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# Microbial Diversity and Geochemical Energy Sources of Tutum Bay, Ambitle Island, Papua New Guinea, an Arsenic-Rich, Shallow-Sea Hydrothermal System

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# MICROBIAL DIVERSITY AND GEOCHEMICAL ENERGY SOURCES OF TUTUM BAY, AMBITLE ISLAND, PAPUA NEW GUINEA, AN ARSENIC-RICH, SHALLOW-SEA HYDROTHERMAL SYSTEM

by

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ABSTRACT OF THE DISSERTATION

Microbial Diversity and Geochemical Energy Sources of Tutum Bay, Ambitle Island, Papua New Guinea, an Arsenic-Rich, Shallow-Sea Hydrothermal System

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Professor Jan P. Amend, Chairperson

I investigate the hydrothermal system located in Tutum Bay, Ambitle Island, Papua New Guinea, a shallow-sea system ~5 – 10 meters below sea level that is arsenic-rich. Hydrothermal vents in the bay expel fluids with arsenite ($As^{III}$) concentrations as high as 950 μg/L. To determine the role that Tutum Bay microorganisms might play in mediating As-redox reactions, three approaches were used: analyzing the geochemical environment for energy sources, characterizing the archaeal community composition of the sediments, and conducting culture-dependent As-cycling experiments. The second chapter of this dissertation discusses an energetic study of potential chemolithotrophic metabolic reactions, including As-redox reactions. Results show that under the environmental conditions present in Tutum Bay, significant amounts of energy for microbial metabolism could be gained from a number of reactions, including $As^{III}$ oxidizing reactions using oxygen and nitrate as terminal electron acceptors. In the third chapter, a 16S rRNA-based culture-independent investigation of the archaeal community structure of the As-rich sediments shows the presence of diverse uncultured archaea at

sites both near and far from hydrothermal venting. The studies in these two chapters demonstrate that the Tutum Bay hydrothermal system provides an environment hospitable to metabolically and phylogenetically diverse microorganisms. Finally, in chapter four, evidence of functional genes related to both arsenate- and arsenite-redox were recovered from sediments examined via molecular screening. It was also shown that microbial consortia enriched from Tutum Bay sediments and porefluids were able to reduce arsenate ($As^V$) to arsenite when incubated at 30°C in an $As^V$-rich growth medium. These results demonstrate that As-redox microorganisms exist in shallow-sea hydrothermal environments and broaden our understanding of not only the types of microbial species that are capable of As-redox, but also the unique environmental niches in which life can exist and thrive.

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# CHAPTER 1

# INTRODUCTION

## Hydrothermal Systems and Chemosynthesis

Hydrothermal systems, where geothermally heated waters are expelled through fissures in the Earth's crust, are located both on land and under the sea. Most people are familiar with terrestrial hydrothermal systems, such as the hot springs and geysers seen in Yellowstone National Park, USA. Marine hydrothermal systems may be less familiar, but photographs of deep-sea systems with their towering sulfide chimneys and communities of strange tube worms, clams, mussels, and crabs have captured public attention since their discovery in the late 1970s (Lonsdale, 1977; Francheteau et al., 1979; Spiess et al., 1980). Even less widely known than deep-sea systems, however, are shallow-sea hydrothermal systems, located at less than 200 meters below sea level (mbsl). Shallow-sea systems, such as the on-shore seeps and shallow vents located in a few meters of water at Vulcano Island, Italy, have been known for much longer than deep-sea systems due to their proximity to shore and easier accessibility. However, they are not yet as well-studied as their deep-sea counterparts, which in part provided the motivation for this dissertation, which focuses on an arsenic-rich shallow-sea hydrothermal system in Tutum Bay, Papua New Guinea.

Hydrothermal systems in at least 55 deep-sea and 21 shallow-sea settings have been documented and their biota studied in detail (Figure 1). Deep-sea systems are numerous while their shallow-sea counterparts have not been as well studied, although shallow-sea systems are more easily accessible and can often be explored via SCUBA

diving. Like their deep-sea counterparts, many shallow-sea systems occur in volcanically active areas, with systems located along arcs, mid-ocean ridges, and in island arc-related environments. Some shallow vent systems have also been found in continental margins undergoing tectonic extension, lakes in continental rift basins, and crater lakes (Prol-Ledesma et al., 2005). Both deep- and shallow-sea systems are high temperature, reducing environments enriched in gases and heavy metals such as iron (up to 6500 and 875 μM/kg for deep and shallow systems, respectively), manganese (1140, 675), zinc (100, 12.5), copper (40, 2.3), and lead (360, 4350) (Tarasov et al., 2005).

Shallow-sea systems also differ from deep-sea systems in a variety of ways. For example, shallow-sea systems are no larger than several hundred square meters, while deep-sea systems can stretch tens of kilometers along ridges (Mironov et al., 2005). In shallow-sea systems, hydrothermal fluids range from 10 to 135°C, much cooler than deep-sea systems where fluids can surpass 400°C (Baross and Hoffman, 1986; Amend, personal communication). The chemical composition of hydrothermal fluids is reducing, like other hydrothermal sites, but shallow-sea systems can be viewed as a transition between deep-sea and terrestrial systems. Fluids are usually more enriched in N, P, and Si and do not have as high concentrations of $CH_4$ and $H_2$ compared to deep-sea systems (von Damm et al., 1995). Shallow vent fluids often contain a meteoric water component (Prol-Ledesma et al., 2004; Pichler, 2005) and their chemistry can both affect and be affected by near-shore terrestrial activity (Price and Pichler, 2005). Pressures are also not as high due to the shallower depths. The lower boiling temperature of water in shallow systems can lead to subsurface deposition of metals (Prol-Ledesma et al., 2005). Shallow-sea vents typically feature an exsolved gas phase such as carbon dioxide,

hydrogen sulfide, or methane (Pichler et al., 1999a; de Ronde et al., 2001; Prol-Ledesma et al., 2002, Amend et al., 2003; Hirayama et al., 2007).

Biologically, both deep- and shallow-sea systems support a wide variety of organisms, but deep-sea systems display vent-obligate fauna; lack diatoms, algae-bacterial mats, and plankton; and are dominated by symbiotrophic forms of life, while shallow-sea systems tend to display a more diverse array of fauna that are not all dependent on the vents (Tarasov et al., 2005). Shallow systems are also known to be hotspots of eukaryotic biodiversity (Morri et al., 1999). Most importantly, shallow-sea systems exist within the photic zone and both photosynthetic and chemosynthetic organisms can play a role in primary production, while chemosynthetic organisms are the main producers in deep-sea systems. In 1977, when scientists made the discovery of hydrothermal vents located ~2500 meters below sea level (mbsl) along the Galapagos Rift, they were surprised that the ocean floor surrounding the vents was not a barren desert, but instead covered in clams, mussels, and giant tube worms, with larger animals such as crabs, octopi, and rays swimming nearby (Londsale, 1977). Scientists in the deep-ocean submersible *Alvin* had seen for the first time entire biological communities that did not depend on sunlight. Instead, chemosynthetic microorganisms are the primary producers in deep-sea hydrothermal systems, gaining energy from the chemical disequilibria created when hot hydrothermal fluids mix with cooler, oxidized seawater. According to Holger Jannasch, "[Chemosynthesis] was a powerful new concept and, in my mind, one of the major biological discoveries of the 20th century." (Jannasch, 1997). An obligate photosynthetic bacterium was recently discovered at a deep-sea vent site, but it is unclear how large a role photosynthetic organisms play in deep-sea system

productivity and it is assumed that chemosynthesis is still the dominant form of primary production (Beatty et al., 2005). In shallow-sea systems, primary production from both chemosynthetic and photosynthetic organisms is well documented (Sorokin et al., 1998; 2003; Tarasov, 1999). Microbial mats and biofilms are common in shallow-sea systems, and mats composed of photosynthetic diatoms, chemosynthetic and photosynthetic prokaryotes, and prokaryotic-algae photosynthetic-and-chemosynthetic communities have all been observed (e.g., Nesterov et al., 1991; Tarasov 2002; Meyer-Dombard et al., 2005; Hirayama et al., 2007).

Numerous biological studies of deep-sea hydrothermal systems have been conducted (e.g., Takai and Horikoshi, 1999; Takai and Sako, 1999; Schrenk et al., 2003; Huber et al., 2006; Ehrhardt et al., 2007), along with studies of terrestrial systems (e.g., Barns et al., 1994; Thevenieau et al., 2006; Mori et al., 2008), and shallow-sea systems (e.g., Miura et al., 2002; Pichler et al., 2006; Hirayama et al., 2007), all of which have increased the range of environments that we now know can harbor life, as well as our understanding of the capabilities of microbial life. Many of the organisms discovered are "extremophiles," able to live in environments of high temperature, pressure, salt content, acidity or alkalinity, and heavy metal concentrations, including arsenic.

In marine hydrothermal systems, arsenic concentrations can range from around the typical seawater background level of ~2.6 μg/L (Mukhopadhyay et al., 2002) to as high as 824 μg/L (von Damm, 1995; Canet et al., 2005; McCarthy et al., 2005). In hot springs, concentrations as high as 150,000 μg/L have been observed in Yellowstone National Park, USA (Langner et al., 2001). Arsenic-rich hydrothermally-influenced sites may host currently unknown As-redox microorganisms. Very little is currently known

about microbially-mediated As-redox at high temperatures, which has provided the other part of the motivation for this research to study the high-arsenic, shallow-sea hydrothermal system located in Tutum Bay, Ambitle Island, Papua New Guinea.

**A Brief History of Arsenic**

Arsenic (As) is a notorious poison. It has been used since ancient times as a toxin in warfare, and in the Middle Ages it became known in France as *la poudre de succession*, the inheritance powder, due to its popularity among the nobility who used it to dispatch their rivals (Nriagu, 2002). Most arsenic compounds, such as arsenic trioxide ($As_2O_3$), are toxic to humans, with the toxicity level estimated to be around 2 – 3 mg/kg of body weight for arsenic trioxide. Thus, a 55-kg (120 lb) person could die from ingesting as little as 0.11 g of arsenic trioxide (Dueñas-Laita et al., 2005).

Arsenic exists in four oxidation states: $As^{V}$, $As^{III}$, $As^{0}$, and $As^{-III}$. The two most common arsenic oxidation states, arsenate ($As^{V}$) and arsenite ($As^{III}$), are some of the most toxic forms of arsenic. Arsenate is a molecular analog of phosphate, and so it can enter the cell through phosphate transporters and inhibit oxidative phosphorylation, which produces ATP, a key molecule that supplies energy for cellular processes, including catabolic (biomass synthesis) reactions. Arsenite is even more toxic than arsenate because it binds to reactive sulfur atoms (-SH groups) in many enzymes, including those important in respiration (NRC, 1999; Oremland and Stolz, 2005).

Clearly, the presence of arsenic can have a deleterious effect on the health of humans. Arsenic-contaminated food and water can cause many health problems, including skin lesions, diabetes, various types of cancer, cardiovascular and neurological

problems, or even death (Smith et al., 2000; Nriagu, 2002; Le, 2002). Countries around the world, including the United States, Mexico, Bangladesh, India, Nepal, China, Vietnam, Taiwan, Afghanistan, Iran, Argentina, Chile, and Australia all have documented cases of chronic poisoning from arsenic-contaminated waters (Mukherjee et al., 2006). Bangladesh has the most widespread problem, with an estimated 35 and 57 million people at risk of drinking water with arsenic concentrations exceeding 0.05 and 0.01 mg/L, respectively (Brinkel et al., 2009). The World Health Organization's recommended levels for arsenic in drinking water are 0.01 mg/L (WHO, 1996), and millions of people in Bangladesh are at risk of developing chronic arsenic poisoning. Both the United States and Canada lowered the maximum contaminant limit for arsenic in drinking water from 50 to 10 μg/L within the past three years (U.S. EPA, 2001; Health Canada, 2006), and this change in water quality standards has made it even more important to understand how arsenic cycles in the environment.

In spite of its toxicity, arsenic has also been used since ancient times as an ingredient in many medical treatments, because in the right concentrations, arsenic can be used to selectively cleanse and kill unwanted targets. It has been used as a cure for conditions ranging from bad skin, asthma, and head lice to diseases like malaria and cancer. Arsenic was a key component in Salvarsan, the first chemotherapy treatment, created by Paul Ehrlich who later won the Nobel Prize for medicine. Salvarsan and its successor Neosalvarsan were used as treatments for syphilis as late as the 1940s (Nriagu, 2002). Organoarsenicals are still the last resort treatment in a number of diseases such as sleeping sickness, and relapsed acute promyelocytic leukemia (Nriagu, 2002; Tallman, 2007). Metal-arsenic compounds, including lead and copper complexes, were often

sprayed on crops as insecticides from the mid-1800s to the late-1900s, and a solution of copper, chromate, and arsenic was used until 2004 in the treatment of wood to prevent insect rot (Nriagu, 2002; Oremland and Stolz, 2003; Christen, 2006). In addition to its use as an insecticide and rodenticide, arsenic compounds have also been used as a green dye for decorative purposes including wallpaper, artificial flowers, birthday cake decorations, toys, and clothing (Aronson, 2005; Hindmarsh and Savory, 2008). Of course, the use of arsenic in medicines, on crops, and in decoration often inadvertently harmed people. For instance, children in the 19$^{th}$ century were poisoned after eating sweets colored with arsenic compounds (Taylor, 1875). In a recent study, it has been suggested that the madness of King George III of England may actually have been exacerbated by the presence of arsenic in some of the very medicines intended to help cure him (Cox et al., 2005). Similarly, Napoleon Bonaparte may have been chronically poisoned either on purpose, by arsenic administered in his food, or accidentally, by toxic gases exuded from the arsenic-painted wallpaper in his rooms during his exile on St. Helena, although his actual cause of death is still debated (Weider and Fournier, 1999; Lin et al., 2004; Aronson, 2005; Hindmarsh and Savory, 2008).

Because of its high toxicity to humans and its constant presence in the water supply, it is important to try and understand the chemistry of arsenic in the environment. A simplistic view of the arsenic cycle is illustrated in Figure 2. Arsenic can enter the environment through both natural geologic and anthropogenic processes. Arsenic is released into the environment through volcanic and tectonic activity and the weathering of arsenic minerals (Cullen and Reimer, 1989; Newman et al., 1997); anthropogenic sources of arsenic are mainly produced as industrial by-products. For instance,

organoarsenicals are a common addition in chicken feed to prevent intestinal parasites and subsequently high levels of arsenic are released in the chicken effluent (Mukhopadhyay et al., 2002). Arsenic in the water supply can be adsorbed onto sediments, oxidized and reduced by microbes, and also taken up by larger organisms. These organisms may store arsenic in their tissues, or convert it into arsenosugars, arsenolipids, methylated species, or other compounds. These arsenic species are eventually passed up the food chain to larger animals and cycled back into the environment (Mukhopadhyay et al., 2002). The role that microbial species play in the arsenic cycle is complicated and largely unknown, and by studying them we can gain key insights into how microbes can affect water and soil quality and even how to use them to bioremediate contaminated sites.

**Microorganisms and Arsenic**

To date, only a few more than 30 microorganisms are known that can use arsenic as an energy source, while many more microbes can reduce or oxidize arsenic as a detoxification mechanism but do not harvest energy from the process (Oremland et al., 2009). Figure 3 is a phylogenetic tree illustrating the relationships of As-redox microorganisms. As can be seen in the figure, almost all As-redox microbes belong to the Domain Bacteria, one of the three domains of life. The other two Domains are the Archaea and the Eukarya. Members of the Bacteria and the Archaea are prokaryotic organisms, while the Eukarya contain eukaryotic organisms. There are at least 17 major groups (phyla) within the Bacteria. Among the Bacteria, As-redox microbes belong to a variety of these phyla including Deinococcus-Thermus, Deferribacter, Gram-Positives,

and all divisions (alpha, beta, gamma, delta, and epsilon) of the Proteobacteria (Ehrlich, 2002; Oremland and Stolz, 2003). The Domain Archaea contains 3 major phyla: Crenarchaeota, Euryarchaeota, and Korarchaeota. Archaea were originally thought to consist mainly of "extremophiles," with the Crenarchaeota phylum incorporating psychrophiles, sulfur-respiring microbes, and some thermophilic microbes and the Euryarchaeota phylum incorporating methanogens, halophiles, and some thermophilic microbes. However, not all Archaea are extremophiles. They are also present in moderate environments such as marine water, sediments, forests, and freshwater lakes at temperatures and chemical conditions that are not so drastic (Schleper et al., 2005). Indeed, one group of Crenarchaeota have been recognized to constitute a significant proportion, up to 20%, of the marine prokaryotic population (DeLong, 2003).

In arsenic microbiology, much less is known about As-redox organisms within the Archaea. Currently, the only archaeal species known to reduce arsenate for energy gain are *Pyrobaculum arsenaticum* and *P. aerophilum* (Huber et al., 2000), while the archaeon *Sulfolobus acidocaldarus* strain BC is capable of arsenite oxidation, but not for energy gain (Sehlin and Lindström, 1992; Ehrlich, 2002). Due to the diverse habitats of archaea, and also considering how the As-redox capability is widespread in the bacterial domain, it is likely As-redox is more extensive among the archaea than currently documented.

Although arsenic can occur in four different oxidation states, microbes predominantly mediate electron transfer among only two—$As^{V}$ and $As^{III}$; energy gain from redox reactions involving $As^{0}$ and $As^{-III}$ has not yet been observed (Oremland and Stolz, 2003). In aqueous environments, $As^{III}$ and $As^{V}$ are the main oxidations states, but the proportions of these two oxidation states depend on a number of factors including the

biological processes in the surrounding environment, the redox potential, and the pH (Lièvremont et al., 2009). In oxic environments, $As^{V}$ is found predominately in the form of $H_2AsO_4^-$ (at pH below ~6.9) and $HAsO_4^{2-}$ (at pH above ~6.9), while $As^{III}$ is found in the form $H_3AsO_3$ until pH > 9.2 (Francesconi and Kuehnelt, 2002; Inskeep et al., 2002; Le, 2002). Arsenate is less mobile than arsenite (Cullen and Reimer, 1989) and it often co-precipitates (sorbs) with ferric iron or sulfur (Foster, 2003; O'Day et al., 2004). It also adsorbs to clay, calcite, organic matter, and iron and aluminum oxides (Cullen and Reimer, 1989; Morin et al., 2003), making arsenic easily removed from solution and potentially less bioavailable for microbial use. However, some studies have shown that microbes are capable of leaching arsenic from mineral substrates, either by using the arsenic directly or by metabolizing other ions such as $Fe^{III}$ in the minerals and thereby releasing the arsenate into the water (Harrington et al., 1998; Oremland et al., 2002b).

Arsenate reduction to arsenite for energy gain is a relatively recently recognized process, with the first isolate, epsilon-Proteobacterium *Sulfurospirillum arsenophilum* strain MIT-13, isolated by Ahmann et al. in 1994 and subsequently genomically characterized in 1999 (Stolz et al., 1999). MIT-13 was obtained from sediments in the Aberjona River watershed of suburban Boston, MA; the Aberjona is heavily contaminated with arsenic from pesticide production wastes (Ahmann et al., 1997). Since MIT-13's discovery, the arsenate respiratory ability has been found to be widespread throughout different phyla in the Bacteria including the gamma-, delta-, epsilon-Proteobacteria, the Gram-Positives, and the Deinococcus-Thermus (see Figure 3). Arsenate-respiring microbes come from a wide variety of environments, including lakes, soda lakes, salt and freshwater marshes, and contaminated sediments from gold mines

and watersheds (Dowdle et al., 1996; Newman et al., 1997; Stolz et al., 1999; Santini et al., 2002; Oremland et al., 2005; Lear et al., 2007; Switzer Blum et al., 2009), and are adapted to a range of living conditions. For example, *P. arsenaticum* and *P. aerophilum* are adapted to high temperatures (95°C) (Huber et al., 2000), and *Halarsenatibacter silvermanii* strain SLAS-1 is an extreme halophile (a salt-loving microorganism) (Oremland et al., 2005; Switzer Blum et al., 2009).

MIT-13 anaerobically oxidizes lactate using arsenate, nitrate, or fumurate as its terminal electron acceptor (Ahmann et al., 1994; Newman et al., 1998). Almost all arsenate reducers are able, like MIT-13, to use other electron acceptors, such as selenate, nitrate, nitrite, sulfate, $Fe^{III}$, thiosulfate, sulfur, dimethylsulfoxide, and trimethylamine oxide – in fact, all of these electron acceptors, in addition to arsenate, can be used by *Sulfurospirillum barnesii* (Oremland et al., 1994; Laverman et al., 1995). In nature, arsenic often interacts with chemical species such as sulfide, nitrate, and FeOOH, which may help explain why arsenate reducers are often able to use a variety of electron acceptors (Oremland and Stolz, 2003). However, one obligate arsenate reducer has been isolated: the delta-Proteobacterium strain MLMS-1 from Mono Lake, California, which couples sulfide oxidation with arsenate reduction (Hoeft et al., 2004).

Metabolically, two pathways are known for arsenate reduction, one of which is for detoxification purposes, and one of which can be used for energy gain. The detoxification pathway has been well studied and characterized (see Mukhopadhyay and Rosen, 2002; Stolz et al., 2006; and Páez-Espino et al., 2009 for reviews). In brief, the *ars* operon in many microbes is composed of up to 5 genes, *arsRDABC*, but at minimum contains three genes: *arsR*, which encodes a transcriptional repressor, *arsB*, a

transmembrane efflux pump, and *arsC*, an arsenate reductase. When present, the proteins encoded by *arsA* and *arsD* help the efflux pump encoded in *arsB* (Lièvremont et al., 2009). In the respiratory pathway, sometimes called dissimilatory arsenate respiration, a respiratory arsenate reductase consisting of two subunits (ArrA and ArrB) carries out arsenate reduction. The reductase is encoded by the *arr* operon which always includes the *arrA* and *arrB* genes, with some strains containing an additional membrane subunit ArrC (Páez-Espino et al., 2009).

Arsenite oxidation comprises the other half of the microbial As-cycle. Microbial arsenite oxidation is a process that has been known and studied since the early 20$^{th}$ century (Green, 1918), with many microorganisms known to be capable of the process (e.g., Anderson et al., 2002; Gihring and Banfield, 2001; Muller et al., 2006). The arsenite oxidase protein structure has been purified and crystallized from several microbes (Ellis et al., 2001, Santini and vanden Hoven 2004; vanden Hoven and Santini 2004). Three different arsenite oxidases have been identified, but are homologous in structure (Quéméneur et al., 2008). The enzyme is a member of the DMSO reductase family and consists of two subunits, AoxA and AoxB, encoded by the *aoxA* and *aoxB* genes (alternatively known as *asoA* and *asoB* or *aroA* and *aroB*) which are expressed in the presence of arsenic (Inskeep et al., 2007; Lièvremont et al., 2009; Páez-Espino et al., 2009). The arsenite oxidase enzyme may be an ancient protein predating the divergence between Archaea and Bacteria (Lebrun et al., 2003), and it has been suggested that $As^{III}$ may have been one of the main energy sources involved in early earth metabolisms (Lièvremont et al., 2009).

Overall, arsenite oxidizers, like arsenate reducers, have been isolated from a wide variety of environments, including stratified soda lakes, hot springs, gold mines, arsenic-contaminated waters, soils, and animal insect-repellants (Santini et al., 2000; Gihring et al., 2001; Langner et al., 2001; Ehrlich, 2002; Oremland et al., 2002b; Donahoe-Christiansen et al., 2004). Arsenite oxidizers (see Figure 3) can be grouped into heterotrophic oxidizers, which convert $As^{III}$ on the cell's outer membrane into $As^{V}$ as a detoxification technique, and chemoautotrophic oxidizers, which use $As^{III}$ as an electron donor to reduce either oxygen or nitrate, with the energy being harnessed for growth (Lièvremont et al., 2009). Currently, oxygen and nitrate are the only two electron acceptors known for respiratory $As^{III}$ oxidation, but other electron acceptors may be discovered in the future.

Chemoautotrophic arsenite oxidizers have mainly been found in extreme (e.g., high pH, high salt content) environments, but have also been found in non-extreme environments such as freshwater (Garcia-Dominguez et al., 2008). They are mainly members of the alpha-Proteobacteria, with a few examples in the beta-Proteobacteria, including *Azoarcus* sp. DAO1 (Rhine et al., 2006) and *Hydrogenophaga* CL-3 (Garcia-Dominguez et al., 2008), and the gamma-Proteobacterium *Alkalilimnicola ehrlichii* strain MLHE-1 (Hoeft et al., 2002; Oremland et al., 2002a). However, there may be more chemoautotrophic arsenite oxidizers in other bacterial – or even archaeal phyla – that have not yet been discovered or characterized (Garcia-Dominguez et al., 2008; Oremland et al., 2009). The only known arsenite oxidizer in the archaeal domain, *Sulfolobus acidocaldarius* strain BC is, like most arsenite oxidizers, a heterotrophic oxidizer that

does not harvest energy from the oxidation reaction (Lindström and Sehlin, 1989; Sehlin and Lindström, 1992).

Arsenite oxidation takes place both aerobically and anaerobically, and sometimes, as in the case of *Thermus* strain HR13, oxic conditions lead to detoxification of arsenite while anoxic conditions result in respiration of arsenate (Gihring and Banfield, 2001). Recently, microbes conducting anoxygenic photosynthesis using arsenic as the electron donor were isolated from microbial mats in anoxic brine pools fed by high-arsenite, high-sulfide hot spring waters from Mono Lake (Kulp et al., 2008). These purple bacteria are the first reported to use arsenite oxidation for photosynthesis.

In Tutum Bay, the arsenic concentrations in the hydrothermal vent fluids are as high as 950 μg/L (Price and Pichler, 2005). This site is ideal for investigating the role of microorganisms in mediating As-redox reactions, and for broadening our understanding of As-redox at elevated temperatures. Following is a brief overview of the field site. For more in-depth discussions of the geologic and geochemical setting, see, e.g., Wallace et al, 1983; Pichler and Dix, 1996; Pichler and Veizer, 1999; Pichler et al., 1999a,b; Price and Pichler, 2005; Pichler et al., 2006; Price et al., 2007.

**Tutum Bay**

Tutum Bay, off the coast of Ambitle Island, Papua New Guinea (Figure 4a), is a unique, arsenic-rich, shallow-sea hydrothermal system located in a coral reef environment. Ambitle, with its northeastern neighbor Babase Island, make up the Feni Islands, the southeasternmost island group in the 260 km long Tabar-Feni chain of eastern Papua New Guinea. The Tabar-Feni chain is a group of fore-arc alkaline

volcanoes on the North Bismarck Plate (Hamilton, 1979). The Pacific Plate is subducting below the North Bismarck Plate along the West Melanesian Trench (Hamilton, 1979) (Figure 5). Ambitle is part of a Quaternary stratovolcano (Wallace et al., 1983). Rising 450 m above sea level, Ambitle is a diamond-shaped island approximately 12 km wide and 14 km from north to south that is mostly covered in dense rain forest. The human population of the Feni Islands is just over 1000 as of 1983, and the population of Ambitle is mainly subsistence farmers who have minimal impact on the nearshore environment (McCloskey, 2009). Several hot mud pools, fumaroles, and springs with chloride or acid-sulfate waters dot the island (Pichler and Dix, 1996).

Off the west coast of Ambitle, Tutum Bay contains a hydrothermal system located among coral-algal reefs 5 – 10 mbsl (Pichler et al., 1999a). Two types of hydrothermal venting occur in Tutum Bay: focused discharge from discrete vents 10 – 15 cm in diameter, and diffuse discharge of gas bubbles through unconsolidated medium to coarse-grained carbonate sand and gravel on the seafloor (Figures 6 – 8). The vents audibly discharge fluid at an estimated rate of 300 – 400 L/min (Pichler and Dix, 1996), and vent fluid temperatures range from 89 – 98°C (Pichler et al., 1999a).

Compared to ambient seawater, the Tutum Bay hydrothermal fluids are enriched in As, B, Mn, Si, Fe, and $HCO_3^-$, and depleted in Na, Cl, $SO_4^{2-}$, and Mg (Pichler et al., 1999a; Price et al., 2007). Arsenic is the only potentially toxic element to be particularly elevated, with hydrothermal fluids containing up to ~950 µg/L $As^{III}$, compared to the typical seawater background concentration of ~2.4 µg/L arsenic (Price et al., 2007). Rocks and corals near vent orifices were coated in arsenate-rich $Fe^{III}$-oxyhydroxide precipitates (Figure 8) which ranged from soft orange and brown layers to hard greenish-

brown layers (Pichler et al., 1999b). The oxyhydroxides contained 6.2 wt% arsenic when measured by neutron activation (Pichler and Veizer, 1999).

The hydrothermal vent orifices in Tutum Bay expel As exclusively in the form of $As^{III}$ (Pichler et al., 1999a; Price and Pichler, 2005; Price et al., 2007), and as such can be viewed as point sources for the release of $As^{III}$ into the hydrothermal system. It is believed that the source of arsenic in the hydrothermal fluids is due to dissolution of arsenopyrite underneath Ambitle Island, which is then transported by the hydrothermal fluids (Pichler et al., 1999a). In this dissertation, I focus on Transect 4B (Figure 4b), which denotes a 300 m long sampling area starting at Vent 4 and extending southwest for 30 m and then due west (to avoid coral reefs) for an additional 270 m.

Along Transect 4B, the major anion, cation, and arsenic concentrations in the porewaters were determined by Roy Price and Thomas Pichler at the University of South Florida; redox sensitive species were determined on site by D'Arcy Meyer-Dombard and Jan Amend. In the vent fluids, concentrations of arsenite up to 950 μg/L have been measured (Price and Pichler, 2005). With increasing distance from the vent, the hydrothermal influence lessens, and at 300 m distance the $As^{III}$ concentration is 2.01 μg/L, less than the local seawater level of 2.6 μg/L. The $As^{V}$ concentration is ~ 7 – 94 times the local concentration of 2.3 μg/L except for the 20 and 240 m sites where the concentration was approximately ambient. The pH is circumneutral along the entire transect, while the temperature drops off quickly from 95°C at the vent to ambient temperatures around 30 m distance.

Figure 9 illustrates a schematic view of a hydrothermal vent in Tutum Bay and the locations at which we could likely expect to find microbial $As^{III}$ oxidizers and $As^{V}$

reducers. In Tutum Bay, the vents act as point-sources of arsenite, so arsenite oxidizing microorganisms in the water column and also in the sediment-water interface and upper layers of the sediment could take advantage of the chemical disequilibria created where expelled vent fluids mix with oxidized seawater. This microbially-mediated $As^{V}$ precipitation may be incorporated into the ferric-oxy-hydroxide precipitates that coat rocks and corals around the vents (Figure 8). Within the coatings, $As^{V}$ could be reduced in anoxic internal layers, which may or may not be biologically mediated. The microbiological communities in these coatings were investigated recently and distinct communities of bacteria and archaea were recovered from differently colored coatings that grew in close proximity (Meyer-Dombard et al., 2005). My research focuses mainly on the porefluids and sediments found in the upper 10 cm of the seawater-seafloor interface, where microbially-mediated $As^{III}$ oxidation and $As^{V}$ reduction are both possible.

**Outline of Dissertation**

This dissertation discusses the microbial life and energy potential of an arsenic-rich shallow-sea hydrothermal system of Tutum Bay, Ambitle Island, Papua New Guinea. Chapters 2, 3, and 4 are written as manuscripts for submission to scientific journals and each of these chapters contains its own introduction, materials and methods, results, discussion, and conclusion sections. A glossary of terms is provided in Appendix A.

In Chapter 2, I calculate the Gibbs free energy of 19 potential chemolithotrophic reactions at 9 sites in Tutum Bay based on the geochemical composition of the porewaters using geochemical data from Roy Price and Thomas Pichler. To gain energy for growth, chemotrophic microbes catalyze oxidation-reduction reactions. The amount

of energy available from metabolic redox reactions can inform us about the types of microbes that may be found in an environment. The amount of energy available from any given reaction depends on the chemical composition of the system, its temperature, pressure, and the standard free energy of the reaction, and can be calculated by the following equation:

$$\Delta G_r = \Delta G_r^o + RT \ln Q \quad (1)$$

where R is the universal gas constant (8.314 J $K^{-1}$ $mol^{-1}$), T is the temperature (in K), and $\Delta G_r^o$ is the sum of the standard Gibbs free energy of reaction at the temperature and pressure of interest, determined by the equation

$$\Delta G_r^o = \sum \nu_i \Delta G_i^o \quad (2)$$

where the stoichiometric reaction coefficient $\nu_i$ is negative for reactants and positive for products, and Q is the activity quotient,

$$Q = \prod a_i^{\nu_i} \quad (3)$$

where $a_i$ is the activity of species *i* raised to its stoichiometric reaction coefficient $\nu_i$ with $\nu$ negative for reactants and positive for products. If $\Delta G_r$ is negative, the reaction is energy-yielding and organisms can gain energy by catalyzing that reaction. A positive value, on the other hand, means that the reaction requires energy to proceed in the direction written and thus consumes energy. This chapter includes a discussion of the variations in the Tutum Bay energy yields in relation to other systems, and a discussion of potential organisms that may inhabit Tutum Bay based on the energy framework constructed. This work is written for submission to *Geobiology*.

Chapter 3 discusses the variety of archaea present at 5 sites in Tutum Bay. Very little is known about archaea that are capable of arsenic redox, so I chose to examine the

archaeal diversity of Tutum Bay using 16S rRNA clone library construction, a culture-independent technique. The bacterial diversity was investigated by our colleagues at the University of South Florida and is not discussed in detail in this dissertation. The phylogenetic relationships of the 16S rRNA sequences identified here are compared to other archaea and this survey provides a view of life in an extreme environment. This chapter is written for submission to *Environmental Microbiology* and follows the formatting guidelines of the journal.

In Chapter 4, I discuss culture-dependent experiments that investigate the As-redox capabilities of microbes enriched from Tutum Bay sediments with a particular emphasis on $As^V$-reduction. One of the key aims of the NSF proposal that funded our research in Tutum Bay was to determine whether microbes were actively involved in arsenic cycling in the bay. To examine this possibility, bulk DNA extracted from sediments and enrichment cultures was screened for the existence of the arsenate reduction respiratory gene *arrA* and arsenite oxidase genes, and bulk RNA extracted from enrichment cultures grown in $As^V$-rich growth medium were screened for *arrA* gene expression. An incubation experiment to observe microbially-mediated As-reduction is also discussed, along with an isolate obtained from Tutum Bay porefluids and sediments that was grown in $As^V$-rich medium. This chapter is to be submitted to *Applied and Environmental Microbiology.*

Finally, in Chapter 5, I summarize future directions for this research and implications of this work. In particular, I discuss bioremediation and how the search for life in other extreme environments, including those on other planetary bodies in our solar system, may be informed by the work in this dissertation.

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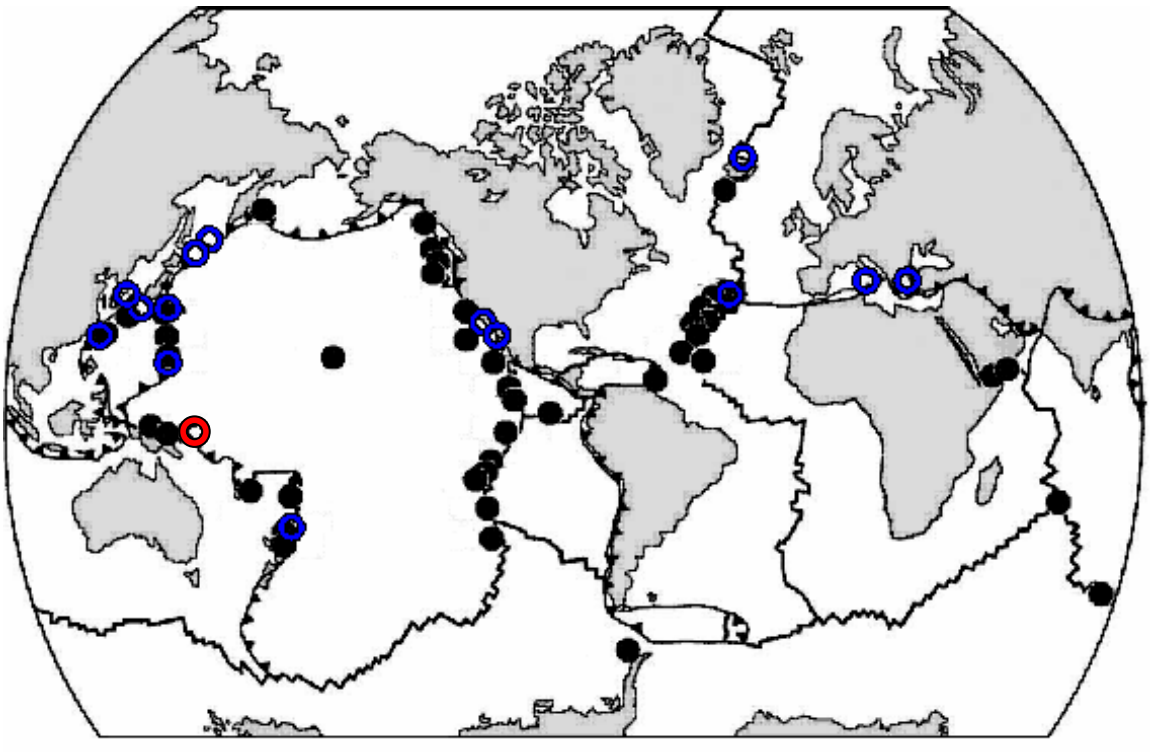

**Figure 1.** Hydrothermal vent systems around the world where biota have been studied. Solid circles indicate deep-sea (>200 m depth) hydrothermal sites; blue rings indicate shallow-sea (<200 m depth) hydrothermal sites. Site indicated with red ring is the shallow-sea hydrothermal system at Tutum Bay, Ambitle Island, Papua New Guinea. Figure adapted from Tarasov et al., 2005.

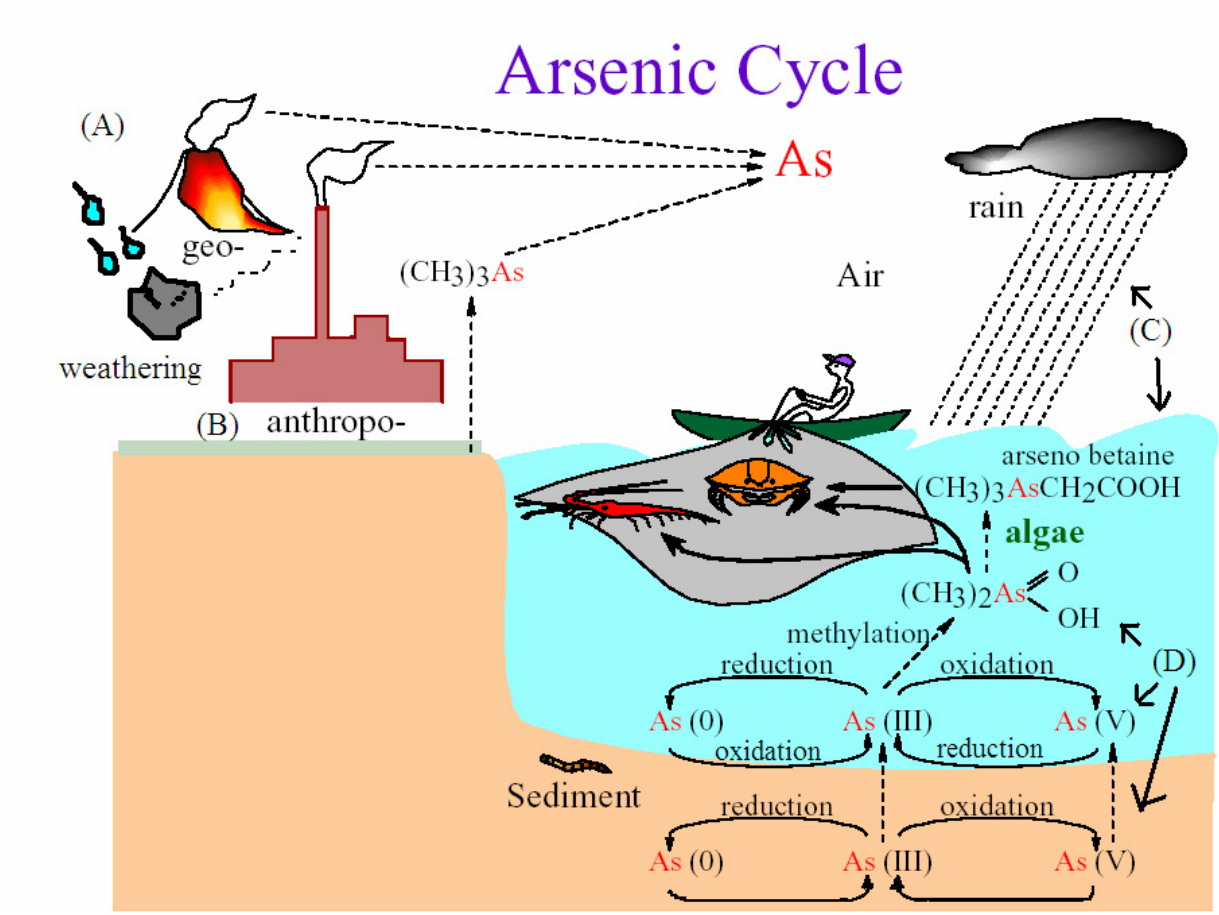


**Figure 2.** The Arsenic Cycle. Arsenic is emitted into the environment through geologic activities (A) and anthropogenic sources (B). Once in the water supply (C), arsenic can be oxidized and reduced by microbes, stored in sediments, or converted into arsenic compounds by algae and other marine organisms that are eaten and returned to the environment (D). Figure adapted from Mukhopadhyay et al., 2002.

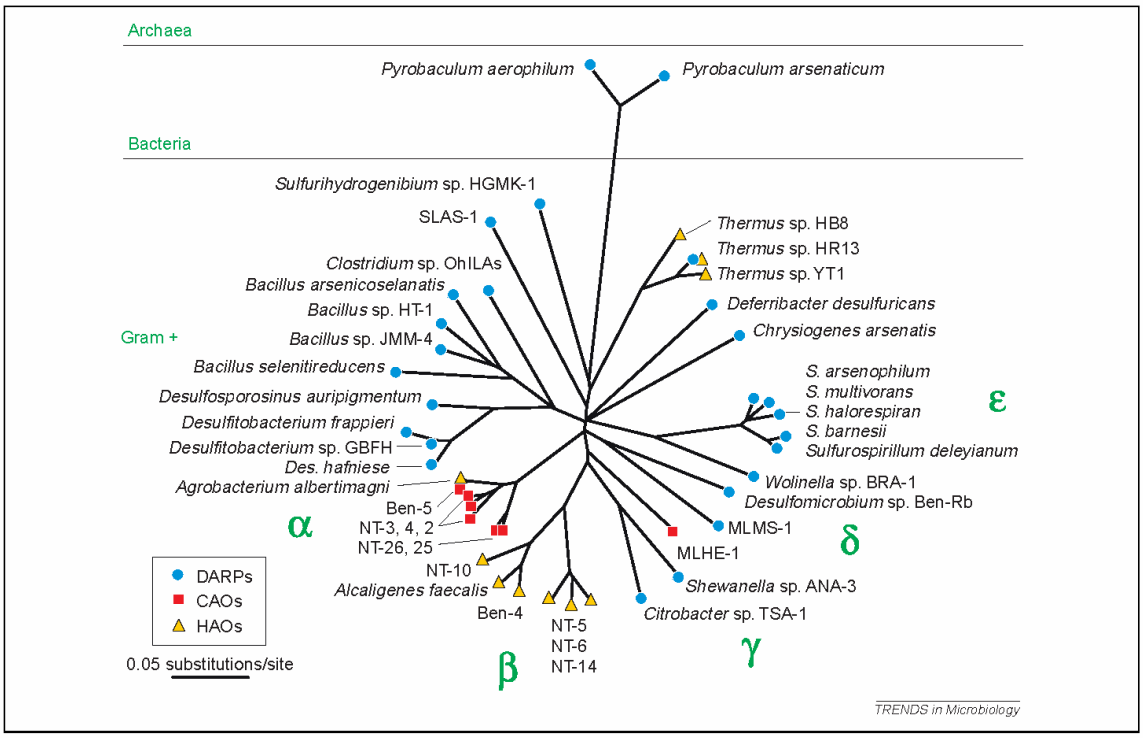


**Figure 3.** Phylogenetic tree showing prokaryotes from the Domain Archaea (top) and Domain Bacteria (bottom) that are capable of As-redox. The dissimilatory $As^{V}$-reducing prokaryotes (DARPs) are indicated by blue circles, chemoautotrophic $As^{III}$ oxidizers (CAOs) are indicated by red squares, and heterotrophic $As^{III}$ oxidizers (HAOs) are indicated by gold triangles. Arsenic-resistant microorganisms are not shown. Figure from Oremland and Stolz, 2005.

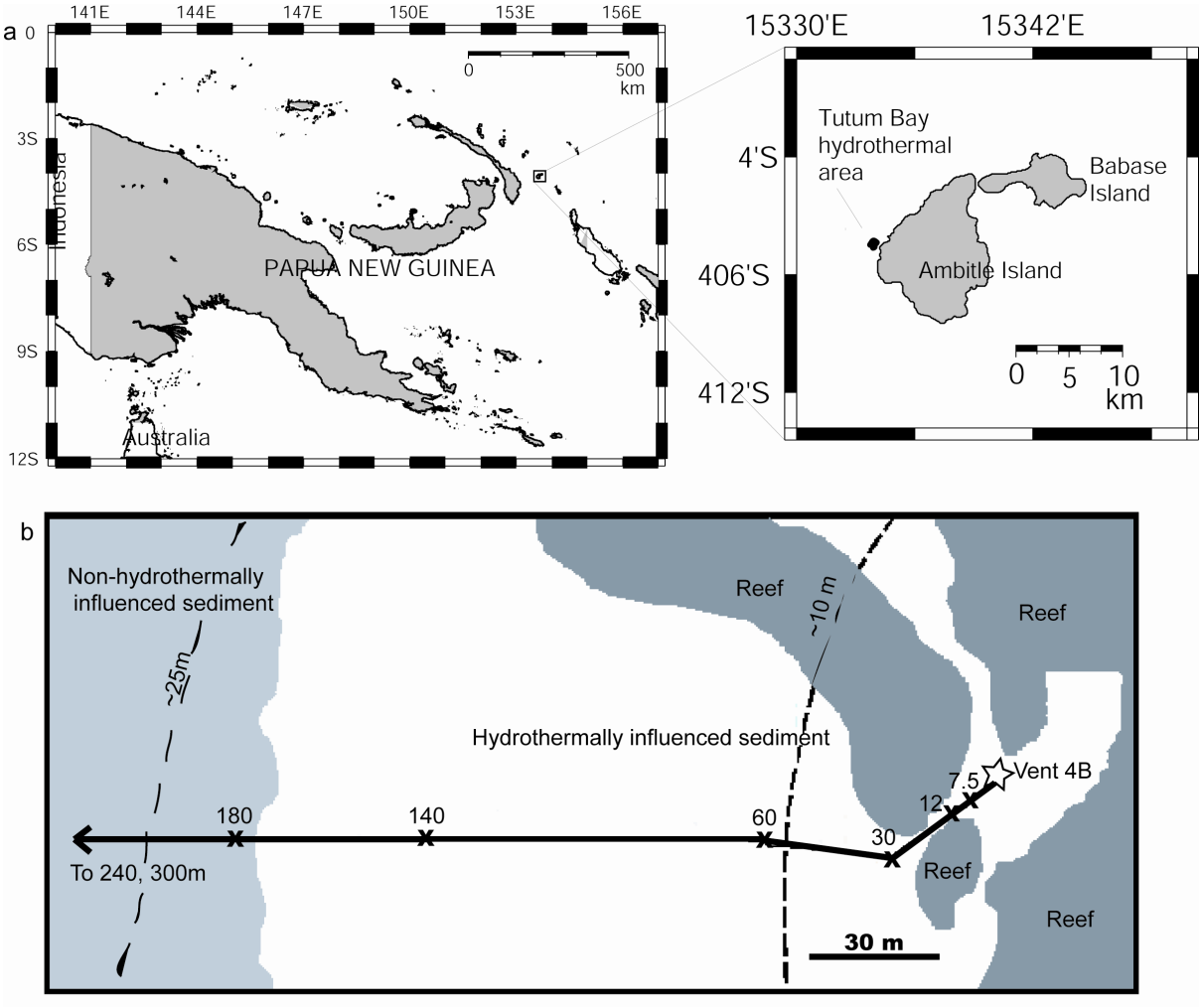


**Figure 4a)** Map showing location of Tutum Bay, Ambitle Island, Papua New Guinea. **b)** Plan view of Tutum Bay hydrothermal area showing sampling transect constructed from Vent 4B. Water depth indicated by dashed lines.

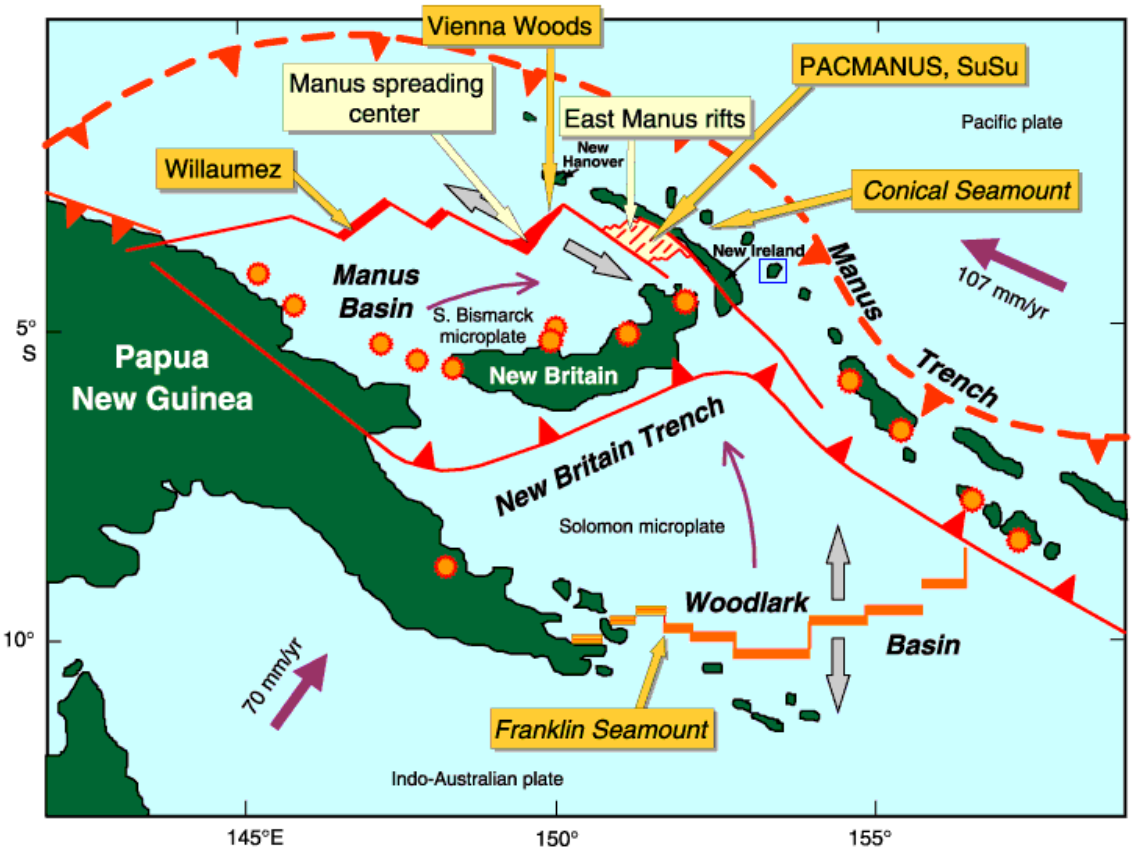


**Figure 5.** Tectonic setting of Ambitle and Babase Islands (indicated by blue-outlined box). The yellow boxes indicate other known hydrothermal sites, the large purple arrows indicate plate motion, and the curved purple arrows indicate the sense of rotation on the microplates as defined by Global Positioning System geodesy (Tregoning et al., 1998) or by the opening and westward propagation of the Woodlark Basin (Taylor et al., 1995). Figure adapted from Shipboard Scientific Party, 2002.

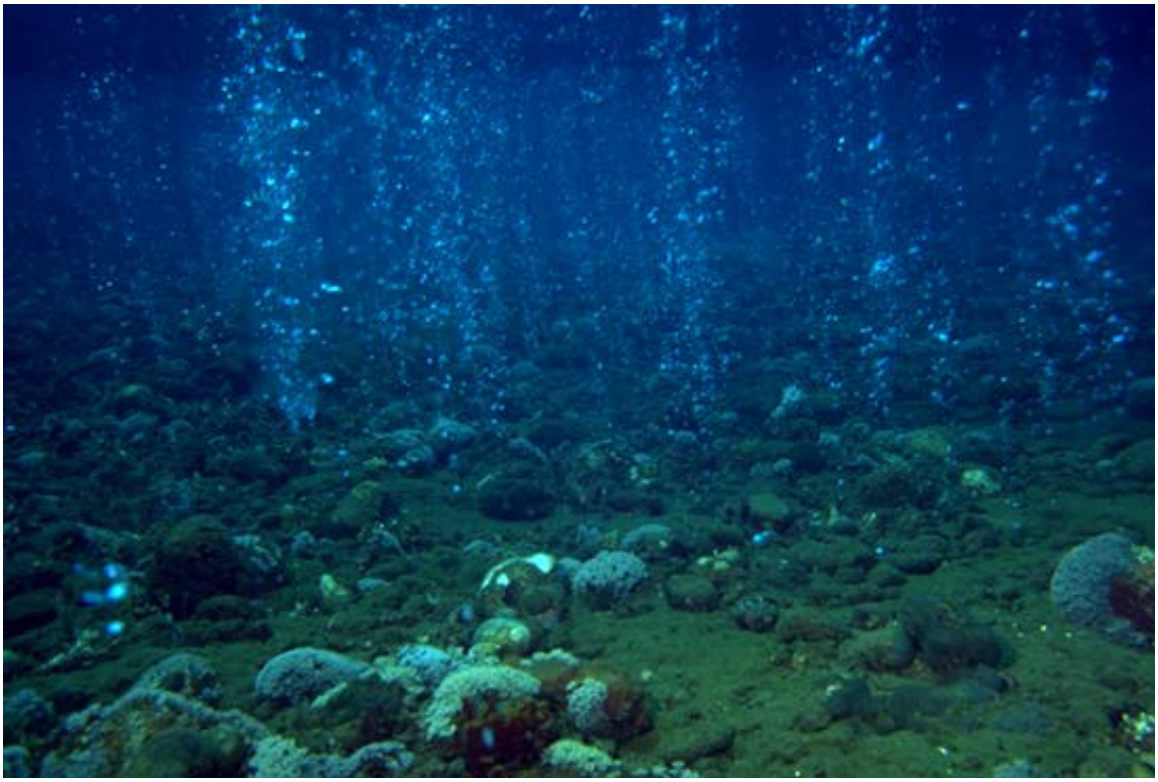

**Figure 6.** Area surrounding Vent 4, Tutum Bay. Diffuse discharge of hydrothermal fluids occurs through a field of volcanic boulders with minor coral growth. Depth is approximately 10 m and width of view is approximately 15 m. Photo courtesy of Thomas Pichler.

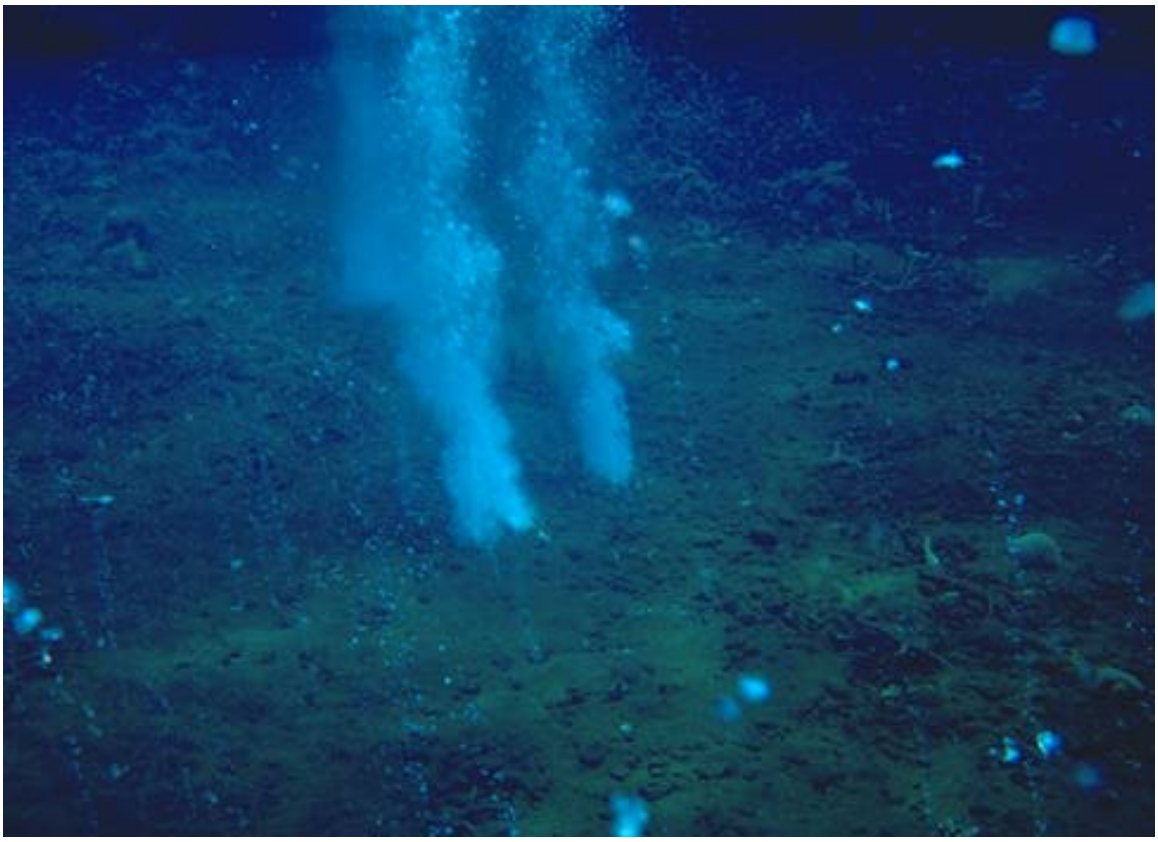

**Figure 7.** Hydrothermal vents in Tutum Bay with diffuse streams of gas bubbles venting from surrounding unconsolidated sediment (field of view ~5 m across). Photo courtesy of Thomas Pichler.

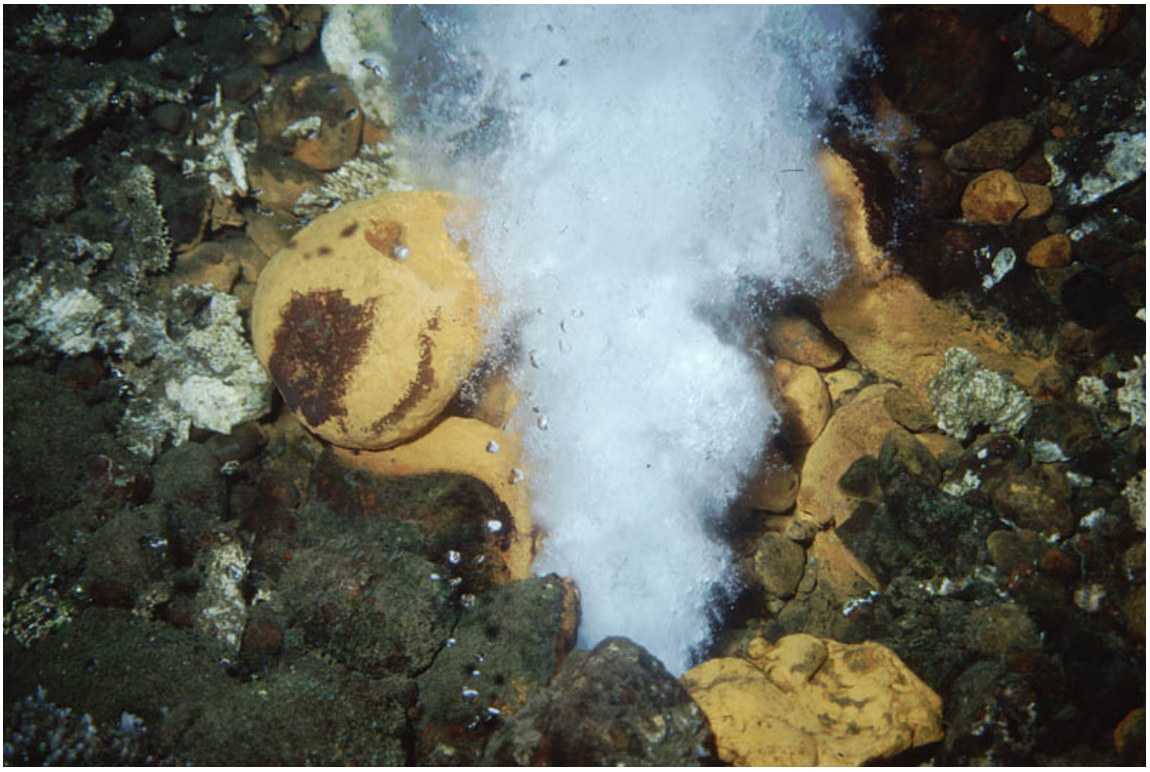

**Figure 8.** Close-up of hydrothermal vent orifice showing orange-colored iron-oxyhydroxide coatings on surrounding rocks and coral. Vent orifice ~5 cm. Photo courtesy of Thomas Pichler.

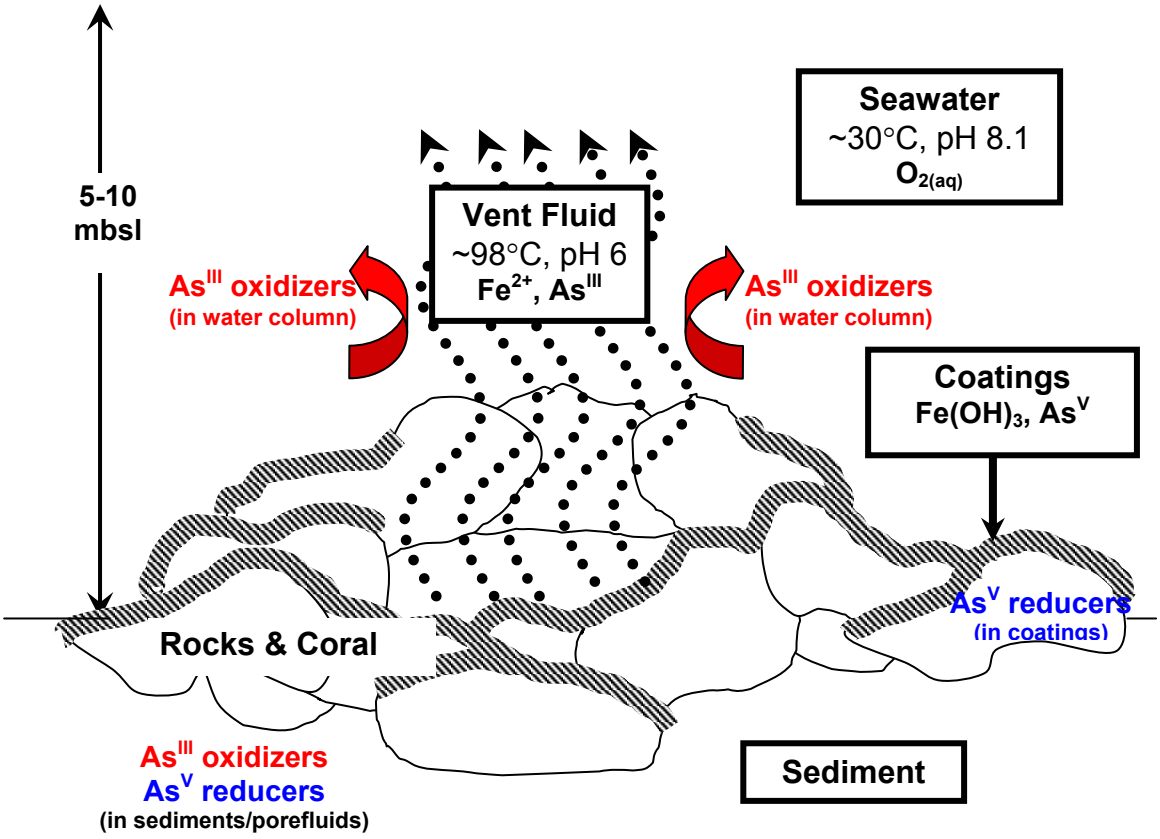


**Figure 9**. Schematic showing hydrothermal Vent 4 in Tutum Bay and its surrounding environment with likely locations of microbial $As^{III}$ oxidizers and $As^V$ reducers indicated.

# CHAPTER 2

## Energy sources for chemolithotrophs in an arsenic- and iron-rich shallow-sea hydrothermal system

Written for *Geobiology*

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**ABSTRACT**

The hydrothermally-influenced sediments of Tutum Bay, Ambitle Island, Papua New Guinea, are ideal for investigating the chemolithotrophic activities of microbes involved in arsenic-cycling because hydrothermal vents in the bay expel fluids with arsenite ($As^{III}$) concentrations as high as 950 µg/L. These hot (~98ºC), slightly acidic (pH ~6), chemically reduced shallow-sea vent fluids mix with cooler, oxidized seawater to create steep gradients in temperature, pH, and concentrations of As-, N-, Fe-, and S-redox species. Near the vents, iron oxyhydroxides precipitate with up to 6.4 wt% arsenate ($As^{V}$). Here, chemical analyses of sediment porewaters from 10 sites along a 300 m transect were combined with standard Gibbs energies to evaluate the energy available ($-\Delta G_r$) from 19 potential chemolithotrophic metabolisms, including $As^{V}$ reduction, $As^{III}$ oxidation, $Fe^{III}$ reduction, and $Fe^{II}$ oxidation reactions. The 19 reactions yielded between −2 kJ/mol $e^-$ and −94 kJ/mol $e^-$, with aerobic oxidation of sulfide and arsenite the two most exergonic reactions. Although arsenate reduction with sulfide as the electron donor and iron reduction with sulfide and arsenite as electron donors were among the least exergonic reactions investigated, they are still potential net metabolisms in this hydrothermal ecosystem.

Energy yields from all but one of the arsenic redox reactions correlate linearly with pH, increasing with increasing pH for $As^{III}$ oxidation and decreasing with increasing pH for $As^{V}$ reduction. Many of the reactions evaluated in this study are known metabolic strategies utilized by microbes in hydrothermal and geothermal sites, and the calculated exergonic energy yields suggest that microorganisms utilizing diverse metabolisms may

be present in Tutum Bay. Studies such as this can help target sampling sites for future microbial collection and cultivation studies.

## INTRODUCTION

Chemolithotrophic microorganisms can be key primary producers in geochemical environments where temperatures are high and light may be scarce (Inskeep et al., 2005). A broad diversity of chemolithotrophic archaea and bacteria have been identified in deep-sea hydrothermal systems (e.g., Reysenbach et al., 2000; Huber et al., 2002; Schrenk et al., 2003; Nakagawa et al., 2006) shallow-sea hydrothermal systems (e.g., Sievert et al., 2000; Miroschnichenko, 2004; Nakagawa et al., 2005; Hirayama et al., 2007; Maugeri et al., 2009), terrestrial hot springs (e.g., Barns *et al.*, 1994; Donahoe-Christiansen *et al.*, 2004; Meyer-Dombard et al., 2005; Wilson et al., 2008; Boyd et al., 2009), and in the deep subsurface (e.g., Stevens and McKinley, 1995; Parkes et al., 2000; Sørensen et al., 2004; Kovacik et al., 2006, Trimarco et al., 2006). Able to metabolize simple inorganic compounds, chemolithotrophs can play an important role in the biogeochemical cycling of many elements, including arsenic and iron (Brock and Gustafson, 1976; Reysenbach et al., 2000; Kulp et al., 2008).

Several recent studies in hydrothermal ecosystems calculated redox reaction energetics to establish a quantitative framework within which to investigate the potential roles of chemolithotrophs. For example, in one recent study of hot springs in Yellowstone National Park, USA (Shock et al., 2009), reactions yielded from around 0 to 150 kJ/mol e- transferred. Another study of four hot springs at acidic and near-neutral pH in Yellowstone showed that 33 chemolithotrophic reactions involving As, Fe, S, N, C,

and $H_{2(aq)}$ species yielded between −95 and +15 kJ/mol $e^-$ transferred (Inskeep et al., 2005). This range in energy was similar to that seen (~120 kJ/mol $e^-$) in a similar study conducted in the hydrothermal system of Vulcano Island, Italy (Rogers and Amend, 2006).

In the shallow-sea hydrothermal fluids at Tutum Bay, Ambitle Island, eastern Papua New Guinea (Fig. 1), arsenic and iron concentrations are highly elevated, with arsenite ($As^{III}$) and $Fe^{2+}$ levels measured as high as 950 µg/L and 0.81 mg/L, respectively. $Fe^{III}$-oxyhydroxides with adsorbed and/or co-precipitated arsenate ($As^{V}$, up to 6.4 wt%) coat many rocks and corals near vent orifices (Pichler & Veizer, 1999). Although high amounts of arsenic can be toxic to many microorganisms, some organisms can perform As-oxidation or As-reduction as metabolisms (for a review see Oremland and Stolz, 2003). Here in Tutum Bay, the co-occurrence of reduced and oxidized forms of As and Fe suggest active microbially mediated cycling of these metal(loid)s. In this study, we calculated the Gibbs free energies for 19 chemolithotrophic reactions using a combination of *in situ* measurements and thermodynamic calculations, and we explore the potential of these reactions to support microbial metabolic activity.

***Study Site***

Ambitle, a volcanic island in eastern Papua New Guinea (Fig. 1), is part of a Quaternary stratovolcano in the Tabar-Feni island arc (Wallace et al., 1983). Several hot mud pools, fumaroles, and springs with chloride or acid-sulfate waters dot the island (Wallace et al., 1983), while Tutum Bay, off the west coast, features shallow-sea hydrothermal vents among coral-algal reefs in 5 – 10 m water depth, circa 150 – 200 m

off shore (Pichler et al., 1999). Two types of hydrothermal venting occur in Tutum Bay: focused discharge from 10 – 15 cm diameter orifices, and diffuse discharge of gas bubbles (94 – 98% $CO_2$) through unconsolidated sediment on the seafloor (Pichler et al., 1999; Price & Pichler, 2005). Compared to ambient seawater, the Tutum Bay hydrothermal fluids are enriched in As, B, Mn, Si, Fe, and $HCO_3^-$, and depleted in Na, Cl, $SO_4^{2-}$, and Mg (Pichler et al., 1999; Price et al., 2007). Arsenic is particularly elevated, with hydrothermal fluids containing up to ~950 μg/L $As^{III}$, compared with ~5 μg/L total arsenic in local seawater. Rocks and corals near vent orifices were coated in arsenate-rich $Fe^{III}$-oxyhydroxide precipitates. These were varied in appearance, from soft orange and brown layers to hard greenish-brown layers; they contained up to 6.4 wt% arsenic, which is two orders of magnitude higher than that found in other marine $Fe^{III}$-oxyhydroxide deposits in hot spots and seamounts in the southwest Pacific (Stoffers et al. 1993; Hein et al., 1994).

## METHODS

### *Sampling and Laboratory Analyses*

A sampling transect was established beginning at one vent (designated Vent 4 in Pichler *et al.*, 1999) and extended southwest for 30 m, then continued west out to 300 m. The change in direction was necessary to avoid coral reef outcrops. A detailed discussion of sampling procedures and water analyses is given in Price *et al.* (2007). In brief, porewaters were collected by SCUBA divers at 10 cm intervals up to 1 m depth with a specially-designed sampler. Porewater temperature and pH were measured *in situ* using hand-held meters and probes. Porewater samples were brought on ship for rapid analyses

of $\Sigma PO_4^{3-}$ and the redox-sensitive $Fe^{2+}$, $NO_3^-$, $NO_2^-$, $NH_4^+$, $\Sigma S^{2-}$, and dissolved oxygen (DO) via portable spectrophotometers (HACH, Colorado), according to the manufacturer's instructions. The pH was re-measured on board using a pH meter with temperature compensation and results were very similar to *in situ* measurements. Water samples were also collected and preserved for later analysis of major elements and arsenic concentration and speciation at the Center for Water and Environmental Analysis at the University of South Florida. Concentrations of Na, Ca, Mg, K, Si, Sr, Mn, and B were measured via inductively coupled plasma-optical emission spectrometry (ICP-OES), and $Cl^-$, $Br^-$, and $SO_4^{2-}$ were measured by ion chromatography (IC). Concentrations of arsenic were measured via hydride generation-atomic fluorescence spectrometry (HG-AFS) and high pressure liquid chromatography (HPLC) was used to separate $As^{III}$, $As^{V}$, dimethylarsinic acid (DMA), and monomethylarsonic acid (MMA) prior to detection by HG-AFS (see Price *et al.* (2007) and Price and Pichler (2005) for details). The compositions of porewaters for the sites investigated are given in Table 1.

***Geochemical Calculations***

The maximum amount of energy available from any chemical reaction is given by the Gibbs energy ($\Delta G_r$), which is a function of the chemical composition of the system, its temperature, pressure, and the *standard* Gibbs energy of reaction ($\Delta G_r°$). The Gibbs energy can be calculated with the equation

$$\Delta G_r = \Delta G_r^o + RT \ln Q_r , \quad (1)$$

where $Q_r$ is the reaction activity quotient, R is the universal gas constant, and T is the temperature in Kelvin. Values of $\Delta G_r^o$ were calculated at the temperatures and pressures

of interest with the computer software package SUPCRT92 (Johnson et al., 1992), and thermodynamic properties given in Diakonov et al., 1994; Helgeson, 1985; Helgeson et al., 1978; King & Weller, 1970; Robie & Hemingway, 1995; Shock & Helgeson, 1988; Shock & Helgeson, 1989; Shock et al., 1989; Shock et al., 1997; Wagman et al., 1982. Values of $Q_r$ can be calculated from the equation

$$Q_r = \prod a_i^{v_i} \quad (2)$$

where $a_i$ is the activity of the *i*th species and $v_i$ is the stoichiometric reaction coefficient, which is positive for products and negative for reactants. Activities were calculated from the measured porewater compositions (Table 1) using the geochemical speciation program module React in The Geochemist's Workbench software package (v.7.0, Rockware, University of Illinois; Bethke and Yeakel, 2008). Log activities of the aqueous species used in the energetics calculations are listed in Table 2. To calculate $\Delta G_r$, activities of the dominant aqueous species were used, except in a few indicated cases where non-dominant species were substituted to keep the reactions consistent as written. Activities of pure minerals were set to unity.

Values of $\Delta G_r$ were calculated for 19 inorganic redox reactions that represent potential chemolithotrophic metabolisms. These reactions, listed in Table 3, involve the following aqueous species and one mineral: $O_2/H_2O$, $H_2AsO_4^-/H_3AsO_3$, $NO_3^-/NO_2^-/NH_4^+$, $SO_4^{2-}/H_2S$, and $Fe(OH)_3$(ferrihydrite)/$Fe^{2+}$. The ferric iron in Tutum Bay is predominately present as 2-line ferrihydrite (Pichler & Veizer, 1999). The structure of ferrihydrite is still debated and thermodynamic data at elevated temperatures and pressures are not yet available; they are likely to be highly dependent on the particle size of ferrihydrite (Navrotsky et al., 2008). To permit energy calculations of reactions with

ferrihydrite, we estimated the corresponding values of $\Delta G^o$ at elevated temperatures using the van't Hoff relation and thermodynamic properties at 25 ºC and 1 bar from Navrotsky et al. (2008). To facilitate direct comparison of energy yields between potential metabolisms, values of $\Delta G_r$ for all redox reactions were normalized per mole of electrons transferred. In addition, all reactions in the direction as written are exergonic.

## RESULTS AND DISCUSSION

### *Composition of Porewaters*

Geochemical analyses of Tutum Bay porewaters are given in Table 1. The temperatures ranged from 69.9 to 81°C within the first 12 m, and then stayed constant at ~30°C at 30 m and further distances. The pH values were circumneutral at all sites, varying from 6.11 to 7.88. The temperature, pH, and concentrations of several species (e.g., $SiO_{2(aq)}$, $Mg^{2+}$, $SO_4^{2-}$) demonstrate that porewaters at 0 – 12 m are dominated by hydrothermal fluid, while those further out are principally seawater. However, As concentrations point to a hydrothermal influence even at 300 m. All 10 sites are characterized by arsenic levels significantly higher than background total arsenic in local seawater (2.4 μg/L; Price et al., 2007). Total arsenic in porewaters ranged from 6.5 to 583.7 μg/L, with arsenite concentrations 1.8 – 395.7 μg/L, and arsenate concentrations 2.4 – 188.0 μg/L. Arsenate dominated arsenite at all sites except at 12, 20, and 240 m. Concentrations of $Fe^{2+}$ were relatively low at all sites (≤ 0.13 mg/L), except at 30 m (0.81 mg/L). Concentrations of other redox species investigated were relatively constant at all sites.

***Gibbs free energy calculations***

The energy yields from the 19 redox reactions listed in Table 3 were calculated at up to 10 sites along the sampling transect. Some reactions could not be evaluated at some of the sites, due to a lack of certain data. For each reaction, the average energy yield (symbol) and range across the sites (horizontal lines) were calculated and plotted in Fig. 2. Values of $\Delta G_r$ are plotted from least exergonic at the top right to most exergonic at the bottom left, with a net range from –2.0 to −94.0 kJ/mol $e^-$. Aerobic sulfide oxidation yielded the highest average energy at –93.2 kJ/mol $e^-$, followed closely by aerobic oxidation of $As^{III}$ (–76.0) and aerobic ferrous iron oxidation (−68.3). The least exergonic reactions were anaerobic reduction of ferrihydrite with $As^{III}$ (−8.6) and ammonium oxidation to nitrite with nitrate as TEA (−14.8). The only $As^V$ reduction reaction investigated, with $H_2S$ as electron donor, also yielded minimal energy (−20.8). Three anaerobic $As^{III}$ oxidation reactions (9, 11, 15), with $NO_3^-$ or $NO_2^-$ as TEA, yielded moderate amounts of energy (−45.7, −34.9, −31.4, respectively), as did the anaerobic oxidation of $H_2S$ with ferrihydrite (−28.0).

Values of $\Delta G_r$ showed a moderately strong correlation with pH of the porefluid ($R^2$ = 0.72 to 0.98) for all of the arsenic reactions except for reaction 18 which involved iron (see Fig. 3). It can be seen that energy yields ($\Delta G_r$) increase with increasing pH for four of the five $As^{III}$ oxidation reactions (2, 9, 11, 15). The opposite trend is true for the $As^V$ reduction reaction (19), where energy yields decrease with increasing pH. Reaction 18 did not display any correlation between pH and $\Delta G_r$ values. No such correlations between energy and pH were observed for other reactions, nor were correlations seen between $\Delta G_r$ values and temperature or $\Delta G_r$ values and distance from the vent.

The energy yields calculated for the Tutum Bay system can be compared with those at other hydrothermal sites. For example, the net range in $\Delta G_r$ for inorganic redox reactions in the present study is 92 kJ/mol $e^-$, compared to ~120 kJ/mol $e^-$ in the shallow-sea hydrothermal system at Vulcano Island, Italy (Amend et al., 2003), and ~150 kJ/mol $e^-$ in an array of terrestrial hot springs at Yellowstone National Park, USA (Shock et al., 2009). It should be pointed out, however, that both the Vulcano study and the Yellowstone study were able to consider a far greater number of potential chemolithoautotrophic metabolisms—90 reactions at Vulcano and 150 at Yellowstone. This expanded coverage was possible there because more redox-sensitive compounds were above measurable detection limits, including $H_2$ and $CH_4$ in the gas phase. In addition, the ranges of pH are broader at Vulcano (2.0 – 6.3) and Yellowstone (<2 to >9) than at Tutum Bay (6.1 – 7.9), which translates to variations in $\Delta G_r$ for many redox reactions. Similar to the $\Delta G_r$-pH trend observed in Tutum Bay (Fig. 3), most reactions investigated at Yellowstone showed this correlation (Shock et al., 2009). Lastly, in the Vulcano system, concentrations of $Fe^{2+}$ varied over 4 orders of magnitude and, consequently, the range of $\Delta G_r$ values for Fe-redox reactions was > ~60 kJ/mol $e^-$. By comparison, at Tutum Bay, the concentrations of $Fe^{2+}$ differed by less than 1 order of magnitude across all sites, and the corresponding $\Delta G_r$ values of the iron redox reactions (3, 10, 12, 16, 17, 18) ranged by 74.1 kJ/mol $e^-$.

***Microbial metabolic strategies***

Relatively few bacteria (and no archaea) are currently known to obtain metabolic energy from $As^{III}$ oxidation (Oremland and Stolz, 2003; Oremland et al., 2009).

Examples include the heterotrophic *Hydrogenophaga* strain NT-14, and the chemolithotrophic α-Proteobacteria NT-26 and BEN-5 isolated from Australian gold mines, all of which couple $As^{III}$ oxidation to $O_2$ reduction (Santini et al., 2000; Santini et al., 2002; vanden Hoven and Santini, 2004), and the Soviet gold mine isolate *Pseudomonas arsenitoxidans* (Ilyaletdinov and Abdrashitova, 1981). The latter species has been lost, preventing its further characterization, but the Australian isolates were grown at 28°C, similar to many of the temperatures recorded in Tutum Bay. While there is no direct evidence of microbially-mediated As oxidation in the Tutum Bay hydrothermal system, we can use the energy framework developed in this study to constrain where this metabolic strategy is most favorable. The high energy yield of reaction 2, aerobic arsenite oxidation, at all sites investigated suggests that microbes could catalyze this reaction for energy gain across Tutum Bay. Reaction 2 was the most exergonic reaction investigated at 180 and 240 m, suggesting these sites in particular may host microbial consortia dominated by aerobic $As^{III}$ oxidizers. Reaction 9, oxidation of $As^{III}$ with nitrate as the TEA, is catalyzed by the chemolithotrophic facultative anaerobe strain MLHE-1, isolated from the arsenic-rich waters of Mono Lake, California (Oremland et al., 2002). The 200μM (0.015g/L) arsenic concentration of Mono Lake is approximately 15 times greater than Tutum Bay waters, but the significant amount of energy that could be gained from catalyzing reaction 9 (–39.0 to –53.5 kJ/mol $e^-$) show that this metabolic process could also support microorganisms in the bay.

Many of the other reactions investigated here are utilized by microorganisms in hydrothermal and geothermal sites and potentially support microorganisms in Tutum Bay as well. Sulfur oxidation and reduction, for example, are widely utilized by

chemolithotrophs, especially thermophiles, as metabolic strategies. Hydrothermal systems in both shallow-sea and deep-sea sites have included sulfide oxidizers. Species of sulfide-oxidizing *Thiomicrospira,* for instance, have been observed in Milos, Greece and at deep-sea vents along the mid-Atlantic ridge, the Izu-Bonin arc in the Western Pacific, and in the Galapagos (Jannasch and Mottl, 1985; Muyzer *et al*., 1995, Brinkhoff *et al.*, 1999; 2005; Kato et al., 2009). Various *Thiobacillus* species are capable of sulfide oxidation coupled with oxygen and nitrate (Robertson and Kuenen, 2006). The sulfide oxidation reactions (1, 7, 8, 14), which used oxygen, nitrate, and nitrite as TEAs, all tended to yield the same amounts of energy (ranging between −48.8 and −93.4 kJ/mol $e^-$) regardless of pH or temperature, and the aerobic sulfide oxidation reaction (1) was the most energetic reaction at all sites where it was evaluated, suggesting that microorganisms capable of any of these reactions could thrive throughout Tutum Bay.

Reaction 5, the aerobic oxidation of ammonium to nitrate, yields −40.7 kJ/mol $e^-$ in Tutum Bay, a significant amount of energy. The first isolated ammonia-oxidizing archaeon, Candidatus "*Nitrosopumilus maritimus"* (Könneke et al., 2005), was recently isolated from a tropical water environment at similar temperatures and pH values to those found in Tutum Bay and is believed to metabolize a reaction similar to reaction 5. Based on 16S gene surveys, organisms similar to *"Nitrosopumilus maritimus"* and the archaeal marine sponge symbiont *Cenarchaeum symbiosum,* which contains putative ammonia-oxidizing genes, have been identified in hydrothermally-influenced Tutum Bay sediment samples (Akerman and Amend, 2009); the energetics data in this study support the hypothesis that populations of microorganisms in the bay may be mediating similar $NH_3/NH_4^+$-oxidizing reactions for metabolic growth.

In Tutum Bay, the reduction of ferric iron produced −28.0 (reaction 17) and −8.6 kJ/mol $e^-$ (reaction 18). Reaction 18, the reduction of ferrihydrite coupled with arsenite oxidation, was the least energy yielding reaction observed in Tutum Bay, although the energy produced is still capable of supporting microbial growth; several studies have shown that some anaerobes can grow at energy yields close to $\Delta G_r = 0$ (Conrad et al., 1986; Wu et al., 1994; Jackson and McInerney, 2002). The reduction of ferric iron has previously been noted as an ability of all hyperthermophilic microorganisms, with many microbes able to harness energy from the reaction (Kashefi et al., 2002). *Thiobacillus* species, in addition to oxidizing sulfur species, are also capable of oxidizing iron and although mainly found in highly acidic environments, some species are capable of growing in neutral pH (Robertson and Kuenen, 2006). Other species capable of aerobic oxidation of ferrous iron include *Sulfobacillus thermosulfidooxidans* and *S. acidophilus* (Norris et al., 1996). In addition, a number of species are able to use hydrogen gas as an electron donor in the reduction of ferric iron; however, $H_{2(g)}$ was measured at <0.01 mmol/mol in Tutum Bay (Pichler et al., 1999), suggesting that reactions using $H_{2(g)}$ in this environment would be less energy-yielding and subsequently microbial species would be less likely to gain energy from such reactions.

## CONCLUSIONS

In Tutum Bay, 19 different potential metabolic reactions were evaluated using *in situ* chemical composition and physical parameters and were found to yield a range of energy (−2.0 to −94.0 kJ/mol $e^-$) across 10 different sample sites. The top five most energy-yielding reactions used $O_2$ and $NO_3^-$ as the terminal electron acceptor. All

reactions yielded significant amounts of energy that could potentially support a variety of microbial life across a range of temperatures and pH values. Although the hydrothermal system contains high amounts of arsenate, arsenite, and iron(III)-oxyhydroxide precipitates (predominately in the form of 2-line ferrihydrite), the most energy yielding reaction on average was aerobic sulfur oxidation. Sulfur reduction and oxidation reactions are typically important and widespread in hydrothermal systems; sulfur redox reactions were the most energetic group of reactions evaluated in Tutum Bay and present a highly advantageous metabolic strategy for microorganisms. Also of interest were the energy-yielding nitrogen redox reactions, which represent metabolic strategies for ammonia oxidizing archaea and bacteria. The reactions involving arsenic and iron species are also known to support numerous microbial species, suggesting that Tutum Bay hydrothermal porewaters can support a variety of microbial communities that may play roles in the redox cycling of As, Fe, N, and S. The pairing of energy with geography presents the possibility of using the calculated energetics as a framework for locating sampling areas where particular energy sources may be utilized by members of the local microbial community, and therefore microbial species with specific metabolic strategies may be targeted for sample collection.

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**Table 1. Composition of hydrothermal pore waters along Transect 4B (distance from vent orifice in m) in Tutum Bay, 2005**

| | 0.5 | 7.5 | 12 | 20 | 30 | 60 | 140 | 180 | 240 | 300 | P.I. [a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **T (°C)** | 69.9 | 73.94 | 81 | 29.69 | 33.22 | 33.9 | 34 | 32.8 | 29.72 | 29.69 | n.a. |
| **pH** | 6.14 | 6.89 | 6.22 | 7.64 | 6.9 | 6.34 | 6.11 | 7.84 | 7.88 | 7.08 | 7.6 |
| **$HCO_3^-$** | 327 | 415 | 605 | 156 | 176 | 264 | 244 | 142 | 154 | 167 | 186.2 |
| **Na** | 975 | 2680 | 2132.5 | 9340 | 10283 | 8687 | 8975 | 9930 | 10300 | 10500 | 10800 |
| **K** | 147 | 234 | 149.8 | 356 | 394 | 351 | 365 | 403 | 387 | 401 | 401 |
| **Mg** | 164 | 636 | 143.8 | 1100 | 1257 | 1067 | 1075 | 1260 | 1260 | 1305 | 1289 |
| **Ca** | 122 | 121 | 223.5 | 372 | 406 | 377 | 369 | 355 | 395 | 411 | 416 |
| **$Fe^{2+}$** | 0.13 | n.a. | 0.04 | 0.11 | 0.81 | 0.05 | n.a. | 0.01 | 0.02 | 0.01 | n.a. |
| **Sr** | n.a. | n.a. | 6.425 | 7 | 7.2 | 6.9 | 6.7 | 10 | 6.9 | 7.2 | 6.9 |
| **$Cl^-$** | 2069 | 6066 | 2753 | 15818 | 19040 | 16198 | 15715 | 18529 | 19350 | 20115 | n.a. |
| **$Br^-$** | 12 | 15.5 | 7.5 | 46.7 | 55.5 | 49.7 | 46.4 | 54.1 | 59 | 60 | n.a. |
| **$SO_4^{2-}$** | 879.5 | 1154.4 | 1174 | 2405.2 | 2734.4 | 2465.6 | 2401.3 | 2688 | 2785 | 2857.1 | n.a. |
| **$PO_4^{3-}$** | 1.43 | 0.19 | 1.48 | 0.67 | 0.23 | 1.8 | 0.67 | 0.08 | 0.03 | 0.2 | n.a. |
| **$As^{(III)}$** | n.a. | 2.16 | 395.72 | 4.19 | 2.31 | 1.97 | 1.8 | 4.89 | 5.07 | 2.01 | 1.9 |
| **$As^{(V)}$** | n.a. | 29.78 | 188 | 2.35 | 18.19 | 49.63 | 45.4 | 14.75 | 2.72 | 12.14 | 1.4 |
| **$NO_3^-$** | 11.8 | 8.3 | 1.3 | 4.1 | 4.3 | 6.4 | 8.5 | 7.5 | 7.1 | 8.4 | n.a. |
| **$NO_2^-$** | 0.008 | 0.0155 | 0.004 | 0.016 | 0.022 | 0.02 | 0.025 | 0.4 | 0.015 | 0.023 | n.a. |
| **$\Sigma S^{2-}$** | 8 | 3 | n.a. | n.a. | 5 | 4 | 3 | n.a. | n.a. | 2 | n.a. |
| **$NH_{3(aq)}$** | 0.38 | 0.02 | 0.35 | 0.11 | n.a. | n.a. | 0.08 | 0.06 | n.a. | 0.07 | n.a. |
| **B** | n.a. | n.a. | 0.75 | 5.2 | 4.8 | 5.6 | 5.9 | n.a. | 4.4 | 4.7 | 0.5 |
| **Si** | n.a. | n.a. | 91.3 | 13.6 | 2.8 | 15.6 | 16.4 | 2 | 0.4 | 1 | 2 |
| **$O_{2(aq)}$** | 3 | 6.9 | 2.4 | 2.8 | 2 | 1.8 | 3.5 | 2.6 | 3.7 | 1.9 | n.a. |

All concentrations are in mg/L except for $As^{(III)}$, $As^{(V)}$, and $\Sigma S^{2-}$ which are in µg/L.
n.a. = not available
[a] P.I. = Picnic Island Control. Values from 2003.

**Table 2. Log activities of aqueous species used in energetic calculations**

| | 0.5 | 7.5 | 12 | 20 | 30 | 60 | 140 | 180 | 240 | 300 |
|---|---|---|---|---|---|---|---|---|---|---|
| $H^+$ | -6.14 | -6.89 | -6.22 | -7.64 | -6.9 | -6.34 | -6.11 | -7.84 | -7.88 | -7.08 |
| $H_2O_{(l)}$ [a] | 0.998 | 0.995 | 0.997 | 0.985 | 0.983 | 0.986 | 0.985 | 0.984 | 0.983 | 0.983 |
| $O_{2(aq)}$ | -4.01 | -3.64 | -4.10 | -4.00 | -4.14 | -4.19 | -3.90 | -4.02 | -3.87 | -4.16 |
| $H_3AsO_{3(aq)}$ | n.a. | -7.54 | -5.26 | -7.27 | -7.51 | -7.58 | -7.62 | -7.21 | -7.20 | -7.57 |
| $H_2AsO_4^-$ | n.a. | -7.19 [b] | -5.93 | -9.15 [b] | -7.60 [b] | -6.73 | -6.65 | -8.56 [b] | -9.34 [b] | -7.93 [b] |
| $NO_3^-$ | -3.83 | -4.03 | -4.81 | -4.37 | -4.36 | -4.18 | -4.06 | -4.11 | -4.14 | -4.07 |
| $NO_2^-$ | -6.87 | -6.62 | -7.19 | -6.65 | -6.52 | -6.55 | -6.46 | -5.26 | -6.68 | -6.50 |
| $NH_4^+$ | -4.77 | -6.11 | -4.83 | -5.41 | n.a. | n.a. | -5.54 | -5.68 | n.a. | -5.61 |
| $SO_4^{2-}$ | -2.64 | -2.78 | -2.63 | -2.60 | -2.57 | -2.58 | -2.60 | -2.57 | -2.56 | -2.56 |
| $H_2S_{(aq)}$ | -6.76 | -7.61 [c] | n.a. | n.a. | -7.20 | -7.05 | -7.12 | n.a. | n.a. | -7.68 [c] |
| $Fe^{2+}$ | -6.19 | n.a. | -6.89 | -6.47 | -5.64 | -6.82 | n.a. | -7.54 | -7.23 | -7.53 |

[a] Represents activities, not log activities
[b] $HAsO_4^{2-}$ dominates, but in the energy calculations the log activities of $H_2AsO_4^-$ were used throughout
[c] $HS^-$ dominates, but in the energy calculations the log activities of $H_2S_{(aq)}$ were used throughout
n.a. = not available

**Table 3. Chemolithotrophic reactions evaluated for energy yield in Tutum Bay**

| | Reaction | $e^-$ transferred |
|---|---|---|
| 1. | $H_2S_{(aq)} + 2O_{2(aq)} \rightarrow SO_4^{2-} + 2H^+$ | 8 |
| 2. | $H_3AsO_{3(aq)} + 0.5O_{2(aq)} \rightarrow H_2AsO_4^- + H^+$ | 2 |
| 3. | $4Fe^{2+} + O_{2(aq)} + 10H_2O \rightarrow 4Fe(OH)_3 + 8H^+$ | 4 |
| 4. | $2NH_4^+ + 3O_{2(aq)} \rightarrow 2NO_2^- + 2H_2O + 4H^+$ | 12 |
| 5. | $NH_4^+ + 2O_{2(aq)} \rightarrow NO_3^- + H_2O + 2H^+$ | 8 |
| 6. | $2NO_2^- + O_{2(aq)} \rightarrow 2NO_3^-$ | 4 |
| 7. | $H_2S_{(aq)} + 4NO_3^- \rightarrow SO_4^{2-} + 4NO_2^- + 2H^+$ | 8 |
| 8. | $H_2S_{(aq)} + NO_3^- + H_2O \rightarrow SO_4^{2-} + NH_4^+$ | 8 |
| 9. | $H_3AsO_{3(aq)} + NO_3^- \rightarrow H_2AsO_4^- + NO_2^- + H^+$ | 2 |
| 10. | $2Fe^{2+} + NO_3^- + 5H_2O \rightarrow 2Fe(OH)_3 + NO_2^- + 4H^+$ | 2 |
| 11. | $4H_3AsO_{3(aq)} + NO_3^- + H_2O \rightarrow 4H_2AsO_4^- + NH_4^+ + 2H^+$ | 8 |
| 12. | $8Fe^{2+} + NO_3^- + 21H_2O \rightarrow 8Fe(OH)_3 + NH_4^+ + 14H^+$ | 8 |
| 13. | $NH_4^+ + 3NO_3^- \rightarrow 4NO_2^- + 2H^+ + H_2O$ | 6 |
| 14. | $3H_2S_{(aq)} + 4NO_2^- + 4H_2O + 2H^+ \rightarrow 3SO_4^{2-} + 4NH_4^+$ | 24 |
| 15. | $3H_3AsO_{3(aq)} + NO_2^- + H_2O \rightarrow 3H_2AsO_4^- + NH_4^+ + H^+$ | 6 |
| 16. | $6Fe^{2+} + NO_2^- + 16H_2O \rightarrow 6Fe(OH)_3 + NH_4^+ + 10H^+$ | 6 |
| 17. | $H_2S_{(aq)} + 8Fe(OH)_3 + 14H^+ \rightarrow SO_4^{2-} + 8Fe^{2+} + 20H_2O$ | 8 |
| 18. | $H_3AsO_{3(aq)} + 2Fe(OH)_3 + 3H^+ \rightarrow H_2AsO_4^- + 2Fe^{2+} + 5H_2O$ | 2 |
| 19. | $H_2S_{(aq)} + 4H_2AsO_4^- + 2H^+ \rightarrow SO_4^{2-} + 4H_3AsO_{3(aq)}$ | 8 |

**Table 4. $\Delta G_r$ of reactions in Tutum Bay (kJ/mol $e^-$ transferred)**

| | 0.5 | 7.5 | 12 | 20 | 30 | 60 | 140 | 180 | 240 | 300 | Avg. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Rxn1** | -91.66 | -92.72 | | | -94.02 | -93.20 | -93.24 | | | -94.03 | -93.15 |
| **2** | | -72.85 | -72.96 | -82.29 | -74.67 | -70.19 | -69.58 | -81.29 | -83.92 | -76.02 | -75.97 |
| **3** | -65.44 | | -62.97 | -76.05 | -72.67 | -59.18 | | -72.64 | -74.60 | -63.14 | -68.34 |
| **4** | -44.13 | -44.67 | -44.49 | -45.91 | | | -42.81 | -44.69 | | -44.26 | -44.42 |
| **5** | -39.62 | -40.48 | -40.22 | -42.18 | | | -39.74 | -42.04 | | -40.78 | -40.72 |
| **6** | -26.10 | -27.91 | -27.40 | -31.01 | -30.95 | -30.20 | -30.54 | -34.12 | -30.42 | -30.33 | -29.90 |
| **7** | -65.56 | -64.82 | | | -63.07 | -63.00 | -62.71 | | | -63.70 | -63.81 |
| **8** | -52.04 | -52.24 | | | | | -53.50 | | | -53.25 | -52.76 |
| **9** | | -44.95 | -45.56 | -51.28 | -43.73 | -39.99 | -39.05 | -47.17 | -53.50 | -45.69 | -45.66 |
| **10** | -39.35 | | -35.56 | -45.04 | -41.72 | -28.99 | | -38.52 | -44.19 | -32.81 | -38.27 |
| **11** | | -32.38 | -32.74 | -40.11 | | | -29.84 | -39.24 | | -35.24 | -34.92 |
| **12** | -28.49 | | -25.28 | -37.01 | | | | -33.70 | | -25.51 | -30.00 |
| **13** | -18.03 | -16.76 | -17.08 | -14.90 | | | -12.27 | -10.57 | | -13.93 | -14.79 |
| **14** | -47.53 | -48.05 | | | | | -50.43 | | | -49.77 | -48.95 |
| **15** | | -28.18 | -28.47 | -36.38 | | | -26.77 | -36.60 | | -31.76 | -31.36 |
| **16** | -21.32 | | -18.48 | -30.14 | | | | -27.95 | | -18.88 | -23.35 |
| **17** | -26.20 | | | | -21.13 | -33.84 | | | | -30.67 | -27.96 |
| **18** | | | -10.00 | -6.24 | -2.00 | -11.00 | | -8.65 | -9.31 | -12.88 | -8.58 |
| **19** | | -19.87 | | | -19.34 | -23.01 | -23.66 | | | -18.01 | -20.78 |

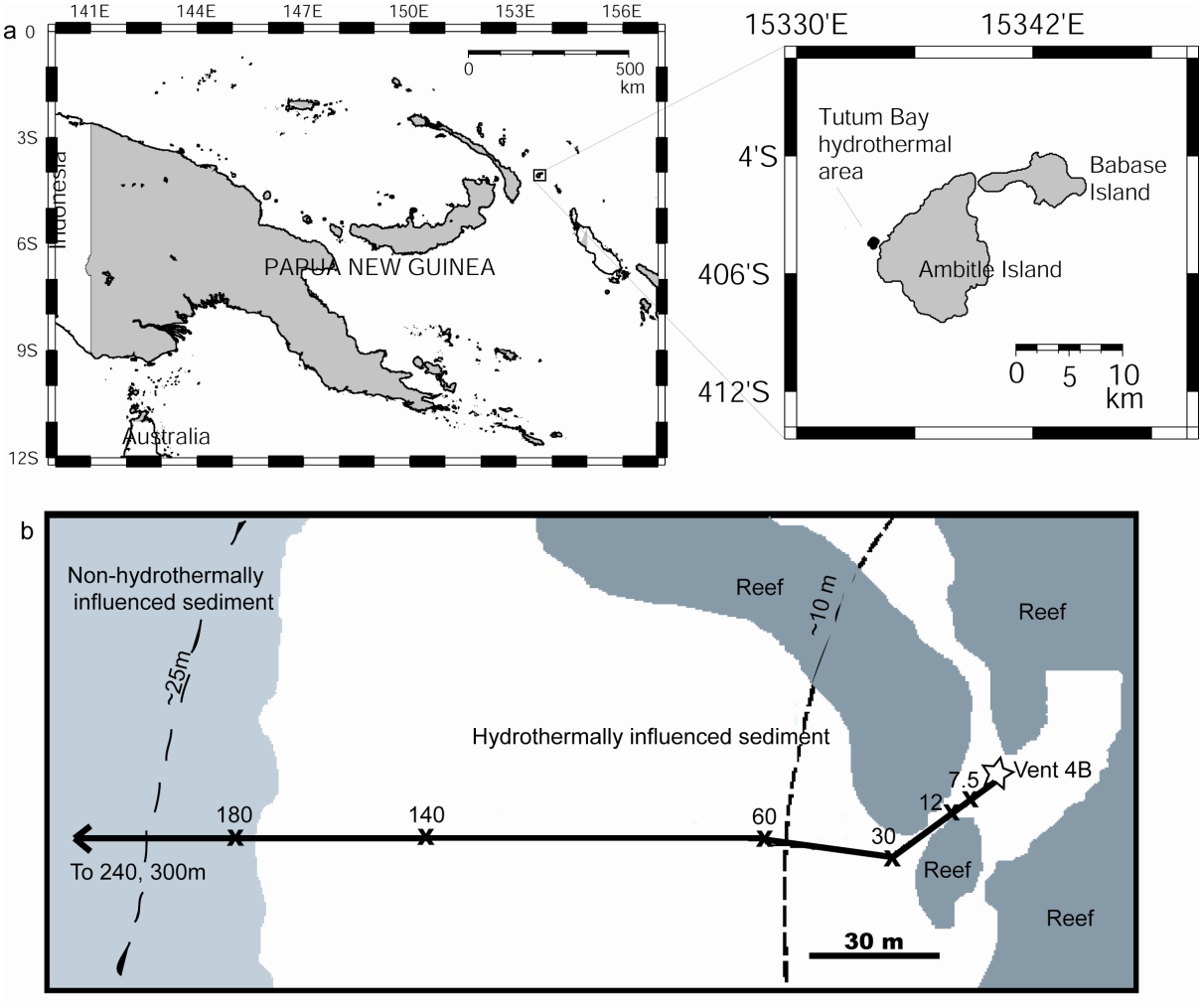


**Figure 1.** (a) Papua New Guinea, with Feni Islands (Ambitle and Babase) enlarged. (b) Plan view of Tutum Bay hydrothermal area with sampling transect constructed from Vent 4B. Sampled sites are indicated by **x**s. Water depth indicated by dashed lines.

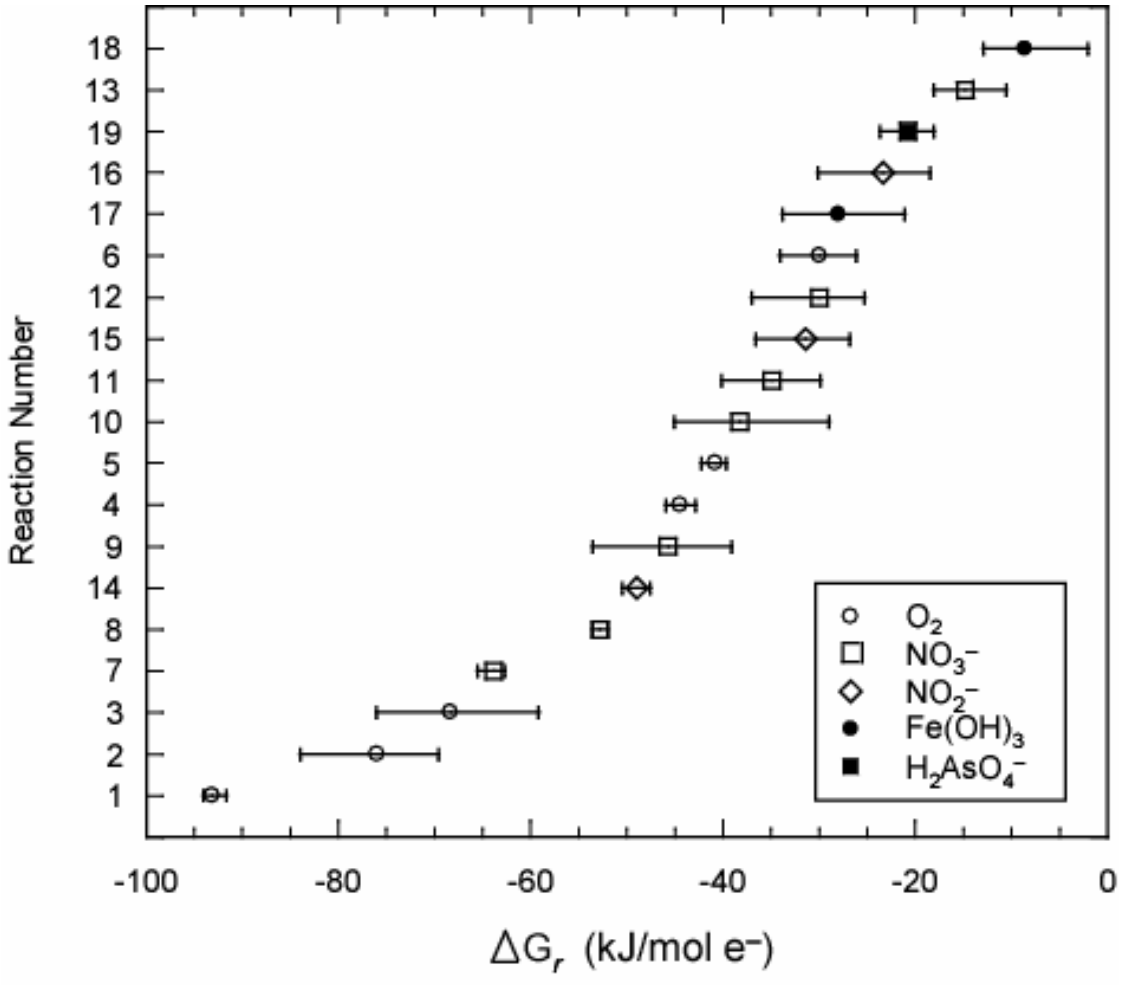


**Figure 2.** Average values of $\Delta G_r$ (kJ/mol $e^-$) of 19 chemolithotrophic reactions (listed in Table 3) in Tutum Bay porewaters. Horizontal lines represent range of values across up to 11 sites. Different symbols represent different TEAs in the reactions.

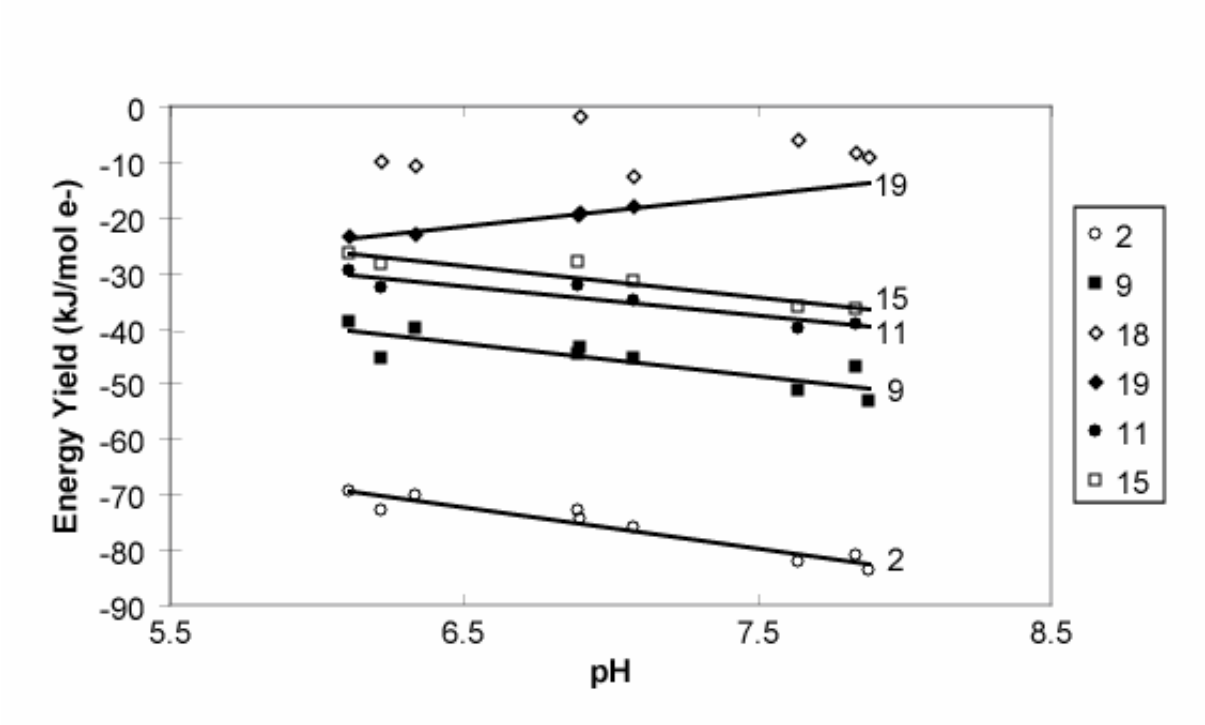


**Figure 3.** $\Delta G_r$ (kJ/mol $e^-$) of arsenic redox reactions as a function of pH. Reaction numbers correspond to those in Table 3.

# CHAPTER 3

## Archaeal community composition in arsenic-rich, shallow-sea hydrothermal sediments

Written for *Environmental Microbiology*

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**ABSTRACT**

Arsenic is a potentially lethal toxin to humans, but a variety of microorganisms can tolerate highly elevated levels of arsenic or even metabolize it for energy gain. Most arsenic microbiology has focused on the role of bacteria with little attention paid to the archaea. Hydrothermal systems are well-known archaeal habitats and often feature high concentrations of arsenic. Here, we investigated the archaeal community structure in the arsenic-rich hydrothermally-influenced sediments of Tutum Bay, Ambitle Island, Papua New Guinea. Arsenic (up to 950 μg/L) is the only elevated toxin in the hot (~98ºC), slightly acidic (pH ~6), chemically reduced shallow-sea vent fluids. Archaeal 16S clone libraries were constructed from bulk DNA extracted from shallow sediment at five sites along a 300 m transect. Phylogenetic analyses showed the presence of archaea at all the sites, with one phylotype of uncultured Crenarchaeota dominating (39 – 90%) at four of them; at the fifth site, a single euryarchaeotal phylotype made up ~50% of the clone library. In addition, the clone libraries featured several clades within the Marine Group 1 Crenarchaeota, a member of the Thermoprotei (which includes the only known As-metabolizing archaeal genera *Sulfolobus* and *Pyrobaculum*), three sequences closely affiliated with the Hot Water Crenarchaeota Group 1, several sequences plotting among known marine hydrothermal vent groups in the Euryarchaeota phylum, and one Korarchaeota-like sequence. In general, the five sediment sites investigated were of low to moderate archaeal diversity with sequences falling into three to eight identified clades.

## INTRODUCTION

Arsenic (As) can be highly toxic to humans, even in relatively small amounts (Dueñas-Laita *et al.*, 2005), but a number of microbial species can tolerate elevated levels of As or even metabolize it. Arsenic-metabolizing microorganisms have been isolated from a variety of environments, including gold mine rocks, muds, and tailings (Santini *et al.*, 2000; Santini *et al.*, 2002; Anderson & Cook, 2004), marshes (Stolz *et al.*, 1999), lake sediments (Niggemyer *et al.*, 2001; Liu *et al.*, 2004; Oremland *et al.*, 2005), and geothermal waters (Salmassi *et al.*, 2002; Donahoe-Christiansen *et al.*, 2004; Kulp *et al.*, 2008). To date, more than two dozen species of microbes are known to be capable of reducing arsenate ($As^{V}$) for energy gain, while other strains gain energy by oxidizing arsenite ($As^{III}$), and a number of strains can reduce or oxidize As as a detoxification mechanism (Oremland et al., 2009). The majority of these species are bacteria, predominately Proteobacteria. Only a few archaea are known to perform arsenic redox: *Sulfolobus metallicus* strain BC (formerly known as *S. acidocaldarius* strain BC) oxidizes arsenite, but does not harvest energy from this process (Sehlin & Lindström, 1992), while *Pyrobaculum arsenaticum* and *P. aerophilum* are both capable of respiring arsenate (Huber *et al.*, 2000). Both *Pyrobaculum* species are hyperthermophiles isolated from thermal sites near Naples, Italy. *P. arsenaticum* was cultivated from slightly acidic sediment (pH 6.0) of the Pisciarelli solfataric area, and *P. aerophilum* was isolated from a marine hot spring on the island of Ischia in the Gulf of Naples (Völkl *et al.*, 1993; Huber *et al.*, 2000).

Fluids, sediments, and mineral precipitates associated with hot springs and marine hydrothermal systems are often enriched in arsenic. For instance, As concentrations in

Yellowstone National Park (USA) hot springs reach ~150,000 μg/L (Langner *et al.*, 2001), although concentrations more typically range from 750 to 3000 μg/L (Stauffer & Thompson, 1984; Ball *et al.*, 1998). Deep-sea hydrothermal vent fluids from Guaymas Basin (Gulf of California) and at 21°N on the East Pacific Rise contained approximately 2 – 80 μg/L of arsenic, while in the Lau Basin the arsenic concentrations were much higher, ranging from approximately 450 to 824 μg/L (von Damm, 1995). Polymetallic massive sulfide structures formed in conjunction with hydrothermal fluids at the Escanaba Trough on the southern part of the Gorda Ridge (in the northeast Pacific Ocean, off the coast of California) contained up to 5 wt% arsenic (Koski *et al.*, 1988). Shallow-sea hydrothermal systems, located at < 200 mbsl, have also been observed to contain elevated levels of As in both vent fluids and nearby precipitates. For example, the Champagne Hot Springs off the coast of Dominica, Lesser Antilles, contained 18 – 80 μg/L of arsenic in 45 – 70°C vent fluids (McCarthy et al., 2005), and in the Bahía Concepción system in the Gulf of California, hydrothermal vent fluids at 0.5 – 15 mbsl contained up to 780 μg/L arsenic (Canet et al., 2005). These two sites also featured sediments and vent precipitates with high As concentrations. In Champagne Hot Springs, hydrous ferrous oxide precipitates contained up to 1880 ppm As, and sediments contained up to 311 ppm As, over 1000 and 100 times the levels found in typical Caribbean sediments, respectively (McCarthy et al., 2005).

The microbial community compositions in several continental hot springs and marine hydrothermal systems have now been investigated via culture-independent techniques (e.g., Barns *et al.*, 1994; Takai and Horikoshi, 1999; Reysenbach *et al.*, 2000; Hirayama et al., 2007; Rusch and Amend, 2008). Connon et al. (2008) studied the

bacterial mat communities in the Alvord hot spring system (Oregon, USA), characterized by 4495 μg/L As at circumneutral pH. They found evidence for microbial arsenite oxidation, but archaeal communities were not identified. Microbial mat communities in an arsenic-rich (~2472 μg/L), acidic hot spring of Norris Geyser Basin in Yellowstone National Park, USA also showed evidence of microbially-mediated arsenic oxidation (Jackson *et al.*, 2001). Intriguingly, the onset of arsenite oxidation in this hot spring coincided with the appearance of archaeal 16S rRNA sequences in the mats. However, the role of non-biofilm forming microbes in both these hot spring environments has received only limited focus.

In marine hydrothermal systems, the majority of microbial surveys are in deep-sea systems, with very little attention paid to their shallow-sea (< 200 m depth) counterparts. In most systems where microbial communities were well characterized, the corresponding fluid composition was not analyzed concurrently or in detail. Notable exceptions include the sites at Vulcano Island, Italy (Rogers & Amend, 2005; Rusch & Amend, 2008), Milos Island, Greece (Sievert *et al.*, 1999; Sievert *et al.*, 2000), Taketomi Island, Japan (Hirayama *et al.*, 2007), and Eyjafjordur, Iceland (Marteinsson *et al.*, 2001b). However, at some of these sites the archaeal community composition was largely ignored. In this study, we investigated the archaeal community structure of arsenic-rich sediments in the shallow-sea hydrothermal system of Tutum Bay, Papua New Guinea (Fig. 1), shedding light on their presence, distribution, and diversity. Archaeal 16S rRNA gene libraries were constructed for 5 shallow (0 – 10 cm deep) sediment sites along a transect leading away from an As-rich vent. A corresponding 16S

rRNA survey of the bacterial communities is being evaluated separately, as was a depth profile for bacteria and archaea at a nearby site (Meyer-Dombard et al., 2009).

## RESULTS

### *Field Site and Geochemical Context*

Ambitle is the southernmost island of the Tabar-Feni Island arc in eastern Papua New Guinea (Fig. 1a). It is part of a Quaternary stratovolcano and features fumaroles, hot mud pools, and hot springs with chloride-rich and acid-sulfate waters (Wallace *et al.*, 1983; Pichler & Dix, 1996). The Tutum Bay hydrothermal system located off the west coast of Ambitle Island features several discrete vents among coral algal reefs. These vents are located ~150 m offshore at a water depth of 5 – 10 m. In addition to focused discharge from discrete orifices, diffuse discharge of gas bubbles (94 – 98% $CO_2$) through unconsolidated seafloor sediment is also present (Pichler *et al.*, 1999a; Pichler et al., 2006). The hydrothermal fluids are predominately of meteoric origin, based on the low total dissolved solids (< 3000 mg/L), molecular ratios indicative of diluted seawater, and $\delta^{18}O$ values that match closely with local precipitation $\delta^{18}O$ records (Pichler et al., 1999b; Pichler, 2005). These hydrothermal fluids contain up to 950 μg/L arsenic, exclusively as $As^{III}$. Arsenite expelled in fluids appears to be quickly oxidized, a process which may be microbially-mediated, and co-precipitated with ferric (oxy)hydroxides that coat sediments, rocks, and coral near vent orifices (Pichler & Veizer, 1999; Pichler *et al.*, 1999b; Pichler *et al.*, 2006). These ferric oxyhydroxide coatings range in color from bright orange to dark brown and green, with As concentrations (as $As^{V}$) measured up to 6 wt% (Pichler & Veizer, 1999).

In this study, porewaters and sediments along a 300 m transect starting at a discrete vent (designated Vent 4B in Pichler *et al.* 1999a) were investigated (Fig. 1b). Geochemical analyses along the transect are discussed in detail elsewhere (Price & Pichler, 2005; Pichler et al., 2006; Price et al., 2007; Akerman et al., 2009), with several key parameters from porewaters at 10 cm depth summarized here (Fig. 2). Note that the temperature was high (~70 – 80°C) along the transect out to a distance of 12 m, but then quickly dropped off to the ambient seawater value (~30°C) and stayed relatively constant along the rest of the transect. The pH was circumneutral along the entire transect, fluctuating between 6 and 6.5 out to ~140 m with one spike to ~7 at 7.5m, and then pH ranged between 7 and 8 beyond 140 m. The total arsenic concentration at all sites was elevated above the local background seawater level of 4.9 μg/L, although $As^{III}$ concentrations fluctuated above and below the local seawater level of 2.6 μg/L while $As^{V}$ remained elevated at all sites above the local level of 2.3 μg/L. The highest values for both oxidation states were recorded at 12 m, with 395.7 μg/L $As^{III}$ and 188 μg/L $As^{V}$. Beyond 12 m, arsenite levels decreased rapidly to < ~5 μg/L, remaining relatively constant out to 300 m. Arsenate concentrations decreased more gradually with distance, reaching < 10 μg/L at 240 m, before increasing again to 12.1 μg/L at 300 m.

***Archaeal diversity***

The archaeal diversity of the Tutum Bay hydrothermal system was investigated by cloning and sequencing 16S rRNA genes from five sites, located at 7.5, 30, 60, 140, and 300 m distance along the Vent 4 transect. In total, 350 archaeal clones were successfully sequenced from the five sites, representing 15 different uncultured

phylotypes. Composite phylogenetic trees from the five clone libraries show 11 Crenarchaeota (Fig 3a.), three Euryarchaeota, and one Marine Hydrothermal Vent Group 1 (MHVG1)-type phylotype that is closely related to, but distinct from, the Korarchaeota (Fig. 3b). The majority of the crenarchaeal clones belonged to the Marine Group 1 archaeal group.

In Fig. 4, archaeal clones are grouped separately for each of the five sediment sample sites. It can be seen that uncultured Crenarchaeota (denoted as CA groups in this study) dominate at each site. All sites, except that at 30 m, were dominated (39 – 90%) by one phylotype, an uncultured Crenarchaeote designated CA5 in this study. At 30 m, an uncultured Euryarchaeote (designated EA1) dominated, contributing 46% of the clone library. It should be noted that Euryarchaeota were only found at the two sites closest to the vent, and in all, only one deep-branching MHVG1-type sequence was found – at the 140 m site. The two sites furthest from the vent displayed the only Hot Water Crenarchaeotic Group I (HWCG I) group sequences.

The 7.5 m site contained eight phylotypes: two uncultured Euryarchaeota and six uncultured Crenarchaeota. The CA5 group represented 40% of all clones at 7.5 m, and the CA4 group represented 25% of the clones. The CA1 group, representing 13% of the clones, contained members which are closely related to the *Cenarchaeales* (97% identical). Some clones in the CA2 group (12%) were 97% identical to the marine archaeon Candidatus *"Nitrosopumilus maritimus*." Eight percent of the clones identified as members of the Euryarchaeota phylum, with all but one clone (7.5_A148) falling in group EA1.

The 30 m site was dominated (46%) by the EA1 group, and contained the largest proportion of CA3 crenarchaeota (39%) of any site investigated. These two groups were followed by clones in group CA1 (8%) and CA2 (5%). This was the only site investigated that did not contain any clones from the CA5 group, and, as noted above, the only site to be comprised of nearly 50% euryarchaeal species. It also contained one clone in the C3 group, a group not seen at any other site.

The 60 m and 140 m sites were both dominated by the same 3 groups: CA5 (71% at 60 m, 60% at 140 m), CA3 (15%, 15%), and CA1 (14%, 13%). While these were the only groups identified at 60 m, however, five other minor groups were also noted at 140 m, including clones in group CA2, the deep-branching clone MHVG1 (140_A053), and one clone (140_A057) affiliated with the HWCG I group.

The 300 m site was dominated (90%) by group CA5 clones. In addition, two clones each of the CA1 (300_A003, 300_A033) and HWCG I (300_A036, 300_A045) groups and one clone (300_A064) affiliating with the Thermoprotei were identified. Unlike the other sites, there were no group CA3 clones observed.

**DISCUSSION**

The clone libraries from Tutum Bay contain clones belonging to 15 phylotypes across the 5 sites studied. Clones representing both of the two main archaeal phyla, the Crenarchaeota and Euryarchaeota, were observed, as well as one clone affiliating with the deep-branching Marine Hydrothermal Vent Group 1 (MHVG1) (Takai et al., 2001). Clones from these three groups were not evenly distributed across the 5 sample sites. All but one of the clones found in this study placed within clades of uncultured organisms

represented only by 16S rRNA archaeal sequences (Fig. 3). That one clone, 300_A064, affiliated phylogenetically with the Thermoprotei, which includes the three known As-redox archaeal species. Clone 300_A064 showed 86% sequenced identity to *Pyrobaculum arsenaticum*, but was more closely related (89%) to *Thermofilum pendens* Hrk 5, a thermophile and moderate acidophile isolated from a solfataric hot spring in Iceland (Zillig et al., 1983). *T. pendens* respires sulfur and grows optimally at 88°C in the laboratory, but is capable of growth at pH ranging from 4 – 6.5 and temperatures from 70 – 95°C (Stetter, 1999). These temperatures are much higher than the 29.7°C recorded at 300 m in Tutum Bay, but do overlap the pH and temperature range measured in our study. *T. pendens* is almost always found associated with *Thermoproteus tenax*, but no clones displaying similarity to *Thermoprot. tenax* were observed in the Tutum Bay clone libraries. Genes associated with specific aspects of arsenic redox, such as an arsenite-transporting ATPase, have been annotated in the complete genome of the organism *T. pendens* (Copeland et al., 2007). While the arsenic oxidizing potential of *T. pendens* has not yet been explored, further work may reveal this metabolic potential. Clone 300_A064's closest relative, however, at 99% sequence identity was the uncultured crenarchaeal clone TB2.5H2_A57, from an ~75°C site along Transect A in Tutum Bay (Meyer-Dombard et al., 2009).

All other phylotypes observed in this study grouped with uncultured archaeal clades, making it impractical to say anything about their physiology or metabolic strategies. Phylogenetic analyses showed that CA5 was the dominant crenarchaeal phylotype (39 – 90% of the clone library) at 4 of the 5 Tutum Bay sites investigated. Only the 30 m site did not contain CA5 clones. CA5 clones are most closely affiliated

with clones Fhm2A82 from a hydrothermal sulfide structure at the southern Mariana Trough (Oba et al., 2007) and fosmid clones HAuD-UA44 and 45-H-12 in the Hot Water Crenarchaeotic Group III (HWCG III), sequenced from the upper layer of a microbial mat community in subsurface geothermal waters in the Hishikari gold mine, Japan (Nunoura *et al.*, 2005). The upper layer of the Hishikari mat consisted almost entirely of the HAuD-UA44 clone, while lower layers displayed mostly bacteria and no evidence of HAuD-UA44. The upper layer of the mat grew *in situ* at 69°C, a temperature that overlaps some of the Tutum Bay temperatures but is hotter than most of the sites investigated here. Furthermore, the Hishikari mat grew in low salinity water with 0.3 mg/L dissolved oxygen and a pH of 5.1 (Hirayama et al., 2005). At Tutum Bay, the dissolved oxygen (~2 – 7 mg/L) and pH values (~6 – 8) were higher, and the porefluids were marine influenced. The Hishikari mat clones affiliate with the Hot Water Crenarchaeotic Group (HWCG), whose members have been identified in a variety of environments including subsurface geothermal fluids, terrestrial hot springs, and deep-sea hydrothermal vents (e.g., Barns *et al.*, 1996; Takai & Sako, 1999; Marteinsson *et al.*, 2001a; Inagaki et al., 2003; Schrenk et al., 2003). Nunoura *et al.* (2005) suggested that the HWCG forms a phylogenetic bridge between the hyperthermophilic *Thermoprotei* and the mesophilic Crenarchaeota and Eukarya. The presence of CA5 clones in both moderate (~30°C) and high (73.9°C) temperature sites at Tutum Bay is consistent with this hypothesis.

The majority of the remaining Crenarchaeota fell into 4 main groups, CA1, CA2, CA3, and CA4. These four groups are tightly clustered within Marine Group I (MG I) on the phylogenetic tree (Fig. 3a). CA1 clones were closely affiliated with Antarctic

bathypelagic sediment clones discovered in the Southern Ocean (Brandt *et al.*, 2007), and clones from the top layer of deep sediment (4500 mbsl) on the NW Atlantic Ocean abyssal plain (Ventriani *et al.*, 1999). CA4 clones were also closely affiliated with deep-sea clones, although they were more closely related to clones from active hydrothermal sulfide chimney structures on the Juan de Fuca Ridge (Schrenk *et al.*, 2003). Clones CA2 and CA3 closely related to microbial species typically associated with sponges including *Aciculites* species (Holmes and Blanch, 2007). CA2 and CA3 were most closely affiliated to clones SeAqRB09 and SeAqRB01, identified in the substratum of a tropical seawater tank in the Seattle Aquarium (Könneke *et al.*, 2005). The species Candidatus *"Nitrosopumilus maritimus*" recently isolated from this tank is closely related to clones in CA2 and CA3, and is the first cultured non-thermophilic chemolithoautotrophic ammonia-oxidizing marine archaeon (Könneke *et al.*, 2005; Wuchter et al., 2005). The average ammonia concentration in Tutum Bay measured ~0.1 mg/L (data not shown), but reached ~0.35 mg/L at several sites, and aerobic ammonia oxidation was calculated to be a thermodynamically favorable reaction at the 7.5, 140, and 300 m sites in Tutum Bay (Akerman et al., 2009), creating a favorable environment for *N. maritimus* and other similar species that are yet to be discovered.

Interestingly, no clones in this study grouped with the *Thermococcales*, even though archaeal communities at deep-sea hydrothermal sites often consist of a high proportion of *Thermococcales* (Reysenbach *et al.*, 2000). The presence of a clone that identified with the Thermoprotei, on the other hand, is an exception to the absence of *Thermoprotei* usually seen in molecular surveys of other marine hydrothermal environments (Nercessian et al., 2004). The phylogenetic affiliation of Tutum Bay

crenarchaeal clones with both deep-sea sedimentary clones and tropical sedimentary clones indicates that similar types of archaea may be present in both deep-sea and shallow-sea sediment environments. This supports the reasoning by Tarasov et al. (2005) that shallow-sea hydrothermal systems should be viewed as a mid-point on the continuum between terrestrial and deep-sea hydrothermal systems. Therefore, CA1 and CA4 clones, although previously found mainly in deep-sea sediment environments, may be important constituents of not only Tutum Bay but also other shallow-sea hydrothermal sediments.

In total, only three euryarchaeotal clades were observed in this study, two of which were represented by a single clone sequence. The dominant EA1 group is closely related to sequences from several hydrothermal systems, including ~100°C fluid discharged from a chimney at the Myojin Knoll, Izu-Ogasawara arc hydrothermal field (Takai & Horikoshi, 1999), and fluids at the southern Mariana Trough (Kato et al., 2009). Group EA1 and these affiliated clones are only distantly related to any isolated organisms, and therefore, their metabolisms are unknown. Phylogenetically, they cluster with thermophilic clades, in particular, with the DHVE1 group (Fig. 3b). The temperature (33°C) at the 30 m site in Tutum Bay, where group EA1 is most abundant, is much cooler than the temperatures recorded at these other sites. It is important to note, however, that even in high-temperature deep-sea systems it was found that clones that identified with hyperthermophilic clades were amplified from the cooler zones around high temperature vents, suggesting that fluid flow and archaeal community expulsion in the subsurface is complex (Kormas et al., 2003; Ehrhardt et al., 2007).

Only one clone (4B140H1_A53) in this study did not affiliate with either Crenarchaeota or Euryarchaeota, plotting closest to the Korarchaeota and MHVG I in Fig.

3b. The closest relatives are clones from deep-sea hydrothermal sulfide structures and from a shallow-sea (22 mbsl) hydrothermal vent fluid (128°C) in Tachibana Bay, Japan (Takai & Sako, 1999). These clones are sometimes erroneously labeled as Korarchaeota, but instead appear to form a separate, marine specific clade, MHVG I (Takai *et al.*, 2001; Auchtung *et al.*, 2006); our data from the shallow-sea system in Papua New Guinea are consistent with this interpretation. Note also that the 140 m clone library was the only one to display sequences of Crenarchaeota, Euryarchaeota, and the deep-branching MHVG1 clade of archaea.

The diversity seen at the 140 m site may reflect the site's geochemical signature. The 140 m porewater contained 47.2 μg/L total As at pH 6.1, compared to the 14.2 μg/L As and 7.1 pH observed at the less diverse 300 m site. The 60 m site had similar pH, temperature, and arsenic concentrations to 140 m and was dominated by the same 3 clades of archaea in similar proportions. The communities closest to the vent (7.5 and 30 m) were the only ones to contain euryarchaeal clones and also had some of the lowest total arsenic concentrations observed along the entire transect, especially in regards to $As^V$ concentrations, which were 29.8 and 18.2 μg/L, respectively. It is important to remember, though, that the physico-chemical parameters in the sediments are constantly in flux due to variable subsurface hydrothermal fluid flow, and that the microbial community composition at any one moment in time is a reflection of these changing parameters.

To place our results into context, we compare them to culture-independent and culture-dependent archaeal surveys of other sites of interest: two depth horizons in a sedimentary core at Tutum Bay (Meyer-Dombard et al., 2009), a shallow-sea coral reef

hydrothermal system in Japan (Hirayama et al., 2007), an As-rich continental hot spring at Yellowstone National Park (Macur et al., 2004), and deltaic sediments of Papua New Guinea (Todorov et al., 2000) (Table 1). The archaeal clones observed in the hotter (~75°C), more acidic (pH < 6), and more As-rich (> 350 μg/L) sediment core depth horizons were overall very different than those observed in this study, with the only overlap being Thermoprotei and Korarchaeota-like sequences were identified in both studies. No euryarchaeota were identified, nor were any Marine Group I crenarchaeota, although 4 clades of MG I were observed in this study (Meyer-Dombard et al., 2009). The geochemical variations between the two transects may account for this high degree of variability. The microbial community survey by Hirayama et al. (2007) at Taketomi Island, Japan, was conducted in the only other presently identified shallow-sea coral reef hydrothermal system. Vent fluids from this system also contained clones from the MG I and DHVE groups, and mats contained MG I and HWCG sequences. However, the Taketomi Island system was more diverse than Tutum Bay, with 25 phylotypes observed in vent fluids, and 39 phylotypes found in microbial mats. The As-rich microbial mats of the Yellowstone hot spring also contained Thermoprotei, including members of the Sulfolobales, Desulfurococcales, and Thermoproteales (Macur et al, 2004). In deltaic sediments from the Gulf of Papua, MG I clones were identified, but no Thermoproteales (Todorov et al., 2000); it is unknown whether these sediments contained arsenic.

Shallow porewaters in Tutum Bay are rich in arsenic, even at several hundred meters from the hydrothermal vent point source. While arsenic metabolism—both the reduction of $As^{V}$ and the oxidation of $As^{III}$—is now well known among the bacteria (see, e.g., Oremland et al., 2009), these metabolic strategies have yet to be assigned to more

than a couple of archaea in laboratory culturing studies. Here, we demonstrated the presence of a number of Crenarchaeota belonging to several different clades in the As-rich sediments of Tutum Bay at both ambient and elevated temperatures. Euryarchaeota were less common overall, but found to be the dominant archaeal phylum at one of the sites investigated. It has also been shown that a variety of As-redox reactions are energetically favorable in the Tutum Bay hydrothermal system (Akerman et al., 2009). However, the role of archaea in As cycling in general, and in marine hydrothermal systems in particular, is entirely unknown. The data presented here, coupled with the energetic framework outlined in Akerman et al. (2009) set the stage for investigations of archaea-mediated As cycling in marine systems.

## EXPERIMENTAL PROCEDURES

### *Collection of sediments*

In May 2005, sediments along transect 4B were cored by SCUBA divers using PVC tubes (1m × 6 cm). At the surface, sediment from the 0 – 10 cm depth range was homogenized, stored in sterile containers, and transported on ice to our laboratory in the USA. There, all samples were stored at –20°C and thawed to room temperature prior to analysis.

### *DNA extraction and 16S clone library construction*

Bulk DNA was extracted from each sediment sample via bead beating, using the FastPrep DNA Soil Extraction Kit (Bio101). Polymerase chain reaction (PCR) of archaeal 16S rRNA genes from bulk DNA was performed using the archaea-targeting

primers 21F (5'–TTC-CGG-TTG-TAC-CYG-CCG-GA–3') and 1391R (5'–GAC-GGG-CGG-TGT-GTR-CA–3') (Lane 1991). The 20 μL PCR reaction mixture consisted of 1.8 μL $MgCl_2$, 2.0 μL GeneAmp 10X PCR buffer, 0.2 μL dNTPs, 0.25 μL AmpliTaq Gold DNA polymerase, 0.5 μL each of forward and reverse primers (1 μM), and 1 μL template DNA. Thermocycling conditions on a Hybaid PCR Express thermalcycler consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1.5 min, followed by a final extension at 72°C for 15 min. PCR products were verified to be the correct size on a 1.5% agarose gel stained with ethidium bromide and photographed under UV light. Products were then cleaned using the QIAquick PCR Purification Kit (Qiagen) using the manufacturer's instructions.

PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen) per manufacturer's instructions. Plasmids were plated on LB agar plates amended with ampicillin and X-gal. Clones were selected randomly and incubated in 3 mL LB with ampicillin liquid growth medium overnight. Plasmid DNA was extracted and purified using the QiaPrep Spin Miniprep Kit (Qiagen). Purified plasmid DNA concentrations were determined via spectrophotometry, diluted to a concentration of 0.04 μg/μL and sequenced at Polymorphic DNA Technologies, Inc. (Alameda, CA) with either the M13 forward and M13 reverse, or the T7 and SP6 sequencing primers.

***Analysis of 16S rRNA clones***

Sequence data were assembled into contigs using Sequencher v.4.7 (Gene Codes) and manually edited. Assembled sequences were aligned using the Greengenes NAST aligner (DeSantis *et al.*, 2006) and checked for chimeras using the Bellerophon3 server at

Greengenes before they were imported into ARB (Swofford, 2003). All sequences were compared to sequences in the NCBI GenBank database via BLAST, and also classified using the Simrank comparison method in the Classify module on Greengenes. These data were used to construct rarefaction curves for each library; all rarefaction curves appeared asymptotic, suggesting the microbial diversity was sufficiently sampled (data not shown). For phylogenetic analysis, closely related sequences were found through BLAST searches and Greengenes. Phylogenetic trees were created using the maximum parsimony insertion tool in ARB. Phylogenetic topologies of 16S rRNA sequences from the 5 clone libraries were constructed in ARB (Ludwig *et al.*, 2004) and PAUP* (Swofford, 2003) via maximum parsimony and neighbor-joining techniques; clone sequences used in the analyses were greater than 1250 nucleotides. The phylogenetic trees show relationships between the clones from each phylotype in Tutum Bay to isolates and clones from other studies. The trees were rooted to an outgroup of 3 bacteria.

***Nucleotide sequence accession numbers***

The sequences reported herein have been deposited in the Genbank database (NCBI) under the following accession numbers: GU137351 through GU137395.

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**Table 1. Comparison of 16S rRNA archaeal clone libraries and cultivation studies from other high-temperature and neighboring geographical sites**

| Study Site and Description | Geochemical Data | Archaeal groups identified | References |
|---|---|---|---|
| Sediments from Tutum Bay, Papua New Guinea: As-rich shallow-sea (~10 mbsl ) hydrothermal system in a coral reef | Temp = 64 – 81°C<br>pH = 5.8 – 6<br>$As^{III}$ = 13 – 95 µg/L<br>$As^{V}$ = 160 – 431 µg/L | Terrestrial miscellaneous crenarchaeal group, Miscellaneous crenarchaeal group, Hyperthermophilic crenarchaeota, Marine hydrothermal vent groups, **Korarchaeota-type, Thermoprotei** | Meyer-Dombard *et al.*, 2009 |
| Vent fluids from hydrothermal system near Taketomi Island, Japan: Methane- and sulfide-rich shallow-sea (23 mbsl) hydrothermal system in a coral reef | Temp ≤ 52°C | Anaerobic methane oxidation group I, Miscellaneous crenarchaeal group**, Deep-sea hydrothermal vent euryarchaeal group**, *Archaeoglobales* | Hirayama *et al.*, 2007 |
| Microbial mats from hydrothermal system near Taketomi Island, Japan: Methane- and sulfide-rich shallow-sea (23 mbsl) hydrothermal system in a coral reef | Temp ≤ 52°C | **Marine Crenarchaeotic Group I, Hot water crenarchaeotic group,** deep-sea archaeal group 6, South African gold mine euryarchaeal group | Hirayama *et al.*, 2007 |
| Succession Spring, Yellowstone National Park, USA: Arsenic-rich hot spring microbial mats | Temp = 48.3 – 79.2°C<br>pH ≈ 3<br>$As_{Total}$ = 5244 – 6743 µg/L | **Thermoprotei** | Macur *et al.*, 2004 |
| Mobile deltaic sediments in the Gulf of Papua, Papua New Guinea: Surface 10 cm of sediment from 50 mbsl | | **Marine Crenarchaeotic Group I**, *Methanobacteria* | Todorov *et al.*, 2000 |

Clone groups in common with those found in this study are indicated in boldface.

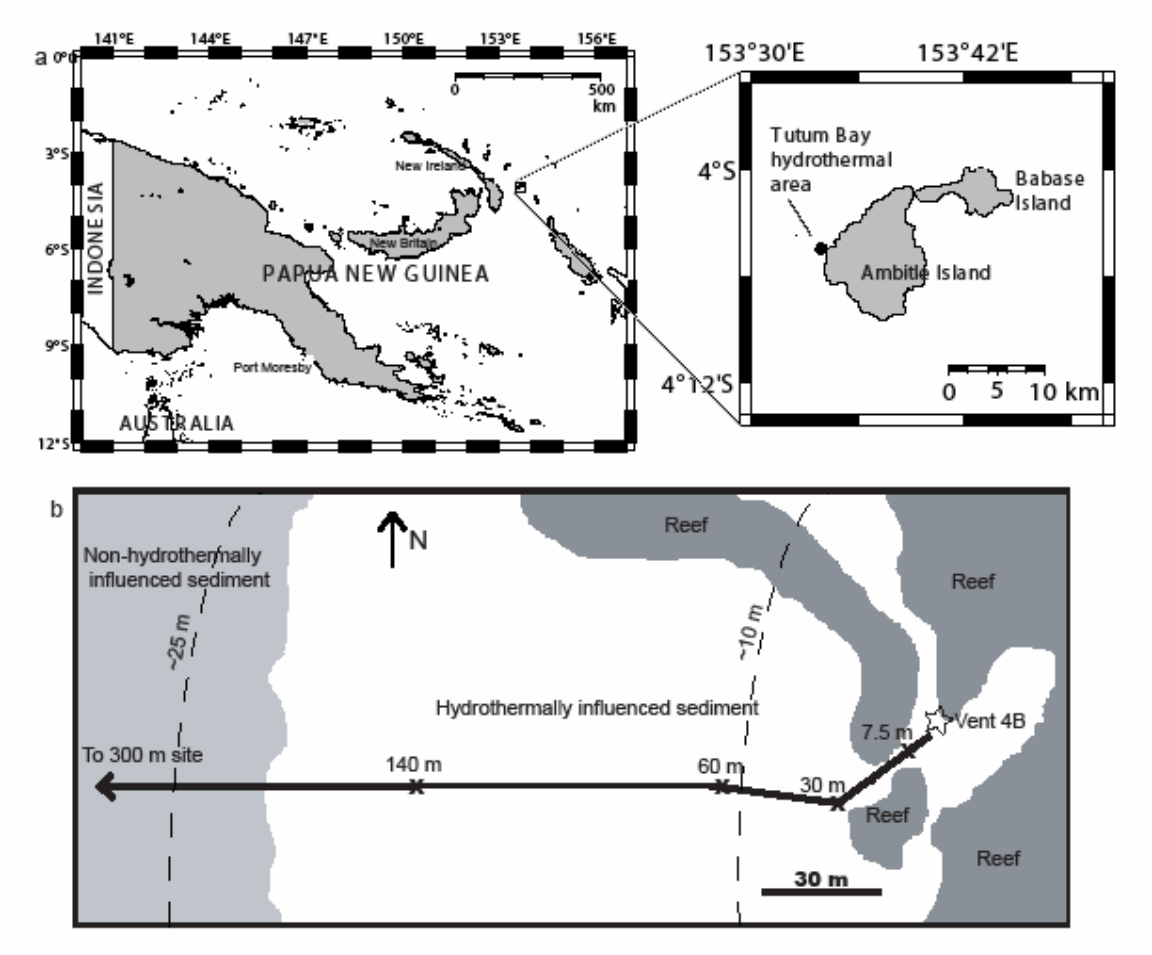



**Figure 1.** (a) Map of Papua New Guinea and Ambitle Island, with the Tutum Bay field site off the western coast. (b) Plan view of Tutum Bay Vent 4B (marked by star) sediment transect, with sampling sites (marked by Xs). Approximate water depths are indicated along the dashed lines.

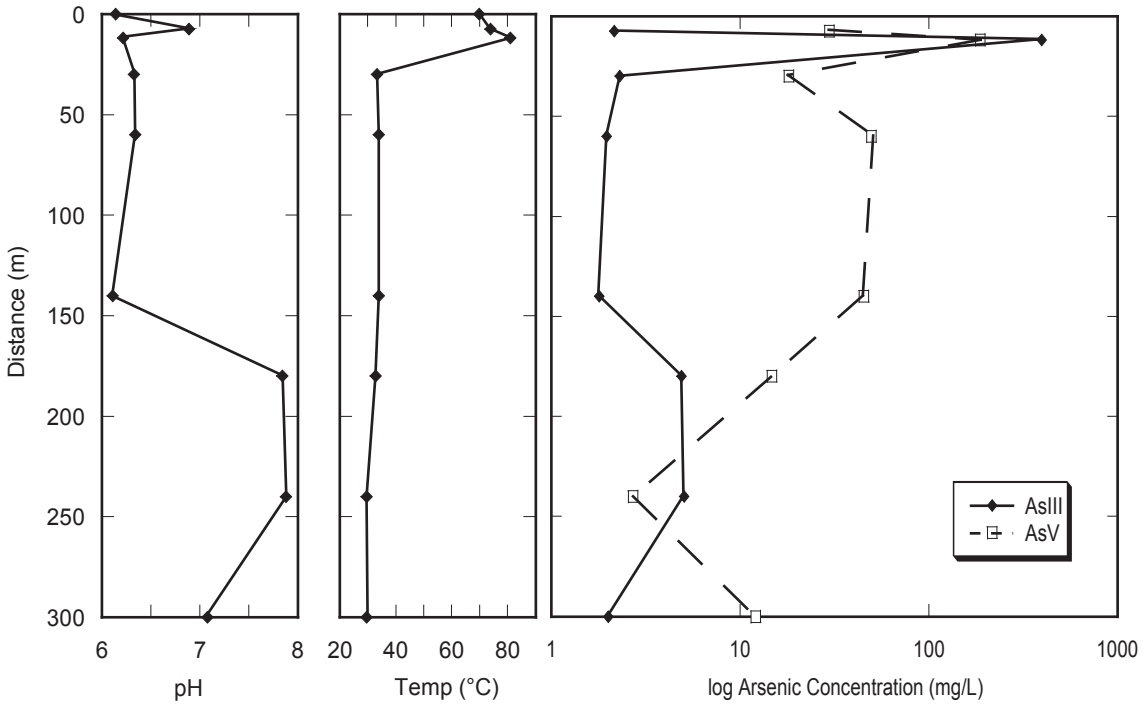



**Figure 2.** Temperature, pH and arsenic concentrations in porefluids (10 cm depth) along transect 4B.

**Figure 3.** Maximum parsimony archaeal phylogenetic trees (a – Crenarchaeota, b – Euryarchaeota and Korarchaeota) rooted to a bacterial outgroup, showing affiliations of clones from the 5 Tutum Bay sample sites. Clones found in this study are indicated in boldface. Scale bar represents 0.10 changes per unit. Numbers in parentheses indicate number of clones in phylogenetic group. *Pyrobaculum arsenaticum*, *P. aerophilum*, and *Sulfolobus metallicus* are the only archaea known to perform arsenic redox.

**Figure 3a.**

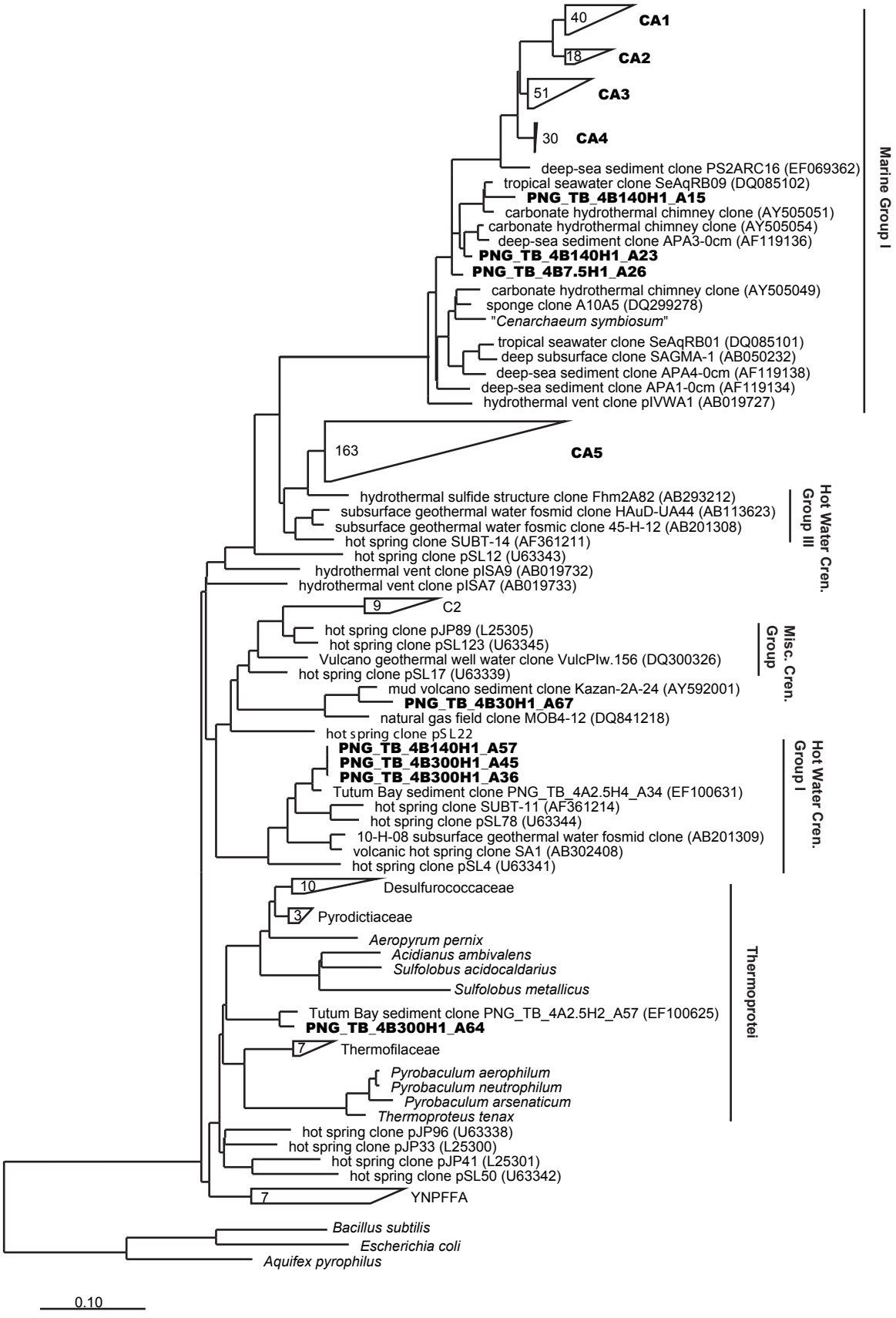

40
CA1
18
CA2
51
CA3
30
CA4
deep-sea sediment clone PS2ARC16 (EF069362)
tropical seawater clone SeAqRB09 (DQ085102)
PNG_TB_4B140H1_A15
carbonate hydrothermal chimney clone (AY505051)
carbonate hydrothermal chimney clone (AY505054)
deep-sea sediment clone APA3-0cm (AF119136)
PNG_TB_4B140H1_A23
PNG_TB_4B7.5H1_A26
carbonate hydrothermal chimney clone (AY505049)
sponge clone A10A5 (DQ299278)
"Cenarchaeum symbiosum"
tropical seawater clone SeAqRB01 (DQ085101)
deep subsurface clone SAGMA-1 (AB050232)
deep-sea sediment clone APA4-0cm (AF119138)
deep-sea sediment clone APA1-0cm (AF119134)
hydrothermal vent clone pIVWA1 (AB019727)
Marine Group I
163
CA5
hydrothermal sulfide structure clone Fhm2A82 (AB293212)
subsurface geothermal water fosmid clone HAuD-UA44 (AB113623)
subsurface geothermal water fosmic clone 45-H-12 (AB201308)
hot spring clone SUBT-14 (AF361211)
hot spring clone pSL12 (U63343)
hydrothermal vent clone pISA9 (AB019732)
hydrothermal vent clone pISA7 (AB019733)
Hot Water Cren. Group III
9
C2
hot spring clone pJP89 (L25305)
hot spring clone pSL123 (U63345)
Vulcano geothermal well water clone VulcPIw.156 (DQ300326)
hot spring clone pSL17 (U63339)
mud volcano sediment clone Kazan-2A-24 (AY592001)
PNG_TB_4B30H1_A67
natural gas field clone MOB4-12 (DQ841218)
Misc. Cren. Group
hot spring clone pSL22
PNG_TB_4B140H1_A57
PNG_TB_4B300H1_A45
PNG_TB_4B300H1_A36
Tutum Bay sediment clone PNG_TB_4A2.5H4_A34 (EF100631)
hot spring clone SUBT-11 (AF361214)
hot spring clone pSL78 (U63344)
10-H-08 subsurface geothermal water fosmid clone (AB201309)
volcanic hot spring clone SA1 (AB302408)
hot spring clone pSL4 (U63341)
Hot Water Cren. Group I
10
Desulfurococcaceae
3
Pyrodictiaceae
Aeropyrum pernix
Acidianus ambivalens
Sulfolobus acidocaldarius
Sulfolobus metallicus
Tutum Bay sediment clone PNG_TB_4A2.5H2_A57 (EF100625)
PNG_TB_4B300H1_A64
7
Thermofilaceae
Pyrobaculum aerophilum
Pyrobaculum neutrophilum
Pyrobaculum arsenaticum
Thermoproteus tenax
Thermoprotei
hot spring clone pJP96 (U63338)
hot spring clone pJP33 (L25300)
hot spring clone pJP41 (L25301)
hot spring clone pSL50 (U63342)
7
YNPFFA
Bacillus subtilis
Escherichia coli
Aquifex pyrophilus
0.10

**Figure 3b.**

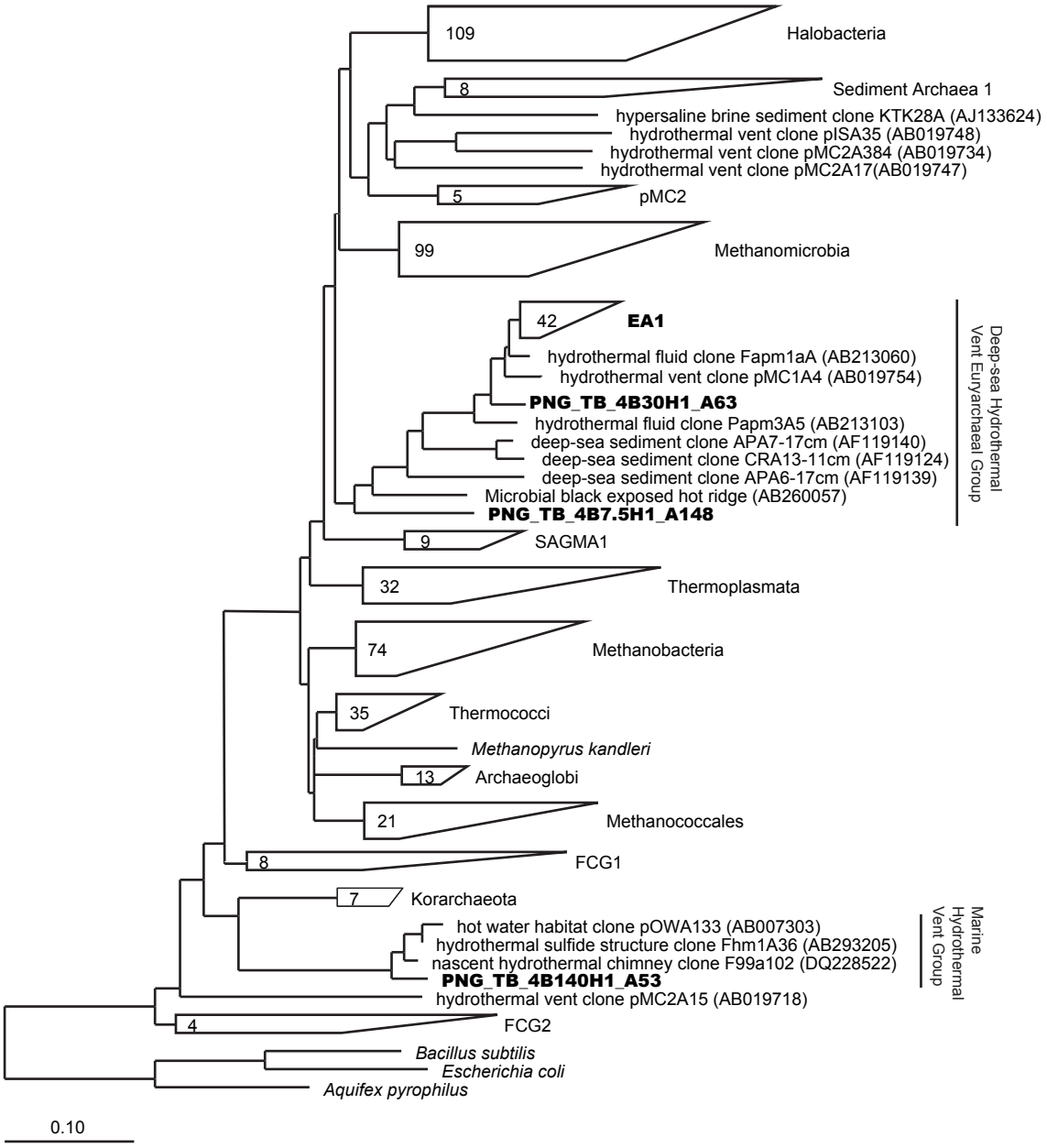
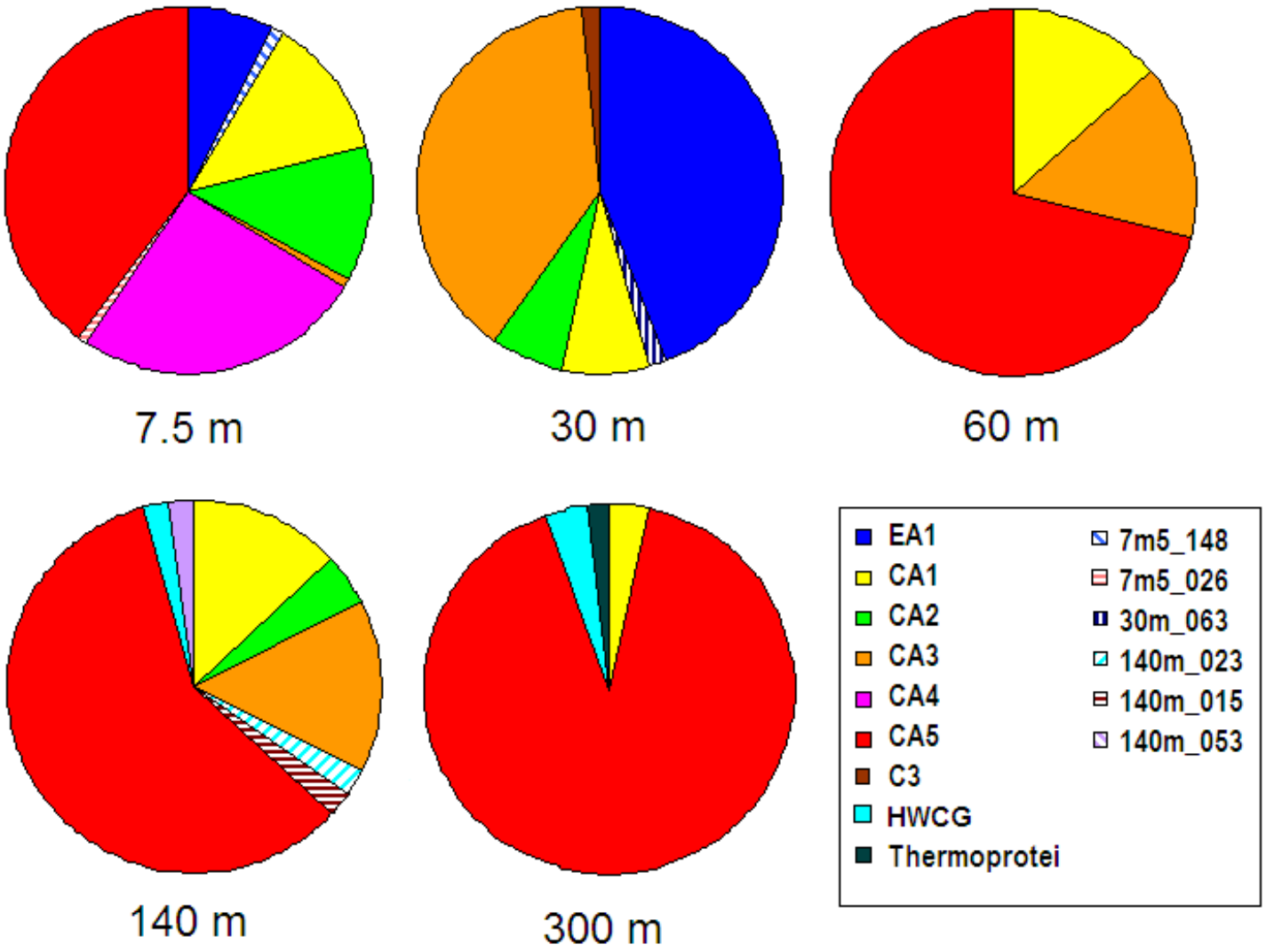

109
Halobacteria
8
Sediment Archaea 1
hypersaline brine sediment clone KTK28A (AJ133624)
hydrothermal vent clone pISA35 (AB019748)
hydrothermal vent clone pMC2A384 (AB019734)
hydrothermal vent clone pMC2A17(AB019747)
5
pMC2
99
Methanomicrobia
42
EA1
hydrothermal fluid clone Fapm1aA (AB213060)
hydrothermal vent clone pMC1A4 (AB019754)
PNG_TB_4B30H1_A63
hydrothermal fluid clone Papm3A5 (AB213103)
deep-sea sediment clone APA7-17cm (AF119140)
deep-sea sediment clone CRA13-11cm (AF119124)
deep-sea sediment clone APA6-17cm (AF119139)
Microbial black exposed hot ridge (AB260057)
PNG_TB_4B7.5H1_A148
Deep-sea Hydrothermal Vent Euryarchaeal Group
9
SAGMA1
32
Thermoplasmata
74
Methanobacteria
35
Thermococci
Methanopyrus kandleri
13
Archaeoglobi
21
Methanococcales
8
FCG1
7
Korarchaeota
hot water habitat clone pOWA133 (AB007303)
hydrothermal sulfide structure clone Fhm1A36 (AB293205)
nascent hydrothermal chimney clone F99a102 (DQ228522)
PNG_TB_4B140H1_A53
Marine Hydrothermal Vent Group
hydrothermal vent clone pMC2A15 (AB019718)
4
FCG2
Bacillus subtilis
Escherichia coli
Aquifex pyrophilus
0.10

**Figure 4.** Pie charts of archaeal clone library data from 5 sample sites in Tutum Bay(118 clones at 7.5 m, 77 at 30 m, 59 at 60 m, 46 at 140 m, 54 at 300 m). Solid colors represent phylotypes found in multiple clone libraries; striped patterns depict phylotypes observed in a single library. All sites (except 30 m) were dominated by CA5 archaea. Euryarchaeota were present at only 7.5 m and 30 m.


7.5 m
30 m
60 m
140 m
300 m
EA1
CA1
CA2
CA3
CA4
CA5
C3
HWCG
Thermoprotei
7m5_148
7m5_026
30m_063
140m_023
140m_015
140m_053

# CHAPTER 4

## Microbially mediated arsenic reduction in hydrothermally-influenced marine fluids and sediments


### ABSTRACT

Tutum Bay hydrothermal porefluids and sediments are rich in arsenic (up to 950 µg/L and 33200 mg/kg, respectively) and present an ideal location in which to study microbially mediated arsenic cycling. In this paper we examine sediments and enrichment cultures to determine whether or not microorganisms in the sediments and porefluids of Tutum Bay, Papua New Guinea are directly involved in As-reduction. Three complementary approaches were used: DNA and RNA screening of arsenic functional genes, analysis of arsenic speciation in a microbial culturing study, and the isolation of strain TB1 in a geochemically-designed As-rich medium. Bulk DNA extracted from sediments in Tutum Bay was screened via PCR for arsenate reductase (*arrA*) and arsenite oxidase (*aroA*-type) genes. Sediments at several sites along a transect to 60 m leading away from a shallow-sea vent orifice contained both genes, but only *arrA* was detected at farther sites, out to 300 m. Bulk DNA and RNA extracted from enrichment cultures at the 30 m site were screened for *arrA* by PCR and RT-PCR. The data indicate active arsenate ($As^{V}$) reduction. Arsenic speciation analyses in incubation experiments with sediment inocula from 7.5 m and 30 m showed a demonstrable decrease of $As^{V}$ and increase of $As^{III}$ over a 48 h period, supporting microbially-mediated arsenate reduction. A novel rod-shaped strain (TB1) belonging to the *Bacillaceae* was isolated at

30ºC in $As^V$-rich medium; its net metabolism has not yet been identified. These molecular and analytical data provide the first evidence of microorganisms in Tutum Bay sediments and porefluids capable of $As^V$ reduction and likely $As^{III}$ oxidation.

## INTRODUCTION

The arsenic-rich hydrothermal vent fluids and sediments of Tutum Bay, Ambitle Island, Papua New Guinea (Figure 1) provide us with an ideal environment in which to study microbially mediated arsenic cycling. The vents discharge hydrothermal fluids with up to 950 µg/L of arsenite ($As^{III}$), and iron-oxyhydroxide coatings on rocks and coral in the bay contain up to 6 wt% of arsenic (Pichler et al, 1999; Pichler et al., 2006), although this arsenic (mainly present in the form of $As^V$ arsenate) is thought to be stable, immobile, and not easily extracted from the sediment, and therefore unlikely to be available for microbial redox (Pichler et al., 2006). As seen in Table 1, concentrations of arsenic in shallow sediments along a transect (referred to as transect 4B) from a hydrothermal vent are very high, ranging from 52 to 1483 mg/kg, compared to 2.2 mg/kg at a local non-hydrothermal control site (Price and Pichler, 2005). Arsenic concentrations decreased with distance from the vent orifice. On average, only ~3.6% of the arsenic in the sediment at any one site in Tutum Bay is "bioavailable," defined as able to freely move into or onto an organism (Price and Pichler, 2005). A previous thermodynamic modeling study of Tutum Bay (Akerman et al., 2009) evaluated the potential energy yields from an array of redox reactions, including those using As and Fe species, that could serve as net microbial metabolisms. In addition, Akerman and Amend (2009) surveyed the archaeal community structure in the Tutum Bay sediments using 16S rRNA

gene sequence analyses and found a variety of archaeal species present, including members of the Thermoprotei. Here, we investigate the role that microorganisms play in cycling the smaller but still significant portion of bioavailable arsenic in Tutum Bay, with a particular focus on arsenate reduction.

Dissimilatory arsenate-reducing bacteria, first discovered in 1994 (Ahmann et al., 1994), gain energy from $As^{V}$ respiration, and they are likely important players in the global arsenic cycle. At least 24 microbial species are known to use arsenate as a terminal electron acceptor (TEA), with two members in the Crenarchaeota, and the rest in the Bacteria among the Aquificae, Chrysiogenes, Deferribacteres, low G+C Gram-Positives, and Proteobactiera (Oremland et al., 2009). Both organic and inorganic compounds serve as electron donors in microbial arsenate reduction. For example, delta-Proteobacterium strain MLMS-1 couples hydrogen sulfide oxidation with arsenate reduction (Hoeft et al., 2004), while the haloalkaliphilic strain SLAS-1 can use either lactate or sulfide as the electron donor (Oremland et al., 2005). Microorganisms capable of arsenate respiration are typically able to use other TEAs, including oxygen, nitrate, nitrite, ferric iron, sulfate and sulfur (Oremland and Stolz, 2003; Laverman et al., 1995); the only obligate arsenate-reducing organism is strain MLMS-1 (Hoeft et al., 2004).

There are two known pathways for $As^{V}$ reduction to $As^{III}$: the respiratory pathway (encoded by the *arrA* gene) couples the oxidation of an organic substrate to $As^{V}$ reduction, resulting in cell growth, while the detoxification pathway (encoded by *arsC*) is used to convert $As^{V}$ to $As^{III}$, which is then transported out of the cell in a process that requires ATP (Campbell et al., 2006). Respiratory arsenate reductases (Arr) have been purified and characterized from *Chrysiogenes arsenatis*, *Bacillus selenitireducens*, and

*Shewanella* sp. strain ANA-3 (Krafft et al., 1998; Afkar et al., 2003; Malasarn et al., 2008); strain ANA-3 also contains arsC genes.

Screening bulk DNA samples for As-redox functional genes helps identify subsets of the microbial community that have the capability of metabolizing arsenic. Additionally, screening samples of bulk RNA extracted from actively-growing enrichment cultures via reverse-transcription PCR (RT-PCR) can reveal whether targeted genes of interest are being expressed, and also helps identify cultures of interest for isolation and metabolism studies. Primers (arrAfwd and arrArev) targeting a ~160–200 bp section of *arrA*, the respiratory arsenate reductase gene, have been designed and tested on a variety of bacterial species (Malasarn et al., 2004). The *arrA* sequence is well conserved, with 61 – 100% amino acid identity between 7 phylogenetically diverse bacterial species, and arrA proteins form a unique group within the family of dimethyl sulfoxide (DMSO) reductases. The arrAfwd and arrArev primers were used successfully to amplify the target genes in 12 of 13 bacterial species, but these primers did not amplify arsenate reductase from archaeal species (Malasarn et al., 2004); at this time no primers are known to target archaeal arsenate reductases.

Three different arsenite oxidases have been identified, but are all very similar in structure (Quéméneur et al., 2008). They belong to the DMSO reductase family, and all consist of two subunits (Páez-Espino et al., 2009). The genes encoding for the different arsenite oxidases are also similar in sequence (Inskeep et al., 2007; Lièvremont et al., 2009). The arsenite oxidase homologues aroA, asoA, and aoxB, are referred to in this paper as aroA-like proteins, while the respective genes are referred to as *aroA*-like genes, following the example of Inskeep et al. (2007). Primers by Inskeep et al. (2007) target

bacterial *aroA*-like gene sequences and have been successful in amplifying over 160 diverse *aroA*-like sequences from 10 geographically isolated, arsenic-contaminated sites and from 13 known arsenite-oxidizing bacteria. Rhine et al. (2007) designed primers to target arsenite oxidase from both heterotrophic and autotrophic archaea and bacteria, with success in amplifying *aroA*-like genes from environmental bacteria.

In this chapter, the role of microorganisms in mediating arsenate reduction was investigated in the shallow-sea hydrothermal system of Tutum Bay, Papua New Guinea. Three complementary approaches were employed: screening cultures and sediments for the presence and expression of functional As genes via PCR and RT-PCR, chemical analyses of $As^{V}$ and $As^{III}$ in a microbial incubation experiment, and culturing enrichments of microbes in a geochemically designed medium targeting As-reduction. Screening cultures for arsenic functional genes can ascertain the existence of strains within the microbial community that have As-redox capabilities, and RT-PCR can reveal whether the functional genes are actively being expressed. In the incubation experiment, enrichment cultures were grown in $As^{V}$-rich medium and subcultures of samples were taken over the course of 48 hours. The $As^{III}$ and $As^{V}$ concentrations in these samples were analyzed and compared to As concentrations in non-inoculated control samples to determine whether or not microbial species were responsible for reducing $As^{V}$ over time. Designed geochemical media provides a growth environment that simulates the *in situ* environmental conditions. This technique has proved successful for culturing novel and previously "unculturable" organisms, often by using media with lower amounts of nutrients that more closely resembles the natural environment (e.g., Rappé et al., 2002; Connon and Giovannoni, 2002; Kaberlein et al., 2002; Amend et al., 2003; Stevenson et

al., 2004). Designed media also can target one particular microbial metabolism by spiking the base medium with one particular electron donor and acceptor pair (Madsen, 2005). This approach has been used successfully to isolate diverse microbes from, for example, hot springs in Yellowstone National Park, USA (Meyer-Dombard, 2004).

## MATERIALS AND METHODS

### *Sample Collection*

Sediment samples for DNA analysis were collected via SCUBA in May 2005 using PVC tubes (1m x 6cm). On ship, sediment from the 0 – 10 cm depth range of the seawater-surface interface was homogenized, stored in sterile containers, and transported frozen to our laboratory where all samples were stored at –20°C and thawed to room temperature prior to analysis.

Sediment and porewater samples for culturing purposes were collected directly from the 0 – 10 cm depth range into sterile containers via SCUBA in May 2003 and stored at 4°C until inoculation.

### *Growth Media Recipes*

Geochemical growth medium "PNG AR2" was designed to simulate Tutum Bay hydrothermal vent fluid compositions as determined by Pichler et al. (1999). The media contained 1.175 mg/L of $As^V$ to act as an electron acceptor, and yeast extract and peptone to act as electron donors. Growth medium "PNG AR2-A" was identical to medium "PNG AR2" except it contained 375,600 mg/L $As^V$; growth medium "PNG AR2-B" was also identical but contained 4.49 mg/L $As^V$. The PNG base solution for all three media

contained 0.19 g KCl, 1.69 g NaCl, 1.1 g $NaHCO_3$, and 0.5 g $MgCl_2 \cdot 6H_2O$ per liter of MilliQ distilled water. One liter of PNG base solution was amended with 3 g yeast extract (Difco), 3 g peptone, from meat (Sigma), either 10 mL concentrated $As^V$ solution (0.21 g $Na_2HAsO_4 \cdot 7H_2O$ per L of water) for "PNG AR2," 20 mL concentrated $As^V$ solution A (7.8 g $Na_2HAsO_4 \cdot 7H_2O$ per L of water) for "PNG AR2-A," or 10 mL concentrated $As^V$ solution B (1.68 g $Na_2HAsO_4 \cdot 7H_2O$ per L of water), and 10 mL concentrated N/P solution (17.4 g $NH_4Cl$, 3.6 g $KH_2PO_4$ per L of water), 0.5 mL 0.2% rezasurin as a redox indicator, 3 g PIPES sodium salt (1,4-Piperazinediethanesulfonic acid sodium salt, Sigma) as a pH buffer, and 10 mL trace element solution (1.03 g NaBr, 4.95 g $H_3BO_3$, 0.62 g LiCl, 0.17 g $MnCl_2 \cdot H_2O$, 0.05 g RbCl, 2.05 g $SrCl_2 \cdot 6H_2O$, 2.28 mg $SbCl_3$, 0.31 g $AsHNa_2O_4 \cdot 7H_2O$, 0.06 mg $CoCl_2 \cdot 6H_2O$, 3.3 mg $ZnCl_2$, 7.7 mg $CuCl_2 \cdot 2H_2O$, 1.32 g $AlCl_3 \cdot 6H_2O$, 0.18 mg $Na_2MoO_4 \cdot 2H_2O$, and 0.44 mg $NiCl_2 \cdot 6H_2O$). The pH was adjusted to 6.5 using 1.0 N NaOH or 1.0 N HCl. After autoclaving at 121°C for 20 minutes, the medium was amended via filter-sterilization (0.22 μm diameter) with 2 mL concentrated $Fe^{II}$ solution (1.53 g $FeCl_2 \cdot 4H_2O$, 1.6 g EDTA [disodium ethylenediaminetetraacetate dehydrate] per L of water), 10 mL concentrated $CaCl_2$ solution (74.98 g $CaCl_2$ per L of water), and 0.5 mL *Archaeoglobus fulgidis* vitamin solution (0.04 g biotin, 0.04 g folic acid, 0.1 g Pyridoxine-HCl [vitamin B6], 0.1 g Thiamine-HCl $\cdot 2H_2O$ [B1], 0.1 g riboflavin [B2], 0.1 g nicotinic acid, 0.1 g D-Ca-pantothenate, 0.1 g cobalamin [B12], 0.1 g p-aminobenzoic acid, and 0.1 g lipoic acid per L of water). The medium was then heated to boiling under $N_{2(g)}$ and 10 mL at a time were pipetted into Balch tubes (Bellco Glass, Vineland, N.J.) from which the air was previously exchanged with $N_{2(g)}$ using a purpose-built gassing station (Balch and Wolfe,

1976). The tubes were then sealed with gas impermeable butyl rubber stoppers, crimped with Al seals, and overpressured to 3 bar with $N_{2(g)}$. Prior to inoculation, the medium was further reduced by the addition of 0.3 mL 2.5% $Na_2S$ solution (2.5 g $Na_2S \cdot 9H_2O$ per 100 mL water).

***Inoculation and Incubation***

Tubes of "PNG AR2" and "PNG AR2 A" growth medium were inoculated with 1 mL each of a sediment and porewater fluid mixture from 7.5, 12, or 30 m. The tubes were incubated in 30°, 50°, and 80°C water baths and checked for growth via phase microscopy. Tubes were pulled from the water bath and stored at 4°C when growth was visually verified.

***Growth of Shewanella species ANA-3 and ARM-1***

Cultures of Shewanella species ANA-3 and ARM-1 were gifts of Chad Saltikov. Strain ANA-3 is a respiratory As-reducing prokaryote, and strain ARM-1 is a genetically modified version of ANA-3 that no longer contains the arrA gene which encodes the As-reducing respiratory reductase. Cultures of both microorganisms were grown overnight on LB agar plates and in 5.0 mL of liquid LB medium at room temperature.

***RNA and DNA extraction***

Bulk DNA was extracted from sediments using the Fast DNA Soil Kit (MP Biomedicals) following the manufacturer's protocol. Initial enrichments of tubes inoculated with fluid and sediment slurries from 7.5, 12, and 30 m were successful at 30

and 50°C. Actively growing cultures were immediately placed on ice for RNA and DNA extraction following visual verification of growth via phase microscopy. RNA was extracted using the SV Total RNA Isolation System (Promega) generally following the manufacturer's protocol for isolation of RNA from Gram-Positive and Gram-Negative bacteria, although cultures incubated overnight were used because even cultures grown overnight did not typically reach an $OD_{600}$ of 0.6 – 1.0. Also, it was assumed that the enrichment could contain a mixture of Gram-Positive and Gram-Negative Bacteria as well as Archaea, so 3 mL were extracted from each tube and 1.5 mL were treated following the protocol for Gram-Positive bacteria and 1.5 mL were treated as for Gram-Negative bacteria. In some cases, DNA was extracted (from the same sample from which RNA was extracted) using a modified protocol (Otto et al., 1998) of the SV Total RNA Isolation System. Successful RNA and DNA extraction was verified using the appropriate Nucleic Acid Analysis method on a DU 800 Beckmann Coulter spectrophotometer.

Bulk DNA was also extracted from cultures of Shewanella sp. ANA-3 and Shewanella sp. ARM-1 following the protocol for Gram-negative bacteria in the Wizard Genomic DNA Purification Kit (Promega), for use as positive and negative controls in functional gene analyses. Successful DNA extraction was verified via spectrophotometer.

***Functional gene analyses***

DNA extracted from enrichments and bulk DNA extracted from sediments were screened for the *arrA* gene using the ArrAfwd (5'-AAG-GTG-TAT-GGA-ATA-AAG-CGT-TTgtbgghgaytt-3') and ArrArev (5'-CCT-GTG-ATT-TCA-GGT-GCC-caytyvggngt-

3') primers (Malasarn et al., 2004). The 20 μL PCR reaction mixture consisted of 2.8 μL $MgCl_2$, 2.0 μL GeneAmp 10X PCR buffer, 0.2 μL dNTPs, 0.25 μL AmpliTaq Gold DNA polymerase, 1 μL each of forward and reverse primers (0.5 μM), and 2 μL template DNA. Thermocycling conditions on a Hybaid PCR Express thermalcycler consisted of initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 50°C for 40 seconds, and 72°C for 1min. A positive control of *Shewanella* ANA-3 and a negative control of *Shewanella* ARM-1 were included for all PCR runs. PCR products were verified to be the correct size (~160 – 200 bp) on a 1.5% agarose gel stained with SYBR Green and photographed under UV light. PCR products or bands excised directly from the gel were cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega) using the manufacturer's instructions.

DNA extracted from enrichments and bulk DNA from sediments was also screened for the arsenite oxidase gene using two primer sets targeting *aroA*: set #1 forward primer 5'-GTS-GGB-TGY-GGM-TAY-CAB-GYC-TA-3' and reverse primer 5'-TTG-TAS-GCB-GGN-CGR-TTR-TGR-AT-3' and set #2 forward primer 5'-GTC-GGY-TGY-GGM-TAY-CAY-GYY-TA-3' and reverse primer 5'-YTC-DGA-RTT-GTA-GGC-YGG-BCG-3' (Inskeep et al., 2007). Both primer sets amplify an ~500bp segment of *aroA*. The 20 μL PCR reaction mixture consisted of 1.8 μL $MgCl_2$, 2.0 μL GeneAmp 10X PCR buffer, 0.2 μL dNTPs, 0.25 μL AmpliTaq Gold DNA polymerase, 2 μL each of forward and reverse primers (1 μM), and 1 μL template DNA. Thermocycling conditions on a Hybaid PCR Express thermalcycler for primer set #1 consisted of initial denaturation at 95°C for 4 min, followed by 9 cycles of 95°C for 45 s, 50°C for 45 s (decreased by 0.5°C for each cycle), and 72°C for 50 s, followed by

25 cycles of 95°C for 45 s, 46°C for 45 s, and 72°C for 50 s, followed by a final extension time of 72°C for 5 min. For primer set #2, the PCR reaction mixture was the same, and thermocycling conditions were 90°C for 3 min, followed by 40 cycles of 92°C for 1 min, 50°C for 1.5 min, and 72°C for 1 min, followed by a final extension time of 72°C for 5 min. Thermocycling conditions were taken from Inskeep et al. (2007). Positive control consisted of bulk DNA extracted from Octopus Pool, Yellowstone National Park, USA. PCR products were verified to be the correct size on a 1.5% agarose gel stained with SYBR Green and photographed under UV light. Products were then cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega) using the manufacturer's instructions.

Reverse-transcription PCR (RT-PCR) was performed on enrichment samples which displayed correctly-sized bands in the screening of the bulk DNA for arsenate reductase. RT-PCR was performed using the Access RT-PCR System (Promega) following the manufacturer's instructions.

***Microbial incubation and arsenate reduction***

Two tubes of "PNG AR2" medium were inoculated with 1 mL each of enrichments cultured from the 7.5 and 30 m sites. Tubes were incubated overnight in a 30°C water bath. Following visual verification via phase microscopy of microbial growth, each tube was well homogenized and 1 mL each used to inoculate 10 tubes. Five of these tubes were reduced with 0.3 mL 2.5% $Na_2S$ prior to inoculation. An additional 10 tubes, 5 of which were amended with 0.3 mL 2.5% $Na_2S$, served as Control tubes. Immediately after inoculation, one tube each of 7.5 m, 30 m, and Control both with and

without $Na_2S$ (6 tubes total) were flash frozen in liquid nitrogen. All remaining tubes were then incubated in a 30°C water bath.

At 24 and 48 hours after inoculation, one tube of each sample with and without $Na_2S$ were removed from the water bath, and 0.3 mL was withdrawn from each tube and stored in 0.15 mL formalin at 4°C before the tube was flash frozen in liquid nitrogen. Following freezing, all tubes were wrapped completely in aluminum foil and stored at −20°C until analysis.

***Total arsenic and arsenic speciation measurements***

For samples that were unfiltered at collection, ~2 mL of sample were filtered through a cellulose 0.22 μm filter. High pressure liquid chromatography (HPLC) was used to separate $As^{V}$, $As^{III}$, DMA (dimethylarsenic acid), and MMA (monomethylarsonic acid) prior to measurement of concentration with hydride generation-atomic fluorescence spectrometry (HG-AFS). The arsenite and arsenate were separated on an anion exchange column and a volatile arsenic hydride generated, which was then detected via AFS.

***Isolation of Microbial Species***

Successful enrichments cultured in "PNG AR2" medium that were inoculated with sediment and porewater slurries from 7.5 m, 12 m, and 30 m, and an enrichment cultured in "PNG AR2 A" inoculated from a porewater and sediment slurry of 7.5 m were identified as candidates for isolation of microbial species. These samples were used as inoculum tubes in a dilution-to-extinction isolation technique. A series of 10 tubes of growth medium was prepared and 1 mL of the inoculum was transferred to tube 1 in the

dilution series. Tube 1 was then homogenized and 1 mL was transferred to tube 2 in the series, and so on until the last tube. All tubes were then incubated in a 30°C water bath and microbial growth was observed via phase microscopy. When growth was visually verified, tubes were stored at 4°C. The last tube in each series that was observed to have microbial growth was used as the inoculum for a new dilution series of 10 tubes. This procedure was followed for a total of 3 dilution series for all 4 samples.

The dilution-to-extinction method successfully yielded an isolate from the 7.5 m enrichment cultured in PNG AR2 medium at 30°C. Bulk DNA and RNA were extracted from a tube of this isolate using the procedures outlined above. PCR was performed to amplify the 16S gene from the bulk DNA. The 20 μL PCR reaction mixture consisted of 1.8 μL $MgCl_2$, 2.0 μL GeneAmp 10X PCR buffer, 0.2 μL dNTPs, 0.25 μL AmpliTaq Gold DNA polymerase, 0.5 μL each of forward and reverse primers (0.25 μM), and 1 μL template DNA. PCR was performed using both 21F (5'–TTC-CGG-TTG-TAC-CYG-CCG-GA–3') and 1391R (5'–GAC-GGG-CGG-TGT-GTR-CA–3') primers targeting archaea, and 27F (5'–AGA-GTT-TGA-TCC-TGG-CTC-AG–3') and 1492R (5'–GGT-TAC-CTT-GTT-ACG-ACT-T–3') primers targeting bacteria. Thermocycling conditions on a Hybaid PCR Express thermalcycler were 95°C for 5 min, followed by 30 cycles at 95°C for 0.5 min, 55°C for 0.5 min, and 72°C for 1.5 min, followed by a final extension at 72°C for 15 min for archaeal primers, and 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by a final extension time of 72°C for 5 min for bacterial primers.

### *Microbial Isolate 16S rRNA Gene Sequencing and Analysis*

16S rRNA DNA amplified from the microbial isolate was cleaned using the Wizard® SV Gel and PCR Clean-Up System (Promega) using the manufacturer's instructions. DNA sequencing was completed at both Protein and Nucleic Acid Chemistry Laboratory (Washington University School of Medicine, St. Louis, MO) and Molecular Cloning Laboratories (South San Francisco, CA).

Sequence data were manually edited and assembled into contigs using Sequencher v.4.7 (Gene Codes). Assembled sequences were aligned using the Greengenes NAST aligner (DeSantis *et al.*, 2006) and checked for chimeras using the Bellerophon3 server at Greengenes. All sequences were compared to sequences in the NCBI GenBank database via BLAST.

## RESULTS AND DISCUSSION

### *Functional gene screening and expression*

Bulk DNA extracted from sediments at 7.5, 30, 60, 140, and 300 m was screened for arsenate reductase (*arrA*). Four samples (7.5, 30, 60, and 300 m) were positive for *arrA*. This is manifested as correctly-sized bands in the gel electrophoresis photograph (Figure 2) in lanes 4 (7.5 m sample), 5 (30 m), 6 (60 m), and 8 (300 m). Bulk DNA extracted from *Shewanella* sp. ANA-3 was used as a positive control for respiratory arsenate reductase (lane 1), while bulk DNA extracted from *Shewanella* sp. ARM-1 was used as a negative control (lane 2). Lane 2 shows the absence of a band at the size location of the positive control and strong banding in two places well above the control band location, verifying its negative control status. Bands in lanes 4, 5, 6, and 8 at the

size fragment of the positive control indicate the presence of amplified *arrA*; the brightness in 4, 5, and 6 indicate that more DNA was amplified in those reactions than in lane 8, which shows a dimmer band. Bulk DNA from the 5 sample sites was also screened for arsenite oxidase (*aroA*-like) genes. Three samples (7.5, 30, and 60 m) were positive for arsenite oxidase using primer set #2, as seen by the bands in lanes 6 (7.5 m), 9 (60 m), and 10 (30 m) which are the same size as the control band in lane 2 (Figure 3). These gene screening results demonstrate that the sites closest to the vent (7.5, 30, and 60 m) feature microbial populations apparently capable of both $As^{V}$-reduction and $As^{III}$-oxidation. Despite elevated As concentrations even beyond 60 m along the transect, sedimentary microbial communities capable of As metabolism are less likely. At the 140 m site, both As functional genes were absent, and at the 300 m site *aroA*-type genes were absent—the *arrA* screen, however, was positive.

Bulk DNA extracted from several laboratory enrichment cultures was also screened for *arrA* and *aroA*-type genes. Cultured enrichments from 7.5 m, 12 m, 30 m at 30°C and enrichments from 12 m at 50°C, all cultured in "PNG AR2" medium, were all screened for both *arrA* and *aroA*-type genes. Only the 30 m enrichment showed a positive band result during PCR screening for the *arrA* gene. Bulk RNA extracted from an actively growing aliquot of the 30 m culture was then used in the RT-PCR reaction. Figure 4 shows the ladder (DNA size marker) band in lanes 1 and 5, the negative control (no band) in lane 2, the band in lane 3 identifies the amplification of the positive control, and the band in lane 4 represents the positive result of RT-PCR for the 30 m enrichment. The positive bands were excised from the gel, sequenced, and compared to other gene sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov; Benson et al.,

1999). The band representing the 30 m enrichment identified 100% with an *arrA*-like gene from an uncultured bacterium (Genbank accession number AY707767; Malasarn et al., 2004), and 97% with the *arrA* gene from the uncultured bacterium clone HRR23 (AY707770; Malasarn et al., 2004). This is robust evidence that microorganisms in the enrichment culture were actively reducing arsenate for respiration at the time of sacrifice. It should be noted however, that these data cannot provide information regarding the relative proportion of respiratory $As^{V}$-reducing organisms compared to microorganisms utilizing other metabolic strategies within the sediments and porefluids of Tutum Bay.

***Arsenic speciation in microbial incubations***

Enrichment cultures from 7.5 m and 30 m were inoculated in fresh PNG AR2 medium and incubated, along with a Control sample (un-inoculated PNG AR2 medium) at 30 ºC for 48 h. Growth medium samples of the 7.5 m, 30 m, and Control samples were taken at 0, 24, and 48 hours and analyzed for $As^{III}$ and $As^{V}$ by high pressure liquid chromatography-hydride generation atomic fluorescence spectroscopy (HPLC-HG-AFS). The speciation data are listed in Table 2 and plotted as a function of time in Figure 5. In Table 2, the $As^{III}$ and $As^{V}$ concentrations, total arsenic (sum of the two species), and percentage of each species are listed for each sample at the 3 time points, in addition to the $As^{III}$ and $As^{V}$ concentrations that were analyzed in a sample of un-inoculated, non-incubated PNG AR2 growth medium (labeled Medium). Only one sample that was amended with $Na_2S$ prior to inoculation was analyzed; these values are also given in Table 2.

It can be seen in Table 2 and Figure 5 that the inoculated tubes from both sites showed a marked decrease in $As^{V}$ levels with a concurrent increase in $As^{III}$. For the 7.5 m samples over a 48 h period, $As^{V}$ decreased from 3.25 to 1.23 mg/L and $As^{III}$ increased from 0.58 to 1.94 mg/L. For the 30 m samples, $As^{V}$ showed an even larger decrease, from 3.29 to 0.54 mg/L while $As^{III}$ increased from 0.41 to 1.81 mg/L. In the non-inoculated Control samples, there was no change in the $As^{V}$ or $As^{III}$ levels with time. Similar values of arsenic concentrations were observed in the Control and Medium samples. The data clearly indicate microbially mediated $As^{V}$ reduction in samples collected from 7.5 m and 30 m.

Arsenic mass balance in the inoculated samples was not achieved (see Table 2); the PNG AR2-B medium contained ~4.5 mg/L, as observed in the Control and Medium samples. However, the inoculated samples displayed lower total concentrations of arsenic than the expected ~4.5 mg/L at all time points. The cause of this phenomenon is currently unclear, but may be due to the fact that the growth medium in the injected inoculum had already been incubated with active microbial populations for ~18 hours, so $As^{V}$ concentrations were already likely to be depleted and $As^{III}$ already present, which may have affected the overall speciation in the freshly-inoculated tubes. In addition, some of the arsenic may have precipitated out of solution and subsequently could have been filtered out of the sample prior to HPLC-HG-AFS analyses. Alternatively, organoarsenical species may have formed; non-methylated organic species were not detectable via HPLC-HG-AFS.

Arsenic speciation in samples that were amended with sodium sulfide prior to inoculation was not analyzed in detail. Initially, the concentration of arsenate detected

was much lower than expected and arsenite was not detected in the Control 0 hr with $Na_2S$ sample, but after treatment with $H_2O_2$, a second analysis of Control 0 hr was able to detect higher amounts of both species (Table 2). It is believed that the addition of $Na_2S$ to the medium had caused the formation of As-S thiocompounds, which cannot be analyzed with HG-AFS. The addition of $Na_2S$ likely affected arsenic speciation greatly, so no further analyses of the samples amended with $Na_2S$ before inoculation were undertaken.

***Isolation of Strain TB1***

A novel bacterium, designated Strain TB1, was isolated in "PNG AR2" growth medium at 30 ºC using the dilution-to-extinction method. The starting inocula were enrichments grown from sediment and porewater slurries from the 7.5 m site. Strain TB1 is rod-shaped with rounded ends, ~1.2 – 1.5 μm in width and ~2 – 8 μm in length (Figure 6a, b). Observations by phase microscopy show that the organism forms sub-terminally-located endospores (Figure 6c) as well as free-floating mature endospores (not shown).

A bacterial 16S rRNA gene was amplified successfully from bulk DNA extracted from a culture of TB1. DNA sequencing of the 16S rRNA gene revealed TB1 to be most closely related (97%) to an uncultured clone identified as a *Bacillaceae* bacterium, in the "low G + C" phylum. Many species of *Bacillus* form endospores, and several *Bacillus* species have been identified as arsenate-respiring, including *Bacillus arsenicoselenatis*, *B. selenitireducens, B. benzoevorans* str. HT-1, and *B. macyae*. *B. arsenicoselenatis* and *B. selenitireducens* are both anaerobic haloalkaliphiles isolated from arsenic-rich Mono Lake, California; the former species forms endospores, but the latter species does not.

They both can grow via dissimilatory reduction of $As^{V}$ to $As^{III}$ with the simultaneous oxidation of lactate to acetate and $CO_2$ (Switzer Blum et al., 1998). The anaerobic *B. benzoevorans* HT-1 was isolated from hamster feces, and is capable of growth using arsenate as the electron acceptor and $H_2$ as the electron donor. It was the first instance of an arsenate-reducer found inside a living creature (Herbel et al., 2002). *B. macyae* is a strictly anaerobic, endospore-forming species, capable of respiring using arsenate and nitrate as terminal electron acceptors coupled with a variety of substrates as the electron donor, including acetate (Santini et al., 2004). Based on 16S rRNA sequence comparisons, none of these particular *Bacillus* species were very closely (> 96%) related to TB1, however, and because the *Bacillaceae* that the isolate was most closely related to is uncharacterized, it is unclear whether this isolate is capable of respiratory arsenate reduction, or is arsenic tolerant.

While the cause for the sporulation seen by Strain TB1 is currently unknown, limitations in metabolizable forms of carbon, nitrogen, or phosphorus, as well as extremes in metal concentrations and temperature can produce endospores (Madigan et al., 2003). Here, it is not known if the high As concentrations in the growth medium were a trigger for sporulation. Further work needs to be done to definitively determine whether or not TB1 is capable of respiratory arsenate reduction, and what factors may act as stressors in its environment to trigger sporulation.

## CONCLUSIONS

The results of the functional gene screening, the RT-PCR gene expression screening, and the incubation experiments all show that microorganisms extant in Tutum

Bay sediments/porefluids are capable of respiratory arsenate reduction and arsenite oxidation. This is the first coupled microbiological and geochemical evidence of active microbially mediated As-cycling in shallow-sea hydrothermal environments. Additional work is needed to fully characterize Strain TB-1, especially to determine its metabolic pathways and potential arsenic-respiration and resistance capabilities. Phylogenetic comparison of this isolate to known As-redox microorganisms may also shed light on its role in As-cycling in Tutum Bay. Other shallow-sea hydrothermal systems that are rich in arsenic, including Champagne Hot Springs in Dominica (Lesser Antilles) and the Hellenic Volcanic arc (McCarthy et al., 2005; Varnavas and Cronan, 2005), contain elevated levels of arsenic and are potential targets for further As-redox culturing studies, including isolation of thermophilic As-redox microorganisms. It is clear that microbes play an important role in As-cycling and studying these organisms can provide greater insights into the biogeochemical cycling of arsenic in diverse environments around the world.

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**Table 1. Arsenic concentrations in surface sediments along the 4B transect (data from Price and Pichler, 2005)**

| Distance from vent (m) | Depth (cm) | As (ppm) |
|---|---|---|
| 0 | 0 | 33200 |
| 1 | 0 | 1483 |
| 7.5 | 0 | 783 |
| | 5 | 872 |
| 12 | 0 | 680 |
| | 2 | 473 |
| 30 | 0 | 539 |
| | 2 | 483 |
| 60 | 0 | 614 |
| | 5 | 635 |
| 90 | 0 | 443 |
| | 5 | 464 |
| 125 | 0 | 468 |
| 150 | 0 | 402 |
| | 5 | 411 |
| 175 | 0 | 360 |
| 200 | 0 | 163 |
| 225 | 0 | 52 |
| | 5 | 72 |

**Table 2. Results of As-Reduction Incubation Experiment**

| | Time (Hrs) | $As^{III}$ (mg/L) | $As^{V}$ (mg/L) | Sum of species (mg/L) | $As^{III}$ % | $As^{V}$% |
|---|---|---|---|---|---|---|
| **Without $Na_2S$** | | | | | | |
| Control | 0 | 1.41 | 2.98 | 4.39 | 32.1 | 67.9 |
| Control | 24 | 1.40 | 3.12 | 4.52 | 31.0 | 69.0 |
| Control | 48 | 1.26 | 2.97 | 4.23 | 29.8 | 70.2 |
| | | | | | | |
| 7.5 m | 0 | 0.58 | 3.25 | 3.83 | 15.1 | 84.9 |
| 7.5 m | 24 | 1.08 | 2.06 | 3.14 | 34.4 | 65.6 |
| 7.5 m | 48 | 1.94 | 1.23 | 3.17 | 61.2 | 38.8 |
| | | | | | | |
| 30 m | 0 | 0.41 | 3.29 | 3.70 | 11.1 | 88.9 |
| 30 m | 24 | 1.53 | 0.99 | 2.52 | 60.7 | 39.3 |
| 30 m | 48 | 1.81 | 0.54 | 2.35 | 77.0 | 23.0 |
| | | | | | | |
| Medium | | 1.29 | 3.29 | 4.58 | 28.2 | 71.8 |
| | | | | | | |
| **With $Na_2S$** | | | | | | |
| Control | 0 | n.d. | 2.95 | 2.95 | | |
| Control with $H_2O_2$ | 0 | 0.08 | 4.16 | 4.24 | | |

n. d. = not detected

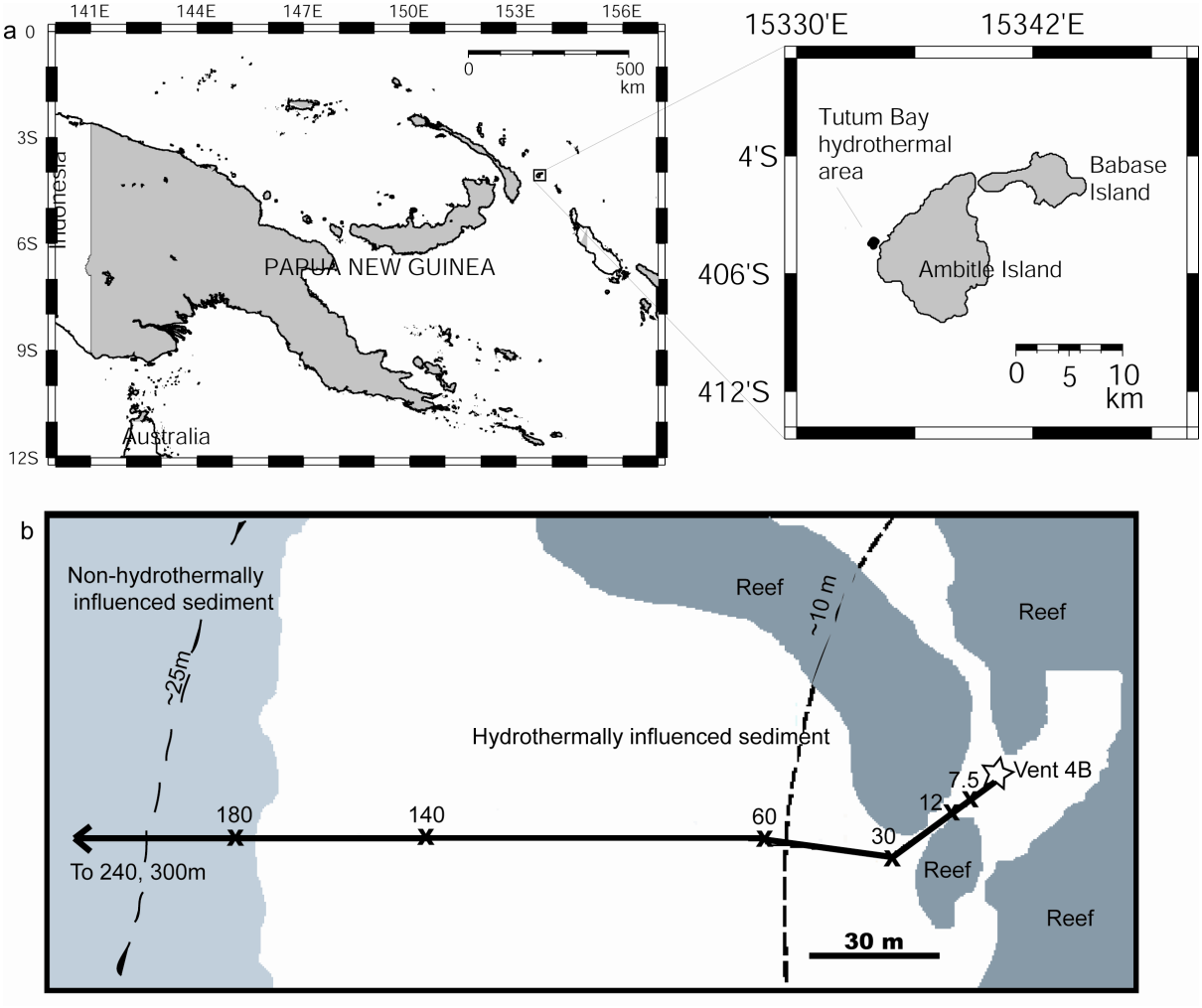


**Figure 1.** (a) Papua New Guinea, with Feni Islands (Ambitle and Babase) enlarged. (b) Plan view of Tutum Bay hydrothermal area with sampling transect constructed from Vent 4B. Sampled sites are indicated by **x**s. Water depth indicated by dashed lines.

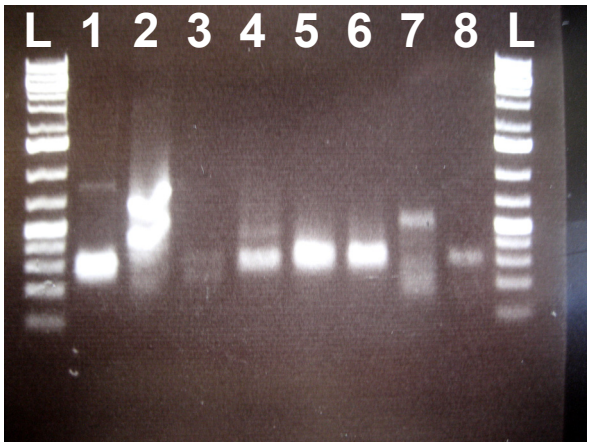


**Figure 2.** PCR results for arsenate reductase (*arrA*) gene amplification. L = ladder. 1: Positive control (ANA-3) 2: Negative control (ARM-1) 3: 30 m enrichment culture (+) 4: 7.5 m sediments (+) 5: 30 m sediments (+) 6: 60 m sediments (+) 7: 140 m sediments (–) 8: 300 m sediments (+).

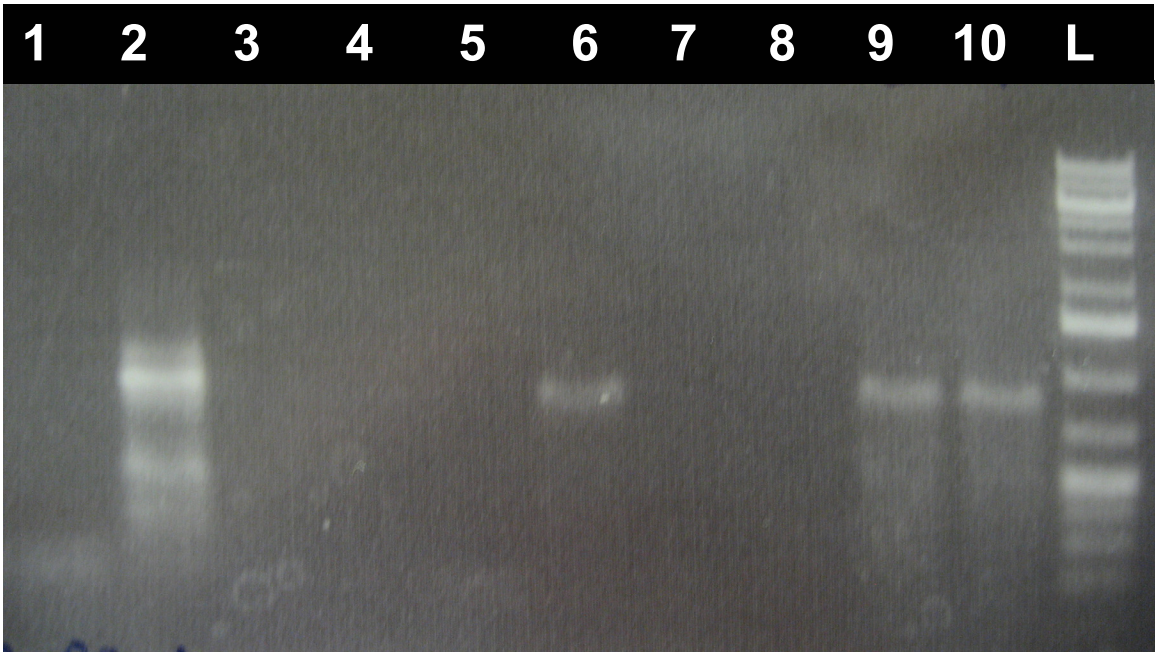


**Figure 3.** PCR results for arsenite oxidase (*aroA*-like) gene amplification. Lane 1: Negative control. 2: Positive control (Octopus Pool) 3, 4, 5: The Grip (sample from Sicily) (all –) 6: 7.5 m sediments (+) 7: 300 m sediments (–) 8: 140 m sediments (–) 9: 60 m sediments (+) 10: 30 m sediments (+)

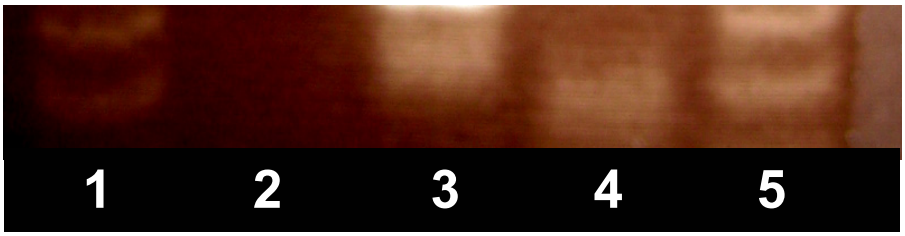


**Figure 4.** RT-PCR results for *arrA* cDNA. Lanes 1, 5 = ladder. 2: Negative control (no amplifiable DNA present). 3: Positive control (+) 4: 30 m enrichment (+).

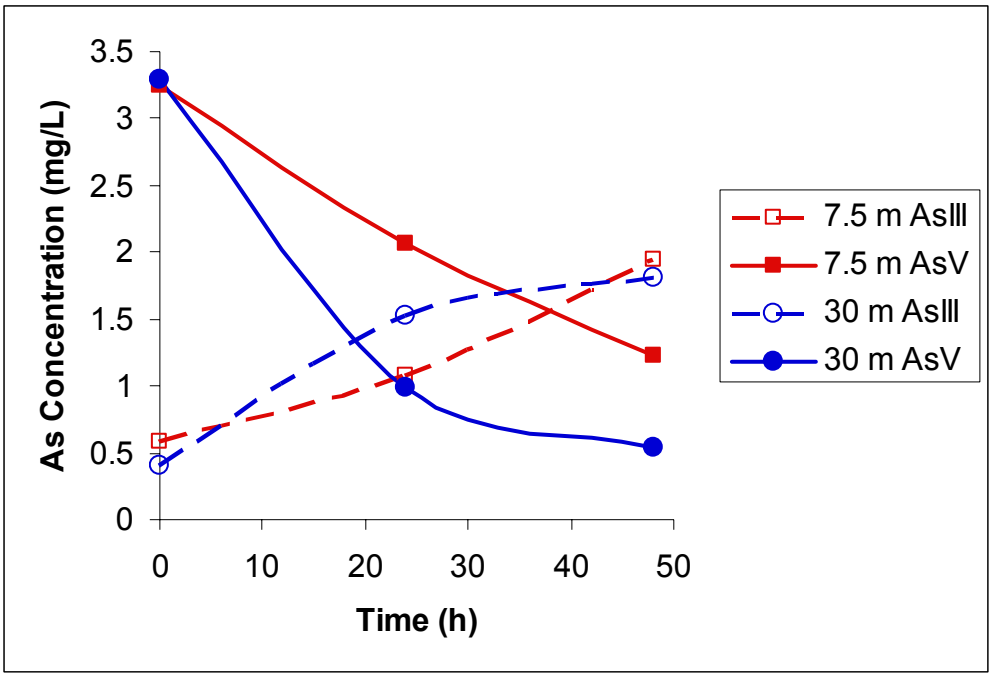


**Figure 5.** Arsenic concentration versus time for 7.5 and 30 m inocula.

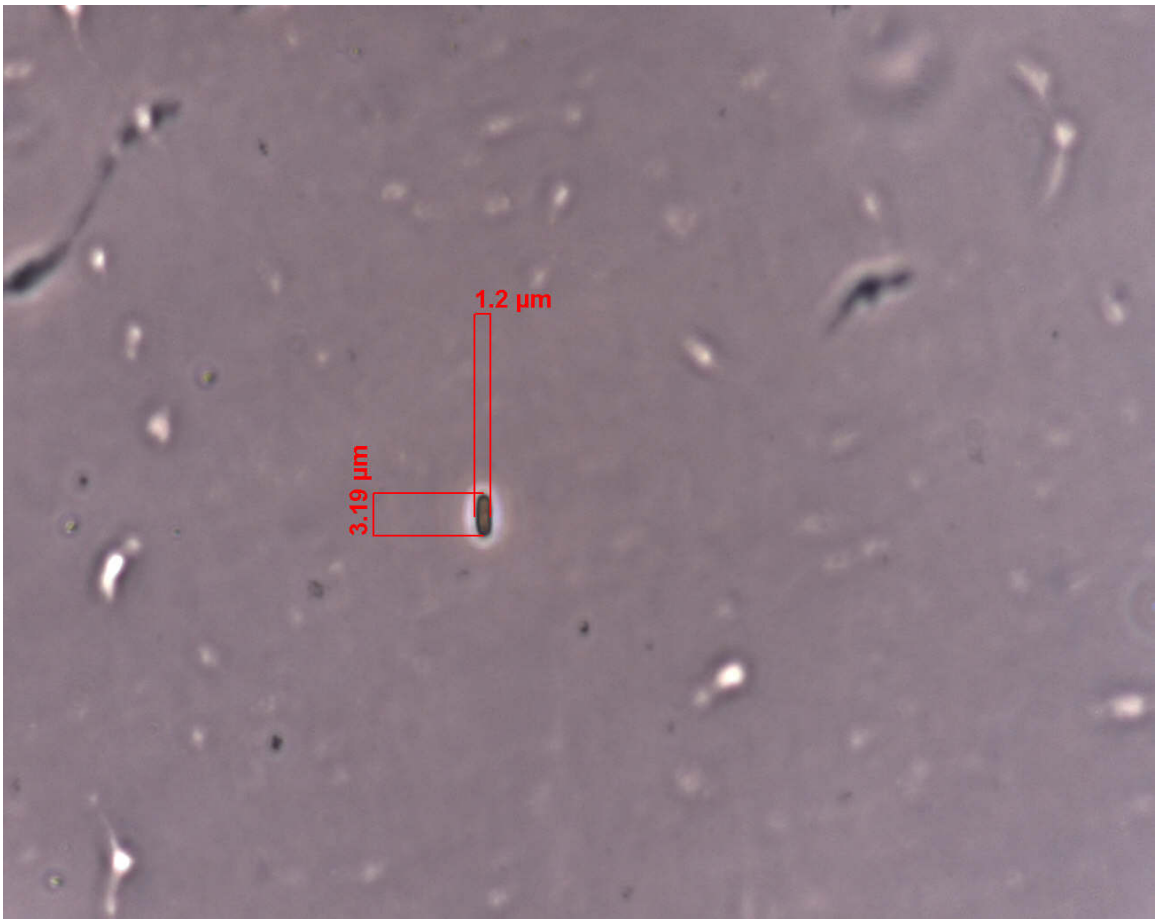


**Figure 6a.** Phase microscopy photograph of isolate TB1, a rod-shaped microorganism closely related to *Bacillus* sp.

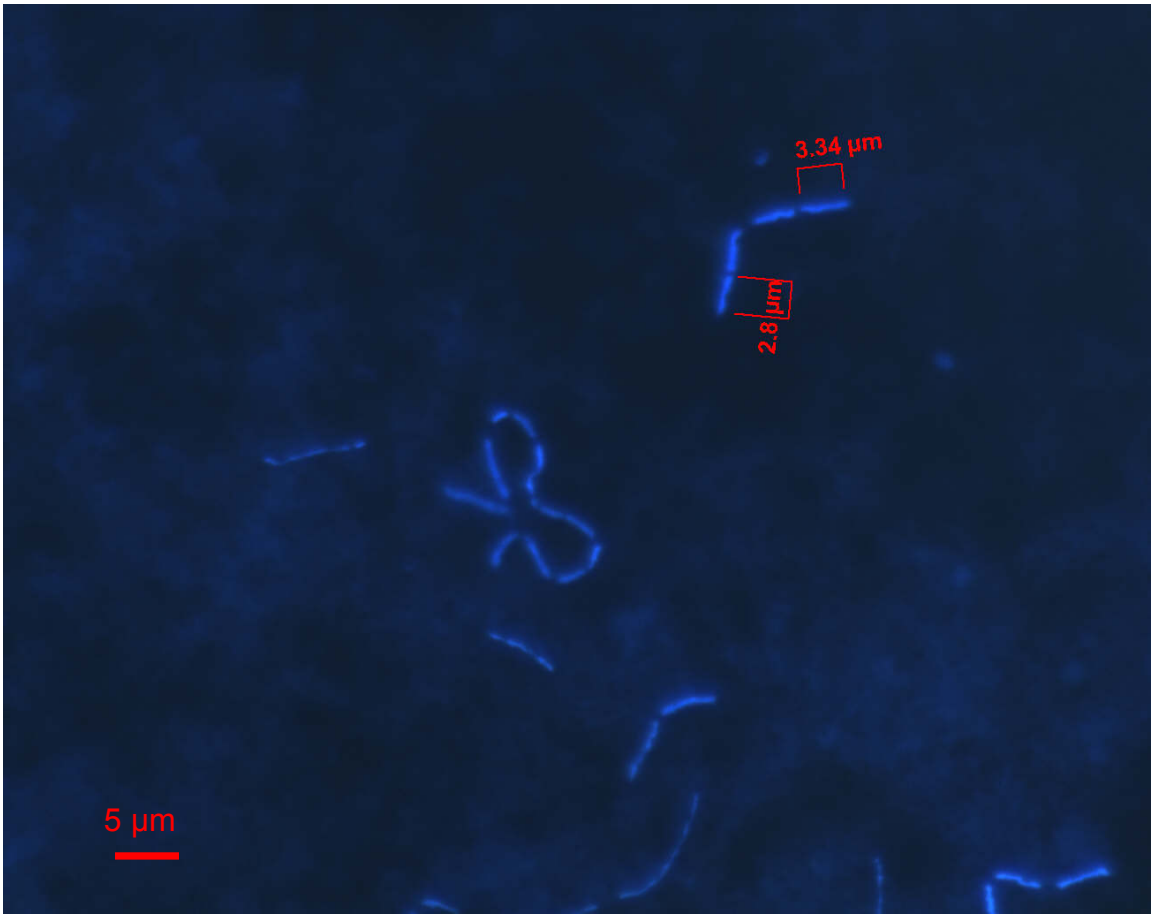


**Figure 6b.** Photograph of strain TB-1 stained with DAPI.

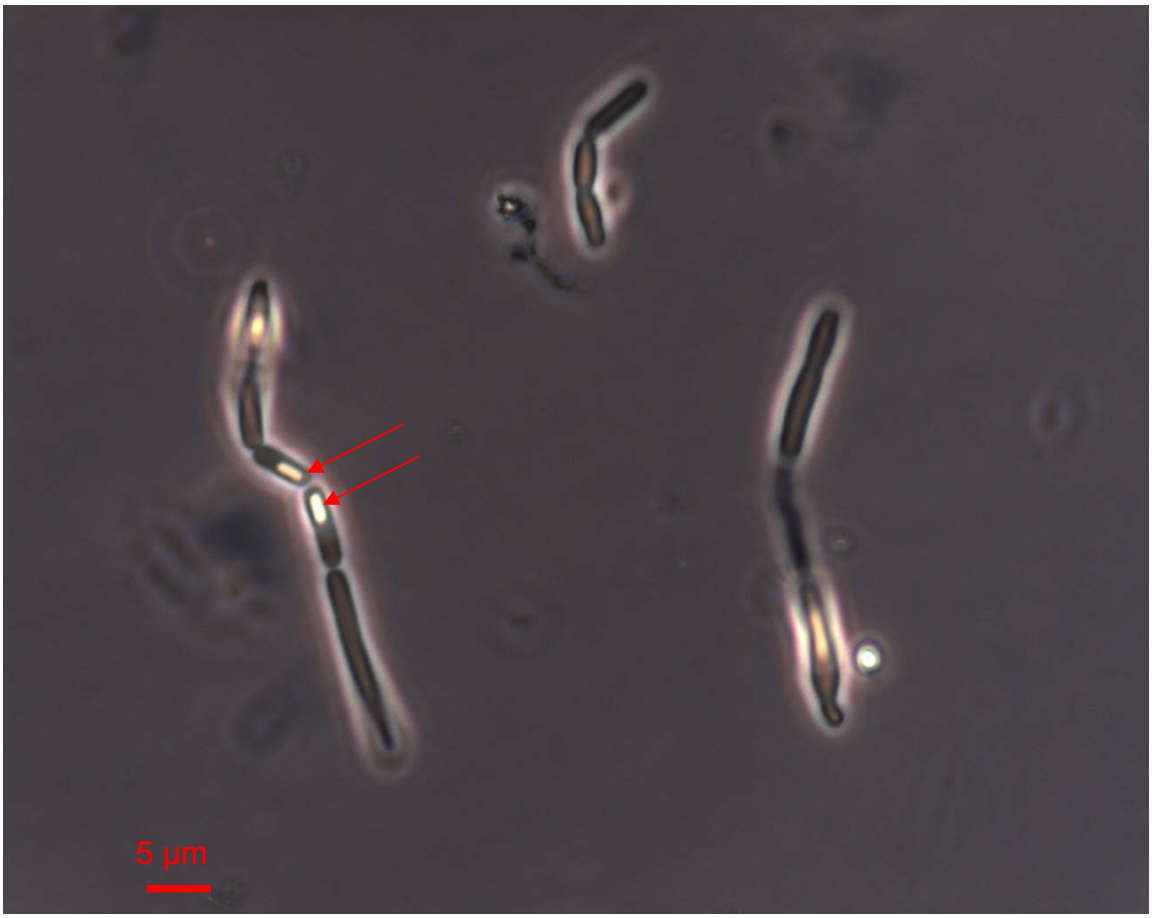


**Figure 6c.** A culture of isolate TB1 with sub-terminal endospores (red arrows indicate their location).

# CHAPTER 5

# CONCLUSIONS

The Tutum Bay hydrothermal system provided an ideal location to study the effects of high concentrations of arsenic in a shallow marine environment. In this environment, a number of chemolithotrophic metabolic reactions, including those involving the oxidation and reduction of arsenic species, were found to be energy yielding, and diverse groups of archaea were identified from the As-rich sediments of Tutum Bay at both ambient and elevated temperatures. Laboratory experiments revealed that microorganisms capable of both $As^{V}$ reduction and $As^{III}$ oxidation were extant in Tutum Bay sediments and porefluids. Microbial consortia enriched from Tutum Bay were actively involved in $As^{V}$ reduction, providing evidence for microbially-mediated As cycling in marine environments.

Experiments to isolate additional microbial species from Tutum Bay are currently underway in the laboratory. Further characterization experiments need to be done to determine the optimal growth conditions of the strain TB1 which has been isolated in this work, including determining its metabolism, phylogenetic relationship to other known organisms, and its arsenic-reducing or arsenic-tolerance potential. In addition, two techniques that can be used in future research to learn more about the Tutum Bay microbial communities are high-throughput sequencing and gene screening (Sogin et al., 2006; Huber et al., 2007), which can exponentially increase our knowledge of presently unculturable microorganisms, and high-throughput culturing techniques which can be used to more quickly isolate microbial species under environmental conditions of interest,

such as high temperature or pressure (Connon et al., 2002; Bollman et al., 2007; Giovannoni et al., 2007).

By understanding the role of microbes like strain TB1 in oxidizing and reducing arsenic, we can better predict the mobilization and transport of arsenic in natural systems. We can harness the arsenic-redox ability of microorganisms to help bioremediate ecosystems that have been contaminated with arsenic from mine waste or other manufacturing byproducts. For example, in France a bioreactor containing a consortium of bacterial species known to be potential $As^{III}$ oxidizers and $As^{V}$ reducers was used to treat arsenic-contaminated mine drainage water (Battaglia-Brunet et al., 2004; 2006), and the French village of Ambacourt has erected a biological processing unit to treat arsenic- and iron-contaminated water with natural biofilms (Casiot et al., 2006). Other microorganisms, such as the highly arsenic-resistant *Corynebacterium glutamicum*, are being genetically engineered to help remove arsenic from contaminated waters (Mateos et al., 2006).

In addition to their application in bioremediation techniques, the study of As-redox microorganisms provides us with a greater understanding of the habitats within which life on Earth and even other planets can exist. Life has an amazing ability to thrive in even the harshest of environments. On Earth, it has been postulated that "weird life" which substitutes arsenic for phosphorus could exist in unusual niche environments (Wolfe-Simon et al., 2009). It has also been hypothesized that on the early Earth, $As^{III}$ may have been one of the main energy sources for chemolithotrophic organisms, and that the microbial transformations of $As^{III}$ to $As^{V}$ may have made early environments more hospitable for other forms of life (Lièvremont et al., 2009).

The potential for life also exists on other planetary bodies in what may prove to be challenging environments. Explorations of our solar system have shown that the planet Mars, the Jovian moon Europa, and the Saturnian moon Enceladus are all believed to have or have had liquid water and ice in the subsurface (Chyba and Phillips, 2001; Hansen et al., 2006; Matson et al., 2007). Europa in particular is believed to have a large liquid water ocean beneath its shell of ice, and potential heating sources that could promote submarine hydrothermal systems (Carr et al., 1998; Pappalardo et al., 1998). Recent studies have also pointed to evidence of ice at the poles and small amounts of water on the surface of our Moon (Pieters et al., 2009; Sunshine et al., 2009). Water is a necessary component for life (at least as we know it) to exist, and these planetary bodies are all sites where we can explore for the existence of life outside of Earth. The work discussed in this dissertation not only broadens our understanding of the environments that life can tolerate or even thrive in on Earth, but can help serve as a potential ground truth for exploring for life in challenging environments elsewhere in our solar system.

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## APPENDIX A.

GLOSSARY OF TERMS

All definitions taken from *Brock Biology of Microorganisms*, by M. T. Madigan, J. M. Martinko, J. Parker. 10th ed. 2003. Prentice Hall: Upper Saddle River, New Jersey.

Aerobe = An organism that grows in the presence of $O_2$; may be facultative, obligate, or microaerophilic.

Anaerobe = An organisms that grows in the absence of $O_2$; some may even be killed by O2.

Anaerobic respiration = Use of an electron acceptor other than $O_2$ in an electron transport-based oxidation and leading to a proton motive force.

Anoxic = Absence of oxygen. Usually used in reference to a microbial habitat.

Archaea = A phylogenetic domain of prokaryotes consisting of the methanogens, most extreme halophiles and hyperthermophiles, and *Thermoplasma.*

ATP = Adenosine triphosphate, the principal energy carrier of the cell.

Autotroph = An organism able to utilize $CO_2$ as a sole source of carbon.

Bacteria = All prokaryotes that are not members of the domain Archaea.

Bioremediation = Use of microorganisms to remove or detoxify toxic or unwanted chemicals in an environment.

Chemolithotroph = An organism obtaining its energy from the oxidation of inorganic compounds.

Chemoorganotroph = An organism obtaining its energy from the oxidation of organic compounds.

Clone = A number of copies of a DNA fragment obtained by allowing an inserted DNA fragment to be replicated by a phage or plasmid.

Culture = A particular strain or kind of organism growing in a laboratory medium.

Culture medium, or growth medium = An aqueous solution of various nutrients suitable for the growth of microorganisms.

Deoxyribonucleic acid (DNA) = A polymer of nucleotides connected via a phosphate-deoxyribose sugar backbone; the genetic material of cells and some viruses.

Domain = The highest level of biological classification. The three domains of biological organisms are the Bacteria, the Archaea, and the Eukarya.

Electron acceptor, or terminal electron acceptor = A substance that accepts electrons during an oxidation-reduction reaction.

Electron donor = A compounds that donates electrons in an oxidation-reduction reaction.

Electrophoresis = Separation of charged molecules in an electric field.

Endospore = A differentiated cell formed within the cells of certain Gram-Positive Bacteria that is extremely resistant to heat as well as to other harmful agents.

Enrichment culture = Use of selective culture media and incubation condition to isolate microorganisms from natural samples.

Eukarya = The phylogenetic domain containing all eukaryotic organisms.

Eukaryote = A cell or organism having a unit membrane-enclosed (true) nucleus and usually other organelles.

Extremophile = An organism that grows optimally under one or more chemical or physical extremes, such as high or low temperature or pH.

Facultative = A qualifying adjective indicating that an organism is able to grow in either the presence or absence of an environmental factor (for example, “facultative aerobe”).

Gene = A unit of heredity; a segment of DNA specifying a particular protein or polypeptide chain, a tRNA or an RNA.

Gram-Positive cell = A prokaryotic cell whose cell wall contains relatively little peptidoglycan but has an outer membrane composed of lipopolysaccharide, lipoprotein, and other complex macromolecules.

Growth medium = see “culture medium.”

Heterotroph = Chemoorganotroph.

Hydrothermal vents = Warm or hot water-emitting springs associated with crustal spreading centers on the sea floor.

Hyperthermophile = A prokaryote having a growth temperature optimum of 80°C or higher.

Inoculum = Material used to initiate a microbial culture.

Mesophile = Organism living in the temperature range near that of warm-blooded animals, and usually showing a growth temperature optimum between 25 and 40°C.

Messenger RNA (mRNA) = An RNA molecule transcribed from DNA that contains the genetic information necessary to encode a particular protein.

Microaerophilic = Requiring O2 but at a level lower than atmospheric.

Microorganism = A microscopic organism consisting of a single cell or cell cluster, also including the viruses.

Molecular cloning = Isolation and incorporation of fragment of DNA into a vector where it can be replicated.

Obligate = A qualifying adjective referring to an environmental factor always required for growth (for example, "obligate anaerobe").

Operon = A cluster of genes whose expression is controlled by a single operator. Typical of prokaryotic cells.

Oxic = Containing oxygen; aerobic. Usually used in reference to a microbial habitat.

Oxidation = A process by which a compound gives up electrons (or H atoms) and becomes oxidized.

Oxidation-reduction (redox) reaction = A pair of reactions in which one compound becomes oxidized while another becomes reduced and takes up the electrons released in the oxidation reaction.

PCR = see "Polymerase chain reaction."

Phototroph = An organism that obtains energy from light.

Phylogeny = The ordering of species into higher taxa and the construction of evolutionary trees based on evolutionary (natural) relationships.

Polymerase chain reaction (PCR) = A method used to amplify a specific DNA sequence *in vitro* by repeated cycles of synthesis using specific primers and DNA polymerase.

Primer = A molecule (usually a polynucleotide) to which DNA polymerase can attach the first deoxyribonucleotide during DNA replication.

Prokaryote = A cell or organism lacking a nucleus and other membrane-enclosed organelles, usually having its DNA in a single circular molecule.

Redox = see "Oxidation-reduction reaction."

Reduction = A process by which a compound accepts electrons to become reduced.

Respiration = Catabolic reactions producing ATP in which either organic or inorganic compounds are primary electron donors and organic or inorganic compounds are ultimate electron acceptors.

Reverse transcription = The process of copying information found in RNA into DNA.
Ribonucleic acid (RNA) = A polymer of nucleotides connected via a phosphate-ribose backbone; involved in protein synthesis or as a genetic material of some viruses.

Ribosomal RNA (rRNA) = Type of RNA found in the ribosome; some rRNAs participate actively in the process of protein synthesis.

Ribosome = A cytoplasmic particle composed of ribosomal RNA and protein, which is part of the protein-synthesizing machinery of the cell.

Screening = Any of a number of procedures that permits the sorting of organisms by phenotype or genotype by allowing growth of some types but not others.

16S rRNA = A large polynucleotide (~1500 bases) that functions as a part of the small subunit of the ribosome of prokaryotes (Bacteria and Archaea) and from whose sequence evolutionary relationships can be obtained; the eukaryotic counterpart is the 18S rRNA.

Strain = A population of cells of a single species all descended from a single cell; a clone.

Thermophile = An organism with a growth temperature optimum between 45 and 80°C.