Temperature Dependence of Phosphate Content in *Thermus thermophilus:*
Characterizing the Source of Temperature Signatures Recorded in Dissolved Phosphate Oxygen Isotope Ratios

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Abstract

Estimates of an organism's growth temperature have previously been made using oxygen isotope analyses of only hard-tissue biomass (carbonates/phosphate minerals), and recent methods for estimating growth temperatures of microorganisms such as Tex^{86} are limited to only one or a few microbial groups. However, *in situ* growth temperatures of microbes have recently been demonstrated to also be reflected in the ^{18}O:^{16}O ratio of phosphate ($\delta^{18}O_P$) in soft-tissue microbial biomass. Furthermore, because dissolved phosphate in the oceans, which is deposited in marine sediments, originates as organic phosphate from marine microbial biomass, these findings have been applied to sedimentary phosphates and phosphate-rich rocks (e.g. Banded Iron Formations) and have yielded valuable paleoclimate data based on measurement of $\delta^{18}O_P$ (Blake et al., 2010).

Calibration of the temperature dependence of $\delta^{18}O_P$ values of bacterial biomass is under investigation and a strong correlation between ambient growth temperature and $\delta^{18}O_P$ values extracted from total microbial biomass has been observed between 12^{o}C and 70^{o}C. However, for some thermophiles and hyperthermophiles, organisms grown above 70^{o}C, much heavier oxygen isotopic compositions than expected have been observed. This divergence from the trend is hypothesized to be due to the production of intracellular compounds called compatible solutes, which protect cells against heat stress. These compounds are often phosphate-rich or have biosynthetic pathways that require large amounts of phosphate, which could lead to fractionation of the intracellular phosphate pool and thus, the observed offset of $\delta^{18}O_P$ values from the trend line. This hypothesis was tested in the present study by determining whether there is an increase in cellular phosphate concentration at the temperature where the offset from the trend line is observed. The thermophilic bacterium *Thermus thermophiles,* which is known to produce compatible solutes, was grown at five different temperatures between 60^{o}C and 80^{o}C to determine the weight percent phosphate of cellular biomass at each temperature and thus, to detect more precisely the temperature at which intracellular phosphate concentrations increase, and to also look for correlation between increased weight percent phosphate and offset from the trend line. Results show that weight percent phosphate of *T. thermophilus* increases over the same temperature range that offset in $\delta^{18}O_P$ values was observed. Thus, this observation supports the hypothesis that fractionation of the intracellular phosphate pool associated with the production of compatible solutes could be responsible for the anomalously heavy $\delta^{18}O$ values of phosphates observed in biomass of *T. thermophilus* and other thermophiles. Correlating the temperature at which the offset in $\delta^{18}O_P$ and the increase in weight percent phosphate are observed in *T. thermophilus* sets the stage for future gene expression and 31P NMR studies to confirm that the offsets in isotopic composition and the elevated weight percent phosphate are due to the synthesis of compatible solutes.

Introduction

Dissolved inorganic phosphate (DIP) serves as the source of phosphate in the rock record; it precipitates or is adsorbed from the water column onto sediments. DIP is derived from the release of phosphate moieties from organic compounds, so DIP records the phosphate oxygen isotope ratio ($^{18}O/^{16}O$) from the organic phosphate (Colman et al., 2005). Thus, the phosphate oxygen isotopic ratio of organic phosphate is preserved in the rock record (Figure 1). Recent studies have suggested that ambient growth temperature is reflected in the oxygen isotope ratio of phosphate in biomass. Blake et al. (2010) used oxygen isotope analysis of phosphate-rich sediments deposited under ancient oceans to estimate paleo-sea water temperatures.

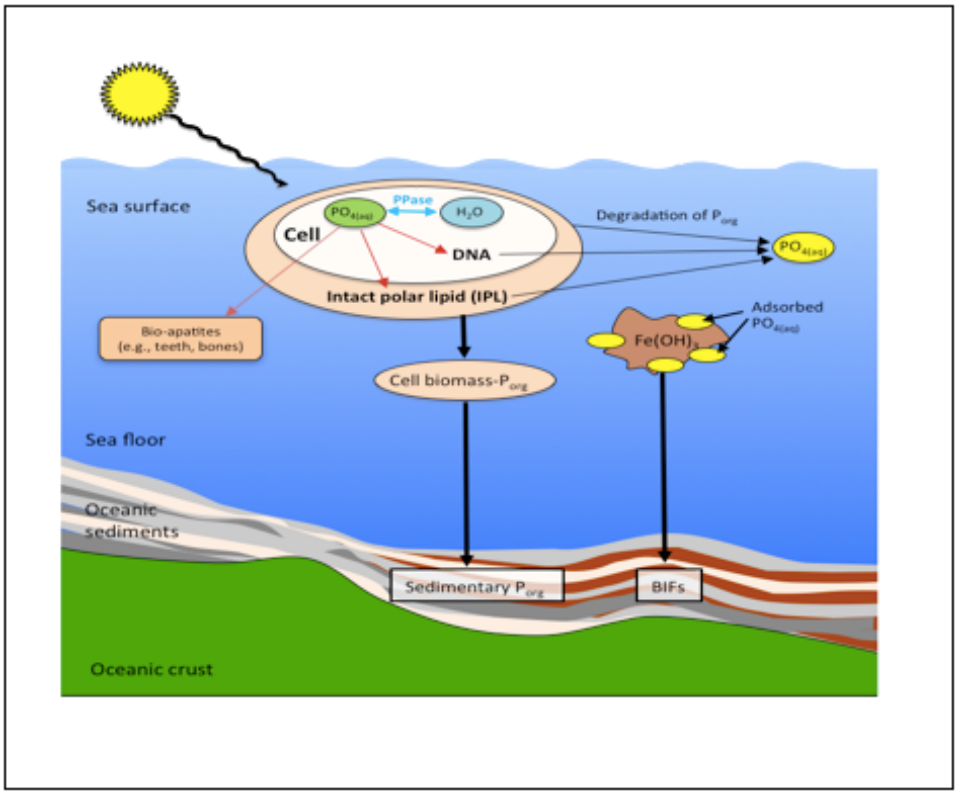


Figure 1. Diagram of organic and inorganic phosphate cycling and deposition in ocean sediments (Blake et al., pers. comm.)

Intracellular pyrophosphatase is responsible for the temperature-dependent fractionation of phosphate oxygen isotopes (Blake et al., 2005). Pyrophosphatase is

the enzyme responsible for catalyzing the complete exchange of oxygen between dissolved phosphate and water in organisms. Blake et al. found that the temperature signal recorded in phosphate oxygen isotopes was due to phosphate's increased preference for the heavier ^{18}O isotope at lower temperatures (2005). Although calculating temperature from the oxygen isotope ratios of phosphate derived from hard biomass (e.g. apatite from bones and teeth) has long been accepted practice, the method through which the temperature signal was produced was not understood until the oxygen fractionation process that accompanied pyrophosphatase catalysis was described (Blake et al., 2005). It is now clear that all biomass phosphate is synthesized from a pool of intracellular phosphate derived from pyrophosphatase catalysis. Thus, all biomass phosphate that is synthesized from this intracellular phosphate pool should reflect the temperature-dependent fractionation of oxygen isotopes at the time of the production of the phosphate. This means that ambient temperature should be observable from the phosphate oxygen isotope ratios of soft biomass, such as DNA, phospholipids, etc.

Previous studies have established a clear trend between the values of phosphate oxygen isotope ratios and growth temperature in mesophilic microbes (solid line in Figure 2). The phosphate oxygen isotope ratios from both biomass and DNA of numerous bacteria and archaea, grown between 12^{0}C and 70^{0}C, have been observed to fall along the calibration line depicted in Figure 2. Because neither bacteria nor archaea possess hard tissue, this corroborates the hypothesis that ambient temperature can be estimated from the oxygen isotope signature from phosphate from soft biomass. However, the oxygen isotope fractionation measured from phosphate within several strains of thermophiles and hyperthermophiles cultured above 70^{0}C do not follow the expected relationship with temperature and plot significantly off of the calibration line (Figure 2). Their phosphate oxygen isotope ratios are observed to be much heavier than expected.

The bacteria *Thermus thermophilus,* one of the three thermophilic outliers depicted in Figure 2, was the focus of this experiment. In a previous study of phosphate oxygen isotope ratios, *T. thermophilus* was cultured at temperatures at the low end of its growth range, from 60^{0}C down to 52^{0}C (Blake et al., pers. comm.).

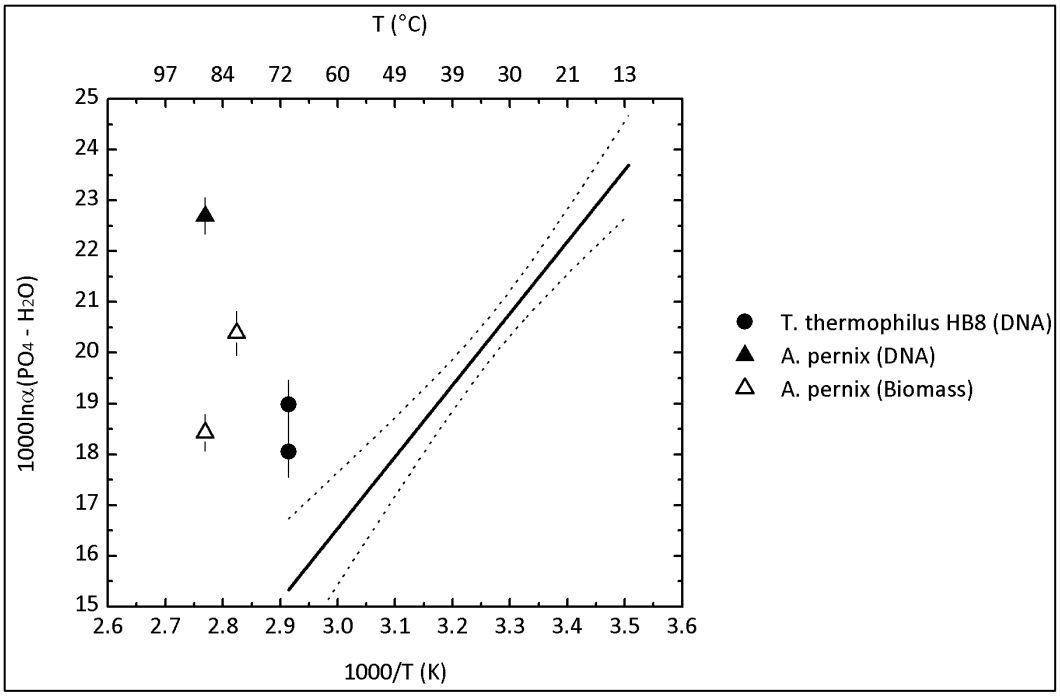


Figure 2. Temperature dependence of phosphate oxygen isotope ratios in microbes. Solid black line represents thermometry calibration from biomass and DNA isotopic ratios. (Blake et al., pers. comm.)

The phosphate oxygen isotope ratios observed for the *T. thermophilus* cultures at cooler temperatures fell along the established calibration line. Thus, the offset from the calibration line in the phosphate oxygen isotope ratios measured for *T. thermophilus* must occur at a temperature between 60^{O}C and 72^{O}C. At 60^{O}C, *T. thermophilus* phosphate oxygen isotope ratios are observed at expected values but by 72^{O}C they are anomalously heavy.

The production of compatible solutes may be responsible for the heavier phosphate oxygen isotopes observed in thermophiles and hyperthermophiles. Compatible solutes, also known as osmoprotectants, are molecules produced by extremophiles to help them counter osmotic stress; most commonly, microbes produce compatible solutes to survive in extremely salty environments and extremely high temperatures (Santos and da Costa, 2002). Production of compatible solutes generally requires microbes to significantly increase their concentrations of intracellular phosphate (Empadinhas and da Costa, 2006). Typically, this is because the compatible solutes are phosphate-rich molecules (e.g, Santos and da Costa, 2002). However, in *T. thermophilus* and in some other thermophilic organisms, the

compatible solutes themselves do not contain phosphate moieties, but higher intracellular phosphate concentrations are needed because the biosynthetic pathways through which compatible solutes are produced require significant quantities of phosphate-rich molecules (Empadinhas and da Costa, 2011).

The biosynthetic pathways involved in the production of compatible solutes likely include intracellular phosphate fractionations that have not yet been detected or explained. If these hypothesized pathways involved in the production of compatible solutes preferentially use lighter oxygen to build phosphate, then that would explain the heavier phosphate oxygen isotope ratios observed in thermophiles. The fractionation processes involved in compatible solute production would leave heavier oxygen for pyrophosphatase catalysis and thus result in the creation of heavier phosphate in cellular biomass. That is, compatible solute biosynthetic pathways would deplete the cell's reservoir of phosphate that contains light oxygen, meaning that phosphate moieties in microbial biomass would be synthesized from phosphate with heavier oxygen, regardless of temperature effects on the process of pyrophosphatase catalysis.

Because microbes need to ingest significant quantities of phosphate to produce compatible solutes, the total phosphate content of the biomass should increase when compatible solutes are synthesized by the organism. Thus, an increase in the concentration of intracellular phosphate can indicate microbial production of compatible solutes. The purpose of this study was to observe the temperature dependence of concentrations of intracellular phosphate in *T. thermophilus,* in order to gain a better understanding of the temperature dependence of compatible solute production in *T. thermophilus. T. thermophilus* was cultured across a twenty degree temperature range, from 60^{0}C to 80^{0}C, biomass was extracted from each culture, and weight percent phosphate was measured for each sample of biomass. Ultimately, the temperature at which changes in phosphate concentration occurred was compared to the temperature at which the offset from the phosphate oxygen isotope calibration line appeared. A correlation between the two temperatures would support the hypothesis that the isotopic signature is affected by the production of compatible solutes by bacteria.

Methods

***Thermus thermophilus* Growth:**

The optimal growth temperature of *Thermus thermophilus* is 68^{O}C, but it can survive in temperatures ranging from 50-82^{O}C (Nunes et al., 1995). For this experiment, *T. thermophilus* was initially grown at 60^{O}C, 70^{O}C, and 80^{O}C. Based on the results at these temperatures, cultures were subsequently grown at 65^{O}C and 75^{O}C to more precisely constrain the temperature at which change in phosphate concentration occurs.

Initially, a growth medium was prepared using accepted nutrients for *T. thermophilus* (Nunes et al., 1995). Sodium chloride was added in a ratio of 2 grams per liter of distilled deionized (DDI) water, peptone in a ratio of 4 grams per liter DDI water, yeast extract in a ratio of 4 grams per liter DDI water, and tryptone in a ratio of 4 grams per liter DDI water. The pH of the medium was then raised to 7.5 via additions of 0.1M sodium hydroxide solution. The medium was autoclaved at 121^{O}C. Then the medium was thermally equilibrated, uninoculated, at 70^{O}C, so that when the *T. thermophilus* was transferred into the medium, the medium would be at the bacteria's optimal growth temperature.

During the inoculation of the growth medium, the lab bench was wiped down using 70% ethanol and all work was done next to a Bunsen burner flame to ensure sterile conditions. The 70^{O}C growth medium was decanted into test tubes with 7ml of medium in each test tube. *T. thermophilus* was kept in the laboratory in a -80^{O}C freezer and a frozen sample of the bacteria was used to inoculate each tube. A loop of ice shavings from the *T. thermophilus* sample was added to each sample test tube. Two test tubes of *T. thermophilus* were grown at each temperature (60^{O}C, 70^{O}C, and 80^{O}C) and an uninoculated test tube was also incubated at each temperature to serve as a control. To measure the cultures' growth progress, turbidity readings were taken approximately every 12 hours with a spectrophotometer, measuring light absorbance at 600nm. (All growth curves can be found in Appendix I.) When the cultures reached stationary growth phase, each of the two tubes was transferred

to 1-liter flasks containing 500ml of the same medium, and incubated at the same temperature as each tube. 30ml of medium from each flask were extracted prior to inoculation and incubated at the same temperature as the flask from which it was extracted to serve as controls. Turbidity was again measured approximately every 12 hours to monitor culture growth.

Growth was measured in both the tubes and the flasks of the *T. thermophilus* cultures at both 60^{O}C and 70^{O}C. Although the tubes of *T. thermophilus* that were incubated at 80^{O}C showed increasing turbidity with time, indicating normal growth, transferring the contents of the tubes to the flasks appeared to kill the bacteria. Neither of the 80^{O}C flasks experienced any change in turbidity after inoculation. Initially, it was believed that the microbes had failed due to a slow transfer, allowing the temperature of the flasks to drop significantly below 80^{O}C, and so *T. thermophilus* was regrown. Two new tubes were inoculated, incubated until they reached stationary growth phase and then each was transferred to a flask containing 500ml of growth medium at 80^{O}C. The time each flask spent outside the incubator was minimized and no significant temperature drop was measured. Nevertheless, as before, once transferred to the flasks, neither *T. thermophilus* culture survived.

Based on the Blake lab's past experiences growing *T. thermophilus*, it was suggested that it would be easier to grow *T. thermophilus* in the flasks if a greater volume of healthy culture was initially transferred into each flask. Three new tubes were filled with 12ml of growth medium each and inoculated with *T. thermophilus*. Once the bacteria in the three tubes reached stationary growth phase, the three tubes were combined and 18ml of solution containing stationary-phase *T. thermophilus* were transferred into each flask. After adding this greater volume of *T. thermophilus* suspension to each flask, the bacteria grew successfully in both flasks at 80^{O}C.

Based on the results of the *T. thermophilus* cultures grown as 60^{O}C, 70^{O}C, and 80^{O}C, cultures were subsequently grown at 65^{O}C and 75^{O}C. Similar procedures were followed to those used at the first three temperatures studied. However, because a smaller volume of cultures was needed, cultures were not transferred from tubes to

flasks. Instead, six tubes with 15ml of growth medium in each were inoculated with *T. thermophilus* and incubated at each temperature until they reached stationary growth phase; this provided enough biomass for weight percent phosphate analysis.

Biomass Extraction:

Once the final cultures of *T. thermophilus* successfully reached stationary growth phase, the bacteria were harvested. The cultures grown at the same temperature were combined and centrifuged at 4,500rpm for 30 minutes at 4^{o}C. The supernatant was removed and the pellet (i.e., the biomass) was rinsed with 10ml of 0.85% saline solution. Then, the biomass was centrifuged at 4,500rpm for 30 minutes at 4^{o}C to pellet the biomass again. The supernatant (i.e., the saline solution) was removed; the biomass was dried and weighed. Then the samples were suspended in 2ml of DDI water and stored in a -80^{o}C freezer until the weight percent phosphate analysis could be performed.

Phosphate Extraction:

To extract the phosphate from the *T. thermophilus* biomass, the biomass samples were completely digested in solution using an oxidation procedure via the addition of hydrogen peroxide and treatment with ultraviolet (UV) light. Approximately 1gram of biomass from each temperature was used, but the exact mass of each sample was measured gravimetrically. The biomass samples were thawed until they reached room temperature and decanted into quartz tubes for the procedure. To serve as controls, standards with known concentrations of phosphate were prepared from potassium monophosphate. Both samples and standards were oxidized by the addition of a 30% hydrogen peroxide solution and exposed to UV light. (Specifically, the device used was an Ace Glass Photooxidation Apparatus equipped with a 1500-watt mercury lamp.) Initially, 1ml of hydrogen peroxide was added to each sample and standard. This produced bubbles from the oxidation of the biomass in the samples. Then, if the sample stopped bubbling gently while biomass was still visible, 0.2ml more of the hydrogen peroxide solution would be added to the sample until the biomass was completely oxidized and digested (i.e. no

longer visible). The time needed for the complete digestion of each sample's biomass varied from 7 to 22 hours of exposure to the UV light. When the biomass from each sample was completely oxidized, the volume in each sample and standard tube was measured.

Phosphate Analysis:

The phosphate concentration in each sample and standard was measured using an ammonium molybdate spectrophotometry method. To begin, ingredients for the ammonium molybdate reagent were prepared. Ammonium molybdate was dissolved in a ratio 3.0g per 100ml DDI water; sulfuric acid was dissolved in a ratio of 70ml per 500ml DDI water; 1.32g L-ascorbic acid was dissolved in a ratio of 1.32g per 50ml DDI water; and antimony potassium tartrate was dissolved in a ratio of 0.034g per 25ml DDI water. The reagent was mixed by combining 5ml of ammonium molybdate, 12.5ml of sulfuric acid, 10ml of L-ascorbic acid and 2.5ml of potassium tartrate (in that order). Then, the six samples and standards from the UV digestion were diluted 100 times by first transferring 50µl of the sample or standard into 0.5ml of DDI water, and then taking 50µl of that solution and transferring it into 0.5ml of DDI water. 500µl of each sample and standard were added to a test tube, followed by 5.500ml of DDI water and 960µl of the ammonium molybdate reagent. Three spectrophotometer standards were also prepared using solutions with known (and unique) phosphate concentrations, ranging from 1µM to 200µM phosphate; measured 500µl of each spectrophotometer standard were added to a test tube, followed by the additions of 5.500ml of DDI water and 960µl of the ammonium molybdate reagent.

Using the spectrophotometer, light absorption by each sample and standard was measured at 880nm. The spectrophotometry standards with known phosphate concentrations were then used to calibrate the relationship between light absorption and phosphate concentration. Each calibration line was produced using Microsoft Excel to calculate the best-fitting linear trend based on the absorption measured for three spectrophotometer standards; the linear trend lines fit well, with R^2 values higher than 0.999. (Trend lines can be found in Appendix II.) The

calibration line was then used to calculate the unknown phosphate concentration of each sample and standard from the UV digestion based on the measured light absorption reading of each. The phosphate concentration of each sample was used to calculate the weight percent phosphate in the biomass of each sample. The calculations involved the conversion from moles of phosphate per liter of solution to grams of phosphate per gram of biomass, and details can be found in Appendix III.

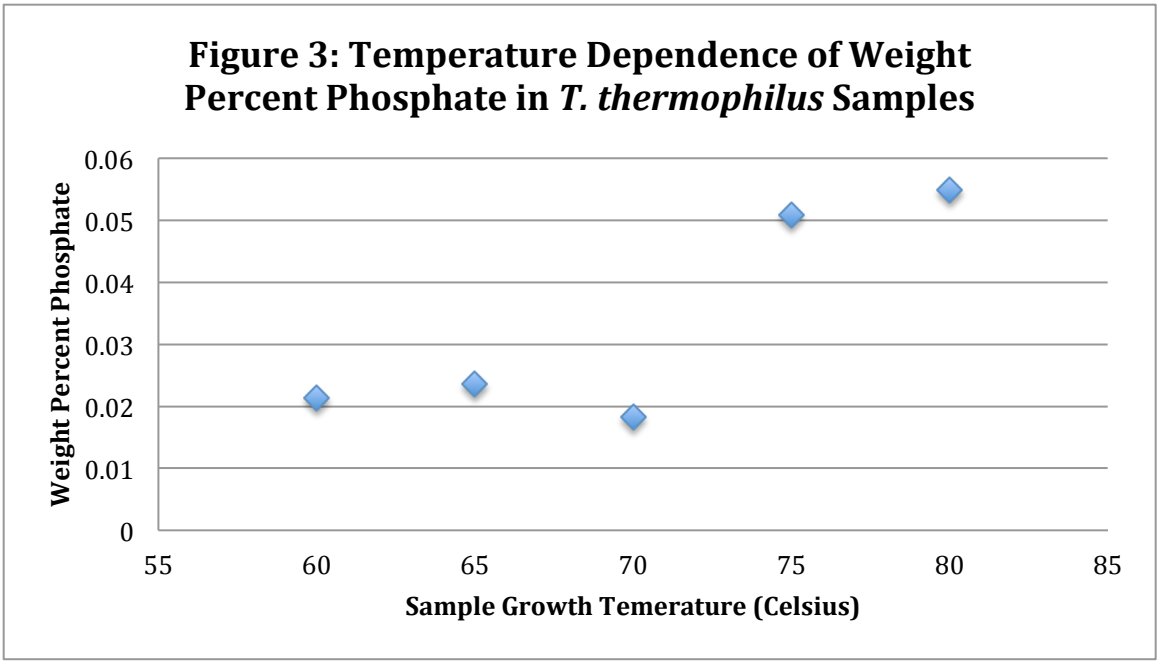
Results

All the successful *Thermus thermophilus* cultures, at all temperatures, in both the tubes and flasks, exhibited classically shaped microbial growth curves. (All growth curves are included in Appendix I.) The growth curves begin with a lag phase as the bacteria adjust to their new environment (i.e., new growth medium, new temperature, etc.). During lag phase there is little microbial growth, indicated by little turbidity; without significant microbial growth, the medium is transparent. For the *T. thermophilus* cultures, there was a relatively large range in the duration of the lag phase, from approximately 20 hours to approximately 70 hours. After the bacteria adjusts to the new environment, it enters a period of exponential growth, during which growth rates significantly outpace death rates and thus turbidity increases rapidly. The length of the exponential growth phase ranged from 90 hours to 130 hours in the *T. thermophilus* cultures. As the microbes begin to deplete their environment of nutrients, the exponential phase ends and the cultures enter a stationary growth phase in which growth rates and death rates are approximately equal and the turbidity of the suspension remains relatively unchanged over time. For this experiment, cells were harvested when the cultures reached stationary phase. Most cultures reached stationary phase after 160 hours of growth. Final turbidity was generally higher in the tubes than in the flasks incubated at the same temperature. The average final turbidity for *T. thermophilus* cultures grown in the tubes was 0.798 and in the flasks it was 0.347. Final turbidity did not vary systematically with temperature.

The potassium monophosphate solutions used as standards in the UV digestion served as a measure of analytical uncertainty. Actual and measured phosphate concentrations can be found in Table 1. Analytical uncertainty was low. Even the least accurate standard, Standard 1, was within 1.2% of its expected value. Standards 2 and 3 were within 0.6% and 0.3% of their expected values, respectively.

Table 1: Phosphate Concentrations from Potassium Monophosphate Standards		
Standard	Actual Phosphate Concentration (µmol)	Measured Phosphate Concentration (µmol)
1	15.0	14.82
2	20.0	19.88
3	20.0	19.94

Figure 3 shows the relationship between the measured weight percent of phosphate in the biomass from the *T. thermophilus* cultures and the temperature at which the *T. thermophilus* cultures were grown. At all temperatures, phosphate makes up a very small portion of the total weight of *T. thermophilus* biomass. The phosphate percentages range from 0.018% to 0.055% of the total biomass weight.



Because the analytical uncertainty of the phosphate measurement is at most ±1.2% of the value of each weight percent phosphate measurement, error bars on the weight percent phosphate measurements, if included in the graph, would be smaller than the height of the plotted data points. Thus, the measured values are significantly above the detection limit of the technique, and even the relatively small variations between measurements are significant.

In Figure 3, the weight percent of phosphate in *T. thermophilus* biomass exhibits a clear step-function relationship with temperature. In the samples of *T. thermophilus* incubated at 60^{O}C, 65^{O}C, and 70^{O}C, phosphate content is relatively constant as a function of temperature and makes up approximately 0.02% of the bacterial biomass. Between 70^{O}C and 75^{O}C, the weight percent phosphate increases markedly to approximately 0.05% of the weight of *T. thermophilus* biomass. The 80^{O}C sample exhibits a slightly higher weight percent phosphate than the 75 ^{O}C sample, with a value of 0.055%.

Discussion

The increase in weight percent phosphate that was observed in this study is consistent with increased production of compatible solutes in *T. thermophilus* in response to increasing incubation temperatures above 70 ^{O}C. To produce compatible solutes, *T. thermophilus* must increase its intake of phosphate because the molecule plays an important role in the biosynthetic pathways used to produce the compatible solutes most common to *T. thermophilus* (Nuno and da Costa, 2006). Thus, the weight percent that phosphate makes up of the total biomass of *T. thermophilus* increases if compatible solutes are produced. In this study, weight percent phosphate of the biomass was observed to increase sharply between the *T. thermophilus* samples incubated at 70^{O}C and 75^{O}C, suggesting that compatible solute production in *T. thermophilus* also increased sharply between 70^{O}C and 75^{O}C. This observation is expected if microbes use compatible solutes to survive osmotic stresses such as extreme heat. At 70^{O}C, *T. thermophilus* is relatively close to its

optimal temperature of 68^{O}C, and so should experience little osmotic stress (Nunes et al., 1995). There is little need for it to produce additional protection against thermal stress. However, as the temperature increases, so does the osmotic stress that *T. thermophilus* experiences from the higher temperature, and thus compatible solutes are needed for the bacteria to survive (Nunes et al., 1995).

The sharp increase in phosphate concentration observed between 70^{O}C and 75^{O}C also suggests that the presence of compatible solutes in *T. thermophilus* is controlled by temperature-triggered gene expression. Compatible solute production is likely controlled by expression of specific genes (Santos and da Costa, 2002). The data suggest that at temperatures below 70^{O}C the necessary genes are not activated, but after some temperature threshold is reached between 70^{O}C and 75^{O}C, the gene becomes activated and compatible solutes are produced, causing the observed increase in phosphate content of the bacteria. Such a process could explain the step-function shape of the relationship between temperature and phosphate concentration. A gradual increase in phosphate content would not be expected if compatible solute synthesis is triggered at a specific temperature, and under conditions where gene expression is triggered (e.g. in this study, apparently between temperatures of 75^{O}C and 80^{O}C), the production of compatible solutes, and hence intracellular bacterial phosphate concentrations, would be elevated but relatively constant.

The temperature range in which phosphate production became elevated is comparable to the temperature at which the phosphate oxygen isotope offset was recorded. Heavier-than-expected isotope values were observed in *T. thermophilus* when grown at 72^{O}C (Figure 2). This study found that *T. thermophilus* begins to exhibit elevated phosphate concentrations at a temperature between 70^{O}C and 75^{O}C. This temperature overlap suggests that *T. thermophilus* may begin producing compatible solutes at the same temperature at which the offset isotope ratios were measured. This temperature correlation supports the hypothesis that production of compatible solutes is responsible for the anomalously heavy phosphate oxygen isotope ratios found in thermophiles.

The results of this study serve as an important foundation for additional experiments to further confirm the synthesis of compatible solutes as the source of the heavier phosphate oxygen isotope ratios observed in thermophiles. Based on the findings of this research, phosphorus-31 nuclear magnetic resonance (31P NMR) spectroscopy and gene expression studies would be useful to confirm that the elevated phosphate concentrations detected in this study actually are caused by the production of compatible solutes in *T. thermophilus*. Gene expression studies could be used to detect activity in the genes needed to produce compatible solutes in *T. thermophilus* and to test whether a temperature trigger exists that controls gene expression. 31P NMR spectroscopy could be used to search for the presence of the compatible solutes most common in *T. thermophilus.* This would directly relate the elevated phosphate concentrations that were measured in this study and the heavier phosphate oxygen isotope ratios observed above 70^{O}C with increased production of compatible solutes.

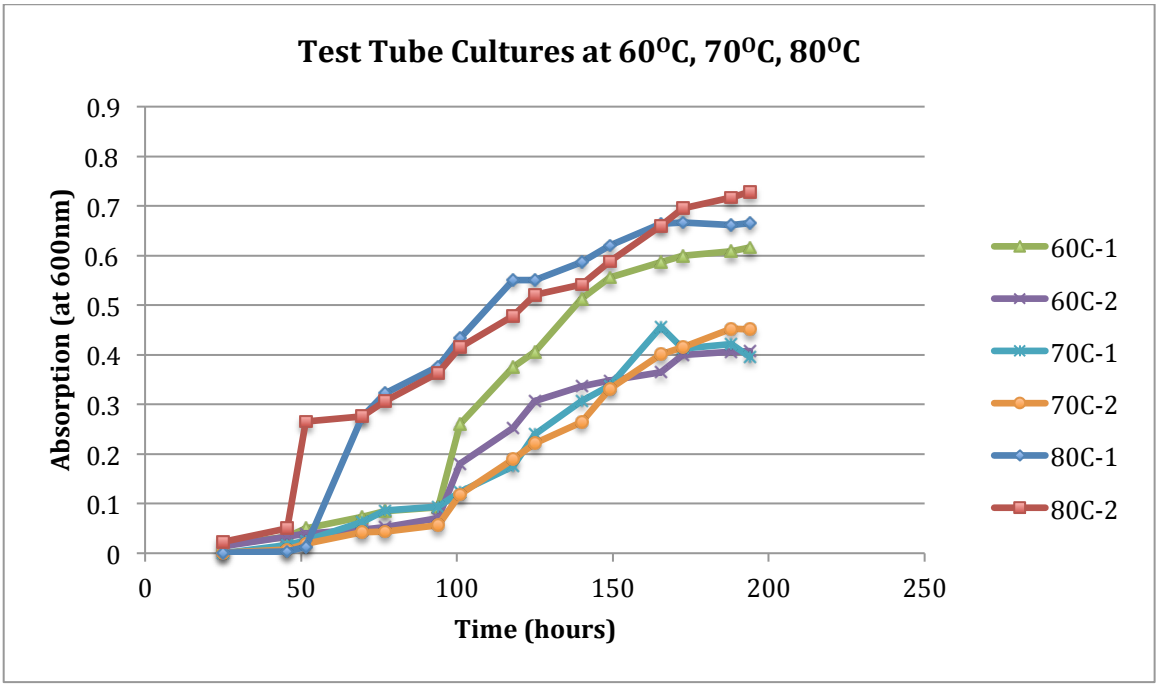
Summary

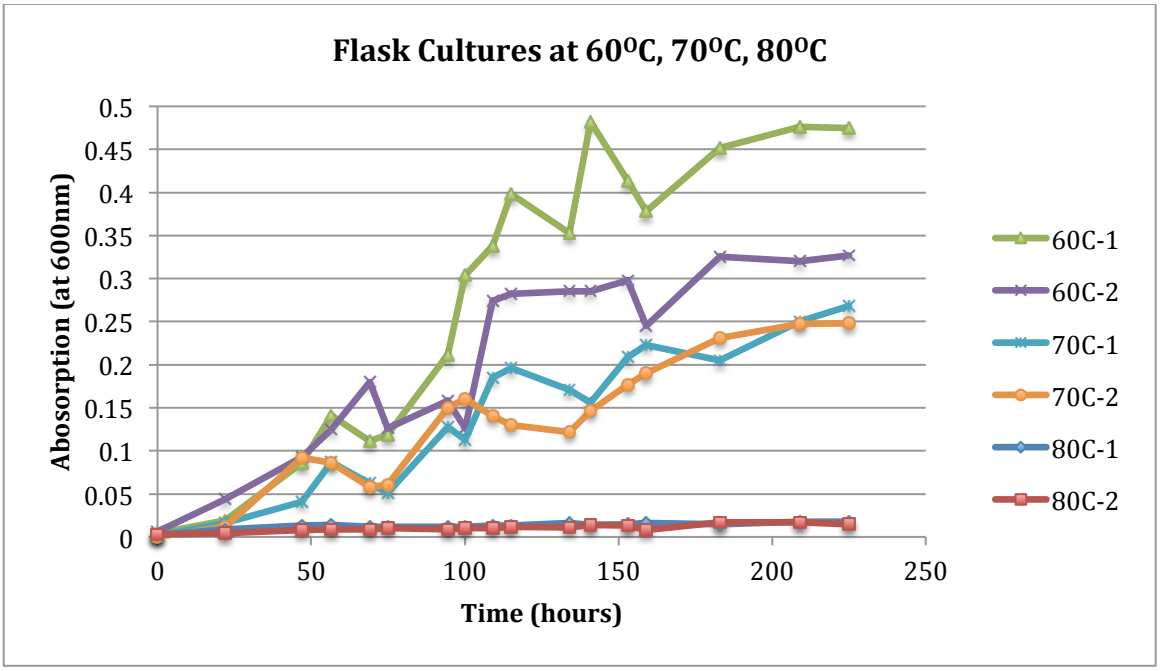
The hypothesis that the anomalously heavy values of phosphate oxygen isotope ratios observed in *Thermus thermophilus* are due to the bacteria's production of compatible solutes was supported by the results of this study. The deviation of phosphate oxygen isotope ratios in *T. thermophilus* from the trend line relating mesophile growth temperature to phosphate oxygen isotope ratios appears above 70^{O}C (Figure 1) and this study found that phosphate concentrations in *T. thermophilus* biomass increase sharply between 70^{O}C and 75^{O}C, a result consistent with a thermal trigger for gene expression control on compatible solute synthesis. Elevated biomass phosphate levels likely are caused by the production of compatible solutes by the bacteria. Therefore, the coincidence of the temperatures at which biomass phosphate concentrations increase markedly and at which anomalously heavy phosphate oxygen isotope ratios in *T. thermophilus* occur suggests that the anomalously heavy phosphate oxygen isotope ratios are caused by

the bacterial synthesis of compatible solutes. This study is an important first step in confirming that the heavier than expected phosphate oxygen isotope values seen in many thermophiles and hyperthermophiles are due to the production of compatible solutes by these species in response to thermal stress. If confirmed, this information could be used to calibrate isotopic trends for hyperthermophiles and hence could help pave the way for the creation of an oxygen isotopic thermometer for phosphate from relatively high temperature geologic systems.

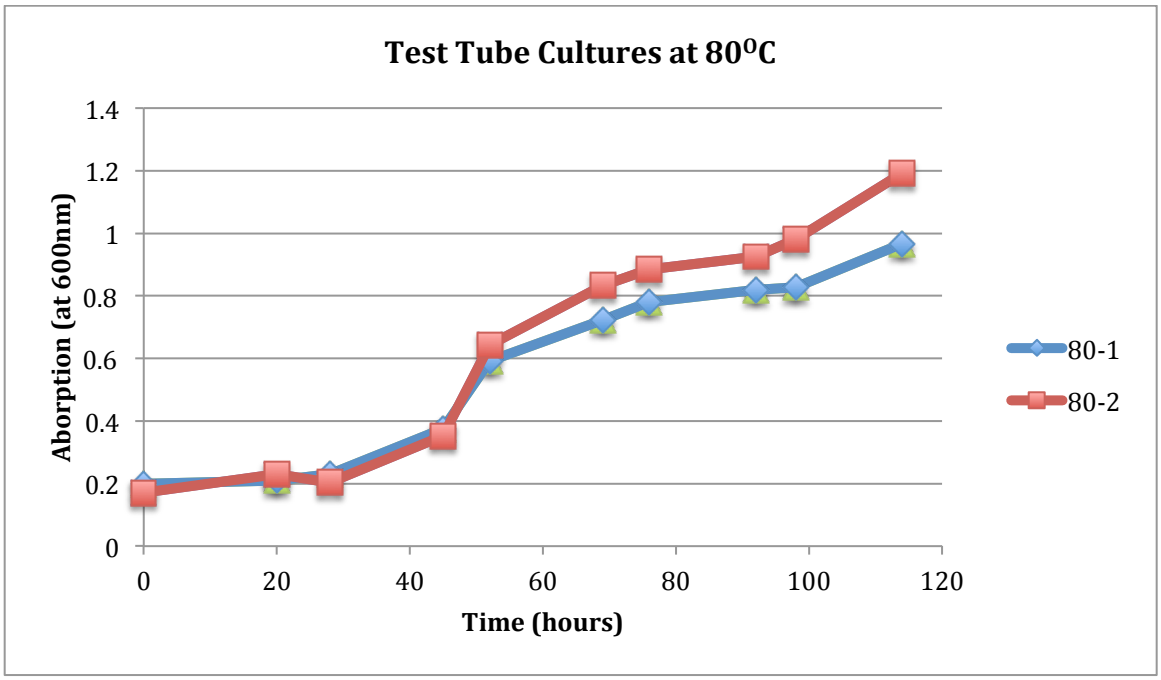
Appendix I – *Thermus thermophilus* Growth Curves

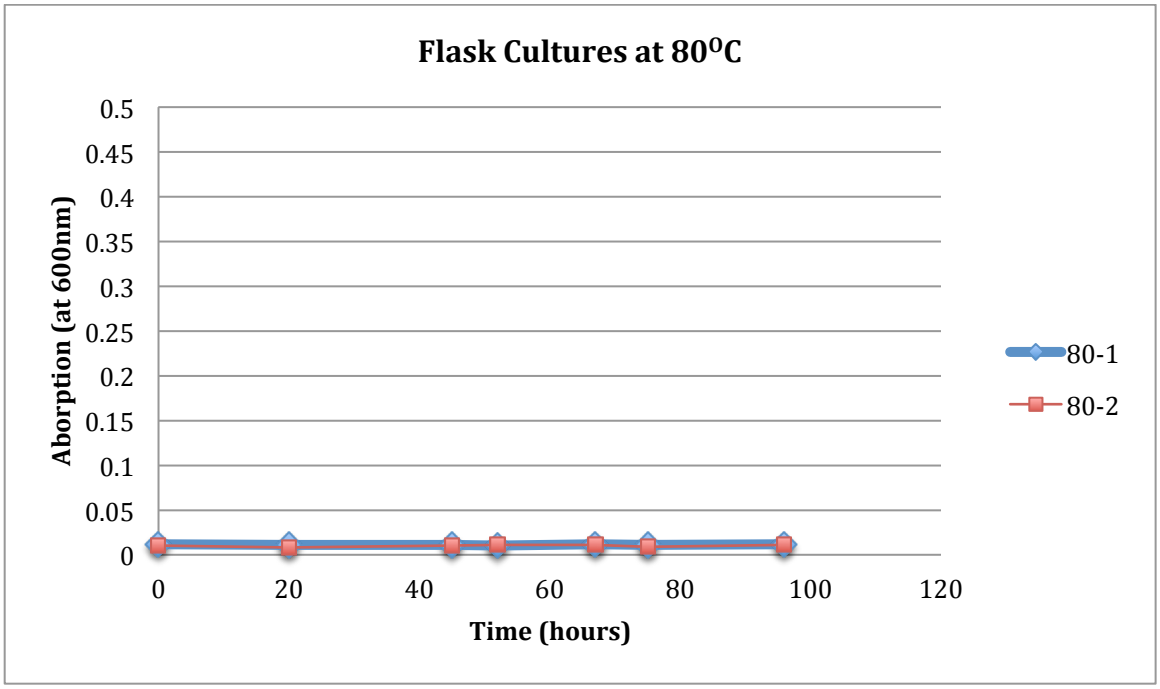
First Culture: six samples, two grown at each 60°C, 70°C, and 80°C



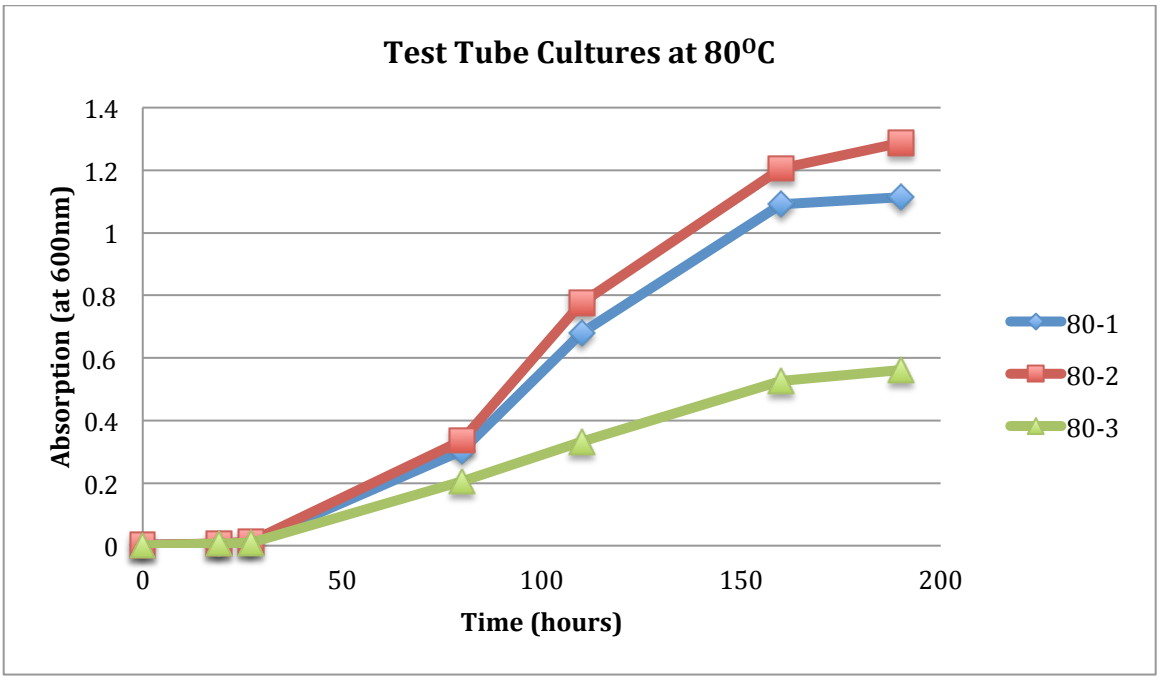


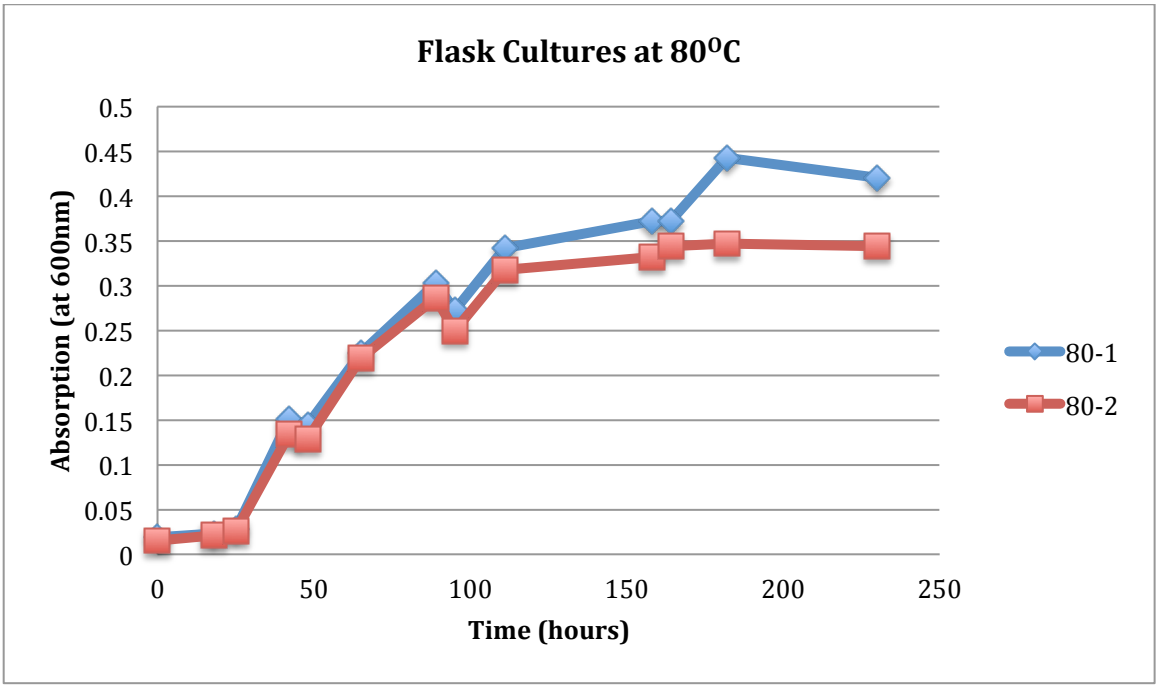
Second Culture: two samples grown at 80^{O}C



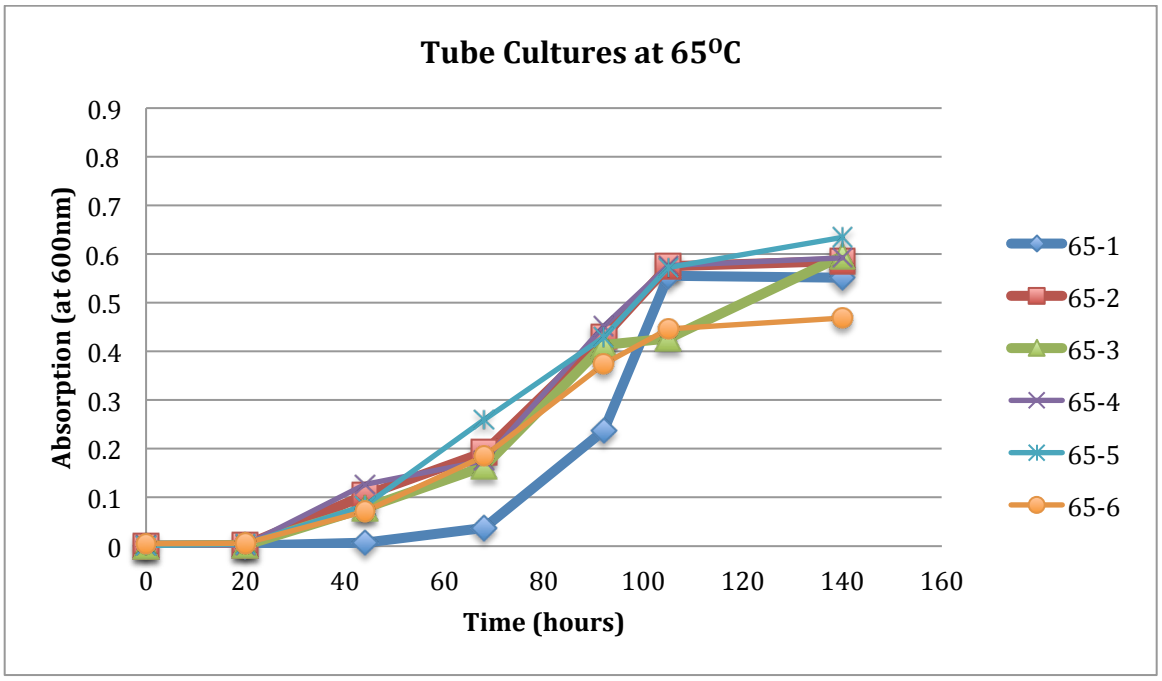


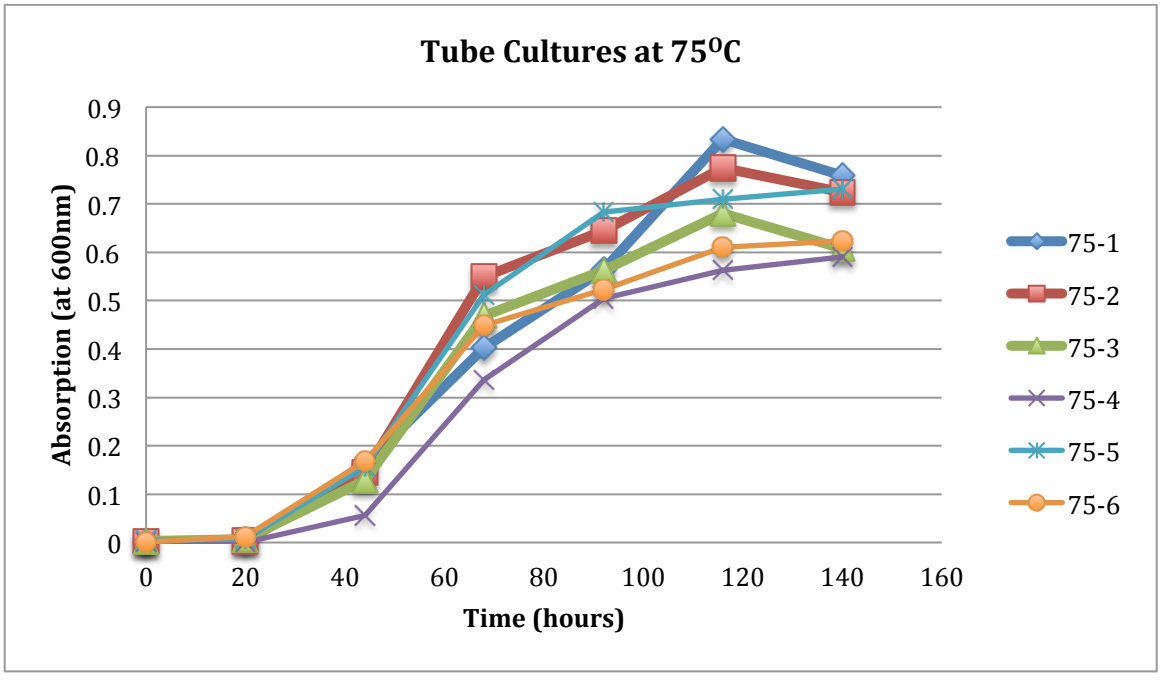
Third Culture: initially three samples, combined into two samples grown at 80^{O}C





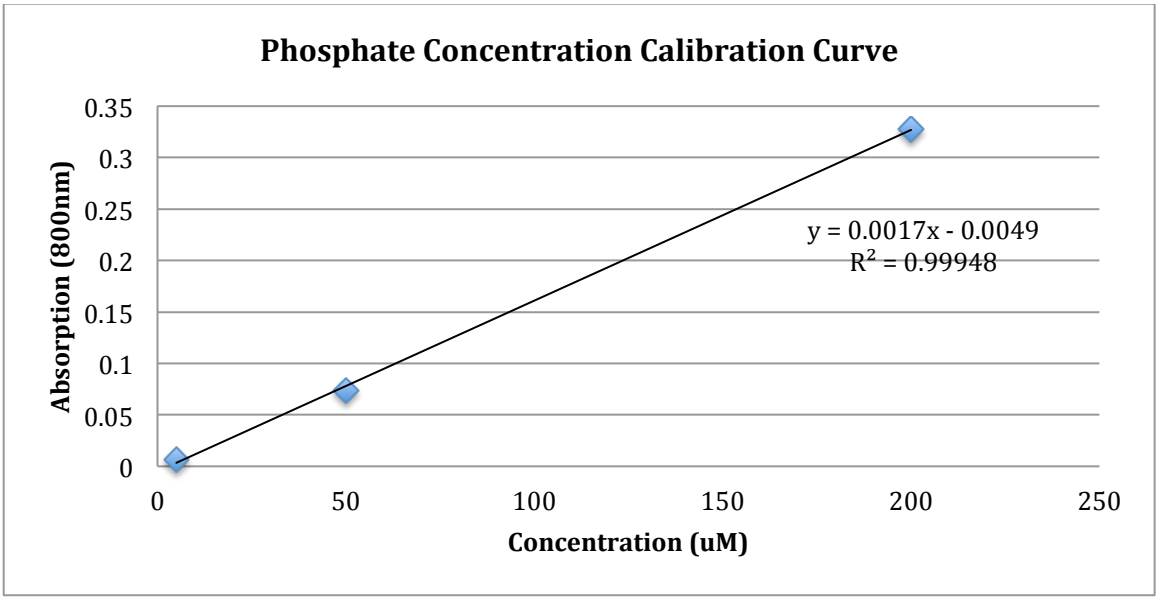
Fourth Culture: twelve samples, six grown at each 65°C and 75°C



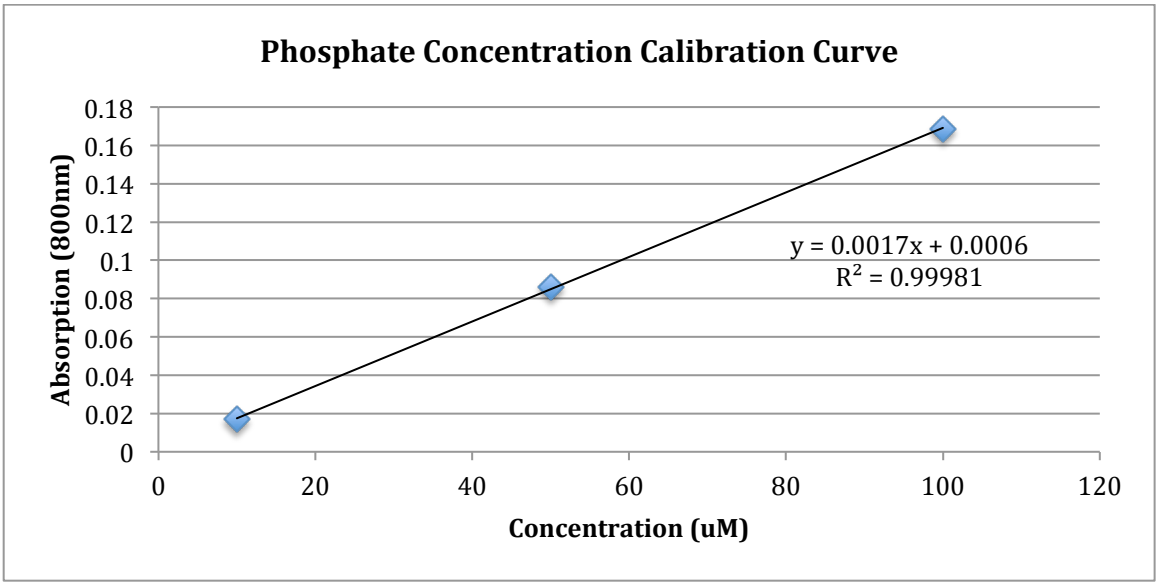


Appendix II – Phosphate Concentration Calibration Lines

First Spectrophotometric Analysis: calibration used for samples grown at 60^{O}C, 70^{O}C, and 80^{O}C



Second Spectrophotometric Analysis: calibration used for samples grown at 65^{O}C and 75^{O}C



Appendix III – Weight Percent Phosphate Conversions

Calculations begin with phosphate molarity of the sample, measured via spectrophotometry:

$$\frac{[\text{sample } PO_4]\ \mu\text{ mol}}{\text{L}} \times \text{UVR sample volume (L)} = [\text{sample } PO_4]\ \mu\text{ mol}$$

$$[\text{sample } PO_4]\ \mu\text{ mol} \times \frac{94.97136\ \mu\text{ gram } PO_4}{\mu\text{ mol } PO_4} = \text{sample } \mu\text{ gram } PO_4$$

$$\frac{\text{sample } \mu\text{ gram } PO_4}{\text{total biomass } \mu\text{ gram}} \times 100 = \text{Weight percent } PO_4$$

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