

MINIREVIEW

An emerging view of the diversity, ecology and function of Archaea in alkaline hydrothermal environments

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One sentence summary: A synthesis of the distribution, diversity and function of Archaea in high pH, high temperature geothermal systems.

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ABSTRACT

The described diversity within the domain Archaea has recently expanded due to advances in sequencing technologies, but many habitats that likely harbor novel lineages of archaea remain understudied. Knowledge of archaea within natural and engineered hydrothermal systems, such as hot springs and engineered subsurface habitats, has been steadily increasing, but the majority of the work has focused on archaea living in acidic or circumneutral environments. The environmental pressures exerted by the combination of high temperatures and high pH likely select for divergent communities and distinct metabolic pathways from those observed in acidic or circumneutral systems. In this review, we examine what is currently known about the archaea found in thermoalkaline environments, focusing on the detection of novel lineages and knowledge of the ecology, metabolic pathways and functions of these populations and communities. We also discuss the potential of emerging multi-omics approaches, including proteomics and metabolomics, to enhance our understanding of archaea within extreme thermoalkaline systems.

Keywords: thermoalkaline; hot springs; metagenomics; metatranscriptomics; metabolomics; geothermal

BACKGROUND

Technological advances have led to a wide array of high throughput methods for documenting the abundance, diversity and functional potential of the domain Archaea. Despite this progress, archaeal communities in key environments remain poorly understood. Sites characterized by high temperatures coupled with pH conditions that range from near-neutral (pH 6.5–7.5) to alkaline (pH > 7.5) represent environments known to host novel lineages within the archaea (Hensel et al. 1997;

Colman et al. 2015; Daebeler et al. 2018), and have been a rich resource for thermostable, alkaline tolerant enzymes for biotechnology (Horikoshi 1999; López-López et al. 2015; Sahay et al. 2017). Archaea that can survive under the polyextreme conditions of both elevated temperature and high pH conditions have unique functional pathways, which in turn represent novel biochemical functions (Wemheuer et al. 2013; López-López et al. 2015). In this review, we examine patterns of microbial diversity within thermal (temperatures > 50°C) and circumneutral (pH > 6.5) and alkaline ecosystems (pH > 7.5). In

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an effort to better understand the ecology of the archaea, we included studies that targeted the total microbial communities in these sites (the prokaryotes bacteria and archaea), as well as both natural (hot springs, serpentinizing marine vents) and engineered (bioreactors, drilled wells) systems. Further, to examine differences among low and high pH sites, we included studies conducted across gradients of pH and temperature to identify abiotic drivers of community composition and function. Using currently available literature, we: (i) explore the ecology of archaeal communities, including the likely environmental drivers of community assembly and co-existence; (ii) review metabolic and functional pathways found in archaeal taxa in thermoalkaline systems, including how functional diversity responds to changing environmental conditions and (iii) outline the progress made in identifying novel lineages, including the use of single cell genomics, metagenomic assembly and metagenome informed culturing methods. Based on our assessment of the ecological characteristic required to survive in thermoalkaline environments, we postulate that these ecological systems host still uncharacterized archaeal lineages with novel functionalities and metabolic adaptations. However, we are still in the early stages of understanding how thermoalkaline archaeal species adapt and survive.

Undersampled thermoalkaline systems

Thermoalkaline environments are found in terrestrial and marine ecosystems, including liquid-phase influenced hot springs (Colman et al. 2019) and serpentine marine vents formed when ultramafic rock is exposed to water (Shrenk et al. 2004; Brazelton et al. 2010a; Sojo et al. 2020). The microbes that inhabit hot springs are perhaps the best studied of these high pH environments, and are found globally (Fig. 1), but molecular surveys of microbial communities have been focused on a limited number of sites (Table S1, Supporting Information). Of the 400 documented hot springs in India, 28 have been sampled using either culture-based or high-throughput sequencing methods (Poddar and Das 2018). Similarly, in YNP, a subset of alkaline hot springs have been sampled extensively, such as Octopus and Mushroom Springs. Two regions in YNP have a large number of thermoalkaline springs, including Heart Lake Geyser Basin (De León et al. 2013, De la Torre et al. 2008, Mohamed et al. 2019) and Lower Geyser Basin (Thiel et al. 2017, Colman et al. 2016, Inskeep et al. 2010), which are often sampled as part of larger-scale surveys that span temperature and pH gradients found within the park. The Uzon Caldera in Russia hosts springs with a wide range of pH (3.1–9.8) (Burgess et al. 2011), but most hot springs sampled in Kamchatka are acidic to circumneutral (pH 3.1–7.0) (Merkel et al. 2016; Wilkins et al. 2019; Eme et al. 2013; Burgess et al. 2011), or are locations with pH conditions that span acidic to alkaline (Burgess et al. 2011). Sites with geothermal activity in central and South America, such as those in Costa Rica, Peru and Argentina, also represent potential reservoirs for taxonomic novelty in the archaea, but to date, studies have focused on acidic and circumneutral sites (Paul et al. 2016; Uribe Lorio et al. 2019; Willis et al. 2019), and the density of higher pH springs is unclear. In addition, there are geothermal regions that are just beginning to be explored, such as the estimated 240 hot springs in Algeria (Moussa et al. 2020). In marine systems, serpentinizing thermal vents support archaeal communities dominated by methanogens (Schrenk et al. 2004; Brazelton et al. 2010b), but a smaller number of alkaline sites, such as the Lost City hydrothermal field, have been examined relative to cold or acidic marine

sites. Clearly, there is more to be discovered on the diversity and function of archaea in thermoalkaline systems.

Polyextremophile archaea: Challenges of high pH and temperature

Archaea that live in thermoalkaline environments are polyextremophiles, able to withstand both high temperatures and high pH (Wiegel 2011; Krulwich et al. 2011). Adaptations to temperature include modifications to the cell membrane, changes to DNA repair pathways, and protein stabilization. Organisms that survive and thrive in extreme pH conditions generally maintain internal pH that varies drastically from their environments. Under alkaline conditions, the best documented mechanism for maintenance of near-neutral internal pH is the use of a antiporters (Na^+/H^+ and K^+/H^+) that exchanges ions for protons (Krulwich et al. 2011). Under the polyextreme conditions of both high pH and temperature, the trade-offs between investment into tolerance and metabolic pathways can alter patterns of growth and activity. For example, the alkaliphile *Natronobacterium cellulositrophum* which could grow at up to pH 9.9 at low temperature could only grow at the lowest pH values included in the study, 8.5 when temperatures exceeded 43°C (Sorokin et al. 2018).

ECOLOGY AND DIVERSITY OF ARCHAEA IN ALKALINE HYDROTHERMAL SITES

Biogeography of Archaea

Biogeography, the study of the distribution of species and communities over geographic, environmental or temporal gradients, can be used to understand the mechanisms determining microbial community abundance, diversity and composition (Ramette and Tiedje 2006). By identifying the range of conditions in which a particular taxon inhabits, studies conducted across broad geographic and environmental gradients provide insights into whether certain archaeal taxa occupy specific niches, thus increasing our understanding of environmental factors which limit archaeal species distribution. Large scale studies, such as those conducted across 925 hot springs in New Zealand (Power et al. 2018) and 118 hot springs in the Tibetan plateau (Zhang et al. 2018), can begin to identify the environmental breadth of given taxonomic groups of archaea, as well as the dispersal limitations that may constrain their geographic ranges. Comparisons of archaeal operational taxonomic units (OTUs) detected in geothermal hot springs that varied in both their temperature (7.5°C to 99°C) and pH (1.8 to 9.0) across Canada and New Zealand found that the sequences detected had close matches to those found from geothermal sites in Japan, Thailand, Tibet and YNP (Sharp et al. 2014). This indicates that archaeal taxa found in hot springs have broad geographic ranges, suggesting dispersal limitation is not a predominant factor driving archaeal distribution in alkaline hydrothermal systems (Sharp et al. 2014). Similarly, based on metagenomic sequencing, a number of uncultivated Aigarchaeota, Korarchaeota and Nanoarchaeota archaeal phyla were distributed across four continents (Hedlund et al. 2015).

Large-scale studies have revealed that functional classifications based on the presence of organisms under specific pH and temperature conditions, such as surveys that attempt to categorize lineages as 'acidophilic' or 'thermophilic', may reflect less robust sampling rather than true physiological constraints. For example, recent genomic surveys of several alkaline springs documented archaeal taxa that were previously thought to be

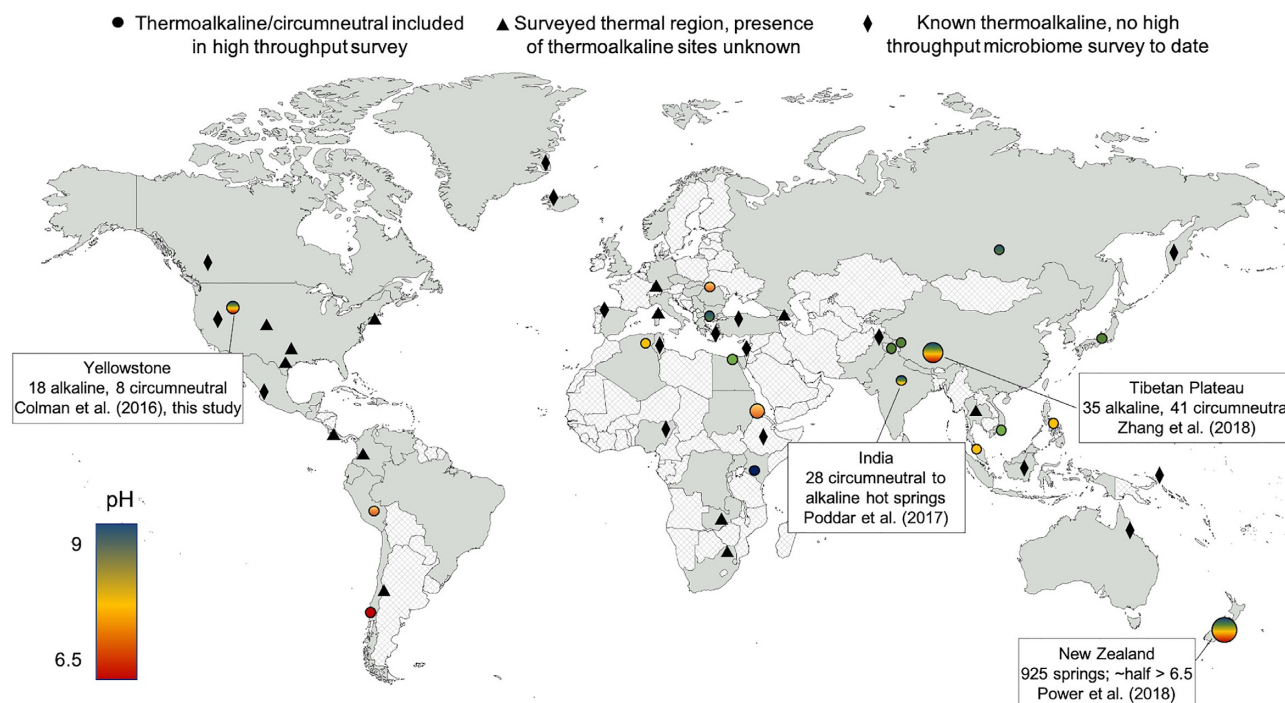


Figure 1. Archaeal communities in circumneutral and alkaline pH terrestrial geothermal systems across the globe. Hot springs have been documented in multiple countries (solid grey). A small number of regions, including China, New Zealand and the USA (primarily YNP), have been more robustly sampled using current -omic approaches such as metabarcoding and shotgun metagenomics; circles). Other geothermal sites have documented thermoalkaline sites but have not been sampled using modern methods (diamonds), and others have not been described in terms of temperature and pH ranges (triangles). Colors of symbols correspond to pH of the hot springs sampled, scaling from circumneutral to alkaline. Circle size corresponds to the number of circumneutral to alkaline hot springs sampled. Data was drawn from published papers, as well as sequence data available in public databases, including GenBank (NCBI).

localized only to acidic systems. In one study, amplicon and metagenome surveys from a moderately alkaline (pH 8) hot spring in Malaysia identified sequences most closely related to *Aciduliprofundum boonei* and *Sulfolobus acidocaldarius* (Chan et al. 2015), taxa considered to be acidophiles. As the number of environmental genomic surveys expands, so does our ability to identify the geographic and environmental ranges of archaeal taxonomic groups. This same trends holds true for high pH geothermal habitats, where a recent 16S rRNA survey conducted in an alkaline (pH ~8.7) hot spring in Russia detected an environmental sequence identified as a species in the genus *Fervidococcus* (Perevalova et al. 2008) that was nearly identical to a phylotype originally detected in a circumneutral (pH 6.7–7.6) hot spring in YNP (Barns et al. 1994). Additional studies conducted across both large spatial gradients, particularly those that target more than phylogenetic markers, could provide additional insights into patterns of evolution and adaptation across both spatial and environmental gradients.

Survey of thermoalkaline sites

In order to understand the roles of archaea in these systems, a better understanding of the range of diversity present is needed. To gain a more holistic understanding of the archaeal diversity present in thermoalkaline sites, and to examine the phylogenetic distribution of environmental sequences from thermoalkaline sites, we downloaded raw 16S rRNA sequence data from NCBI for hot spring sites from China (Jiang et al. 2016; Zhang et al. 2018), YNP (Colman et al. 2016), Russia (Zaitseva et al. 2017), India (Najar et al. 2020), Japan (Nakagawa and Fukui 2002), and Romania (Coman et al. 2013). We also included additional sequences

generated from sediment slurry collected in YNP, including the Five Sisters, and sites previously surveyed in Heart Lake Geyser Basin (De León et al. 2013). In order to compare among sites, we limited our analysis to studies that targeted the V3 or V3-V4 region of the 16S using paired-end sequencing on the Illumina MiSeq platform. Fastq files were filtered using USEARCH (Edgar and Flyvbjerg 2015) with an expected value of 0.5 and operational taxonomic units (OTUs) were delineated using UNOISE2 (Edgar 2016). Environmental OTUs were aligned to full-length and near full-length 16S sequences from archaea downloaded from NCBI and the GTDB (Parks et al. 2018). Sequences were aligned using mafft (Katoh and Standley 2013) and a maximum likelihood phylogeny was inferred using RAxML (Stamatakis 2014) with the GTR+gamma model. The phylogenetic and taxonomic classifications of archaea continue to shift, and so we assigned taxonomic identification of reference sequences based on Baker et al. (2020). For example, based on NCBI taxonomy, the phylum Nanohaloarchaeota is part of the superphylum Euryarchaeota, but is placed with DPANN in later classifications. The phylogeny was annotated using the Interactive Tree of Life (Letunic and Bork 2016) (Fig. 2). Although universal 16S primers may not detect all members of the archaea (Colman et al. 2015), later iterations of commonly used primers, such as the 515F-806R Earth Microbiome primers, have been modified to reflect additional diversity.

The phylogenetic placement of the several archaeal phyla from publicly available sequences was not monophyletic, possibly due to the use of 16S rRNA sequences from MAGs and SAGs, but from this analysis, several patterns emerged. First, there were large numbers of OTUs that were closely related to phyla in the DPANN superphylum, particularly in the Woesarchaeota

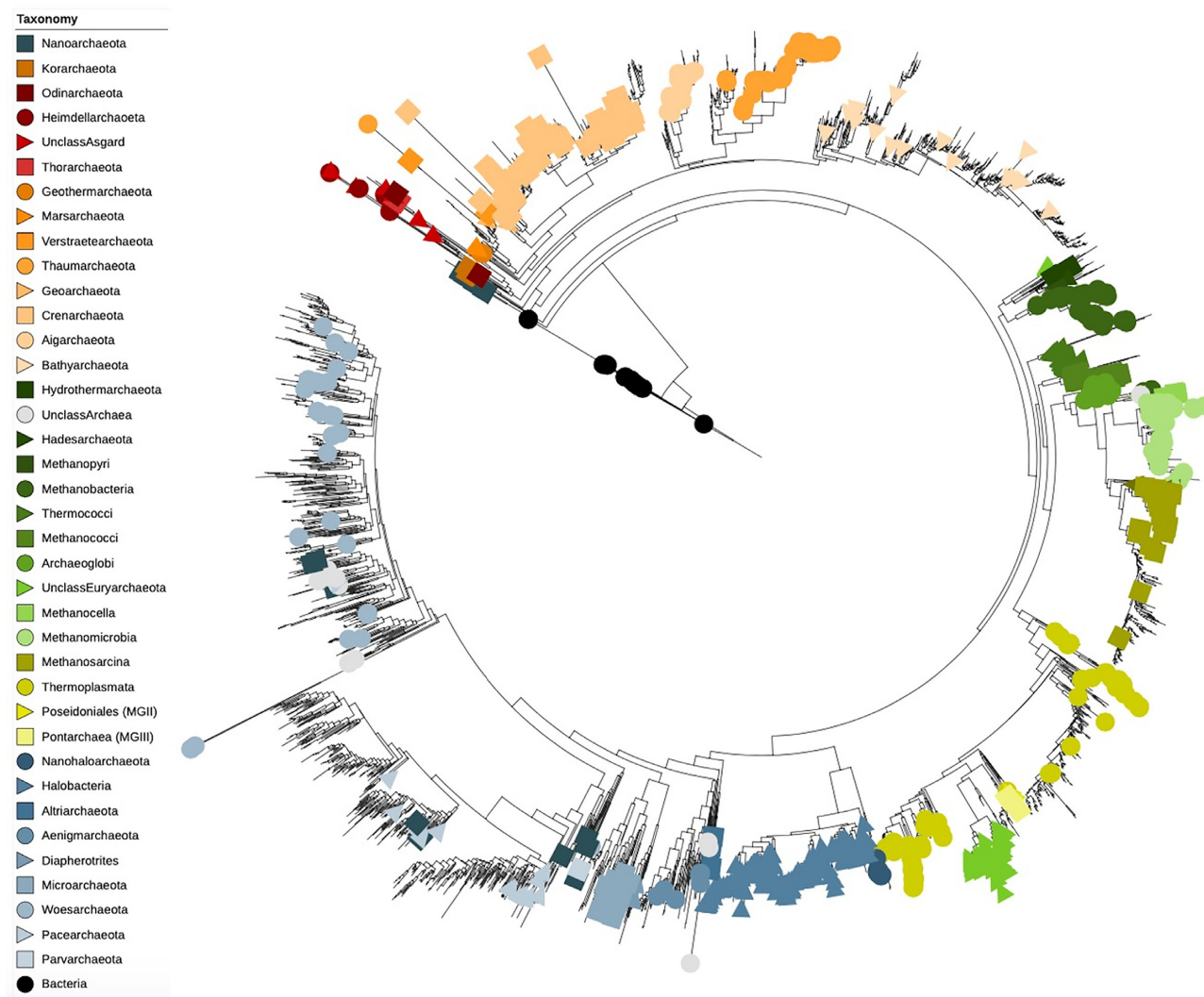


Figure 2. A phylogenetic tree based on full-length and near-full length archaeal sequences from NCBI and the GTDB and environmental OTUs generated from publicly available MiSeq data across a range of locations, including YNP, China (Tibetan Plateau and Zimequan), India (Garampani and Polok), Japan, and Russia (Baikal) and drilled well in Romania. Reference sequences are colored according to their current phylum level classification (DPANN: blue, Euryarchaeota: green, Asgard: red, TACK: orange), environmental sequences are not colored. Likely due to the use of 16S sequences from MAGs or SAGs, the tree is not monophyletic, particularly for the DPANN superphylum where the number of cultured representatives is minimal (Baker et al. 2020).

and Pacearchaeota, as well as the TACK phylum Bathyarchaeota. To date, none of these groups have a cultured representative (Baker et al. 2020), and thus these sites could be targets for culturing efforts of these groups. Second, the number of OTUs from known methanogenic groups was comparatively low; however, given the expansion of methanogenic pathways in taxa outside of the Euryarchaeota, methanogenesis may be undertaken by other groups. Finally, only a small number of OTUs were placed with members of the Asgard superphylum, which could be due to the lower number of reference sequences available, but could also be indicative of lower abundance of this group in alkaline sites.

Environmental drivers of archaeal abundance and diversity: Temperature

Temperature is known to be a strong driver of archaeal abundance in hydrothermal environments, with high temperature (>80°C) conditions favoring the formation of ecological systems

with higher proportions of archaea (Song et al. 2010; Coman et al. 2013; Sharp et al. 2014; Stefanova et al. 2015). For example, a comparison of several sites in New Zealand and Canada that represented a gradient in both pH (~2 to 9) and temperature (~10 to 100°C) found that the relative abundance of the archaeal phylum Crenarchaeota increased at higher temperature, from 1% at 0°C–20°C to nearly 50% at 80°C–100°C (Sharp et al. 2014). Correspondingly, a qPCR-based analysis of three abandoned geothermal oil wells in Romania with high levels of bicarbonate (HCO_3^-) and temperatures of 32°C, 49°C and 65°C noted that the relative abundance of archaea was 36% of the total community in the 65°C well, but less than 1% at cooler temperatures (Coman et al. 2015). When relative abundance was examined using qPCR, the relative abundance of archaea increased to 45% in the highest temperature well, and less than 2% in the 32°C and 49°C well.

The relative abundance of archaea is nearly always maximized at high temperatures, but diversity patterns are less clear. Ribosomal genes detected in shotgun metagenome surveys generally indicate that the archaea constitute a lower proportion

of the microbial diversity compared to bacteria (Mangrola et al. 2015; Valle, Morales-Vale and Ríos-Velázquez 2017), regardless of temperature, and this pattern is also generally found in targeted surveys performed alongside shotgun sequencing (Chan et al. 2015). Two studies that examined changes in microbial (bacteria plus archaea) across broad temperature gradients found curvilinear patterns, where diversity peaked at mesic temperatures (~21°C) and plateaued above 65°C (Sharp et al. 2014; Power et al. 2018), a pattern also observed at the Lost City vent (Brazelton et al. 2006). Archaeal-focused studies have revealed less consistent patterns. In Iceland, a comparative study of archaea across four circumneutral hot springs with identical pH (6.5) found the highest diversity at 88°C, but the lowest at 90°C, with intermediate values observed at lower temperatures (65°C and 67°C) (Mirete, de Figueras and González-Pastor 2011), suggesting an abrupt threshold at extreme temperatures. In contrast, Song et al. (2010) found that moderate temperature springs hosted the highest levels of archaeal diversity, but were dominated by members of a single phylum, the Crenarchaeota (Song et al. 2010). In Romanian wells, the number of OTUs was similar across different temperature wells, but a single genus, *Methanobacteria*, dominated at high temperatures (Coman et al. 2015), resulting in shifts in community composition due to the high dominance of putative methanogens at higher temperatures. Although function is not always predicted by phylogenetic diversity, a better understanding of the phylogenetic breadth of communities could provide insights into the potential functional diversity (e.g. Tucker et al. 2018) across temperature and pH gradients.

Environmental drivers of archaeal abundance and diversity: pH

As shown in soil across numerous ecosystems (Rousk et al. 2010), pH can be a driver of microbial abundance in hydrothermal systems (e.g., Sharp et al. 2014). In focused studies of alkaline springs, archaea rarely exceed 10% of the overall microbial population abundance (e.g. López-López et al. 2015), and general 16S rRNA gene surveys have concluded that archaea are more abundant in low pH hot springs in comparison to their alkaline counterparts (Li et al. 2015b). This pattern is consistent across regional geographic gradients as well, as Power et al. (2018) found that archaea exceeded 1% relative abundance only in acidic to circumneutral springs. This was corroborated by a study conducted in Costa Rica that did not detect archaea in any hot springs with a pH > 6 (Uribe Lorío et al. 2019).

While the abundance of archaea may be lower in alkaline hot spring systems relative to their acidic counterparts, as with the effects of temperature, there is some evidence that archaeal diversity increases at high pH. 16S rRNA gene sequences analyzed from one acidic (pH 3.6) and three alkaline (pH 7.5–9.3) geothermal springs in China found that archaeal phylogenetic diversity increased as a function of elevated pH (Li et al. 2015b). In this system, the acidic spring was dominated by a single archaeal lineage, Sulfolobales, while three alkaline springs supported a broad phylogenetic range of archaeal populations including the Desulfurococcales, Euryarchaeota and Korarchaeota, along with several other unclassified archaeal phyla. These findings come with a caveat, however, as the acidic spring also had the highest temperature (93.6°C), while the alkaline springs ranged between 48.7 to 88.3°C. Further, a separate comparative study of thirty-eight hot springs across China found that over a temperature range spanning from 55.1°C to 93.6°C,

archaeal taxonomic diversity decreased with decreasing pH (pH range 2.5 to 9.4); (Hou et al. 2013). As seen in other studies (Li et al. 2015b), the lower diversity was driven by the high dominance of the Sulfolobales in low pH springs, while numerous circumneutral to alkaline (pH > 6) hydrothermal sites hosted a mixture of Desulfurococcales and Thermoproteales. This study also suggested there are interactive effects of temperature and pH on archaeal diversity, where at lower temperatures, Euryarchaeota and Thaumarchaeota archaeal lineages dominated in both alkaline and acidic habitats. The pattern of decreasing diversity with decreasing pH reported by (Hou et al. 2013) was subsequently confirmed in a PhyloChip analysis of a subset of these sites (Briggs et al. 2013). A comparative study of two high temperature springs (51°C and pH 3, and 93°C and pH 8) in the Azores Islands off of Portugal based on pyrosequencing and domain-specific FISH probes found that a single genus in the Euryarchaeota, *Thermoplasma*, comprised nearly 90% of the archaeal community in the acidic spring, while a broader range of genera, including *Pyrobaculum*, *Sulfophobococcus* and *Desulfurococcus*, in the phylum Crenarchaeota were detected in the alkaline spring (Sahm et al. 2013). Comparisons between acidic and non-acidic hot springs in China also found that archaeal communities were simplified in acidic springs, hosting a restricted range of phyla (Song et al. 2013). Together, these studies indicate that the phylogenetic breadth of archaea in alkaline springs exceeds that of acidic springs, potentially indicating a larger range of functional niches in these systems.

Studies using primer-independent sequencing techniques have found similar patterns to those that employ targeted sequencing approaches. A shotgun metagenome study of thermophiles from a variety of hydrothermal environments across four continents found that genera in the uncultivated archaea phyla Aigarchaeota, Korarchaeota and Nanoarchaeota were widely distributed in geothermal sites ranging in pH from 3.4 to 9.3 (Hedlund et al. 2015), although their relative abundance varied among the sites. For example, the obligate endosymbiont Nanoarchaeota was present at >1% abundance in the four acidic habitats (pH 2.9–4.4) included in the analysis, but was detected at <0.1% relative abundance in three alkaline (pH 6.4–8.0) YNP springs, suggesting that pH likely acts as a secondary selection filter relative to temperature. The patterns observed for Nanoarchaeota, which is an extremely small, obligate archaeal symbiont, can also be linked to the distribution of their host organism, which comprise members of either the Sulfolobales or the Acidobiales taxonomic orders, and tend to be more abundant in acidic springs (e.g. Hou et al. 2013; Li et al. 2015b). This body of work suggests that although the relative abundance of archaea in alkaline hot springs is lower than that of acidic geothermal environments, the phylogenetic, and potentially the functional, diversity of archaea may be higher in alkaline systems. Further work is needed to elucidate the effects of temperature and pH in driving patterns of archaeal abundance and diversity, especially in alkaline thermal systems. Based on current studies, it seems likely that these two critical factors interact to drive archaeal community patterns. Additional investigations, including broad-scale global studies and meta-analysis, would likely be beneficial in providing further insights.

Outside of the strong selective pressure of temperature and pH, other factors such as organic carbon inputs, sulfide concentrations, salinity and/or dissolved oxygen, are known to be important drivers of diversity and abundance in other hydrothermal systems (Purcell et al. 2007), and may exert significant selection pressures on archaeal communities in thermoalkaline environments. Insights into the drivers of archaeal

community composition can be gained by identifying the metabolic pathways used across a range of environmental conditions.

FUNCTIONAL ROLES OF ARCHAEA

Metabolic pathways of archaea in thermoalkaline systems

Temperature and pH are commonly examined as potential drivers of microbial community assembly and are often closely linked to both taxonomic and functional differences among hot springs, but factors outside of these parameters also play critical roles in driving community assembly (Massello et al. 2020). Archaea play critical roles in global biogeochemical cycles, and are particularly important in the global nitrogen, sulfur, and carbon cycles through ammonia oxidation, sulfate reduction and methanogenesis, respectively (Offre, Spang and Schleper 2013). As a result, in addition to temperature and pH, available energy sources play an important role in driving both taxonomic and functional composition (Inskeep et al. 2010), and the metabolic pathways of organisms found in a particular site can often be predicted based on the geochemical makeup of the spring. In general, energy yields follow predictable paths based on available electron donors and acceptors, but metabolic reactions favored under one set of geochemical conditions are not energy sources at other conditions (Shock et al. 2010; Cox, Shock and Havig 2011). In general, redox reactions that are more energetically favorable will be preferentially utilized by microbes, and detailed geochemical analysis coupled with thermodynamic modeling can be used to predict the most likely redox coupled reactions (Dodsworth, McDonald and Hedlund 2012), which can in turn be linked to the functional pathways of the microbiome. These models do not account for biotic interactions, such as syntrophy and competition, and may be difficult to apply to populations with metabolic flexibility in carbon and energy metabolism (Schubotz et al. 2013), and mixotrophy observed in alkaline microbial communities (Schubotz et al. 2015). Further, cross comparisons among studies can be limited as high resolution measures of geochemistry represent a significant monetary investment, and they are often not completed, particularly when a large number of springs are included in the study.

Carbon cycling: heterotrophic and autotrophic pathways

Archaea utilize metabolic pathways that diverge from those of known bacteria (Wang et al. 2019a). In extreme hydrothermal systems, archaea are theorized to drive anaerobic carbon cycling through fermentation-based metabolisms (Castelle et al. 2015). Heterotrophic archaea have central carbon metabolic pathways that are distinct, including modified variants of sugar degradation pathways found in bacteria (Brasen et al. 2014). Archaeal utilization of organic carbon depends upon inputs into the system. Genome comparisons of archaeal phyla using the database of carbohydrate-active enzymes (CAZymes) found large differences in the number and type of CAZymes detected, with particularly high numbers of GH in the newly described TACK phylum Brockarchaeota (De Anda et al. 2020), and there is evidence that some archaeal taxa in the Bathyarchaeota utilize lignin (Yu et al. 2018). While lignin deconstruction is energetically costly, organisms able to utilize this form of organic carbon should be favored

in springs with woody inputs, as seen in a comparative study of springs with and without plant biomass inputs (Lee et al. 2018).

Archaea also play critical roles in chemolithotrophic carbon fixation. The upper temperature limit for photosynthesis is pH dependent (as well as dependent on sulfide concentrations). In hot springs, photosynthesis occurs at up to 72°C in alkaline geothermal environments (Cox, Shock and Havig 2011); above this threshold, non-phototrophic carbon fixation by chemolithoautotrophs provides critical inputs of organic carbon into thermal systems. To date, two CO₂ fixation pathways, DC/4-HB and 3-HP/4-HB have been found only in hyperthermic archaea (Straub et al. 2018). In sites where DIC made up a large proportion of total carbon, stable isotope probing with ¹³C showed that DIC-derived microbial carbon, driven in part by the archaeal community, was an important input across a range of conditions (Jennings et al. 2017). A survey of hot springs in YNP that included two alkaline springs found autotrophic archaea with the DC/4-HB pathway at high pH, (Thermoproteales, likely *Pyrobaculum* or *Thermococcus*), but found no organisms with the 3-HP/4-HB pathway (Jennings et al. 2017).

Methanogenesis

The methane cycle is broadly driven by members of the archaea. Our understanding of the molecular pathways that underlie methanogenesis has expanded through genomics to reveal novel metabolisms, including hydrogen-dependent methylotrophic methanogenesis (Söllinger and Urich 2019). Methanogens are widely distributed in anoxic environments (Söllinger and Urich 2019), but the energetic costs of reducing CO₂ increase at higher temperatures. Known methanogenic taxa are commonly detected in alkaline hot springs (e.g. De León et al. 2013) (also see Fig. 2), but enrichments for methanogens from two circumneutral (pH 7.2) hot springs in Armenia found no evidence of activity at 65°C (Hedlund et al. 2013). This suggests that the polyextreme conditions of both high pH and high temperature could limit the importance of methanogenesis in thermoalkaline sites, but known functional marker genes have been detected across a range of conditions. Targeted analysis of the methyl coenzyme M reductase (*mcrA*) gene across multiple hot springs in Tunisia ranging from slightly acidic (pH 6.2) to moderately alkaline (pH 8) successfully detected the *mcrA* only in alkaline hot springs with temperatures above 50°C (Sayeh et al. 2010). More recent metagenomic surveys found evidence of archaea with *mcrA* across multiple hot springs in the Yunnan province in China (Hua et al. 2019). Closer examination of the alkaline (pH 7.6–9.6) hot springs showed MAGs from the Nezharchaeota, Verstraarchaeota and Archaeoglobales (Euryarchaeota).

In addition to widespread distribution, recent studies have revealed pathways for methanogenesis beyond the well known hydrogenotrophic pathway, which is strictly anaerobic in which CO₂, formate or CO reduction is coupled with oxidation of hydrogen. Based on the presence of methyltransferase/coronoid proteins and the absence of a complete Wood-Ljungdahl carbon dioxide reduction pathway, the Verstraearchaeota were classified as H₂-dependent methylotrophic methanogens, whereas the other MAGs contained *mcr* genes associated with methanogenesis or methanotrophy and encoded a conventional Wood-Ljungdahl pathway (Hua et al. 2019). Alkaline methanogens may also oxidize alkanes; examination of the methyl-coenzyme M reductase gene for anaerobic methane oxidation showed the potential for alkane oxidation, generally coupled to dissimilatory sulfate or sulfite reduction or nitrate reduction, was present

across a range of phyla, including the Archaeoglobi, Hadesarchaeota, Nezaarchaeota, Korarchaeota and Verstaetarchaeota (Wang et al. 2019b). Further analysis of *mcr* across archaeal lineages have identified *mcr*-like genes that drive the oxidation of short-chain alkanes, suggesting that genes linked to methanogenesis are involved in multiple metabolic pathways (Evans et al. 2019). The expanding number of taxa found to contain genes for methanogenesis beyond the hydrogenotrophic Euryarchaeota, and the emerging complexity of the methanogenesis molecular pathways suggest that additional surveys in understudied environments will further increase our understanding of substrate utilization by archaea.

Archaea in sulfur and nitrogen cycling

Archaea are known to have unique pathways for sulfur metabolism coupled to methanogenesis, including respiratory reduction of the heterodisulfide of coenzyme M and coenzyme B in methanogens (Liu, Beer and Whitman 2012). Archaea also appear to have unique pathways for Fe-S cluster biogenesis, although the proteins required are still mostly unidentified. Sulfide-rich alkaline hot springs are thought to be relatively rare in most geothermal basins (Skirnisdottir et al. 2000), and are found primarily in Iceland and India (Skirnisdottir et al. 2000; Tripathy et al. 2016), but also in YNP (Härtig et al. 2012), Papua New Guinea (Meyer-Dombard and Amend 2014) and China (Jiang et al. 2016). Alkaline springs not characterized as sulfidic can also show high levels of variation in sulfur compounds. For example, in the Lower Geyser Basin of YNP, total sulfide (HS^- plus H_2S ; μmol) in hot springs with pH > 7.5 in the Mounds region ranged from 3.65 to 19.5 (mean 15.1), but ranged from below detection limit to 0.593 (mean 0.176) in the White Creek/Octopus Spring region (Shock et al. 2010). A survey of four hot springs representing the four main geochemical classifications in YNP (alkaline-chloride, mixed alkaline chloride, acid-chloride-sulfate and travertine precipitation) found that 80% contained sulfur, even sites with low amounts of hydrogen sulfide (e.g. alkaline chloride springs) (Gonsior et al. 2018). In contrast, sulfate concentrations in hot springs surveyed in New Zealand were high only in acidic sites; sulfate in most alkaline and circumneutral springs was less than 100 ppm (Power et al. 2018). In a survey of metagenomes from YNP, genes for sulfur metabolism were generally more prevalent in acidic springs (Colman et al. 2019), but sulfur oxidation pathways have been documented in Aigarchaeota (Hua et al. 2018), which inhabit circumneutral to alkaline hot springs. Sulfur cycling appears to be absent in two superphyla, the DPANN and the Asgard, which have no documented phyla with even partial metabolic pathways for sulfur cycling, as identified based on the absence of key genes (Baker et al. 2020).

Ammonia oxidizing archaea (AOA) tend to be found in acidic soils and sites (Hatzenpichler 2012) and have historically been thought to play critical roles in the nitrogen cycle in acidic, low nutrient content sites with at least minimal concentrations of sulfide (Erguder et al. 2009). However, in soils, both the abundance and diversity of AOA are positively correlated with increasing pH, and adaptation to pH appears to drive niche specialization in AOA (Gubry-Rangin, Hai and Quince 2011), suggesting that AOA play key roles under alkaline conditions. Further, AOA have higher temperature optima, and higher nitrification activity at high temperature than ammonia oxidizing bacteria (Taylor et al. 2017), suggesting that they have a competitive advantage under high temperature conditions. These general patterns hold in high temperature sites. In an alkaline

spring (pH ~8) in Tengchong, a survey of *amoA* genes found that bacteria dominated at lower temperatures, (<75°C), archaea were more abundant at temperatures up to 90.8°C and AOA were more active under higher temperatures (Li et al. 2015a). Targeted sequencing of archaeal *amoA* genes showed that AOA were present across a range of hot springs, including alkaline sites (pH 7.6–9) (Zhang et al. 2008; De la Torre et al. 2008). AOA have also been isolated from non-acidic sites, including *Nitrosocaldus yellowstonii* from multiple hot springs in YNP (pH 6.2–9, 54°C–90°C) (De la Torre et al. 2008) and an obligately thermophilic ammonia oxidizer, '*Candidatus Nitrosocaldus islandicus*' from a circumneutral hot spring in Iceland (pH 6.5, 73°C) (Daebeler et al. 2018). Based on high throughput sequencing of the 16S rRNA gene, '*Candidatus Nitrosocaldus*', a putative AOA, was found in a thermoalkaline spring (pH 8.5, 75°C) in YNP (De León et al. 2013), and multiple OTUs similar to *Nitrosocaldus* were found across a range of thermoalkaline sites (Fig. 2). Again, as compared to acidic environments, relatively less is known regarding the role nitrification by archaea in alkaline environments (Lehtovirta-Morley 2018) but given the higher temperature and pH ranges of AOA, is it likely that they play key roles in nitrogen-rich thermoalkaline springs.

Archaea in thermoalkaline systems clearly play key roles in nutrient cycles, but in many cases, functions are inferred from organisms found in other conditions. Continued understanding of the true functional pathways, as well as how organisms will interact (Hug and Co 2018), requires additional focus on thermoalkaline sites. Even in YNP, arguably the best studied hydrothermal system, metagenomic sequencing has focused primarily on acidic and circumneutral hot springs (Data from NCBI Short Read Archive, November 2019; Fig. 3). The limited coverage of high pH sites suggests that additional high-coverage metagenomics could reveal additional insights into archaeal populations, communities and metabolic pathways.

Key taxa from thermoalkaline sites: Metagenomes and isolates

Alkaline hydrothermal systems are known to be reservoirs for novel uncultured archaea, as well as other microbial taxa with distinct metabolic pathways (López-López et al. 2015), and novel archaea are still being discovered in hot springs (John et al. 2019; Kato et al. 2019). Recent advances in sequencing technologies, including metagenomics and single cell genomics, have uncovered many new archaeal lineages in extreme environments, and have helped improve our understanding of their ecology and function. Despite the limited focus on thermoalkaline environments, metagenomic studies from other types of extreme environments have uncovered a broad range of phyla in the archaea. Early applications of metagenomic sequences detected the uncultivated archaeal lineages Thaumarchaeota, Korarchaeota and the Aigarchaeota (Schleper, Jurgens and Jonuscheit 2005). Analyses of these genomes have found that the newly described lineages often contain genes that code for novel metabolic pathways (Nunoura et al. 2010). By expanding environmental surveys to include a broader range of conditions, we would likely gain insights into the factors that structure the metabolic makeup of archaea, since geochemical parameters have been shown to closely follow predicted functional pathways in archaeal metagenomes from other systems (Inskeep et al. 2010).

To date, culturing efforts for archaea have yielded uneven results. Currently the majority of isolates are methanogenic members of the Euryarchaeota, and most phyla are known only

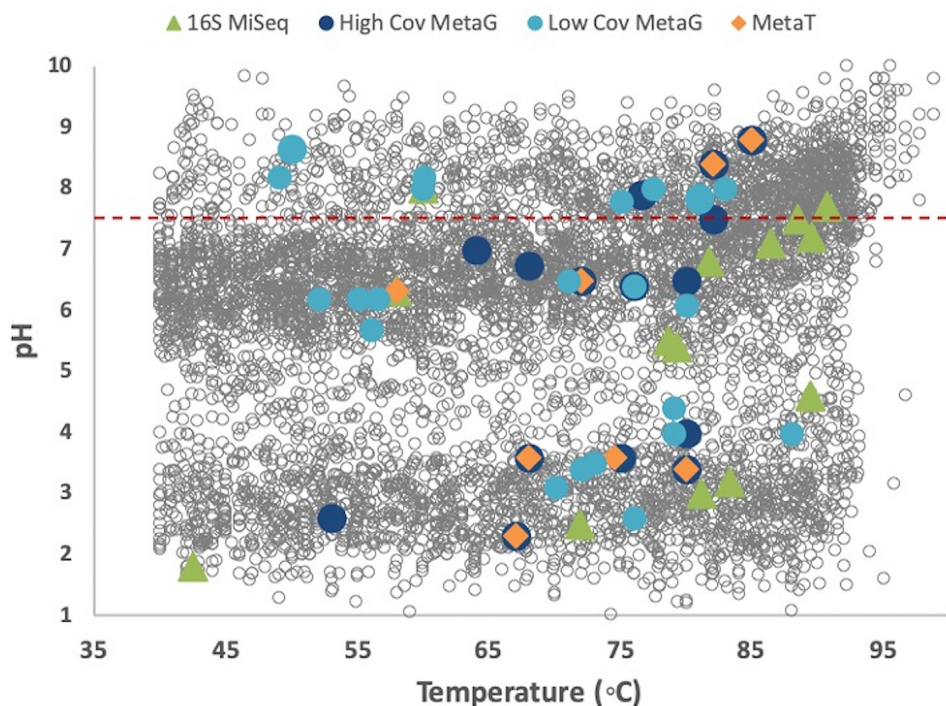


Figure 3. The number of thermal features (grey circles) in YNP relative to studies of their microbial communities. Temperature and pH data were downloaded from the Yellowstone National Park Research Coordination Network (rcn.montana.edu). Colored points represent studies that used 16S sequencing with the Illumina MiSeq platform (16S MiSeq), low coverage shotgun metagenomics (Sanger, 454, single-end Illumina; Low Cov MetaG), high coverage metagenomics (>10 million reads per sample; primarily paired-end Illumina; High Cov MetaG) and metatranscriptomes (MetaT). Data are taken from Coleman et al. (2016), Inskeep et al. (2013), Inskeep et al. (2010), McKay et al. (2019), Coleman et al. (2019) and additional data with no corresponding authorship in NCBI. The microbial community data are based on publicly available sequencing data from GenBank (NCBI) that also had corresponding feature information (e.g. feature name or geochemical data). Due to limited reporting of pH and temperature, or lack of named hot spring, this does not capture all hot springs surveyed to date, but those that have whole-community microbial surveys paired with environmental measures. A number of sites were sampled in multiple studies using different methodologies (e.g. Monarch Geyser, Octopus Spring) and so have overlapping points. The dashed line shows the transition to alkaline sites.

from SAG and MAG reconstructions (Baker et al. 2020), but these reconstructions have yielded important insights into archaeal diversity and function. A number of recently described lineages have been found in thermoalkaline hot springs. Aigarchaeota (TACK Superphylum) are distributed globally, and are found across a range of environmental conditions, but appear to be most prevalent at circumneutral to alkaline and moderately high temperatures (75°C–85°C) (Hedlund et al. 2015). Increasing resolution in metagenomic and single cell genomic studies (Rinke et al. 2013), has shown the potential for autotrophic metabolism, dissimilatory sulfite reduction and potentially carbon monoxide oxidation (Hua et al. 2018), as well as the ability to utilize a wide array of organic carbon substrates, including acetate, fatty acids, amino acids and sugars, but are likely oligotrophic (Beam et al. 2016). Sequences with high similarity to Aigarchaeota dominated the archaeal community in a thermoalkaline spring, but only in a single year (De León et al. 2013), and in a metagenomic survey of an alkaline hot spring in Spain, '*Candidatus Caldiarchaeum subterraneum*' was the dominant archaeal organism detected, and made up 4% of the classified sequences in the fosmid library (López-López et al. 2015).

Culturing from thermal systems has generally been skewed toward acidic or circumneutral sites (Blöchl et al. 1997; Daebeler et al. 2018; Kato et al. 2019). Archaea have been successfully isolated from alkaline geothermal systems, but as with the majority of archaeal isolates (Baker et al. 2020) have generally been organisms with previously characterized metabolic pathways (e.g. Hensel et al. 1997). Metagenome sequencing, assembly and annotation using homology of observed protein-coding

genes against databases of better characterized isolates can be improved by with the addition of single cell genomics and metatranscriptomics to identify the poorly understood pathways of novel organisms (Rinke et al. 2013). This vast volume of sequence data could in turn be used to inform new culturing strategies to isolate novel archaeal lineages from the environment (Hug et al. 2016; Johanna et al. 2018) by informing cultivation techniques (Hedlund et al. 2014). For example, metagenomics and lipid profiles revealed novel *amoA*-like sequences that formed the basis of culturing efforts that led to the successful growth of a novel lineage, the '*Candidatus Nitrosocaldus yellowstonii*' enriched from an alkaline (pH 8.3) spring in YNP (De la Torre et al. 2008). Additional *Nitrosocaldus* isolates have been identified in a mildly alkaline hot spring (pH 7–8) in Italy (Abby et al. 2018) and a circumneutral site in Iceland (Daebeler et al. 2018). Based on metagenome surveys, we found that a number of recently described taxa in the TACK superphylum have been documented in higher temperature systems under alkaline conditions (Table 1), although some recently described lineages, such as the Marsarchaeota, have only been found in acidic sites (Jay et al. 2018). Increasing attention to these systems will likely uncover additional taxonomic and functional diversity of archaea, and also provide insights into the mechanisms of tolerance to polyextreme conditions.

Marine thermoalkaline systems: Serpentinizing sites

We have focused primarily terrestrial thermoalkaline systems, and hot springs in particular, but thermoalkaline systems can

Table 1. Archaea from the TACK superphylum and selected members of the DPANN and Asgard with representative genomes generated from metagenomic assembly or single-cell assembled genomes. The pH max represents the environmental measures of the sites examined. All sites had measured temperatures >65°C. The observed high pH of newly described phyla, such as the Marsarchaeota and Brockarchaeota, could expand with additional sampling.

	Phylum	pH max	Location(s)	Putative metabolism	Study
TACK	Nezhaarchaeota	9.6	Gumingquan, Tengchong	Hydrogenotrophic methanogenesis	Hua et al. (2019)
	Verstraetearchaeota	9.6	Gumingquan, Tengchong	Methylotrophic methanogenesis	Hua et al. (2019)
	Marsarchaeota	3.4	Beowulf, Echinus, One Hundred, YNP	Chemoorganotrophy, Fe(III) reduction	Jay et al. (2018)
	Aigarchaeota	9.3	Gumingquan, Tengchong	Multiple (see Key taxa section)	Hedlund et al. (2015)
	Bathyarchaeota	5.5–7.5	Zavarzin Spring, Kamchatka	Methylotrophic methanogenesis ^a	Wilkins et al. (2019)
	Thaumarchaeota (Nitrosocaldus)	8.3	Heart Lake Geyser Basin, YNP	Ammonia oxidation	De la Terre et al. (2008)
	Geoarchaeota	7.2	Great Boiling Spring	Facultative nitrate reduction	Becraft et al. (2017)
	Geothermarchaeota	NR	Juan de Fuca Ridge		Jungbluth, Amend and Rappé (2017)
	Korarchaeota	9.3	Gumingquan, Tengchong	Anaerobic methane/dissimilatory sulfur metabolism ^b	Hedlund et al. (2015)
	Crenarchaeota (Sulfolobus)	8.2	Lobios, Spain		López-López et al. (2015)
DPANN	Brockarchaeota	7.3	Jinze, Tengchong	Methylotrophy	De Anda et al. (2020)
	Nanoarchaeota	8.0	Octopus Spring, YNP	Endosymbiont	Hedlund et al. (2015)
Asgard	Lokiarchaeota	8.6	YNP		Saw et al. (2015) ^{SAG}

Geoarchaeota was previously NAG1; Korarchaeota observed was 0.01% of recruited reads; Geothermoarchaeota has been observed to date only in Juan de Fuca (pH and temperature not reported).

^aHua et al. 2019.

^bMcKay et al. 2019.

also be found in marine environments, particularly in serpentinizing locations. Serpentinizing systems have a high abundance of electron donors, including H and CH₄, but are often limited in electron acceptors (Brazelton et al. 2006). Although limited microbial surveys have been performed in these extreme systems, the geochemistry of alkaline hydrothermal vents have been well studied as the potential site for the origin of life (Sojo et al. 2016), and have provided insights into the mechanistic underpinnings of critical geochemical cycles, both biotic and abiotic, including methanogenesis and iron cycling (Martin and Russell 2007; Camprubi et al. 2017; Martin 2020). Continental serpentine springs tend to be lower temperature (Brazelton et al. 2017) and microbial biomass in marine systems is often low, making comparisons with terrestrial hot springs difficult (Schrenk, Brazelton and Lang 2013). In addition, unlike many thermoalkaline hot springs, the DIC and ΣCO₂ in these systems tends to be low, particularly in deep marine systems (Miller et al. 2018), potentially limiting chemolithoautotrophs (Lang and Brazelton 2020). In the Lost City, targeted sequencing found taxa in the Crenarchaeota and Euryarchaeota, as well as members of the ANME-1 (Brazelton et al. 2010b), and communities are often dominated by a single phylotype of methanogenic archaea (Schrenk et al. 2004). The lower diversity is likely due to the more extreme environmental conditions of deep sea environments, with higher temperatures and pH, as well as the absence of light may represent a stronger environmental filter than seen in most thermoalkaline hot springs.

Future directions for understanding archaea in thermoalkaline systems

A large number of archaeal lineages have not been cultured to date (Baker et al. 2020). The metabolic pathways inferred

using genomics have provided critical insights into the niches occupied by archaea in thermoalkaline systems but culturing these organisms can provide additional knowledge that can be gleaned only through experimental manipulations. The vast volume of sequence data available can be used to inform new culturing strategies to isolate novel archaeal lineages from the environment (Hug et al. 2016; Johanna et al. 2018). Using information from genome reconstruction approaches to better design cultivation techniques are promising (Hedlund et al. 2014), and a number of these studies have been conducted in acidic (Wurch et al. 2016) and thermoalkaline (De la Torre et al. 2008) environments. For these approaches to become more broadly feasible, additional genomic studies in thermoalkaline environments must be undertaken to provide baseline information on taxonomy, metabolic pathways and likely community interactions (Johanna et al. 2018).

The use of more recently developed omics approaches, such as metatranscriptomics, metaproteomics and metametabolomics have only recently been applied in *in situ* thermoalkaline systems. Metagenomics provides a baseline for predicted function but understanding ‘realized’ function requires measures of transcription and post-translational products, including proteins and metabolites. Metatranscriptomic surveys allow for patterns of gene expression under varying environmental conditions to be quantified, as well as assisting in filling in gaps in metagenome assemblies (Brown et al. 2015). Transcriptomic studies have also been used to quantify the likely functional pathways and niches of isolated organisms. For example, Beam et al. (2016) generated a metatranscriptome for a novel ‘*Candidatus* Calditenus aerorheumensis’ (Phylum Aigarchaeota) and paired this data with extensive environmental measurements to better understand its metabolic adaptations. To our knowledge, metaproteomics have not been reported for

hot spring environments, but analyses of individual proteins have been undertaken (Schröder et al. 2014). These types of surveys, particularly when conducted across environmental gradients, or with experimental manipulations, enhance our understanding of the functional roles filled by taxa in the archaea.

CONCLUSIONS

Thermoalkaline sites represent extreme environments that are significantly understudied. Patterns found across the currently available literature reveal high pH habitats likely host high levels of uncharacterized archaeal diversity. Analyses of published studies indicate that temperature is the primary driver of archaeal abundance, diversity and composition, but there is also evidence that pH, particularly above circumneutral, plays a critical role in determining the taxonomic and functional makeup of microbial communities. Additional studies that focus on high pH systems, particularly those that sample across broad geographic ranges, with geochemical variability, as well as temperature, have the potential to improve our understanding of which environmental parameters drive patterns of archaeal community assembly and function in extreme environments. Combined omics approaches that employ multiple methods, including metagenomic and metatranscriptomics surveys, which give a broad picture of the likely function, coupled with metaproteomic and metabolomics analyses, which provides a measure of the realized function within a given site, would be particularly powerful. These combined approaches not only quantify the function of a given microbial community, but potentially provide a foundation for culturing of novel organisms. In addition, experimental manipulations could provide valuable insights into the cellular mechanisms that drive archaeal communities, which would not only test fundamental hypotheses in theoretical ecology but could also uncover novel metabolic pathways and identify enzymes with applications to biotechnology.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://academic.oup.com/femsec/) online.

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