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TRACE METAL BIOSIGNATURES

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ABSTRACT

Trace metals are vital components of biological systems. Microbial trace metal utilization, as observed in extant microorganisms, can provide information regarding metabolic processes, environmental conditions and potential biosignatures. Additionally, inferences may be possible regarding the nature and evolution of early life on Earth.

This thesis investigates microbial trace metal utilization via a series of laboratory growth experiments involving pure cultures of primarily archaeal hyperthermophiles, with the aim of discovering potential biosignatures. Trace metal contents of investigated microorganisms revealed a distinct metallome pattern and possible biosignature for the methanogen *M. jannaschii* in which high cellular concentrations of Ni, Co and W were observed. Microorganisms can alter mineral substrates through scavenging of bioessential trace metals, which may produce biomarkers. To investigate whether hyperthermophiles produce ligands under metal-limited conditions, supernatant growth experiments were conducted with hyperthermophiles in the presence of powdered basalt. Tungsten (W) was targeted as the metal of interest as it has been shown to be an important bioessential nutrient required for the growth of many hyperthermophiles. Bulk results from all experiments suggest hyperthermophiles do not produce W ligands however, due to reproducibility issues, the role of W as a possible hyperthermophile biosignature remains unresolved.

Finally, novel Ni stable isotopic investigations were performed for geological and biological materials. Mass spectrometry, column chemistry and microbiological protocols specific to Ni and hyperthermophiles, were developed and established. Terrestrial and extraterrestrial geological materials vary within a small range of Ni isotopic compositions: -0.04

to +0.36 per mil. Relative to the starting composition of the growth media, diverse metabolic groups of methanogens impart distinct and significant mass-dependent fractionations on Ni isotopes, preferentially sequestering the lighter isotopes which results in an isotopic fractionation on the order of ~ 0.8 - 1.0 per mil. Non-methanogenic cells also produced a small fractionation. Organisms in general may fractionate Ni isotopes but not as significantly as methanogens. Ni isotopes may be useful, not only as a general methanogenic biomarker but also as a marker for distinguishing between diverse metabolic groups. Ni stable isotopes may have important implications in the fields of transition metal isotopes, geomicrobiology and astrobiology.

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Chapter 1

Introduction

1.1 Bioessential Trace Metals

The biological chemistry of life is based on and is a reflection of, the physical abiotic chemistry of the natural environment. As has been amply demonstrated by the studies of Frausto da Silva and Williams (for e.g. SILVA and WILLIAMS, 2001; WILLIAMS, 2002; WILLIAMS, 2007; WILLIAMS and DA SILVA, 2000) the contents of a cell can be broadly defined by three related and dependent systems: the genome (DNA), the proteome (proteins) and the metallome (element content). The metallome is the simplest of the systems and in addition to the basic (C, N, O, H) and essential elements (Na, K, Ca, Mg, P, S, Cl) necessary for all life, there are approximately eleven transition metals (Fe, Zn, Ni, Mn, Co, Mo, Cu, V, Cr, W, Cd) which generally occur in trace amounts in the natural environment and hence, are correspondingly required by biology in varying trace quantities. All biological groups do not need all of these bioessential trace metals. For example, Fe is generally universally required but a few metals like W and Cd are only utilized by specific organisms.

Microbial trace metal utilization is a function of many factors that include availability and environmental constraints (e.g. redox conditions, pH, temperature) as well as the response of the microorganism to changes in these conditions. Furthermore, it is probably to a large extent, a direct result of these factors which has dictated the evolution of trace metal utilization in microbial metabolisms and consequently, the course of biological evolution. Evidence of the role and impact of trace metals in life's evolution can be found in groups of modern extant organisms where most function primarily as catalysts in a wide range of metabolic reactions.

However, an examination of the correlations between trace metal utilization, microbial metabolisms and the microorganisms themselves can be used to infer information regarding environmental changes through geologic time and could possibly be exploited as potential biosignatures. For instance, trace elements such as Ni, W, V and Co *generally* have greater importance to certain anaerobic, phylogenetically ancient but extant microorganisms in very specific metabolisms (e.g. FRAUSTO DA SILVA and WILLIAMS, 2001; STIEFEL, 2002), which has been inferred to be a result of the availability of these metals on an early reducing Earth (KLETZIN and ADAMS, 1996; WILLIAMS and DA SILVA, 2002). The utilization of other trace metals like Mo, Zn and Cu, may only have attained a greater level of importance after oxygenation of the Earth. The ability of early microorganisms to adapt and take advantage of varying metal conditions would have been beneficial in that, it could have allowed the optimization of metabolic processes through improved enzyme function and, allowed the adaptation of microorganisms to new aerobic environments.

1.2 Biosignatures

A vast array of morphological, chemical and isotopic evidence has been described as markers or potential markers of life (BROCKS et al., 1999; BUICK, 1992; DOUGLAS, 2005; DOUGLAS et al., 2008; FURNES et al., 2001; JAVAUX, 2006; SCHOPF and PACKER, 1987; SHEN et al., 2001). Some are definitive indicators of life but others remain ambiguous (BRASIER et al., 2002; HORITA, 2005; HORITA and BERNDT, 1999; UENO et al., 2006). These markers have provided valuable information concerning in particular, the nature of Earth's early environment as well as the subsequent changes that have taken place through geological time. Additionally,

biosignatures have imparted important clues as to the evolution and impact of life in the geosphere.

A primary component of current research, driven to a large extent by the present unmanned voyages to Mars and the general search for life in the universe, has been geared towards finding new methods of detecting and identifying potential biosignatures (e.g. EDWARDS et al., 2007; SOBRON et al., 2008). Such studies are important but innovative methods also need to be developed, applied and understood within the context of biosignatures on Earth, particularly for those markers that remain controversial. For example, stable isotopes are perhaps one of the more robust tools applied for discriminating between biotic and abiotic processes. Yet, the application of isotopic systems such as Fe isotopes has been equivocal. Iron is probably the most important trace metal on the Earth as well as to almost all forms of life and it was thought that biological fractionation of its stable isotopes might potentially prove to be a valuable biomarker. Early studies showed Fe-reducing bacteria produced residual ferrous iron that was 1.3 per mil lighter than the starting iron substrate, which was also within the range of fractionation observed for ferromanganese nodules and BIF samples (BEARD et al., 1999). In growth experiments with soil bacteria and hornblende, Brantley et al. (2001) showed that relative to the bulk mineral, Fe dissolved from the hornblende was lighter by as much as 0.8 per mil. More recent studies involving anoxygenic Fe(II)-oxidizing photoautotrophic bacteria (CROAL et al., 2004) demonstrated that hydrous ferric oxide metabolic products were isotopically enriched in the heavier isotope relative to aqueous ferrous iron by approximately 1.5±0.2 per mil. Similar effects were shown by WASYLENKI et al. (2007) in growth experiments with the diazotroph, *A. vinelandii*, in which isotopically lighter residual medium was produced as the cells assimilated the heavier Fe isotopes ($\Delta\ ^{56/54}Fe_{cells\text{-}medium} = +1.1$ per mil). Biological

uptake of Fe isotopes into an *A. vinelandii* protein also produced a mass fractionation between the protein and Fe source of 13.2 ε^{57}Fe units (ZHU et al., 2002). However, despite these studies, it is the overriding larger, more variable and comparable fractionation (~ 3.5 per mil) produced by natural materials and nonbiological processes, which have diminished the role of Fe isotopes as a potential biosignature (ANBAR, 2004; ANBAR et al., 2000; BEARD et al., 2003; BULLEN et al., 2001; JOHNSON et al., 2005).

1.3 Summary

Trace metals are vital components of biological systems. Interactions between the biosphere and geosphere have occurred throughout geological time, remnants of which may be imprinted in the rock record. Microbial trace metal utilization, as observed in extant microorganisms, may therefore provide information regarding metabolic processes, environmental conditions and potential biosignatures, in modern settings and allow for extrapolations to be made vis-à-vis the evolution of these factors through time.

The research outlined in this thesis explores these characteristics of microbial trace metal utilization via a series of experimental growth experiments conducted with pure cultures of laboratory-grown microorganisms. In Chapter 2, the trace metal contents of three microorganisms, *Methanococcus jannaschii*, *Pyrococcus furiosus* and *Escherichia coli*, were determined in order to characterize their metallome content and to establish if the cellular metallomes contain potential metal biosignatures. The results from three physical lysis techniques and whole cell digests were consistent. Metallome patterns were observed for all cells but was most distinct for the hyperthermophilic methanogen, *M. jannaschii*. A potential biosignature is also suggested for this microorganism based on the elevated cellular

concentrations of the trace metals Ni, Co and possibly W. These metals are also utilized in unique metabolisms specific to *M. jannaschii* and as such, may indicate an ancient origin of its metabolism as well as for methanogens.

The capability of many microorganisms to physically or geochemically alter the mineral substrates with which they interact was investigated in Chapter 3. Microorganisms can scavenge bioessential trace metals from the environment through the production of ligands, such as siderophores, especially during times of nutrient limitation. However, with the exception of siderophores, the nature and identity of other metal ligands have yet to be determined. To investigate whether hyperthermophiles produce ligands under metal-limiting conditions, biotic and abiotic supernatant experiments were conducted with a suite of hyperthermophilic Archaea in the presence of powdered basalt. Tungsten (W) was targeted as the metal of interest as it has been shown to be an important bioessential nutrient required for the growth of many hyperthermophiles. Bulk results from all experiments suggest hyperthermophiles do not produce W ligands. However, due to issues regarding the reproducibility of experiments, the role of W as a possible hyperthermophile biosignature remains unresolved but the research is still relevant in regards to the function of specific trace metals and the nature and evolution of primitive organisms on the early Earth.

In Chapter 4, I present the results from novel Ni stable isotopic investigations of geological materials and from biological growth experiments. Protocols were developed and established for mass spectrometry, for dealing with hyperthermophilic growth processes and for separating and purifying Ni from complex media. The results are remarkable. Geological materials vary within a small range of Ni isotopic composition: samples representing the bulk solid Earth have variations in the range -0.04 to +0.34 per mil while meteorites display values of +0.19 to +0.36

per mil. Relative to the starting composition of the growth media, methanogens impart a distinct and significant mass-dependent fractionation on Ni isotopes, preferentially sequestering the lighter isotopes which results in an isotopic fractionation on the order of ~ 0.8 - 1.0 per mil. Non-methanogenic cells also produced a small fractionation (average of 0.07 per mil). The results suggest Ni isotopes are probably fractionated by biology, in general but not as significantly as the fractionation produced by methanogens. Additionally, the data suggests different groups of methanogens may fractionate Ni to varying degrees, with the largest fractionation being produced by the hyperthermophiles. Ni isotopes may be useful, not only as a general methanogenic biomarker but also as a marker for distinguishing between diverse metabolic groups. Compared to other transition metal isotope systems, Ni stable isotopes may well prove to be the first true unambiguous trace metal biomarker and as such, may have important implications in the fields of transition metal isotopes, geomicrobiology and astrobiology.

Lastly, the research presented in this thesis is collaborative but represents my own independent and original efforts. Chapter 3 was originally conceived by C. H. House and S. L. Brantley and Z. Zhang collected the initial data set however, I implemented and collected the second half of the data for this chapter. Chapters 2 and 4 were conceived by me and I was responsible for the majority of the data collection. I was also responsible for most of the data analyses and the writing of all chapters. All work for Chapter 4 was carried out at the University of Bristol, UK and under the guidance of D. Vance. Chapter 4 will be edited and submitted for review as a multi-authored work to *Science* (Cameron, Vance, Archer & House; Ni Stable Isotopes: A Novel Isotope Biomarker).

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Chapter 2

Metallome Biosignatures of Hyperthermophilic Archaea

2.1 Abstract

The trace metal contents of three microorganisms, the hyperthermophilic Archaea *Methanococcus jannaschii* and *Pyrococcus furiosus* and the mesophilic Bacterium *Escherichia coli*, were grown under conditions of varying metal concentrations in order to characterize the metallome content and hence, metal utilization of the cells as well as to determine if the cellular metallomes contain potential microbial metal biosignatures. Metal concentrations were analyzed from whole cell digests and cellular lysates evolved from three physical cell lysis techniques: ultrasonication, freeze-thaw and bead beating. The results show consistency in all of the methods employed as evident by trends in the similarity of metal concentrations and order of importance/utilization. Metallome patterns for the microorganisms were observed but was especially distinctive for the hyperthermophilic methanogen, *M. jannaschii*. A potential biosignature is also suggested for this microorganism based on the elevated cellular concentrations of the trace metals Ni, Co and possibly W. The pattern of metal utilization observed for *E. coli* is more variable and the metallome of *P. furiosus* is closer to that of *M. jannaschii* but displays some similarities in metal usage with *E.coli* and therefore, conveys a less distinctive biosignature than the methanogen. The bioavailability of trace metals more than likely has varied through time. Evolving microorganisms may have taken advantage of such geochemical changes by adapting and incorporating more available metals especially for improved metabolic and/or cellular functions or as a means of survival, which could have

facilitated their expansion into new environments and habitats. Therefore, in addition to providing specific metal biosignatures, microbial utilization of certain trace metals may give information regarding the antiquity and evolution of microbial groups as well as their metabolisms.

2.2 Introduction

Trace metals are vital components of all living systems and are required for a host of metabolic and structural functions. Primary amongst these is a catalytic role in a vast array of cellular reactions including energy generation. Apart from the eleven elements required for all biology, there are approximately ten bioessential transition metals that include Fe, Mn, Co, Mo, Zn, Ni, Cr, Cu, V and W. Some metals such as Fe and Zn have a universal utility in most organisms while others are restricted to particular groups and/or perform specific functions in certain types of organisms (FRAUSTO DA SILVA and WILLIAMS, 2001). As shown in Figure 2.1 (WILLIAMS and DA SILVA, 2003), the availability of inorganic elements and especially trace metals almost certainly have varied through time. These variations were facilitated by changing environmental conditions on the Earth and most likely directly impacted the evolution of life, evidence for which can be seen in metal utilization amongst specific extant groups of organisms. For instance, Ni and W are fundamental requirements for the growth of primarily anaerobic archaeal methanogens and hyperthermophiles, respectively, where they operate in metabolisms that are generally thought to be quite primitive (KLETZIN and ADAMS, 1996; FRAUSTO DA SILVA and WILLIAMS, 2001). On the other hand, the present-day widespread use of Mo in bacteria and eukarya for nitrogen fixation and nitrate reduction probably occurred after the development of an

oxygenated Earth at ~ 2.2 Ga, a situation that allowed soluble Mo oxides to become a more biologically available species (CANFIELD, 1998; STIEFEL, 2002; WILLIAMS and DA SILVA, 2002).

Hyperthermophiles are prokaryotic microorganisms that grow optimally at temperatures > 80 ºC. They are found predominantly within the Archaeal domain but also within the Bacteria. There are two primary Archaeal groups: the largely hyperthermophilic Crenarchaeota and the methanogen-containing Euryarchaeota. Most hyperthermophiles occupy a place in all of the deepest and shortest lineages in both domains (STETTER, 1996), are strict anaerobes and encompass a range of chemolithotrophic (autotrophs utilizing CO_2 as a carbon source) and chemoorganotrophic (heterotrophs that use organic compounds for carbon) metabolisms (MADIGAN et al., 2000). Hyperthermophiles are found in unique and specific high temperature environments such as at deep ocean hydrothermal vents or geothermal terrestrial sites (e.g. hot springs) (STETTER, 1996; STETTER et al., 1990). These natural environments typically contain relatively high, and in some cases, toxic amounts of metals that paradoxically promote and sustain the growth of the microorganisms which are present. For example, the concentration of Fe in seawater is ~ < 1 nM but can commonly exceed values of 10 mM in hydrothermal vent fluids (KELLEY et al., 2002; VONDAMM et al., 1985a; VONDAMM et al., 1985b). However, all organisms including hyperthermophiles have specific strategies and evolved mechanisms for coping with environmental stressors such as the lack or overabundance of chemical elements that can involve for instance, the production of metal chelators which function to both secure metals for the cell or conversely, bind and remove harmful species intracellularly or extracellularly (for e.g. BRULAND, 1989; EDGCOMB et al., 2004; HOLDEN and ADAMS, 2003; NEILANDS, 1995; SANDER et al., 2007; WILLIAMS, 2001).

Microbial studies investigating trace metals are usually carried out in relation to determining some sort of specific information concerning for example, cellular components like enzymes (ERMLER, 2005; KLETZIN and ADAMS, 1996), particular metabolic functions such as methanogenesis (DIEKERT et al., 1981; ZHANG et al., 2003) or for other cellular processes, for instance optimizing growth yields or determining metal concentrations under variable environmental factors (FORTIN et al., 1994; OUTTEN and O'HALLORAN, 2001; SCHONHEIT et al., 1979). Many of these studies have focused on individual species or type microorganisms like *E. coli* for which there would already be other relevant information. Element data especially for trace metals is lacking for most hyperthermophiles. Additionally, as a result of explicit metal utilization in unique metabolisms, the potential exists for finding metal biosignatures in hyperthermophilic systems. To this end, lysis experiments were conducted in order to determine the trace metal content within hyperthermophilic cells, to investigate the cell's uptake of metals under variable metal concentrations and to establish whether certain physical lysing methods differ in their ability to break open hyperthermophilic cells. As a baseline and in order to make comparisons and inferences, element contents were also determined for *E. coli*.

2.3 Materials and Methods

Microorganisms

Three microorganisms were used in this study: *Escherichia coli*, *Pyrococcus furiosus* and the hyperthermophilic methanogen, *Methanococcus jannaschii*. The latter two are strict anaerobes; *E. coli* is a facultative aerobe and was grown aerobically and anaerobically. The optimum growth temperatures for these organisms are: *E. coli*, 37 ºC; *P. furiosus*, 100-113 ºC; and *M. jannaschii*, 85 ºC. Organisms were originally obtained from the Deutsche Sammlung von

Mikroorganismen und Zellkulturen (DSMZ) and are maintained in the laboratory of C. H. House (Penn State University, University Park, PA.). *M. jannaschii* was recently reclassified to *Methanocaldococcus jannaschii* (WHITMAN, 2002).

Growth Media

Labware used in media preparation and sample collection was cleaned by soaking in 10% HCl followed by thorough rinsing in 18 MΩ water. Reagents were supplied from Fisher or Sigma-Aldrich Chemicals. Savillex PFA labware (Minnetonka, MN, USA) was used to collect and digest all final cell and cell lysate samples.

E. coli (per liter): 10.0 g LB broth, ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA), *5x $Na_2WO_4 \bullet 2H_2O$ solution (0.02 g L^{-1}), *5x mineral solution containing (per liter) 2.0 g $MgSO_4 \bullet 7H_2O$, 0.2 g $MnSO_4 \bullet H_2O$, 0.2 g $FeSO_4 \bullet 7H_2O$, 0.02 g $CoSO_4 \bullet 7H_2O$, 0.1 g $ZnSO_4 \bullet 7H_2O$, 0.02 g $CuSO_4 \bullet 5H_2O$, 0.01 g $Na_2MoO_4 \bullet 2H_2O$, 0.02 g L^{-1} $NiCl_2 \bullet 6H_2O$, 0.01 g L^{-1} $Na_2SeO_3 \bullet 5H_2O$, 0.01 g $VOSO_4$, 0.01 g $CrK(SO4)_2 \bullet 12H_2O$, 0.008 g H_3BO_3; concentrated HCl was used to keep metals in solution (pH ~ 3). This medium was made under aerobic and anaerobic conditions. The LB powder and Chelex was stirred for 1 hr then gravity filtered to remove the resin. Trace metal solutions were added and the medium was brought up to volume and pH (~ 7) adjusted. Aerobic medium (500 ml) was dispensed into 1 liter Erlenmeyer flasks that were capped with silicone plugs. Anaerobic medium was degassed by bubbling with a stream of N_2 for ~ 20 min; pH adjustment (~ 7) with concentrated HCl or NaOH (10%) and dispensation into serum bottles was carried out in an anaerobic chamber. The headspace in the anaerobic bottles was flushed three times with N_2, followed by a final addition of the same gas to 0.5 bar pressure. All media was sterilized by autoclaving before being used for the growth

experiments. * For low metal concentration experiments, 2.0 ml L^{-1} was used; 7.0 ml L^{-1} was used for the high concentration. Also, trace metals are not required for the growth of E. coli but were used for these experiments.

P. furiosus (per liter): 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.3 g $MgCl_2 \cdot 6H_2O$, 20.0 g NaCl, 5.0 g peptone, 1.0 g yeast, *5x mineral solution(s) (see E. coli), ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA). Prior to making up the medium and to remove any metals present in the organic substrates, a solution of peptone, yeast and Chelex was stirred for 1 hr then gravity filtered to remove the resin-bound metals. The medium was made by mixing in solution all reagents and degassed by bubbling with a stream of N_2 for ~ 20 min; pH adjustment (~ 7) with concentrated HCl or NaOH (10%) and dispensation (250 ml) into 0.5 liter serum bottles was carried out in an anaerobic chamber. The headspace in the experiment bottles was flushed three times with N_2, followed by a final addition of the same gas to 2 bars pressure. An autoclave was used for media sterilization.

M. jannaschii (per liter): 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.3 g $MgCl_2 \cdot 6H_2O$, 20.0 g NaCl, 3.0 ml NaOH solution (10%), *5x mineral solution(s) (see E. coli), 0.5 g cysteine•HCl. Medium was made by mixing in solution all reagents except cysteine•HCl and degassed by bubbling with a stream of $H_2 + CO_2$ (80% + 20%) for ~ 20 min. The addition of cysteine•HCl, pH adjustment (~ 7) with concentrated HCl or NaOH (10%) and dispensation (200 or 250 ml) into 1 liter serum bottles was carried out in an anaerobic chamber. The headspace in the experiment bottles was flushed three times with $H_2 + CO_2$, followed by a final addition of the same gas mix to 2 bars pressure. An autoclave was used for media sterilization.

Growth & Lysis Experiments

Stock cultures of all microorganisms, grown in the base media without trace metals or very small amounts carried over during transfer, were established prior to being used as innoculum for the growth experiments. Cells were grown under three separate trace metal conditions: (1) no metals, (2) low metal concentration, (3) high metal concentration (Table 2.1). *M. jannaschii* cannot grow without trace metals so the medium for the 'no-metals' experiment was made with the same concentration of trace metals as the low concentration batch but was filtered (0.1 μm) after autoclaving and before inoculation with the cells. Also, low metal experiments were not performed for *E. coli*. Media samples were taken prior to inoculation at the beginning of growth. All batches were run at the same time for each cell and depending on the microorganism, growth typically proceeded over a period of 2 to 5 days. Following growth, the bulk volume of cells and spent medium was gently mixed and poured into centrifuge tubes. Cell count and supernatant samples were removed before centrifuging (5000 rpm, 1 hr, 10 ºC). Another supernatant sample was removed after centrifuging, the medium was decanted and the cell pellet washed 3x with a NaCl solution. The salt solution used for washing was analogous to the salt concentration in the medium for each cell (*M. jannaschii* & *P. furiosus*, 2.5 %; *E. coli*, 0.5 %). During the wash steps, cells were centrifuged at 8000 rpm for 15 min at 10 ºC. After washing, the bulk cell pellet was slurried in ultrapure water and divided into equal fractions for lysis.

Four lysis techniques were employed: ultrasonication, freeze-thaw, bead beating and whole cell, acid digestion. The conditions for each technique are as follows. A Branson Sonifier 450 (output ~ 3) was used for ultrasonication. The tip was cleaned with ethanol and distilled water before being used. Samples were sonicated for 15 sec followed by a cooling period of 45

sec in an ice water slurry; this was repeated 3x. For the freeze-thaw method, the cell pellet slurry was frozen at –80 ºC overnight, removed and allowed to thaw at room temperature, gently mixed and frozen again; this was repeated 3x. Zirconium oxide beads (low-binding, 200 μm, OPS Diagnostics LLC, NJ) were used in the bead beating technique. The beads were cleaned in 50% HNO_3, rinsed with double distilled water and dried prior to being used. The cell pellet was 'beaten' (setting used was 'homogenize') for 5 min then cooled for 3 min in an ice bath; this was repeated over a period of 20 min. The lysed cells from the above three methods were centrifuged (5000 rpm, 30 min), the metal containing lysates filtered through 0.1 μm Acrodisc syringe filters and collected directly into PFA beakers. The acid digest fraction was centrifuged to remove most of the liquid and the pellet was transferred into PFA beakers and dried (50 ºC) to obtain a dry cell pellet weight.

Medium, supernatant and experimental blank samples were filtered (0.1 μm Acrodisc filters) and acidified with 2.5 % HNO_3 + 0.1 % HF for analysis. The lysates and dried acid digest pellet were taken up in ultrapure concentrated HNO_3, dried down, digested overnight in concentrated HNO_3, dried again and finally taken up in 2.5 % HNO_3 + 0.1 % HF. Indium (In) was used as an internal standard and was added to all samples and standards to a concentration of 20 ppb before analysis. Three sets of matrix-matched standards (2.5, 0.5 & 0 % NaCl) for major elements (cations at high concentration, e.g. Mg) and for trace metals (low element concentration) were made and analyzed to determine element concentrations. Individual cation stocks (High-Purity Standards) and a multi-element trace element stock (PSU-CAL-1, Inorganic Ventures, Inc.) were used to make the standard solutions. High resolution inductively coupled plasma mass spectrometry (ICP-MS - Finnigan Element 1) was used to measure metal concentrations in the samples.

2.4 Results and Discussion

The total growth volumes with resulting cell numbers is shown in Table 2.2. The largest cell pellet representing the greatest number of cells was obtained for the *E. coli* cultures and the smallest for the hyperthermophiles, *P. furiosus* and *M. jannaschii*. As would be expected, cell numbers for all microorganisms were lower for the experiments conducted with no trace metals. As shown in Table 2.1, even though the organic media for both *E. coli* and *P. furiosus* are treated with Chelex to remove metals before being used to make up the media for the growth experiments, there is still sufficient residual amounts of metals remaining to facilitate microbial growth, particularly metals such as Fe and Zn which are probably more abundant in the organic substrates. The effects may be especially true in the case of *E. coli* which does not require the addition of a trace metal solution for growth and obtains all bioessential materials from the organic medium in which it is grown. By comparison, most hyperthermophiles, including the two in this study, are typically grown in an inorganic medium or an organic-supplemented inorganic medium containing sufficient levels of trace metals that are required for optimum growth. The lower cell numbers for these microorganisms in the trace metal limiting experiments may therefore be a direct result of the extremely low trace metal medium in which they were grown. The conditions of growth are also relevant and possibly has an affect on facultative microbes, as shown by the higher cell numbers obtained for *E. coli* under aerobic versus anaerobic growth.

Results from all experiments are presented in Figures 2.2 to 2.5. Whole cell trace metal concentrations representing the base metallome content of the cells are shown for the acid digest fractions of the experiments conducted without trace metals in Figure 2.2. Some trends are immediately observed. Not surprising, Fe was the most abundant metal in all cells however there

is a marked contrast in the other metal contents between cells particularly for the methanogen *M. jannaschii*. Excluding Fe and Mn which for unknown reasons could not be measured for this fraction, the order of metal abundance is Ni > Co > Zn > W > Cu > Mo for *M. jannaschii* while that of *E. coli* is almost the reverse; Zn > Cu > Mo > Ni > W > Co. The metal contents measured for *P. furiosus* lies between the trends observed for the other two microorganisms: Zn > Ni > W > Cu > Co > Mo. The trend observed for *E. coli* grown under the presence or absence of oxygen was essentially the same; the only variations noted are a slightly higher metal concentration, particularly for Co, in the anaerobically grown cells. Generally, hyperthermophile metal concentrations are to a small degree, enhanced over *E. coli*.

Metal concentrations measured for the three physical lysis techniques (ultrasonication, freeze-thaw, bead beating) under each of the three trace metal conditions (Figures 2.3, 2.4 & 2.5) for all cells show small and generally, insignificant variations. The lysis methods were all effective in breaking open cells. Deviations in any of the methods would have been evident in the behavior of the metals and this was only seen primarily for Cu. A few differences are noted in the hierarchy of metals for the lysis techniques when compared to the acid digest results but the trends are similar. Variations are most likely attributable to differences imparted by the lysis methods as well as to growth under diverse metal conditions. This is demonstrated particularly by *E. coli* and the change in abundance of Mn which under metal-limited conditions, is moderate but becomes the most abundant, surpassing Fe, when the cell is exposed to high levels of trace metals, either aerobically or anaerobically. This microbe also displayed some other subtle metal variations. Disregarding Fe and Mn, the trace metal trends for the no-metal (Figure 2.3) and high-metal (Figure 2.5) experiments for aerobic and anaerobic growths, respectively, are identical. Also, Ni concentrations increased in the no-metal, anaerobic cells yet remained

relatively low under all other growth conditions. Concentrations of metals in the lysis experiments were in the range $1x10^{-12}$ to $1x10^{-17}$ μ g/cell. Based on these observed concentrations, the metals in order of importance to *E. coli* regardless of oxic conditions are Fe > Zn > Mn >/= Cu > Mo > Ni > W > Co.

Metal trends were more consistent amongst the lysis and trace metal experiments for the hyperthermophiles. *M. jannaschii* displayed the most unambiguous results, showing a clear preference for metals in the order Fe > Ni > Zn=Co > W >/= Mo > Cu. Manganese had the least importance under low metal conditions (Figure 2.4) but attained more moderate concentrations under high metal concentrations (Figure 2.5). Once again, *P. furiosus* had metal preferences in common with both of the other microorganisms though these were more distinct than of *E. coli*. The metals of particular importance to *P. furiosus* seemed to be Fe > Zn > W >/= Ni, Cu > Mo > Co. As observed with *M. jannaschii* but in an opposite trend, Mn concentrations varied under differing metal conditions, with the lowest concentrations occurring under higher metal conditions. Also for *P. furiosus*, Ni potentially has an important role and may be expressed in certain enzymes during periods of metal limitation as suggested by the obvious increase in concentration in only the acid digest (Figure 2.2) and lysis fractions of the no-metal experiment. Trace metal concentrations for all hyperthermophile experiments were in the range ~ $1x10^{-11}$ to $1x10^{-15}$ μg/cell.

In addition to the metal preferences and trends observed for the individual microorganisms, the data also indicate that metallome concentrations are conserved and in general, are not particularly affected by fluctuations in trace metal availability. Studies such as Zhang et al. (2003) have measured specific trace metal concentrations in methanogens with the aim of improving cell yields for methane production through the provision of optimal metal

conditions. Others (EDGCOMB et al., 2004) have shown the effective capabilities of high temperature vent microorganisms such as *M. jannaschii* and *Pyrococcus* to utilize metal complexation processes to reduce the effects of highly toxic levels of metals to the cells. Regardless of the extremes of these conditions, studies such as WILLIAMS (2002, 2007) and WILLIAMS and DA SILVA (2003) have shown that the cellular free metal ion concentration of bioessential elements are controlled, at values in consort with the ocean and effectively maintained in the cytoplasm at very low levels. Additionally, these constraints have been in place since the metallome system in primitive cells evolved with cells responding to the uptake and utilization of different and specific trace metals as (1) microorganisms evolved and (2) in response to the changing environment of metal availability and concentrations. For instance, a constant Fe^{2+} concentration of 10^{-7} M is required by modern organisms and is believed to have been required throughout life's evolution even when conditions on the Earth changed and the atmosphere became oxygenated. This concentration would have been established early in life's evolution as a result of factors such as the abundant Fe concentration present at the time as well as the binding constants for the metal established internally within the cell and externally in the ocean (SAITO et al., 2003; WILLIAMS, 2002; WILLIAMS and DA SILVA, 2003). Other elements such as Zn and Cu previously sequestered in the pre-oxygen sulfidic ocean as inorganic sulfide precipitates became available after oxygenation at much higher toxic concentrations and early microorganisms adapted by constraining the cytoplasmic levels of these metals (10^{-10} M and 10^{-15} M for Zn and Cu, respectively) (WILLIAMS, 2002).

The changes occurring in Earth's early environment from reducing to oxidizing states also allowed for adaptations to new uses of previously unavailable metals, either in the capacity of coping with the changing environment and/or to optimizing metabolic functions previously

met by other trace metals which could have facilitated the organism's evolution and radiation into other habitats. Mo and W are two metals that particularly exemplify these ideas. Hot environments like deep ocean hydrothermal vents are natural habitats for hyperthermophilic microorganisms. These environments, thought to be a possible cradle of life (NISBET and SLEEP, 2001; RUSSELL and MARTIN, 2004; WACHTERSHAUSER, 1993) are characterized by reducing conditions and abundant trace metals such as W (ARNORSSON and OSKARSSON, 2007; KELLEY et al., 2002; KISHIDA et al., 2004). The behavior of Mo and W in the environment are dissimilar due to the redox chemistry of both metals; for example, under sulfidic conditions, MoS precipitates while WS remains soluble but under oxidizing conditions, the reverse takes place (IVANOVA, 1986). Due to these properties, as well as its known utilization in anaerobic carbon metabolism and probably, high temperature enzyme stability, it has been postulated (e.g. KLETZIN and ADAMS, 1996) that W may have been biologically available and thus had an important role for primitive prokaryotes on the pre-oxygenated Earth. Mo would have been unavailable in the oceans and would only have achieved its modern-day biological status and abundance after oxic conditions reigned. In modern environments, Mo is a key metal required for nitrogen, sulfur and carbon metabolism, amongst others. The Mo-containing nitrogenase is the most important of the three enzymes known to carry out nitrogen fixation; the other two (containing V and Fe) are less-efficient, not expressed when Mo is abundant and, are not used exclusively by any known organism (STIEFEL, 2002). Additionally, several molybdo- and tungsto- enzymes and isoenzymes exist however, 'true' tungstoenzymes which can only utilize W have only been found in hyperthermophilic Archaea while analogous Mo enzymes are found in microorganisms like *E. coli* and in such cells, W substitution renders the enzymes inactive (KLETZIN and ADAMS, 1996). Relative to concentration, the results of this work shows W to be

of some importance over Mo to the hyperthermophiles and Mo to *E. coli*. Even though the dataset is small, taken together with the studies cited above, the order of importance of trace metals indicated by their relative concentrations and hence, utilization in the cells could be used to infer and provide support for the evolution of these microorganisms.

Of the three microorganisms, the most promising biosignature may be the metallome trend observed for the hyperthermophile, *M. jannaschii*. As shown in Figure 2.6, the high percentage of Ni, Co and possibly W is distinctive for this methanogen when compared to the metallome percentages and trends of the other cells. Similar results were shown in the work of ZERKLE et al. (2005). Nickel is required by all methanogens specifically for carrying out methanogenesis. To date, at least three of the seven known Ni enzymes are found in methanogens and two are absolutely critical. The Ni-containing cofactor, F_{430} of MCR (methyl-coenzyme M reductase) catalyzes the terminal step in methanogenesis, the reduction of a methyl group to methane while the CODH/ACS (carbon monoxide dehydrogenase/acetyl coenzyme A synthase) catalyzes the oxidation of CO to CO_2 as well as the formation or degradation of acetyl-CoA (FERRY, 1993). The cofactor F_{430} is unique to methanogens and has not been found in any other organism. Interestingly, hyperthermophilic methanogens also contain another exclusive enzyme, the W-containing FMDH (formylmethanofuran dehydrogenase) which catalyzes the initial conversion of CO_2 at the beginning of methanogenesis. Mo-containing FMDHs are also found in some mesophilic to thermophilic methanogens (KLETZIN and ADAMS, 1996). Cobalt is another trace metal that has importance in metabolisms that have a probable primitive origin. The metal is found in the vitamin B12 cobalamin cofactor, in the same enzyme family as the Ni-containing F_{430} and is utilized primarily in anaerobic systems for metabolizing H_2, CO and $-CH_3$ groups (FRAUSTO DA SILVA and WILLIAMS, 2001). Not surprising then that it displays such an

enhanced concentration in *M. jannaschii*. There are two complex pathways for the *de novo* synthesis of cobalamin: the anaerobic route which does not require oxygen and the aerobic route in which molecular oxygen is required. These systems are only utilized by a few groups of prokaryotes and are not known to be present in eukaryotes (FRANK et al., 2005; HELDT et al., 2005). In fact even microorganisms like *E. coli* only induce the synthesis of cobalamin as well as Ni uptake under anaerobic conditions (FRAUSTO DA SILVA and WILLIAMS, 2001), evidence for which is clearly found in this work (see Figures 2.2, 2.3, 2.5 and 2.6).

2.5 Conclusion

The trace metal contents of three distinctive microorganisms grown under conditions of varying metal concentrations were determined, with the aim of characterizing the metallome content and hence, metal utilization of the cells as well as trying to determine if the cellular metallomes contain potential microbial metal biosignatures. Metal concentrations were analyzed from whole cell digests and cellular lysates evolved from three physical cell lysis techniques applied to hyperthermophilic Archaea and the mesophilic bacterium, *E. coli*, which was grown under aerobic and anaerobic conditions. The methods employed were all consistent as is evident by trends in the similarity of metal concentrations and order of importance/utilization. The results show a conspicuous metallome pattern for the microorganisms but particularly for the hyperthermophilic methanogen, *M. jannaschii*. A potential biosignature is also suggested for this microorganism based on its unique metabolism that most likely relies to a great extent on the presence and availability of the trace metals Ni, Co and possibly W. The pattern of metal utilization observed for *E. coli* is more variable and may be a consequence of particular enzyme expression under different growth conditions. The metallome of the heterotrophic

hyperthermophile *P. furiosus* is closer to that of *M. jannaschii* but displays some similarities in metal usage with *E.coli* and therefore, conveys a less distinctive biosignature than the methanogen. The bioavailability of trace metals more than likely have varied through time yet the microbial free ion metallome concentrations observed in modern extant cells may have been set at the time and in the environment, of the primitive ocean. Evolving microorganisms may have taken advantage of such geochemical changes by adapting and incorporating more available metals especially for improved metabolic and/or cellular functions or as a means of survival which could have facilitated their expansion into new environments and habitats. Therefore, in addition to providing specific metal biosignatures, microbial utilization of certain trace metals may give information regarding the antiquity and evolution of microbial groups as well as their metabolisms.

2.6 References

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2.7 Figures and Tables

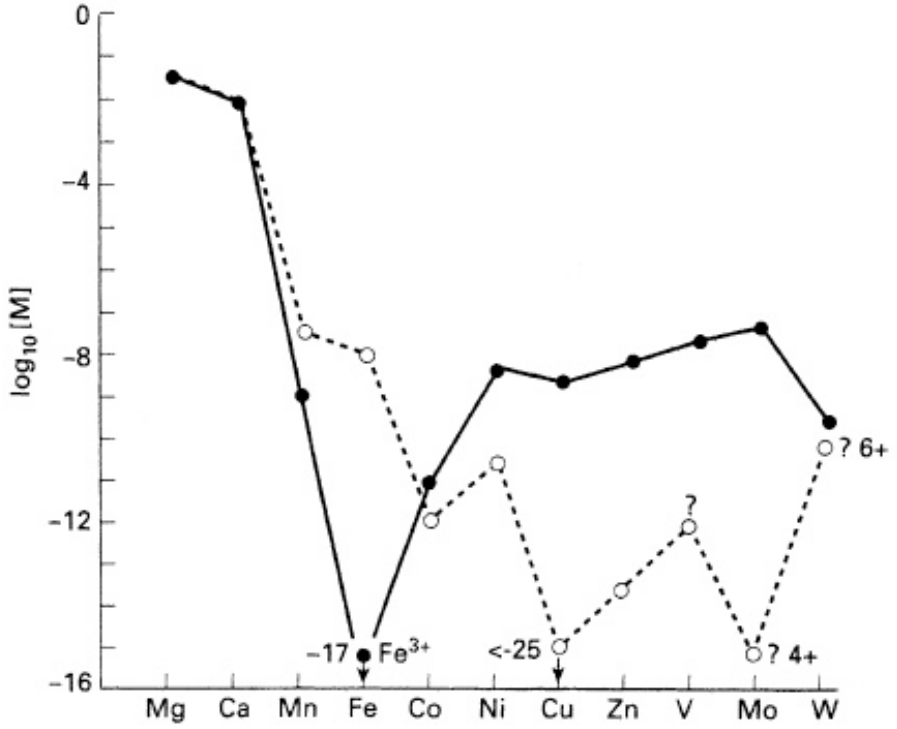

Figure 2.1: Trace metal composition of the modern ocean (solid black line) compared with that of the probable primitive sea ~ 4.5 Ga (broken line). The abundance of trace metals may have varied through time due to prevailing redox conditions, which could have impacted the availability of bioessential metals to the microorganisms that were present. The values reported for the primitive ocean are based on the assumption of a reducing sulfide system. From Williams and Frausto da Silva (2003).

Table 2.1: Experimental trace metal conditions (L, H) and metal concentration in the various microbial media (see Methods) after treatment with Chelex. The organic substrates used in the media for *E. coli* and *P. furiosus* were treated with the metal-binding resin, Chelex, to remove any metals that are present prior to making up the media with a known quantity of trace metals. Values reported for L (low trace metal condition) and H (high trace metal condition) are the concentrations of each metal added to the respective metal experiments and under which the cells were grown. The metal concentrations listed for *E. coli* and *P. furiosus* are the concentrations still present in the no-metal experimental condition *after* treatment with Chelex. The methanogen, *M. jannaschii* is unable to grow without trace metals. Therefore, its medium was made up with the same concentration of metals as L and then filtered *after* autoclaving, the values for which are reported in the last column. The filtered *M. jannaschii* medium represents the no-metals condition for this cell.

Metal	**L (ppm)**	**H (ppm)**	***E. coli* (ppm)**	***P. furiosus* (ppm)**	***M. jannaschii* (ppm)**
Mg	20.06	70.22	8.5500	32.7900	37.3200
Mn	2.01	7.02	0.0080	0.0020	1.2300
Fe	2.10	7.34	0.2600	0.0250	0.6140
Co	0.21	0.74	0.0040	0.0020	0.0800
Zn	1.05	3.68	0.4900	0.3500	BD
Cu	0.24	0.84	0.0060	0.0002	BD
Mo	0.15	0.52	0.0050	0.0100	0.0700
Ni	0.22	0.77	0.0040	0.0030	0.0900
Se	0.13	0.44	-	-	-
V	0.13	0.44	0.0030	0.0020	0.0400
Cr	0.11	0.38	0.0060	0.0070	0.0200
W	0.23	0.79	0.0009	0.0003	0.0009

Table 2.2: Growth volumes, associated cell count numbers and dry pellet weight. *E. coli* aerobic [Ec (a)]; *E. coli* anaerobic [Ec (an)]; *P. furiosus* [Pf]; *M. jannaschii* [Mj]. Experiment trace metal conditions: N=no trace metals; L=low metal conc; H=high metal conc; F=filtered (only for *M. jannaschii*; see Methods).

Microorganism Condition	Growth vol (ml)	Cell count (cells/ml)		Dry cell pellet weight (g)
		bef. washing	aft. washing	
Ec (a) - N	1000	3.20E+08	1.09E+11	0.4392
Ec (a) - H	1000	7.87E+08	1.05E+11	-
Ec (an) - N	1000	9.60E+07	7.52E+09	0.0792
Ec (an) - H	1000	1.24E+08	7.62E+09	-
Pf - N	1000	3.44E+07	2.31E+09	0.0296
Pf - L	1000	1.47E+08	7.93E+09	-
Pf - H	1000	6.80E+07	5.68E+09	-
Mj - F	500	3.60E+07	3.92E+09	0.0428
Mj - L	1000	2.36E+07	6.16E+08	-
Mj - H	1000	6.01E+07	2.91E+09	-

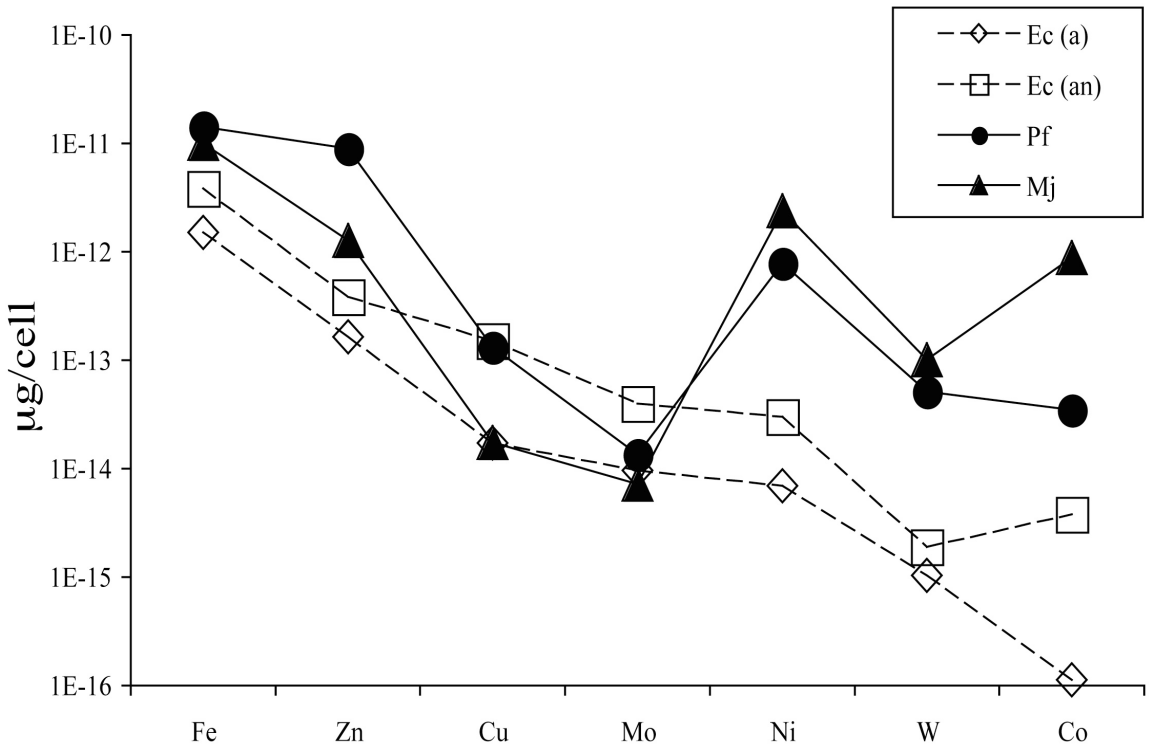

Figure 2.2: Results for all microorganisms from the whole cell acid digest fraction of the no-trace metal experiment. Trace metals on the x-axis are plotted against the metal amounts per cell on the y-axis. The metals are placed in order of decreasing concentration measured for *E. coli*. Metal contents for the other two cells are plotted relative to the order of the *E. coli* values. *E. coli* aerobic [Ec (a)]; *E. coli* anaerobic [Ec (an)]; *P. furiosus* [Pf]; methanogen, *M. jannaschii* [Mj]. Concentration is in logarithmic values.

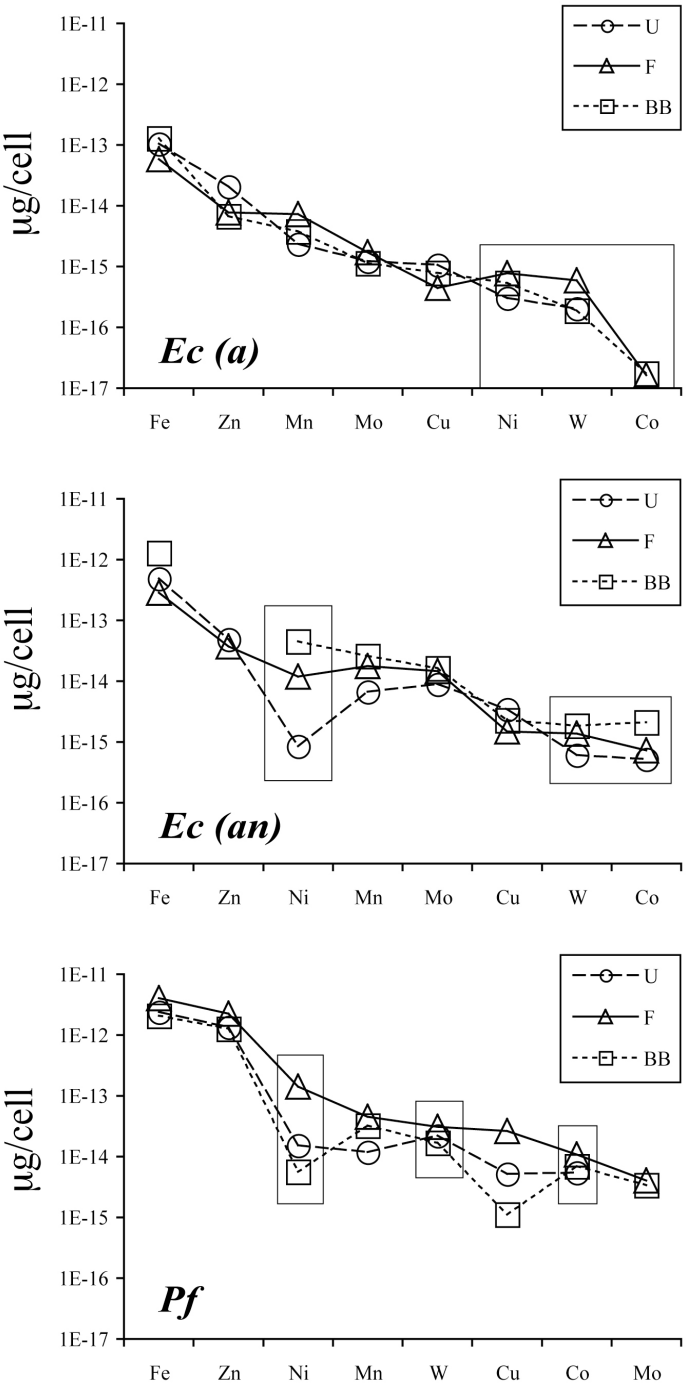

Figure 2.3: Results from the no-trace metal lysis experiments. Metals are placed in order of decreasing concentrations in the cell for each of the lysis techniques. The three metals of importance (Ni, Co, and W) noted for the methanogen, *M. jannaschii* in Figure 2.2 are outlined in boxes. *E. coli* aerobic [Ec (a)]; *E. coli* anaerobic [Ec (an)]; *P. furiosus* [Pf]. Lysis methods are: U=ultrasonication; F=freeze-thaw; BB=bead beating. Concentration is in logarithmic values.

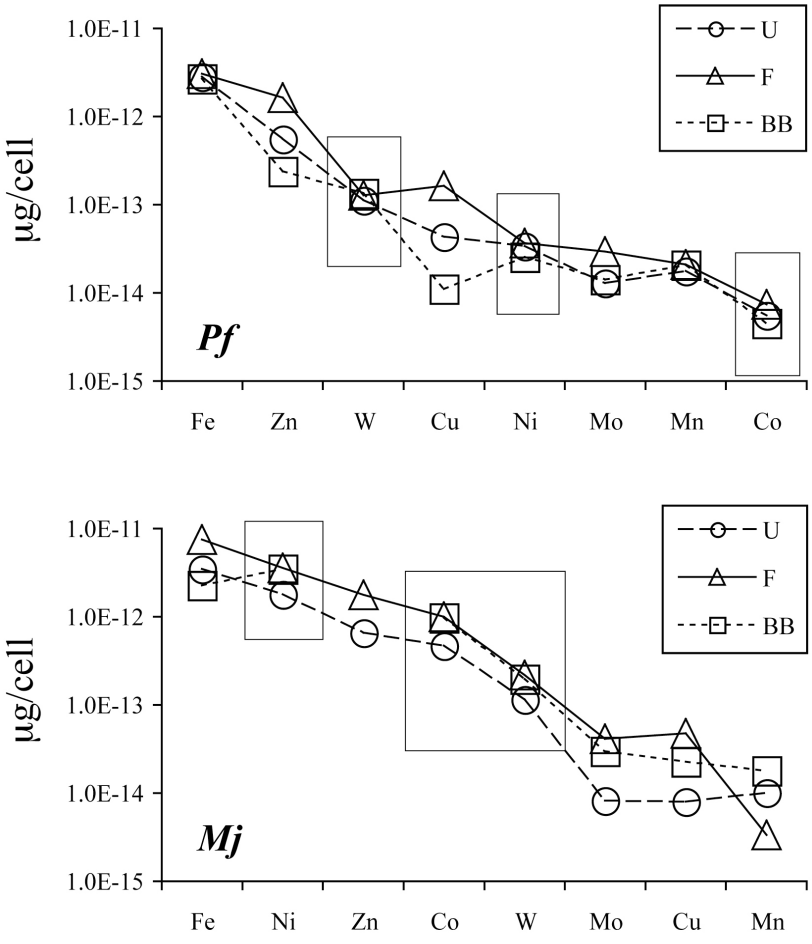

Figure 2.4: Results from the low metal concentration (L) lysis experiments. Metals are placed in order of decreasing concentrations in the cell for each of the lysis techniques. The three metals of importance (Ni, Co, and W) noted for the methanogen, *M. jannaschii* in Figure 2.2 are outlined in boxes. *P. furiosus* [Pf]; *M. jannaschii* [Mj]. Lysis methods are: U=ultrasonication; F=freeze-thaw; BB=bead beating. Concentration is in logarithmic values.

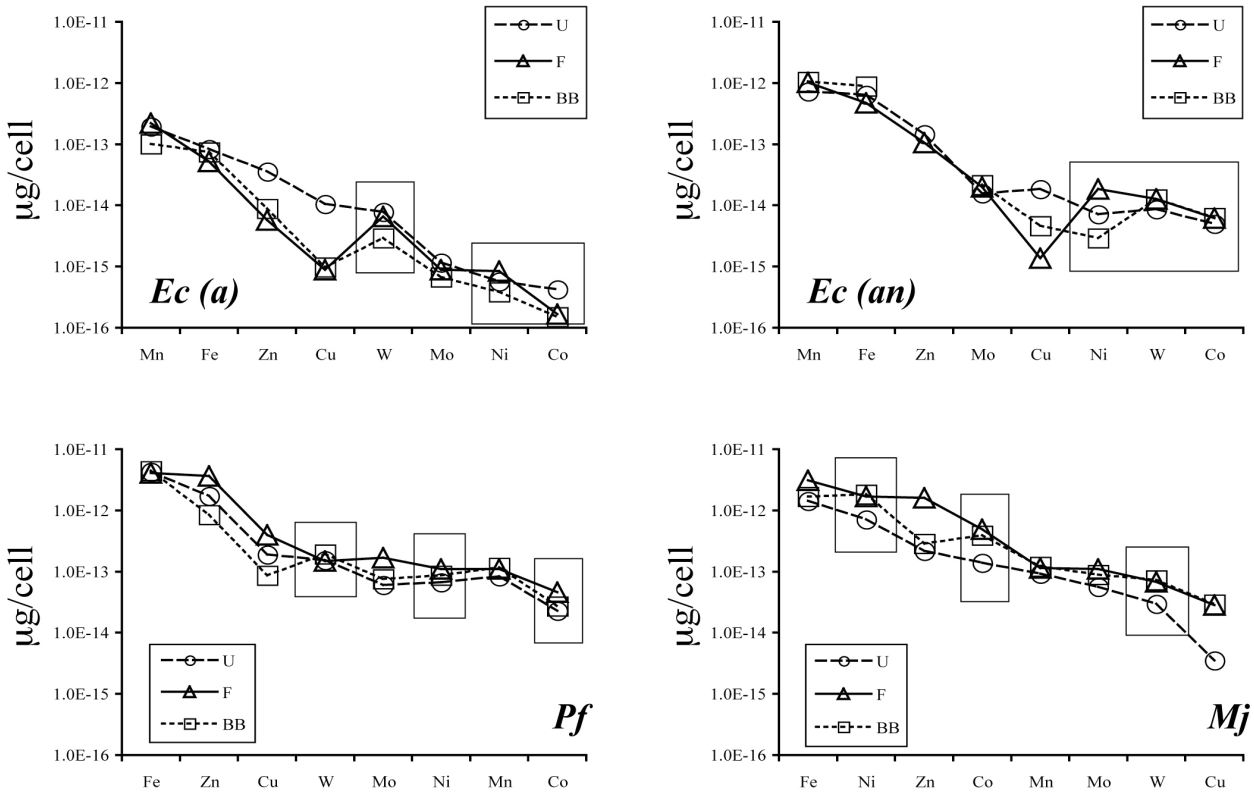

Figure 2.5: Results from the high metal concentration (H) lysis experiments. Metals are placed in order of decreasing concentrations in the cell for each of the lysis techniques. The three metals of importance (Ni, Co, and W) noted for the methanogen, *M. jannaschii* in Figure 2.2 are outlined in boxes. *E. coli* aerobic [Ec (a)]; *E. coli* anaerobic [Ec (an)]; *P. furiosus* [Pf]; *M. jannaschii* [Mj]. Lysis methods are: U=ultrasonication; F=freeze-thaw; BB=bead beating. Concentration is in logarithmic values.

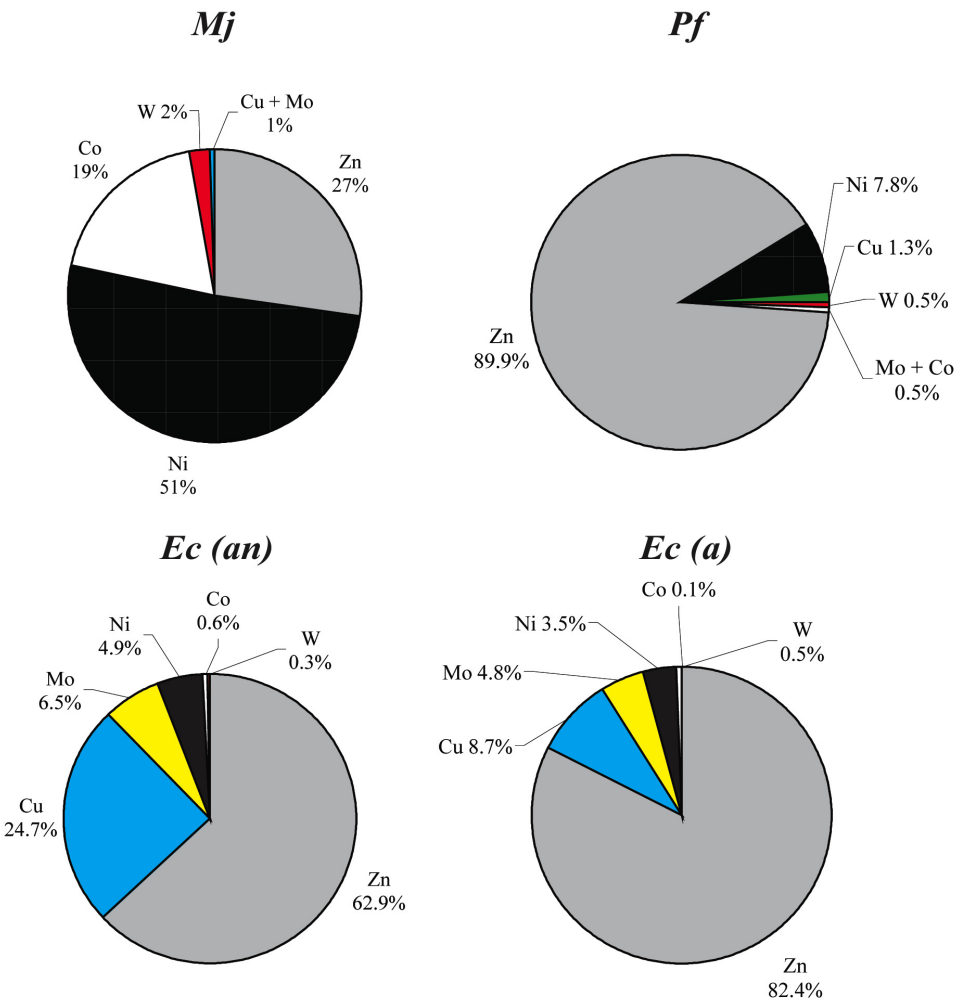

Figure 2.6: Pie charts depicting relative abundance of all metals except Fe, for all microorganisms based on the whole cell acid digest fraction of the no-trace metal experiment. Note that Fe is not included as it is the most abundant metal for all cells. The trace metal pattern for the methanogen *M. jannaschii* is distinct when compared to the other two cells and may be useful as a potential biosignature. *M. jannaschii* [Mj]; *P. furiosus* [Pf]; *E. coli* anaerobic [Ec (an)]; *E. coli* aerobic [Ec (a)].

Chapter 3

A Search for Hydrothermal Tungsten Ligands

3.1 Abstract

Trace metals are required for cellular processes by all hyperthermophilic microorganisms. Recent studies suggest microorganisms produce ligands, such as siderophores, to scavenge metals from the environment especially during times of nutrient limitation. However, with the exception of siderophores, the nature and identity of most metal ligands have yet to be determined. Hydrothermal fluids contain large quantities of trace metals compared to seawater. While hyperthermophilic microorganisms will scavenge metals from hydrothermal fluids, it is not known if they can preferentially leach trace metals from basalts and minerals by production of a metal-specific ligand. To investigate whether hyperthermophiles produce ligands under metal-limiting conditions, biotic and abiotic supernatant experiments were conducted with a suite of hyperthermophilic Archaea in the presence of powdered basalt. Tungsten (W) was targeted as the metal of interest as it has been shown to be an important bioessential nutrient required for the growth of many hyperthermophiles. Bulk results from all experiments suggests hyperthermophiles do not produce W ligands. Given the reproducibility of the experiments, evidence for the production of a W ligand was not observed even though positive indications for possible ligands were demonstrated in biogenic experiments for two microorganisms. Additionally, hydrothermal fluids and associated water-rock reactions are probably sufficient supplies of essential micronutrients for the microbes inhabiting hydrothermal environments. The role of W as a possible hyperthermophile biosignature remains unresolved but the research is still

relevant in regards to the function of specific trace metals and the nature and evolution of primitive organisms on the early Earth.

3.2 Introduction

Microorganisms produce and secrete specific ligands for the acquision and uptake of bioessential trace metals. Leaching of trace metals from geological materials by an assortment of microbes has been shown to be an important biogeochemical process and is due in part to the relatively low trace metal concentrations typically found in many environments. For example, the most commonly known ligands are siderophores which are specialized ferric iron chelating agents produced by aerobic bacteria and fungi during periods of low iron availability (LIERMANN et al., 2000; NEILANDS, 1995). Other researchers (BRULAND, 1989; BRULAND, 1992; CROOT et al., 2003; SAITO et al., 2002) have demonstrated the existence of possible Co, Cu, Cd and Zn-specific ligands in seawater but the source (biologically mediated or natural organic complexation) and nature of these ligands have not been determined. Laboratory experiments with microorganisms in pure culture have also demonstrated the existence of a possible molybdophore secreted by N_2-fixing soil bacterium (LIERMANN et al., 2005). In addition, with the exception of siderophores, specific ligands for other trace metals have yet to be *identified*.

To date, very few studies (SANDER et al., 2007) have searched for probable bio-organic ligands in hydrothermal environments such as those found at deep-marine vent sites. With the exception of molybdenum, anaerobic hydrothermal fluids generally contain significantly greater concentrations of trace metals compared to seawater (Table 3.1). If required, hyperthermophilic microorganisms will transport metals from hydrothermal fluids into their cells but it is not known if they leach trace metals from basaltic rock. Furthermore, if this scavenging process takes place,

it is unknown if metal-specific ligands would be produced to facilitate metal extraction. As shown in Table 3.1, microorganisms use a variety of transition metals as micronutrients. Some such as Cu, Mo and Fe are well known and have important biological roles, primarily in enzymatic functions (MADIGAN et al., 2000). The requirement of others such as Cr, Se and W (tungsten) is less well characterized.

To further investigate the question of microbial metal leaching, particularly in high temperature environments, a series of laboratory growth and rock leaching experiments involving hyperthermophiles was conducted. In this work, the trace metal of interest is tungsten as it is a necessary nutrient for most hyperthermophiles in hydrothermal settings and is becoming increasingly recognized as having an important functional role in biological systems.

Tungsten is a lithophile element with atomic number 74, an atomic mass of 184 and the highest known melting point of all metals (6192 ºF). It is also possibly the heaviest element known to have biological relevance. From a geochemical and biological perspective, W is commonly compared to Mo as both elements share similarities in regards to their chemical properties, behavior and interactions (STIEFEL, 2002). However, the utilization and biological preference for W over Mo is unique and is a characteristic restricted primarily to prokaryotic microorganisms (Archaea and some Bacteria). Tungsten is scarce in nature and is typically found as oxo-rich tungstate minerals [W(VI)] such as scheelite ($CaWO_4$) or wolframite ($[Fe/Mn]WO_4$). Concentrations of tungsten in freshwaters range from < 0.5 to 20 nM (IVANOVA, 1986; KRAUSKOPF, 1972). Deposits rich in W are usually formed in felsic crust by hydrothermal mineralization, metamorphic processes and at the discharge site of thermal waters. Sulfur-containing deposits are rare in part because of the solubility of tungsten-sulfides (GIBERT et al., 1992; IVANOVA, 1986). Significant concentrations (> 50 nM) of W are found in four types of

natural waters: [I] groundwaters directly associated with W-containing ore deposits; [II] thermal waters of alkaline, nitrogenous fissure-veins of crystalline rocks; [III] alkaline waters of lakes in arid zones and hot-spring waters; and [IV] hydrothermal vents (CARPENTER and GARRETT, 1959; IVANOVA, 1986; SO and YUN, 1994). Not surprising then that hydrothermal vents may prove to be especially interesting environments for tungsten. One study carried out on the amount of W in deep-sea sites found concentrations in samples of vent fluids about one thousand times greater than the concentration found in seawater (KELLEY et al., 2002). Also, W concentrations in samples of chimney vents and flanges (porous metal sulfide-silica structures deposited from the flow of white smoker fluid) were ten times the concentration of Mo. Notable as well, was the presence of large numbers of microorganisms found within the same samples (KLETZIN and ADAMS, 1996). More recent work by KISHIDA et al. (2004) documents large variations in the W concentration of vent fluids from different vents (0.21-123 nmol/kg) and in comparison to seawater (0.070 nmol/kg).

An excellent review of the importance of tungsten in biology has been presented in KLETZIN and ADAMS (1996) and much of the information presented below has been summarized from that work. Tungsten was generally considered a biological antagonist to Mo and was often used in studies of the properties and functions of Mo in Mo-enzymes, where it was used to form inactive or very low activity enzyme analogs. Presently, four disparate groups consisting of a total of approximately ten W-enzyme members, so called tungstoenzymes, are known but it is thought that many more of these enzymes are yet to be discovered. The four tungstoenzyme groups are: formate dehydrogenase (FDH), formylmethanofuran dehydrogenase (FMDH), acetylene hydratase (AH) and aldehyde-oxidizing enzymes (e.g. AOR/CAR). The enzymes were isolated and purified from a variety of microorganisms, primarily thermophilic to

hyperthermophilic archaea and bacteria but some are also present in mesophilic sulfate-reducing bacteria (SRB) and various aerobic methylotrophs (HEIDER et al., 1995). FDH and FMDH are both used in CO_2 fixation, respectively catalyzing the reversible oxidation of formate and N-formylmethanofuran/methanofuran. Interestingly, FDHs purified from aerobic microorganisms do not contain metals or other cofactors; instead, the enzyme uses NAD(H) as the electron carrier. Conversely, anaerobic FDHs contain metal cofactors. FMDH functions in methanogenesis, producing the aforementioned methanofuran groups, which are eventually reduced to methane but notably, this enzyme is found only in methanogens and *Archaeoglobus fulgidus*, a hyperthermophilic sulfate reducer. Additionally, the electron carrier for FMDH is unknown. AH is the most recently discovered group, the least studied and the only tungstoenzyme catalyzing a hydration reaction, the fermentation of acetylene to acetate and finally, acetaldehyde. The hydration reaction of AH is believed to take place in aerobic acetylene-oxidizing bacteria but the enzyme itself has only been purified from an anaerobe.

The last tungstoenzyme group of aldehyde-oxidizing enzymes is perhaps the best known and studied. The members of this group include AOR (aldehyde ferredoxin oxidoreductase), CAR (carboxylic reductase), FOR (formaldehyde ferredoxin oxidoreductase), GAPOR (glyceraldehydes-3-phosphate ferredoxin oxidoreductase), ADH (aldehyde dehydrogenase) and the most recent members, WOR4 and WOR5 (BEVERS et al., 2005; ROY and ADAMS, 2002; ROY et al., 1999). All of these enzymes, except GAPOR, catalyze the oxidation of various types of aliphatic and aromatic aldehydes coupled to the reduction of ferredoxin, providing the cell with a carbon and energy source. AOR and FOR have important roles in peptide fermentation as well as optimal enzyme activity at temperatures > 95 ºC however, while AOR has broad substrate specificity, utilizing a range of peptides and sugars, FOR can only oxidize C_1-C_3 aldehydes.

Additionally, AOR is generally abundant in hyperthermophilic archaea, comprising approximately 1% of the total protein content (ROY et al., 2001). CAR is different from the other enzymes in that it can catalyze the reversible reduction of carboxylic acid to aldehydes and thus far, has only been obtained from mesophilic to thermophilic acetogens. GAPOR functions in a modified Embden-Meyerhof pathway for glucose catabolism and can only use glyceraldehydes-3-phosphate as a substrate (MA et al., 1997). WOR5 has been identified as the fifth type of aldehyde oxidoreductase (BEVERS et al., 2005) but the exact function of WOR4 remains unknown though it is thought the enzyme may have a role in sulfur metabolism ((ROY and ADAMS, 2002). At least four members of this tungstoenzyme group - AOR, FOR, GAPOR and WOR - have been found in the heterotrophic hyperthermophile, *Pyrococcus furiosus*.

The organisms utilizing W or Mo can generally be divided into three groups: (1) organisms preferring Mo to W, (2) organisms capable of using both metals and, (3) organisms preferring W to Mo. In the first group (animals, plants, fungi, algae, bacteria such as *E. Coli*), W easily replaces Mo in Mo-enzymes creating inactive tungsten analogs; the reaction is reversible. Conversely, in the second group (e.g. *Methanobacterium wolfei*, *Methanobacterium thermoautotrophicum*) Mo can be replaced by W, which results in a functional enzyme with decreased activity. 'True' tungsten enzymes belong to the last group (hyperthermophilic Archaea, e.g. *Pyrococcus furiosus*), in which W cannot be replaced by Mo. More importantly, tungstoenzymes are constitutive enzymes while Mo-analogs are stimulated only in the presence of Mo (L'VOV et al., 2002). In addition, all tungstoenzymes (with the exception of AH) catalyze two electron redox reactions, carry out catalysis at much lower redox potentials in comparison to most biological reactions [equal to or more negative than the H electrode; E_o'= - 420 mV, pH 7, 25 ºC] and most are extremely oxygen-sensitive (KLETZIN and ADAMS, 1996).

The obligate requirement for tungsten by primarily one particular group of microorganisms, the hyperthermophiles, offers a unique system for geochemical and geomicrobiological investigation. Using an experimental approach, we tested whether hyperthermophilic microbes produce ligands in order to leach metals from basalt, the type of rock that dominates marine hydrothermal environments. This is a concept which could provide insight into microbe-metal utilization and the effect of such processes in natural hydrothermal environments. For instance, this research could be the first to provide evidence that hyperthermophiles alter the geochemistry of their environment by direct microbe-rock interactions. Hydrothermal interactions with crustal materials are well documented. Studies (e.g. ALT et al., 1986; HART, 1970) have shown weathering of basalts by the passage of seawater increases the formation of hydrated minerals and chemical species (e.g. Si, Ca, Mg, K). Similarly, basaltic glass is chemically unstable in seawater and its alteration leads to important and significant geochemical fluxes between oceanic crust and seawater (STAUDIGEL and HART, 1983). While these abiotic alteration processes are well known, the magnitude of contributions from biogenic reactions that alter ocean chemistry have not been determined. Results from this study may therefore allow a more comprehensive model of the alteration of oceanic crust and the evolution of hydrothermal fluids to be determined.

Previous work by other researchers has documented the occurrence of microbial alteration in basaltic glass from pillow lavas of the upper oceanic crust (FISK et al., 1998; FURNES et al., 1999; FURNES et al., 2001a; FURNES and STAUDIGEL, 1999; FURNES et al., 2001b; TORSVIK et al., 1998). Why the biogenerated textures have been produced is unknown but a metal leaching process may be one reason. More importantly, microbial alteration and the

resultant physical textures and geochemical byproducts may function as biosignatures of microbial processes.

With respect to W, life and the early Earth, this research may be especially significant. ANBAR and KNOLL (2002) have postulated that the Proterozoic ocean was moderately oxic at the surface and sulfidic at depth, which would have resulted in the scarcity of biologically important trace metals such as Mo. Two conclusions can be drawn from this scenario: organisms, for example those that depend on Mo, could possibly have needed to develop mechanisms to scavenge required metals. As stated earlier, WS_2 is easily solubilized; this leads to the second and more profound possibility. The earliest forms of life could have been hyperthermophilic as well as W-dependent (DANIEL and COWAN, 2000; KLETZIN and ADAMS, 1996; NISBET and SLEEP, 2001).

3.3 Materials and Methods

Microorganisms

Five microorganisms, all from the domain Archaea, were used in this study: *Pyrobaculum islandicum*, *Pyrobaculum aerophilum*, *Pyrobaculum calidifontis*, *Pyrococcus furiosus* and a methanogen, *Methanococcus jannaschii*. All are anaerobic hyperthermophiles however, *P. aerophilum* and *P. calidifontis* can also grow under microaerobic and aerobic conditions, respectively. The optimum growth temperatures for these organisms are: *P. furiosus*, 100-113ºC; *P.aerophilum* and *P. islandicum*, 100 ºC; *P. calidifontis*, 90 ºC and *M. jannaschii*, 85 ºC. *P. calidifontis* was originally furnished to the House laboratory by Professor Tadayuki Imanaka (Department of Synthetic Chemistry and Biological Chemistry, Kyoto University, Japan) (AMO et al., 2002). The other organisms were originally obtained from the Deutsche

Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and are maintained in the laboratory of C. H. House (Penn State University, University Park, PA.). *M. jannaschii* was recently reclassified to *Methanocaldococcus jannaschii* (WHITMAN, 2002).

Growth Media

Labware used in media preparation and sample collection was cleaned by soaking in 10% HCl followed by thorough rinsing in 18 MΩ water. Reagents were supplied from Fisher or Sigma-Aldrich Chemicals.

P. islandicum: This medium is a modification of the DSMZ medium 390. The medium contains (per liter) 0.5 g peptone, 0.2 g yeast, ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA), 1.3 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$, 2.0 g $Na_2S_2O_3 \cdot 5H_2O$ and 10 ml of 100x stock mineral elixir. The mineral elixir contains (per liter) 0.0018 g $MnCl_2 \cdot 4H_2O$, 0.0045 g $Na_2B_4O_7 \cdot 10H_2O$, 0.00022 g $ZnSO_4 \cdot 7H_2O$, 0.00005 g $CuCl_2 \cdot 2H_2O$, 0.00003 g $NaMoO_4 \cdot 2H_2O$, 0.00001 g $CoSO_4$, 0.00003 g $VoSO_4 \cdot 2H_2O$. In sulfidic experiments, 0.5 g L^{-1} Na_2S was also added. Prior to making up the medium and to remove any metals present in the organic substrates, a solution of peptone, yeast and Chelex was stirred for 1 hr then gravity filtered to remove the resin-bound metals. The medium was made by mixing in solution all reagents and degassed by bubbling with a stream of N_2 for ~ 20 min. The addition of Na_2S, pH adjustment (~ 7) with concentrated HCl or NaOH (10%) and dispensation into serum bottles was carried out in an anaerobic chamber. The headspace in the experiment bottles was flushed three times with N_2, followed by a final addition of the same gas to 2 bars pressure. The same medium was made for pre-experiment innoculum cultures and in this instance, an autoclave was used for media sterilization.

P. aerophilum: This medium is a slight modification of the *P. islandicum* medium above and DSMZ medium 611. The medium contains (per liter) 0.5 g peptone, 0.2 g yeast, ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA), 1.3 g $(NH_4)_2SO_4$, 0.28 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 1.5 g NaCl, 0.1 ml Na_2SeO_4 (1%) and 10 ml of 100x stock mineral elixir. In sulfidic experiments, 0.5 g L^{-1} Na_2S was also added. Same procedures outlined above for *P. islandicum* were followed for making this medium with the following exceptions. For anaerobic experiments, 0.2 ml of 0.1 M KNO_3 (per 20 ml medium) was added prior to addition of the cells. In the microaerobic experiments, 2.0 g L^{-1} $Na_2S_2O_3 \cdot 5H_2O$ was added during medium preparation and 7.5 ml air (per 100 ml bottle volume) added to the bottle headspace before inoculation with cells.

P. calidifontis (per liter): 10.0 g tryptone, 1.0 g yeast, 3.0 g $Na_2S_2O_3 \cdot 5H_2O$, ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA). This medium was made under aerobic conditions. A solution of tryptone, yeast and Chelex was stirred for 1 hr then gravity filtered to remove the resin. Thiosulfate was added before the volume and pH (~ 7) were adjusted. The medium was dispensed into Erlenmeyer flasks that were capped with silicone plugs and autoclaved before being used for the growth experiments.

P. furiosus (per liter): 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.3 g $MgCl_2 \cdot 6H_2O$, 20.0 g NaCl, 5.0 g peptone, 1.0 g yeast, 1.0 ml 10x modified Wolfe mineral solution (WOLIN et al., 1963), ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA). Modifications to the Wolfe solution include the addition of 0.025 g L^{-1} $NiCl_2 \cdot 6H_2O$ and 0.0003 g L^{-1} $Na_2SeO_3 \cdot 5H_2O$, removal of Na_2MoO_4 and concentrated HCl was used in place of nitrilotriacetic acid. Medium preparation was carried out in the same manner as *P. islandicum*.

M. jannaschi (per liter): 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.2 g $CaCl_2 \cdot 2H_2O$, 0.3 g $MgCl_2 \cdot 6H_2O$, 20.0 g NaCl, 3.0 ml NaOH solution (10%), 1.0 ml 10x modified Wolfe mineral solution (see *P. furiosus*), 0.5 g cysteine•HCl. Medium was made by mixing in solution all reagents except cysteine•HCl and degassed by bubbling with a stream of $H_2 + CO_2$ (80% + 20%) for ~ 20 min. The addition of cysteine•HCl, pH adjustment (~ 7) with concentrated HCl or NaOH (10%) and dispensation into serum bottles was carried out in an anaerobic chamber. The headspace in the experiment bottles was flushed three times with $H_2 + CO_2$, followed by a final addition of the same gas mix to 2 bars pressure.

Basalt Leaching Experiments

The experimental setup, as follows, was the same for all microorganisms, regardless of variations in growth conditions. Three experimental conditions were set up: (A) media and cells (biotic control); (B) media, basalt powder and cells; (C) media and basalt powder (abiotic control). Cells and basalt powder (USGS Icelandic basalt standard, BIR-1) were added to the respective bottles (1 ml cells and/or 0.5 g basalt per 100 ml medium) at the beginning of the experiments. Stock cultures of all microorganisms, grown in the base media without trace metals or very small amounts carried over during transfer, were established prior to being used as innoculum for the growth experiments. All three conditions were run at the same time for each cell. Depending on the microorganism, growth typically proceeded over a period of 3 to 8 days during which supernatant samples were removed with sterile, O_2-free syringes and collected. Following growth, each batch was micro-filtered three times to remove the cells and basalt powder: twice through 0.2 μm syringe filters and once through an 0.1 μm Acrodisc disposable filter. The supernatant, possibly containing organic ligands produced by the organism, was

adjusted to the starting pH (if required), divided into several serum bottles and reincubated with fresh basalt powder under the same initial experiment parameters (growth temperatures, headspace gas, pressure) for a further period of 12 to 35 days. Individual bottles were sacrificed for supernatant samples that were collected during the period after filtration. Collected samples were centrifuged (8000 rpm, 15 min), syringe filtered through 0.1 μm Acrodisc filters and acidified to 2.5% with ultra pure HNO_3. High resolution inductively coupled plasma mass spectrometry (ICP-MS - Finnigan Element 1) was used to measure metal concentrations in the samples. Tungsten and the concentrations of other metals were analyzed to determine if metal leaching occurred.

Additional information specific to each microorganism is as follows. *P. islandicum*: three sets of experiments under sulfidic and non-sulfidic conditions; initial growth volume of 150 ml for each batch; 100 ºC and 50 ºC. *P. aerophilum* (a: micro-aerobic): five rounds of experiments; initial growth volume of 150 ml for each batch; non-sulfidic; 100 ºC and 50 ºC. *P. aerophilum* (an: anaerobic): three rounds of experiments; initial growth volume of 150 ml for each batch; sulfidic and non-sulfidic; 100 ºC. *P. calidifontis*: two sets of experiments; initial growth volume of 200 ml for each batch; 90 ºC and 50 ºC. *P. furiosus*: two sets of experiments; initial growth volume of 125 ml for each batch; 100 ºC and 70 ºC. *M. jannaschii:* two sets of experiments, initial growth volume of 200 ml for each batch; 85 ºC and 50 ºC.

3.4 Results

The results of all experiments are presented in Tables 3.2 - 3.7. Element concentrations are shown only for batches B (media+basalt+cells) and C (media+basalt: abiotic control) in experiments for *P. islandicum* and *P. aerophilum* since batch A (media+cells) samples were not

analyzed. Also, in some experiments, such as the 4th round for *P. aerophilum* (a) (Table 3.2), batches would have initially been started at the optimum growth temperature for the cell and after filtration to remove the cells and/or basalt, were incubated for a further period under a lower temperature or split into two sets with one set being incubated at a lower temperature and the other, at the initial growth temperature (for instance, see *M. jannaschii*, Table 3.6). This was done in order to test if ligands could also be produced at temperatures lower than the optimum growth temperatures for the cells. With the exception of *P. calidifontis* (Table 3.7), tungsten concentrations were obtained for all other microorganisms though not for every series of experiments (compare 2nd & 3rd round experiments for *P. aerophilum* (an); Table 3.3).

Tungsten concentrations measured in the supernatant samples collected over the duration of the experiments are presented in Figures 3.1 through 3.5. Evidence for possible ligand activity indicated by enhanced concentrations of W in batch B bottles were found for *P. aerophilum* (a) and *P. islandicum* and are shown in Figures 3.1 and 3.3, respectively. In the *P. aerophilum* (a) experiments, which were all conducted under non-sulfidic conditions, increased W concentrations (~ 0.1 to 0.3 ppb) relative to the abiotic control were found only in rounds 1 & 2 after filtration of cells and basalt powder. Round 3 results are also interesting as greater amounts of W (~ 0.7 ppb) are present in B for 18 days before decreasing, which is the same trend observed in round 2. Round 5 was a continuous culture experiment in that the cells and basalt were not removed after growth; the production and presence of ligands are not indicated and in fact, the data suggest that there may have been problems with this W analysis. A difference of ~ 0.7 ppb between B and C after filtration and under sulfidic conditions, also suggest possible leaching of W from the basalt powder in the 2nd round experiment for *P. islandicum* (Figure 3.3). However, additional support for this observation was not provided by the other sets of

experiments carried out for the cell. Negative results, i.e. no difference in values between the biotic and abiotic experiments or a marked increase in values for the abiotic control over biotic, were obtained for the other microorganisms. Unlike the micro-aerobic experiments, *P. aerophilum* (an) values for B and C, shown in Figure 3.2 were essentially the same, reaching a maximum of ~ 0.6 ppb in the 2nd round experiments. Regardless of temperature, the experiments conducted with *P.furiosus* (Figure 3.4) and *M. jannaschii* (Figure 3.5) were dominated by the abiotic control and with the exception of the high temperature *M. jannaschii* control, W values for all experiments were generally lower (maximum ~ 0.25 ppb) than was measured for *P. aerophilum* and *P. islandicum*. Concentrations of W measured for *P. calidifontis* (Table 3.7) were all below detection; this may have been a result of a greater degree of precipitation imparted by the aerobic conditions of growth which could facilitate the formation of stable insoluble $CaWO_4$ (notice that Ca was also below detection in most experiments).

The results were also scrutinized for evidence of other potential trace metal ligands, particularly Fe, Ni and Mo. Figures 3.6 through 3.11 shows Fe and Ni concentrations measured for all experiments. The range of concentrations is considerable and variable with maximum values from ~ 12 to > 11,000 ppb for Fe and ~ 1.7 to 61 ppb for Ni. However, the abnormally high values found only in *M. jannaschii* (Figure 3.10) and *P.furiosus* (Figure 3.9) experiments are artificial since both metals were added to the trace metal solution used in the growth medium for each microorganism. The other three microorganisms (Figures 3.6 & 3.7, 3.8, 3.11) have a range of maximum values of ~12 to 400 ppb and ~ 1.7 to 16 ppb for Fe and Ni, respectively. Evidence for possible Fe leaching in the biotic experiments was seen in the 2nd and 3rd round experiments for *P. aerophilum* (a) (Figure 3.6). Also, Fe concentrations were higher in experiments without sulfide, however, no trends were observed under conditions of lower

temperatures. Nickel leaching is indicated in the 1st round experiment for *P. aerophilum* (an) (Figure 3.7) and possibly, the 50 ºC 3rd round batch of *P. islandicum* (Figure 3.8). Nonetheless, Ni generally behaves conservatively in all experiments, displaying a similar trend in both the biotic and abiotic batches under all conditions.

Mo results are presented in Figures 3.12 to 3.17. Leaching of this metal from the basalt powder in the presence of cells is only indicated in the micro-aerobic, sulfide-free 2nd and 4th round batches for *P. aerophilum* (Figure 3.12). Interestingly, the highest concentration of Mo (10 ppb) was attained in the 4th round batch B which, after filtration to remove cells and basalt, was re-incubated with fresh basalt at the lower temperature of 50 ºC. Mo values for the abiotic controls in both sets of experiments were between 1 to 2 ppb. Concentrations of Mo in all other abiotic batches for the different microorganisms were the same as or higher than values in the biotic batches regardless of experimental conditions. Additionally, values were generally higher under non-sulfidic conditions.

3.5 Discussion

Supernatant experiments designed to investigate biological production of W-specific complexing ligands by hyperthermophilic Archaea during growth and under limited W availability revealed that these microorganisms most likely do not produce such ligands. There is a suggestion in some of the experiments that the presence of microorganisms such as *P.aerophilum* and *P. islandicum* may facilitate the production of metal binding ligands however, additional growth experiments intended to verify and support these initial positive results proved to be distinctly negative. Iron chelators or siderophores are typically produced by aerobic and facultatively anaerobic microorganisms (NEILANDS, 1995) so it is perhaps not surprising that

some evidence for W, Fe, Ni and Mo leaching were only found for the facultatively microaerobic *P. aerophilum*. Furthermore, the nature of the ligand (e.g. low affinity chelators such as organic acids or stronger, high affinity Fe-specific ligands) produced by the same organism can be variable depending on the level of nutrient limitation (KALINOWSKI et al., 2000; LIERMANN et al., 2000; ROGERS and BENNETT, 2004). It is tempting to believe that the positive results of enhanced metal concentrations in the biogenic experiments are due to the action of metal-specific ligands however, apart from the additional growth experiments, tests to verify the presence of organic ligands or to demonstrate the influence of other physico-chemical factors which could also produce such results, were not carried out. For instance, possible changes in pH occurring after filtration and potentially mediated by enhanced dissolution of the basalt powder under high temperatures, was not monitored. The experiments were also conducted as separate individual sets, not replicates (for e.g. 5 rounds or sets of *P. aerophilum* (a), with each set consisting of batch A, B & C) and under differing experimental conditions, which makes comparison between experimental sets as well as between microorganisms, difficult. Given these circumstances, we cannot unequivocally say that the positive W results are evidence of a W-specific ligand.

There are several reasons which may account for the negative findings obtained in experiments for all microorganisms. The most obvious is that a W ligand was not produced during growth. Support for this is provided by the abiotic controls (batch C) which have metal concentrations that are rarely lower compared to the respective biotic experiments and in some cases, such as the abiotic W concentrations for *P. furiosus* and *M. jannaschi* (Figures 3.4 & 3.5) are more elevated and/or constant throughout the entire period of the experiment, even under lower temperatures. Another factor inhibiting ligand production may be attributed to ineffective metal removal from the organic substrates used in the growth media. Studies investigating the

effects of W or Mo during microbial growth (MUKUND and ADAMS, 1996; WHITE and SIMON, 1992) have reported that organic substrates like yeast and tryptone contain 3 to 10 nmol W and between 14 to 20 nmol Mo, respectively per gram dry weight. Indubitably, these organics will also have other contaminating trace metals such as Fe and Ni and most likely at much higher quantities. With the exception of the strict chemolithoautotroph *M. jannaschii*, organic substrates are used in the growth media of all microorganisms examined and even though metals are removed from the organics with the metal-binding resin Chelex before incorporation into the media, it is plausible that the resin did not efficiently remove all metals. Previous work utilizing Chelex to concentrate trace metals in seawater (RILEY and TAYLOR, 1968) have demonstrated that metals like W and Mo will only be quantitatively removed at a very restricted pH range of 5 – 6 and it is uncertain whether this requirement was met for the experiments in this study. Such contamination would also be quite severe in the experiments for *P. furiosus* and *P. calidifontis*, as their growth media requires 5 – 20x more organic substrates compared to the other Pyrobaculum species.

Another issue that must be taken into account is the metabolic and physiological differences between hyperthermophiles, which can impact their utilization of required nutrients. The non-methanogenic hyperthermophiles in this study are primarily facultative autotrophs (*P. islandicum*, *P. aerophilum*) or heterotrophs (*P. furiosus*, *P. calidifontis*). *P. furiosus* does not require sulfur for growth and gains energy primarily by fermenting organic substrates while the sulfur respirer, *P. islandicum* reduces elemental sulfur or oxidized sulfur compounds (e.g. thiosulfate) coupled to H_2 oxidation (STETTER, 1996; STETTER et al., 1990) as well as a range of metals (KASHEFI and LOVLEY, 2000). *P. aerophilum* and *P. calidifontis* are more similar in that they can use O_2 as an electron acceptor during microaerobic and aerobic growth, respectively

and both carry out nitrate reduction under anaerobic conditions however, while the former can utilize thiosulfate the latter cannot, unless under aerobic growth (AMO et al., 2002; VOLKL et al., 1993). These variable metabolic conditions can, in some cases affect cellular expression of specific W-enzymes, induce the expression of alternate Mo-isoenzymes where known to exist or, have no affect at all. For instance, AOR is one of the most important of the three tungstoenzymes required for growth by *P. furiosus* (the other two are GAPOR and FOR) but it is also significant to sulfur-utilizing hyperthermophiles such as *P. aerophilum* (MA et al., 1997). Similarly, a GAPOR homolog is thought to exist in *M. jannaschii*, which grows autotrophically and not heterotrophically (ROY et al., 1999). Tungsten is so critical to the growth of *P. furiosus* that the cell when grown in a 10,000 fold excess of Mo over W scavenged the required amount of W to produce AOR and FOR and did not express Mo-isoenzymes (KLETZIN and ADAMS, 1996) and at present, Mo-isoenzymes of AOR, FOR and GAPOR are not known to exist. These observations may explain the results obtained in the biotic batch A and B tungsten experiments for both *P. furiosus* (Figure 3.4) and *M. jannaschii* (Figure 3.5), which show W concentrations steadily declining before filtration over the period of growth. By contrast, the abiotic batches remain at constant elevated levels before and after filtration. The class of oxotransferase enzymes to which tungstoenzymes belong is comprised predominantly of molybdoenzymes including those involved in sulfur metabolism and nitrate reduction, amongst others (CHAN et al., 1995). The majority of these enzymes, particularly nitrate reductases, are inactivated in the presence of W. However, a Mo-containing nitrate reductase highly active in the presence of elevated W concentrations has been isolated and purified from *P. aerophilum* (AFSHAR et al., 2001). Results of the Mo biotic experiments in the 2nd and 4th round for *P. aerophilum* (a) (Figure 3.12) and the 2nd round for *P. islandicum* (Figure 3.14) suggests a Mo ligand may have been produced, which

is surprising considering Mo was supplied in the growth media for both microorganisms. The Mo data trend for *P. islandicum* is also identical to the corresponding W data (Figure 3.3) the only difference being the concentrations of Mo after filtration is ~ 3x greater than W. Transport systems for intracellular and extracellular binding and uptake of W and Mo have not been identified though genes encoding high affinity proteins for Mo are known to exist in microorganisms such as *E. coli* (RECH et al., 1996) and *A. vinelandii* (LUQUE et al., 1993). Such transport systems for both metals may be quite similar in nature and a protein which displays high affinity for Mo and W has been purified from *E. coli* (HAGEN and ARENDSEN, 1998; RECH et al., 1996). Given these facts, it may be conceivable that the Mo and W results for *P. islandicum* as well as *P. aerophilum* could possibly be due to the presence of some type of organic ligand which facilitated selective leaching of these metals, though preferentially for Mo over W.

The lack of direct evidence for hyperthermophilic W ligands to promote W-specific leaching of basalt under pseudo hydrothermal conditions suggests that such processes may not be operating in environments containing hyperthermophiles. As shown by the abiotic experiments, water-rock reactions and the natural enrichment of W and other metals in hydrothermal fluids (e.g. KISHIDA et al., 2004) probably obviate the need for high-temperature microorganisms to produce such specific metal chelators. However, the results of this study do not mean that these microorganisms and the environments they inhabit are not important in regards to mineral-microbe interactions, a source of markers for life or to investigations of early life on Earth. At deep ocean hydrothermal vent sites, hyperthermophiles like the ones in this study are typically found within the higher temperature niches such as the subsurface rocks, vent smoker fluids and the inner hotter recesses of precipitated sulfide structures (the chimney vent) (KELLEY et al.,

2002). In some cases, organisms are found in intimate association with specific areas of metal enrichment. For instance, horizontal sulfide structures called flanges which form from the chimney are commonly partitioned into particular mineral and temperature gradients; high concentrations of W were found in the layer that consisted primarily of Zn-Fe-S minerals that had precipitated from < 250 ºC fluids. More important is that this layer also correlated with the presence of high numbers of hyperthermophilic Archaea and whether abiotic W precipitation resulted in the presence of the microorganisms or vice versa, remains to be answered (HOLDEN and ADAMS, 2003). It is also not known whether hyperthermophiles affect direct microbe-mineral interactions that can produce evidence for other types of biomarkers such as etch pits similar to those documented in basaltic glass (FURNES et al., 1999; FURNES et al., 2001b) or biofilms resulting from colonization. However, unless freely floating, it is expected that microorganisms are most likely attached to a surface in some fashion and that the interaction, either direct or indirect (for example, pH) will have an effect on the mineral. Additionally, it is thought that extracellular polymeric substances (EPS) may be important to the survivability of microorganisms in the vent environment specifically for physical and chemical protection from fluctuating vent conditions (EDWARDS et al., 2005).

The unique place that W occupies in biology has led to many questions regarding the role of the metal and that of the microorganisms that utilize it in the origin of early life on Earth. The tungstoenzymes (AOR, FOR & GAPOR) that are found in the strictly anaerobic heterotrophic hyperthermophiles such as *P. furiosus* have no Mo analogs and are the only enzymes capable of catalyzing extremely low potential (- 580 mV) carbon chemistry at 100 ºC or higher. By contrast, Mo enzymes catalyze higher potential reactions and are dominant in oxic environments and in the mesophilic to thermophilic microorganisms, some of which can utilize both Mo and W

analogs (KLETZIN and ADAMS, 1996). Hyperthermophiles are found amongst the deepest and slowest evolving lineages in the tree of life and as such, are thought to be evolutionarily primitive and it is even thought that the last common ancestor could have been a hyperthermophile (STETTER, 1996). The concentration of W in the modern and ancient oceans are thought to be quite similar but unlike Mo which is abundant today, W would have been more available in the reducing sulfidic early environment on Earth (WILLIAMS and DA SILVA, 2002). Hence, from a geochemical, metabolic and phylogenetic perspective, W enzymes and hyperthermophiles may not only be primitive but also evolutionarily linked.

3.6 Conclusion

Tungsten is a vital micronutrient required for the growth of anaerobic heterotrophic hyperthermophiles. However, bulk results of supernatant experiments conducted with hyperthermophilic Archaea indicate these microorganisms do not produce W-specific ligands when grown under limited W availability. Alternatively, it is possible that evidence for biological production of W ligands were not observed given the reproducibility of the experiments. In spite of this, it is not expected that in natural hydrothermal environments microbial metal chelators are important for leaching bioessential trace metals from rocks or minerals as hydrothermal fluids are typically enriched in metals (Table 3.1). Additionally, microbe-mineral interactions may not significantly impact the geochemistry of hydrothermal fluids and as shown by a multitude of studies, abiotic water-rock reactions are invariably dominant processes in hydrothermal settings. However, microorganisms respond to environmental stressors such as exposure to high metal concentrations which may result in metal toxicity to the cells, by various methods. For example, they can produce ligands that chelate and

transport metals away from the organism, thereby maintaining intracellular metal concentrations (EDGCOMB et al., 2004; HOLDEN and ADAMS, 2003). The rock record is replete with the products and effects of microbial processes. As shown by extant microorganisms, tungsten has an important role in biology and further studies will be required to evaluate the impact of this trace metal in modern and ancient environments. Whether such evidence can be detected in the rock record as a biomarker remains to be seen however, W and hyperthermophiles may have been important in the evolution of early life.

3.7 References

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3.8 Figures and Tables

Table 3.1: Bioessential trace metal concentrations in geological environments.

Trace Metal	Hydrothermal Fluids	Seawater	Crustal Abundance	Basalt	Granite
	ppm (μg/g)	ppm (μg/g)	ppm (μg/g)	ppm (μg/g)	ppm (μg/g)
Chromium (Cr)	?	0.0006	100	9 - 412	3.7 - 4.9
Cobalt (Co)	0.0022 - 0.084	0.00008	25	37 - 53	2.1
Copper (Cu)	0.006 - 2.22	0.003	50	19 - 136	11
Manganese (Mn)	0.55 - 63250	0.002	1000	2000	600
Molybdenum (Mo)	0 - 0.0032	0.01	1.5	1.11	2.48
Nickel (Ni)	0.00059 - 3.11	0.000176	75	12 - 175	3.7 - 4.4
Selenium (Se)	0.000047 - 0.0081	0.00045	0.1	0.054 - 0.15	0.006 - 0.01
Tungsten (W)	**0.000039 - 0.023**	**0.000013**	**1**	**0.23**	**1.46**
Vanadium (V)	?	0.0015	150	321 - 405	12
Zinc (Zn)	0.04578 - 202.74	0.005	80	65 - 129	30
Iron (Fe)	0 - 75999.6	0.003	46500	105400	24400

Data Sources:

Eggins et al. (1997); Hall et al. (1987); Hall & Pelchat (1997); Kelley et al. (2002); Kishida et al. (2004); Kletzin & Adams (1996); Rose et al. (1979); Sclater et al. (1976); Von Damm (1995); Winter (2001)

Table 3.2: Element concentrations measured for *P. aerophilum* (a). Experiments with sulfide are not in italics; those without sulfide are in italics. All at 100 ºC unless indicated otherwise.

GROWTH CONDITION	SAMPLING PERIOD (DAY)	ELEMENT CONCENTRATION (ppb)										
		Mg	Si	Ca	Cr	Fe	Ni	Sr	Mo	W	Al	Cu
1st Round												
	1	*2423.5*	-	-	-	*6.160*	*0.300*	*1.370*	*1.430*	*0.041*	*8.7*	*8.7*
	3	*2661.4*	-	-	-	*8.820*	*0.670*	*1.570*	*1.550*	*0.005*	*118.0*	*7.6*
B	4	*2789.5*	-	-	-	*3.480*	*8.760*	*3.080*	*2.480*	*0.121*	*16.9*	*26.0*
(med+bas+cells)	12	*2914.1*	-	-	-	*11.710*	*11.470*	*3.230*	*1.740*	*0.296*	*11.5*	*34.4*
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
	1	*2628.0*	-	-	-	*3.080*	*5.870*	*1.420*	*1.780*	*0.035*	*5.3*	*29.9*
C	3	*2878.9*	-	-	-	*6.610*	*0.660*	*1.590*	*1.670*	*0.011*	*6.2*	*32.4*
(med+bas)	4	*3060.8*	-	-	-	*6.050*	*13.650*	*2.930*	*2.370*	*0.024*	*10.1*	*52.6*
	12	*3101.4*	-	-	-	*10.700*	*5.520*	*2.960*	*2.350*	*0.011*	*1505.3*	*59.6*
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
2nd Round												
	1	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-
B	4	-	-	-	-	-	-	-	-	-	-	-
(med+bas+cells)	9	*1780.1*	*2564.8*	*1341.3*	*0.099*	*4.128*	*0.833*	*1.852*	*1.454*	*0.080*	-	-
	12	-	-	-	-	-	-	-	-	-	-	-
	18	*1923.5*	*3062.5*	*1379.8*	*0.136*	*3.890*	*1.615*	*3.131*	*6.201*	*0.302*	-	-
	30	*1996.7*	*3562.8*	*1594.7*	*0.907*	*24.533*	*1.578*	*3.064*	*1.362*	*0.283*	-	-
	1	-	-	-	-	-	-	-	-	-	-	-
C	3	-	-	-	-	-	-	-	-	-	-	-
(med+bas)	4	-	-	-	-	-	-	-	-	-	-	-
	9	*1751.5*	*1934.1*	*1283.9*	*0.322*	*5.644*	*1.131*	*1.510*	*1.476*	*0.023*	-	-
	12	-	-	-	-	-	-	-	-	-	-	-
	18	*1880.6*	*2526.5*	*1406.9*	*0.815*	*11.363*	*0.883*	*2.921*	*1.664*	*0.096*	-	-
	30	*1829.5*	*2830.6*	*1290.2*	*0.213*	*17.188*	*1.727*	*3.145*	*1.448*	*0.214*	-	-
3rd Round												
	1	*1987.8*	*749.2*	*1597.5*	*0.146*	*BD*	*0.423*	*0.805*	*2.157*	*2.120*	-	-
	3	*2076.5*	*1067.9*	*1617.9*	*0.103*	*21.521*	*0.468*	*0.834*	*0.825*	*BD*	-	-
B	4	*2018.4*	*2397.0*	*1554.1*	*0.273*	*9.747*	*6.630*	*2.306*	*1.366*	*0.788*	-	-
(med+bas+cells)	12	*2029.6*	*3815.4*	*1658.9*	*0.184*	*BD*	*4.960*	*3.049*	*1.377*	*0.581*	-	-
	18	*2111.2*	*4016.4*	*1794.6*	*0.367*	*36.390*	*5.579*	*2.596*	*1.589*	*0.709*	-	-
	30	*2262.1*	*4887.3*	*1952.0*	*0.190*	*289.118*	*4.399*	*2.561*	*1.297*	*BD*	-	-
	1	*2066.0*	*794.0*	*1652.7*	*0.129*	*BD*	*0.526*	*0.826*	*1.170*	*1.105*	-	-
C	3	*1996.8*	*1014.1*	*1607.9*	*0.072*	*BD*	*0.887*	*1.443*	*0.784*	*BD*	-	-
(med+bas)	4	*2210.8*	*2198.1*	*1800.0*	*0.171*	*26.827*	*0.830*	*2.111*	*0.826*	*BD*	-	-
	12	*2111.3*	*3622.3*	*1907.5*	*0.097*	*BD*	*0.911*	*2.554*	*1.002*	*BD*	-	-
	18	*2193.6*	*4390.2*	*1953.4*	*1.356*	*55.630*	*7.118*	*2.531*	*1.613*	*0.665*	-	-
	30	*2364.9*	*5822.1*	*2072.8*	*0.091*	*111.755*	*1.019*	*2.965*	*0.974*	*2.808*	-	-
4th Round												
	1	*1689.6*	*646.0*	*1621.0*	*BD*	*BD*	*0.314*	*0.878*	*4.164*	*2.201*	-	-
	3	*1876.2*	*1059.2*	*1629.9*	*0.107*	*61.441*	*0.276*	*1.449*	*1.755*	*0.706*	-	-
B	4	-	-	-	-	-	-	-	-	-	-	-
(med+bas+cells)	12	-	-	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
	1	*1869.8*	*705.3*	*1638.2*	*BD*	*BD*	*0.275*	*1.412*	*2.135*	*1.150*	-	-
C	3	*1972.9*	*1099.2*	*1720.2*	*0.152*	*BD*	*0.230*	*1.523*	*1.660*	*BD*	-	-
(med+bas)	4	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
4th Round - 50ºC												
	1	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-
B	4	*1930.6*	*1229.8*	*1711.8*	*BD*	*BD*	*0.948*	*2.147*	*1.545*	*BD*	-	-
(med+bas+cells)	12	*1939.3*	*1371.5*	*1630.1*	*0.276*	*133.823*	*6.945*	*1.892*	*10.079*	*BD*	-	-
	18	*1913.0*	*1334.5*	*1703.2*	*0.478*	*BD*	*1.250*	*1.841*	*1.563*	*BD*	-	-
	30	*2022.8*	*1358.0*	*1743.8*	*BD*	*BD*	*0.867*	*1.960*	*1.485*	*BD*	-	-
	1	-	-	-	-	-	-	-	-	-	-	-
C	3	-	-	-	-	-	-	-	-	-	-	-
(med+bas)	4	*1889.6*	*1271.7*	*1783.8*	*0.123*	*BD*	*1.497*	*2.059*	*1.506*	*BD*	-	-
	12	*1981.6*	*1406.1*	*1796.0*	*0.222*	*BD*	*1.670*	*1.914*	*1.717*	*BD*	-	-
	18	*2017.6*	*1511.0*	*1792.4*	*0.157*	*BD*	*2.676*	*1.934*	*2.172*	*BD*	-	-
	30	*1922.2*	*1466.3*	*1737.4*	*0.169*	*203.449*	*5.041*	*1.893*	*1.642*	*BD*	-	-
5th Round												
	1	*2.7*	*1.6*	*1.5*	*0.500*	*19.517*	*2.387*	*1.989*	*2.457*	*1.000*	-	-
B	3	*2.6*	*2.2*	*1.4*	*0.500*	*10.000*	*0.920*	*1.989*	*2.457*	*1.000*	-	-
(med+bas+cells)	7	*2.8*	*2.5*	*1.4*	*0.500*	*10.000*	*4.380*	*1.989*	*3.888*	*1.000*	-	-
	15	*2.7*	*3.9*	*1.2*	*0.500*	*12.660*	*3.065*	*2.995*	*2.739*	*1.000*	-	-
	21	*2.6*	*4.3*	*1.3*	*0.500*	*17.937*	*1.279*	*3.728*	*2.379*	*1.000*	-	-

Table 3.3: Element concentrations for *P. aerophilum* (an). Experiments with sulfide are not in italics; those without sulfide are in italics. Experiments were all done at 100 ºC.

GROWTH CONDITION	SAMPLING PERIOD (DAY)	ELEMENT CONCENTRATION (ppb)										
		Mg	Si	Ca	Cr	Fe	Ni	Sr	Mo	W	Al	Cu
1st Round												
	1	2684.9				5.840	1.710	1.340		0.018	13.2	3.8
	3	2691.1				14.260	1.890	1.490		0.006	85.3	4.1
B	4	2566.3				10.990	2.780	3.700		0.018	77.1	5.0
(med+bas+cells)	12	2453.8				11.460	10.190	4.090		0.016	73.1	4.0
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
	1	2762.1				11.290	2.060	1.540		0.024	8.3	5.7
C	3	2730.3				11.770	2.220	3.580		0.013	12.4	4.1
(med+bas)	4	2636.0				6.750	6.100	8.020		0.015	24.5	4.0
	12	2466.9				7.750	1.440	3.290		0.013	14.9	2.9
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
2nd Round												
	1	1594.1	815.7	1207.2	0.214	5.204	0.559	0.804	1.586	0.417	-	-
	3	1645.5	1065.6	1153.2	0.253	6.495	0.525	0.857	1.137	0.173	-	-
B	4	2623.0	4210.5	1157.4	1.751	14.630	5.971	1.595	1.531	0.537	-	-
(med+bas+cells)	9	1776.1	2291.7	1006.7	0.122	5.624	1.116	1.458	1.152	0.017	-	-
	12	2635.9	4638.0	1197.6	0.276	4.285	7.012	2.137	3.511	0.333	-	-
	18	1680.6	2579.2	938.2	0.385	5.529	1.070	2.845	2.347	0.584	-	-
	30	1792.2	3317.5	971.3	0.091	6.747	1.294	3.164	1.158	0.144	-	-
	1	1621.3	798.7	1233.9	0.324	4.457	0.630	0.839	1.190	0.254	-	-
C	3	1652.4	1094.2	1238.1	0.104	2.972	0.354	0.898	1.071	0.155	-	-
(med+bas)	4	1621.3	2411.0	1236.6	0.392	4.491	1.240	2.155	1.280	0.294	-	-
	9	1804.9	2395.2	1086.2	0.582	3.369	2.422	1.560	1.249	0.066	-	-
	12	1643.1	3207.9	1279.1	0.419	4.366	1.455	2.433	1.463	0.394	-	-
	18	1718.1	2694.5	962.0	0.385	6.513	3.684	2.198	3.188	0.571	-	-
	30	1826.9	3513.8	1000.6	0.066	7.782	1.174	3.403	1.187	0.176	-	-
3rd Round												
	1	*2059.0*	*1009.8*	*1506.4*	*0.121*	*2.690*	*0.452*	*0.709*	*1.572*	*BD*	-	-
	3	*2087.9*	*1233.2*	*1398.8*	*0.095*	*21.056*	*0.354*	*1.353*	*1.682*	*BD*	-	-
B	4	*2290.0*	*2434.4*	*1517.5*	*0.100*	*59.015*	*1.119*	*2.135*	*1.771*	*BD*	-	-
(med+bas+cells)	12	*2223.6*	*3184.6*	*1494.5*	*0.053*	*171.387*	*0.877*	*1.917*	*1.709*	*BD*	-	-
	18	*2147.9*	*3405.6*	*1502.8*	*0.093*	*141.974*	*1.186*	*1.994*	*1.638*	*BD*	-	-
	30	*2309.1*	*5091.1*	*1688.0*	*0.093*	*353.180*	*0.860*	*1.790*	*1.738*	*BD*	-	-
	1	*2235.4*	*1049.9*	*1635.6*	*0.129*	*2.900*	*0.565*	*0.812*	*1.596*	*BD*	-	-
C	3	*2079.4*	*1295.1*	*1502.6*	*0.069*	*BD*	*0.417*	*1.545*	*1.483*	*BD*	-	-
(med+bas)	4	*2305.4*	*2780.8*	*1612.1*	*0.103*	*376.618*	*2.680*	*2.003*	*1.632*	*BD*	-	-
	12	*2338.8*	*4098.0*	*1742.6*	*0.104*	*403.727*	*2.001*	*2.358*	*1.689*	*BD*	-	-
	18	*2101.9*	*3750.1*	*1689.5*	*0.092*	*24.584*	*1.602*	*2.140*	*1.725*	*BD*	-	-
	30	*2266.5*	*5383.9*	*1784.9*	*0.160*	*402.458*	*1.587*	*2.006*	*1.806*	*BD*	-	-

Table 3.4: Element concentrations for *P. islandicum*. Experiments with sulfide are not in italics; those with sulfide are in italics. Experiments were all at 100 ºC unless otherwise indicated.

GROWTH CONDITION	SAMPLING PERIOD (DAY)	ELEMENT CONCENTRATION (ppb)										
		Mg	Si	Ca	Cr	Fe	Ni	Sr	Mo	W	Al	Cu
1st Round												
	1	2109.4	-	-	-	5.46	1.610	1.480	0.011	-	13.370	6.500
	3	2228.5	-	-	-	4.65	0.740	1.690	0.004	-	173.040	6.490
B	4	2337.7	-	-	-	18.78	5.810	2.360	0.016	-	36.89	6.48
(med+bas+cells)	12	2219.0	-	-	-	7.95	4.590	2.560	0.004	-	44.67	5.03
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
	1	2327.7	-	-	-	3.95	0.810	1.690	0.011	-	33.47	8.02
C	3	2486.1	-	-	-	8.90	2.750	1.840	0.009	-	11.25	7.06
(med+bas)	4	2388.6	-	-	-	5.83	8.640	3.100	0.024	-	15.61	18.94
	12	2387.0	-	-	-	11.16	13.840	4.400	0.025	-	17.1	22.48
	18	-	-	-	-	-	-	-	-	-	-	-
	30	-	-	-	-	-	-	-	-	-	-	-
2nd Round												
	1	*965.5*	*922.5*	*1270.7*	*0.431*	*6.39*	*0.740*	*0.871*	*1.276*	*0.033*	-	-
	3	*1620.0*	*1315.7*	*1085.8*	*0.634*	*9.32*	*0.485*	*0.800*	*1.385*	*0.056*	-	-
B	4	*1634.3*	*2527.4*	*743.0*	*0.725*	*15.18*	*2.275*	*1.562*	*2.048*	*0.197*	-	-
(med+bas+cells)	9	1725.1	2190.4	710.8	0.144	8.25	0.587	1.214	1.003	0.015	-	-
	12	1684.1	3020.3	753.4	0.227	11.12	2.726	2.078	3.433	0.905	-	-
	18	1579.5	2395.2	633.3	0.077	2.34	0.534	2.068	1.278	0.218	-	-
	30	1521.4	2966.7	587.1	0.152	3.62	2.049	2.527	2.703	0.688	-	-
	1	*1992.8*	*1010.7*	*1442.7*	*2.493*	*27.71*	*2.343*	*1.050*	*1.532*	*0.114*	-	-
	3	*2042.9*	*1340.7*	*1488.9*	*0.481*	*7.62*	*7.880*	*1.162*	*1.702*	*0.065*	-	-
C	4	*1869.0*	*2719.9*	*1359.3*	*0.348*	*5.97*	*1.504*	*1.888*	*1.727*	*0.422*	-	-
(med+bas)	9	1986.9	2317.3	1257.1	0.090	7.05	1.192	2.121	1.357	0.039	-	-
	12	2064.2	3592.7	1425.7	0.210	14.24	3.928	2.795	2.522	0.272	-	-
	18	1812.0	2531.5	1159.0	0.621	9.27	0.991	2.527	1.473	0.220	-	-
	30	1864.2	3137.0	1211.5	0.059	10.29	1.196	3.477	1.690	0.333	-	-
3rd Round												
	1	*2174.0*	*836.3*	*1753.6*	*BD*	*BD*	*0.278*	*0.798*	*1.089*	*BD*	-	-
	3	*2113.5*	*1314.0*	*1353.7*	*2.905*	*96.59*	*2.198*	*0.686*	*BD*	*BD*	-	-
B	4	*2134.2*	*2522.0*	*1038.0*	*0.270*	*BD*	*3.280*	*1.585*	*0.901*	*BD*	-	-
(med+bas+cells)	12	*1957.1*	*3467.3*	*959.5*	*0.139*	*53.97*	*2.146*	*2.261*	*0.847*	*BD*	-	-
	18	*1942.0*	*4070.6*	*939.8*	*0.134*	*BD*	*1.398*	*1.973*	*1.876*	*BD*	-	-
	30	*1997.4*	*4607.5*	*966.9*	*0.094*	*77.48*	*2.461*	*1.862*	*0.871*	*BD*	-	-
	1	*2170.2*	*793.4*	*1804.7*	*BD*	*BD*	*0.348*	*1.355*	*1.505*	*BD*	-	-
C	3	*2103.2*	*1127.4*	*1787.2*	*BD*	*BD*	*0.329*	*1.005*	*1.493*	*BD*	-	-
(med+bas)	4	*2023.2*	*2380.2*	*1563.3*	*0.062*	*BD*	*1.495*	*1.814*	*1.887*	*BD*	-	-
	12	*2187.6*	*3578.6*	*1593.6*	*0.306*	*94.67*	*2.978*	*2.303*	*2.414*	*BD*	-	-
	18	*2238.1*	*4386.4*	*1665.7*	*0.128*	*166.10*	*1.773*	*2.481*	*2.448*	*BD*	-	-
	30	*2309.9*	*5422.8*	*1774.2*	*0.066*	*266.55*	*1.327*	*2.445*	*2.320*	*BD*	-	-
3rd Round - 50°C												
	1	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-
B	4	*1956.6*	*1253.4*	*1221.4*	*1.057*	*87.82*	*1.983*	*1.549*	*3.285*	*1.902*	-	-
(med+bas+cells)	12	*2141.5*	*1374.4*	*1339.9*	*1.130*	*129.85*	*2.709*	*1.124*	*1.444*	*0.931*	-	-
	18	*2135.6*	*1523.4*	*1349.1*	*1.735*	*104.60*	*3.034*	*1.323*	*1.153*	*0.511*	-	-
	30	*2161.4*	*1614.0*	*1351.8*	*1.170*	*84.70*	*2.594*	*1.750*	*0.876*	*BD*	-	-
	1	-	-	-	-	-	-	-	-	-	-	-
C	3	-	-	-	-	-	-	-	-	-	-	-
(med+bas)	4	*2131.0*	*1233.3*	*1620.6*	*0.197*	*BD*	*2.108*	*1.657*	*2.671*	*1.301*	-	-
	12	*2137.9*	*1420.1*	*1698.1*	*0.318*	*BD*	*1.466*	*1.468*	*2.165*	*0.580*	-	-
	18	*2184.2*	*1494.1*	*1733.2*	*0.235*	*BD*	*1.502*	*1.400*	*2.142*	*0.526*	-	-
	30	*2151.8*	*1480.4*	*1691.0*	*0.070*	*BD*	*1.142*	*1.289*	*1.850*	*BD*	-	-

Table 3.5: Element concentrations for *P. furiosus*. Experiments were conducted at 100 & 70 ºC.

GROWTH CONDITION	SAMPLING PERIOD (DAY)	ELEMENT CONCENTRATION (ppb)											
		Sr	Mo	W	Al	V	Cr	Mn	Fe	Co	Ni	Cu	Zn
A - 100 ºC (med+cells)	0	9	1.8	0.19	16	1.6	5.4	662	104	157	27	17	418
	1	10	1.6	0.01	17	1.9	5.8	694	105	162	28	8	770
	3	10	1.7	0.03	32	1.6	6.1	726	90	167	30	4	696
	4	21	1.8	0.13	28	3.9	5.6	621	581	162	33	10	466
	12	26	1.8	0.12	42	2.9	4.5	468	840	166	33	9	99
	18	27	1.6	0.13	46	2.6	4.1	427	895	163	33	8	207
	30	31	1.6	0.14	58	2.2	3.8	393	1,233	148	30	10	402
B - 100 ºC (med+bas+cells)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	19	1.9	0.01	26	3.5	4.4	664	432	160	33	38	441
	3	18	1.6	0.05	24	2.3	3.4	541	1,071	140	31	5	407
	4	26	1.7	0.09	19	4.0	3.4	417	557	146	36	13	523
	12	27	1.5	0.09	36	2.7	2.5	267	846	129	32	10	107
	18	33	1.8	0.08	53	2.8	2.7	297	1,048	155	38	11	182
	30	36	1.5	0.10	58	2.1	3.4	244	1,147	128	32	8	243
C - 100 ºC (med+bas)	0	12	1.8	0.20	74	1.8	5.1	681	113	162	28	31	374
	1	18	1.6	0.20	21	3.9	4.0	608	388	148	31	57	666
	3	19	1.6	0.20	24	2.7	3.4	566	903	144	31	78	544
	4	30	1.6	0.23	13	3.4	2.9	453	521	130	31	78	519
	12	33	1.8	0.20	29	2.6	2.5	411	897	143	36	94	110
	18	33	2.1	0.19	30	2.9	2.4	411	1,053	148	43	97	182
	30	41	1.8	0.22	48	2.8	1.7	299	1,322	120	33	4	550
A - 70 ºC (med+cells)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	10	1.7	0.20	15	2.2	5.9	674	105	159	27	14	610
	4	10	2.2	0.02	19	3.6	6.8	788	128	187	33	14	1002
	6	10	1.9	0.02	23	2.4	6.7	776	106	182	31	11	862
	7	17	1.4	0.18	29	3.7	5.5	649	700	144	29	39	444
	15	17	1.9	0.13	16	2.2	5.4	645	838	146	32	18	128
	21	24	2.0	0.10	27	3.1	6.0	714	1,242	177	37	14	142
	33	26	2.2	0.11	33	3.3	6.9	714	1,208	182	38	14	1050
B - 70 ºC (med+bas+cells)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	19	1.7	0.21	22	3.8	3.8	642	338	150	32	115	798
	4	30	1.7	0.06	8	4.0	3.7	634	1,100	148	37	73	302
	6	22	1.8	0.01	55	3.7	3.7	685	2,240	154	35	102	336
	7	21	1.7	0.03	30	3.6	3.6	622	765	147	32	99	513
	15	30	1.9	0.07	7	3.0	3.6	625	1,000	160	40	26	69
	21	33	2.2	0.06	9	3.3	3.3	589	959	156	40	17	73
	33	35	2.0	0.07	9	3.3	3.1	552	809	166	50	12	75
C - 70 ºC (med+bas)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	17	1.8	0.19	30	3.3	4.3	698	380	161	33	99	641
	4	19	2.1	0.18	26	4.0	4.1	677	644	161	33	93	614
	6	19	1.9	0.18	25	3.5	4.2	706	832	168	35	106	706
	7	28	1.8	0.19	19	4.4	4.2	661	873	161	37	134	571
	15	26	1.5	0.20	12	2.8	3.0	507	734	134	33	97	73
	21	30	2.1	0.19	15	2.4	3.4	556	767	152	38	114	87
	33	29	1.7	0.22	9	1.9	2.6	464	596	130	34	99	95

Table 3.6: Element concentrations for *M. jannaschii*. Experiments conducted at 85 & 50 ºC.

GROWTH CONDITION	SAMPLING PERIOD (DAY)	ELEMENT CONCENTRATION (ppb)											
		Sr	Mo	W	Al	V	Cr	Mn	Fe	Co	Ni	Cu	Zn
A - 85 °C (med+cells)	0	9	0.50	0.11	0	0.2	7.4	784	79	194	36	17.7	483
	1	BD	BD	BD	5	0.4	9.5	600	134	144	30	4.3	814
	6	10	0.30	0.02	21	1.3	13.1	798	172	189	35	0.2	1,260
	8	9	0.20	0.02	25	2.1	13.7	808	152	191	31	0.8	83
	9	27	0.20	0.05	75	2.5	12.7	819	2,072	187	48	12.0	180
	17	26	0.60	0.04	241	2.6	14.3	848	4,769	203	51	1.0	140
	23	23	BD	0.06	360	3.2	12.6	765	5,281	179	40	0.1	107
	35	26	0.10	0.05	517	4.2	12.5	705	5,953	167	39	BD	721
B - 85 °C (med+bas+cells)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	18	0.30	0.12	7	1.3	3.3	716	1,954	164	39	20.3	763
	6	21	0.20	0.06	49	1.0	5.0	726	3,923	163	42	BD	583
	8	19	BD	0.04	79	0.5	4.9	659	3,840	149	36	BD	51
	9	30	0.20	0.05	86	2.3	5.5	718	5,463	156	42	4.3	125
	17	35	0.20	0.05	247	2.4	6.2	718	6,755	163	44	0.4	108
	23	36	0.30	0.04	426	2.1	6.8	777	7,288	183	51	0.4	71
	35	38	0.10	0.05	505	2.8	6.0	674	7,126	159	44	1.1	822
C - 85 °C (med+bas)	0	11	0.20	0.12	4	0.3	3.1	678	138	163	32	40.0	361
	1	18	0.50	0.11	8	1.1	4.2	775	2,097	179	43	17.5	1,063
	6	20	0.20	0.13	46	1.4	5.0	734	4,046	164	42	BD	838
	8	21	0.50	0.11	109	0.8	5.6	814	4,893	187	47	0.1	164
	9	28	0.30	0.15	82	1.9	6.0	743	5,648	164	46	4.4	129
	17	33	0.30	0.13	281	2.6	6.9	802	7,247	188	52	BD	134
	23	35	0.40	0.12	474	2.3	7.4	795	7,913	185	53	0.4	132
	35	36	0.30	0.51	595	2.2	6.4	730	8,181	168	51	BD	79
A - 50 °C (med+cells)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	9	18	0.20	0.03	203	1.7	11.7	747	2,204	167	35	92.7	119
	17	22	0.60	0.04	77	3.2	12.2	896	6,894	192	48	134.1	115
	23	22	0.60	0.03	86	2.9	12.1	919	8,469	201	52	91.2	101
	35	24	0.40	0.05	63	1.6	10.6	813	7,980	174	47	85.2	186
B - 50 °C (med+bas+cells)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	9	29	0.40	0.05	462	2.4	6.6	850	6,399	185	49	46.5	117
	17	33	0.50	0.05	262	3.8	8.3	867	8,361	190	53	29.6	96
	23	34	0.70	0.05	249	3.9	7.2	900	9,447	196	56	16.2	104
	35	35	0.30	0.06	197	3.3	6.0	750	8,617	160	48	5.7	158
C - 50 °C (med+bas)	0	-	-	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	9	30	0.50	0.12	363	3.0	6.5	830	6,334	186	51	97.4	142
	17	32	0.70	0.13	213	3.8	6.3	870	8,805	186	56	77.7	126
	23	31	0.40	0.13	231	4.1	6.3	882	9,417	187	56	31.3	169
	35	34	0.60	0.12	243	4.0	7.0	921	10,849	197	61	28.6	456

Table 3.7: Element concentrations for *P. calidifontis*. Experiments conducted at 90 & 50 ºC.

GROWTH CONDITION	SAMPLING PERIOD (DAY)	ELEMENT CONCENTRATION (ppb)								
		Ca	Mg	Mo	Sr	Ni	Fe	W	Cr	Si
A - 90 ºC (med+cells)	0	136.4	137	1.11	1.6	0.6	4	0.17	1.3	174
	1	BD	459	1.7	1.3	4.0	14	BD	1.6	BD
	3	BD	182	1.06	1.6	0.6	6	BD	1.2	BD
	5	0.8	200	1.05	1.7	2.2	4	BD	1.4	BD
	7	BD	323	1.41	1.2	0.6	2	BD	0.5	BD
	12	BD	187	1.46	1.9	3.5	7	BD	0.8	BD
	15	BD	361	0.96	2.1	1.2	26	BD	0.7	BD
	21	BD	619	0.84	3.2	0.8	47	BD	0.8	BD
	32	BD	886	1.5	3.7	2.7	51	BD	0.8	BD
B - 90 ºC (med+bas+cells)	0	-	-	-	-	-	-	-	-	-
	1	3.6	182	1.09	1.9	0.5	24	BD	1.2	BD
	3	1.6	262	1.32	1.7	4.6	12	BD	8.0	BD
	5	4.0	260	1.92	1.8	8.6	10	BD	8.3	BD
	7	BD	291	0.86	1.4	2.1	4	BD	3.6	BD
	12	3.8	257	0.9	2.3	4.6	15	BD	7.2	BD
	15	1.8	407	1.01	3.2	4.5	37	BD	6.3	BD
	21	BD	746	0.84	3.5	3.1	55	BD	5.1	BD
	32	BD	672	0.79	3.1	3.2	38	BD	3.5	BD
C - 90 ºC (med+bas)	0	172.2	1015	4.22	2.1	13.9	41	0.01	3.7	84
	1	6.3	149	1.1	1.9	0.5	26	BD	1.3	BD
	3	3.9	190	1.28	2.0	2.0	13	BD	3.7	BD
	5	BD	275	1.12	1.7	1.9	7	BD	2.9	BD
	7	BD	367	1.96	2.2	2.2	11	BD	2.8	BD
	12	BD	223	1.87	2.1	2.4	20	BD	2.5	BD
	15	BD	502	1.03	2.7	2.5	44	BD	2.2	BD
	21	4.7	781	0.88	5.1	2.3	67	BD	3.0	BD
	32	BD	1083	1.26	3.9	1.4	53	BD	1.8	BD
A - 50 ºC (med+cells)	0	-	-	-	-	-	-	-	-	-
	1	BD	105	1.01	0.7	0.5	5	BD	1.6	BD
	3	1.4	148	1.19	1.8	1.1	10	BD	1.5	BD
	5	BD	157	0.95	1.6	1.4	9	BD	1.1	BD
	7	BD	185	1.03	1.6	0.6	8	BD	1.1	BD
	12	BD	188	1.12	1.5	0.4	7	BD	0.9	BD
	15	BD	154	1.65	1.1	0.8	5	BD	0.7	BD
	21	BD	252	1.09	1.2	0.4	5	BD	0.8	BD
	32	BD	196	1.01	1.1	0.5	3	BD	0.6	BD
B - 50 ºC (med+bas+cells)	0	-	-	-	-	-	-	-	-	-
	1	0.2	232	0.7	1.0	1.8	21	BD	1.1	BD
	3	2.5	281	0.84	1.9	4.8	29	BD	8.6	BD
	5	6.7	269	1.48	2.0	7.3	28	BD	9.7	BD
	7	BD	335	1.12	1.9	5.4	23	BD	8.2	BD
	12	BD	235	1.1	1.6	4.6	18	BD	6.4	BD
	15	BD	233	0.81	1.4	3.1	13	BD	5.2	BD
	21	BD	279	1.05	1.3	2.9	10	BD	5.0	BD
	32	BD	296	0.85	1.0	2.3	8	BD	3.6	BD
C - 50 ºC (med+bas)	0	-	-	-	-	-	-	-	-	-
	1	0.5	175	0.94	0.9	0.4	27	BD	1.1	BD
	3	0.9	280	1.31	1.8	2.8	21	BD	4.0	BD
	5	BD	348	0.88	1.1	0.9	8	BD	1.8	BD
	7	BD	304	1.18	1.6	1.7	14	BD	3.3	BD
	12	BD	242	1	1.4	2.3	14	BD	2.7	BD
	15	BD	375	0.88	1.2	1.5	10	BD	2.2	BD
	21	BD	269	1.08	1.2	1.6	8	BD	2.4	BD
	32	BD	333	1.53	1.0	1.2	6	BD	1.7	BD

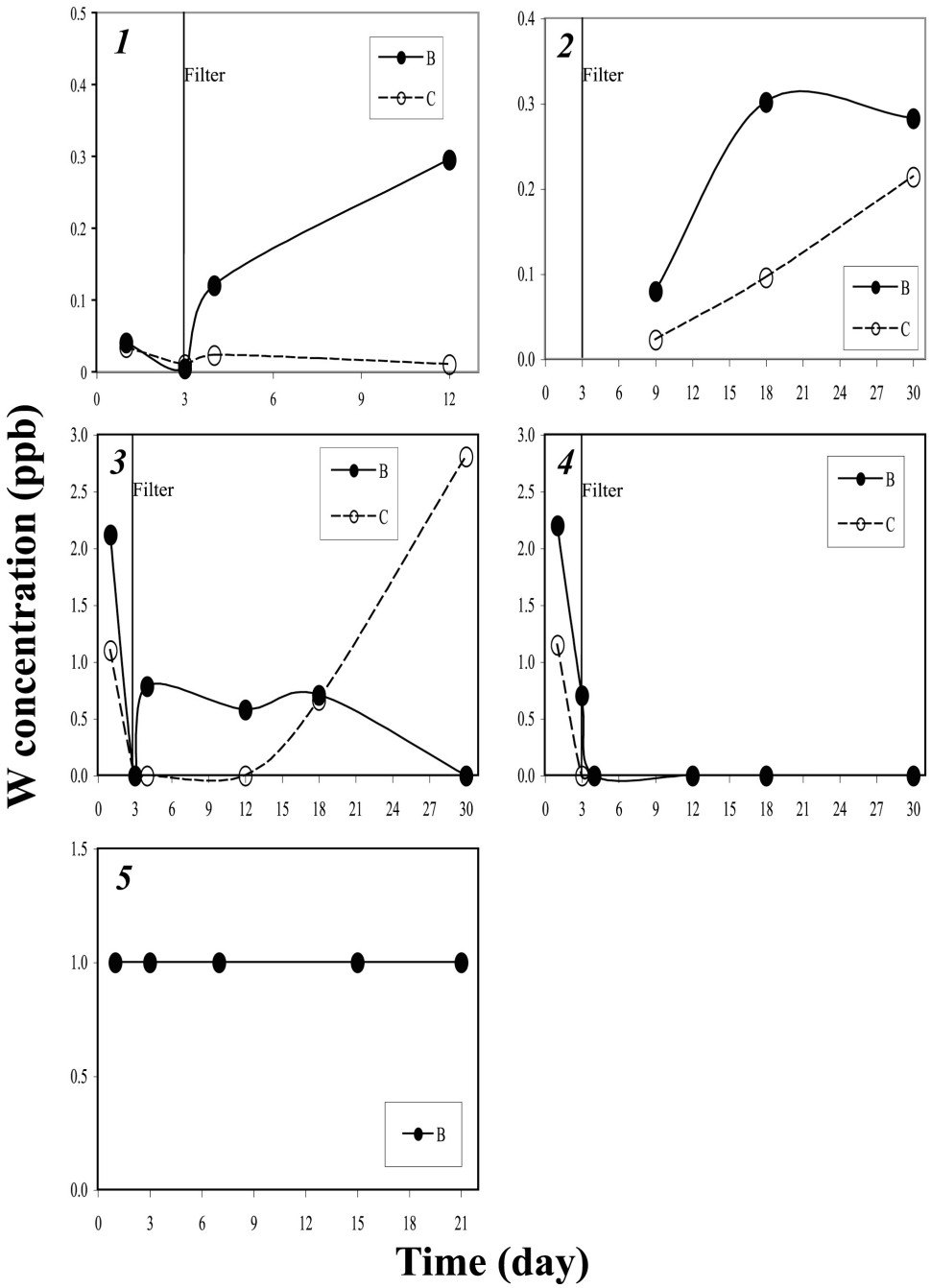

Figure 3.1: W concentration for *P. aerophilum* (a). Numbers (***1***, ***2***, etc.) indicate each round of experiments. All experiments started at 100 ^{O}C; round 4 after filtration was incubated at 50 ºC. Evidence for the production of a W ligand is only observed in experiment 1 in which the concentration of W in the biotic experiment is greater than the abiotic experiment. B (medium+cells+basalt); C (medium+basalt).

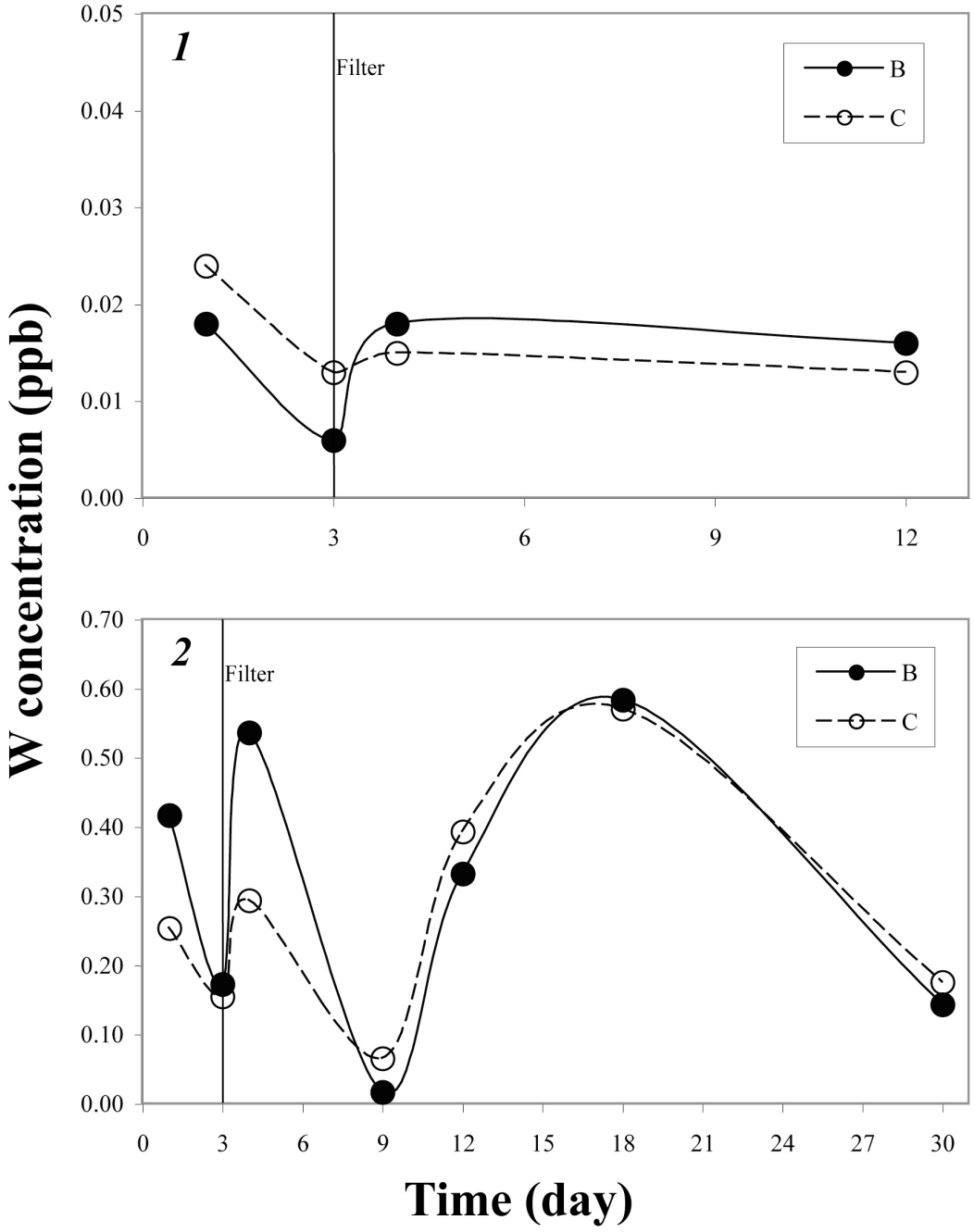

Figure 3.2: W concentration for *P. aerophilum* (an), rounds 1 & 2. Values for round 3 were below detection. All experiments done at 100 ºC. B (medium+cells+basalt); C (medium+basalt).

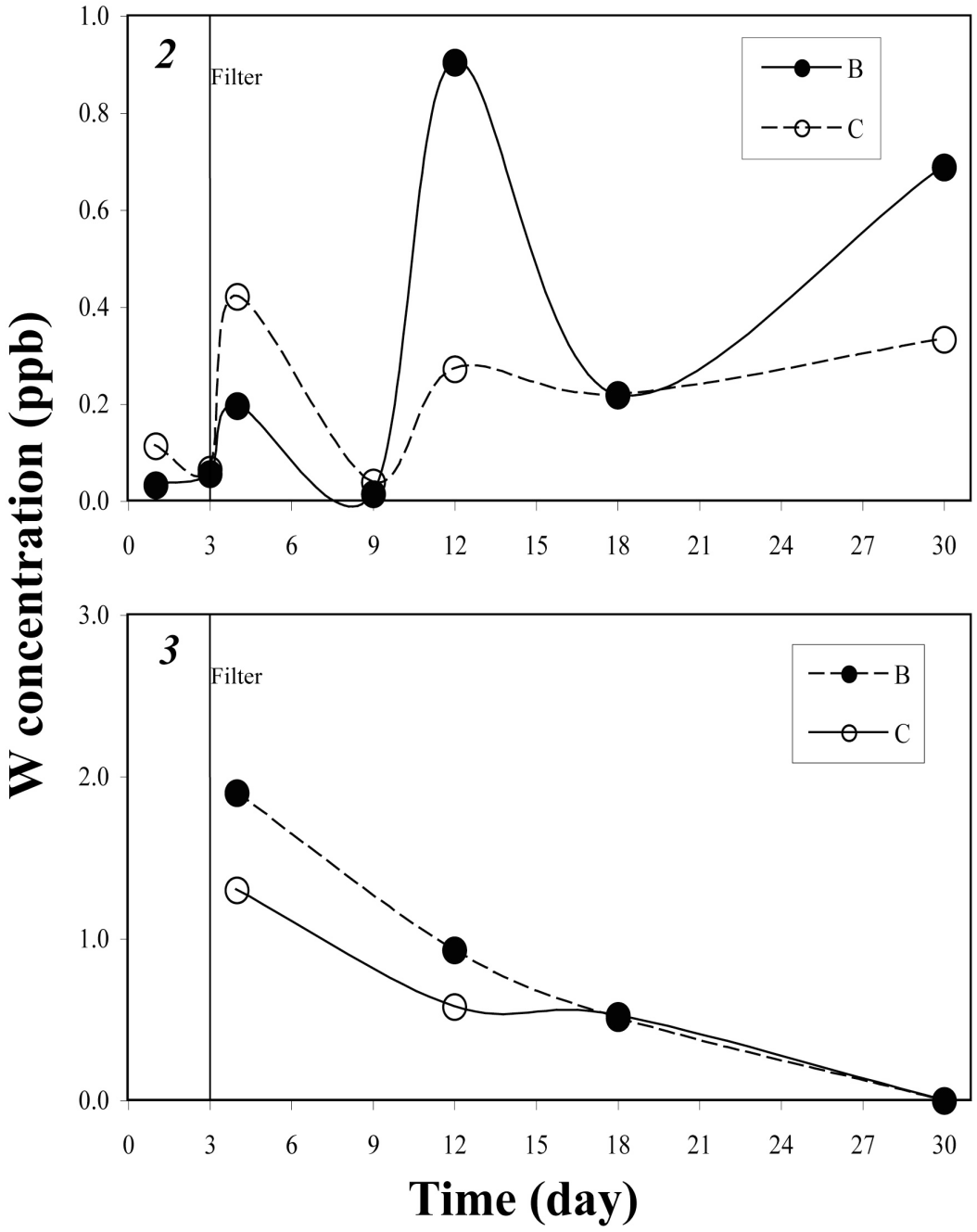

Figure 3.3: W concentration for *P. islandicum* for rounds 2 & 3 (50 ºC). Values for rounds 1 & 3 (100 ºC) were below detection. All experiments started at 100 ºC. B (medium+cells+basalt); C (medium+basalt).

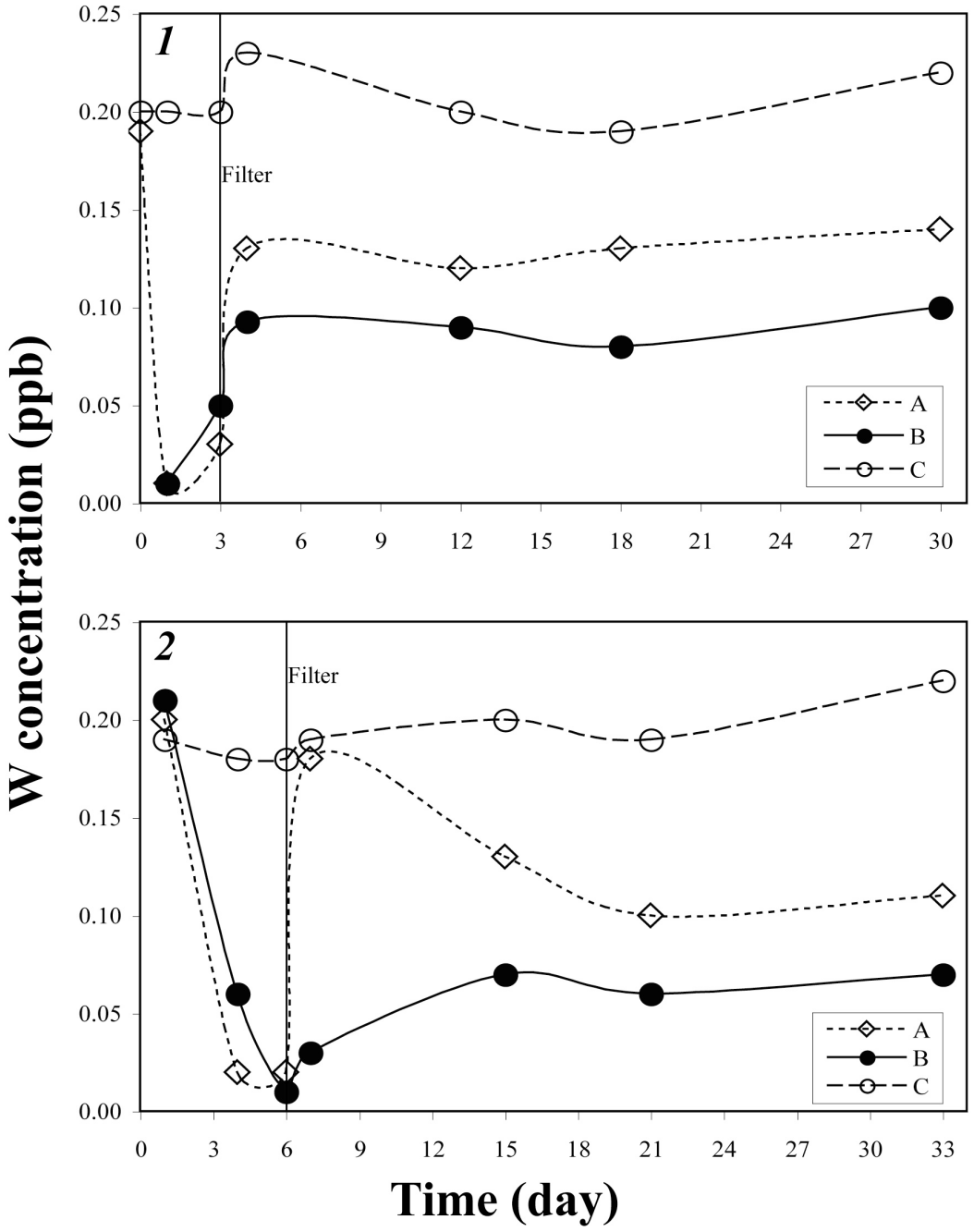

Figure 3.4: W concentration for *P. furiosus*. Round ***1*** (100 ºC); ***2*** (70 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

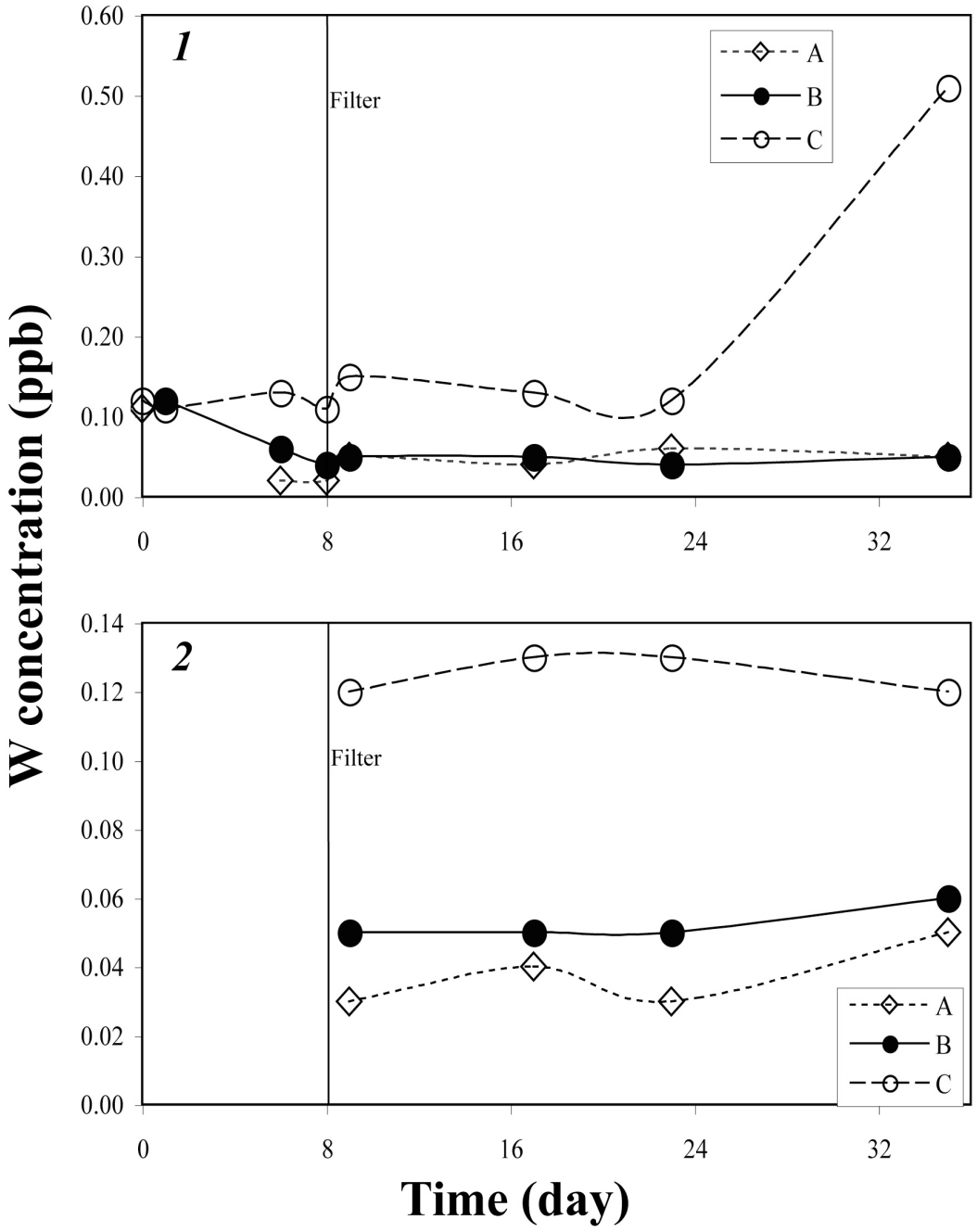

Figure 3.5: W concentration for *M. jannaschii*. Round ***1*** (85 ºC); ***2*** (50 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

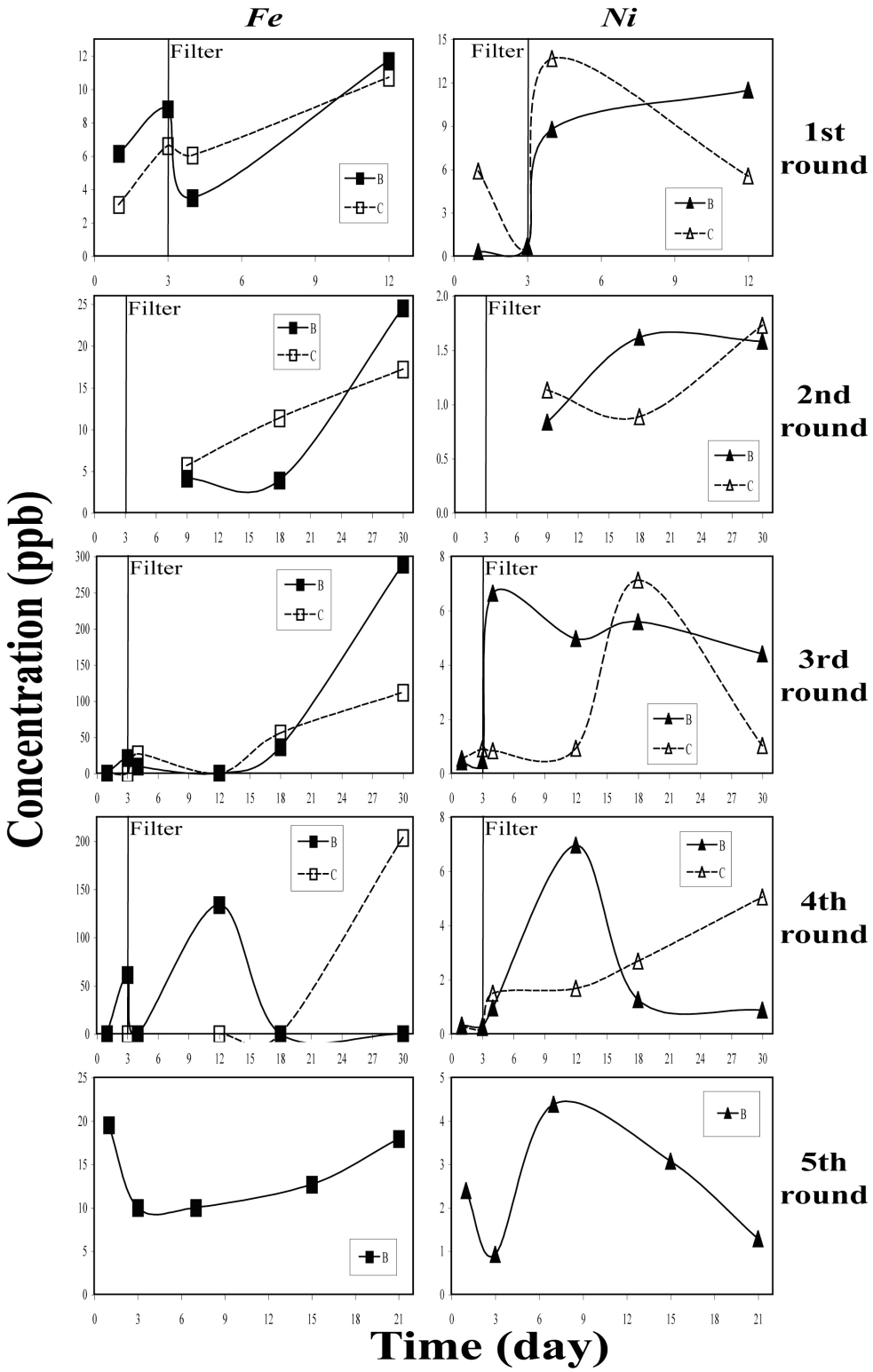

Figure 3.6: Fe & Ni concentrations for *P. aerophilum* (a). All experiments started at 100 ºC; round 4 after filtration was incubated at 50 ºC. B (medium+cells+basalt); C (medium+basalt).

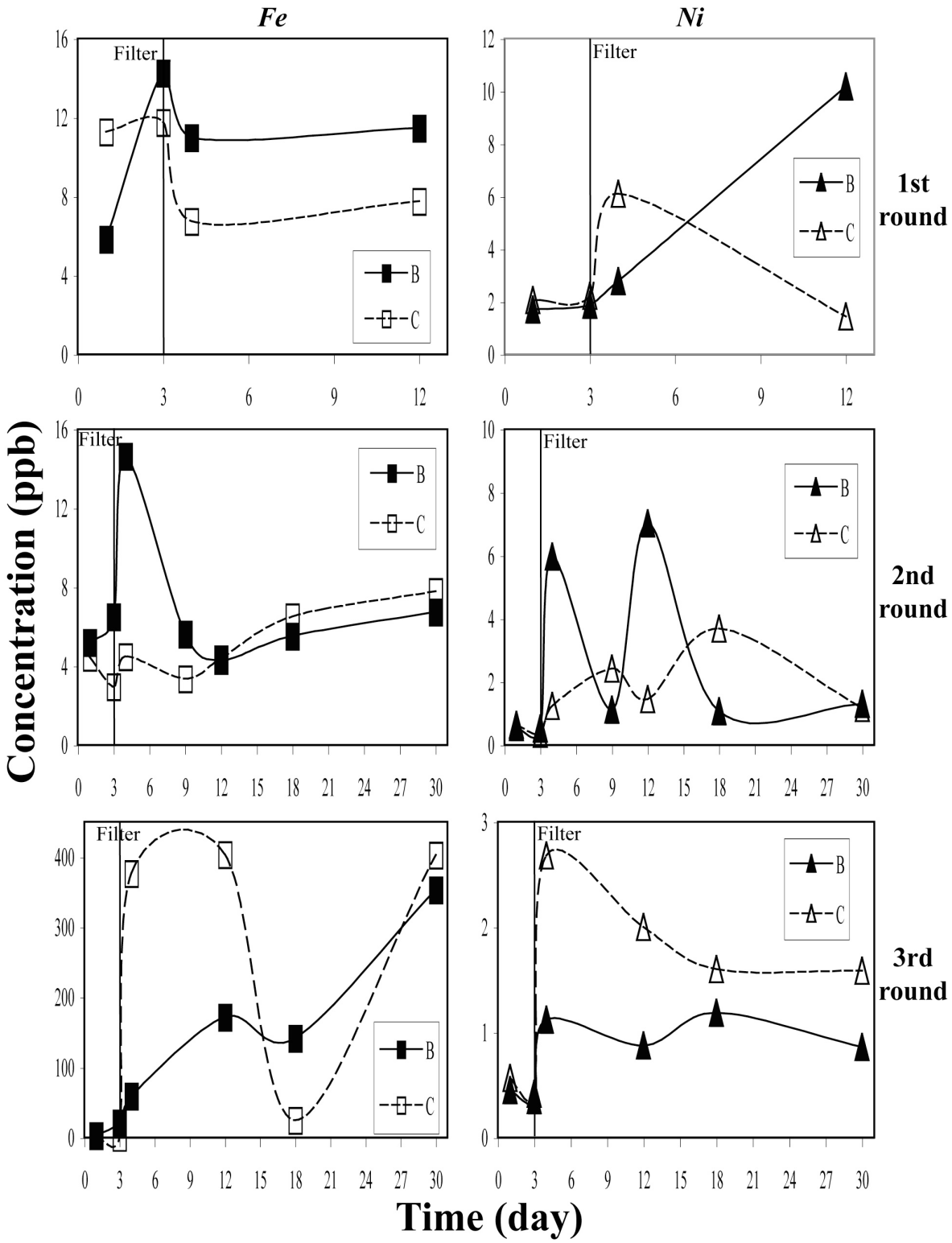

Figure 3.7: Fe & Ni concentrations for *P. aerophilum* (an). All experiments done at 100 ºC. B (medium+cells+basalt); C (medium+basalt).

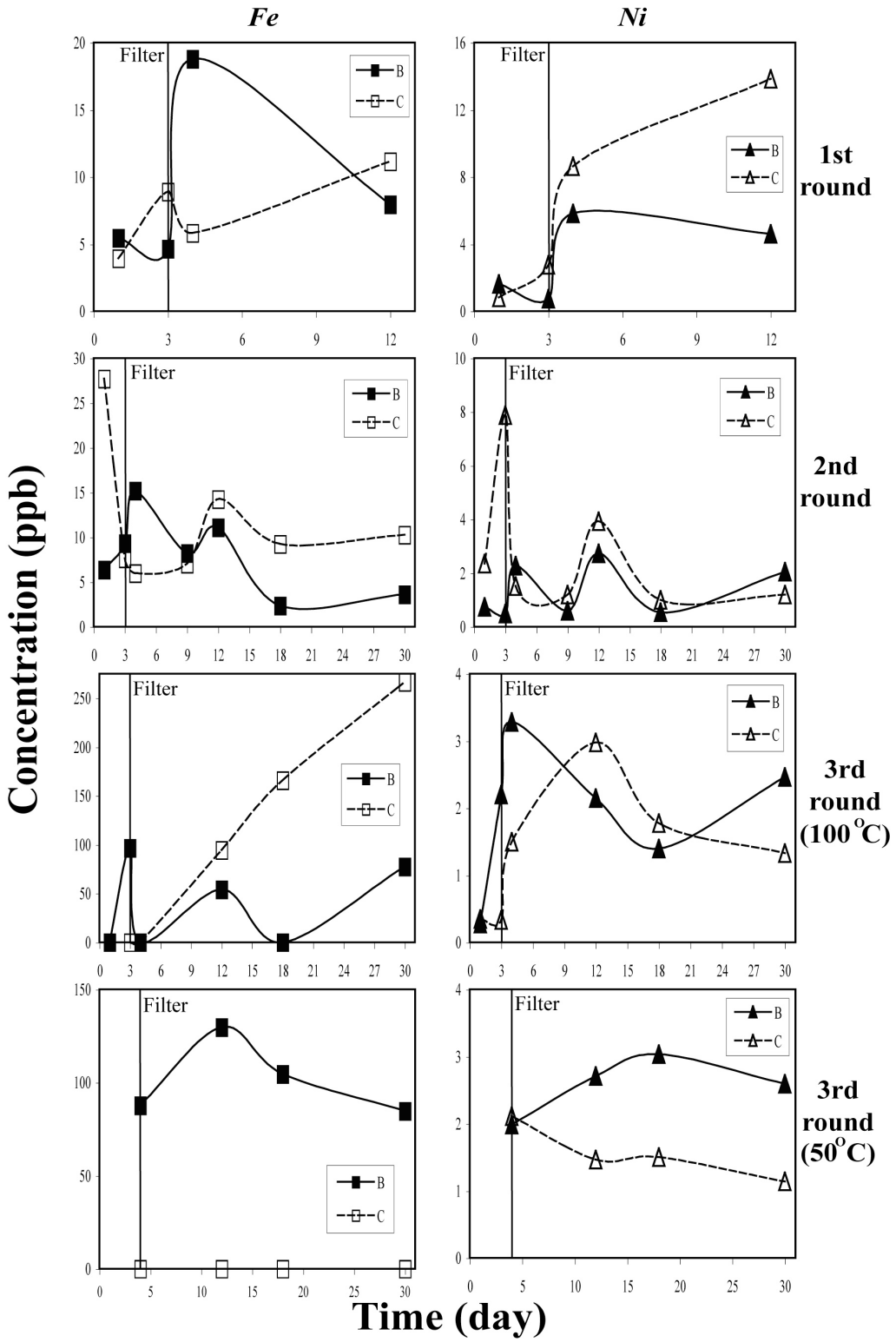

Figure 3.8: Fe & Ni concentration for *P. islandicum*. B (medium+cells+basalt); C (medium+basalt).

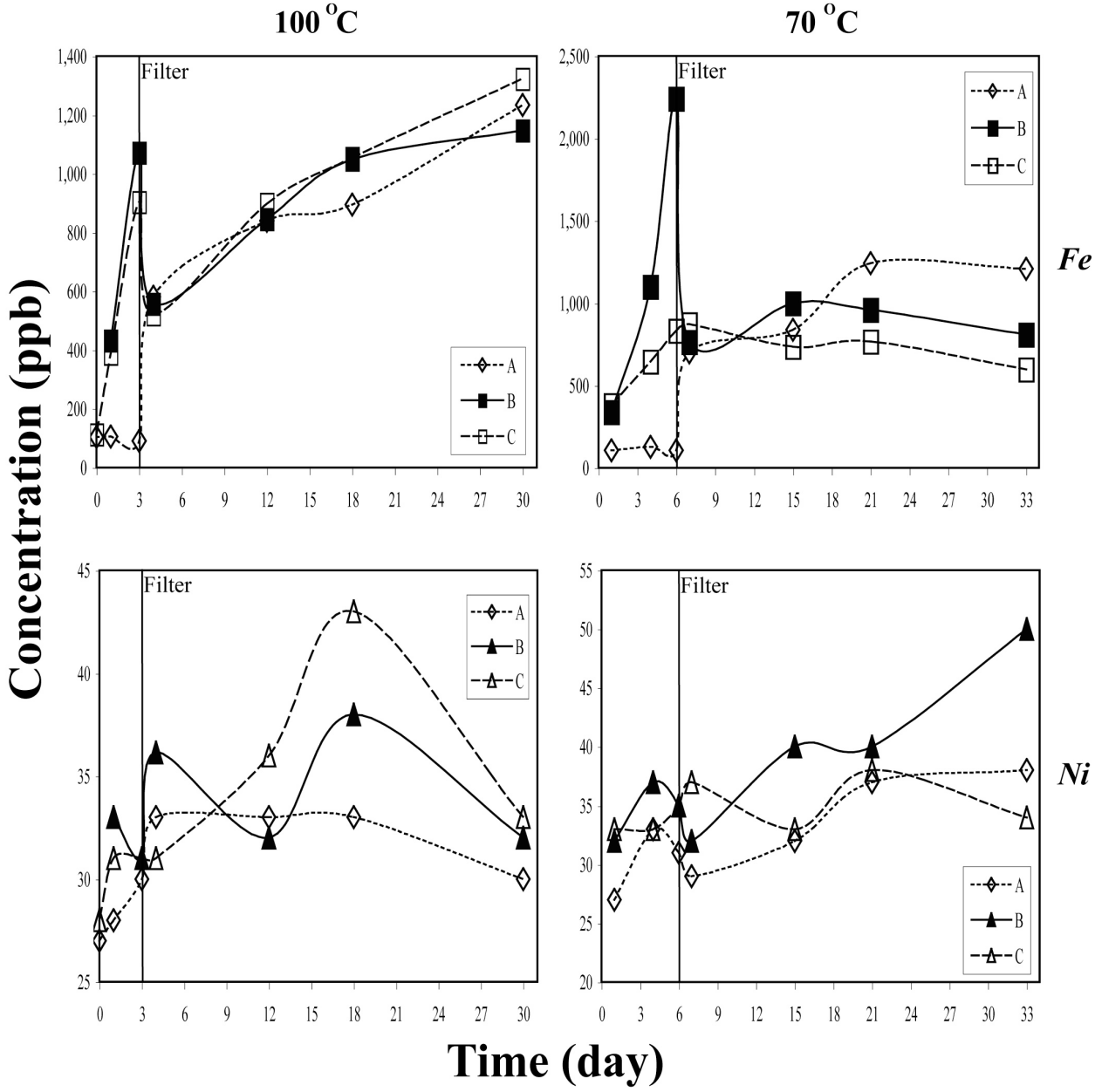

Figure 3.9: Fe & Ni concentrations for *P. furiosus*. Round *1* (100 ºC); *2* (70 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

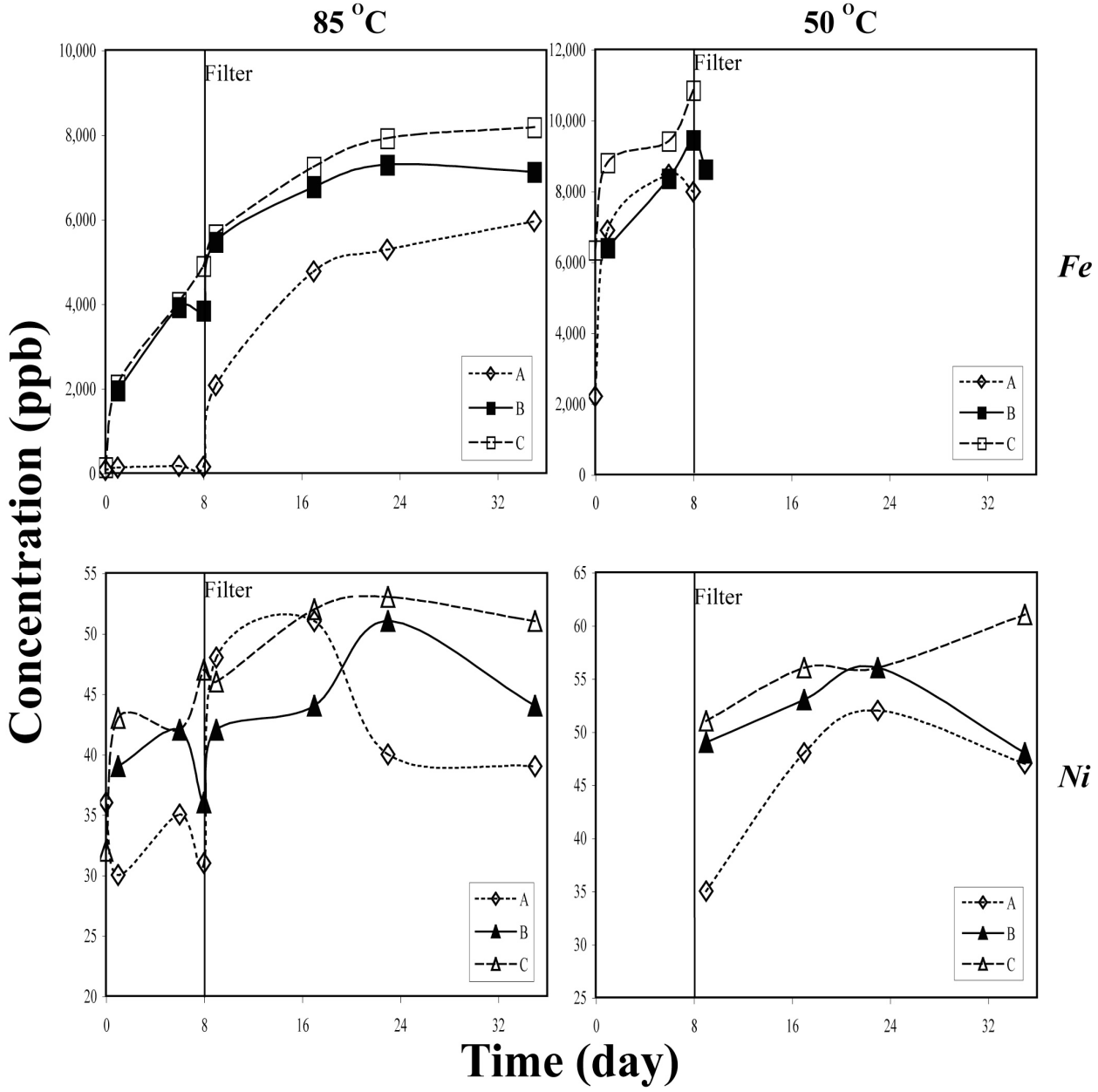

Figure 3.10: Fe & Ni concentrations for *M. jannaschii*. Round *1* (85 ºC); *2* (50 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

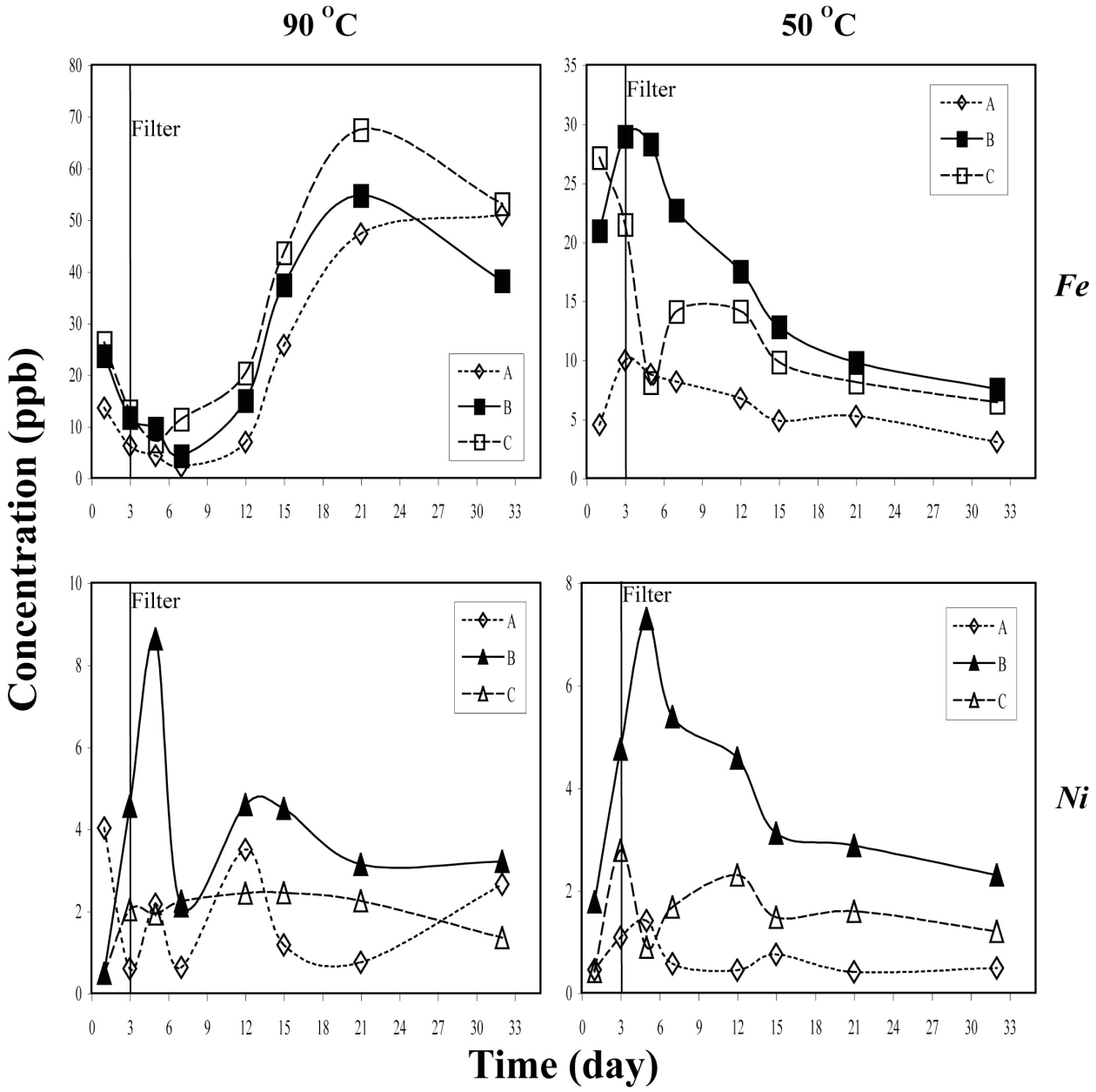

Figure 3.11: Fe & Ni concentrations for *P. calidifontis*. Round *1* (90 ºC); *2* (50 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

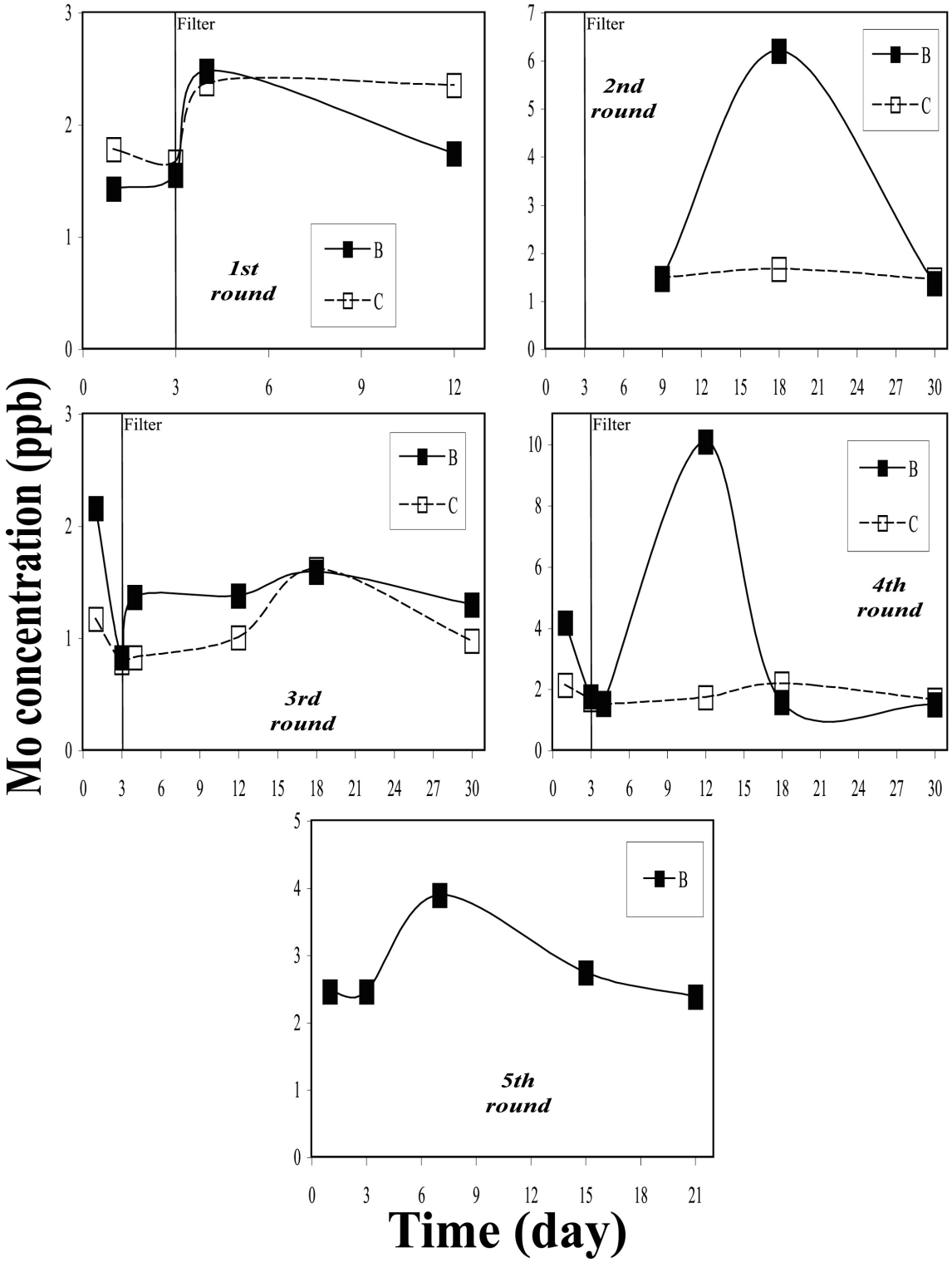

Figure 3.12: Mo concentration for *P. aerophilum* (a). All experiments started at 100 ºC; round 4 after filtration was incubated at 50 ºC. B (medium+cells+basalt); C (medium+basalt).

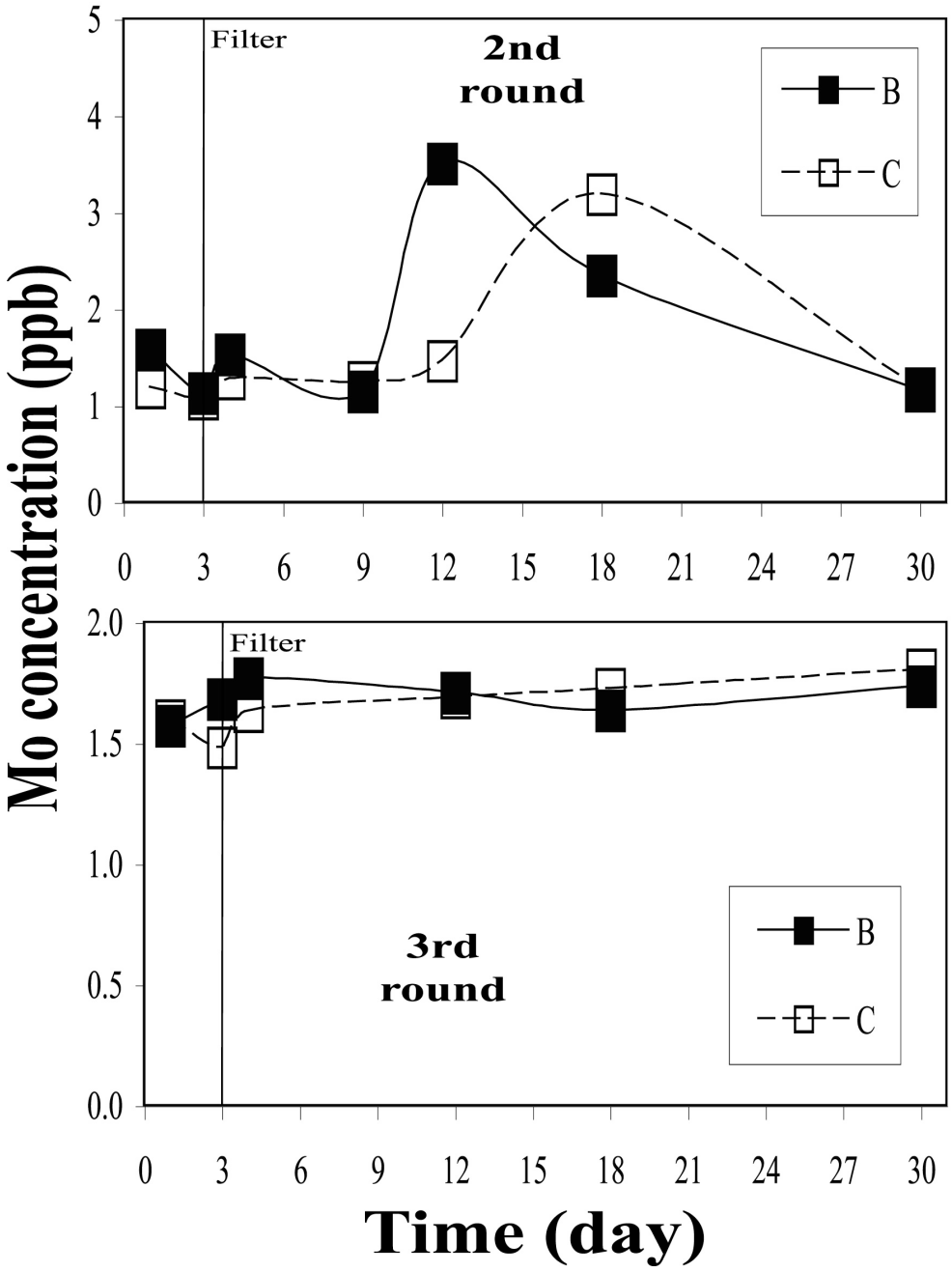

Figure 3.13: Mo concentration for *P. aerophilum* (an). All experiments done at 100 ºC. B (medium+cells+basalt); C (medium+basalt).

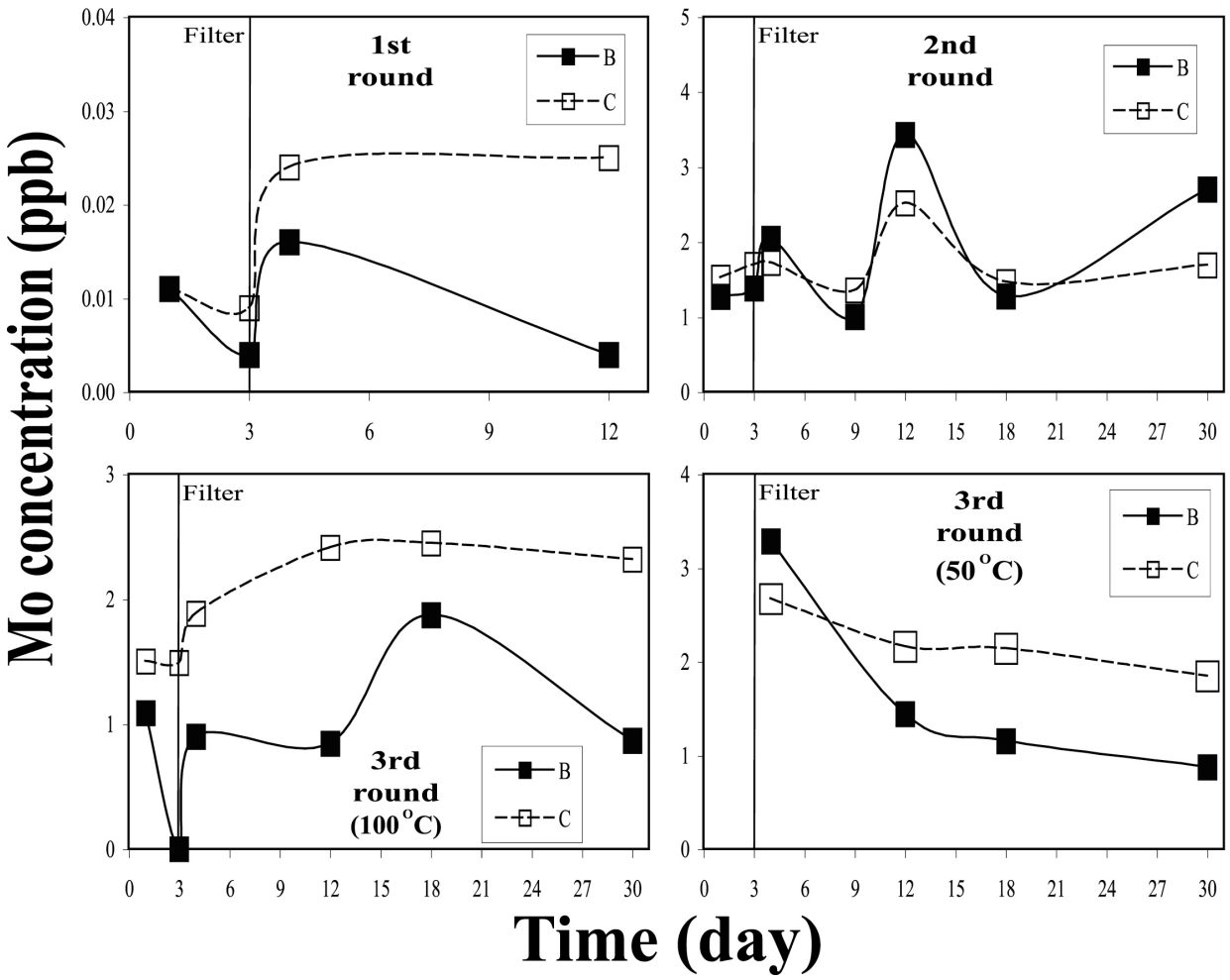

Figure 3.14: Mo concentration for *P. islandicum*. B (medium+cells+basalt); C (medium+basalt).

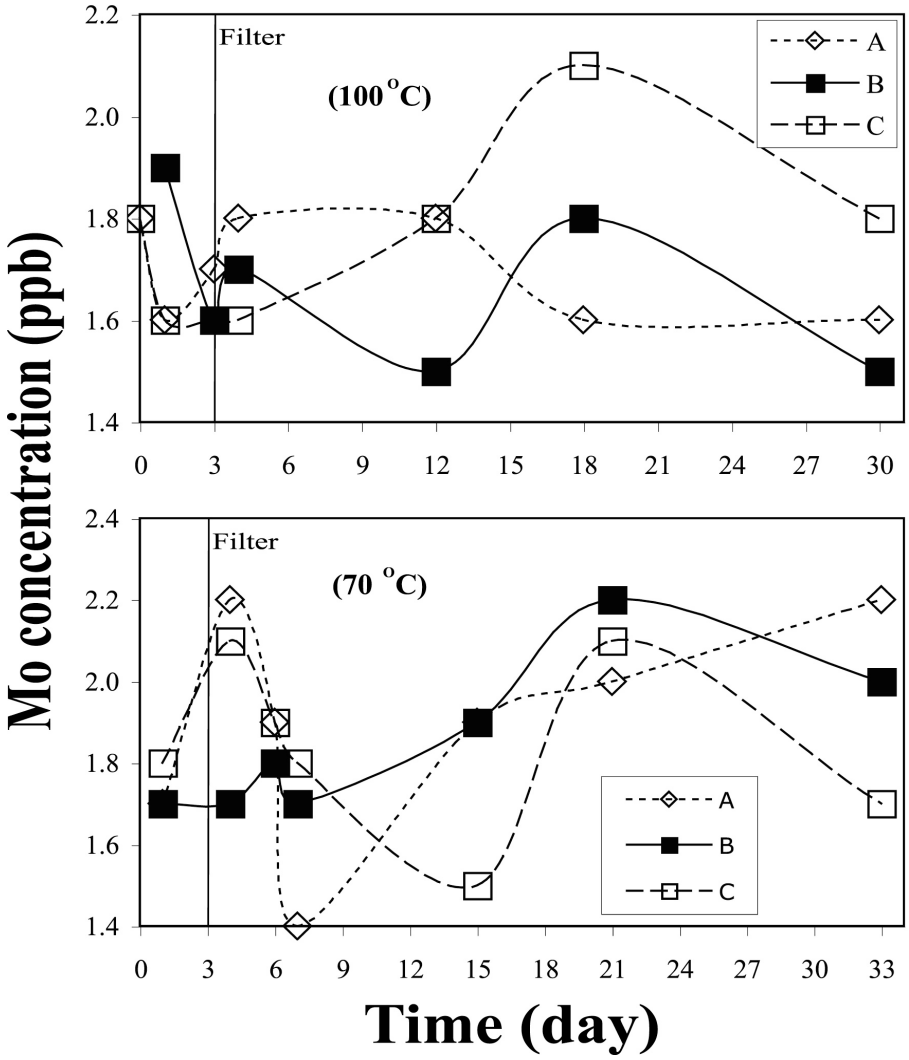

Figure 3.15: Mo concentration for *P. furiosus*. Round *1* (100 ºC); *2* (70 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

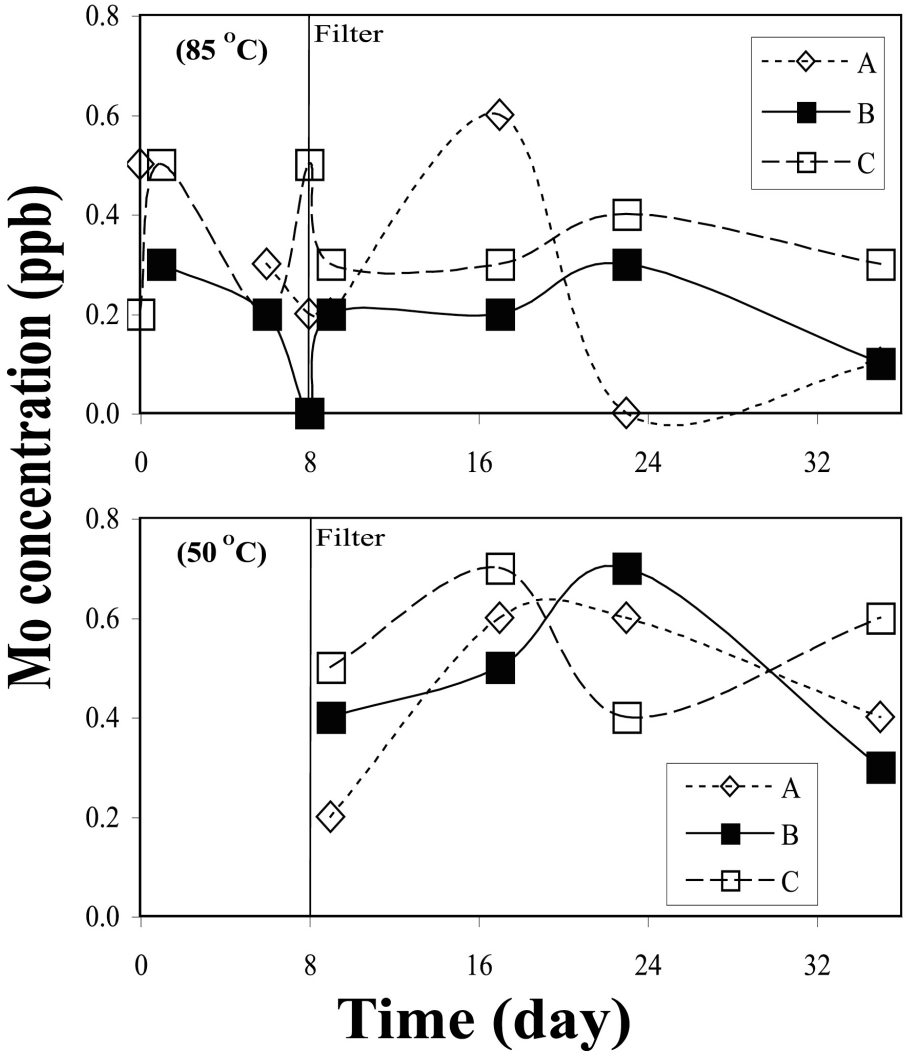

Figure 3.16: Mo concentration for *M. jannaschii*. Round *1* (85 ºC); *2* (50 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

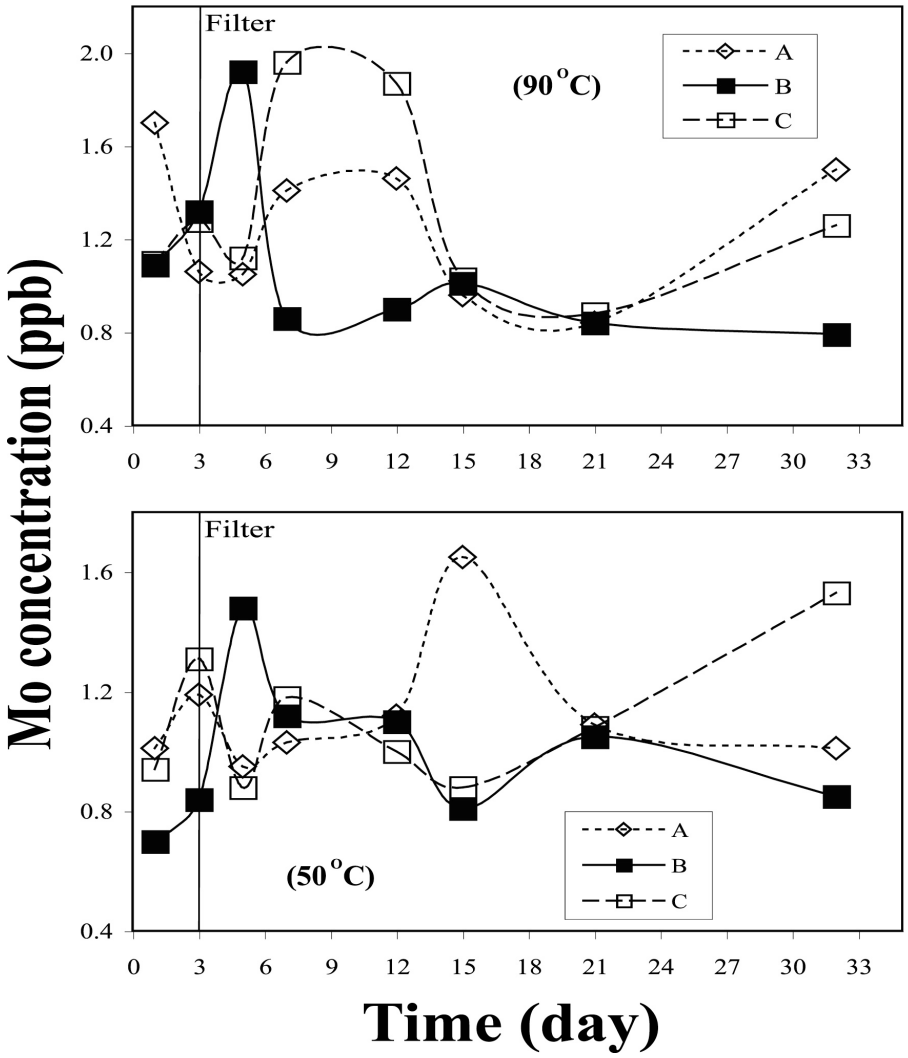

Figure 3.17: Mo concentration for *P. calidifontis*. Round *1* (90 ºC); *2* (50 ºC). A (medium+cells); B (medium+cells+basalt); C (medium+basalt).

Chapter 4

Nickel Stable Isotopes: A Novel Isotope Biomarker

4.1 Abstract

Novel Ni stable isotope investigations were carried out in geological and biological materials. In addition to establishing mass spectrometry protocols for analyzing Ni, our methods also involved the development and application of procedures for hyperthermophilic culturing as well as the separation and purification of Ni from complex media. Compared to other transition metal isotope systems, biological Ni stable isotope fractionation may well prove to be the first true unambiguous trace metal biomarker. The results are remarkable and can be summarized as follows. (1) Basalt standards, mid-ocean ridge basalts, loess and river samples representing the bulk solid Earth vary within a very small range of Ni isotopic composition (-0.04 to +0.34) and have an average δ^{60}Ni of 0.15 ± 0.24 ‰. (2) Ni stable isotopic compositions for a suite of stony and iron meteorites are less variable (0.17 ‰) than the terrestrial samples (0.38 ‰) displaying values of +0.19 to +0.36 ‰ and, taken together with the terrestrial samples, provides additional evidence for a homogenous Ni reservoir in the early solar system and in the silicate portion of the Earth. (3) In stark contrast to the geological materials and relative to the starting composition of the growth media, methanogens (*M. barkeri*, *M. acetivorans* and *M. jannaschii*) impart a distinct and significant mass-dependent fractionation on Ni isotopes, preferentially sequestering the lighter isotopes which results in an isotopic fractionation on the order of ~ 0.8 - 1.0 ‰. Cells of the hyperthermophile *P. calidifontis* also produced a small fractionation on Ni isotopes (average of 0.07 ‰). The results of the growth experiments suggest Ni isotopes are probably

fractionated by life, in general but not as significantly as the fractionation produced by methanogens. Additionally, our data suggests different groups of methanogens may fractionate Ni to varying degrees, with the largest fractionation being produced by the hyperthermophiles. Further work will be needed before this can be unequivocally substantiated but if true, Ni isotopes may be useful, not only as a general methanogenic biomarker but also as a marker for distinguishing between diverse metabolic groups. This work will add to ongoing studies that continue to expand the relatively recent area of transition metal isotopes. Ni isotope fractionation may have the potential to become an important marker or tracer for biological and geological processes or geochemical/biochemical interactions, in modern and ancient environments. The Ni isotope system might also be useful in pinpointing which metabolisms and hence, microorganisms were important on the early Earth and allow for extrapolations to be made in determining the possibility of life on other planets, such as Mars.

4.2 Introduction

The impact of life in the ~ 4.5 Ga of Earth's geological evolution and the resulting biosignatures revealed for deciphering and understanding this history, the terrestrial evolution of life itself and as a tool in facilitating the search for life elsewhere in the universe, cannot be overstated. In addition to a multitude of physical and morphological evidence (AWRAMIK, 1992; AWRAMIK and BARGHOORN, 1977; BARGHOORN and SCHOPF, 1965; BRASIER et al., 2002; SCHOPF et al., 2002; SCHOPF and PACKER, 1987; WESTALL, 1999; WESTALL et al., 2001), life has imprinted the geological record with a variety of chemical and isotopic markers (BROCKS et al., 2003; BROCKS et al., 1999; HINRICHS, 2002; MOJZSIS et al., 1996; ONO et al., 2003; SCHIDLOWSKI, 2001; SHEN et al., 2001; SUMMONS et al., 1999; TICE and LOWE, 2004; TICE and

LOWE, 2006). In this respect, the application and role of transition metal isotopes as biosignatures is relatively new and thus far, intensely studied systems endeavoring to be potential markers for life, such as Fe (ANBAR, 2004; BEARD et al., 1999; WASYLENKI et al., 2007) and Mo (BARLING et al., 2001), have proven ambiguous.

Nickel is a first row transition metal (d-block) and can occupy a range of oxidation states from 0 to +4, though +2 is by far the most common and, essentially the only natural oxidation state. It has five naturally occurring stable isotopes ^{58}Ni, ^{60}Ni, ^{61}Ni, ^{62}Ni, and ^{64}Ni with respective relative abundances of 68.08%, 26.22%, 1.14%, 3.63% and 0.93%. Nickel is typically partitioned into the metal-rich phase of iron-type meteorites and, to date, Ni isotope studies have focused exclusively on extraterrestrial materials and the search for radiogenic ^{60}Ni, the decay product of extinct ^{60}Fe (BIRCK and LUGMAIR, 1988; COOK et al., 2008; COOK et al., 2006; MORAND and ALLEGRE, 1983; MOYNIER et al., 2007; QUITTE and OBERLI, 2006; SHIMAMURA and LUGMAIR, 1983). Within analytical uncertainties, these studies have demonstrated a fairly homogenous Ni isotopic composition for the early solar system, though two studies report moderate stable isotope variability for specific meteoritic fractions (COOK et al., 2008; MOYNIER et al., 2007). The Earth's primary Ni reservoir is found in the core of the planet and the metal's emplacement in the crust occurs principally through magmatic processes. On account of its dual siderophile (iron) and chalcophile (sulfur) properties, Ni commonly forms two types of ores, sedimentary laterites produced from the weathering of ultramafic rocks and magmatic sulfide deposits.

Inputs of Ni to the ocean are mainly through rivers and dust (BOYLE et al., 1981; Bruland 1980). With a relatively short residence time of 10,000 years (SCLATER et al., 1976), Ni displays a nutrient-like vertical distribution in the open ocean and has a regeneration cycle at shallow

depths and in the deep ocean. Surface seawater has <1 to 3 nmol/kg Ni and deep waters, ~ 11 nmol/kg. These variations correlate with patterns observed for phosphate and silicate which reach maximum values at shallow and deep waters, respectively (BRULAND, 1980; NOLTING et al., 1999; SCLATER et al., 1976). Deep ocean hydrothermal sites hosting ultramafic rocks undergoing serpentinization processes, such as the Rainbow vent field (MARQUES et al., 2007) on the Mid Atlantic Ridge may also be important systems for Ni investigations as large concentrations have been documented for the evolved fluids, altered minerals and sediments. Reported values for hydrothermal fluids primarily associated with high temperature black smoker vents are greatly enriched, ranging from 0.01 to 53 mmol/kg Ni (VON DAMM, 1995; KELLEY et al., 2002).

Life requires metals. A few of the ~ 10 bioessential trace metals (MADIGAN et al., 2000; FAUSTO DA SILVA and WILLIAMS, 2001) like Fe and Zn, are universal. Others tend to have greater significance to particular organisms or in regards to specific functions. Nickel falls within this latter category. Microorganisms utilize trace metals typically as micronutrients and notably, for vital roles in enzymes and the amounts needed are analogous to the metal concentration in seawater (FAUSTO DA SILVA and WILLIAMS, 2001). Trace metals make enzymes function: they are commonly the active sites in enzymes and are also needed for coenzymes and cofactors. There are seven known Ni enzymes, six of which have been identified (WATT and LUDDEN, 1999). All function as catalysts in very specific metabolisms, predominantly in prokaryotic microorganisms. Only one, urease, is utilized by higher-order organisms (plants, fungi) for nitrogen fixation (FAUSTO DA SILVA and WILLIAMS, 2001). From a functional and evolutionary perspective, the absolute requirement for Ni by methanogens and particularly by one very special enzyme, exemplifies what could be termed, a class-specific metal dependency. At least three Ni

enzymes, methyl-coenzyme M reductase (MCR), carbon monoxide dehydrogenase/acetyl coenzyme A synthase (CODH/ACS) and hydrogenase are found in methanogens. Briefly, methanogenesis is the process by which methanogens generate energy via a stepwise reduction of single carbon substrates; concomitantly, methane is produced as a waste product. Regardless of the reductive pathway used, the terminal process of methane formation involves the MCR catalyzed reaction of two coenzymes, methyl-coenzyme M (CH_3-SCoM) and coenzyme B (HS-CoB). The active site of MCR is a unique Ni cofactor, F_{430}, which is found only in methanogens and whose only known function is the catalytic activity within MCR (ERMLER, 2005; THAUER, 1998; WATT and LUDDEN, 1999). An analogue of MCR that catalyzes reverse methanogenesis, the anaerobic oxidation of methane (AOM), has been proposed (KRUGER et al., 2003) but its identity, structure and function remains unknown. Another intriguing Ni enzyme is the bifunctional CODH/ACS cluster which catalyzes the reversible oxidation of CO to CO_2 as well as the synthesis and/or degradation of acetyl-CoA (FERRY, 1993). Microorganisms possessing these enzymes utilize the reductive acetyl-CoA pathway (Wood-Ljungdahl) for growth and are thought to be evolutionarily primitive, a concept for which there are several lines of support (HUBER and WACHTERSHAUSER, 1997; LINDAHL and CHANG, 2001).

The advent of high-precision multi collector inductively coupled plasma mass spectrometry (MC-ICP-MS) has facilitated remarkable growth in the field of stable isotope geochemistry. In particular, multiple studies have been conducted of the more common transition metals such as Fe, Mo, Zn, Cu, and Se. The relative importance of these investigations to cosmo-, geo- and biochemical processes is wide-ranging and is based to a large extent on the chemistry, behavior and interactions of each element in the environment of interest. Less routine and perhaps more slow to develop has been the application of stable isotope measurements in

geomicrobiological studies. Progress in this area has been hampered for a variety of reasons, including instrumental and chemical processing limitations as well as problems associated with microbial growth experiments or manipulation of environmental samples. The very few but notable transition metal stable isotopes documenting fractionations in biological systems include Fe (ARCHER AND VANCE, 2006; BEARD et al., 1999; BRANTLEY et al., 2001), Se (HERBEL et al., 2000) and Cu (ZHU et al., 2002).

Here, I present novel measurements of Ni stable isotopes in geological and biological materials. Preliminary results suggest a role for Ni isotopic fractionation as the first true unambiguous trace metal biomarker. Additionally, the Ni isotope system may have significant potential for studies investigating terrestrial materials as well as interactions between the geosphere and biosphere.

4.3 Materials and Methods

All work was conducted at the University of Bristol, using facilities housed within the Department of Earth Sciences. Complete descriptions of all procedures employed in this study follows.

All sample digestions and chemical procedures were carried out under clean laboratory conditions in "Class 100" cleanhoods utilizing only Savillex (Minnetonka, MN, USA) PFA labware that was thoroughly cleaned in hot concentrated nitric acid between samples. Cell growth experiments and collection of all biological samples were carried out in the geomicrobiological laboratory of Dr. Edward Hornibrook (Department of Earth Sciences, University of Bristol), prior to digestion and chemical processing. Further details of these procedures are provided below. The acids used in this study were all double-distilled (Teflon,

sub-boiling) from a Merck AnalaR grade starting reagent. 18 MΩ water was obtained from a MQ (Millipore) system.

Abiotic rock and sediment samples

Powdered basalt and river sediment samples were available in-house. Loess samples were provided by Professor Kenneth Pye (Kenneth Pye Associates Ltd., Berkshire, UK). Concentrated stock samples of previously digested meteorites were supplied by Dr. Tim Elliot (Department of Earth Sciences, University of Bristol). Sample preparation for all terrestrial and extraterrestrial materials (see Table 4.1) was carried out as follows. Approximately 50 mg of each basalt, loess and river sediment sample was weighed directly into pre-weighed PFA beakers. In order to get rid of organics, particularly in the river and loess sediments, the samples were treated multiple times with concentrated HNO_3 (15.3M). Basalts were dried down in concentrated HNO_3. Concentrated HNO_3 was added to the river and loess samples, the vials capped after ~ 15 min, the samples ultrasonicated in order to react any remaining carbonates, and the acid dried down. The terrestrial samples were digested in closed PFA beakers for 48 hr (140ºC) in a mixture of concentrated HNO_3 + HF (0.5 ml + 2 ml). After digestion, samples were uncapped, dried down overnight and then treated three times with 15.3 M HNO_3. Finally, samples were dissolved in 7M HCl, resulting in stock solutions of these materials. Stocks of meteorite samples were also established by diluting an aliquot of each concentrated digested meteorite solution in 7M HCl.

Biological Samples

Microorganisms

Samples of cell cultures (see Table 4.2 for summary) were obtained from established and maintained cultured stocks in the laboratory of Dr. Christopher House (Department of Geosciences, Pennsylvania State University, USA). The microorganisms used in this work are all from the domain Archaea and include several types of methanogens (*Methanosarcina acetivorans*, *Methanosarcina barkeri*, *Methanococcus jannaschii*) and a facultatively aerobic heterotrophic hyperthermophile (*Pyrobaculum calidifontis*) which was originally furnished to the House laboratory by Professor Tadayuki Imanaka (Department of Synthetic Chemistry and Biological Chemistry, Kyoto University, Japan) (AMO et al.). *M. acetivorans* was provided by Dr. J. G. Ferry (Department of Biochemistry, Pennsylvania State University). *M. jannaschii* was recently reclassified to *Methanocaldococcus jannaschii* (WHITMAN, 2002). All microorganisms, with the exception of *P. calidifontis*, are strict anaerobes.

Growth Media

All labware used in media preparation was additionally cleaned by soaking in 20% HCl followed by thorough rinsing in 18 MΩ water. Reagents were supplied from Fisher Chemicals.

M. barkeri (per liter): 0.23 g K_2HPO_4, 0.23 g KH_2PO_4, 0.23 g $(NH_4)_2SO_4$, 0.45 g NaCl, 0.09 g $MgSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 2H_2O$, 1.0 ml $NiCl_2 \cdot 6H_2O$ solution (0.2%), 1.0 ml $FeSO_4 \cdot 7H_2O$ solution (0.2%), 4.0 ml NaOH solution (10%), 6.0 ml vitamin solution, 1.0 ml 10x modified Wolfe mineral solution (WOLIN et al., 1963), 0.5 g cysteine•HCl. Modifications to the Wolfe solution include the addition of 0.025 g $NiCl_2 \cdot 6H_2O$ and 0.0003 g $Na_2SeO_3 \cdot 5H_2O$; concentrated HCl was used in place of nitrilotriacetic acid. Medium was made by mixing in

solution all reagents except cysteine•HCl and degassed by bubbling with a stream of H_2 + CO_2 (80% + 20%) for ~ 20 min. The addition of cysteine•HCl, pH adjustment (~ 7) with concentrated HCl or NaOH (10%) and dispensation into serum bottles was carried out in an anaerobic chamber (Thermo Forma), followed by flushing the bottle headspace three times with H_2 + CO_2 and final addition of the same gas mix to 2 bars pressure. An autoclave was used for sterilization.

M. acetivorans (per liter): 23.4 g NaCl, 11.0 g $MgCl_2$•$6H_2O$, 1.0 g NH_4Cl, 1.0 g KCl, 3.8 g $NaHCO_3$, 0.6 g Na_2HPO_4•$12H_2O$, 0.15 g $CaCl_2$•$2H_2O$, 2.0 ml vitamin solution, 1.0 ml 10x modified Wolfe mineral solution (WOLIN et al., 1963), 5.0 ml methanol, 0.5 g cysteine•HCl. The same procedures outlined above were followed for making this medium with the following exceptions: methanol, in addition to cysteine•HCl was added in the anaerobic chamber; the gas mix used was N_2 + CO_2 (80:20); final addition of 0.5 bar pressure to bottle headspace.

M. jannaschii (per liter): 3.0 g Na_2SO_4, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.2 g $CaCl_2$•$2H_2O$, 0.3 g $MgCl_2$•$6H_2O$, 20.0 g NaCl, 3.0 ml NaOH solution (10%), 0.5 ml $(NH_4)_2Fe(SO_4)_2$•$6H_2O$ solution (0.2%), 1.0 ml 10x modified Wolfe mineral solution (WOLIN et al., 1963), 0.5 g cysteine•HCl. Same procedures followed as for *M. barkeri*.

P. calidifontis (per liter): 10.0 g peptone, 1.0 g yeast, 3.0 g $Na_2S_2O_3$•$5H_2O$, 1.0 ml 10x modified Wolfe mineral solution (WOLIN et al., 1963), ~ 3.0 g Chelex 100 resin, 200-400 mesh size (Biorad, CA, USA). This medium was made under aerobic conditions. A solution of peptone, yeast and Chelex was stirred for 1 hr then gravity filtered to remove the resin. Thiosulfate and Wolfe solution were added before the volume and pH (~ 7) were adjusted. The medium was dispensed into Erlenmeyer flasks that were capped with silicone plugs and autoclaved.

Cell cultivation and sample collection

Stock cultures of all microorganisms were established prior to being used as inoculum for the growth experiments. In order to reduce metal-sulfide precipitation during growth, 0.3 mM Ti-NTA (titanium (III) complexed with nitrilotriacetic acid) made according to the methods of Moench & Zeikus (1983) was added to some of the bottles before inoculation with cells. Culture bottles were typically inoculated with 0.5 ml cells per 50 ml medium under sterile and strict anaerobic conditions (for the anaerobes). Growth temperatures were: *M. barkeri*, 33ºC; *M. acetivorans*, 37ºC; *M. jannaschi*, 85ºC and *P. calidifontis*, 90ºC. Growth was determined visually (turbidity), by light microscope and for methanogen cultures, by gas chromatographic measurement of evolved CH_4 (Perking Elmer Clarus 500; Elite-Q Plot phase capillary column; flame ionization detector for CH_4 analysis).

Starting medium samples for isotopic analysis (2 ml aliquots) were collected prior to inoculation with cells. At the end of growth and before the cells were harvested, residual media samples (2 ml aliquots) were removed and filtered through 0.2 μm syringe filters. For comparison, unfiltered residual media samples were also collected for some experiments. Cells were pelleted by centrifugation (4500 rpm, 15 min, 4ºC), resuspended and washed three times in phosphate-buffered saline (PBS). Depending on the microorganism, either a high salt PBS (308 mM NaCl, 6 mM $Na_2HPO_4 \bullet 12H_2O$, 7.5 mM KH_2PO_4) or low salt PBS (7 mM KCl, 1.5 mM $Na_2HPO_4 \bullet 12H_2O$, 7.5 mM KH_2PO_4) solution was used. The final cell pellets were suspended in PBS and transferred to PFA beakers. Pellets were dried and weighed to determine dry weights, but this proved impossible for some samples due either to a small pellet size and/or too much precipitated salts from the PBS solution.

The final samples from the growth experiments were precipitates that formed during cell growth. Two types were collected: black precipitate from *M. acetivorans* and white precipitate from *M. jannaschii* where Ti-NTA was used. *M. acetivorans* growth seems to produce a mucousy substance, most likely a type of EPS (extracellular polymeric substances), in which much of the black precipitate and cells are entrained. After pelleting, the mucous-containing precipitates separate as a distinct layer on top of the cell pellet. This was removed as a separate sample and washed in the same manner as the cells but with 18 MΩ water. At temperatures above 65°C, Ti-NTA apparently produces a fine-grained white residue that adhered to the bottom of the serum bottles (Ti-NTA was also used for *M. acetivorans* and *M. barkeri*; residues were not produced). After the contents were removed, the bottles were thoroughly washed with 18 MΩ water, 7 M HCl added and the dissolved residue collected for analysis.

In all but one case these precipitates contain only 1-3% of the total starting Ni in the experiment, so that their impact on the mass balance and the isotopic data for the cultures is negligible. In one case, *M. acetivorans* - exp. 3 (see Table 4.3), the amount of Ni in the precipitate is significant, at ~13% of the total starting Ni. In this case the cells and the precipitate proved impossible to separate so that this analysis is almost certainly dominated by cells. This is supported by the fact that the Ni in the analysis has an isotopic composition that is identical to the corresponding cell. Quantitative analysis of the precipitates by SEM and ICP-MS (results not reported here) show that they consist primarily of Mn and Ca, for the black and white precipitates, respectively.

Samples were also collected from abiotic controls (Table 4.4) that were run for *M.acetivorans* and *M. jannaschii*. For these experiments, growth media made as described above, was incubated at the growth temperatures of the cells for a period of five days. In the case

of the medium for the latter microorganism, Ti-NTA was also added. Samples were collected and processed in the same manner as the biotic samples. However, there were some analytical complications during analysis of the *M. jannaschii* starting medium; the values are therefore not reported in Table 4.4.

In a similar fashion to the geological samples, the biological samples were digested in the clean hood with a series of 15.3M HNO_3 treatments. Aliquots (2%) of all final sample volumes were taken and diluted to determine Ni concentrations (on a Finnigan Element 2 high-resolution inductively coupled plasma mass spectrometer (HR-ICPMS) at the University of Bristol) for spiking. The appropriate volume of double spike (see next section) was added to the remaining 98% of the sample, which was then dried down and treated with HCl (0.5 ml 10.7M HCl, 2 ml 7M HCl, 0.5 ml 1M HCl).

Column Chemistry

Nickel separation and purification was achieved through a two-column ion exchange procedure, modified from Quitte & Oberli (2006). Microbial growth media are rarely simple solutions. Additionally, a number of the media used in this study are analogous to seawater and thus contain significant quantities of Na, Mg and other constituents, which must be removed to eliminate matrix effects and isobaric interferences. We tested several chemical procedures for this task (KORKISCH and AHLUWALI.S, 1967; MORAND and ALLEGRE, 1983; STRELOW et al., 1972; VICTOR, 1986; WU and BOYLE, 1997; WU and BOYLE, 1998) but, while all can successfully separate Ni from simple solutions, they were unsuccessful when applied to the complex growth media. The technique employed has been adapted for a relatively small sample volume. After sample digestion, the dried residue is taken up and dissolved in 1 M HCl (2 ml).

Ammonium citrate (0.4 ml, 1M) is added and the pH adjusted to 8-9 with 5M NH_4OH. Samples are loaded on to columns (Teflon, made in house) pre-filled with Ni resin (Eichrom Technologies Inc.). Prior to sample loading, the resin is washed with 18 MΩ water (2 ml) and 0.2 M ammonium citrate (1.5 ml), the latter also being used for resin conditioning (2 ml). The Ni-DMG complex forms and is retained, on the resin. Matrix elements are washed off with 0.2M ammonium citrate (6 ml) and Ni is collected in 3M HNO_3 (3 ml). After addition of 15.3M HNO_3 (2 ml), the Ni solutions are dried down overnight. The residue is dissolved in 15.3M HNO_3 (2 ml) and the solution digested overnight at 170°C in closed beakers. This process, which separates the Ni-DMG complex and oxidizes DMG, is repeated twice more.

Fe and Zn are the major atomic interferences on Ni (^{58}Fe on ^{58}Ni and ^{64}Zn on ^{64}Ni, though the latter is not relevant here and will not be discussed further) and, though these elements are removed in the first column step, a second column filled with the macroporous anion resin, AG MP-1M (Biorad, CA, USA) was used to ensure their complete removal. Previous use of this resin to separate Fe and Zn is described elsewhere (ARCHER and VANCE, 2004; MARECHAL et al., 1999). The dried, digested samples from the first column are treated with 10.7M HCl (0.3 ml) followed by 7M HCl + 0.01% H_2O_2 (0.3 ml); this last step is repeated twice. The residue is dissolved (0.75 ml), loaded and eluted from the second column in 7M HCl + H_2O_2. At acid concentrations above 2M HCl, Fe and Zn are retained on the resin while Ni passes through. ^{56}Fe was routinely checked before the full Ni isotopic analysis and the possible interference on ^{58}Ni was never significant. The purified Ni fractions are dried down, treated with 15.3M HNO_3 and finally dissolved in 2% HNO_3 for analysis.

The yield for the column chemistry, as obtained from the double spike-derived Ni amount *before* the separation procedure and the Ni signal size relative to a standard *after* the separation

procedure, is close to 100% - this approach does not give precise yields because signal size is dependent on tuning of the mass spectrometer and even minor quantities of residual matrix. Since samples are spiked before chemical separation, quantitative yields are not required. Also, the yield is only 100% if care is taken to break down the Ni-DMG complex (as above) after the first column. If this is not complete Ni is lost on the second column. Total procedural blanks are about 2 ng and are ~5% of the smallest sample. No corrections were done to the isotopic data. In an extreme case of a Ni blank isotopic composition 1.5‰ different from the sample, the size of the blank would suggest that the maximum magnitude of the correction would be 0.075‰, or the same as the reproducibility of std-spike mixtures.

Mass Spectrometry

Of the three methods available for correcting instrumental mass discrimination, the double spike approach has been shown to be a robust and rigorous technique when applied to isotope systems that have four or more isotopes (BERMIN et al., 2006; BERMIN, 2006; DODSON, 1963; JOHNSON and BEARD, 1999). In this regard, the five stable isotopes of Ni make it an ideal candidate for this approach. The advantages of using the double-spike method are well known and include elimination of issues concerning differences in the behavior of samples and dopant or standards, used respectively in the doping and standard-bracketing methods, as well as removing potential fractionation problems induced due to incomplete yield from the column chemistry (BERMIN et al., 2006; BERMIN, 2006).

The quality of isotopic analyses depends critically on the exact composition of the double spike and the optimum composition was first established using simulations of mass spectrometer runs for various combinations of double spike isotopes. The optimum spike was found to be a

mixture of ^{61}Ni and ^{62}Ni. These isotopes were obtained from Oak Ridge National Laboratories (^{61}Ni, batch #127890; ^{62}Ni, batch #233026). Each isotope was weighed and digested separately with concentrated HNO_3 (0.5 ml) in sealed PFA beakers. The solutions were then transferred to individual 125 ml FEP bottles and diluted with 18 MΩ water to 2% HNO_3. These solutions were then mixed, resulting in a ^{61}Ni-^{62}Ni double spike with sub-equal proportions of the two isotopes. The isotopic composition of the spike was obtained via isotopic analysis of a series of standard-spike mixtures, with std/spike ratios ranging from 2 to 0.1. NIST SRM 986 Ni standard was used to determine the isotopic composition and all isotopic data are thus reported relative to a value of 0‰ for the isotopic composition of this standard. The CRM 1000 ppm single element Ni standard (Peak Performance) was used to calibrate the total Ni concentration of the spike. The double spike reduction procedure has been described previously for Zn (BERMIN et al., 2006; BERMIN, 2006) and the procedure used here is identical to that, with ^{60}Ni/^{58}Ni, ^{61}Ni/^{58}Ni and ^{62}Ni/^{58}Ni as the three isotope ratios used in the double spike calculation. As well as the composition of the double spike, the sample/spike ratio of the mixture measured is also critical for obtaining precise and accurate isotopic analyses. At extreme sample/spike ratios error magnification during the reduction procedure increases, and accuracy is also compromised. The behaviour of the spike in terms of these issues was characterized using spike/standard mixtures and is illustrated in Figure 4.1. All samples measured here had sample/spike mixtures with ratios between 0.1 and 1. Non-exponential mass discrimination was dealt with in exactly the same way as described by Bermin et al. (2006) for Zn.

All analyses were carried out at the University of Bristol using a ThermoFinnigan Neptune multi-collector (MC) ICPMS coupled to an Aridus desolvating nebuliser system (CETAC, Omaha, NE, USA) fitted with a PFA nebuliser and spray chamber (CPI, Amsterdam,

Netherlands). Nickel cones were used in this work and, as described in QUITTE and OBERLI, (2006), contributed very little instrumental blank to our measurements. Sweep gas was typically ~ 4.25 L min^{-1} Ar + 10 ml min^{-1} N_2, with the latter being critical to the reduction of a background at mass 58, presumably $^{40}Ar^{18}O$. All isotopes were measured simultaneously in static mode using a multiple Faraday collector array, but ^{64}Ni is not used in the calculation. Data collection consisted of 15 4-s integrations of the 2% HNO_3 blank followed by measurement of standards or samples for 30 4-s integrations. Blank contributions were subtracted from the standards and samples and generally accounted for less than 0.02% (for ^{58}Ni) of the ion beams. Typically 150 to 300 ng Ni was used for each measurement with an associated internal error on the $^{60}Ni/^{58}Ni$ ratio of 0.001-0.002% at the 2σ level. The amount of Ni measured in some of the biological samples (see Table 4.3) fell outside this range, with associated internal errors of up to 0.0035% at the 2s level.

All Ni data are reported relative to NIST SRM 986, in the standard delta notation:

$$\delta^{60}Ni = \left[\frac{^{60}Ni/^{58}Ni_{sample}}{^{60}Ni/^{58}Ni_{SRM986}} - 1 \right] \text{x } 1000$$

All reported uncertainities are 2σ.

4.4 Results and Discussion

The results, which are summarized in Tables 4.1 and 4.3 and shown in Figures 4.2 and 4.3, are striking and several important features are immediately observed. Firstly, terrestrial samples representing the bulk solid Earth show very little variation in Ni isotopic composition

(average δ^{60}Ni of 0.15 ± 0.24 ‰). Secondly, within the group of analyzed meteorites, there is even less deviation from the average value of δ^{60}Ni = 0.27 ± 0.06 ‰. Taken together with the terrestrial samples, these data provide additional evidence for a homogenous Ni reservoir in the early solar system and in the silicate portion of the Earth. Thirdly, and in stark contrast to the geological materials and relative to the starting composition of the growth media, microorganisms, particularly methanogens, impart a distinct and significant mass-dependent fractionation on Ni isotopes, preferentially sequestering the lighter isotopes which results in an isotopic fractionation on the order of ~ 1.0 ‰.

A central aim of this research was to investigate Ni isotope fractionation during microbial assimilation of Ni and to evaluate Ni isotope systematics as a potential biomarker and biogeochemical tool. Primary microorganisms studied were laboratory-grown strains of methanogens, the group of microorganisms that arguably have the greatest requirement for Ni. The source of Ni in the starting media for all methanogens was very similar and the analyses of Ni separated from these media yielded a δ^{60}Ni from +0.42 ± 0.10 to +0.60 ± 0.06 ‰. These results are virtually indistinguishable from Ni analyzed in the trace metal solution added to all growth media (0.47 ± 0.0 ‰). In contrast, the methanogen cells are universally light in Ni relative to the starting media value. Ni was fractionated irrespective of phylogenetic differences between methanogens. As shown in Table 4.3, *M. barkeri* experiments 1 & 3 yielded cells with δ^{60}Ni that are identical (-0.83 ± 0.09 and -0.86 ± 0.09 ‰), while experiment 2 had a smaller fractionation but larger uncertainty (-0.44 ± 0.20). Five *M. acetivorans* experiments had a range of $\Delta\delta^{60}Ni_{\text{starting medium-cells}}$ of -0.63 to -1.07. Two of the *M. jannaschii* experiments yielded analytically identical values of -0.96 ± 0.09 and -0.81 ± 0.06, while the third gave the largest fractionation of -1.46 ± 0.06. Residual media for the culture experiments are variable heavy in

response to the preferential removal of isotopically light Ni into the cells. Some of the residual media have Ni isotopic compositions that are within the analytical uncertainties of the starting Ni since the amount of Ni in the cells represents quite a small proportion of the total Ni in the system. In a few experiments (e.g. *M. acetivorans* exps. A, B & 2) substantial proportions of the total starting Ni in the system is contained within the cells and as a result, the residual media are, as expected from mass balance, significantly heavier than the starting Ni. The Ni uptake experiment conducted with *M. acetivorans* was specifically designed to monitor the isotopic evolution of the residual medium as more cells are grown, removing and sequestering more Ni from the system. The results (Table 4.3) are presented in Figure 4.3. Cells (~ 0.0221 g) were harvested during the exponential phase of growth, as indicated by the large volume of CH_4 evolved during the final stage of the growth period. The maximum amount of Ni taken up (1.2 μg), representing ~ 25% of total Ni available in the growth medium, correlate extremely well with the isotopic fractionation produced. Based on these results and on a closed system Rayleigh fractionation model, a fractionation factor of 0.7 - 1.0 ‰ has been calculated for $^{60}Ni/^{58}Ni$ during biological Ni uptake (Figure 4.4).

The production of mineral precipitates during microbial growth is a frustrating and ever-present issue for many trace metal studies. In addition to naturally occurring colloidal particles formed during preparation of the growth medium, microorganisms directly or indirectly affect extracellular precipitation or formation of mineral species during metabolism (EHRLICH, 1999) that can include metal sulfides, carbonates, phosphates and oxyhydroxides. In contrast to nickel, Fe has a significant role in many of these species. In addition, I have found that published methods (WASYLENKI et al., 2007; ZEHNDER and WUHRMANN, 1976) commonly cited for preventing microbially-mediated mineral precipitation are ineffective for microorganisms

growing at thermophilic to hyperthermophilic temperatures. Similarly, methods (PERTOFT, 2000; PUTZER et al., 1991) for separating cells from the minerals they produce were unsuccessful for microorganisms such as *M. acetivorans* but more importantly, undesirable for isotope work. The method employed in an attempt to prevent mineral formation was successful in that mineral production was lessened and for some growths, allowed the formation of a completely different type of white precipitate that, in many regards, was easier to deal with. Two types of precipitates were produced in this study. Black precipitates produced by *M. acetivorans*, regardless of the volume of growth (see for e.g., exp. 3), have an identical or very similar δ^{60}Ni to the cells. White precipitates formed by the addition of Ti-NTA during high temperature growth of *M. jannaschii*, have isotopic values in the range –0.27 ± 0.12 ‰ to –0.97 ± 0.25 ‰. However, these samples were not pure mineral separates but an intimate agglomeration of cells and ultra fine mineral particles that have thus far proved impossible to separate. This was particularly true for the large precipitate sample in exp. 3 for *M. acetivorans* which when separated from the corresponding cell pellet, visually had a substantial number of entrained cells. The dry weight (cells + precipitate) of this sample was comparable to the total weight of the cell pellet collected in the uptake experiment (Table 4.2). However, the equivalent precipitate sample separated in the uptake experiment (exp. 2) had no visible cells and had a dry weight that was essentially immeasurable (< 0.00001 mg). With the exception of the sample in exp.3, all precipitate samples have an extremely low Ni content relative to all other samples, for e.g., ~ 8% of cell & < 4% of the final residual medium for *M. acetivorans* exp. 2 (Table 4.3). If inorganic metal precipitation or microbial redox processes were significant contributors, it would be expected that these values would be much higher.

The most important aspect of these precipitates as well as the abiotic controls shown in Table 4.4, is the very small amount of Ni they contain. In most cases this is on the order of 1-2% of the total Ni available in the experiments. As such, their isotopic compositions were very difficult to measure and, is certainly one factor in the large apparent range of measured Ni isotopic compositions, particularly in the white precipitates. Importantly, the small amount of Ni in the precipitates also means that they are insignificant for mass balance and therefore, irrelevant in the growth experiments as they do not affect the values for the isotopic composition of the cells.

Metal precipitates in pure culture experiments are commonly Fe, Zn and Cu sulfides (EHRLICH, 1999) and it would be conceivable that Ni-S also forms. However, laboratory studies conducted with methanogenic and non-methanogenic cultures (FORTIN et al., 1994; JANSEN et al., 2007) have shown that contrary to NiS precipitation, soluble Ni-S complexes, which have large stability constants, are significant and dominant forms in the respective media and that increasing the concentration of Ni actually promotes a reduction in Fe precipitation (FORTIN et al., 1994). Natural settings also provide support for such findings: trace metal surveys in anoxic and sulfidic marine environments such as the Black Sea, show dissolved Ni concentrations are virtually constant with depth and that the metal is not affected by redox processes (HARALDSSON and WESTERLUND, 1988; LEWIS and LANDING, 1992). Given these facts and the results of our experiments, we conclude the $^{60}Ni/^{58}Ni$ isotopic composition of both types of precipitate is overwhelmingly representative of biogenically produced fractionated Ni from the entrained cells and not precipitated Ni.

In addition to the methanogens, isotope measurements were also made of cultures of the heterotrophic archaeal hyperthermophile, *P. calidifontis* (Table 4.3 & Figure 4.2). In contrast to

the methanogens and within analytical error, very little fractionation is observed relative to the starting medium, with measured δ^{60}Ni being analytically identical for cells and both starting and residual media (note that the Ni source in experiment 4 is different from exps. 1 and 2). Nickel is an important bioessential trace metal even to non-methanogens yet not to the same level as methanogens. *P. calidifontis* grows in an organic medium comprised of peptone (or tryptone) and yeast. For trace metal studies, the metals found in these substrates are first removed, commonly by using a metal-binding resin such as Chelex (Biorad), prior to making up the medium with the addition of a known quantity of trace metals (as in exps. 1, 2). Typically, *P. calidifontis* obtains bioessential metals from the organics (when Chelex is not used) and the addition of trace metals is not required for growth (as in exp. 4). The results show that even under conditions of potential metal-limitation or instances where only small quantities of nutrients may be required, microorganisms will preferentially take up the lighter isotope, inducing a slight mass-dependent fractionation on Ni isotopes. However, the small dataset for non-methanogens represented by *P. calidifontis* needs to be expanded by analysis of other microorganisms and at this time, the two conclusions which can be drawn from the results are: (1) microorganisms, in general, probably fractionates Ni isotopes and, (2) this fractionation may not be as significant for most microorganisms as the fractionation produced by methanogens.

Ni isotope data for several meteorites and a suite of terrestrial materials are presented in Table 4.1 and Figure 4.2. Meteorites analyzed are carbonaceous chondrites and irons. All vary within a range of 0.17 ‰ (+0.19 to +0.36). Terrestrial samples taken to be representative of the bulk silicate Earth, have Ni isotopic variations within 0.38 ‰ (–0.04 to +0.34). The continental crust represented by the limited loess and river sediments, are isotopically closer in value to the SRM 986 standard, while the basalts have slightly heavier δ^{60}Ni more in line with meteoritic

values. Young MORBs (< 10-200 yr) from Axial Summit, East Pacific Rise (SIMS et al., 2002) overlap both groups. These samples are analogues for inorganic or abiogenic processes occurring under diverse redox and temperature gradients and altogether, show relatively insignificant Ni isotopic variations. The dataset thus far also supports two characteristics of Ni that have been demonstrated by previous works: a source of Ni derived from a homogenous reservoir as well as its effective conservative behavior and resistance to redox processes in the natural environment.

The combined results of the biological experiments and analyses of natural samples suggest a powerful and vital role for Ni stable isotopes as a biomarker and as a biogeochemical tracer for understanding interactions between the geosphere and biosphere. Nickel's inimitable and specific niche in life may have important implications for understanding biogeochemical processes not only in modern environments but perhaps critically, those occurring back through time to the very early stages of life on Earth. Singular, atypical but ubiquitous - methanogens impact virtually every environment and their biosignatures (extremely light C isotopes; CH_4) have become unique indicators for life. This study demonstrates that archaeal groups such as methanogens and hyperthermophiles and perhaps organisms in general, actively promote mass fractionation of Ni isotopes and that isotopically distinct signatures are produced and detected under the physico-chemical conditions necessary for the growth of these microbes. Particularly, the extent of the fractionation produced by methanogens may result in an additional role for Ni isotopes as a class-specific indicator for these microorganisms and possibly, for other microbial groups as well. Furthermore, the extent of Ni isotopic fractionation produced by specific groups of methanogens (contrast *M. acetivorans* and *M. jannaschii*) may be potentially exploited as a signature of metabolic processes.

The probable antiquity of Ni enzymes (FRAUSTO DA SILVA and WILLIAMS, 2001) as well as the microorganisms that utilize them, coupled to the fractionation imparted by biology, could allow the full realization of this isotope system as a marker for ancient life. Huber and Wachtershauser (HUBER and WACHTERSHAUSER, 1997) proposed a chemoautotrophic model for a high temperature origin of life involving the reduction of CO_2 on the surface of an ancient Fe-Ni-S metalloenzyme that resulted in the catalytic synthesis of the acetyl-CoA synthase (ACS) proto-ancestor. ACS evolved and catalyzed the synthesis of other enzymes that led eventually to the evolution of the first proto-life. Direct descendants of these primordial life-forms are unknown but methanogens are postulated candidates based on phylogenetic (position close to the root of the tree of life) and metabolic (primarily inorganic, C_1 substrate utilization; Acetyl-CoA pathway) evidence (LINDAHL and CHANG, 2001). Modern links to the abiotic importance of Ni minerals can be observed in hydrothermal environments where Fe-Ni alloys possibly act as catalysts in Fischer-Tropsch reactions during serpentinization of the ocean crust, which leads to the generation of significant amounts of methane, hydrogen and other hydrocarbons. One study has estimated that through such processes, the plutonic section of the ocean crust (representing ~60% of the ocean crust) may contain ~10^{19} g of abiogenic methane and that even larger reservoirs may be contained in the shallow mantle (KELLEY et al., 2002). Interestingly, some of these environments are also dominated by methanogens (SCHRENK et al., 2004).

In addition to influencing Earth's modern environment, particularly the carbon cycle (RAGSDALE, 2007), methanogens may have had a profound and significant impact on the early Earth, prior to the evolution of oxygen. Carbon isotope evidence suggests life arose before ~ 3.8 Ga (MOJZSIS et al., 1996; SCHIDLOWSKI, 2001) and the presence of several prokaryotic groups in the Archaean including sulfate reducers (SHEN et al., 2001), iron reducers (ARCHER and VANCE,

2006) and methanogens (BAPTESTE et al., 2005; BATTISTUZZI et al., 2004; UENO et al., 2006) have been proposed. Geological processes contribute to questionable and unresolved ambiguities in the fossil record further back in time one travels but the application of novel and rigorous multi-disciplinary tools such as Ni isotopes which have specific and intimate connections to probable ancient microorganisms may be vital for elucidating the nature and impact of early life.

Finally, the efficacy of a proven Ni biomarker bodes well not only for the field of transition metal isotopes but also for other interdisciplinary endeavors. Fundamental indicators of life on Earth, for instance carbon isotopes or the requirement of an aqueous medium, are driving the search for life elsewhere in the universe and perhaps optimistically, in addition to current approaches in astrobiology of "follow the water" and "follow the energy" (HOEHLER et al., 2007) future models and criteria that define and dictate the search for life will also include "follow the metal isotopes."

4.5 Conclusion

Novel isotope investigations were carried out of Ni stable isotopes in geological and biological materials. In addition to establishing mass spectrometry protocols for analyzing Ni, methods also involved the development and application of procedures for culturing hyperthermophiles as well as the separation and purification of Ni from complex media. The results are remarkable and can be summarized as follows: (1) terrestrial samples representing the bulk solid Earth show very little variation in Ni isotopic composition and have an average δ^{60}Ni of 0.15 ± 0.24 ‰; (2) Ni stable isotopic compositions for a suite of stony and iron meteorites are less invariant (0.17 ‰) than the terrestrial samples (0.38 ‰) displaying an average value of δ^{60}Ni = 0.27 ± 0.06 ‰ and, taken together with the terrestrial samples, provides additional

evidence for a homogenous Ni reservoir in the early solar system; (3) in stark contrast to the geological materials and relative to the starting composition of the growth media, methanogens impart a distinct and significant mass-dependent fractionation on Ni isotopes, preferentially sequestering the lighter isotopes which results in an isotopic fractionation on the order of ~ 0.8 - 1.0 ‰. Additionally, the data suggests different groups of methanogens may fractionate Ni to varying degrees, with the largest fractionation being produced by the hyperthermophiles. Further work will be needed before this can be unequivocally substantiated but if true, biological Ni isotopic fractionation may be useful, not only as a general methanogenic biomarker but also as a marker for distinguishing between diverse metabolic groups. This work will add to ongoing studies that continue to expand the relatively recent area of transition metal isotopes. Fractionation of Ni isotopes may have the potential to become an important marker or tracer for biological and geological processes or geochemical/biochemical interactions, in modern and ancient environments. The Ni isotope system might also be useful in pinpointing which metabolisms and hence, microorganisms were important on the early Earth and allow for extrapolations to be made in determining the possibility of life on other planets, such as Mars.

4.6 References

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4.7 Tables and Figures

Table 4.1: Ni stable isotope data for abiotic terrestrial and solar system samples. [1] All isotope ratios expressed as per mil deviation from NIST SRM986 Ni isotopic standard. Quoted uncertainties in this table are the measurement uncertainties propagated through the double spike algebra. Reproducibility of std-spike mixtures suggests an overall reproducibility of 0.08‰ (see methods description and Figure 4.1)

Meteorites					
Sample	Type	Class	Group	$\delta^{60}Ni^{1}$	2 sigma
Murchison	Stone	Carbonaceous chondrite	CM	0.21	0.03
Leoville	Stone	Carbonaceous chondrite	CV	0.3	0.05
Orgueil	Stone	Carbonaceous chondrite	CI	0.21	0.07
Felix	Stone	Carbonaceous chondrite	CO	0.31	0.07
Chainpur	Stone	Ordinary chondrite	LL	0.28	0.10
Abee	Stone	Enstatite chondrite	EH	0.19	0.05
Coahuila	Iron	Hexahedrite	IIAB	0.36	0.04
Henbury	Iron	Medium octahedrite	IIIAB	0.24	0.07
Bristol	Iron	Octahedrite	IVA	0.28	0.05
Hoba	Iron	Ataxite	IVB	0.33	0.06
Basalts					
Sample	Details				
BCR-2	Columbia River basalt, USGS ref std			0.2	0.07
BHVO-2	Hawaiian Basalt, USGS ref std			0.13	0.03
PCC-1	Peridotite, USGS ref std			0.34	0.08
JA-1	Japanese basalt, GSJ ref std			0.33	0.10
JB-2	Japanese basalt, GSJ ref std			0.26	0.03
JG-2	Japanese basalt, GSJ ref std			-0.03	0.07
JR-2	Japanese basalt, GSJ ref std			-0.02	0.05
2359-4	MORB, East Pacific Rise			0.01	0.07
2372-1	MORB, East Pacific Rise			0.24	0.09
2351-2	MORB, East Pacific Rise			0.13	0.06
Continental sediments					
CL1	Loess, Lanzhou, China			-0.04	0.02
KRM-93-56	Loess, Karamaidan, Tajikistan			0.17	0.06
SCOT 1	Loess, Scottsdale, Arizona			0.1	0.03
V2B	Loess, Vicksburg, Mississippi			0.23	0.08
ATB01/2	River sediment, main Nile, Sudan			0.13	0.03
RAB01/4	River sediment, White Nile, Sudan			0.19	0.07
Average and 2 std dev for terrestrial rocks				**0.15**	**0.24**

Table 4.2: Additional information regarding microorganisms utilised in this study. [1]Ti(III)-NTA is a solution of titanium (III) complexed with nitrilotriacetic acid (MOENCH & ZEIKUS, 1983) and, as indicated, was only used in five experiments.

Microorganism	Medium volume (ml)	Ti(III)-NTA [1]	Dry pellet mass (g)
Methanosarcina barkeri - exp. 1	35	No	-
Methanosarcina barkeri - exp. 2	35	Yes	-
Methanosarcina barkeri - exp. 3	35	No	0.0094
Methanosarcina acetivorans - exp. A	50	No	0.0399
Methanosarcina acetivorans - exp. B	50	No	0.0394
Methanosarcina acetivorans - exp. 1	40	Yes	-
Methanosarcina acetivorans - exp. 2 - Ni uptake	50	Yes	0.0221
Methanosarcina acetivorans - exp. 3	260	No	0.0757
Methanococcus jannaschii - exp. 1	40	Yes	-
Methanococcus jannaschii - exp. 2	42	Yes	-
Methanococcus jannaschii - exp. 3	43	No	0.0114
Pyrobaculum calidifontis - exp. 1	46	No	0.004
Pyrobaculum calidifontis - exp. 2	47	No	0.004
Pyrobaculum calidifontis - exp. 4	22	No	0.009

Table 4.3: Ni stable isotope data for cultures of methanogens and one heterotrophic Archaea.

					Mass balance[1]	
Microorganism	**Experimental product**	**δ^{60}Ni (‰)**	**$2\sigma^2$**	**Total Ni (μg)**	**Amount**	**Isotopic**
Ni trace metal source[3]		0.47	0.07			
M. barkeri - exp. 1	Starting medium	0.55	0.02	20.7		
	Residual medium - filtered	0.48	0.03	17		
	Cells	-0.28	0.09	0.08	0.83	-0.07
M. barkeri - exp. 2	Starting medium	0.46	0.07	20.7		
	Residual medium - filtered	0.46	0.06	20.1		
	Cells	0.02	0.2	0.06	0.97	0
M. barkeri - exp. 3	Starting medium[4]	0.55	0.02	20.7		
	Residual medium - filtered	0.49	0.05	17.7		
	Residual medium - unfiltered	0.47	0.06	18		
	Cells	-0.31	0.05	0.04	0.87	-0.08
M. acetivorans - exp. A[5]	Starting medium	0.57	0.07			
	Residual medium - filtered	1.52	0.07			
	Cells	-0.35	0.04			
M. acetivorans - exp. B[5]	Starting medium	0.6	0.06			
	Residual medium - filtered	1.45	0.04			
	Cells	-0.47	0.05			
M. acetivorans - exp. 1	Starting medium	0.44	0.07	2.64		
	Residual medium - filtered	0.54	0.06	2.23		
	Cells	-0.19	0.08	0.08		
	Black ppt	0.39	0.08	0.05	0.89	0.07
M. acetivorans - exp. 3	Starting medium	0.42	0.1	17.1		
	Residual medium - filtered	0.8	0.05	14.6		
	Cells	-0.42	0.05	4.7		
	Black ppt[o]	-0.44	0.03	2.35	1.27	-0.02
M. jannaschii - exp. 1	Starting medium	0.52	0.09	2.81		
	Residual medium - filtered	0.57	0.07	2.77		
	Cells	-0.44	0.09	0.09		
	White ppt	-0.27	0.12	0.04	1.03	0.01
M. jannaschii - exp. 2	Starting medium	0.43	0.06	2.88		
	Residual medium - filtered	0.46	0.06	2.41		
	Cells	-0.38	0.06	0.1		
	White ppt	-0.97	0.25	0.08	0.9	-0.05
M. jannaschii - exp. 3	Starting medium[7]	0.52	0.09	2.64		
	Residual medium - filtered	0.47	0.05	1.9		
	Residual medium - unfiltered	0.43	0.06	1.88		
	Cells	-0.94	0.06	0.08	0.74	-0.15

Table 4.3 cont'd.

					Mass balance[1]	
Microorganism	**Experimental product**	**δ^{60}Ni (‰)**	**$2\sigma^2$**	**Total Ni (μg)**	**Amount**	**Isotopic**
P. calidifontis - exp. 1	Starting medium	0.14	0.09	4.41		
	Residual medium - filtered	0.19	0.06	5.31		
	Cells	0.08	0.1	0.05	1.22	0.05
P. calidifontis - exp. 2	Starting medium	0.47	0.07	4.92		
	Residual medium - filtered	0.36	0.08	6		
	Cells	0.36	0.09	0.06	1.23	-0.11
P. calidifontis - exp. 4[8]	Residual medium - filtered	-0.3	0.07	0.48		
	Residual medium - unfiltered	-0.28	0.06	0.38		
	Cells	-0.35	0.03	0.15		
M. acetivorans - exp. 2: Ni uptake	Starting medium	0.43	0.09	4.78		
	Residual medium 1	0.4	0.09	4.66		
	Residual medium 2	0.49	0.09	4.35		
	Residual medium 3	0.74	0.08	4.22		
	Final residual medium 4	0.72	0.05	2.8		
	Final residual medium 4 -unfil.	0.8	0.09	2.8		
	Final cells	-0.32	0.07	1.16		
	Black ppt	-0.44	0.36	0.1	0.85	0.02
Average					**0.98**	**-0.03**
Standard deviation					**0.18**	**0.07**

[1] Ni amount mass balance = total Ni in all final experimental products (residual media, cells, precipitate where present) normalized to the amount in the starting medium. Thus, the expected value is 1. The isotopic mass balance = $(\sum P_i \delta^{60}Ni_i) - \delta^{60}Ni_{starting}$ where P_i is the proportion of the total Ni in the i final experimental products and $\delta^{60}Ni_{i,\ starting}$ is the isotopic composition of an experimental product, starting medium. The expected figure for perfect mass balance is zero.
[2] Quoted uncertainties in this table are the measurement uncertainties propagated through the double spike algebra. Reproducibility of std-spike mixtures suggests an overall reproducibility of 0.08‰ (see methods description and Figure 4.1).
[3] Analysis of the Ni standard added to all of the media except some of those for the *P.calidifontis* cultures. The result, for this pure Ni standard before addition to media, is isotopically identical to the Ni separated from the media, and yields confidence in our ability to measure Ni from complex matrices.
[4] Same starting medium, and same data listed, as *Methanosarcina barkeri* exp 1.
[5] Ni amounts not measured - these were early experiments and volumes of media taken for analysis were not precisely controlled.
[6] An attempt to measure the black precipitate, but in this case the precipitate for analysis was mixed with some cells that could not be separated. This is reflected in the large amount of Ni compared to other precipitate measurements and the Ni isotopic composition that is also identical to the cell value.
[7] Same starting medium, and same data listed, as *Methanococcus jannaschi* - exp. 1.
[8] Mass balance calculation not possible as starting medium not measured.

Table 4.4: Ni data for the abiotic controls.

Microorganism	Experimental product	Medium volume (ml)	δ^{60}Ni (‰)	2σ	Total Ni (μg)
M. acetivorans	Starting medium	48	0.58	0.03	7.31
	Residual medium - filtered		0.67	0.04	7.12
	Black ppt		5.27	0.07	0.03
M. jannaschii	Starting medium	125			
	Residual medium - filtered		0.64	0.02	3.28
	White ppt		-10.90	0.10	0.05

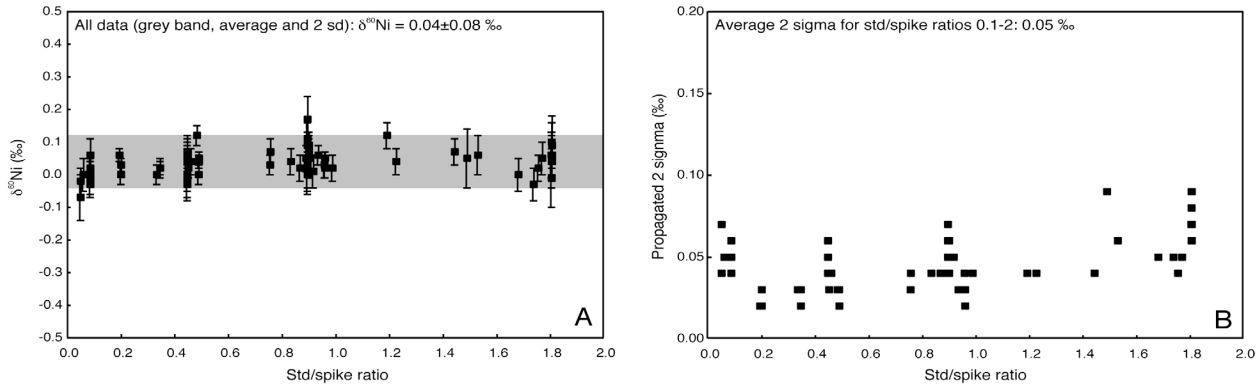

Figure 4.1: Characteristics of Ni double spike for obtaining precise and accurate Ni stable isotope ratios. **A**: data obtained for mixtures of NIST SRM986 (treated as an unknown) and the ^{61}Ni-^{62}Ni double spike; 4 std-spike mixtures (std/spike ratios 0.1, 0.5, 1 and 2) are run during each analytical session to ensure that the correct value of 0 ‰ is obtained for the standard. The data in A represent a compilation of these analyses obtained during numerous analytical sessions over a 12 month period. The average value for the NIST standard is accurate, and precise to 0.08‰, for the whole range of standard-spike ratios. Samples are spiked to obtain a ratio around 0.5, for which data may be more precise than ±0.08‰. **B:** analytical precision for the same std-spike mixtures, obtained by propagation of mass spectrometric uncertainties on all measured isotope ratios used in the calculation through the double spike data reduction procedure (for typical internal mass spectrometric uncertainties of 0.01-0.02 ‰ on all Ni isotope ratios).

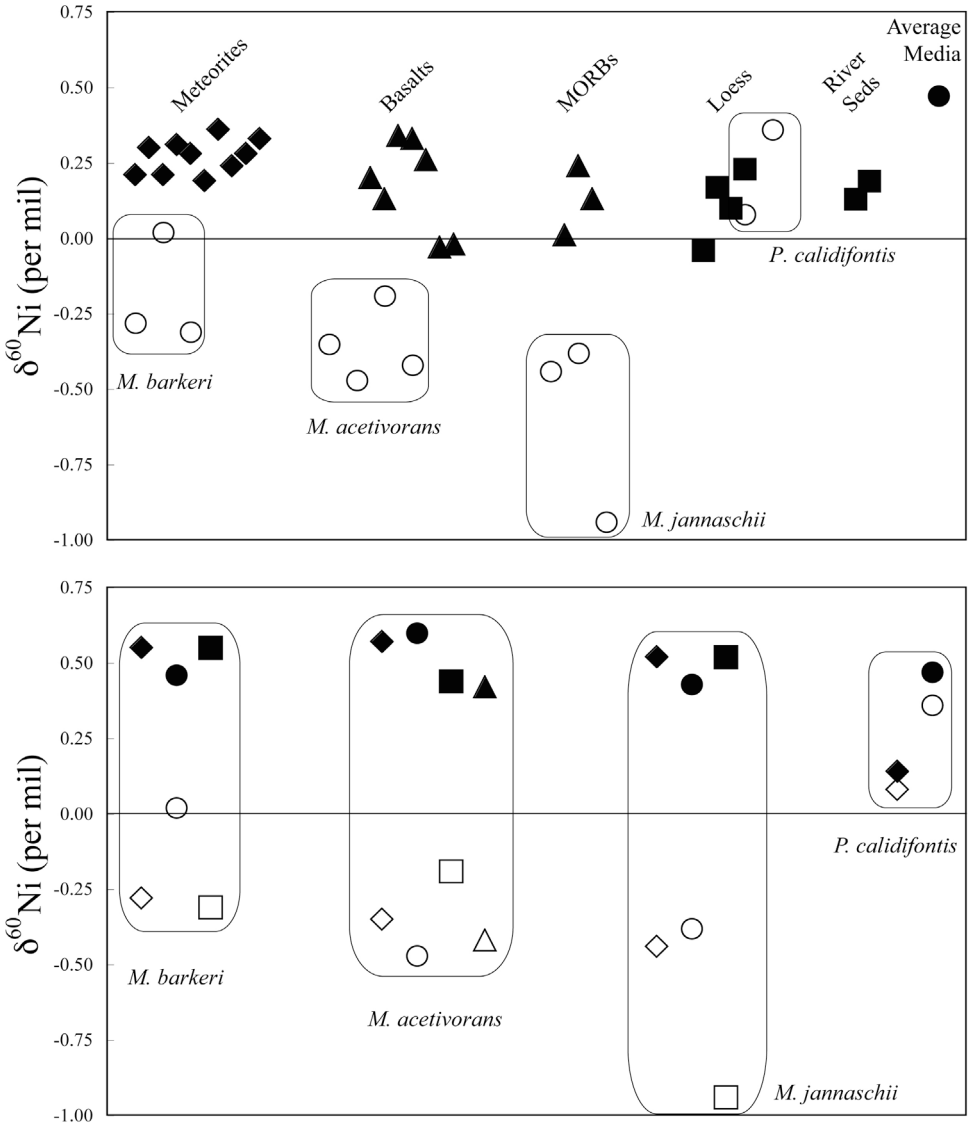

Figure 4.2: ***Top graph*** - δ^{60}Ni values measured for geological and biological samples. Cell pellet values from each experiment are shown for the biotic samples. Average growth media is the mean value (+0.47 ‰) of all media. Relative to a NIST SRM 986 value of ~ 0 ‰, a spread of 0.40 per mil is observed for all measured natural abiotic materials. In contrast, biology imparts a distinct mass-dependent fractionation on Ni isotopes. The largest range in fractionation (~ -1.5 per mil) was measured for the hyperthermophilic methanogen, *M. jannaschii*. ***Bottom graph*** – isotopic data for the biotic samples plotted in the top graph. The cell samples are plotted in relation to the isotopic values for the corresponding starting growth medium (see Table 4.3). Note that one of the measurements (exp. 4) for *P. calidifontis* in both graphs has not been plotted as the growth medium at the start of growth was not measured.

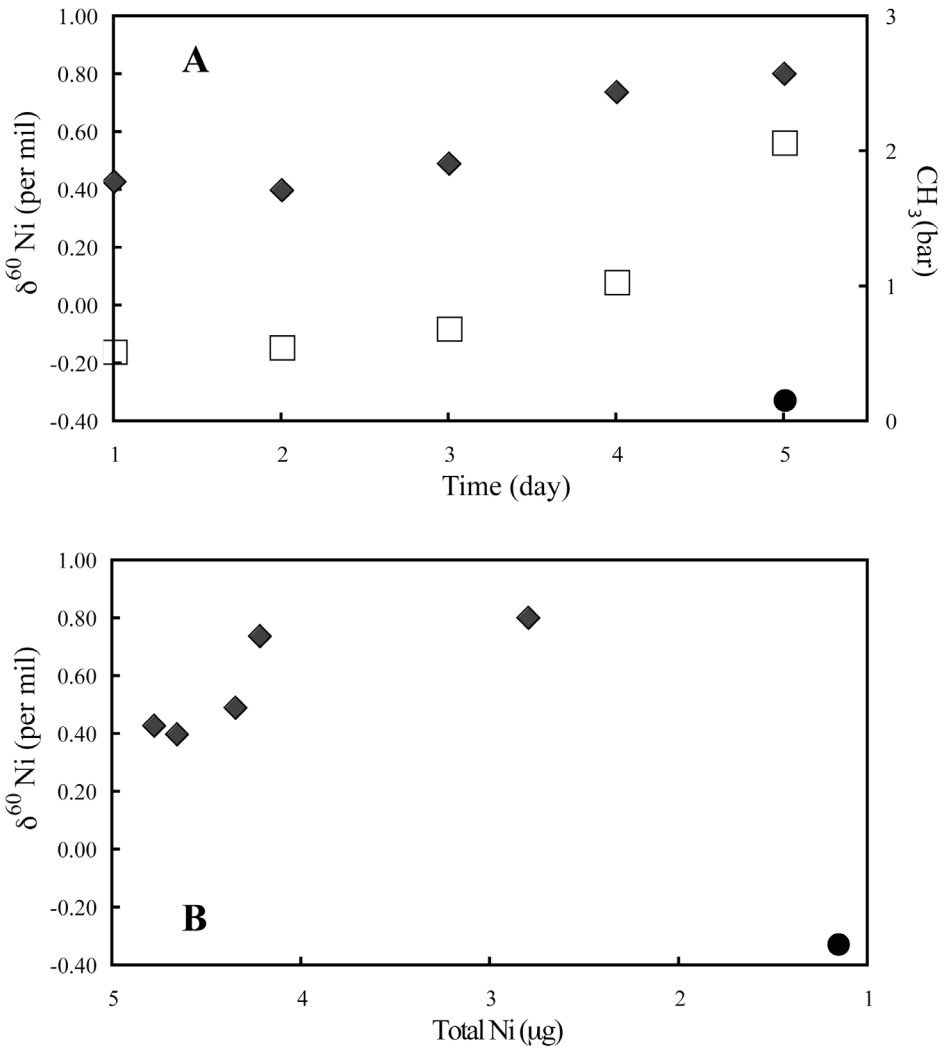

Figure 4.3: Results of the Ni uptake experiment conducted with the methanogen *M. acetivorans*. **A**: individual bottles of growth were sacrificed for each residual media sample collected for isotope analysis (♦) and headspace gas measurement (□) made over the 5-day period. Delta ^{60}Ni values and the amount of CH_4 evolved over the growth period, as a function of headspace pressure, is plotted on the y-axes against time on the x-axis axis. The CH_4 value on day 1 (~ 0.5 bar) is the amount added to the bottles at the beginning of the experiment. Cells (●) were harvested for isotopic analysis only on day 5 of the experiment. As would be predicted, a positive trend is observed for cell growth with increasing amounts of CH_4 being produced as growth proceeds. Correlating with the production of CH_4 is the increasingly heavier isotopic values in the same residual media samples. **B**: total Ni amounts measured in the residual media are plotted on the bottom axis against δ^{60}Ni values on the left-hand axis. A correlating effect is seen with the isotope values and Ni amounts; i.e., the residual medium becomes increasingly heavier and depleted as the cells grow by preferential removal of the lighter Ni isotope.

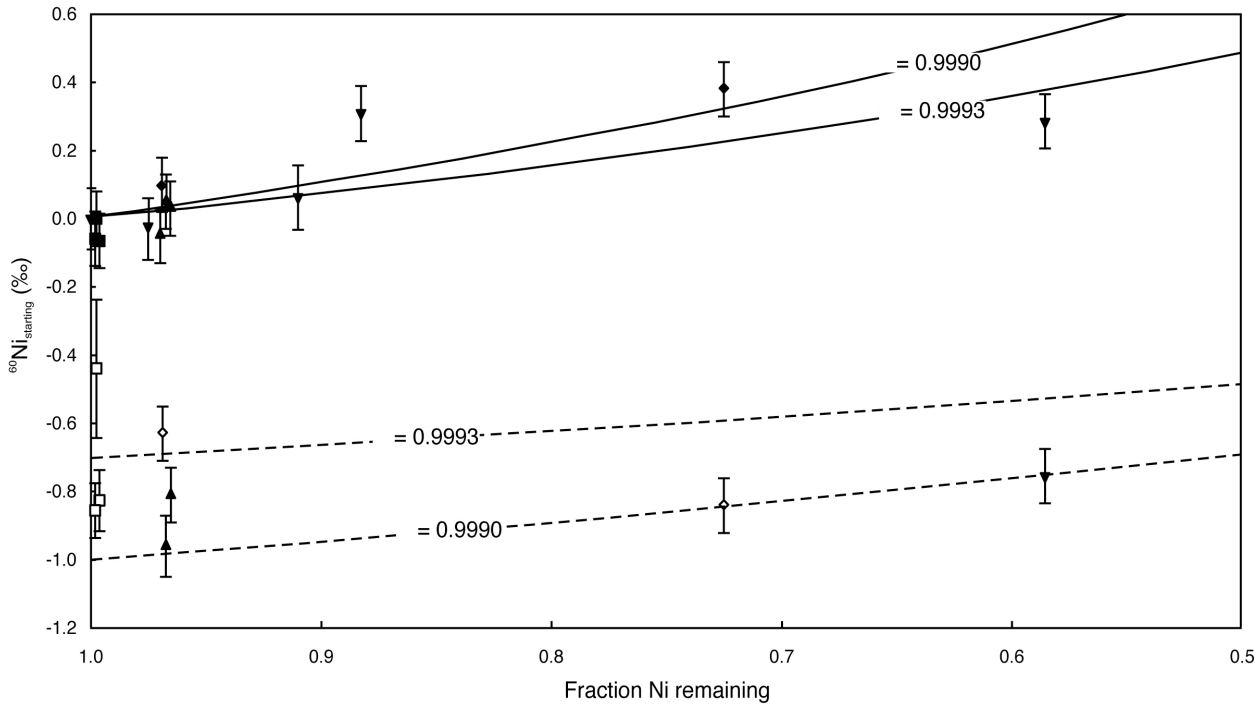

Figure 4.4: Rayleigh fraction curve for Ni. Nickel stable isotope data for all methanogen cultures for which the total Ni mass balance is also available (but omitting one extreme value for *M. jannaschii* cells which plots at -1.4 ‰ – see Figure 4.2), plotted as a function of the fraction of total starting Ni remaining in the medium at the end of the experiment. All Ni isotope data for residual media (filled symbols) and cells (open symbols) are plotted relative to the starting medium for the respective experiments. Symbols for different species (*M. barkeri* ■, *M. acetivorans* ◆, *M. jannaschii* ▲) except that the non-methanogen is not plotted and the inverted triangles represent data for the *M. acetivorans* Ni uptake experiment. Also plotted are model closed system Rayleigh fractionation curves for residual media (solid lines) and accumulated cells (dashed lines) assuming fractionation factors for Ni uptake into cells of 0.9990 and 0.9993.

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