Preservation of detrital uraninite in sediments older than 2.2 Ga suggests widespread anoxia during the Archean.<sup>53, 54</sup>

However, the detrital origin of these heavy metals have come into question. Further investigation of these detrital minerals points instead towards a hydrothermal

origin.<sup>55</sup> Additionally, Ohmoto questions the validity of detrital uraninite as a paleoproxy for O<sub>2</sub>. He points out that, contrary to popular belief, uraninite dissolution occurs as fast in anoxic water as oxic water. Survival of detrital uraninite in Archean sediments can therefore not be used to infer pO<sub>2</sub>.<sup>56</sup> Additionally, uranium enrichment in >3.7 Ga sediments in West Greenland indicates mobilization and transportation of uranium at the time of deposition, requiring oxidizing conditions.<sup>13</sup>

A second detrital mineral deposited during the Archean is pyrite (FeS<sub>2</sub>). Oxidative weathering and dissolution of pyrite occurs in an O<sub>2</sub>-rich atmosphere. Some researchers have interpreted pyrite grains of apparent detrital origin as evidence of an anoxic Archean atmosphere.<sup>26, 57</sup> However their detrital origin has come under fire. Upon further investigation, Ohmoto *et al* presented evidence showing detrital pyrite grains formed through reactions with H<sub>2</sub>S-rich hydrothermal fluids and detrital grains of other Fe-rich minerals, thereby concluding these “detrital” pyrite minerals can not be used to constrain the redox state of the Archean atmosphere.<sup>58</sup>

Additionally, recent microprobe analysis of sulfur isotopes in pyrite grains from weather-free drill cores, recovered from the Archean Biosphere Drilling Project, point towards SO<sub>4</sub><sup>-2</sup>-rich Archean oceans and an oxygenated atmosphere.<sup>17</sup> Further, the higher pyrite content and variable S/C ratios of the 2.92 Ga marine shales suggests that SO<sub>4</sub><sup>-2</sup> concentrations were very similar to that of modern euxinic seas under an oxidizing atmosphere.<sup>9</sup>



## Chemical Makeup of Early Archean Oceans

Prior to the evolution of oxygenic photosynthesis, oceans were devoid of O<sub>2</sub> and rich in reducing compounds, including hydrogen sulfide (H<sub>2</sub>S) and ferrous iron (Fe<sup>+2</sup>), that originated primarily from mid-ocean ridge systems.<sup>41</sup> Concentrations of these major oceanic constituents are still debated. Mounting evidence suggests Archean oceans were dominated by ferrous iron.<sup>8, 42, 59</sup> Proponents of iron-rich Archean oceans argue that increased mid-ocean ridge activity on early Earth led to increased oceanic input of reduced iron. According to this theory, iron concentrations ranged from 50 μM to 200 μM Fe<sup>+2</sup>.<sup>26</sup> Since sulfide release from mid-ocean ridges would immediately precipitate iron-sulfides, sulfide levels would be minimal.<sup>42</sup> Some researchers instead suggest Archean oceans were rich in sulfur species including sulfates and sulfides.<sup>17, 18</sup>

The chemical makeup of the Archean ocean may have had a significant effect on the timing of the rise of oxygen. Iron and sulfide may have served as the primary sink for oxygen following the evolution of cyanobacteria.<sup>60</sup> Yet evidence exists suggesting O<sub>2</sub> removal by reducing oceans would not have been sufficient to account for a long delay in oxygen accumulation.<sup>61</sup>

## Chapter 2

### Experiment

As outlined in the background, evidence exists to support both an early and late rise of O<sub>2</sub>, as well as both an iron-rich and sulfur-rich ocean prior to its accumulation. Although, little work has been done linking effects the chemical make-up of Archean waters had on cyanobacteria and preexisting bacteria with the evolution of O<sub>2</sub>. The broad objective of this study was to test how conditions proposed for early-Archean oceans influenced cyanobacterial development and proliferation, preexisting green sulfur bacteria, and the effect these influences had on the timing of the rise of oxygen.

I analyzed the growth *Ananbaena variabilis* (a freshwater cyanobacterium), *Synechococcus sp. strain 7002* (a marine cyanobacterium), and *Chlorobium phaeobacteroides* (a green sulfur bacterium) under increasing sulfide, iron, and oxygen levels. Each phototroph was individually grown with *either* H<sub>2</sub>S, Fe<sup>+2</sup>, or O<sub>2</sub> at concentrations ranging from zero to levels well above those proposed for the early Archean.

Several key questions were addressed. First, how might the evolution and proliferation of oxygenic photosynthesis been affected by sulfide and iron levels proposed for early Archean oceans? How might these effects contribute to an early or a late rise of oxygen? Did increasing O<sub>2</sub> levels alone increase the rate of cyanobacterial proliferation? Second, how could have preexisting green sulfur bacteria responded to rising O<sub>2</sub> following the evolution of oxygenic photosynthesis? How might have different iron-rich and sulfide-rich conditions proposed for early Archean oceans influenced this

response? What conditions would have promoted an immediate rise in oxygen, or a late rise?

I began this research with several general hypotheses. First, the chemical make-up of early Archean waters, whether iron or sulfide-rich, would have affected the development and geographic distribution of oxygenic photosynthesis as well as the timing of the rise of oxygen. Second, following the evolution of cyanobacteria, rising O<sub>2</sub> would likely have promoted increasing rates of cyanobacterial growth and oxygen accumulation. Furthermore, I suspect that preexisting green sulfur bacteria responded very negatively to the introduction of cyanobacteria and rising oxygen, and the degree of this response may have been affected by the composition of Archean oceans.

## **Cyanobacteria**

To better understand the nature of cyanobacterial proliferation immediately following their introduction into anoxic Archean oceans, *A. variabilis* and *Synechococcus* sp. Strain 7002 were grown anaerobically. These strains of cyanobacteria were also grown with initial O<sub>2</sub> concentrations of 0.20%, 1.0%, and 10.0%. The objective was to test the effect of oxygen accumulation on early earth. For reference, these two strains were also grown with 21% and 35% O<sub>2</sub>. 21% oxygen represents present atmospheric levels (PAL), while 35% O<sub>2</sub> simulates Carboniferous oxygen concentrations, the highest in Earth's history.

Since Archean oceans were rich in reduced compounds prior to the evolution of cyanobacteria, part of this study focused on the toxic affect of increasing sulfide and iron

on cyanobacterial growth and proliferation. *Anabaena variabilis* and *Synechococcus sp. Strain 7002* were grown under anaerobic conditions with either 0 mM, 0.156 mM, 0.312 mM, 0.468 mM, or 0.624 mM H<sub>2</sub>S. The goal was to examine how sulfide may have affected the growth of cyanobacteria in euxinic Archean oceans, relative to anaerobic, sulfide-free conditions (0 mM H<sub>2</sub>S).

In order to study the toxicity of reduced iron on cyanobacterial growth, *A. variabilis* and *Synechococcus sp. Strain 7002* were grown at increasing Fe<sup>+2</sup> concentrations. *Anabaena variabilis* and *Synechococcus sp. Strain 7002* were cultured anaerobically with 50 μM and 0.20 mM Fe<sup>+2</sup> to simulate iron-rich Archean oceans. These cyanobacteria were also grown under 0 mM, 5.0 μM, and 2.0 mM Fe<sup>+2</sup>. Low concentrations of Fe<sup>+2</sup> represent oceans with little to no reduced iron, and 2.0 mM Fe<sup>+2</sup> represents iron output from modern mid-ocean ridges. A second iron series was run, identical to the first with the exception that Fe<sup>+2</sup> was chelated. Since chelators bind to ions like iron, chelated Fe<sup>+2</sup> may be less toxic to cyanobacteria.

### **Green Sulfur Bacteria**

Another component of this research considered how preexisting green sulfur bacteria may have responded to the evolution of cyanobacteria and the accumulation of O<sub>2</sub>. Understanding how preexisting metabolisms responded to increasing O<sub>2</sub> is necessary in understanding the nature and timing of Earth's oxidations. Like the experiments with cyanobacteria described above, *Chlorobium phaeobacteroides* was cultured anaerobically with either 0%, 0.20%, 1.0%, 10.0%, 21%, or 35% oxygen. Here the purpose was to study toxicity of increasing O<sub>2</sub> on green sulfur bacteria.

The poisonous effects of reduced iron on green sulfur bacteria were also examined. *Chlorobium phaeobacteroides* was grown at 0 mM, 5.0  $\mu$ M, 50.0  $\mu$ M, 0.20 mM, and 2.0 mM  $\text{Fe}^{+2}$ . Considering how green sulfur bacteria tolerate oxygen and iron may be important in understanding the degree of competition between preexisting sulfur bacteria and newly evolved cyanobacteria.

Because green sulfur bacteria metabolize reduced sulfur compounds, Earth's oxidation would have led to their decreasing productivity. *Chlorobium phaeobacteroides* was grown with 0 mM, 0.15 mM, 0.62 mM, 2.5 mM, and 5.0 mM  $\text{H}_2\text{S}$ . The objective was to test the effect of decreasing sulfide concentrations on the growth of green sulfur bacteria.

# Chapter 3

## Materials and Methods

### Geologically Significant Microorganisms

I performed growth curves on three strains of geologically significant phototrophs: *Anabaena variabilis* (freshwater cyanobacterium), *Synechococcus sp. strain 7002* (marine cyanobacterium), and *Chlorobium phaeobacteroides*, a sulfide oxidizer. The *Anabaena variabilis* strain originated from the Pasteur Culture Collection (PCC). Both the *Synechococcus sp. strain 7002* and *Chlorobium phaeobacteroides* strains were obtained from stock cultures in Dr. Don Bryant's laboratory at The Pennsylvania State University. All three photosynthetic microorganisms were grown at their optimal temperature and pH. Both cyanobacterial strains and the green sulfur bacterium were grown at 33°C. *Anabaena variabilis* and *Chlorobium phaeobacteroides* were grown at a pH 7.3 and *Synechococcus sp. strain 7002* at pH 7.8.

### General Media Bottle Set-Up

Media for each organism was prepared in bulk and degassed with N<sub>2</sub> to remove O<sub>2</sub>. After autoclaving, bulk media was placed in the anaerobic chamber where 75 mL aliquots were allocated into 455 mL media bottles. Hydrogen sulfide, ferrous iron, and chelator were added to the appropriate bottles before the pH of each was adjusted to ~7.3-7.7. Bottles were capped, removed from the anaerobic chamber, and their headspace was vacuumed flushed with N<sub>2</sub> three times. Approximately 1.25 bars N<sub>2</sub> were added to all culture bottles at the gas station, and each were injected with 2.0% (9.4 mL) CO<sub>2</sub>.

Cultures examining the affect of oxygen were injected with desired levels of O<sub>2</sub>. Finally, each bottle was inoculated with 0.5 mL stock culture of one of the three phototrophs described above. In order to prevent the interaction of variables, most likely affecting my results, sulfide, oxygen, and iron were never together in the same culture bottle. Following inoculation, cultures were placed in the light box at a constant temperature of 33<sup>0</sup>C.

### **Sulfide, Ti-citrate, Fe(II), and NTA Stock Solutions**

All stock solutions were prepared using sterilized materials, such as glassware and water, to reduce the chance of contamination. Additionally, in order to prevent significant inconsistencies in the volume of cultures, the maximum amount of any stock solution added to the 75 mL media was only 1.0 mL. Consequently, two stock solutions of Na<sub>2</sub>S were used as my sulfide source. The first, a 0.187 M sulfide solution, was prepared by dissolving 1.68g Na<sub>2</sub>S in 37.5 mL H<sub>2</sub>O. This sulfide solution was used only primarily when looking at how *Chlorobium phaeobacteroides* responded to very high levels of sulfide. The second was a 0.046M solution made by dissolving 0.42g Na<sub>2</sub>S in 37 mL H<sub>2</sub>O. In the absence of sulfide, *Chlorobium phaeobacteroides* requires a reductant, where then thiosulfate can be used as its electron source. Titanium citrate was the reductant I used in this study. It was prepared by the addition of 3.75 mL of a 20% Ti(III)Cl solution into 37.5 mL of 0.20M Na-citrate. This mixture was sealed in a 100 mL serum bottle, capped, and degassed with N<sub>2</sub> to remove all free oxygen.

My source of reduced iron came from a 0.15 M stock solution of FeCl<sub>2</sub>, prepared by first dissolving 1.49g FeCl<sub>2</sub> into 10 mL of 6N HCl. This solution was then mixed with

40 mL H<sub>2</sub>O and immediately placed in the anaerobic chamber to prevent iron oxidation. Those cultures in which chelated iron was the variable, nitrotriloacetic acid (NTA) served as the chelator. A 0.15 M NTA solution was made by dissolving 1.43g NTA in 50 mL of 1 M NaOH.

### **Sulfide Series**

Growth media for all three phototrophs was prepared in bulk without sulfide. Once in the anaerobic chamber, the media was divided up into individual bottles. Next, specific concentrations of sulfide from the 0.05 M or 0.1875 M sterile stock solutions of Na<sub>2</sub>S were injected into each culture bottle. The two cyanobacterial strains in this study were grown in 0 mM, 0.156 mM, 0.324, 0.468mM, or 0.625 mM H<sub>2</sub>S. *Chlorobium phaeobacteroides* was injected with either 0.156 mM, 0.624 mM, 2.5 mM, or 5 mM H<sub>2</sub>S. Since growth of *Chlorobium phaeobacteroides* is inhibited in the absence of a sulfide, which served as the reductant, .25 mL of a 0.2 mM Ti-citrate solution was added in cultures inoculated with *Chlorobium phaeobacteroides* containing 0 mM sulfide. Following the addition of these solutions, the pH of each individual bottle was adjusted, the bottles were capped and removed from the glove box. As described above, the headspace of each bottle was vacuumed flushed with N<sub>2</sub>, the pressure was brought up to 1.25 bars N<sub>2</sub>, 2% CO<sub>2</sub> was injected into each bottle. Finally, each was inoculated with 0.5 mL stock culture of either *Chlorobium phaeobacteroides*, *Anabaena variabilis*, or *Synechococcus sp. strain 7002*.



### **Iron Series: Chelated vs. Unchelated**

Once the bulk media was inside the anaerobic chamber, reduced iron and chelator from stock solutions were added to individual media bottles. For each organism, media was divided into two sets. One set of bottles remained unchelated and received only iron, while the other set were chelated and received both iron and NTA. Each phototroph was grown at 0 mM, 5  $\mu$ M, 50  $\mu$ M, 0.2 mM, or 2.0 mM Fe<sup>+2</sup>. All chelated cultures received 2.0 mM NTA to reduce parameters and to ensure that iron was completely chelated. All bottles were capped, removed from the glove box, vacuumed flushed, and the standard N<sub>2</sub>/CO<sub>2</sub> headspace was added to each. Last, they were inoculated.

Media for *Chlorobium phaeobacteroides* was made in the absence of sulfide to prevent precipitation of FeS. As a consequence, all cultures in the iron series inoculated *Chlorobium phaeobacteroides* received 0.25mL Ti-citrate to function as the reductant.

### **Oxygen Series**

Again, bulk media for the three organisms were prepared, degassed, autoclaved, and placed in the anaerobic chamber. Inside the glove box, the pH of each bottle was adjusted before being capped and removed from the anaerobic chamber. Bottles were vacuumed flushed and the standard N<sub>2</sub>/CO<sub>2</sub> headspace was added. Next, unlike the previously described sulfide and iron series, desired amounts O<sub>2</sub> were injected into the headspace from a sterile, sealed bottle of O<sub>2</sub>. Each phototroph was grown under 0%, 0.2%, 1.0%, 10.0%, 21%, or 35% O<sub>2</sub>. To finish, they were inoculated with one of the three microorganisms of interest.

Once more, Ti-citrate was added to all cultures inoculated with *Chlorobium phaeobacteroides* since the media for this organism was prepared without sulfide. In this case, I did not want sulfide oxidation by O<sub>2</sub> occurring.

### **Growth Analysis**

The number cells per mL versus time for every variable was plotted to generate growth curves. I used a spectrophotometer to track the optical density of cultures. For every duplicate in each series, I took measurements by drawing out 1.0 mL aliquots, from which two separate readings were taken. 0.5 mL was injected into a 2 mL cuvette and three or more measures of absorbency were taken. This was repeated for the second 0.5 mL aliquot. The values from both aliquots and both duplicates were averaged to get a final measure of absorbency, for a particular parameter, at a specific time. Absorbencies were measured at 400 nm and 600 nm wavelengths. These measures of absorbency were converted into cells/mL after generating a calibration curve for each organism that related absorbency and number of cells/mL.

### **Tracking Oxygen and Sulfide in *A. variabilis* and *Synechococcus sp. strain 7002***

In order to track to the concentration of oxygen in *Anabaena variabilis*, I periodically withdrew 10 mL samples of headspaces using an airtight syringe. These samples were injected into a Gas Chromatograph Mass Spectrometer (GCMS) with a Pulse Discharge Detector (PDD), also called a PDD-GCMS. This instrument provided the concentration of O<sub>2</sub> in the headspace of each bottle, so oxygen concentration through time could be tracked. Change in O<sub>2</sub> through time was tracked in cultures injected with

initial O<sub>2</sub> concentrations of 0% and 35%. Standards were prepared and run intermittently between samples to ensure the instrument was working properly.

Sulfide levels were monitored in cultures inoculated with *Anabaena variabilis* and *Synechecoccus sp. strain 7002* containing 0.156 mM and 0.624 mM sulfide. At random times I randomly withdrew 0.5 mL aliquots of the culture solution and added it to a 2 mL cuvette. After adding 0.25 mL of reagents #1 and #2, from the Sulfide Hach Kit, to the cuvette, the sample was inverted 3 times and spectroscopically measured at 680 nm. A calibration curve was generated for this experiment that related sulfide concentration to absorbency at 680nm.

# Chapter 4

## Results and Discussion

### Oxygen

Both *Anabaena variabilis* and *Synechococcus sp. strain 7002* grew at all initial atmospheric oxygen concentrations examined, from 0% O<sub>2</sub> to 35% O<sub>2</sub> (Fig 1 and 2). Anaerobic growth of these modern cyanobacteria was previously demonstrated in a study in which *Anabaena* and *Synechococcus* grew without oxygen under a N<sub>2</sub> and CO<sub>2</sub>-rich atmosphere.<sup>62</sup> This suggests that external O<sub>2</sub> is not a limiting growth factor for these modern cyanobacteria, and enough internal O<sub>2</sub> exists to support respiration.

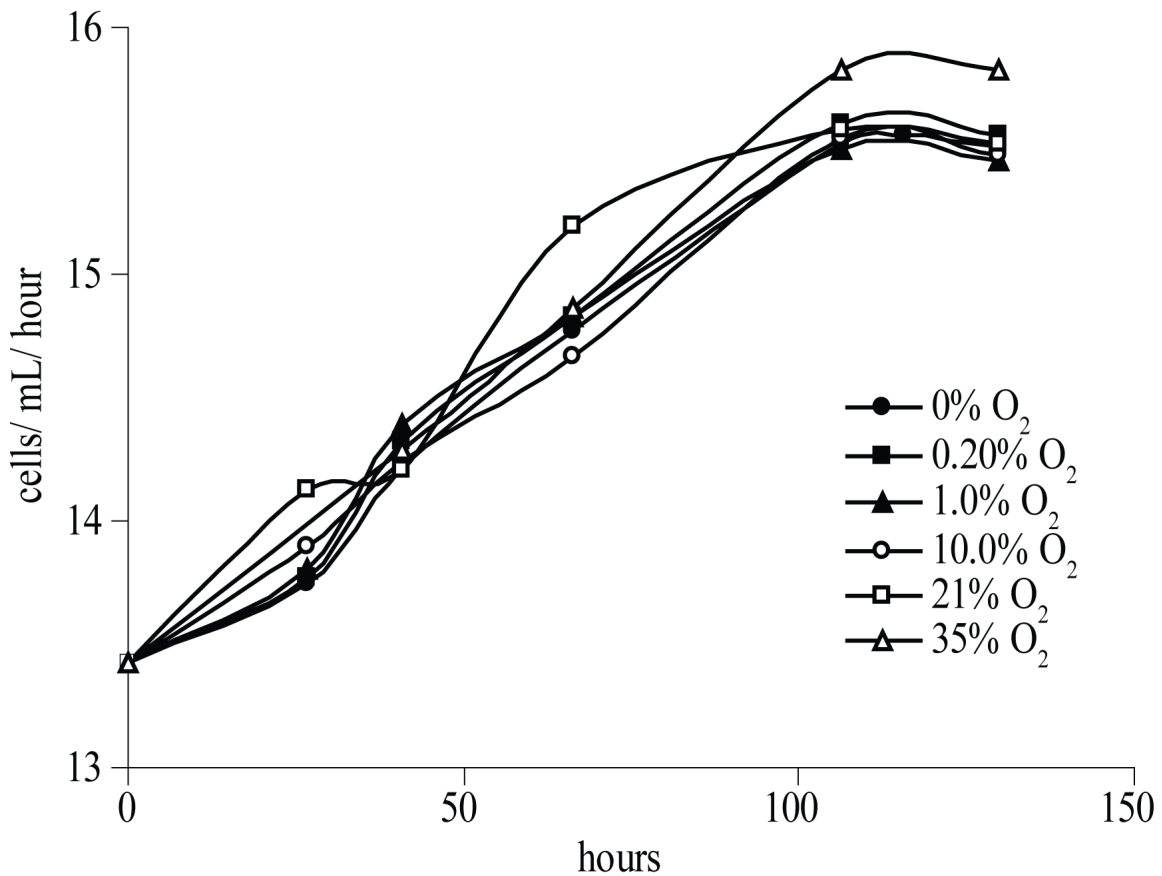
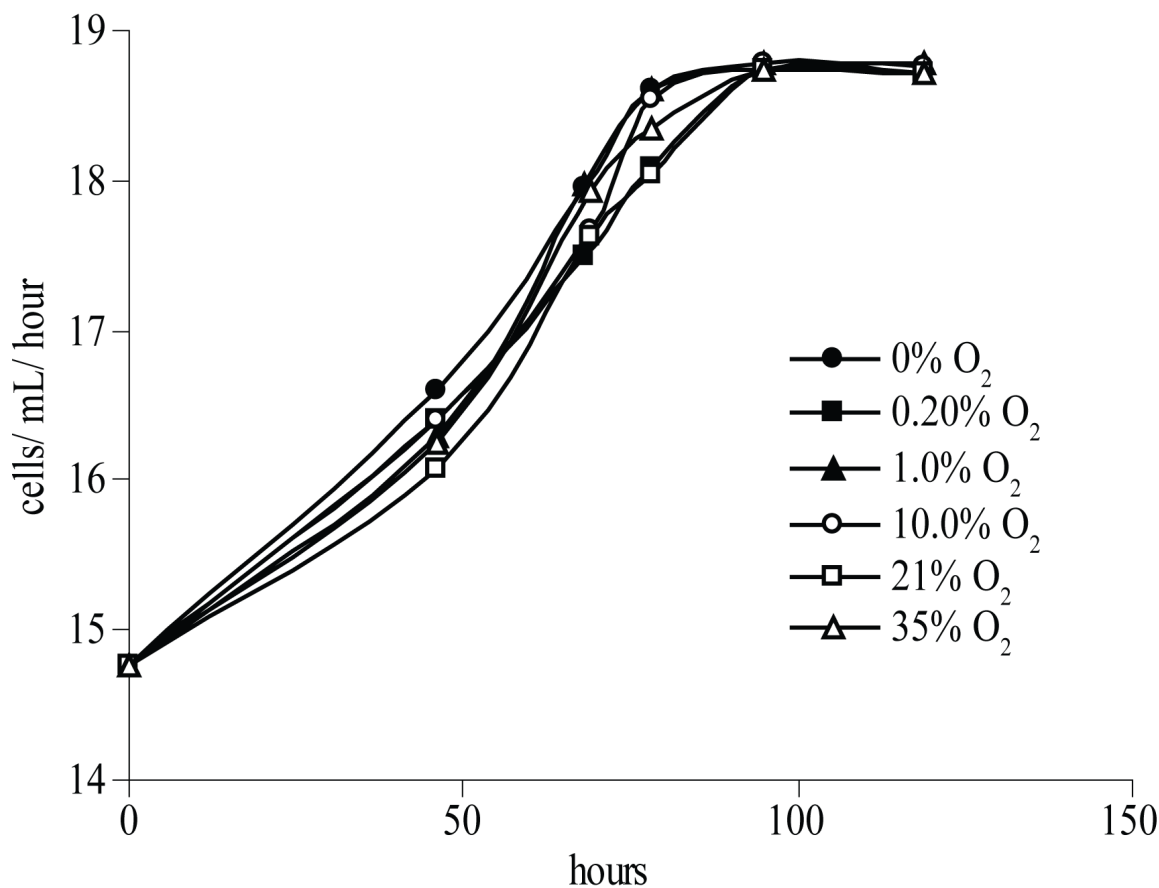


Figure 1. Effect of increasing atmospheric oxygen on growth of *Anabaena variabilis*.



**Figure 2. Effect of atmospheric oxygen on growth of *Synechococcus* sp. strain 7002.**

Furthermore, individual growth characteristics of *Anabaena variabilis* and *Synechococcus* sp. strain 7002 were similar at all oxygen levels, including lag time, rate constants/doubling times, and maximum turbidity (Table 1 and 2). The sole exception was a duplicate culture of *A. variabilis* initially injected with 35% O<sub>2</sub>. This anomaly was not detected with *Synechococcus* sp. strain 7002.

O <sub>2</sub> concentration	lag time	rate constant	doubling time (hours)	maximum turbidity (cell number x 10 <sup>6</sup> )
0%	0	.0206	33.6	5.7
0.20%	0	.0213	32.5	5.7
1.0%	0	.0201	34.4	5.7
10.0%	0	.020	34.6	5.7
21%	0	.0211	32.8	5.7
35%	0	.0226	30.6	5.7

**Table 1. Growth characteristics of *Anabaena variabilis* under increasing atmospheric oxygen.**

O <sub>2</sub> concentration	lag time	rate constant	doubling time (hours)	maximum turbidity (cell number x 10 <sup>8</sup> )
0%	0	.042	16.5	1.4
0.20%	0	.045	15.4	1.4
1.0%	0	.044	15.7	1.4
10.0%	0	.044	15.7	1.4
21%	0	.044	15.7	1.4
35%	0	.044	15.7	1.4

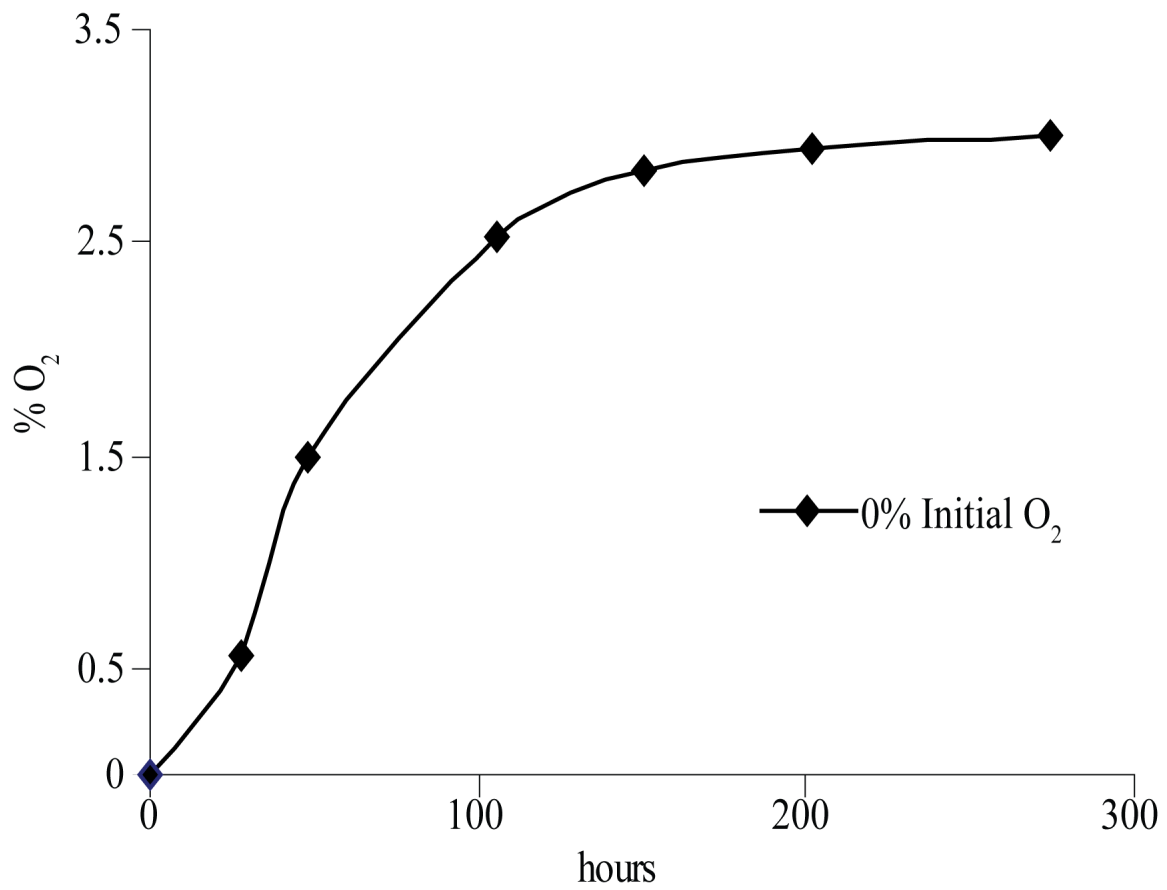
**Table 2. Growth of *Synechecoccus sp. strain 7002* under increasing oxygen concentrations.**

No lag time was observed in cultures of *A. variabilis* and *Synechecoccus sp. strain 7002* with initial O<sub>2</sub> concentrations of 0%, 0.2%, 1.0%, 10.0%, 21%, and 35%. The absence of any discernable lag time indicates *A. variabilis* and *Synechecoccus sp. strain 7002* began growing immediately following inoculation. Rate constants calculated for the exponential phase of *A. variabilis* vary by only .0005 between 0% and 21% O<sub>2</sub>, from 0.0206 to 0.0211 respectively (Figure). A duplicate culture inoculated with *A. variabilis* and grown under 35% O<sub>2</sub> provides an exception; the rate constant calculated for the exponential phase of was 0.0226. Rate constants of *Synechecoccus sp. strain 7002* varied between .042 and .044 from 0% to 35% O<sub>2</sub>. Similar rate constants translate

into similar doubling times. Between 0% and 35% initial oxygen, the doubling times of *Synechococcus sp. strain 7002* ranged from 15.4 to 16.5 hours. Doubling times during the exponential phase of *A. variabilis* fluctuated from 32.5 hours 34.6 hours between 0% and 21% O<sub>2</sub>. Again, a duplicate culture of *A. variabilis* grown with 35% initial oxygen is an exception with a doubling time of 30.6 hours.

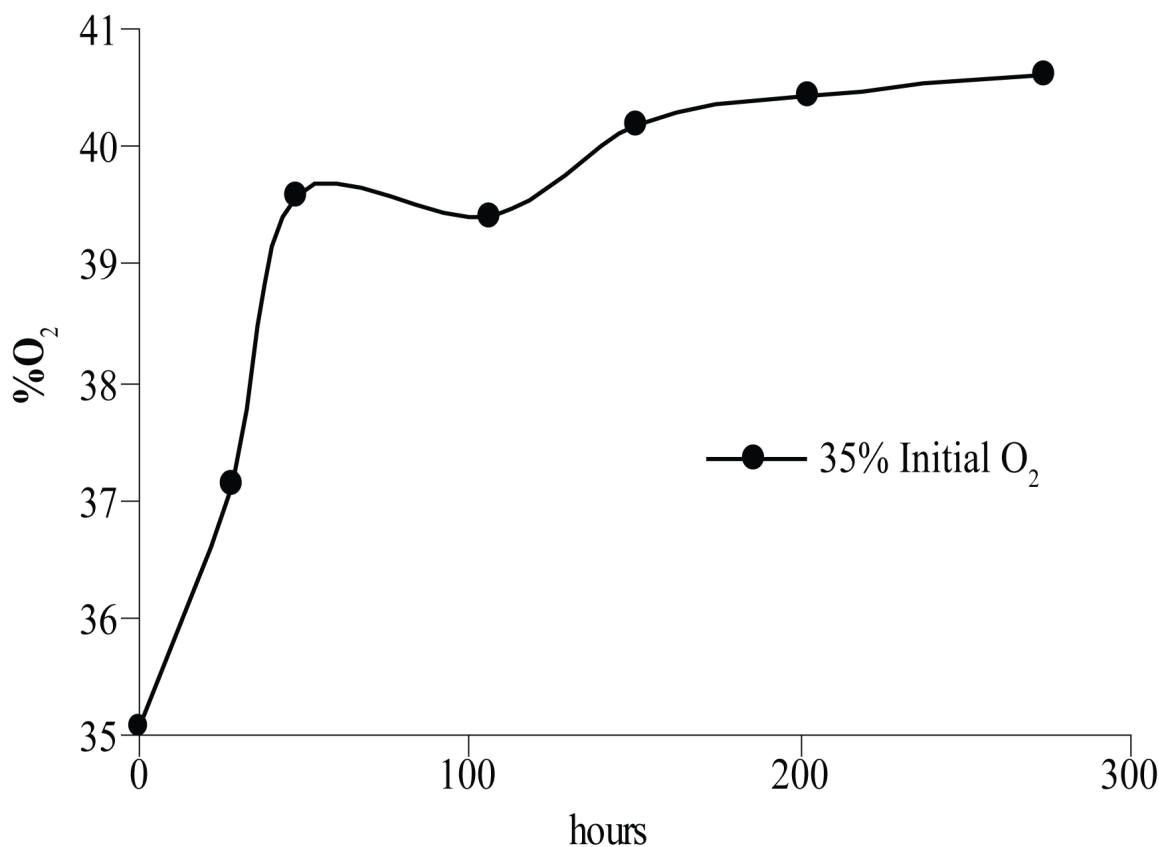
The maximum turbidity these cyanobacteria reached were also similar under increasing O<sub>2</sub> concentrations. With the exclusion of 35% oxygen, *A. variabilis* reached a maximum turbidity of  $\sim 5.7 \times 10^6$  cells/mL. Under all O<sub>2</sub> levels examined, the turbidity of *Synechococcus sp. strain 7002* peaked at  $\sim 1.4 \times 10^8$  cells/mL.

The most significant information inferred from these results is the absence of a trend between increasing initial oxygen levels and lag time, rate constants/doubling time, or maximum turbidity and *A. variabilis* and *Synechococcus sp. strain 7002*. This indicates no significant advantage is provided for growth of modern cyanobacteria as oxygen levels increase. Previous studies actually reveal inhibition of oxygen production reactions in photosynthesis by negative thermodynamic feedback from oxygen build-up and generation of reactive oxygen species (ROS).<sup>63</sup> Clausen *et al* demonstrate high O<sub>2</sub> concentrations impede oxygen production,<sup>64, 65</sup> and the inhibitory effects of O<sub>2</sub> on PSII and O<sub>2</sub> production would have been a contributing component of the mechanisms yielding an upper limit on oxygen accumulation.<sup>66</sup> Although, the small increase in atmospheric oxygen through time in cultures of *A. variabilis* does not support this idea and instead indicates O<sub>2</sub> concentration has no effect on the rate of O<sub>2</sub> production by cyanobacteria (Fig 3 & 4).



**Figure 3. Increase in  $O_2$  concentration through time of *Anabaena variabilis* under anaerobic starting conditions.**





**Figure 4. Oxygen increase through time in cultures of *Anabaena variabilis* containing 35% initial O<sub>2</sub>.**

Under highly reducing conditions, here provided by Ti-citrate instead of sulfide, growth of *Chlorobium phaeobacteroides* at very low oxygen concentrations was observed (Figure 5). This green sulfur bacterium was tolerant of initial O<sub>2</sub> concentrations of 0.20% and 1.0% O<sub>2</sub> but was completely inhibited at 10%, 21%, and 35% oxygen. Under anaerobic conditions, no lag time was observed in growth of *Ch. phaeobacteroides*. Cultures instead moved immediately into exponential phase following inoculation. The rate constant during exponential growth was .0297, doubling every 23 hours.

Conversely, following the introduction of 0.20% and 1.0% O<sub>2</sub> in cultures of *Ch. phaeobacteroides*, a 2 day lag period was observed. The rate constant and doubling times of *Ch. phaeobacteroides* during exponential growth at 0.20% and 1.0% O<sub>2</sub> was .0406, .0327 and 17, 21 hours respectively (Table 3). Growth of *Chlorobium phaeobacteroides* under slightly aerobic conditions may reflect a defense mechanism against O<sub>2</sub> harbored by green sulfur bacteria.

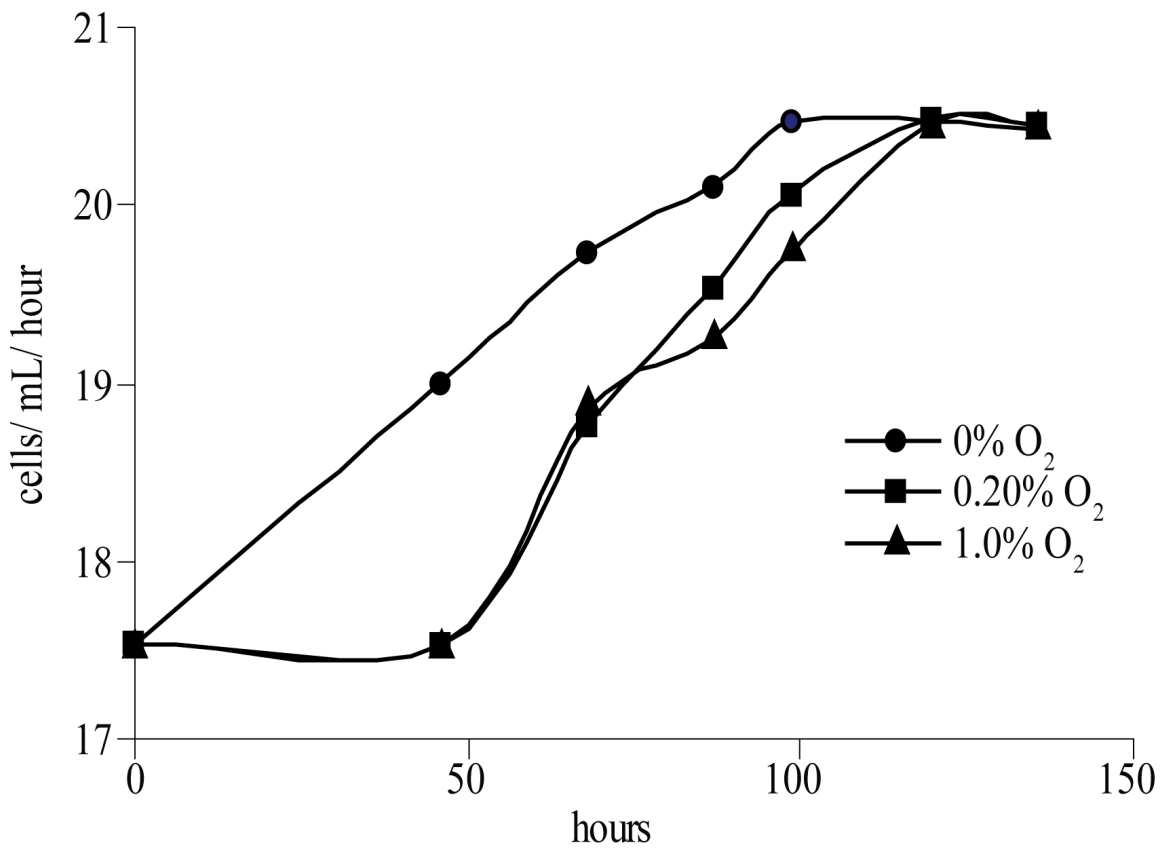


Figure 5. Effect of increasing atmospheric oxygen on growth of *Chlorobium phaeobacteroides*.

<b>O<sub>2</sub> concentration</b>	<b>lag time (hours)</b>	<b>rate constant</b>	<b>doubling time (hours)</b>	<b>maximum turbidity (cell number x 10<sup>8</sup>)</b>
0%	0	.0297	23.3	7.7
0.2%	50	.0406	17.0	7.7
1.0%	50	.0327	21.2	7.7

**Table 3. Growth characteristics of *Chlorobium phaeobacteroides* under increasing oxygen concentrations.**

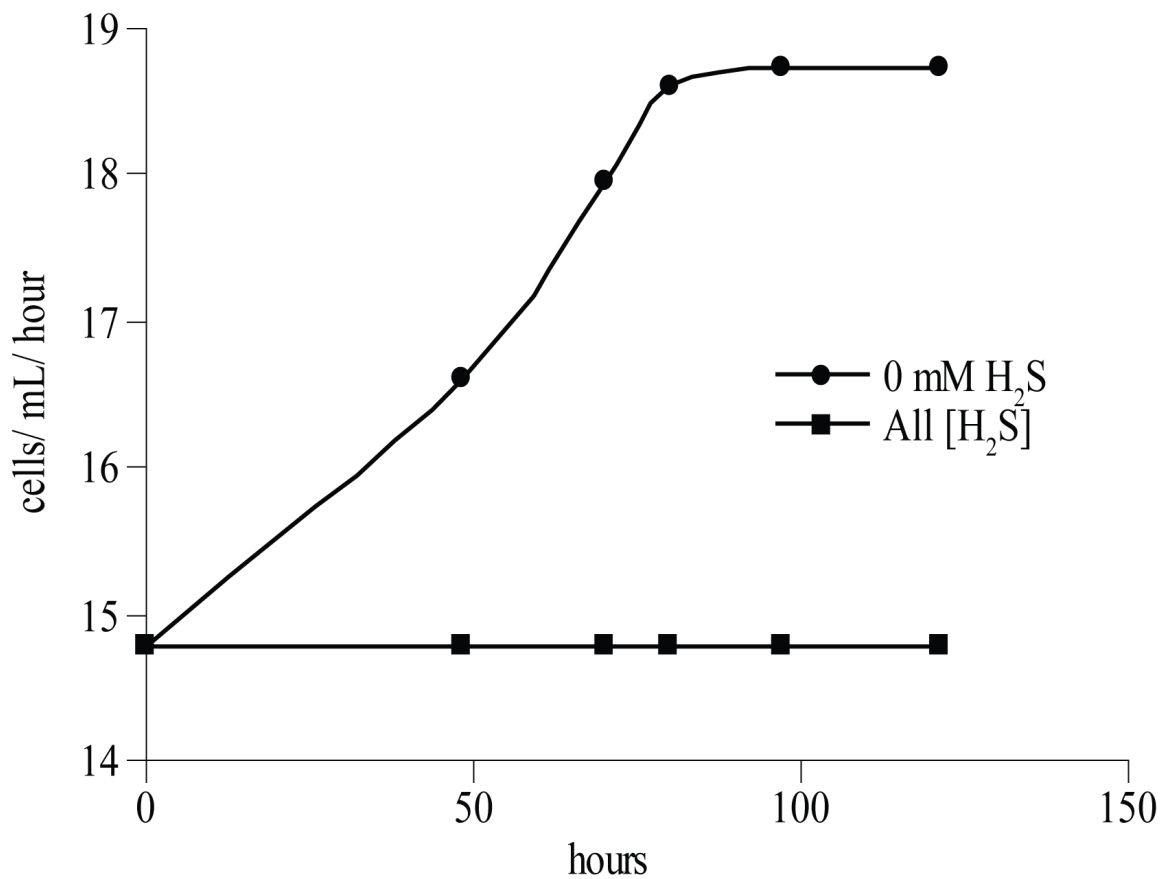
Oxygen toxicity arises from reactions between O<sub>2</sub> and the low potential reductants produced by the reaction center of green sulfur bacteria to form damaging ROS.<sup>67,68</sup> Frigaard and Matsuura found that O<sub>2</sub> uncouples chlorosome light absorption and photosynthetic electron transfer in green sulfur bacteria as a way to prevent oxidation of low potential reductants and the subsequent generation of harmful ROS.<sup>69</sup> Such a defense mechanism enhances the chance of survival of green sulfur bacteria under aerobic conditions. In this study, *Ch. phaeobacteroides* survived following the introduction of 0.20% and 1.0% O<sub>2</sub> by utilizing this defense mechanism during the first 48 hours lag phase. Ti-citrate presumably removed O<sub>2</sub>, allowing the return of anaerobic conditions and growth of this green sulfur bacterium.

### **Sulfide**

Sulfide is very toxic to maladapted cyanobacteria<sup>70</sup>. It inhibits photosystem II by binding to and reacting with cytochromes, hemoproteins, and other metal proteins thereby shutting down the electron transport chain.<sup>71</sup> Despite general sulfide toxicity to oxygenic photosynthesis, there exists variation in sulfide tolerance among modern cyanobacteria.<sup>72</sup> Previous work shows the degree of adaptation to sulfide ranges from complete inhibition of oxygenic photosynthesis at very low sulfide levels to only partial inhibition of PSII at

concentrations  $< 1\text{mM}$ .<sup>72</sup> Variations in the ability of cyanobacteria to deal with sulfide are demonstrated in this study.

The negative effect of sulfide on oxygenic photosynthesis in *Synechococcus sp.* strain 7002 was much more severe than in *Anabaena variabilis*. Growth of *Synechococcus sp.* strain 7002 was completely inhibited by  $\text{H}_2\text{S}$  (Figure 6), yet an earlier study showed different species of *Synechococcus* are more tolerant of sulfide.<sup>72</sup>



**Figure 6.** Effect of  $\text{H}_2\text{S}$  on growth of *Synechococcus sp.* strain 7002.

*A. variabilis* responded significantly better to sulfide toxicity, growing at all sulfide concentrations (Figure 7). Results demonstrate a correlation between increasing sulfide concentration and decreasing sulfide tolerance. The exception was maximum turbidity. At every sulfide concentration examined, *A. variabilis* achieved a maximum cell density of  $5.7 \times 10^6$  cells/mL, the same reached in the oxygen series. Other growth characteristics varied as sulfide increased (Table 4).

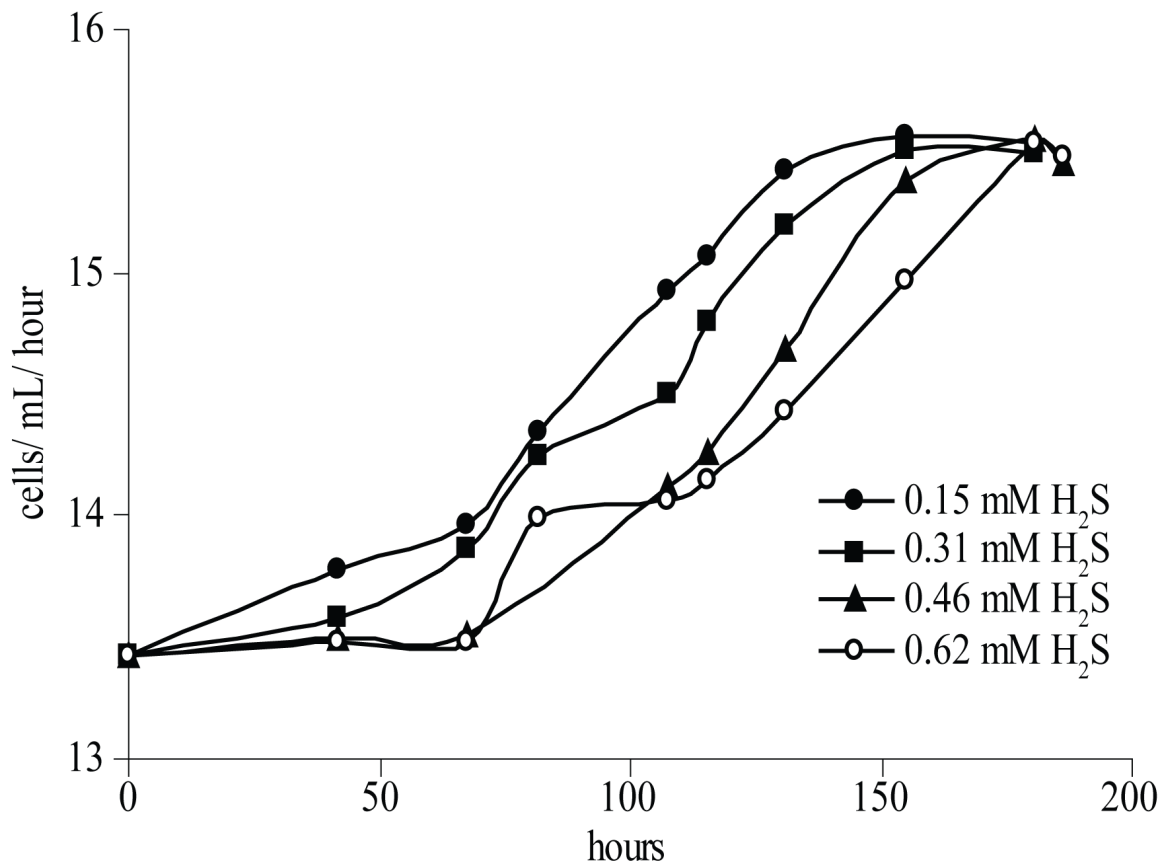


Figure 7. Effect of hydrogen sulfide on growth of *Anabaena variabilis*.

<b>sulfide concentration</b>	<b>lag time (hours)</b>	<b>initial doubling time (hours)</b>	<b>rate constant</b>	<b>doubling time (hours)</b>	<b>Maximum turbidity (cell number x 10<sup>6</sup>)</b>
.15 mM	0	86.6	.0226	30.6	5.7
.31 mM	0	110	.0187	37	5.7
.46 mM	68	0	.0198	35	5.7
.62 mM	68	0	.0167	41.5	5.7

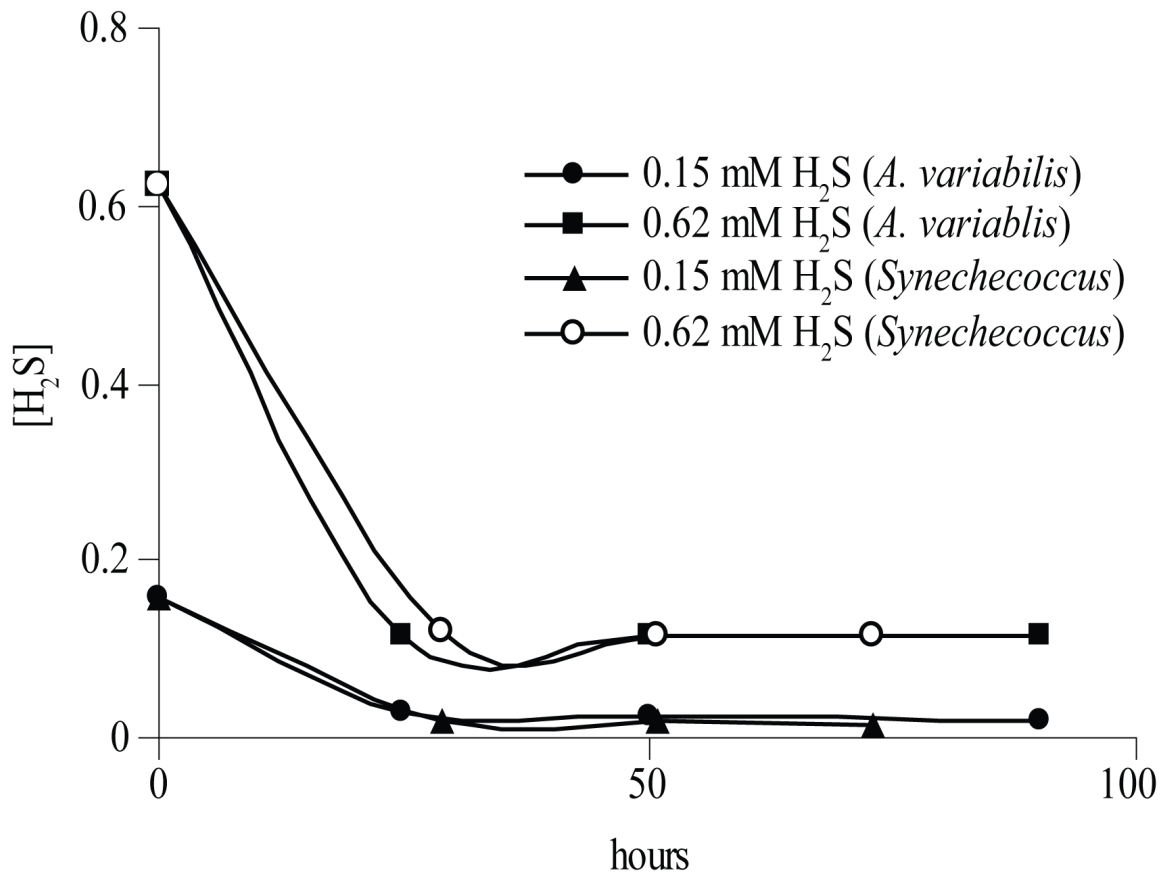
**Table 4. Growth characteristics of *Anabaena variabilis* under varying sulfide levels.**

Cultures containing 0.15 mM and 0.31 mM sulfide displayed no lag time while a significant lag time was observed in those with 0.46 mM and 0.62 mM sulfide. Instead of proceeding into exponential phase after inoculation, at 0.15 mM and 0.31 mM H<sub>2</sub>S *A. variabilis* exhibited an initial linear growth phase of 7000 cells per mL/hr and 5000 cells per mL/hour respectively. These cultures subsequently advanced into exponential phase. The exponential growth rate of *A. variabilis* under 0.15 mM H<sub>2</sub>S was 0.0266 with a doubling time of 30.6 hours. At 0.31 mM H<sub>2</sub>S the exponential growth rate decreased to 0.0817 and doubling time increased to 37.0 hours.

Following a 68 hour lag period, cultures of *A. variabilis* containing higher concentrations showed no initial linear growth before moving into exponential growth. The rate during this phase was 0.0198, with a doubling time of 35 hours, at 0.46 mM sulfide. Under the highest sulfide level tested, exponential growth rate of *A. variabilis* was 0.0167 and doubled every 41.5 hours. Considering growth at all sulfide concentrations, the average doubling time was 36 hours.

A caveat of these results stems from a significant decrease in sulfide concentration of all cultures after only 48 hours (Figure 8). Sulfide diffusion out of the bottles and/or photochemical reactions may be responsible for this phenomenon.

Therefore, it can only be correctly concluded that in this study, *A. variabilis* can grow in sulfide levels as high as 62  $\mu\text{M}$  sulfide. This more closely agrees with a study by Cohen *et al* where they showed PSII inhibition of this cyanobacterium at 50  $\mu\text{M}$  sulfide.<sup>72</sup>



**Figure 8.**  $\text{H}_2\text{S}$  concentration through time in cultures of *Anabaena variabilis* and *Synechecoccus*.

Sulfide tolerance in *A. variabilis* and other cyanobacteria is not unusual. Miller and Bebout showed sulfide tolerance is not unique to a few species of cyanobacteria but is widespread and exists among many different evolutionary lineages.<sup>73</sup>

*Chlorobium phaeobacteroides* thrived under extremely euxinic conditions. As sulfide concentrations increased, there was a general trend towards increasing viability of this green sulfur bacterium (Figure 9). Under 0.15 mM and 0.62 mM sulfide, there was a one day lag phase, yet at 2.5 mM and 5.0 mM H<sub>2</sub>S no lag was observed. Additionally, exponential growth rates increased and doubling times decreased as sulfide levels were raised. *Ch. phaeobacteroides* doubled about every 30 hours at 0.15 mM and 0.62 mM H<sub>2</sub>S. Cultures containing 2.5 mM and 5 mM sulfide exhibited doubling times of 26.5 and 19 hours, respectively (Table 5).

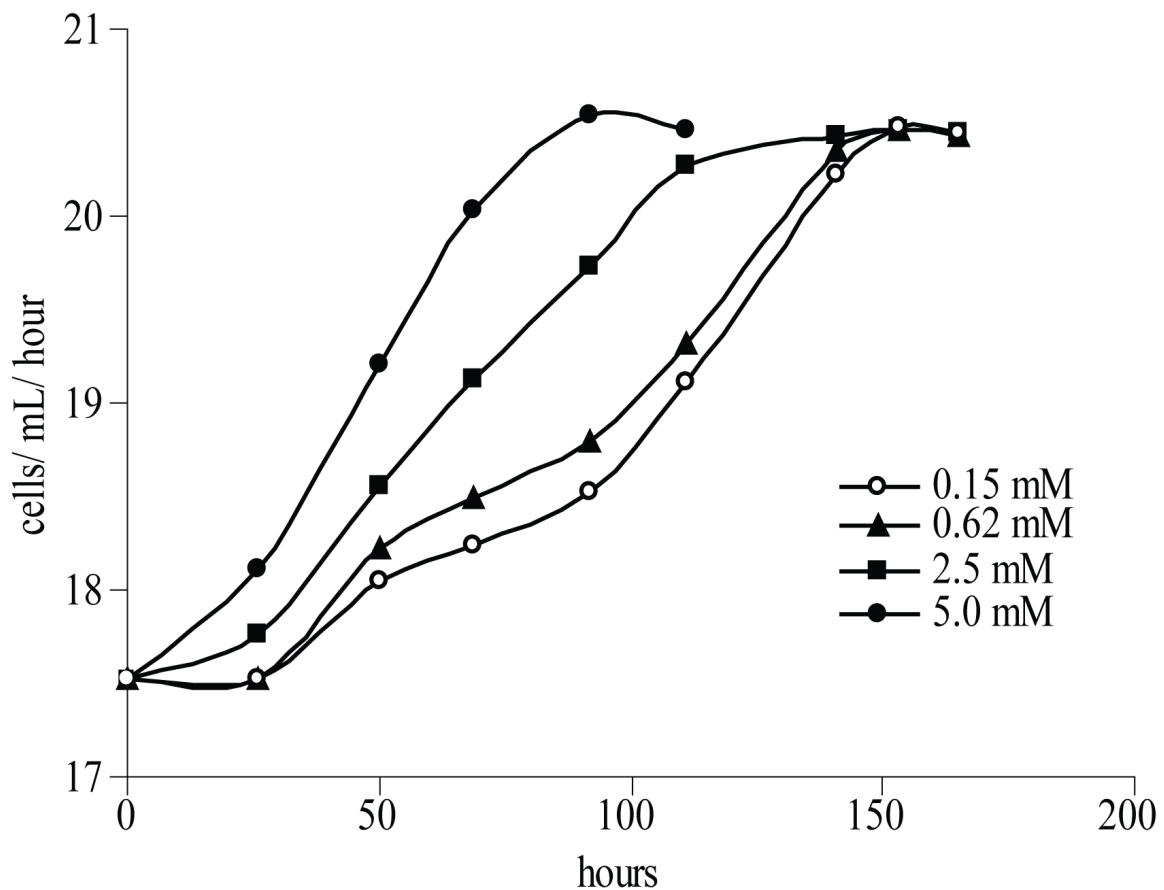


Figure 9. Effect of decreasing H<sub>2</sub>S concentration on *Chlorobium phaeobacteroides*.



<b>sulfide concentration</b>	<b>lag time (hours)</b>	<b>rate constant</b>	<b>doubling time (hours)</b>	<b>maximum turbidity (cell number x 10<sup>6</sup>)</b>
0 mM	0	.0297	23.3	7.7
5 mM	0	.035	19.8	7.7
2.5 mM	0	.0261	26.5	7.7
.62 mM	26	.023	30.1	7.7
.15 mM	26	.0233	29.7	7.7

**Table 5. Growth characteristics of *Chlorobium phaeobacteroides* under different sulfide concentrations.**

Most noteworthy, under anaerobic conditions and in the absence of any sulfide, there was significant growth of *Ch. phaeobacteroides*. In fact, *Ch. phaeobacteroides* grew better under 0.20% and 1.0% O<sub>2</sub> than 0.15 mM and 0.62 mM sulfide. This indicates reduction potential has a greater affect on photosynthetic growth of *Ch. phaeobacteroides* than does sulfide availability. As reducing power decreased, viability of this green sulfur bacterium decreased. Supporting evidence comes from previous studies demonstrating energy transfer and photosynthetic growth is highly dependent on the redox potential of the medium. As redox potential increases, photosynthetic energy transfer shuts down, thereby preventing oxidation of low potential electron acceptors produced in reaction centers of green sulfur bacteria.<sup>73</sup> Here, Ti-citrate in cultures containing 0.2% and 1.0% O<sub>2</sub> maintained lower redox potential than those cultures containing 0.15 mM and 0.62 mM sulfide.

## Iron

Iron is critical for growth since it is present in heme and iron-sulfur proteins involved in photosynthesis.<sup>74</sup> However at high levels, iron and other heavy metals can become toxic or lethal.<sup>75</sup> Generally, growth of *Anabaena variabilis* and *Synechecoccus sp. strain 7002* were negatively affected in the presence of ferrous iron, both chelated and unchelated. Only at the lowest concentration of unchelated  $\text{Fe}^{+2}$  did these two cyanobacteria grow (Figure 10 and 11). *A. variabilis* displayed a significant tolerance to chelated iron while *Synechecoccus sp. strain 7002* was growth completely inhibited under chelated conditions.

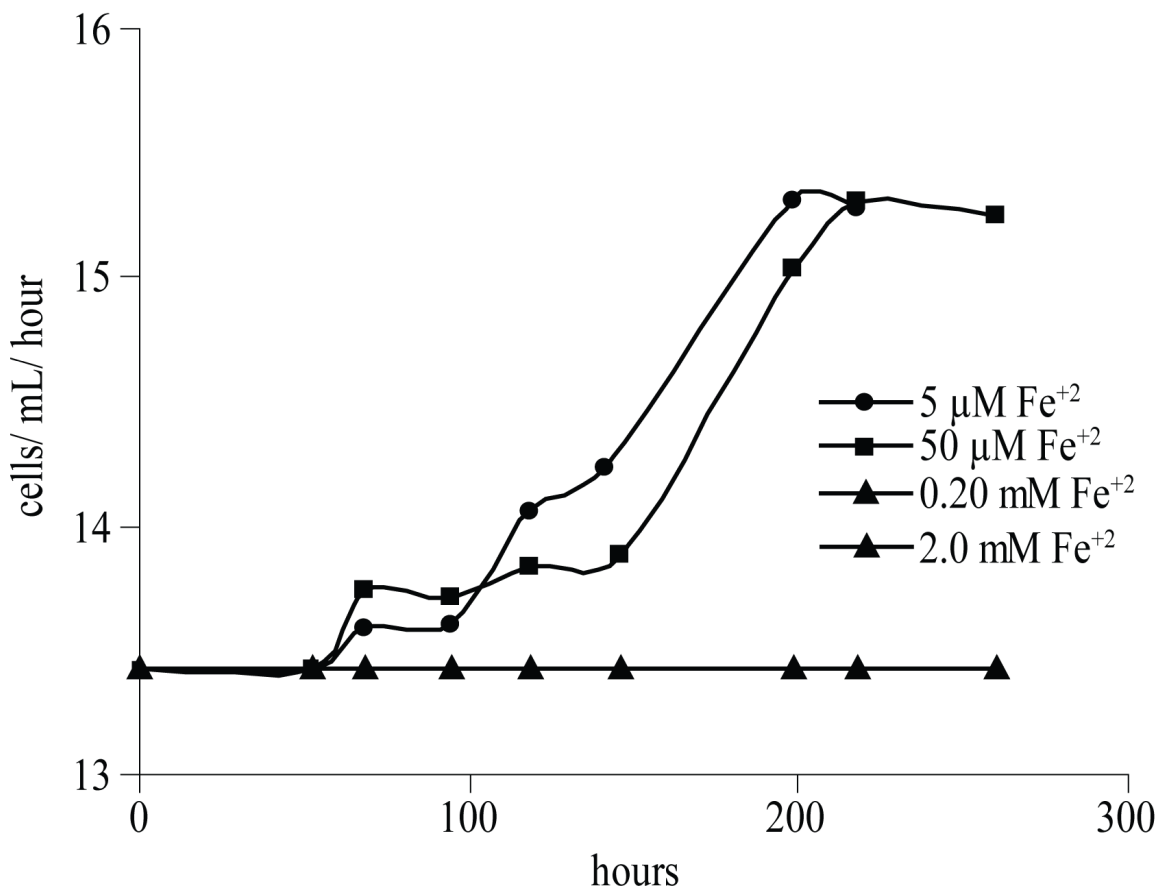


Figure 10. Effect of unchelated  $\text{Fe}^{+2}$  on growth of *Anabaena variabilis*.

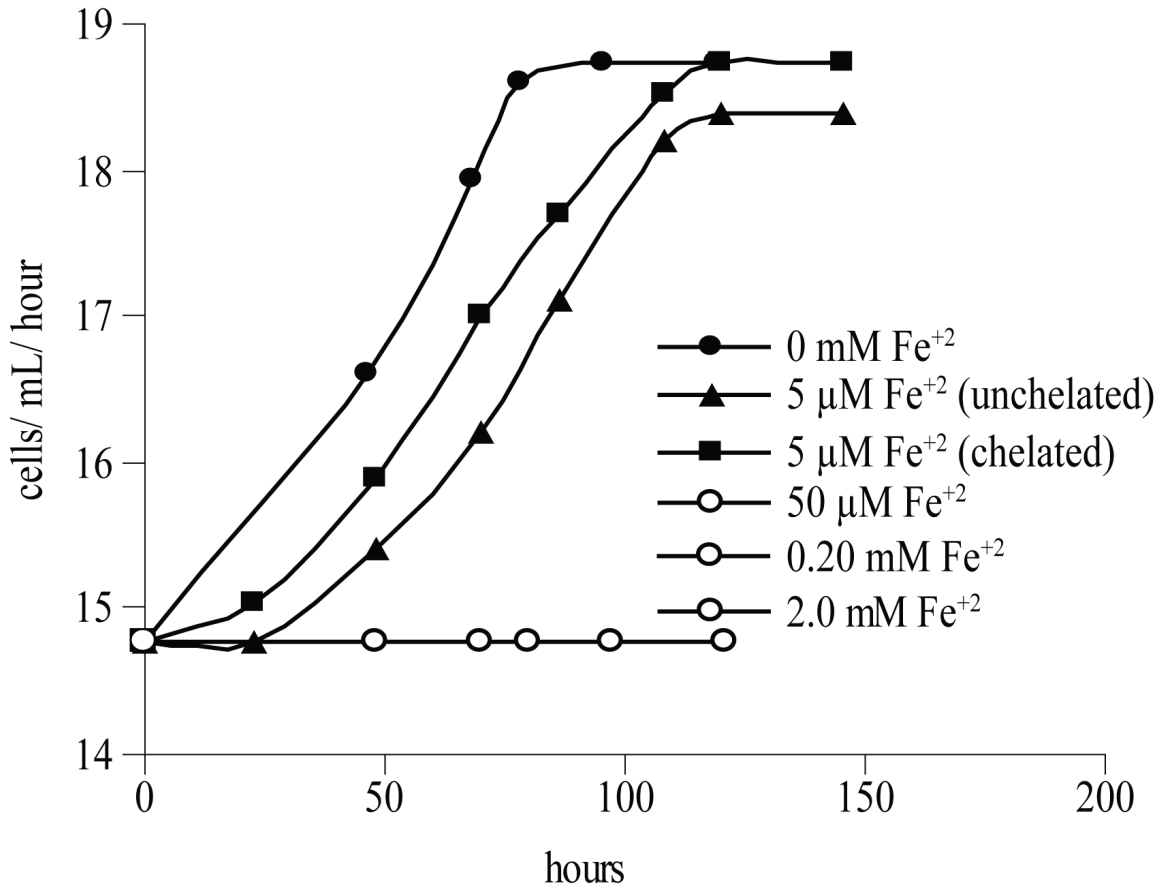
Growth of *Anabaena variabilis* was observed under 5  $\mu\text{M}$  and 50.0  $\mu\text{M}$  unchelated  $\text{Fe}^{+2}$  and displayed almost identical growth characteristics. Before growth was detected, there was a 53 hour delay. Following this lag phase and preceding exponential phase, *A. variabilis* exhibited a linear mode of growth. The linear growth rates at 5  $\mu\text{M}$  and 50  $\mu\text{M}$   $\text{Fe}^{+2}$  were greater than 3,000 cells per mL/hour. The length of this phase was the only significant difference observed in growth between 5  $\mu\text{M}$  and 50.0  $\mu\text{M}$  unchelated  $\text{Fe}^{+2}$ , lasting 50 hours and 92.5 hours respectively. At both  $\text{Fe}^{+2}$  concentrations, exponential growth rate of this freshwater cyanobacterium was 0.0162, doubling every 42.6 hours (Table 6).

<b>Fe concentration</b>	<b>lag time (hours)</b>	<b>initial doubling time (hours)</b>	<b>rate constant</b>	<b>doubling time (hours)</b>	<b>maximum turbidity (cell number x 10<sup>6</sup>)</b>
0 mM	0	0	.0206	33.6	5.7
5 $\mu\text{M}$	53	50	.0163	42.5	4.4
50 $\mu\text{M}$	53	92.5	.0163	42.7	4.4

**Table 6. Characteristics of growth of *Anabaena variabilis* under varying unchelated  $\text{Fe}^{+2}$  levels.**

Growth of *Synechococcus sp. strain 7002* was observed only under 5  $\mu\text{M}$   $\text{Fe}^{+2}$  and indicates very low iron tolerance of this marine cyanobacterium. Previous studies have on the other hand demonstrated high iron tolerance of different *Synechococcus sp.* This modern cyanobacterium was found thriving in near-neutral iron-depositing hot springs in Yellowstone National Park with anoxic source waters comprised of 45-200  $\mu\text{M}$   $\text{Fe}^{+2}$ .<sup>76</sup> Ferrous iron (> 40  $\mu\text{M}$ ) was shown to actually enhance productivity of thermophilic cyanobacteria in these iron-rich springs.<sup>77</sup> Oxidation by  $\text{O}_2$ , microbial consortia with

anoxygenic photosynthesizers that take up  $\text{Fe}^{+2}$ , and even direct oxidation by Photosystem I or II have been put forth as potential mechanisms by which iron-tolerant cyanobacteria protect themselves from elevated intracellular  $\text{Fe}^{+2}$ .<sup>78, 76</sup> However,  $\text{Fe}^{+2}$  concentrations higher than 40  $\mu\text{M}$  were proven toxic to mesophilic cyanobacteria,<sup>78</sup> consistent with results of this study.



**Figure 11. Effect of both chelated and unchelated  $\text{Fe}^{+2}$  on growth of *Synechococcus sp. strain 7002*.**

<b>Fe<sup>+2</sup> concentration</b>	<b>lag time (hours)</b>	<b>rate constant</b>	<b>doubling time (hours)</b>	<b>Maximum turbidity (cell number x 10<sup>8</sup>)</b>
0 mM	0	.042	16.5	1.4
5.0 μM (NO NTA)	0	.04	17.3	1.4
5.0 μM (NTA)	0	.043	16.1	1.4
50 μM (+/- NTA)	NA	NA	NA	NA
0.20 mM (+/- NTA)	NA	NA	NA	NA
2.0 mM (+/- NTA)	NA	NA	NA	NA

**Table 7. Growth characteristics of *Synechococcus* under increasing Fe<sup>+2</sup> concentrations.**

Under chelated conditions, *A. variabilis* was able to grow in Fe<sup>+2</sup> concentrations up to 2 mM (Fig 12). At 5 μM Fe<sup>+2</sup> there was no discernable lag phase while there was a 53 hour lag in cultures supplied with 50.0 μM and 200 μM chelated Fe<sup>+2</sup>. At 100 hours, the longest lag time occurred in cultures containing 2 mM Fe<sup>+2</sup>. *A. variabilis* did not display linear growth before proceeding into exponential phase when Fe<sup>+2</sup> was chelated. The exponential growth rate at 5 μM chelated iron was 0.0231 with a doubling time of 30 hours. At 50 μM, 200 μM, and 2 mM Fe<sup>+2</sup>, *A. variabilis* grew at an exponential rate of 0.0197 and doubled every 35 hours. High iron tolerance of this cyanobacterium simply indicates partiality to chelating conditions (Table 8). Since NTA binds Fe<sup>+2</sup>, leaving it unavailable to cause intracellular damage, this result is not unexpected.

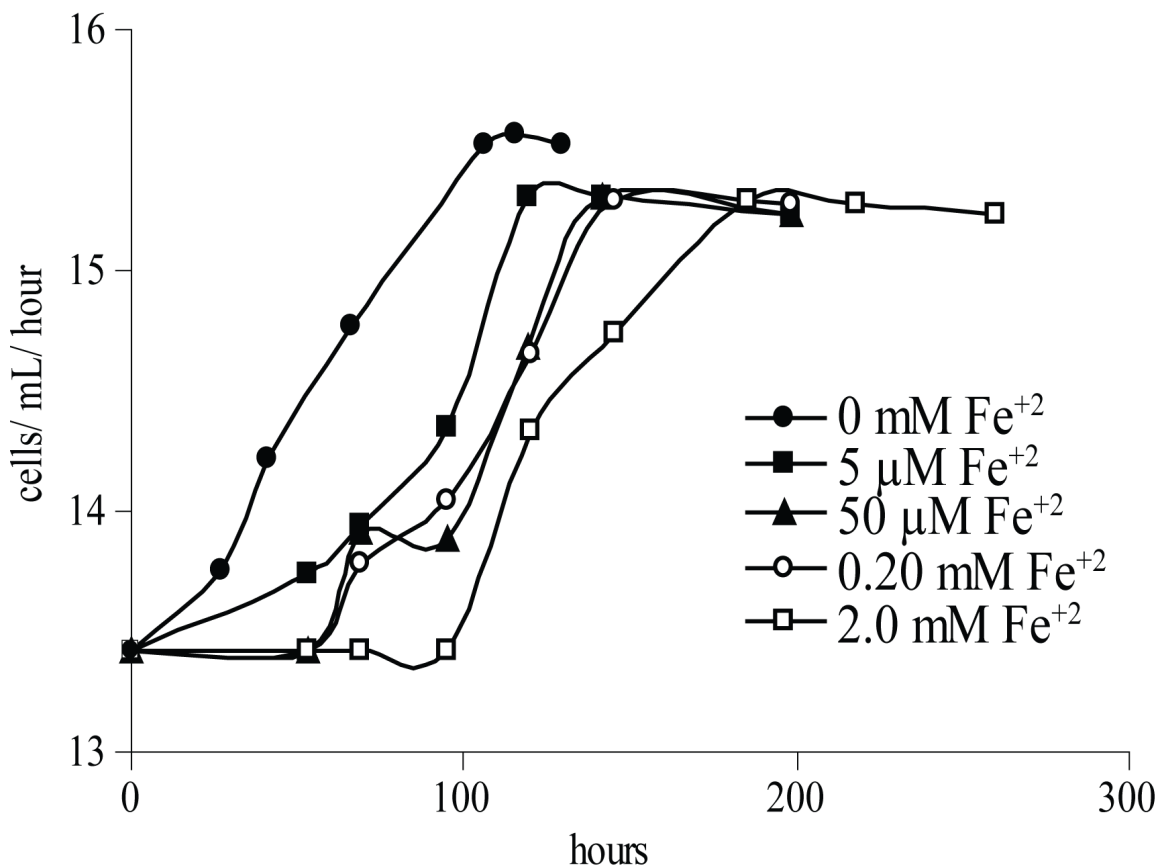


Figure 12. Effect of chelated  $\text{Fe}^{+2}$  on growth of *Anabaena variabilis*.

Fe concentration	lag time (hours)	rate constant	doubling time (hours)	maximum turbidity (cell number $\times 10^6$ )
0 mM	0	.0206	33.6	5.7
5.0 $\mu\text{M}$	0	.0231	30	4.4
50 $\mu\text{M}$	53	.0198	35	4.4
0.20 mM	53	.0195	35.5	4.4
2.0 mM	100	.0199	34.8	4.4

Table 8. Growth characteristics of *Anabaena variabilis* under increasing chelated  $\text{Fe}^{+2}$  levels.

Reduced iron was extremely toxic to *Chlorobium phaeobacteroides*. This green sulfur bacterium grew only in 5  $\mu\text{M}$  unchelated  $\text{Fe}^{+2}$  and was completely inhibited under chelating conditions (Fig 13). While there was no discernable lag phase following inoculation, growth during exponential growth was fairly slow. The rate constant during

this phase was 0.0186 with a doubling time of 37 hours (Table 9). While *Chlorobium* displays severe inhibition when exposed to ferrous iron, Heising *et al* discovered a novel phototroph that oxidizes ferrous iron in a coculture with a *Geospirillum* sp. strain. This new microbe was classified as part of the *Chlorobium* genus and named *Chlorobium ferrooxidans*.<sup>79</sup>

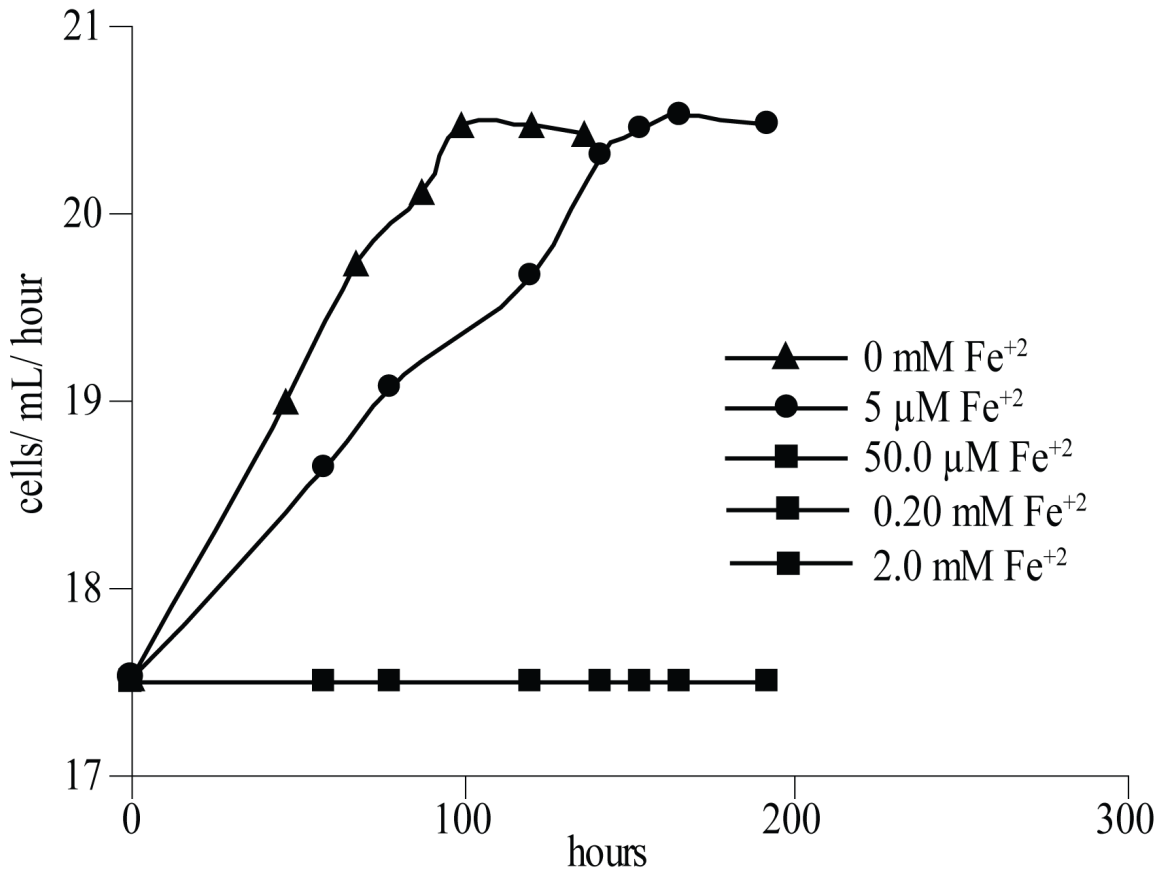


Figure 13. Effect of unchelated  $\text{Fe}^{+2}$  on growth of *Chlorobium phaeobacteroides*.

<b>Fe<sup>+2</sup> concentration</b>	<b>lag time (hours)</b>	<b>rate constant</b>	<b>doubling time (hours)</b>	<b>maximum turbidity (cell number x 10<sup>6</sup>)</b>
0 mM	0	.0297	23.3	7.7
5.0 μM	0	.0186	37.2	8.1
50 μM	NA	NA	NA	NA
0.20 mM	NA	NA	NA	NA
2.0 mM	NA	NA	NA	NA

**Table 9. Growth characteristics of *Chlorobium phaeobacteroides* under unchelated Fe<sup>+2</sup>.**

Interestingly, two weeks after inoculation, growth of *Ch. phaeobacteroides* in 50 μM and 200 μM unchelated Fe<sup>+2</sup> was observed, each called mutant a' and b' respectively. After transferring these two mutants to fresh cultures, both grew almost identically under 50 μM unchelated Fe<sup>+2</sup> (Fig 12). While there was growth of mutant a' and b', these cultures exhibited slightly longer doubling times and decreased maximum turbidity (Table 10).



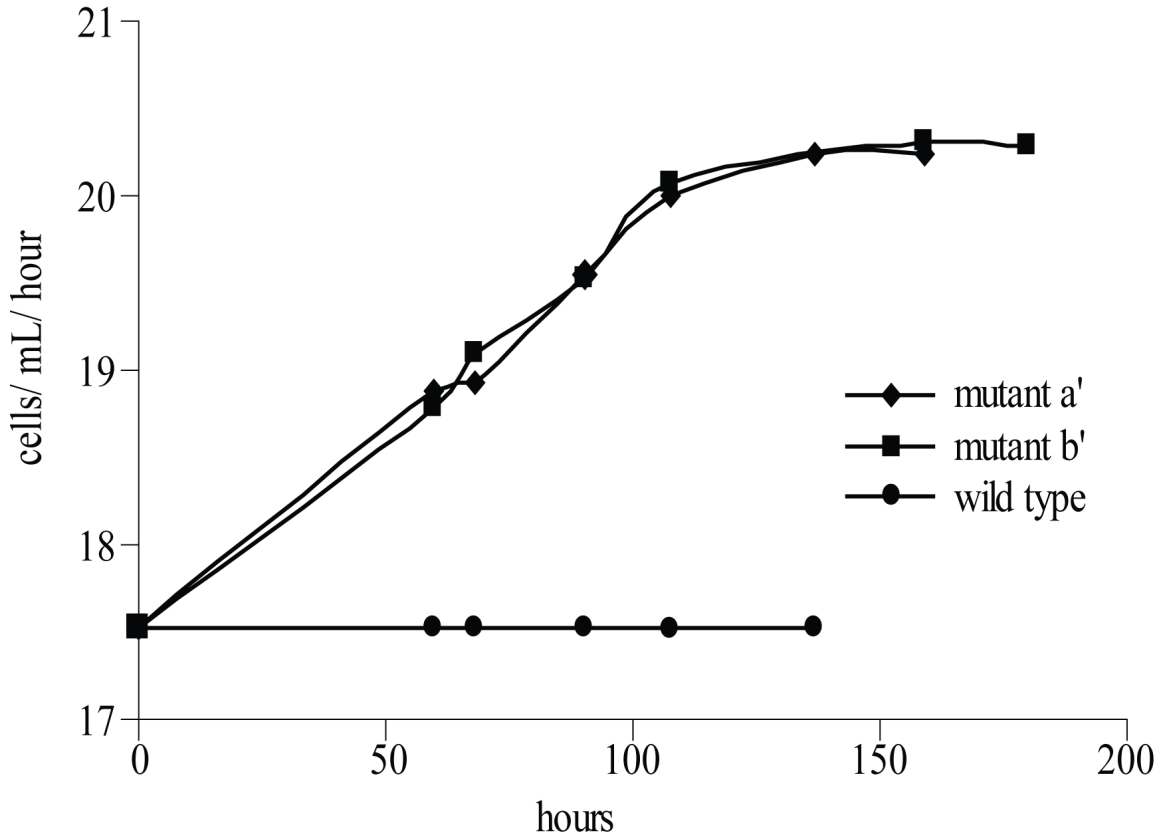


Figure 12. Growth of wild type vs. mutant *Chlorobium phaeobacteroides* under 50 μM Fe<sup>2+</sup>.

Fe <sup>2+</sup> -tolerant mutant	lag time (hours)	rate constant	doubling time (hours)	maximum turbidity (cell number x 10 <sup>8</sup> )
a'	0	.022	31.5	6.6
b'	0	.023	30.1	6.2

Table 10. Growth characteristics of mutant *Chlorobium phaeobacteroides* a' and b' under 50μM unchelated Fe<sup>2+</sup>.

Mutant b' was transferred into fresh cultures containing either 0 mM, 5.0 μM, 50.0 μM, 0.20 mM, and 2 mM (Figure 15). There was significant growth in all Fe<sup>2+</sup> concentrations except 2.0 mM (Table 11). Collectively, data supports that under iron-rich conditions, modern green sulfur bacteria have the ability to adapt to and thrive.

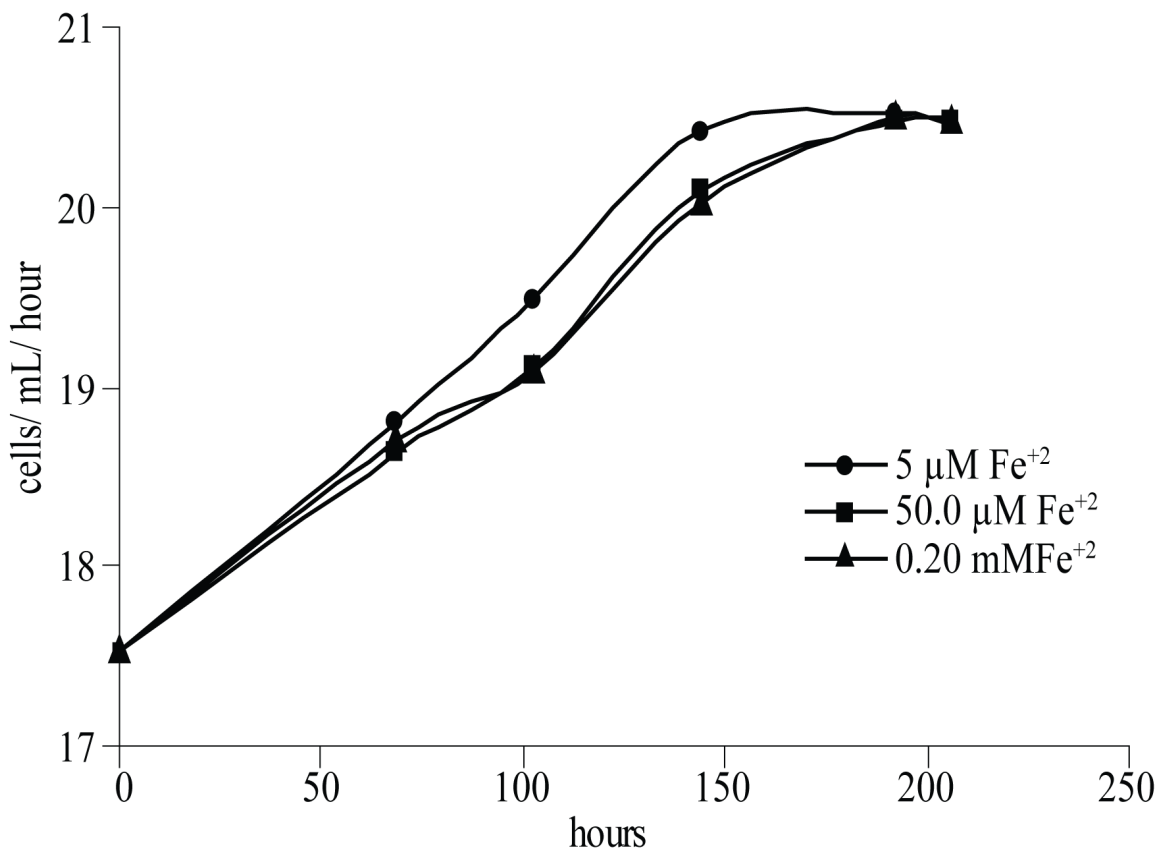


Figure 15. Growth of mutant *Chlorobium phaeobacteroides* b' under increasing unchelated  $\text{Fe}^{+2}$  conditions.

$\text{Fe}^{+2}$ concentration	lag time (hours)	rate constant	doubling time (hours)	maximum turbidity (cell number $\times 10^8$ )
5.0 $\mu\text{M}$	0	.020	34.4	7.7
50 $\mu\text{M}$	0	.016	43.3	7.7
0.20 mM	0	.015	46.2	7.7

Table 11. *Chlorobium phaeobacteroides* mutant b' under increasing unchelated  $\text{Fe}^{+2}$  concentrations.

## Chapter 5

### Conclusions

As previously mentioned, results of this study reveal increasing oxygenation affords no advantage to growth of modern cyanobacteria. This has important implications for the evolution of oxygenic photosynthesis and Earth's oxidation. The evolution and proliferation of cyanobacteria and subsequent accumulation of free oxygen would not have generated a positive feedback mechanism between rising O<sub>2</sub> and viability of oxygenic photosynthesizers. As O<sub>2</sub> rose in Earth's early atmosphere and oceans, increasing availability of O<sub>2</sub> did not by itself promote growth of cyanobacteria and a faster rate of oxygen production. In conjunction O<sub>2</sub> removal by reducing Archean oceans, these results support a sizeable delay between the evolution of oxygenic photosynthesis and the rise of atmospheric oxygen to appreciable levels.

In this study, growth characteristics of *A. variabilis* under oxygenated and euxinic conditions were analogous and are important when considering how cyanobacteria may have fared in euxinic Archean oceans. Under both a sulfide and O<sub>2</sub>-rich environment, this modern cyanobacterium reached a maximum turbidity of  $\sim 5.7 \times 10^6$  cells/mL and exhibited similar behavior during exponential growth (Figure 16). These results, in combination with pervasive sulfide tolerance among several evolutionary lineages, suggest the metabolic precursor to oxygenic photosynthesis may have evolved in a sulfidic environment.

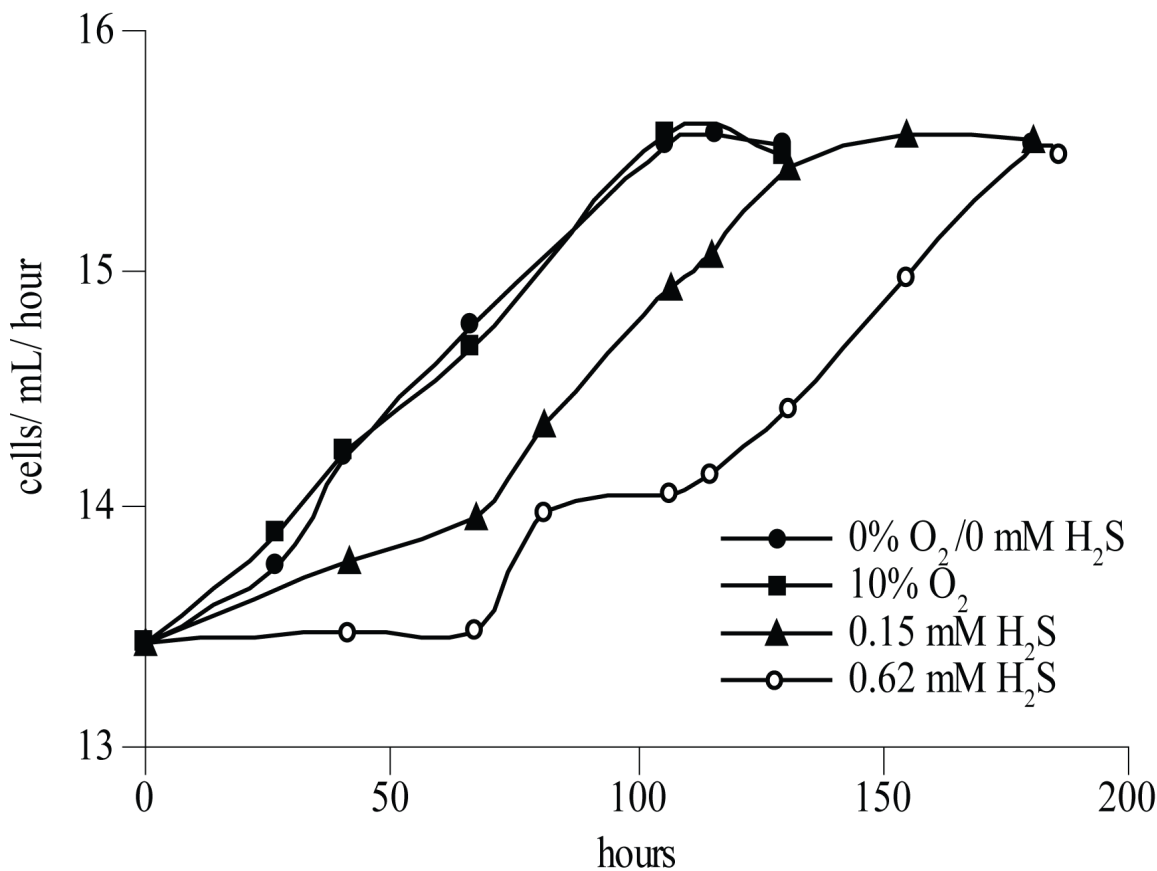


Figure 16. Growth curves of *Anabaena variabilis* under oxygenic vs. sulfidic conditions.

Furthermore, this study demonstrates the dependency of green sulfur bacteria on redox potential of the environment (Figure 17) and supports rapid global cyanobacterial proliferation and an early rise of O<sub>2</sub> through negative feedback. As sulfur-rich Archean oceans became more oxidizing, redox potential increased and availability of reduced sulfur compounds decreased. Consequently, populations of green sulfur bacteria would have quickly diminished. The resulting decrease in competition for limiting nutrients would have helped pave the way for immediate global proliferation of cyanobacteria, rapidly increasing O<sub>2</sub>, and further decline of green sulfur bacteria and other preexisting photosynthesizers.

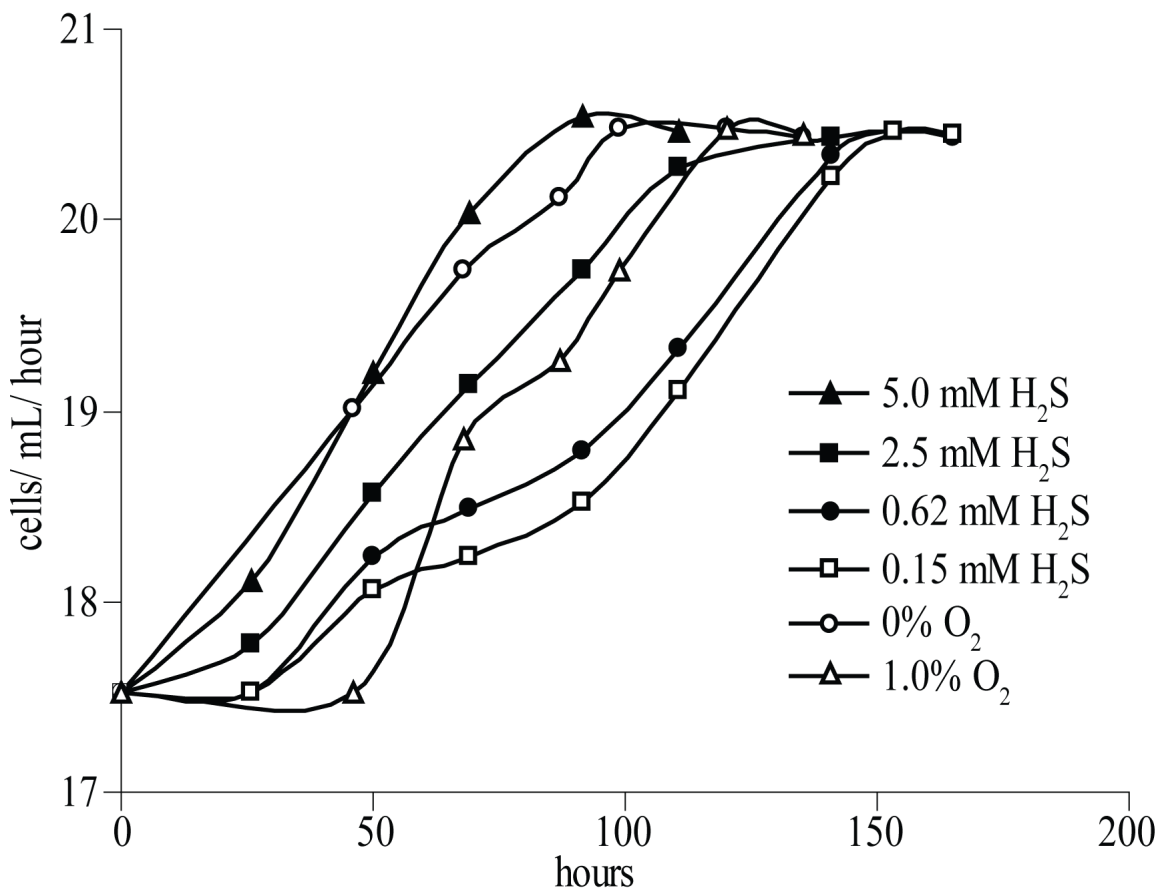
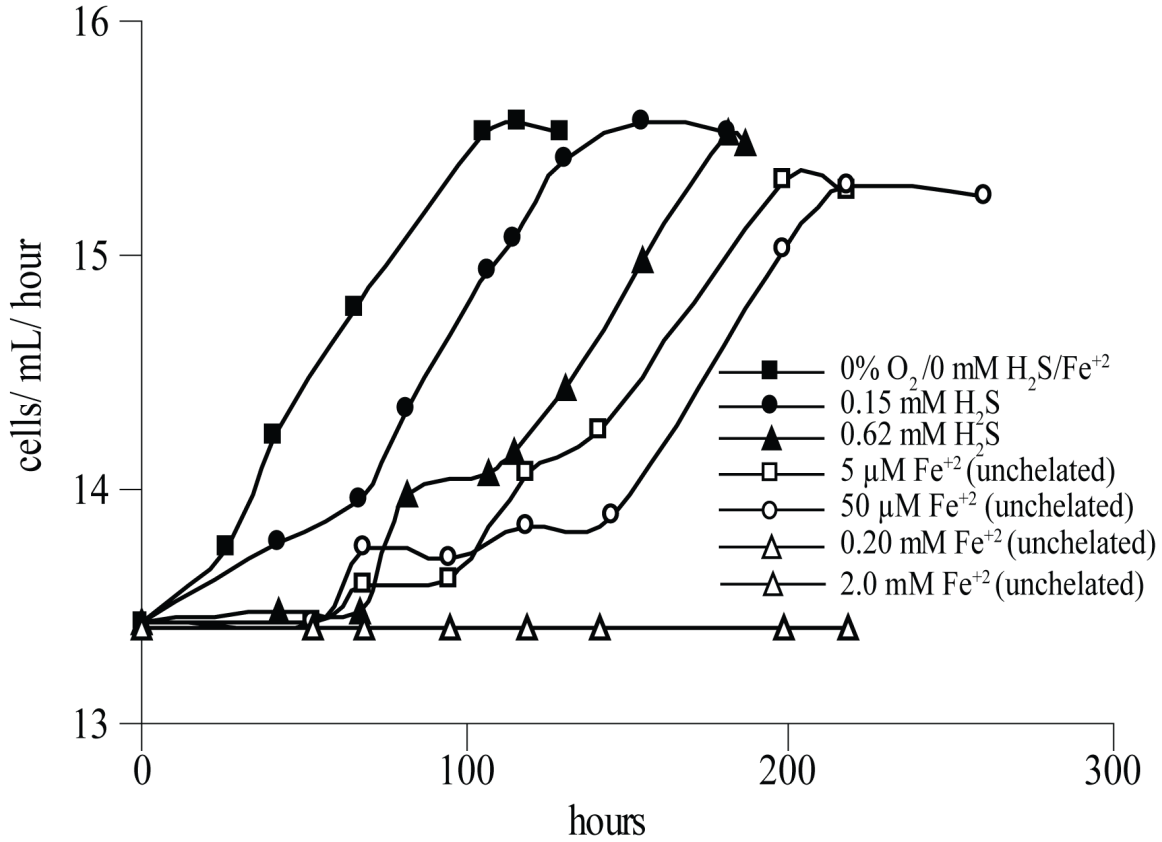


Figure 17. Growth of *Chlorobium phaeobacteroides* under decreasing sulfide and increasing O<sub>2</sub>.

In contrast, in reference to growth under oxygenated conditions, modern cyanobacteria demonstrated a lower tolerance to reduced iron compared to sulfide. Relative to oxygenated and sulfidic conditions, there was a 23% decrease in maximum turbidity of Fe<sup>+2</sup>-containing (chelated and unchelated) *A. variabilis* cultures. Furthermore, only under the lowest concentrations of unchelated Fe<sup>+2</sup> was growth observed, and it occurred at a slower rate (Figure 18). This indicates cyanobacteria may have had considerable difficulty evolving and flourishing in iron-rich Archean oceans, leading to a significant delay in oxygen accumulation. Results of this study instead suggest that, if early oceans were rich in iron, it's more likely that oxygenic

photosynthesis developed in waters, such as lakes and ponds, not subject to continuous iron input from mid-ocean ridges. Consequently, global proliferation of cyanobacteria and the subsequent rise of atmospheric O<sub>2</sub> would have been delayed.



**Figure 18.** Graph showing growth of *Anabaena variabilis* under increasing O<sub>2</sub>, H<sub>2</sub>S, and Fe<sup>+2</sup> concentrations.

On the other hand, high tolerance of some modern cyanobacteria to chelated Fe<sup>+2</sup> indicates that complete iron chelation in Archean oceans would have more easily allowed the evolution and global proliferation of oxygenic photosynthesis. However, complete iron chelation in early oceans is unlikely. Chelators are not specific to iron and would have bound to the multiple reducing compounds abundant in Archean oceans. Therefore, large quantities of chelating agents would have been required to tie up all the oceanic

iron. Small amounts of ferrous iron left unchelated would have been very inhibitory to cyanobacteria and led to slow oxygen accumulation.

Iron adaptation and tolerance by modern green sulfur bacteria was exhibited in this study, providing further support for delayed global cyanobacterial proliferation and oxygen accumulation in iron-rich Archean oceans. Competition with copious green sulfur and other photosynthetic bacteria, in addition to difficulty posed by  $\text{Fe}^{+2}$ , would have strongly inhibited immediate global proliferation of cyanobacteria and rapid oxygen accumulation under iron-rich Archean conditions.

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