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Earth's most needed uncultivated aquatic prokaryotes

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ABSTRACT

Aquatic ecosystems house a significant fraction of Earth's biosphere, yet most prokaryotes inhabiting these environments remain uncultivated. While recently developed genome-resolved metagenomics and single-cell genomics techniques have underscored the immense genetic breadth and metabolic potential residing in uncultivated *Bacteria* and *Archaea*, cultivation of these microorganisms is required to study their physiology via genetic systems, confirm predicted biochemical pathways, exploit biotechnological potential, and accurately appraise nutrient turnover. Over the past two decades, the limitations of culture-independent investigations highlighted the importance of cultivation in bridging this vast knowledge gap. Here, we collected more than 80 highly sought-after uncultivated lineages of aquatic *Bacteria* and *Archaea* with global ecological impact. In addition to fulfilling critical roles in global carbon, nitrogen, and sulfur cycling, many of these organisms are thought to partake in key symbiotic relationships. This review highlights the vital contributions of uncultured microbes in aquatic ecosystems, from lakes and groundwater to the surfaces and depths of the oceans and will guide current and future initiatives tasked with cultivating our planet's most elusive, yet highly consequential aquatic microflora.

1. Introduction

Aquatic ecosystems constitute more than 90 % of Earth's habitable space (UNEP, 2006), yet only a minor fraction of their biodiversity has been explored. Despite accounting for only 0.01 % of our planet's total water, freshwater ecosystems alone harbor 6 % of Earth's biomass (Dudgeon et al., 2006). The diversity assessments that lead to the aforementioned estimation are skewed heavily in favor of Eukarya, as most of the prokaryotic diversity (*Bacteria* and *Archaea*) in freshwater ecosystems is only now beginning to be catalogued. Recently, deep genomic sequencing of shallow aquifers revealed that these ecosystems harbor representatives of nearly all known bacterial phyla (Anantharaman et al., 2016). In marine ecosystems, approximately half of the global primary production is taking place (Field et al., 1998). A large portion of nutrients is dissolved and primarily available to Archaea and Bacteria as DOM (dissolved organic matter). This process greatly

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influences global biogeochemical cycles (Azam, 1998; Azam and Malfatti, 2007). Globally, aquatic ecosystems render countless prokaryotic-mediated essential services, from primary production and decomposition to biomineralization and biogeochemical cycling (Singh, 2015).

Nearly four decades ago, prior to the advent of advanced molecular techniques in microbial ecology, cell counts obtained via cultivation-based methods were consistently orders of magnitude lower than those observed via microscopy. This discrepancy came to be dubbed the great plate count anomaly (Staley and Konopka, 1985). From this point forward, the limitations of culture-dependent laboratory techniques in elucidating the breadth of microbial diversity from a given environment became increasingly clear. Today, it is well accepted that cultivation-based assays fail to adequately appraise the broad spectrum of microbial diversity present in most environmental samples. A number of technical and biological factors causes the inability to cultivate the majority of microorganisms in the laboratory. Physical and chemical conditions of natural habitats, including (but not limited to), *e.g.,* temperature, pH, pressure, salinity, gas-phase composition, or trace elements, have to be replicated fastidiously. Furthermore, essential growth factors and metabolites produced by other organisms are often absent in artificially prepared media. Obligate symbionts require specific partners for growth, while others may face competition from fast-growing species or growth-inhibition by toxic by-products in enrichment cultures. From a technical standpoint, aspects such as media preparation need to be carefully looked at, as processes such as autoclaving can unintentionally trigger inhibitory reactions (Tanaka et al., 2014). To overcome these challenges, strategies such as co-cultivation and single-cell sorting are employed. Co-cultivation allows partner organisms to supply necessary metabolites (Park et al., 2011), while single-cell sorting helps bypass competitive interactions. Genome-guided approaches are also widely used to predict growth requirements, high-throughput culturing enables simultaneous screening under diverse conditions (Connon and Giovannoni, 2002; Tillich et al., 2014).

As global biodiversity surveys estimate that only 0.1–1 % of all prokaryotes are capable of being cultivated under defined laboratory conditions (Amann et al., 1995; Colwell, 1997; Tyson and Banfield, 2005), many ecosystem processes, *e.g.*, anaerobic oxidation of methane (Boetius et al., 2000), are carried out by microorganisms that have not been isolated in pure culture. In lieu of techniques to study non-cultivable representatives, however, the cultivation of amenable organisms and subsequent physiological, enzymatic, and genetic interrogations have enabled the development of biotechnological applications for decades. Recent successes in enriching hitherto non-cultivable microorganisms have resurrected cultivation strategies in modern microbiology, highlighted by the cultivation of *Candidatus* Lokiarchaeia, member of a phylum bridging the gap between *Archaea* and Eukarya (Imachi et al., 2020)], *Ca.* Micrarchaeota [small archaeal symbionts (Sakai et al., 2022)], bacterial representatives of the Candidate Phyla Radiation [*i.e.*, phylum *Ca.* Patescibacteria; small bacterial symbionts (Cross et al., 2019)], and chemolithoautotrophic manganese oxidizing bacteria (Yu and Leadbetter, 2020). While decades ago, the phrase “cultivation independent” was used to advertise microbiome studies based on molecular assessment of biodiversity, microbiological sciences are now transitioning to an era in which meta-omics data does not only provide information on uncultivated key species but can also be leveraged to support cultivation-focused research.

Looking at the frequency of publications describing novel bacterial species (based on data from the LPSN database at DSMZ (Parte et al., 2020) (https://lpsn.dsmz.de/; accessed 11.01.2024; Fig. 1), there is almost an exponential increase in literature entries from 1990 until 2006, in part bolstered by the ability to delineate species based on their 16S rRNA gene sequence. The frequency remained fairly constant until 2012 and has been declining in the past three years. While the underlying reasons for the strong increase in 2008/9 and the recent decline of descriptions of novel microbial species in the recent years remains unknown, it is evident that there exists a current backward trend in isolating and validly describing novel microbial species. The prominent peak of newly published phyla in 2021 is due to the official recognition of the phylum rank by the ICNP (Oren and Garrity, 2021). Nevertheless, more systematic screenings and identifications of uncultivated key species are as much needed as funding agencies providing the respective resources to eventually bring those species in culture.

In the meantime, scientists defined specific target microbes that are

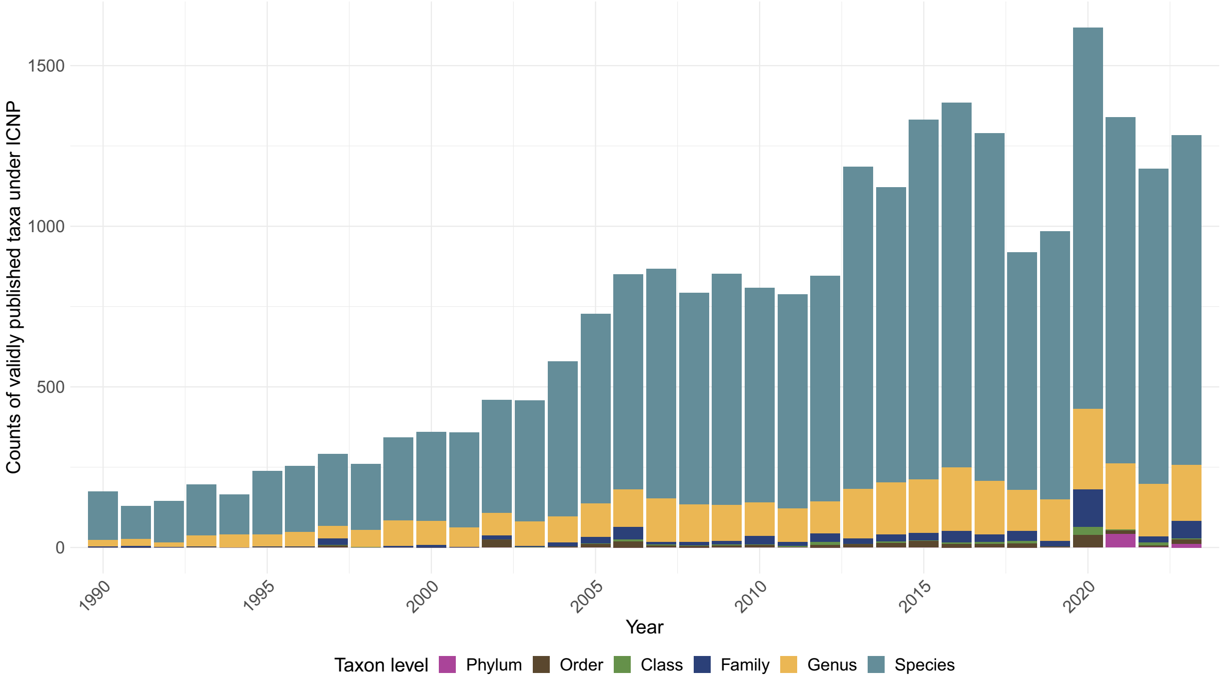

Fig. 1. Enumeration of taxonomically recognized bacterial and archaeal entities according to the International Code of Nomenclature for Prokaryotes (ICNP) from 1990 to 2023. For an individual graph per taxon level please see Supplementary Fig. S1.

essential for certain ecosystems. The “most wanted” uncultivated species included microbes from soil (Delgado-Baquerizo et al., 2018), wastewater treatment plants (Dueholm et al., 2022; Wu et al., 2019; Zhang et al., 2023), and also ‘spookmicrobes’, chemolithoautotroph microorganisms, involved in the methane and nitrogen biogeochemical cycles (in‘t Zandt et al. 2018), and a cross-ecosystem list of yet-to-be cultivated taxa (Lewis et al., 2021). Fodor et al. (2012) have made a similar list targeting the human microbiome, although with a specific focus on missing whole genome sequences, but also indicating the need of their cultivation. Lacking in the current body of literature is a guide to identifying non-cultivable aquatic microorganisms bearing the most ecosystemic relevance and/or high biotechnological potential. To this end, we present a catalog, sorted by aquatic ecosystems, of more than 80 hitherto uncultivated bacterial and archaeal aquatic taxa (Fig. 2) that are either intimately involved in biogeochemical cycling of carbon, nitrogen, sulfur, and/or phosphorus, globally distributed, relevant to biotechnological advancement, and/or germane to the study of evolution of life on Earth. Supplementary Table S1 gives an quick overview of all most-wanted taxa. This compendium across all major aquatic biomes on Earth (Fig. 3) may serve as a resource to guide researchers, funding agencies, and large-scale initiatives in their endeavors to cultivate novel prokaryotes from aquatic ecosystems.

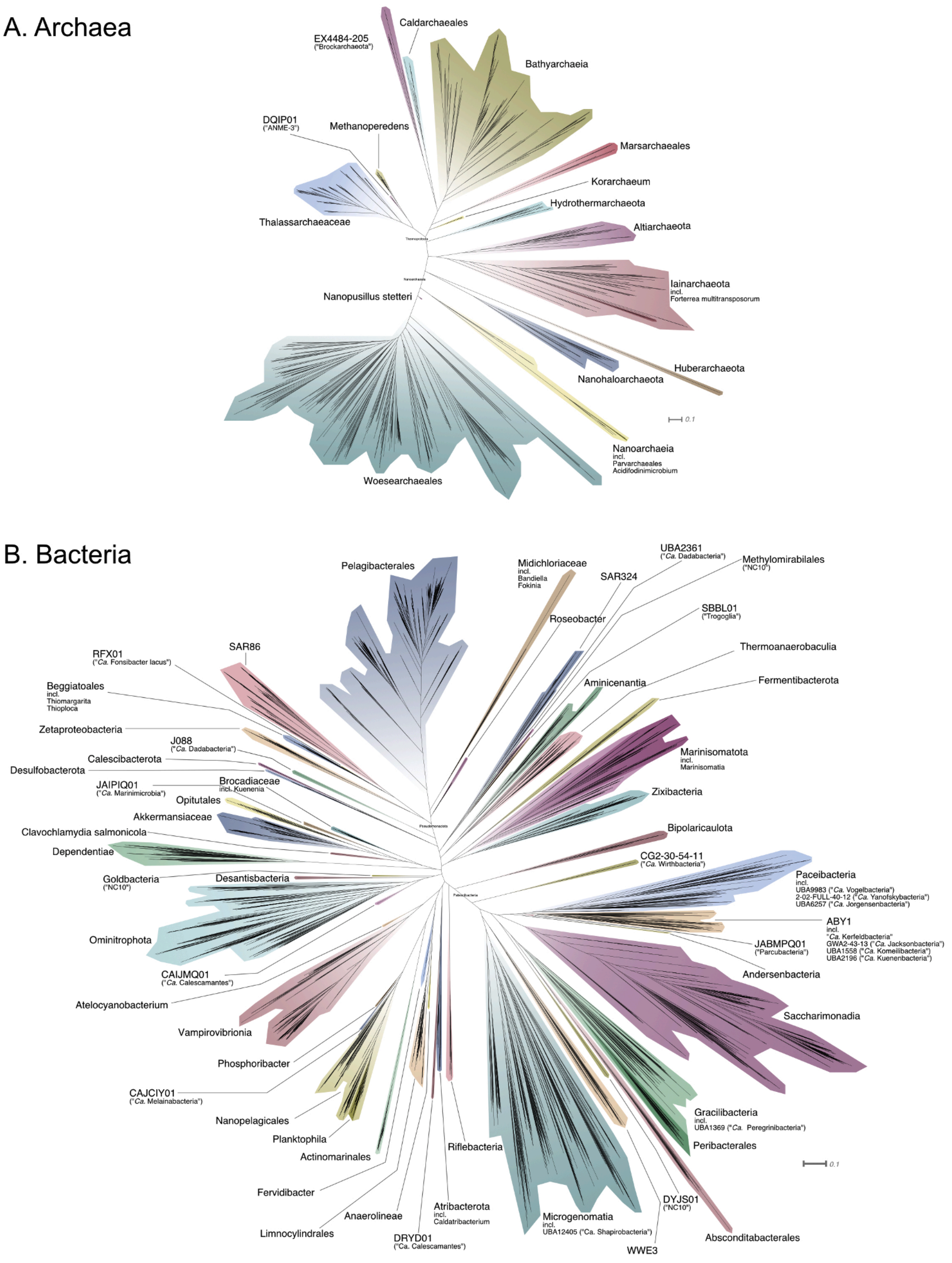

Fig. 2. Most wanted prokaryotes displayed as unrooted phylogenetic trees (A, Archaea; B, Bacteria) which are based on the GTDB bacterial reference tree showing the bacterial clades with missing cultivated representatives. Some of the "most wanted" taxa mentioned in the text do not appear in the phylogenetic trees, for example because they are only known by 16S rRNA gene sequences and thus do not appear in the GTDB reference trees. Other taxa occur in the trees as interesting targets for cultivation-based studies but are not discussed further in the manuscript. Colors delineate branches on the tree that we refer to as the most sought-after bacteria and archaea from aquatic systems. The bacterial and archaeal trees are available in Newick format in Supplementary Dataset 1 and 2. Methods are given in Supplementary Methods.

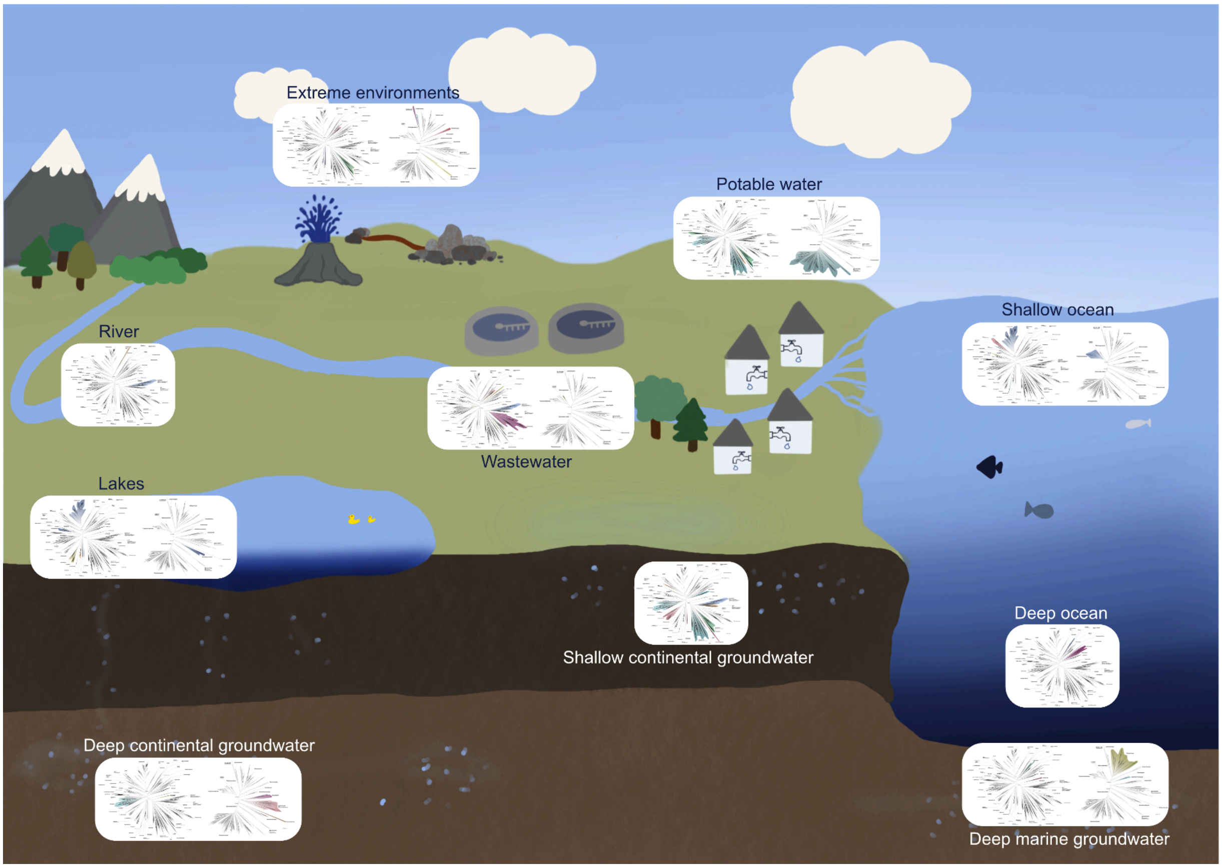

Fig. 3. Illustration of all aquatic ecosystems covered in this compendium with phylogenetic trees highlighting the ecosystem's key taxa eluding cultivation: surface waters such as lakes and rivers, continental (shallow and deep) and deep marine groundwaters, oceans divided into shallow and deep, man-made ecosystems such as potable water and wastewater and extreme aquatic ecosystems exemplified here by a geyser and acid mine drainage. The individual highlighted trees are available as Supplement Figs. S2–S18.

2. Main

2.1. *Shallow ocean – the sunlit area of marine microbial diversity*

The marine pelagic zone is one of the largest ecosystems on Earth, connected worldwide by currents that form a highly dynamic system resulting in an intensive transport of nutrients and microorganisms. The upper part of the water column is characterized by the availability of sunlight which can penetrate down to a depth of about 200 m and is named the euphotic zone. Here, photosynthetic prokaryotic microorganisms and eukaryotic algae convert sunlight into chemically bound energy. The oceanic primary production by photosynthetic organisms is almost equal to the primary production by plants on the land masses (Field et al., 1998). Photosynthetic organisms produce not only biomass but release a high quantity of various smaller organic compounds. Both, complex biopolymers and DOM represent the energy and carbon source of chemoorganoheterotrophic microorganisms which remineralize these nutrients in a process defined as the microbial loop (Azam et al., 1983).

Certain characteristics have been identified to be shared across highly abundant microorganisms that are well adapted to pelagic conditions. In addition to bearing streamlined genomes, indicative of adaptation to oligotrophic pelagic habitats, the vast majority of such taxa can proliferate at extremely low substrate concentrations (Giovannoni et al., 2005b; Martens-Habbena et al., 2009; Voget et al., 2015). For instance, certain planktonic chemoheterotrophic groups have in common that distinct ecotypes found in the euphotic zone contain genes for proteorhodopsin Liu et al. (2023b) suggesting an additional light-driven energy metabolism next to chemoorganotrophy, while such genes are missing in the genomes of ecotypes from the same clade found in greater depth without any sunlight; *e.g., Ca.* Pelagibacterales ("SAR11 clade"), *Flavobacteriales, Porticoccaceae* ("SAR92 clade") or *Ca.* Poseidoniales ("Marine Group II") (Fuhrman et al., 2008; Giovannoni et al., 2005a; Gómez-Consarnau et al., 2007; Stingl et al., 2007; Tully, 2019).

The introduction of novel medium compositions, mainly with reduced substrate concentration to previous studies, and new cultivation techniques resulted in the isolation of various highly abundant and ecological relevant microorganisms from the oceanic waters (Giovannoni and Stingl, 2007) like members of the ubiquitous *Ca.* Pelagibacterales (Rappé et al., 2002), *Thermoproteota* (previously "Thaumarchaeota") (Könneke et al., 2005), or the *Roseobacter* clade (Giebel et al., 2011). However, several sub-groups of these ubiquitous clades still miss cultured members (like *Ca.* Pelagibacterales "SAR11-IIIb" or uncultured clusters / clades "CHAB-I-5″, "NAC 11–7″, "SAG-O19" of the *Roseobacter* group). Another important group of oceanic picoplankton without cultured members are marine phylotypes that share high similarities with cultured terrestrial microorganism groups like the genus *Ca.* Fucivorax of the family *Puniceicoccaceae* within the *Verrucomicrobiota* (Orellana et al., 2022).

Among many potentially chemotrophic uncultured pelagic microorganism groups, the unicellular cyanobacterium UCYN-A (*Candidatus* Atelocyanobacterium thalassae (Zehr et al., 2001)) is an abundant phototrophic and widely distributed diazotrophic bacterium with extremely reduced metabolic capacities (Farnelid et al., 2016). This symbiotic nitrogen-fixing cyanobacterial group has been found as one of the most important nitrogen-fixers in oceanic water, consists of four distinct phylogenetic subgroups, and all members live in symbiotic association with eukaryotic hosts (Zehr et al., 2016). A very recent study even suggests that *Ca.* Atelocyanobacterium thalassae is integrated in marine algae as a N_2-fixing cell organelle called "nitroplast" (Coale et al., 2024).

Candidatus Actinomarinales was initially discovered in the chlorophyll maximum zone (110 m depth) of the Mediterranean Sea and subsequently identified in various oceanic waters around the globe (Ghai et al., 2013; Morris et al., 2005). Members of this group can be distinguished from terrestrial *Actinobacteria* by a very low GC content and are characterized by their small dimension and an estimated genome size of about 1 Mb. Based on assembled contigs from different metagenomic databases, members of *Candidatus* Actinomarinales are chemoorganoheterotrophs containing light-harvesting proteorhodopsin (López-Pérez et al., 2020).

Members of the bacterial clade SAR86 are ubiquitous and highly abundant in surface ocean waters. Although they were already discovered in the early 1990s by Britschgi and Giovannoni (1991), no representative has yet been cultivated and questions about their ecological role can at present only be discussed based on metagenomics. The SAR86 cluster shows a high phylogenetic diversity and metagenomic analysis suggest a chemoorganoheterotrophic physiology (Dupont et al., 2012).

The first sequences of the phylum *Desulfobacterota* (previously "*Ca.* Dadabacteria") were described by Hugenholtz et al. (2001) as SBR1093. Based on metagenomic data, the marine members of the *Desulfobacterota* can be divided into two ecological subgroups, one with the potential of photoheterotrophy that occurs in surface waters, and another group that lacks proteorhodopsin and is more abundant in the deep euphotic zone (Graham and Tully, 2021).

An abundant planktonic archaeal group in oceanic water lacking a cultured member is the archaeal Marine Group II (MG II). The MG II was originally discovered by DeLong (1992) and is ubiquitous in the marine water column (Massana et al., 2000). Phylogenetic studies based on metagenome-assembled genomes (MAG) proposed the order-level lineage *Candidatus* Poseidoniales which should be divided in the families *Candidatus* Poseidonaceae, *Candidatus* Thalassarchaeceae, and *Candidatus* Qianlongarchaeaceae (Liu et al., 2023a; Rinke et al., 2019). MAGs suggest that *Ca.* Poseidoniales grow chemoorganoheteterotrophically on dissolved or particulate organic matter and, in combination with time-series data, indicate the presence of distinct ecotypes in different water layers and specific season-dependent distribution patterns (Orellana et al., 2019).

2.2. *Deep ocean – a connected but extremely heterogeneous ecosystem*

The deep, aphotic zone (> 200 m depth) of the ocean is the largest connected habitat on Earth (Orcutt et al., 2011a). Covering more than 70 % of the Earth's surface with an average depth of 4000 m, oceanic waters encompass a volume of 1.3×10^{18} m^3 (Arístegui et al., 2009). While cell abundances decrease with depth to a mere 10^3 cells/ml (Nagata et al., 2000; Patching and Eardly, 1997; Reinthaler et al., 2006), the deep ocean habitat harbors upwards of 6.5×10^{28} cells, accounting for 75 % of the global oceans' total prokaryotic biomass (Arístegui et al., 2009; Whitman et al., 1998). While minor variations arise due to the productivity of the euphotic zone, depth, and distance from shore, deep ocean waters are typically characterized by permanent darkness, high pressure, low temperatures, and a paucity of organic nutrients (Arístegui et al., 2009; Orcutt et al., 2011b). Nearly all the energy supporting life in the dark realm of the deep ocean originates from organic carbon fixed via photosynthesis in the upper euphotic zone. Only a small fraction of this habitat's biomass is formed by chemolithoautotrophic *Bacteria* and *Archaea* that use reduced inorganic compounds as electron donors and energy sources (Orcutt et al., 2011a). Chemolithotrophic processes play a particularly important role in marine oxygen minimum zones (OMZ), which are characterized by vertical gradients of dissolved oxygen and redox potential (Bertagnolli and Stewart, 2018; Canfield et al., 2006). Additional redox gradients occur on micro-scale levels, *e.g.,* about the surfaces of sinking organic rich aggregates. Seafloor hydrothermal vent and seep systems provide reduced compounds, enabling chemosynthetic organisms to sustain life webs *sans* chemically bound energy stored in organic matter from photosynthesis (Lutz and Kennish, 1993).

The physico-chemical heterogeneity of the deep ocean water column is reflected by the complex diversity of free-living and/or particle-associated microorganisms. Sogin et al. (2006) reported more than a thousand distinct microbial species in a single liter of seawater. The largely unexplored "rare biosphere" likely accounts for a significant fraction of the observed phylogenetic diversity and provides an enormous reservoir of genetic variability (Sogin et al., 2006). More recently, advances in sequencing technologies and bioinformatic utilities have helped several environmental metagenomic initiatives to unravel the functional diversity and biotechnological potential of deep-ocean organisms. However, all of the uncultured deep ocean prokaryotic taxa discussed here were previously detected via comprehensive 16S rRNA gene sequencing surveys in the 1990s and have eluded cultivation for more than 30 years now.

One cosmopolitan group of deep ocean microorganisms lacking a cultured representative is the deep branching SAR324-cluster (Brown and Donachie, 2007; Pommier et al., 2005). A cluster of sequences belonging to members of the SAR324 clade was initially detected at 250 m depth in the Sargasso Sea (Wright et al., 1997). Subsequently, studies have reported the global distribution of these taxa ranging in ocean depth from the surface to 5000 m (Brown and Donachie, 2007; Nunoura et al., 2016). In deep waters these microbes account for up to 5 % of the total picoplankton (Schattenhofer et al., 2009) and associate strongly with particles (Li et al., 2023; Swan et al., 2011). Recently, Boeuf et al. (2021) described different ecotypes with distinct depth and temporal distribution profiles. This comprehensive pangenomic ecological study identified at least four distinct ecotypes, underscoring elevated divergences in genomic inventory and metabolic capacities that have led to the numerical dominance and ubiquity of the SAR324 clade. This conclusion corroborates the findings of other studies that describe the metabolic flexibility of this clade as ranging from a chemolithoautotrophic physiology to a purely heterotrophic metabolism (Georges et al., 2014; Sheik et al., 2014).

The pelagic bacterial lineage MGA/SAR406 was originally detected by Gordon and Giovannoni (1996) in the Atlantic and the Pacific Oceans via 16S rRNA gene sequence-based biodiversity surveys. Initial phylogenetic analyses showed that members of this clade were most closely related to species of *Chlorobium* and *Fibribacter*. As such, taxa in this lineage are now arranged in the phylum *Ca.* Marinisomatota (previously "*Ca.* Marinimicrobia") within the superphylum-level group "*Fibrobacterota-Bacteroidota*" (previously "*Fibrobacterota, Chlorobiota,* and *Bacteroidota*") (Rinke et al., 2013). *Ca.* Marinisomatota taxa occur ubiquitously throughout the marine water column, occurring in greatest abundance (up to 8 %) across oxygen minimum zones (Allers et al., 2013; Schattenhofer et al., 2009). As with many other (yet uncultured) pelagic bacterial groups, metagenomic and metatranscriptomic analyses revealed immense phylogenetic and functional diversity within this phylum, suggestive of niche separation of distinct subgroups (Bertagnolli et al., 2017). Metagenome-assembled genomes of *Ca.* Marinisomatota unveiled metabolic links to anaerobic metabolism and physiological links to nitrogen, sulfur, and carbon cycling in OMZ habitats (Bertagnolli et al., 2017). A first representative ("IA91") belonging to *Ca.* Marinisomatota has been recently successfully isolated, which overcomes energy limitations by relying on peptidoglycan intermediates produced from other bacteria (Katayama et al., 2024).

Another group of bacteria flourishing in OMZ environments, *e.g.*, the eastern tropical South Pacific (Stevens and Ulloa, 2008) and Monterey Bay onthe west coast of the US (Suzuki et al., 2004), are members of the Agg47 cluster within the *Gammaproteobacteria*. The acronym Agg47 was bestowed upon this group as its members prefer an aggregate-attached lifestyle (DeLong et al., 1993). Later, Eilers et al. (2000) discovered an Agg47-related clone belonging to the phylogenetic group NOR4 and Stevens and Ulloa (2008) proposed the ESOA cluster II, comprising members of the Agg47 clade. Subsequently, members of this clade were detected throughout the water column at the Japan Trench (Nunoura et al., 2016), as well as in cold deep waters of the North Pacific (DeLong et al., 2006). Agg47 taxa were one of the three most abundant groups in samples collected from the North Pacific Subtropical Gyre (Pham et al., 2008). Metagenomic analyses of Agg47 revealed genes for carbon fixation (ribulose-1,5-bisphosphate carboxylase-oxygenase) and energy generation via sulfur oxidation (Swan et al., 2011). These chemolithotrophs encode an APS reductase phylogenetically distinct from those involved in sulfate reduction and assimilatory APS pathways (Meyer and Kuever, 2007; Swan et al., 2011) which suggests an integral role of these microbes in the global sulfur cycle.

2.3. Lakes – global hotspots of uncultivated prokaryotes

Among the environments with most microbes on Earth, lakes (freshwater and saline) harbor approximately 2.3×10^{26} prokaryotic cells globally, 1.3×10^{26} of which are exclusive to bodies of freshwater (Whitman et al., 1998). Freshwater ecosystems including lakes claim the lowest fractions of cultivated microorganisms anywhere, with only 11 % of cells detected by metagenomics represented in axenic monocultures at genus level and below (Lloyd et al., 2018). Consequently, the majority of dominant and/or widely distributed prokaryotic species in lakes are also yet to be cultivated. Many of these organisms are devoid of cultured relatives of the same class, including members of phylum *Chloroflexota* ("Chloroflexi"), class *Actinobacteria* (phylum *Actinomycetota*), and order *Ca.* Pelagibacterales (also known as SAR11; phylum *Pseudomonadota*).

Within the phylum *Chloroflexota*, members of the order *Ca.* Limnocylindrales (class *Ca.* Limnocylindria, also known as the SL56 cluster) are commonly found about the epilimnion of stratified lakes and throughout the water column of holomictic lakes during mixing seasons (Mehrshad et al., 2018). *Ca.* Limnocylindrus can reach relatively high local abundances (up to 5 % relative abundance), while closely related *Ca.* Aquidulcis is less abundant but widely distributed in lakes globally (Mehrshad et al., 2018; Rodriguez-R et al., 2020). Members of the family *Ca.* Profundisolitareaceae (class *Anaerolineae*, CL500–11) dominate the hypolimnion of stratified lakes, as well as deep lakes during partial or complete mixing (Mehrshad et al., 2018). Also highly abundant in lakes around the world, particularly in freshwater bodies, are members of the order *Ca.* Nanopelagicales (acI lineage) in the class *Actinobacteria* (Allgaier and Grossart, 2006; Newton et al., 2011; Warnecke et al., 2005). This order envelops several lineages (considered families and/or tribes), that exhibit closely knit phylogenetically conserved associations with distinct ecological niches within and between lakes (Newton et al., 2007; Tran et al., 2021). Within this order, the family *Ca.* Nanopelagicaceae, encompassing small bacteria with extremely streamlined genomes, harbors significant diversity with most of its members occurring in elevated abundance in lake ecosystems (Neuenschwander et al., 2018). While previous efforts toward isolating representatives of these lineages have resulted in 16 axenic cultures from which high-quality genomes have been generated, monocultures had until recently survived no more than three propagations (Neuenschwander et al., 2018). More recently, mixed cultures guided by network analysis achieved stability in the maintenance of *Ca.* Nanopelagicaceae (*Ca.* Planktophila, also known as acI-A), but the order *Ca.* Nanopelagicales continued to elude stable axenic monoculture (Mondav et al., 2020). However, the discovery of widespread heme auxotrophy in this group and many other abundant aquatic groups have now resulted in two stable cultures from the genus *Ca.* Plantophila (Kim et al., 2021; 2019) and is likely to permit the future isolation of other members of the order.

Yet another group of small bacteria with condensed genomes dominating lake environments is *Ca.* Allofontibacter (LD12) of the order *Ca.* Pelagibacterales (SAR11; *Pseudomonadota*) (Henson et al., 2018; Salcher et al., 2011). While the genus *Ca.* Allofontibacter (originally named "Fonsibacter") does have a cultured representative [*i.e.*, *Ca.* Allofontibacter communis (originally named "Fonsibacter ubiquis", (Henson et al., 2018); orthographic corrections proposed by Oren et al. (2020)], the source of this isolate was a saltwater lagoon. *Ca.* Allofontibacter communis has been reported to be completely absent or present only at low abundances in freshwater lakes. However, lineages of *Ca.* Pelagibacterales, such as *Ca.* Allofontibacter lacus, are consistently observed at elevated abundance in lakes but remain to be cultured (Rodriguez-R et al., 2020; Tsementzi et al., 2019)

We posit that significant efforts toward the cultivation of representatives of these groups are warranted given their ecological importance, unique metabolic and physiological potential, and high abundance and cosmopolitan distribution in lakes globally. In addition, intriguing features exhibited by less abundant lake bacteria also warrant attention, as their isolation bears immense potential toward advancing the current understanding of microbial physiology and evolution in these ecosystems. One such group, the giant polyploid *Achromatiuym oxaliferum* (*Thiotrichaceae, Gammaproteobacteria*), was first described in 1893 (Schewiakoff, 1893). This group of bacteria continues to reveal surprising biological features despite eluding all attempts at cultivation. Cells of *Achromatium* spp. are 15 × 125 μm in size, which corresponds to interior volumes ten thousand times larger than that of most known bacteria. These bacteria accumulate crystalline calcite and sulfur globules internally, and have been reported to carry hundreds of distinct chromosomes (Babenzien, 1991; Ionescu et al., 2017; Salman et al., 2015).

Finally, we opine that culturing extremophilic prokaryotes that flourish in lakes bearing one or more extreme environmental characteristics significantly bolsters the likelihood of unveiling novel ecophysiological adaptations, evolutionary strategies, and biotechnological applications. The most salient examples include *Ca.* Nanohaloarchaeota and members of *Atribacterota* ("OP9"). The former, a group of extremely halophilic archaea of the DPANN superphylum which thrive in hypersaline habitats (Hedlund et al., 2014; La Cono et al., 2020; Rinke et al., 2019), includes *Ca.* Nanohaloarchaeum antarcticus, which has been successfully cultured in conjunction with *Halorubrum lacusprofundi* but not in pure culture (Hamm et al., 2019). The latter, including the sole isolated species *Atribacter laminatus* from pond sediments (Katayama et al., 2020), is a diverse lineage of thermophilic bacteria found in naturally occurring geothermal habitats, thermal bioreactors, high-temperature oil reservoirs, and wastewater treatment facilities (Dodsworth et al., 2013; Hedlund et al., 2014).

2.4. Streams – the blank spot in biodiversity surveys

Freshwater streams, here defined as a body of water that flows on Earth's surface,account for a small percentage of the total water on Earth, yet their importance cannot be overstated. Streams connect ecosystems, impact critically important biogeochemical cycles on a global scale (Battin et al., 2009; Fasching et al., 2020; Savio et al., 2015) and harbor a wealth of biodiversity. Recently, it has been shown that the role of streams regarding primary production and carbon respiration may even be underestimated in traditional metabolism models (Tromboni et al., 2022). Streams are characterized by a high heterogeneity concerning size and shape or surrounding landscape, not only comparing different streams, but also within the same system (Hassell et al., 2018). This has direct effects on the microbial community of the specific section. For example, while nitrogen metabolism has been shown to dominate wetland-dominated streams, agricultural streams were driven by monomer uptake and carbohydrate metabolism (Fasching et al., 2020). In addition, humans rely on streams for recreational activities, transportation of goods, and most importantly – a source of fresh drinking water. Despite all of this, very little is known about the microbial community composition of freshwater streams. To date, the ecological contributions of only a few uncultivated microbial lineages have been reported. Yet, unraveling so far uncultivated key players would be crucial having in mind that organisms surviving in this ecosystem must be adapted to ever-changing conditions, *e.g.*, temperature and sunlight on a daily basis or chemical pollution by humans.

In a study investigating the aquatic bacterial community structure of a riverine system via next generation sequencing techniques, Staley et al. (2013) found *Ca.* Paceibacteria (OD1; phylum *Ca.* Patescibacteria) to be among the most abundant taxa. The presence of these parasitic, symbiotic, and/or scavenging bacteria is likely to yield profound effects on other organisms, not to mention corresponding ecosystem functions (Nelson and Stegen, 2015). Very prominent were members of *Ca.* Pelagibacterales (SAR11), one of the most abundant aquatic bacterial orders that includes the *Ca.* Allofontibacter (belonging to the subclade SAR11-IIIb; LD12) found in freshwater systems (Haro-Moreno et al., 2020; Herlemann et al., 2014).

In addition to large taxonomic clades, highly relevant individual uncultivated species have also been documented for river ecosystems. The impact of individual uncultivated species is typically not considered until a stream system is impacted by anthropogenic pollution or distinct physical parameters, *e.g.*, extreme nutrient deficiency or low/high pH, draw attention. Also important is the untapped potential for advancing biotechnological applications, such as the removal of excess phosphorus by the polyphosphate-accumulating organism *Ca*. Accumulibacter. This organism has been detected in freshwater river systems at varying distances from wastewater treatment plants (WTP) (Peterson et al., 2008). Negative economic implications also prompt investigations into uncultivated stream prokaryotes. In Europe, the infectious presence of *Ca*. Clavichlamydia salmonicola (originally spelled *Ca.* Clavochlamydia) and *Ca*. Piscichlamydia salmonis in salmonids caused major damage to aquacultures (Karlsen et al., 2007; Schmidt-Posthaus et al., 2012; Zezekalo et al., 2021). As presented, only little is known about the uncultured microbial diversity of stream ecosystems. Although it is established that lotic ecosystem processes are sensitive to climate variations and anthropogenic processes (Jones et al., 2020; Picazo et al., 2020), research questions regarding impacts that are a result of climate change remain unanswered. To what extent and how do droughts or salinity and temperature increase impact microbial communities and their ecological functions in streams? Is the loss of biodiversity of uncultivated microbes a driver for pathogenic organisms in streams? Unraveling those research gaps could lead to the discovery of key and keystone species useful in biotechnological processes or human health research.

2.5. Shallow continental groundwater – the biggest limnic ecosystem

The terrestrial subsurface biome is complex and full of highly active microorganisms constituting upwards of 15 % of the planet's total biomass (Bar-On et al., 2018). Terrestrial groundwater accounts for approximately 95 % of Earth's total freshwater (Griebler et al., 2014) and is easily distinguishable from deep groundwater in that it is in direct contact with water bodies on the surface (Lovley and Chapelle, 1995). Microbial cell densities in pristine groundwater range from 10^4 to 10^5 organisms/mL (Farnleitner et al., 2005; McMahon and Parnell, 2014), while much greater densities can be found in contaminated aquifers. Yet, microbial populations are not evenly distributed throughout groundwater and the number of attached cells is commonly assumed to be 100 to 1000 times higher compared to unattached cells (Flemming and Wuertz, 2019; Griebler et al., 2002; McMahon and Parnell, 2014). Due to the absence of sunlight, photosynthetic primary production, and diurnal rhythms, subsurface habitats tend to be more stable than surface systems. Shallow aquifers, however, are significantly affected by regular environmental fluctuations throughout the year, especially varying influxes of water (Yan et al., 2021). The microbial community structure varies with the water chemistry of the respective aquifer, particularly with regard to oxygen content and redox potential. As shallow groundwaters are fueled by percolating precipitation and surface waters, oxygen and organic carbon contents tend to decrease with increasing depth, while levels of dissolved iron and manganese increase. Groundwater food webs rely primarily on the influx of organic carbon, as rates of light-independent chemoautotrophic primary production are presumably low. However, there is growing evidence that chemolithoautotrophic processes play a significant role in groundwater ecosystems, even in aquifers that are organically contaminated (Kellermann et al., 2012; Kirs et al., 2020). Except for a few capstone model study areas, the groundwater microbiome is so far grossly understudied, and many important groups of microorganisms lack cultured representatives.

In one groundwater monitoring transect in Germany, for instance, dominant phyla included *Bacteroidota, Pseudomonadota*, , *Planctomycetota, Thermoproteota* (previously *Ca.* Thaumarchaeota), *Omnitrophota, Bacillota* (previously "Firmicutes"), and *Ca.* Patescibacteria (Candidate Phyla Radiation, CPR) (Herrmann et al., 2019; Perez-Molphe-Montoya et al., 2022; Wegner et al., 2019; Yan et al., 2020). Microbial groups of interest without or with only few cultured representatives include *Ca.* Zixiibacteriota (originally spelled "Zixibacteria"), *Omnitrophota, Vampirovibprionia* (formerly *Ca.* Melainabacteria, phylum *Cyanobacteriota*), *Nitrospirota* and several taxa withinthe phylum *Ca.* Patescibacteria (*Ca.* Paceibacteria, *Ca.* Absconditabacterales, *Ca.* Gracilibacteria and *Ca.* Saccharimonadia).

Ca. Zixiibacteriota (RBG-1) exhibit a broad repertoire of metabolic pathways that may allow them to mitigate fluctuating redox conditions near the oxic/anoxic boundary. In particular, predicted enzymes arsenite oxidase and arsenate reductase, as well as enzymes for iron oxidation and reduction, suggest the involvement of these organisms in the biogeochemical cycling of arsenic and iron (Casar et al., 2021; Castelle et al., 2013). Members of phylum *Omnitrophota* (OP3, PVC supergroup), also putatively involved in arsenic cycling, as they possess genes for arsenic reductase, have been observed in great abundance in arsenic-reducing groundwater communities (Anantharaman et al., 2016; Wang et al., 2021). Overall, this group seems characterized by flexible metabolic traits (Perez-Molphe-Montoya et al., 2022). Potential abounds for biotechnological applications arising from microorganisms involved in arsenic cycling, including the bioremediation of contaminated (ground)water and purification of fresh potable waters.

Vampirovibrionia, a class of *Cyanobacteriota*, have been detected metagenomically in groundwater (Di Rienzi et al., 2013). These organisms are non-phototrophs and likely obligate fermenters, yet they possess genes for nitrogenase and nitrogen fixation. In addition to being cosmopolitan in aquatic habitats, members of this phylum have been found in the gut of humans and animals, where they might benefit their host (Di Rienzi et al., 2013). *Vampirovibrio chlorellavorus,* the first cultivated member of the *Vampirovibrionia,* has been obtained in co-culture with *Chlorella vulgaris*, yet the culture has been lost (Soo et al., 2015). As such, cultivation of these bacteria is warranted not only from an environmental and evolutionary point of view, but also with respect to human health and gut microbiome dynamics.

Iron and manganese oxidizing bacteria are of immense biotechnological interest. These microbes can be used to treat drinking water, but their activities must be monitored as they can rapidly cause detrimental microbially-mediated corrosion. Ecologically speaking, these organisms play important roles globally in the biogeochemical cycling of iron and manganese. In water treatment, they colonize biological filters used to remove iron(II) and manganese(II) from reduced groundwater to yield potable water. While the existence of these groups of bacteria has been known for decades, only a handful of representatives have been isolated. *Gallionellaceae*, autotrophic iron oxidizing *Betaproteobacteria*, are represented by only few isolates, although metagenomic studies suggest a higher diversity (Casar et al., 2021; Hoover et al., 2023; Varaljay et al., 2016). In a similar vein, the first described chemolithoautotrophic manganese oxidizing bacteria, *Ca.* Manganitrophus noduliformans (*phylum Nitrospirota*), couples manganese oxidation to autotrophic CO_2 fixation via the reductive TCA cycle (Yu and Leadbetter, 2020).

Most members of *Ca.* Patescibacteria are yet to be cultivated, having been detected mainly by metagenomic approaches as outlined above

(Brown et al., 2015; Wrighton et al., 2012). These bacteria are overly abundant in certain aquatic environments, *e.g.,* Lake Baikal and Lake Zurich (Cabello-Yeves et al., 2020), howevermost of the genomes assembled have originated in groundwater ecosystems (Chiriac et al., 2022). Several *Ca.* Patescibacteria are ultra-small bacteria bearing streamlined genomes (Luef et al., 2015), suggesting a symbiotic or parasitic lifestyle dependent upon on other organisms (He et al., 2021; Luef et al., 2015; Nelson and Stegen, 2015). Given their limited metabolic capacity, they likely sequester lipids, nucleic acids, and/or amino acids from their hosts or the environment (Chiriac et al., 2022; Kantor et al., 2013; Wrighton et al., 2012). *Ca.* Paceibacteria (previously "Parcubacteria (OD1)") are regularly detected in both oxic and anoxic groundwaters (Herrmann et al., 2019; Luef et al., 2015; Nelson and Stegen, 2015). In addition to possessing genes for sulfur reduction (Wrighton et al., 2012), these microbes are prevalent in (hemi)cellulose-degrading microcosms in groundwater (Danczak et al., 2017). The presence of *Ca.* Paceibacteria differentiates these microcosms from their soil borne counterparts, implicating a role for these microorganisms in subsurface carbon cycling (Herrmann et al., 2019). The genetic capacity of *Ca.* Paceibacteria predisposes them to a symbiotic lifestyle of slow and steady growth, unable to rapidly adapt to changing environmental conditions (Nelson and Stegen, 2015). Other putatively symbiotic CPR organisms include *Ca.* Vampirococcus (*Ca.* Absconditabacterales), which are thought to live in association with *Halochromatium* in salt lakes (Moreira et al., 2021), and *Ca.* Gracilibacteria, which are likely associated with *Campylobacterales* (Jaffe et al., 2021). Both phyla are readily abundant in certain terrestrial groundwaters (Jaffe et al., 2021) and are thus predicted to be dependent upon other organisms as symbionts (reviewed in Castelle et al. 2018). Recent successes in cultivating members of the CPR demonstrated the ability of these bacteria to kill their hosts in co-cultures. Such was the case in *Ca.* Saccharibacteria (Batinovic et al., 2021), a co-culture of *Ca.* Vampirococcus (Moreira et al., 2021) and another lytic CPR bacterium, both of which are members of the *Ca.* Absconditabacterales (Yakimov et al., 2022). While members of the *Ca.* Saccharimonadia exhibited a propensity to live alongside their hosts (Murugkar et al., 2020), all other cultivated organisms killed their hosts, irrespective of nutrient availability. These observations of behavior in six cultivated CPR bacterial lineages considers a mere fraction of the diversity enveloped in the *Ca.* Patescibacteria (Castelle et al., 2018),which accounts for one third of Earth's prokaryotic diversity (Castelle et al., 2018). The SILVA database currently houses 59,916 16S rRNA gene sequences of *Patescibacteria* (accessed Jun 1, 2024), which cluster to 1581 distinct operational taxonomic units at 97 % identity (usearch, default settings (Edgar, 2010)). While many of these sequences were retrieved from groundwater, none of the six isolates originated from this environment (He et al., 2021). This underscores the need to cultivate these and similar microorganisms from their deep subsurface groundwater habitat. Further patescibacterial taxa of interest to biotechnology include UBA12405 ("Ca. Shapirobacteria"), Moranbacterales ("*Ca.* Moranbacteria"), GWA2-43-13 ("Ca. Jacksonbacteria"), UBA2196 ("Ca. Kuenenbacteria"), and UBA6257 ("Ca. Jorgensenbacteria") (He et al., 2021).

Another group without isolates in pure culture is the phylum *Ca.* Rifleibacteriota. The phylum is named after a sampling site near the city of Rifle, Colorado, USA, where they were detected in shallow groundwater samples of four to six meters depth (Anantharaman et al., 2016). They also form part of deep groundwater communities in Japan and Russia (Hernsdorf et al., 2017; Kadnikov et al., 2018). Metagenomic reconstruction of their metabolisms indicates an anaerobic, heterotrophic, and possibly iron reducing lifestyle. In an aquifer of 2 km depth *Ca.* Rifleibacteriota accounted for up to 10 % of the community, even though their growth rates are presumably low, with only a third of cells replicating (Kadnikov et al., 2018). Both their metabolic potential and their nativity to both deep and shallow groundwaters make members of the genus an awarding subject for cultivation and isolation efforts.

2.6. *Deep continental groundwater – the hidden aquatic world below the critical zone*

The deep continental deep biosphere is home to roughly 3 × 10^{29} microbes, a small fraction of which (∼ 0.2 %) inhabit groundwaters (Flemming and Wuertz, 2019). Typically, more accessible than sediments, aquifers facilitate continuous sampling of deep ecosystems with strong contamination control. In most deep aquifers nutrients are scarce, and a recently established paradigm suggests means by which inhabitants endure these oligotrophic challenges (Mehrshad et al., 2021). According to the model, microbes remain metabolically dormant until a surge of nutrients arises, at which time they rapidly metabolize and then return to inactivity (Mehrshad et al., 2021). Metagenome-based predictions for maximum growth (Vieira-Silva and Rocha, 2010) and active genome replication (Brown et al., 2016) confirmed this model, as faster growth and slower replication coincided with increased depth (Bornemann et al., 2022). Mantle-borne bursts of CO_2, H_2, and other nutrients induce microbial activity, resulting in spiking cell densities (Bornemann et al., 2022). As primary production and recycling activities are vital to maintaining nutrient depleted deep groundwater ecosystems, procaryotes involved in these processes are major drivers of deep biosphere energy cycles.

Primary producers in the deep biosphere chemolithoautotrophically fix carbon dioxide primarily via Wood-Ljungdahl (WL) or reverse TCA (rTCA) pathways (Mehrshad et al., 2021; Momper et al., 2017; Probst et al., 2018; 2017; 2014b). Except for methanogens (Tyne et al., 2021), which are well studied, the scientific community lacks cultivated representatives of primary producers in the deep biosphere. One of the best studied examples are species of the uncultivated genus *Ca.* Altiarchaeum. Metagenomic and isotopic analyses, revealing alternate versions of the WL pathway and ^{13}C-depleted lipids, respectively, suggest that these microbes are autotrophic (Probst et al., 2014b). These organisms can account for upwards of 70 % of the relative abundance in deep biosphere communities (Bornemann et al., 2022; Hernsdorf et al., 2017; Probst et al., 2018; 2014b), implicating their integral role in organic carbon acquisition in the deep terrestrial subsurface. Predation of *Ca.* Altiarchaea (and likely other primary producers) by viruses is thought to release significant amounts of organic carbon, thereby stimulating recycling processes in these ecosystems (Rahlff et al., 2021).

Ca. Altiarchaeum hamiconexum cells bear hami, *i.e.*, surface appendages of barbwire-esque basal structure with nano-grappling hooks at their tips (Moissl et al., 2005), which appeal to biotechnological applications like natural hook and loop fasteners. Since its discovery in 2001 (Rudolph et al., 2001), all attempts at cultivating members of this genus have proven futile. The primary reason for this is that the undisturbed ecosystem in which *Ca.* Altiarchaeum spp. live remains poorly understood. Species of *Ca.* Altiarchaeum have been reported to dominate the microbial communities of geysers (Probst et al., 2017; 2014b), artesian wells intersecting multiple aquifers (Probst et al., 2014a), natural springs (including those in marsh environments (Probst and Moissl-Eichinger, 2015; Rudolph et al., 2004)), and standalone aquifers (Bornemann et al., 2022). With respect to the originating source(s) of colonization and proliferation, it is extremely difficult to pinpoint specific aquifers and/or stimulating natural conditions. While pressure and pH cannot be determined in the natural ecosystem, these factors certainly impact cultivation. "Alti clade 2″, a sister clade of *Ca.* Altiarchaeum and based on genomic inferences incapable of fixing carbon, has been detected in marine sediments (Bird et al., 2016). *Ca.* Altiarchaeia (*i.e.,* "Alti clade 1″) and "Alti clade 2″ form the phylum *Ca.* Altiarchaeota (Probst et al., 2018), which is one of the deepest branching Archaeal lineage known (Spang et al., 2017), consisting solely of uncultivated representatives.

Metagenome analyses of deep continental subsurface waters have revealed the presence of uncultivated putatively carbon-fixing bacteria belonging to phyla *Ca.* Desantisiibacteriota and *Omnitrophota*, among others (Probst et al., 2017). Members of these two phyla can reduce sulfur and fix carbon via the WL pathway (Anantharaman et al., 2018), suggesting their integral roles in cycling sulfur in these ecosystems. The rTCA cycle was also identified in *Candidatus* Wirthibacterota, a deep branching clade comprising only a few genomes (Hug et al., 2016). This phylum is a phylogenetic sister to the monophyletic clade of the *Ca.* Patescibacteria (Hug et al., 2016) and it remains unclear if this phylum-level lineage belongs to the CPR branch. While *Ca.* Wirthibacterota are predicted to grow autotrophically (Probst et al., 2017), most members of the CPR are likely symbionts (for details see section *shallow continental groundwater* (Castelle et al., 2018)).

In addition to CPR bacteria, archaea of the DPANN clade have been predicted to behave as symbionts in deep groundwater. The genomes of DPANN archaea are small, rendering their metabolic potential extremely limited (Rinke et al., 2013). While their diversity in the deep subsurface is but a fraction of that observed in shallow groundwaters (see above), several specific lineages lacking cultivated representatives have been identified. A deep-branching member of the *Ca.* Iainarchaeota, a DPANN clade thought to have acquired free-living capabilities via horizontal gene transfer from bacteria, was identified in a deep groundwater aquifer (accessed through a CO_2-driven geyser). This organism, *Ca.* Forterrea multitransposorum, lacks the genetic and metabolic capacity to survive independent of hosts (Probst and Banfield, 2018). Another uncultivated group of archaea residing in the deep biosphere, the genus *Ca.* Huberarchaeum, forms a separate phylum within the DPANN supergroup (*Ca.* Huberarchaeota, (Probst et al., 2018)). Species of *Ca.* Huberarchaeum are posited to be symbionts of *Ca.* Altiarchaeum spp., surviving at the behest of the predominant primary producers in these systems. Like *Ca.* Altiarchaeum spp., species of *Ca.* Huberarchaeum are thought to function as carbon sinks, processing altered carbon forms directly from primary sources of carbon acquisition/assimilation in the system (Esser et al., 2023; Schwank et al., 2019). While deep subsurface aquifer environments remain poorly understood, especially when compared to aquatic systems in the critical zone, most organisms inhabiting this niche bear immense potential to biotechnological advancement, from novel CRISPR-Cas gene editing motifs to biological nano-hooks – and beyond. Cultivating these and other key species from the deep biosphere is of critical importance to understanding the enormous extent of carbon and nutrient turnover in these ecosystems, as well as leveraging their innate biotechnological potential.

2.7. *Deep marine groundwater – aquifers below the ocean*

Located beneath the seafloor and sealed by overlaying sediments of the deep ocean, sub-ocean groundwaters constitute “the crustal aquifer” within the upper volcanic basement. The volume of this sub-seafloor aquifer is comparable to the global riverine influx into the oceans (Fisher, 2005). The lateral flux of seawater through the upper oceanic crust is directed from outcrop to outcrop, where seamounts facilitate recharging and discharging sites (Hutnak et al., 2008). These geological features are typically found about the ridges and flanks of ocean spreading centers.

Microorganisms inhabiting ridge-flank systems are fueled by seawater-rock interactions, *e.g.,* abiotic release of hydrogen following radiolysis of water (Sauvage et al., 2021) and/or other reduced compounds originating from igneous basaltic rocks (Bach and Edwards, 2003; Orcutt et al., 2011b). Recharging seawater parcels support microbial respiration by supplying sulfate, nitrate, and/or even oxygen to the crustal aquifer. DOM is utilized as an electron donor continuously along the flow path of the aquifer, leading to decreased bioavailability near discharge sites (Shah Walter et al., 2018). Partially reduced deep marine groundwater also diffuses into overlaying sediments and stimulates microbial respiration in locations depleted of electron acceptors from the seafloor (Engelen et al., 2008; Fichtel et al., 2012; Mewes et al., 2016; Zinke et al., 2018).

Microbial community composition and metabolic transformation processes were examined in two crustal aquifers of different hydrological regimes. Both systems were accessed via borehole observatories (CORKs) installed in the frame of the integrated ocean discovery program (IODP). The first fully operational circulation obviation retrofit kit (CORK) is located along the eastern flank of the Juan de Fuca Ridge (JdFR, North Pacific), within an anoxic aquifer at a basement temperature of $\sim 64\ ^\circ C$. These conditions are not representative of most crustal aquifers along ridge flanks, in which oxygenated seawater flows at much lower temperatures. Given this discrepancy, the North Pond aquifer located in the North Atlantic was chosen to represent global sub-seafloor hydrogeological settings and be analyzed microbiologically (Edwards et al., 2014). Orcutt et al. (2013) reported that decreasing oxygen concentrations at the sediment-basement interface along the flow path of the aquifer resulted from microbial respiration. This microbial process was confirmed by direct measurements of oxygen and DOM depletion, as well as the detection of active ammonium oxidation in the observatory’s boreholes toward the discharge area of the crustal aquifer (Meyer et al., 2016; Shah Walter et al., 2018) Microbial communities inhabiting the North Pond crustal aquifer, including rock-associated consortia (roughly 10^4 cells/g rock), were dominated by groups of *Pseudomonadota* (Baquiran et al., 2016) that were distinct from those detected in overlaying sediments (Jørgensen and Zhao, 2016). Metagenomic studies indicated the metabolic potential for carbon, nitrogen, sulfur, and iron cycling (Tully et al., 2018).

Recently, a metagenomic analyses of samples collected from Juan de Fuca Ridge (JdFR) observatories yielded 98 distinct metagenome-assembled genomes (MAGs), nearly all of which were very distantly related to cultivated representatives (Jungbluth et al., 2017). The 53 bacterial MAGs included *Chloroflexota, Nitrospirota, Bipolaricaulota,* Acetothermia (OP1), EM3, *Ca.* Aminicenantia (“OP8”), *Gammaproteobacteria* and *Desulfobacterota*. The genomes of uncultured *Chloroflexota, Nitrospirota*, and *Bipolaricaulota* were also detected in alkaline thermal springs of the Baikal rift zone, suggesting a thermophilic lifestyle for these lineages (Korzhenkov et al., 2018). Carbonate and bicarbonate are likely major carbon sources for *Bipolaricaulota* and other primary producers in this system. Members of the uncultured EM3 group are also thought to be moderately thermophilic and rely on autotrophs to provide reduced organic carbon for anaerobic respiration (Colman et al., 2016). Uncultured members of thermophilic *Ca.* Aminicenantia are thought to adhere to fermentative saccharolytic lifestyles. A MAG of *Ca.* Aminicenantia generated from a 2 km-deep subsurface thermal aquifer in Western Siberia (Kadnikov et al., 2019) harbored the genetic potential to facilitate the fermentation of carbohydrates and proteins, as well as nitrite reduction.

The 45 archaeal MAGs generated from JdFR observatories included *Archaeoglobi, Bathyarchaeia* (MCG), and *Ca.* Hydrothermarchaeota (Marine Benthic Group E Archaea (MBG-E)) (Jungbluth et al., 2017). A novel thermophilic, facultatively lithoautotrophic sulfate reducing *Archaeoglobus* (*A. sulfaticallidus*) was previously isolated from a JdFR CORK (Steinsbu et al., 2010). *Bathyarchaeia* is a wide-spread and heterogeneous group of benthic *Archaea* (Yu et al., 2017) thought to metabolize distinct carbon substrates, such as detrital proteins (Lazar et al., 2016). *Bathyarchaeia* are likely organo-heterotrophic and autotrophic acetogens, capable of dissimilatory nitrite reduction. MAGs obtained from a hydrothermal vent system representing *Ca.* Hydrothermarchaeota suggested a versatile carbon metabolism, including CO_2-fixation and the oxidation of C1- and methyl compounds. In addition, these archaea house the genetic potential to facilitate denitrification, sulfate reduction, and the oxidation of sulfur, arsenic, and hydrogen (Zhou et al., 2020).

From a technological perspective, deep crustal aquifers became interesting for geological carbon sequestration due to the huge size of this reservoir and the potential for permanent geochemical CO_2 trapping (Goldberg and Slagle, 2009). In this process, CO_2 reacts with the basalt and forms carbonates under *in situ* pressures and temperatures, mimicking the naturally occurring weathering of basaltic rocks

(Christensen and Salisbury, 1972). Due to the fast clogging of the aquifer pore space by carbonate formation, Wikin and DiGiulio stated that a risk assessment of CO_2 sequestration might include aquifer geology, mineralogy, and groundwater chemistry (Liu et al. 2018). However, the microbial component is not considered even though this habitat harbors a substantial amount of not-yet cultured microorganisms with an undiscovered role for the environment and putative biotechnological potential.

2.8. Potable water – the unknown ecosystem everyone consumes

While drinking water and wastewater both represent artificial aquatic ecosystems, they exhibit remarkably distinct environmental conditions. Drinking water is derived from various sources, such as groundwater or surface waters (*i.e.,* lakes, reservoirs, and rivers). A variety of treatment regimens are required to render hygienic and safe drinking water. While groundwater typically requires little to no treatment (*e.g.*, biofiltration for the removal of reduced iron and/or manganese), surface waters usually require complex treatment, including disinfection for the removal of chemical and fecal microbiological contaminants. Despite efforts toward utter and complete purification, the presence of microbes in drinking waters has been proven and acknowledged (Liu et al., 2018). A healthy microbial population plays a central role in improving and maintaining drinking water quality during treatment, storage, and distribution, as natural biofilms preclude the colonization and proliferation of pathogens (Prest et al., 2016). Besides, the potable water microbiome has been shown to naturally influence the human gut microbiome via horizontal admission (Lugli et al., 2022). Yet, many of these microorganisms so far withstand cultivation efforts.

Taxonomically diverse communities of bacteria, archaea, viruses, protozoa, and fungi routinely inhabit potable water parcels and pipe biofilms (Douterelo et al., 2014). Predominant phyla of the drinking water microbiome include *Pseudomonadota, Actinomycetota*, and *Bacteroidota* (Bae et al., 2019; Brumfield et al., 2020; Henne et al., 2012; Ling et al., 2018). Much like groundwaters, drinking water abounds in bacterial cells small in size and limited in genetic potential, many of which are *Ca.* Patescibacteria (Proctor et al., 2018). Of these, *Ca.* Paceibacteria ("Parcubacteria"), *Ca.* Gracilibacteria and *Ca.* Microgenomatia are commonly detected. Furthermore, the versatile and metabolic flexible phylum *Omnitrophota* (OP3) are uncultivated organisms commonly detected in drinking water and groundwater samples possibly involved in nitrogen, sulfur, and carbon cycling (Perez-Molphe-Montoya et al., 2022; Seymour et al., 2023). *Vampirovibrionia*, a lineage of uncultivated bacteria in the phylum *Cyanobacteriota* (Ling et al., 2018), have also been shown to inhabit drinking waters. Only very recently, a genome was announced from a laboratory co-culture with a diatom (Abresch et al., 2024). Numbers of these bacteria decline in pipes where *Pseudomonadota* become more abundant (Ling et al., 2018). To date, only two species of the phylum *Ca.* Babelota (previously "Dependentiae" or "candidate division TM6") have been isolated, both from free-living amoebae. These metabolically limited taxa infect protozoans and derive energy and metabolites from these protistan hosts (Deeg et al., 2019). Representatives of *Ca.* Babelota have been detected in biofilms within potable water distribution systems (Henne et al., 2012), and they have been posited to play an active role in biofilm development (McLean et al., 2013). Initially detected in wastewaters, uncultivated patescibacterial class WWE3 have now also been documented in drinking water (Guermazi et al., 2008; Kantor et al., 2013; Luef et al., 2015).

Due to their lower abundance, much less attention has been paid to *Archaea* in drinking waters and their role therein. Ammonium-oxidizing archaea, for instance, play a major role in drinking water treatment (Kasuga et al., 2010; van der Wielen et al., 2009). While *Ca.* Woesearchaeales are found in a wide variety of aquatic ecosystems, including non-disinfected drinking waters, members of *Nitrosarchaeum* are more abundant in disinfected drinking waters (Inkinen et al., 2021).

Overall, the natural microbial community presumably plays a central role for hygienic drinking water quality by minimizing the nutrient availability and thus preventing the establishment of potentially pathogenic bacteria. Thus, isolates are required to study their metabolic features, interactions, and ecosystem functions.

2.9. Wastewater – a man-made ecosystem

Sewer systems transporting wastewater provide unique habitats with a distinct and coherent microbial community not resembling other ecosystems (Roguet et al., 2022). Yet, their metabolic potential remains unexplored, also due to a lack of cultivated species. Perhaps the most important step in wastewater processing is the biological treatment with activated sludge. A global study of microbial communities inhabiting activated sludge revealed a highly diverse population of organisms most closely resembling that of freshwater communities (Wu et al., 2019). The study estimated 10^9 different bacterial species in the activated sludge samples assayed, 99.999 % of which lacked cultured representatives. This underscores the enormous gap in knowledge that exists for even a well-controlled artificial system (Wu et al., 2019). Subsequent analyses assigned a high percentage of sequences to new genera, new orders, new classes and new phyla of the *Ca.* Patescibacteria, *Pseudomonadota, Bacillota, Planctomycetota, Verrucomicrobiota, Bacteroidota, Bdellovibrionota*, and *Chloroflexota* (Dueholm et al., 2022). Organisms bearing novel biotechnological potential included polyphosphate accumulating bacteria, such as *Ca.* Accumulibacter (*Pseudomonadota*) and *Ca.* Phosphoribacter (formerly *Tetrasphaera, Actinomycetota*) (Albertsen et al., 2012; Petriglieri et al., 2022; Singleton et al., 2022). While their metabolic capabilities have been extensively studied, pure cultures of anammox bacteria (*Ca.* Brocadiaceae, *Planctomycetota*) are still lacking. Members of *Ca.* Kuenenia stuttgartensis and *Ca.* Brocadia anammoxidans are also dominant species in wastewater treatment environments, where they play an important part in the removal of nitrogen (Jetten et al., 2005)

Microbes catalyzing N-damo processes, *e.g.*, the nitrite-dependent oxidation of methane catalyzed by *Methylomirabilota* ("NC10" phylum) and the nitrate-dependent oxidation of methane catalyzed by archaea belonging to the family *Ca.* Methanoperedenaceae (previously "ANME-2d" lineage), are of particular interest to cultivation endeavors. *Ca.* Methanoperedens manganicus and *Ca.* Methanoperedens manganireducens, which couple anaerobic oxidation of methane to the reduction on Mn(IV) oxides, were already successfully enriched in a freshwater sediment reactor (Leu et al., 2020). Cocultures of N-damo and anammox microorganisms have proven beneficial for sustainable wastewater treatment (van Kessel et al., 2018; Xu et al., 2021). Comammox bacteria, such as *Nitrospira* spp., can completely oxidize ammonium. These taxa have been shown to serve important roles not only in activated sludge and wastewaters, but also in drinking water filtration processes and other aquatic habitats (Daims et al., 2015; Saunders et al., 2016; van Kessel et al., 2015; Xu et al., 2021).

Also of particular interest are members of the class *Ca.* Saccharimonadia (previously "TM7"). While these ultra-small organisms have been shown to be abundant in sewage (Meng et al., 2019), a lone representative has been cultured (from the human oral cavity no less). These morphologically and phylogenetically diverse microbes have been shown to metabolize glucose and other organic compounds in aerobic, nitrate-reducing, and anaerobic conditions, as well as partake in nitrification in acidic environments (Kindaichi et al., 2016). The uncultivated patescibacterial class *Ca.* Yanofskybacteria ("2-02-FULL-40-12″), present in wastewater sludge samples grows attached to *Methanothrix* (methanogenic *Archaea*), presumably in a symbiotic or parasitic relationship (Kuroda et al., 2022).

Thermoanaerobaculia, members of the *Acidobacteriota*, are represented by the lone thermophilic species *Thermoanaerobaculum aquaticum,* which was isolated from a hot spring (Losey et al., 2013; Ruhl et al., 2022). As these microbes have been detected via metagenomics in wastewater treatment plants, it would likely prove worthwhile to interrogate their biotechnological potential using culture-based methods. Members of the phylum *Ca.* Fermentibacterota are also routinely detected in anaerobic sludge, where genomic studies suggest they readily ferment sugars (Kirkegaard et al., 2016). In general, a better understanding of wastewater communities and identification of their metabolic capacities could help future engineering efforts to improve processes by enhancing desired microorganism.

2.10. Extreme aquatic environments – selection for biotechnology's best

Extreme aquatic environments and microorganisms inhabiting them are widely distributed around the globe (Rothschild and Mancinelli, 2001; Shu and Huang, 2022). Extreme environmental factors include temperature, pH value, high salt concentrations, high heavy-metal concentrations, pressure, redox potential, and combinations thereof rendering poly-extreme aquatic ecosystems. Examples of extreme aquatic ecosystem are hot springs, hydrothermal vents, soda lakes, acid mine drainages or hypersaline reservoirs. Enzymes, pigments, and compatible solutes from extremophiles are extremely valuable for biotechnology applications. Prime examples of biotechnologically relevant enzymes from extremophiles are polymerases such as *Taq* and *Pfu*, which paved the way for high-throughput polymerase chain reaction due to their thermostability. The *Taq* polymerase was first retrieved from the organism *Thermus aquaticus* isolated from hot springs in Yellowstone National Park (Brock and Freeze, 1969; Saiki et al., 1988). The *Pfu* polymerase with an additional proofreading activity was isolated from the hyperthermophile archaeal species *Pyrococcus furiosus* (Fiala and Stetter, 1986; Lundberg et al., 1991). Modern cultivation-independent techniques heavily rely on these two enzymes and so does the exploration of uncultivated microorganisms. In addition, extremophilic prokaryotes are used for the production of biofuels (*e.g.,* butanol and ethanol), organic acids, ectoine or for bioremediation, also with the help of genetic engineering. Biotechnological applications of extremophiles have been reviewed elsewhere in detail (*c.f.* (Straub et al., 2018; Van Den Burg, 2003; Ye et al., 2023)).

Currently, sequencing-based studies offer further insight into the genomic diversity and biotechnological potential of aquatic extremophiles that lack cultivated representatives. This includes putative acidophiles like the *Ca.* Parvarchaeales (previously *Ca.* Parvarchaeota, now an order of the phylum *Nanobdellota* – previously *Ca.* Nanoarchaeota) which were originally found in acid mine drainages (pH < 1) (Baker et al., 2010). Despite their small genomes (0.64–0.96 Mb) they encode resistance genes for responding to different stressors and might contribute to carbon, nitrogen, and iron cycling (Chen et al., 2018). Information retrieved from MAGs resulted in the proposal of a novel genus within the *Ca.* Parvarchaeales, *Ca.* Acidifodinimicrobium, which encode for arsenate, mercuric, and chromate reductases enabling reducing metalloids to reduced forms that are less toxic (Luo et al., 2020). Another interesting type of extremophiles, as described above, are thermophilic microorganisms. The phylum *Thermoproteota* includes organisms formerly classified in the "TACK" superphylum (originally standing for "Thaumarchaeota", "Aigarchaeota", "Crenarchaeota" and "Korarchaeota"). *Ca.* Caldarchaeales (previously "Aigarchaeota" or *Ca.* Augarchaeota) are globally distributed in hydrothermal ecosystems. *Ca.* Caldarchaeales genomes have so far been recovered metagenomically in hot springs with temperatures ranging from 70 to 89 °C and a pH varying from acidic (<6) to alkaline (>8) (Beam et al., 2016; Hedlund et al., 2015; Hua et al., 2018; Nunoura et al., 2011), while a minority of 16S rRNA gene sequence hits was also found in moderate and cold-temperature samples (Hedlund et al., 2015). Inter-domain horizontal gene transfer was prevalent in *Ca.* Caldarchaeales MAGs, resulting in detailed evolutionary analyses of these organisms (Hua et al., 2018). Since auxotrophy is known for members of *Ca.* Caldarchaeales (Beam et al., 2016), cultivation without one or more of their community members might be challenging. Recent enrichment efforts revealed Wolfram to be essential for producing a stable cultivation of Caldarchaeles (Buessecker et al. 2022), yet their isolation in pure culture was not successful so far. Hydrothermal vents harbor a high diversity of thermophilic microorganisms, from which many representatives have been already isolated [c.f., (Erauso et al., 1993; Huber et al., 1995; Marteinsson et al., 1999; Takai et al., 2000)]. Recently, previously undescribed high relative abundances of Patescibacteria have been metagenomically been detected in the vicinity of hydrothermal vents (Zhou et al., 2022). Despite their limited metabolic potential, stable enrichments or co-cultures might help to explain their prevalence.

Studying life in extreme environments on Earth does not only bear new insights into the evolution of life and provide novel enzymes and enzymatic pathways for biotechnology, it also defines potential extraterrestrial ecosystems, *e.g.,* in our solarsystem and beyond. Continuous expansion on the limits of life (*e.g.,* 122 °C of *Methanopyrus kandleri*) consequently also continuously widens our horizon when it comes to alien life – may it exist – and is the basis for the discipline of astrobiology. Thus, isolation of novel extremophiles from (poly)extreme ecosystems is a necessary driver for this field of research.

2.11. An engineering perspective on limitations and advances of cultivation strategies

Since the first awareness of the "great plate count anomaly" (Staley and Konopka, 1985), cultivation strategies have become more sophisticated and targeted in recent years. Techniques like reverse genomics or microfluidic cultivation are particular noteworthy. Applying reverse genomics was successfully applied to isolate and cultivate a novel *Ca.* Saccharimonadia, one of the few cultivated *Ca.* Patescibacteria (Cross et al., 2019). Using microfluidics, the first representative of the *Ruminococcaceae* family, a most wanted member of the human microbiome, was cultured (Ma et al., 2014). A detailed overview of the microbiological perspective on innovative cultivation methods has been already compiled by Lewis and colleagues (Lewis et al., 2021). In addition to the biological perspective on cultivation, it is equally important to review the engineering aspects on microbial cultivation. In this context, the successful cultivation of novel aquatic microorganisms suffers two significant shortcomings. First and foremost, aquatic ecosystems tend to host highly adapted specialists oftentimes participating in complex mutualistic relationships and/or phases of transitory dormancy – conditions that cannot be imitated in a laboratory setting. In addition, while most aquatic ecosystems are dominated by oligotrophic microorganisms, many of the recently developed enrichment and isolation paradigms are not transferrable to high-volume, low-cell-density cultivation reactors. Most naturally occurring microorganisms are not adapted to metabolize the complex and often highly concentrated carbon sources provided in frequently used complex culture media, preferring instead minimal or fastidious media (Goh et al., 2019). Furthermore, failing to mimic the natural environment adequately can lead to altered growth, behavior, and/or metabolism (Connon and Giovannoni, 2002). As such, preservation, enrichment, and isolation must be adapted and applied in a cradle-to-grave manner germane to the ecosystem of interest.

We assert that flawed experimental design renders most attempts at cultivating novel aquatic microflora doomed from the outset. It has become overly apparent that disruptive engineering approaches need to be avoided to improve the likelihood of cells surviving beyond enrichment thresholds. Here, we address prerequisites that promote initial cultivation success and highlight recent advances in inoculation techniques and reactor design. First, strategies must be highly reproducible and transferable across various target ecosystems. Sampling devices, and bioreactors alike, must be versatile and amenable to a variety of habitats.

Corrosion resistance and pressure conformity limitations applied to reactor materials might inadvertently obscure the recreation of certain natural habitats (Zhang et al., 2010; 2018). Typically, stainless steel or titanium is used, though some applications require alloying stainless steel with molybdenum to preclude pitting (Zhang et al., 2011). It is important to determine whether the presence of these metals affects the mimicked environment adversely. Furthermore, the ingress of selective nutrient media, growth factors, inhibitors, and/or substrates must be accomplished without altering critically important reactor parameters, *e.g.*, pressure, salinity, temperature, or pH. In a similar vein, any real-time observation of microbial dynamics must be achieved *sans* interference of any kind. Finally, all mechanical stresses on housed cells must be controlled via low-turbulence and uniform mixing unless gradients are necessary.

Since prokaryotes lack pressure-sensitive structures (like cavities), the effects on them are more limited to molecular changes, thus the physical effect of pressure and low temperature are similar on microorganisms. Many isolated piezophiles are also psychrophilic and reported to grow better at lower temperatures, if the pressure below their habitat's ambient pressure (Kato et al., 1995; Yayanos et al., 1982). However, precise control of ambient pressure has been shown to be beneficial for cultivation endeavors: Kallmeyer et al. (2003) studied sulfate reduction within a microbial community at hydrodynamic pressures of 10.00, 220.00, and 450.00 bar, and found that activity at the two higher pressures was at least one order of magnitude greater than at 10.00 bar. Parkes et al. (1995) examined sulfate reduction rates at 1.01 and 45.60 bar. In this study, however, reduction rates at the lower 1.01 bar were roughly an order of magnitude greater than those at 45.6 bar. Allochthonous microorganisms can gain access to deeper waters via sinking and mixing processes and remain inactive at the elevated hydrodynamic pressures and lower temperatures present at these depths. When then cultivated at standard atmospheric pressure and temperature, these transient allochthonous microorganisms can easily be mistaken for being both resident and even dominant about the community (Tamburini et al., 2013).

In addition to metabolic activity, the effects of hydrostatic pressure on microbial growth have also been investigated. In several cases, growth rates were reported to correlate positively with hydrostatic pressure, being significantly greater under elevated pressure corresponding to the natural hydrostatic pressure of the ecosystem (Birrien et al., 2011; Marteinsson et al., 1999). For example, for the piezophilic and hyperthermophilic *archaea Thermococcus* barophilus and *Pyrococcus* yayanosii, the growth rates were reported to be significantly accelerated under elevated pressures (Birrien et al., 2011; Marteinsson et al., 1999).

While the proliferation of microorganisms can remain stable across wide pressure ranges, oftentimes the extent of pressure tolerated is dependent on the metabolic pathways being expressed by the organism (Oliver et al., 2020). Ultimately, hydrostatic pressure can affect a wide variety of microbiological processes, including cell division, RNA and protein synthesis, and the proper functioning of membrane proteins and lipids (Bartlett, 2002). While most high-pressure experiments linked to cultivation focus on hydrostatic pressure, it is also important to monitor the partial pressure of constituent gases and adjust as necessary to best simulate naturally occurring conditions. As partial pressures dictate the concentration of dissolved gases, they largely affect which substrates microorganisms can readily access, which influences a multitude of biological reactions (Sauer et al., 2012; Stief et al., 2023).

In addition to strict process control, sterile barriers, and optimal energy and mass transfer, basic functions such as heat management, waste disposal, maintenance accessibility, and inoculation must be adapted to optimal conditions. This may require individual sealing and novel pumping paradigms for all static and dynamic parts. As constant monitoring of the reactor is required to control environmental conditions, an adequate number of sensors and probes is of paramount importance. Homogeneity, steady hydrodynamic conditions, and time-dependent changes in flow must be ensured, which is why perfusion reactors are preferable for mimicking aquatic habitats. In these types of reactors, low substrate concentrations can easily be maintained while preserving microbial community structure (Flickinger, 2013; Paek et al., 2014). Additional parameters to consider include (i) pressure fluctuation avoidance, particularly for sensitive piezophilic species (Zhang et al., 2015), (ii) external irradiation of photosynthetic species, (iii) implementation of periodic movements or vibrations, and (iv) sustainment of co-culture dependency (Goh et al., 2019).

Pressure management presents the greatest challenge to reactor design. At 11,000 m depth in the "Challenger Deep" zone of the Mariana Trench, hydrostatic pressures reach 1100 bar. Temperatures on this seabed range from 1 to 3 °C, except for hydrothermal vent environments, where temperatures can reach several hundred degrees Celsius (Zhang et al., 2018). As such, while deep sea polyextremophile organisms are of particular interest scientifically, they also present unique challenges to cultivation methodologies. A continuously operated high-pressure reactor was first reported by Zhang et al. (2010) in studying the oxidation of methane by methanotrophic archaea. In this model, isolated sample volumes were incubated in a stainless-steel tank at 80 bar. Another means of rendering elevated hydrodynamic pressures, like those found in deep-sea habitats, is enclosing test tubes in a pressure vessel. In this case, test tubes housing the sample material are enclosed by a flexible lid, which serves as a piston to transfer pressure from the vessel to the inside of the test tube (Oliver et al., 2020; Parkes et al., 1995). Houghton et al. (2007) used a continuous flow reactor to simulate the conditions of a hydrothermal deep-sea spring. This reactor consisted of a column packed with rock samples from the hydrothermal vent, through which liquid medium rich in dissolved gaseous substrates flowed through at 250 bar (Houghton et al., 2007).

So-called "*in situ* reactors" can be used to screen unknown culture conditions. In this case, the reactor is lowered to the seabed, where it remains for several months. To circumvent the need to reproduce the correct nutrient composition in the laboratory, seawater from the sampling location enters the reactor through membranes as byproducts are flushed out (Alain and Querellou, 2009). Selective membranes that allow seawater to flow into the reactor but prevent the egress of byproducts can also be applied (Zhang et al., 2011). The internal pressure of the reactor is increased with increasing sea depth, accordingly, with a piston system. The first complete utility for high-pressure sampling and cultivation from a seabed, the DEEP BATH system, was developed by Kyo et al. (1991) and used to microbiologically assay the Mannequin Valley in 1997. The authors collected a sample from 6300 m depth and performed cultivation techniques at 650 bar. The hyperthermophilic archaea *Thermococcus peptonophilus* and *Pyrococcus horikoshii* were cultivated at temperatures and pressures exceeding 100 °C and 100 bar, respectively (Kato, 2006). Later, Parkes et al. (2009) developed the DeepIsoBUG system to investigate gas hydrate samples, based on the HYACINTH drilling system, by Schultheiss et al. (2006). DeepIsoBUG was the first utility capable of collecting samples from wells exceeding pressures of 250 bar. Using this system, species of *Carnobacterium* and other bacteria were successfully isolated from gas hydrates in the Gulf of Mexico, the Indian Ocean, and the Pacific Ocean.

However, these were exclusively piezo-tolerant, which can survive under high pressure, but do not necessarily need it for growth. Overall, the technical possibilities for research on extremophiles from aquatic ecosystems are still limited (Alain and Querellou, 2009). This applies specifically for piezophilic and polyextremophilic bacteria and archaea. However, the shown unconventional applications for sampling and preserving selected aquatic microorganisms manifest the potential to cultivate yet uncultivated species by increasing the amount and precision of parameter control from the crucial transition of a sample to a reactor. Advances in the engineering of integrated sampler-bioreactor modules are one of the main milestones towards culturing the uncultured, while – as a side effect – constitutes major reduction potential for frequent and costly sampling in hardly accessible aquatic habitats.

3. Conclusion & outlook

Summarizing the roles of aquatic prokaryotes in biogeochemical cycles, makes clear that scientists still have much to learn about the majority of prokaryotes in aquatic ecosystems. While we are gaining better insights through, *e.g.*, metagenomics or single-cell genomics, cultivation and isolation of prokaryotes are still fundamental to verify bioinformatics-based predictions. During the course of revising this review, some taxa, that we also considered in our list of most wanted aquatic microbiota were successfully cultivated. For instance, Korarcheia were successfully cultivated and proven to be methanogens, highlighting methanogenesis outside of the former Euryarchaeota phylum (Krukenberg et al. 2024). Moreover, the hyperthermophile Fervidibacter sacchari was isolated using media prepared based on its genome-predicted metabolism (Nou et al., 2024). Cultivation of the ubiquitously distributed marine clade SAR202 was recently achieved using a dilution-to-extinction approach. The cultivated SAR202 strains show a variety of metabolic pathways (*e.g.,* oxidation of various organic carbons) undermining their role in the marine carbon cycle. Besides their growth is inhibited by light, the possibility of light sensitivity is another factor that should be considered when cultivating environmental microorganisms (Lim et al., 2023). These recent examples highlight the validity of the list proposed herein and the capacity to use environmental genomics to successfully guide cultivation strategies. Consequently, future studies can substantially benefit from the literature resources provided in this review to design their cultivation and isolation endeavors.Despite this ongoing progress, we still face a number of reasons why cultivation attempts and research projects on the isolation of microorganisms fail: inadequate growth conditions (pH, temperature, pressure), low growth rates of microbes that might exceed usual project terms, influence of viral predation in closed system cultivation, auxotrophy, and dependance on other community members, which can also include Eukarya. Some of these challenges might be overcome by using enrichment cultures, whose first goal is not the isolation of a species but merely its enrichment. Using long-term cultivation (up to 1579 days) in continuous reactors, Kato et al. (2018) were able to detect some uncultured *Candidatus* taxa. For example, they enriched organisms belonging to *Ca.* Caldarchaeales, *Ca.* Paceibacteria, or *Desulfobacterota* (although as a phylum not entirely uncultivated), which we classified as "most wanted", rendering continuous cultivation, a technique with great potential for providing the basis for the isolation of novel microorganisms.

Costs for cultivation of microorganisms, particularly if they necessitate large bioreactors over long periods of time (Kato et al. 2018), are usually high and thus require substantial amounts of funding that go beyond basic funding of universities. By investing more funding in cultivation of yet-to-be-cultivated microorganisms, we stand to validate hypotheses derived from meta'omics data and discover novel biochemical pathways, enzymes, and bioactive compounds with applications in various fields (*e.g.,* medicine, agriculture, and biotechnology) and a deeper understanding of microbes' key ecological roles. As target microorganisms may be sourced from diverse ecosystems globally, adherence to the Nagoya Protocol (https://www.cbd.int/abs/) promotes fair and equitable partnerships between researchers and the countries providing the microbial samples, fostering international collaboration in the pursuit of scientific advancement and sustainable development. However, the Nagoya Protocol should entail a responsibility on the part of the countries to financially support local cultivation projects. As an example, we would like to point out the "Culture Challenge" launched by the German Federal Ministry of Education and Research in 2018, during which scientists and engineers were brought together to collect ideas for innovative cultivation techniques. The most creative concepts have already received seed funding of up to 200,000 euros for one year to subsequently develop the idea in detail for large-scale follow-up projects (https://www.gesundheitsforschung-bmbf.de/de/culture-challenge-wer-hat-die-beste-idee-8196.php [20.12.23]). We herewith encourage other funding agencies from all countries to follow this example for bolstering cultivation and isolation of novel, important microbes from aquatic ecosystems across the globe. Aquatic ecosystems surround us and are of immeasurable value in various ways. However, the majority of aquatic microorganisms have not yet been cultivated and there is a lack of knowledge about fundamental metabolic capacities shaping geobiological processes.

CRediT authorship contribution statement

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Declaration of competing interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.122928.

Data availability

We provide all data used alongside the manuscript either by citation in the manuscript itself or by providing the data in the supplementary (*e.g.*, newick tree formats of tress).

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