



MINIREVIEW

Impact of hydrologic boundaries on microbial planktonic and biofilm communities in shallow terrestrial subsurface environments

H.J. Smith^{1,9}, A.J. Zelaya^{1,2,9}, K.B. De León^{3,9}, R. Chakraborty^{4,9}, D.A. Elias^{5,9}, T.C. Hazen^{6,9}, A.P. Arkin^{7,9}, A.B. Cunningham^{1,8} and M.W. Fields^{1,2,9,*†}

¹Center for Biofilm Engineering, Montana State University, Bozeman, MT, ²Department of Microbiology & Immunology, Montana State University, Bozeman, MT, ³Department of Biochemistry, University of Missouri, Columbia, MO, ⁴Climate and Ecosystems Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA, ⁵Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN, ⁶Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN, ⁷Department of Bioengineering, Lawrence Berkeley National Laboratory, Berkeley, CA, ⁸Department of Civil Engineering, Montana State University, Montana State University, Bozeman, MT and ⁹ENIGMA (www.enigma.lbl.gov) Environmental Genomics and Systems Biology Division, Biosciences Area, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, MS:977, Berkeley, CA 94720

*Corresponding author: 366 Barnard Hall, Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717. Tel: 406-994-7340; E-mail: matthew.fields@biofilm.montana.edu

One sentence summary: The current information on the diversity and activity of shallow freshwater subsurface habitats is discussed within the context of the challenges associated with sampling planktonic and biofilm communities across spatial, temporal and geological gradients, and how biofilms may respond and impact shallow terrestrial subsurface aquifers.

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†M.W. Fields, <http://orcid.org/0000-0001-9053-1849>

ABSTRACT

Subsurface environments contain a large proportion of planetary microbial biomass and harbor diverse communities responsible for mediating biogeochemical cycles important to groundwater used by human society for consumption, irrigation, agriculture and industry. Within the saturated zone, capillary fringe and vadose zones, microorganisms can reside in two distinct phases (planktonic or biofilm), and significant differences in community composition, structure and activity between free-living and attached communities are commonly accepted. However, largely due to sampling constraints and the challenges of working with solid substrata, the contribution of each phase to subsurface processes is largely unresolved. Here, we synthesize current information on the diversity and activity of shallow freshwater subsurface habitats, discuss the challenges associated with sampling planktonic and biofilm communities across spatial, temporal and

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geological gradients, and discuss how biofilms may be constrained within shallow terrestrial subsurface aquifers. We suggest that merging traditional activity measurements and sequencing/-omics technologies with hydrological parameters important to sediment biofilm assembly and stability will help delineate key system parameters. Ultimately, integration will enhance our understanding of shallow subsurface ecophysiology in terms of bulk-flow through porous media and distinguish the respective activities of sessile microbial communities from more transient planktonic communities to ecosystem service and maintenance.

Keywords: groundwater; sediment; aquifer; ecology; activity

INTRODUCTION

The terrestrial, shallow subsurface is a complex and microbially-active habitat located beneath the surface soil layers, comprised of sediments (inorganic or organic unconsolidated material that comes from the weathering of rock transported by wind, water or ice), rocks, gas, pore water and groundwater (Atekwana, Werkema and Atekwana 2006). Typically, subsurface environments contain less labile organic matter (OM) compared to surface soils, and the degree of hydrological connectivity to the surface is routinely used to delineate between shallow and deep biospheres rather than depth alone (Lovley and Chapelle 1995). Although water covers 70% of the Earth's surface, roughly 1% is readily available for human use, and a vast majority (~95%) of the Earth's consumable and available freshwater is groundwater (Danielopol et al. 2008; Griebler et al. 2014; Dennehy, Reilly and Cunningham 2015). Despite the importance for the world's population, the role of microbial communities in the maintenance of groundwater ecosystems is not fully understood. Case in point, the recent increase of artificially recharging natural aquifers via managed aquifer recharge to meet the global demand for water availability is concerning because of the potential to drastically alter groundwater systems (Lee and Lee 2017). This mini-review will focus on aspects of 'shallow' freshwater subsurface environments (mainly porous/granular) which typically have higher rates of recharge and flow as well as have a high degree of connectedness with the surface as opposed to 'deep' subsurface environments that are much less connected with the surface and receive limited surficial inputs of water and/or nutrients.

Primary motivations for studying the subsurface are to expand what is known about Earth's microbial diversity and the subsurface microorganisms under low nutrient conditions that significantly impact C, S, N, P and mineral cycles. Microbial life is thought to vary from the terrestrial surface to the deep subsurface dependent upon water, nutrient inputs and environmental stressors. Upwards of 40% of the microbial biomass and 10^{16} – 10^{17} g C on Earth resides within the terrestrial subsurface (Whitman, Coleman and Weibe 1998; Griebler and Lueders 2009; McMahon and Parnell 2014). Over the last 30 years, there has been an increasing interest in surveying the taxonomic and functional biodiversity of subsurface environments, largely due to the concern over biodiversity and subsequent ecosystem function loss (Danielopol et al. 2003; Hancock, Boulton and Humphreys 2005; Wall and Nielsen 2012; Lijzen, Otte and van Dreumel 2014). However, on an ecosystem scale, there is limited information regarding the exact relationship between microbial diversity, environmental parameters and biogeochemical processes between groundwater and subsurface porous media. Studies focusing on subsurface habitats have revealed many significant roles that microorganisms play in shallow subsurface processes (e.g. Chapelle 2000; Atekwana, Werkema and Atekwana 2006; Hwang et al. 2009; Mitchell et al. 2010; Akob and Kùsel 2011; Griebler and Avramov 2015).

In the environment, microorganisms can be observed in two distinct phases: free-living (planktonic) and associated with a surface as single cells to multicellular aggregates (i.e. biofilm). Biofilms are often composed of diverse taxonomic lineages attached to surfaces and each other, typically surrounded by extracellular polymeric substances (Hall-Stoodley, Costerton and Stoodley 2004; Gross et al. 2007; Stewart and Franklin 2008). Biofilms have not been explicitly studied within the subsurface; however, because biofilms have been described at liquid–solid, liquid–gas or solid–solid interfaces, it is becoming increasingly clear that biofilms more closely resemble *in situ* conditions for microorganisms from diverse environments (Hall-Stoodley, Costerton and Stoodley 2004). Therefore, it is likely that attached modes of growth are a universal feature presenting an important physiology to explore within the subsurface in addition to typically conducted planktonic cell studies (Dunne 2002; Kolter 2005).

Cells growing on a surface (i.e. biofilms) are known to have physiologies and properties distinct from planktonic cells including increased resistance to external stresses such as antimicrobials, heavy metals, desiccation and substrate deprivation (e.g. Clark et al. 2012; Kurczyk et al. 2015; Stylo et al. 2015). Most microbial environments are physically dynamic habitats where fluxes in water, nutrients, temperature, pH and osmolarity can create challenges for survival. Altered flow conditions can limit motility/dispersal and nutrient availability can result in decreased microbial activity and altered population distribution (Or et al. 2007). Biofilm matrices can retain water, sorb nutrients and protect against rapid changes in local geochemistry, attributes that significantly improve microbial viability and activity. Additional ecophysiological advantages from residing within biofilms include metabolic cooperation, the exchange of genetic material and the development of regulatory mechanisms and social behaviors (Dang and Lovell 2016 and references therein).

Traditionally, subsurface habitats were analyzed through bulk activity assays and total and viable cell enumerations (mainly with groundwater samples). Recent studies have relied on sequencing and -omics techniques to identify new diversity and functionality. Unfortunately, little overlap exists between more traditional quantitative activity measurements and newer sequencing capabilities. Such overlap is necessary to link phylogenies to quantitative functionality, although systems approaches have been used for bioremediation sites (Chakraborty, Wu and Hazen 2012). The objectives of this review are to synthesize the current understanding of (i) microbial population distributions and activities spanning shallow subsurface habitats (with a focus on freshwater systems when possible), (ii) discuss the challenges associated with sampling planktonic and biofilm communities across spatial, temporal and geological gradients, (iii) identify subsurface geochemical and physical properties that potentially constrain biofilm development and

(iv) give recommendations and considerations for future studies. Additionally, Table S1 (Supporting Information) provides a quick summary of sample and environmental details, including lithologic information when available, for the relevant sources cited in this review.

CHARACTERISTICS OF SHALLOW SUBSURFACE ENVIRONMENTS

Although estimates vary, the shallow subsurface environment can extend from beneath the OM rich soil layers (A and B horizons) to tens of meters (Atekwana, Werkema and Atekwana 2006; Pepper and Brusseau 2006). In the shallow subsurface environment of an aquifer, sediments are assumed to lie below the vertically weathered top soil profiles. In the B horizon (below O, A and E), minerals, clays and organic material are leached from the upper horizons. The C horizon (below B) is characterized by unweathered minerals that were the parent material from which the upper soils were formed while at deeper depths the R horizon is the native bedrock material (Pepper and Brusseau 2006). The shallow subsurface is typically described as being below the surface soil horizons (typically 1–10 cm) and above bedrock (<50 m in depth) (Chu et al. 2016), and can have a high degree of hydrological connectedness with the surface compared to the deep subsurface (Toth 1963; Lovley and Chapelle 1995). By contrast, deep subsurface systems have been distinguished by arbitrary depth measurements ranging from hundreds to thousands of meters below the surface (Balkwill 1989; Lovley and Chapelle 1995; Head, Jones and Larter 2003) or by a lack of surface connectivity (Toth 1963; Lovley and Chapelle 1995). The designation of ‘shallow’ versus ‘deep’ can be variable dependent upon respective geology and environment. Additionally, albeit not within the scope of this minireview, there are different concepts to categorize aquifers (e.g. aquifer, aquiclude and aquitard) in the context of hydrology.

Traditionally, the shallow subsurface can be separated into three distinct zones based on moisture content in relationship to water table configuration termed the vadose, capillary fringe and saturated zones (Fig. 1). The vadose zone represents the upper most boundary of the subsurface comprised of the upper horizons (O–B) and contains unweathered and weathered materials. Following precipitation events, the vadose zone experiences high saturation levels as vertical infiltration proceeds downward to the water table, yet residual pore water can persist creating varying levels of water and gas saturation (Jones and Bennett 2014). The capillary fringe exists at the interface of the saturated and vadose zone and is highly dependent upon fluctuations of the local water table. The capillary fringe is dynamic overtime with varying physicochemical conditions resulting from water table fluctuations (Griebler and Lueders 2009). This fluctuating interface has been shown to be a ‘hotspot’ of subsurface activity especially with respect to biogeochemical cycling (Silliman et al. 2002; Berkowitz, Silliman and Dunn 2004). The saturated zone (i.e. at/below water table) of most aquifers consists of porous parent material (C and R horizons) and voids are filled with water. Generally, the direction of water flow in the saturated zone can be 3-dimensional depending on hydraulic gradients and porous media properties (e.g. clay lenses).

With respect to the impact on microbial communities, much attention has been given to the water table position and sediments transported in the saturated and capillary fringe zones.

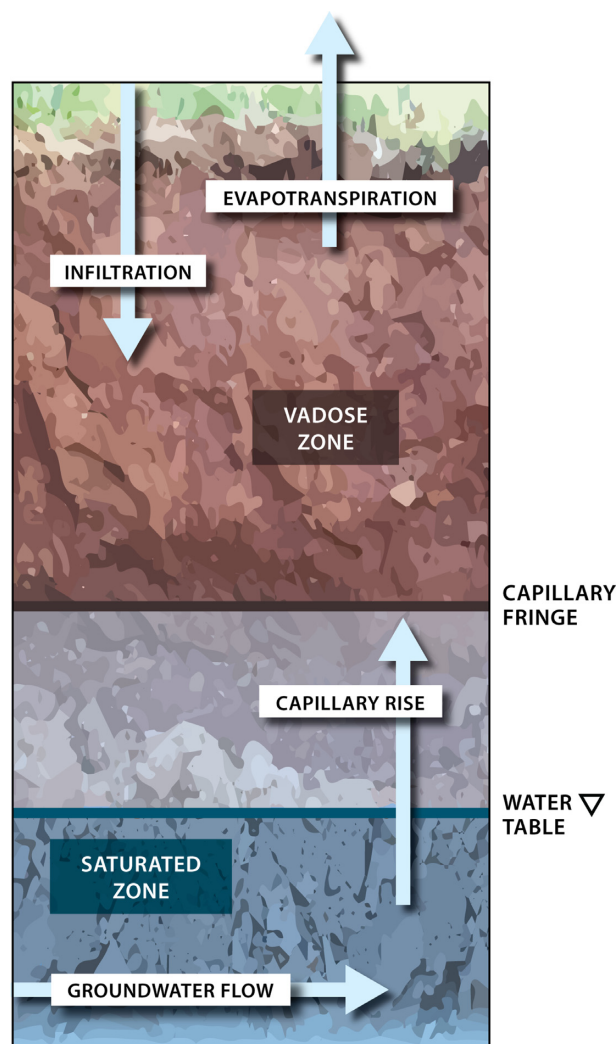


Figure 1. Conceptual illustration of representative shallow subsurface environment that includes the vadose, capillary fringe and saturated zones. Arrows depict the movement of water through infiltration, evapotranspiration, capillary rise and re-charge, and the movement of water within and between these zones creates dynamic conditions for the formation and maintenance of subsurface biofilms.

The latter transitional boundary between the vadose and saturated zones is capable of drastic changes in geochemical parameters [e.g. pH and dissolved oxygen] that impact ecosystem function in terms of geochemical cycling, biotic/abiotic filtering and buffering processes (Rainwater et al. 1993; Reddi, Han and Banks 1998; Dobson, Schroth and Zeyer 2007; Pilloni 2011; Chakraborty, Wu and Hazen 2012). However, the vadose zone can also have soils and weathered particles (sediments) impacted by water movement, likely dictated by surface infiltration and evapotranspiration. In addition, clays and clay lenses are also thought to impact water and gas flow that could significantly impact microbial processes (Faybishenko et al. 2000).

In addition, particle structure can impact community composition and activity, for example, the turnover of matrix-associated NOM (natural organic matter) correlates to the proportion of fine-grained particles (Keil and Mayer 2014). Physical properties of sediments (e.g. particle size) is also thought to impact microbial activity and distribution although most studies have been done with surface or near-surface soils/sediments

(e.g. Jackson and Weeks 2008; Hemkemeyer et al. 2015, 2018). Individual aggregates in groundwater and soil as well as soil pores can have discrete microenvironments with distinct activities and conditions (Keil and Mayer 2014) that likely contribute to spatial and temporal areas of high metabolic activities or 'hot spots and hot moments' (McClain et al. 2003). Pores and aggregates are continuously changing due to biogeochemical and physical processes (Schlüter and Vogel 2016), and wetting/drying cycles (i.e. capillary fringe) can greatly impact pore size distributions (Bodner, Scholl and Kaul 2013). Sediment-groundwater-cell interactions can occur at the pore scale (\leq micrometer) where diffusion and dispersal can be limited. However, little is known about how microbially relevant scales ultimately impact field scale behavior and function, and few studies have determined the proper scale to delineate these relationships.

While the subsurface begins below the humus rich soil horizons, NOM (including particulate and dissolved fractions excluding organic contaminants) is a primary source of C/N that supports microbial life in the shallow subsurface. Despite seasonal shifts, there is a natural gradient of decreasing nutrient and oxygen concentrations with depth leading to oligotrophic and anoxic conditions within the saturated zone (Danielopol, Pospisil and Rouch 2000; Awoyemi, Achudume and Okoya 2014). Additionally, NOM is thought to decline with depth, and recent comparisons of water-extractable organic matter from a shallow subsurface core showed total organic carbon was \sim 19 mg/g and inorganic carbon was 8 mg/g in shallow sediment (Chakraborty et al., unpublished data). Due to nutrient limiting conditions, microorganisms in subsurface habitats have most likely developed strategies to use NOM and other reduced compounds (e.g. Mn(II), Fe(II), ammonia, sulfide, methane and hydrogen) as part of directly or indirectly coupled processes in the groundwater, pore water and sediment surfaces.

SHALLOW SUBSURFACE MICROBIAL BIODIVERSITY: PROGRESS AND CHALLENGES

The relationship between biodiversity and ecosystem functioning has been well studied above ground (Cardinale et al. 2006; Ives and Carpenter 2007); however, similar studies are in the early stages for subsurface environments. While perceived functional redundancy could have a limited role in subsurface ecosystem functioning, studies also indicate that microbial taxonomic diversity plays a role in mitigating ecosystem collapse and contributing to faster functional recovery (Wagg et al. 2014; Delgado-Baquerizo et al. 2016; Louca and Doebeli 2016). Subsurface groundwaters and sediments have been shown to harbor far more taxonomic and functional diversity than previously inferred by cultivation attempts and microscopic observations (Brown et al. 2015; Lynch and Neufeld 2015; Lennon and Locey 2016). In addition, these environments exhibit a wide diversity of previously undescribed bacteria and archaea (Castelle et al. 2013; Brown et al. 2015; Anantharaman et al. 2016; Lazar et al. 2017). While specific taxonomic lineages can be prevalent in several types of underground ecosystems (Griebler and Lueders 2009; Akob and Küsel 2011; Hubalek et al. 2016), thus far no true 'endemic' shallow subsurface populations have been identified (Griebler and Lueders 2009). To date, the debate regarding the influence of biodiversity and ecosystem functioning, especially within the subsurface, has yet to be thoroughly explored. Any resolution will most likely be challenging at best due to extreme spatial heterogeneity.

It remains unresolved whether our current understanding of subsurface microbial biodiversity is real or merely an artifact of the following topics: (i) technological approaches (i.e. short reads lengths from next generation sequencing), (ii) low relative diversity and/or abundances of oligotrophic systems, (iii) the use of bulk sampling techniques compared to the retrieval of samples representing discrete phases (planktonic vs. biofilm) and/or discrete zones (i.e. vadose, capillary fringe, and saturated zones) which could further delineate spatial differences, and/or (iv) temporal dynamics that have been poorly resolved. For example, recent work has shown the potential importance of microbial biomass for protozoan food webs in shallow aquifers (Hutchins et al. 2016) and differences in carbon cycling between groundwater and shallow sediments over time and space (<1 m) (Longnecker and Kujawinski 2013). Given these types of observations, the roles of biofilm diversity in the shallow subsurface for resistance to predation pressures and ultimately on resource allocation are not known. Therefore, as discussed below, future studies should combine technological approaches at appropriate temporal and spatial scales for both groundwater and matrix material.

Technological approaches

Studies of microbial biodiversity have historically been performed via traditional microbiological techniques (Goldscheider, Hunkeler and Rossi 2006 and references therein; Sinclair and Ghiorse 1989). Profound advancements have been made in the application of next-generation sequencing (Tringe and Hugenholtz 2008), high-throughput -omics approaches (López-García and Moreira 2008; Prosser 2015), single-cell methods (Lasken and McLean 2014) and methods encompassing untargeted functional potential (López-García and Moreira 2008; Rajendhran and Gunasekaran 2011). Up until July 2015, a total of $\sim 1.4 \times 10^6$ and $\sim 5.4 \times 10^5$ full-length bacterial and archaeal 16S rRNA reference sequences, respectively, have been deposited into SILVA-ARB (www.arb-silva.de) and IMG (img.jgi.doe.gov), comprising a total of 65 bacterial and 20 archaeal phyla (Schloss et al. 2016). Interestingly, it was estimated that only 7.8% and 16.5% of all reference sequences originated from soil and aquatic environments, respectively (Schloss et al. 2016). As the above estimates include surface waters (e.g. lakes and rivers), marine environments and surface soils, the percentage of sequences specific to groundwater, and more so for shallow subsurface sediments, is quite low. The drastic under-sampling of the subsurface has led to a scarcity of reference sequences specific to these environments, leading to the high risk of mis-identification of retrieved sequences and an under estimation of subsurface biodiversity and biochemical capacity. As the number of non-targeted (DNA/RNA-based) and targeted metagenomes (SIP/activity) are increased for shallow subsurface groundwater and sediments, it is likely that unique lineages with novel capability will be discovered across all three domains, and thus an improved representation of in situ diversity can be achieved.

Low relative diversity/abundances of oligotrophic systems

Based upon a limited number of studies that survey diversity as a function of depth, it has been observed that species richness declines over depth, with transient increases at transition zones. Currently, it remains unclear if this trend is merely a consequence of the combination of limited/recalcitrant resources

(C) and energy restriction (anoxic) or other more specific selection mechanisms that may differ from surface environments (Musslewhite et al. 2003; Lin et al. 2012b; Chu et al. 2016). Recent studies suggest that the large fraction of lowly abundant or 'rare' organisms observed in subsurface environments may play important ecological roles. For example, they may contribute to biogeochemical reactions (Pester et al. 2010) while also serving as a 'microbial bank' that can 'seed' environments when conditions change (Lynch and Neufeld 2015). Biofilms could play a major role across the shallow subsurface zones in which changing conditions (e.g. pH, conductivity and flow) could drive dispersal and/or invasion (discussed below). As oligotrophy is inherent to most subsurface systems, techniques that couple high-throughput manipulation with small volumes and -omics methodology (e.g. micro-droplet fluidics and flow cytometry) should be included in future work to enable cultivation and activity measurements of slower growing microorganisms (e.g. Wilkins et al. 2014).

Spatial variability: diversity of discrete zones

While mechanisms that affect population distributions have been formulated based on surface habitats (i.e. biotic interactions, dispersal limitation and environmental filtering) (Martiny et al. 2006; O'Malley 2007; Griebler and Lueders 2009; Shoemaker, Locey and Lennon 2017), it remains unclear whether these mechanisms hold true for the distribution of microorganisms within the oligotrophic subsurface (Musslewhite et al. 2003; Chu et al. 2016). It is hypothesized that transition zones (macro- as well as micro-transition zones, such as between individual particles and surrounding pore water) are important ecotones or 'hotspots' of microbial diversity and activity (Zhang et al. 1998; McClain et al. 2003; Goldscheider, Hunkeler and Rossi 2006; Bougon et al. 2012; Campbell et al. 2012; Jones and Bennett 2014) and deserve more careful attention. There is evidence of spatial (vertical and horizontal) taxonomic variation of groundwater (Lin et al. 2011; Lin et al. 2012a; Herrmann et al. 2015) and sediments (Lin et al. 2012b). Results typically show a decline in microbial richness and diversity over vertical depth. The extent that microbial communities vary in relation to depth, even with application of newer sequencing technologies, is still poorly resolved for the variety of geological strata that represent the shallow subsurface. Therefore, increased spatial resolution is needed to better understand the implications of micro-scale heterogeneity on microbial population distributions.

It is not known if observed variation is a consequence of geophysical, geochemical or hydrogeochemical constraints, or a combination thereof. Whereas various scales have been surveyed (cm, m, km) when measuring spatial β -diversity of microbial communities, studies that span over several cm are more common, with deeper samplings that span meters being less frequent. Pronounced effects of horizontal spatial dissimilarity on β -diversity increasing with depth have been shown for surface and subsurface soils (Chu et al. 2016), suggesting that, at least down to the saturated zone, subsurface sediment biofilms could be more greatly affected by dispersal limitation than communities of surface soils. Moreover, the proper scale at varied spatial resolution to capture microscale heterogeneity or the proper scale for different geologic strata is currently unknown.

Spatial variability: diversity of discrete phases

In order to further investigate microbial diversity in the subsurface, it is essential to differentiate between planktonic and

attached populations. Historically, the ease of groundwater sampling via well-pumping has resulted in the majority of subsurface datasets. However, inferences made about subsurface communities based solely on the planktonic fraction may not adequately represent all microbial members of the subsurface ecosystem (Hug et al. 2015).

Studies that have attempted to compare planktonic versus biofilm communities have resorted to the use of surrogate sediments (native and/or artificial material incubated *in situ* down well) that represent the geology of the aquifer (Reardon et al. 2004; Flynn, Sanford and Bethke 2008; Flynn et al. 2012; Converse et al. 2015; Graham et al. 2017). These surrogates include laboratory microcosms (Lee and Lee 2017), in field biofilm reactors (King et al. 2017; Christensen et al. 2018), or sediment fines from backwashed pumps (Cardenas et al. 2008; Li et al. 2018). Early studies that compared the planktonic versus attached fractions have generally observed a subset of the planktonic community in the attached fraction (Hazen et al. 1991). Several studies have corroborated these findings over the years (Reardon et al. 2004; Brad et al. 2008; Anneser et al. 2010; Zhou, Kellermann and Griebler 2012).

Studies to differentiate planktonic versus biofilm functions may be able to capture the transitional states of planktonic communities (from planktonic to biofilm and vice versa), but there are unique limitations to each approach. For example, samplers (e.g. sampling coupons) typically contain unconsolidated sediments that may not accurately mimic the hydrological effects of consolidated or saturated sediments. Thus, borehole artifacts must be considered (Lehman 2007a). In addition, colonization of the native matrix material is dependent upon surrounding groundwater/porewater. The colonization no doubt occurs *in situ*, but the studies are over short time periods compared to *in situ* conditions and may not achieve the diversity of the natural setting. However, the down-well incubations of solid material does enable the capture of some microbial populations typically missed by groundwater sampling and could capture interaction dynamics across the aqueous/solid matrix boundary under *in situ* conditions (Barnhart et al. 2013).

We recently used a revised microbial sampler (patent pending) in a coal-bed aquifer packed with native coal material incubated down-well for ~ 3 months and compared the bacterial communities (SSU rRNA gene libraries) between sampled groundwater, native coal core and coal material from the same formation incubated in microbial samplers (Schweitzer et al., unpublished data). Preliminary analyses suggest that some family-level operational taxonomic units were common to all three samples while other operational taxonomic units were common to groundwater and the surrogate matrix material ($n = 3$). Two operational taxonomic units were unique to the sampled groundwater and four unique to the surrogate matrix material. Not surprisingly, the native coal material had the most unique operational taxonomic units ($n = 6$). Whereas differences between the samples were expected, surrogate matrix material could be used in future studies to capture additional diversity from the subsurface and delineate ecology dynamics in terms of core (i.e. consistent) and transient populations between groundwater and matrix material.

The inability to distinguish between different phases (i.e. groundwater vs. sediments) for key biogeochemical processes poses a challenge for answering basic ecological questions. Recent -omics approaches applied to samples from the subsurface are revealing the range of possible activities within the subsurface and the potential for broad biochemical functionalities (Griebler and Lueders 2009; Akob and Küsel 2011; Flynn et al.

2013; Hubalek et al. 2016). Through genomic surveys, microorganisms have been linked to the transformations of carbon compounds, the nitrogen cycle and sequestration of greenhouse gases (Hemme et al. 2015; Trivedi, Delgado-Baquerizo and Trivedi 2016). Typically, functionality has been inferred from the presence of specific functional genes in a sample set (e.g. Yan et al. 2003; Fields et al. 2006; Winderl, Schaefer and Lueders 2007) or, more holistically, across a subsurface ecosystem. Usually the larger scale sampling needed to characterize the potential microbial function in an ecosystem relies on testing the more easily accessible groundwater (Hug et al. 2015; Smith et al. 2015; He et al. 2018). Therefore, despite any novel and potential activity identified in groundwater, the full extent of possible differences in the sediments are unknown. Due to the challenges of getting intact sediment samples, previous studies have used surge blocks (Wu et al. 2013) on groundwater pumps to collect sediment fines. This procedure allowed delineation of the groundwater populations from those associated with the sediment fines (Cardenas et al. 2008; Li et al. 2018), and the reported results showed definite differences between the groundwater and sampled sediments. The potential differences in functionality between the planktonic and sediment microorganisms have implications (e.g. selection pressures and dispersal) for ecosystem stability and resiliency, particularly with dynamic hydrological cycles that can change over varying time-scales.

Effects of temporal fluctuations on biodiversity

Taxonomic diversity in relation to spatiotemporal fluctuations in physicochemical and geochemical properties of the shallow subsurface is largely understudied. Physicochemical and geochemical relationships over time may drive phylogenetic and functional microbial diversity changes that are often associated with both short-term (e.g. diel cycles; extreme weather) and long-term (e.g. seasonal) alterations. Spatiotemporal fluctuations in conjunction with porosity and permeability modifications can lead to varying degrees of hydrogeochemical mixing. The role of mixing in aquatic systems has been implicated in the creation of patchy distributions of both nutrients and biomass (Ebrahimi and Or 2016; 2018). Depending on the aquifer system, highly mixed waters can be found in shallow areas, where there are higher rates of infiltration from rain or surface waters. Alternatively, at the water table, seasonal fluctuations can result in mixing groundwater with sediments of the variably saturated zone (Fig. 2). In the shallow subsurface, mixing is thought to cause instability due to faster and shorter local flow paths; whereas, more stable and predictable diversity may result from slower regional flow significantly below the water table (Ben Maamar et al. 2015).

Most spatiotemporal studies of subsurface environments focus on the changes of geochemistry and corresponding groundwater communities collected via sampling wells over time across different depths in the water table (Lin et al. 2012a; Brad et al. 2013; Sirisena et al. 2013; Ben Maamar et al. 2015; Schwab et al. 2016). Studies have consistently shown that mixed groundwaters have higher diversity and variability than groundwater that undergoes less mixing (Bougon et al. 2012; Hug et al. 2015; Danczak et al. 2016; Hubalek et al. 2016). Additionally, it has been shown that the degree of hydrogeochemical mixing can greatly impact microbial assemblage compositions due to the influx of nutrients and migration of transient populations (Haack et al. 2004; Fields et al. 2006; Hwang et al. 2009; Velasco-Ayuso et al. 2009; Lin et al. 2012a; Hubalek et al. 2016).

The impact of mixing on *in situ* sediment biofilms is largely unknown, mainly because temporal sampling in the same location is nearly impossible for native matrix material. Surrogate sediments incubated down-well have enabled some degree of temporal sampling of attached microorganisms (Zhou, Kellermann and Griebler 2012), as well as successional events when monitored longer times. Comparisons of cataloged cored sediments separated by months or years have also been performed (Hug et al. 2015). It is likely that feedbacks between resident microorganisms and hydrogeochemistry exist and impact subsurface ecosystem structure and function at a larger-scale (Mendoza-Lera and Mutz 2013; Lee and Lee 2017) particularly for sediment biofilms where impacts at the microscale are more likely compared to bulk-phase changes.

ACTIVITY IN THE SHALLOW SUBSURFACE

There are significantly fewer studies that have simultaneously compared microbial activities in the sediment and groundwater fractions. Quantifying activity from subsurface samples is a non-trivial task, as the retrieval of 'undisturbed' samples in combination with 'representative' incubation times necessary for activity assays can potentially lead to artifacts which greatly influences downstream analyses and interpretations. While sequencing capabilities have produced substantial insight about the potential functionality of porous subsurface aquifers, traditional -omics studies struggle to make quantitative estimations about activity (Hemme et al. 2015; Hug et al. 2015). Subsurface activity is typically measured utilizing traditional approaches (e.g. extracellular enzyme assays, radioisotope tracers, viable plate counts, most probable numbers and direct counts with fluorescent compounds indicative of activity), all of which have been shown to have inherent biases (Kepner and Pratt 1994; Stewart et al. 1994). Historically, not only were there greater densities of total cells in the sediments, but a higher proportion of active cells are associated with sediment compared to planktonic cells (Hazen et al. 1991; Alfreider, Krössbacher and Psenner 1997). However, the contribution of free-living and biofilm cells to subsurface processes on a per cell basis is unclear. Recently, it has been proposed that microbial competition selects against rapid growth in biofilm populations (Coyte et al. 2016). These findings offer a unique and contradictory perspective as to the role of free-living organisms compared to biofilms that may alter our current understanding of colonization, maintenance and dispersal of microbial populations in porous environments.

Activity in groundwater

Most researchers have now concluded that attached bacteria dominate oligotrophic subsurface environments in terms of biomass and activity and that most planktonic cells are 'inactive' subsets of benthic organisms (Goldscheider, Hunkeler and Rossi 2006 and references therein). Initially, indications that groundwater samples had a low proportion of active cells came from microscopic evaluation of pristine aquifers which observed cells between 0.4 and 0.9 μm in size, suggesting that these bacteria were in a starved state with reduced activity (Balkwill and Ghiorse 1985). However, a recent study identified novel ultramicrobacteria that are inherently small (<0.1 μm) in groundwater but activity was not reported (Luef et al. 2015).

Groundwater habitats have been shown to be able to vary drastically over time and space. For example, in a two-year study, all tested extracellular enzyme assays were found to vary significantly both spatially and temporally (Velasco-Ayuso et al. 2011).

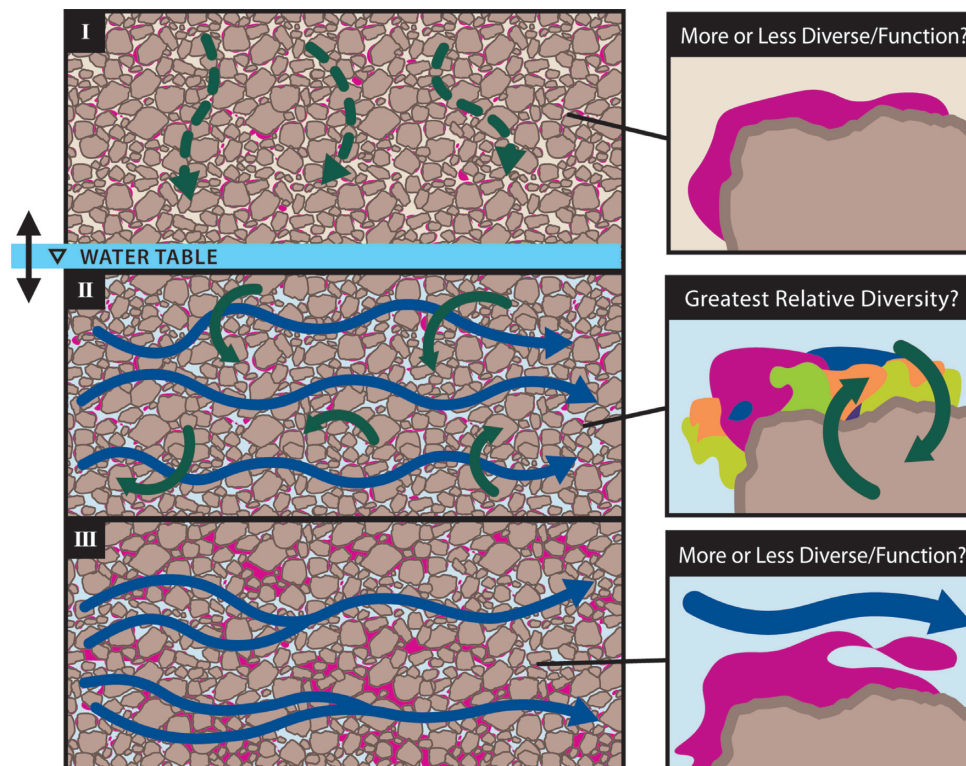


Figure 2. Conceptual model of subsurface flow and mixing zones and potential effects on biofilm life-cycle dynamics. Subsurface porous media habitats can be conceptually divided into three zones (I, II and III) with respect to ground water flow and mixing. (I) The vadose zone (including the capillary fringe) is variably saturated depending on infiltration episodes and degree of vertical water table fluctuation, (II) Zone II is the 'shallow' groundwater zone wherein ground water flow, together with seasonal changes in water table elevation, can cause multi-directional flow (i.e. vertical and horizontal fluctuations) that can result in greater mixing, (III) The 'deeper' groundwater zone (zone III) lies below the depth affected by seasonal water table fluctuations. The degree of mixing in zone III is related mainly to the ground water flow field. In zone II the higher level of seasonal mixing could result in a 'hot spot' of greater relative biofilm diversity and activity (represented by multi-color sections; biofilm not depicted at scale) (Bougon et al. 2012). Zone I could have lower biofilm diversity/activity due to limited and transient mixing, although it is possible that diversity and activity in zone I would be more similar to zone II than region III. In zone III, which is deeper and has a more consistent ground water flow regime, biofilms would be less diverse/active. The roles of adhesion/detachment/dispersion could vary with the extent of mixing in the different zones and suggests that different mechanisms of microbial community assembly and diversification impact in situ biofilms.

Recent advances are moving away from relying solely on bulk activity measurements. Quantitative studies that are capable of linking individual microorganisms to biogeochemical processes have been applied to groundwater from carbonate-rock aquifers. Although these results are not from a porous aquifer, the combination of metabolic labeling (i.e. D_2O) with Raman microscopy, metaproteomics and carbon amendments quantitatively showed that naturally occurring heterotrophic organisms preferentially assimilated lignin derivatives over biomass degradation products (Taubert et al. 2017) and are therefore involved in subsurface carbon cycling processes.

Activity in sediment

The overwhelming density of sediment associated organisms presents a compelling case that sediment core samples are likely the most representative samples for biomass analysis in the shallow subsurface. Studies based on cored samples have looked at the microbial activity of attached communities as a function of depth and particle size. Not only are cell numbers higher in shallower depths compared to deeper depths, the same holds true for activity (Beloin, Sinclair and Ghiorse 1988; Martino et al. 1998). This trend has been shown, regardless of the methodology used [ATP assays, MPN, viable plate counts and the tetrazolium reduction method (INT)]; however, activity

(specifically within the saturated zone) was shown to vary seasonally dependent on the method utilized (Beloin, Sinclair and Ghiorse 1988). When comparing similar depth profiles (<50 m), other researchers have observed only slight variations in total cell abundances over depth and the largest differences were observed in the active fraction (determined with viable plate counts) which decreased with depth (Balkwill and Ghiorse 1985; Balkwill 1989). Studies utilizing radioisotope tracers have found higher metabolic activities in shallower depths as well as spikes in activity within the saturated zone (Phelps et al. 1988). Interestingly, anaerobic bacteria have also been found to decrease in viability with depth and have been reported to be a 100-fold lower than aerobic organisms (Balkwill and Ghiorse 1985). Conversely, in low conductivity ecosystems, studies have found that anaerobic microorganisms have greater viability at deeper depths (Martino et al. 1998). The discrepancies between studies are likely attributed to differences in hydraulic conductivity which directly impacts microbial and nutrient sources and local geochemistry, the exclusion of temporal analysis and differences in methodologies.

Activity measurements comparing biofilm and planktonic populations within sediment mesocosms observed a higher proportion of activity in sediments (0.25 m columns with shallow sediments) compared to the planktonic communities (Longnecker and Kujawinski 2013), a finding that corroborates results from field studies (Thomas, Lee and Ward 1987; Hazen et al.

1991; Holm et al. 1992; Alfreider, Krössbacher and Psenner 1997; Anneser et al. 2010). Some studies have observed total cellular abundances to be highest with coarse particles (Albrechtsen 1994), while others have demonstrated a greater number of microorganisms associated with fine silt particles (<20 μm) (Harvey, Smith and George 1984). However, the study that found higher total densities of organisms associated with coarse particles showed with multiple methodologies that the greatest proportion of active organisms (91.9–100% of viable bacteria) were associated with smaller size particles (1.2–100 μm) (Albrechtsen 1994). An additional attribute that is largely unknown for sediment biofilms is cell density per given surface area and/or co-occurrence of cells or populations in more oligotrophic environments.

Paired studies comparing activity in groundwater to sediment

In order to accurately determine the contribution of free-living and attached, paired groundwater and core samples are necessary albeit these studies are significantly fewer in number. Multiple methodologies have shown that total measured activity (e.g. general metabolic activity, degradation of a specific compound) is greater per gram of sediment than for comparable adjacent groundwater (per mL or L) for both contaminated and pristine aquifers (Thomas, Lee and Ward 1987; Hazen et al. 1991; Holm et al. 1992; Alfreider, Krössbacher and Psenner 1997; Anneser et al. 2010). A variety of radioisotope tracers are routinely used to infer activity from environmental samples, and a study on a sandy aquifer that used ^3H -thymidine in combination with ^{14}C -leucine was not able to detect significant activity in any tested groundwater samples while activity was readily quantified for colonized material from down-well sampling devices (Alfreider, Krössbacher and Psenner 1997). The greatest activity was observed for smaller grain size particles (125–250 μm) compared to larger particle sizes (250–500 μm) (Alfreider, Krössbacher and Psenner 1997). The percentage of respiring bacteria as determined with INT has been shown to range between 1.0–24.9% of total cells for pumped groundwater and 6.0–41.1% of total cells for sandy sediments with on average three-fold fewer active cells in groundwater than associated with sediment. While this study demonstrated higher totals and higher numbers of metabolically active cells as well as greater rates of general metabolic activity in sediment samples, the results potentially stem from an artifact due to the use of sterile sediments suspended down-well rather than the use of actual sediment cores.

An early study used direct counts (cellular DNA stained via acridine orange) and viable plate counts to compare subsurface sediment to adjacent groundwater. Total and viable bacterial abundances for sediments collected from three individual boreholes were higher than for any adjacent groundwater samples from discrete depths (Hazen et al. 1991). The study also observed higher densities of total and active cells associated with aquifer sediments than groundwater and concluded that attached bacterial communities are not reflected in groundwater samples. In addition, recovered sediment isolates were capable of utilizing a broader range of carbon sources than planktonic cells from groundwater which the authors concluded relevant for *in situ* bioremediation efforts.

More recently, from a tar oil contaminated aquifer, sediments contained greater than 97.7% of all bacterial cells and displayed six-fold greater enzyme activities than groundwater, although

groundwater dominated more specific processes such as sulfate and iron reduction (Anneser et al. 2010). These observations corroborate previous studies: the highest density of organisms is associated with sediments but groundwater communities have the potential to dominate certain redox reactions when sampled at the appropriate resolution. The idea that some microorganisms reside mainly in the planktonic phase of subsurface porous environments is supported by the predominance of methanogenic microorganisms observed in the planktonic phase compared to sediments (Lehman 2007b). Using a combination of laboratory and field-based studies Holm et al. (1992) examined the role of planktonic and biofilm associated cells on the biodegradation of hydrocarbons and concluded that whereas there were substantially lower rates of degradation for groundwater samples, the planktonic phase significantly contributed to the biodegradation of organic contaminants. While reaction rates within the groundwater are typically lower on a per volume basis, the studies demonstrate the importance of sampling and studying both groundwater and subsurface biofilms.

A possible explanation for differences in activities between attached and free-living populations is likely due to differences in cell abundances. However, it remains unresolved whether free-living cells in porous subsurface habitats are in fact metabolically slower or faster. Recently, models have predicted that attached cells can be selected to grow at slower rates as to avoid mass transport limitations (Coyte et al. 2016). While this concept has not been directly shown under natural conditions, a study that combined cellular abundances and volumetric rates of degradation found that some cell specific activities for planktonic bacteria may be higher than for sediment associated organisms (Lehman 2007a).

SUBSURFACE BIOFILMS

Subsurface communities are traditionally discussed in terms of the planktonic or attached phases with little reference to attached communities as 'biofilms'; therefore, the following section aims to merge the available information from biofilm studies (often done in laboratory settings with single model organisms) with properties and constraints relevant to subsurface processes. Within these attached communities, microorganisms with varied metabolic functionalities can coexist and have cell-to-cell contact (Stewart and Franklin 2008), periodically detaching to become part of the planktonic phase (McDougald et al. 2012). It is likely these cells colonize new environments and are a primary mechanism for translocation from one surface to another (Watnick and Kolter 2000). Thus, the solid sediment matrix potentially could act as a seed bank of pelagic bacteria that can then be translocated. Despite the ecological significance of biofilms, the relationships between source diversity (and activity) within the groundwater and local diversity (and activity) in the sediments are difficult to ascertain based upon logistics of sampling intact sediment material. Major logistics include expensive sampling that only provides a single time and space point that cannot be replicated and the subsequent impact on water flow through the disturbed matrix (discussed above).

Life cycle stages of subsurface biofilms

Biofilms appear to be an inherent phenotype for most microorganisms studied to date. The basic cycle of at least bacterial biofilms is attachment or adhesion, maturation and detachment (Hall-Stoodley, Costerton and Stoodley 2004). Typically, surface

properties control cellular attachment while mass transport of substrates (influx) and/or products (efflux) limits overall biofilm maturation/growth. Detachment can be caused by a variety of conditions that result in desorption, detachment and/or dispersion dependent upon varying geochemical and geophysical conditions.

Adhesion

Typically, microbial attachment is reversible (Dowd, Herman and Maier 2000), which could be beneficial for subsurface microorganisms subjected to environmental perturbations. The exact conditions that promote some microorganisms in the shallow subsurface to initiate attachment is unknown, but most likely includes physical (e.g. cell charge and flow) and chemical (e.g. pH and conductivity) parameters as well as biological (e.g. aggregation). While this has not been explicitly explored in shallow, subsurface biofilms, it is likely that the attachment of biofilms within the subsurface enhances survival in nutrient limited conditions by creating a microenvironment distinct from surrounding conditions (Coombs et al. 2010). Previous work mostly with *Pseudomonas* but also others (e.g. *Shewanella*), has compared vertical and horizontal attachment in reference to flow forces and the ability of cells to attach or detach (Conrad et al. 2011; Bennett et al. 2016). Initial attachment and any subsequent cell division has a direct impact on the architecture of the biofilm. For biofilms on porous media similar to shallow subsurface sediments and for those with slow flow regimes that impact mass transport variables (e.g. advection, dispersion and mass flux), the studies are quite limited.

It is likely that biofilms in the oligotrophic subsurface are non-continuous or patchy and that surface substrate and community composition impact adhesion. Biofilms from injections of radiolabeled cells into intact sediment cores dominated by quartz showed cell attachment around particular mineral grains suggesting a mineral preference for the adsorbed bacteria (Dong et al. 1999). Bacterial adhesion has been shown in laboratory studies to increase in areas where quartz sands have been artificially coated with metal oxyhydroxides (Scholl et al. 1990; Mills et al. 1994). Other studies have demonstrated different populations are enriched on different materials (Reardon et al. 2004; Bollmann et al. 2010; Converse et al. 2015). Further work is needed to better understand the physical forces under low fluidization that can promote or deter microbial biofilms under oligotrophic conditions and different mixing regimes (Fig. 2).

Growth/Dispersal

The formation and growth of biofilms in subsurface habitats is likely dependent on the microbial assemblages present, nutrient availability, substrate composition and hydraulic residence time (Coombs et al. 2010). Biofilm thickness is highly variable and ranges from the thickness of single cells to thicker microcolonies that are adhered together by extracellular polymeric substances. Thin and/or patchy biofilms are usually not limited by diffusion (Rittman 1993) and most likely represent biofilms in undisturbed, oligotrophic (i.e. pristine) subsurface environments. However, sediment biofilms can be thicker under different conditions that change over time and space, for instance, during biostimulation when nutrients are added.

Total cell numbers of bacteria that have been documented in groundwater ecosystems have ranged between 10^2 and 10^6 cells/mL of groundwater (Griebler and Lueders 2009); however, these values may be dependent on hydrological fluctuations

overtime (Velasco-Ayuso et al. 2009). While micro-eukaryotes have also been found within subsurface groundwater environments, reported observations have shown that the majority of cellular biomass in the subsurface is bacterial and archaeal (Griebler and Lueders 2009; Valster et al. 2009; Zinger, Gobeta and Pommiers 2012).

In comparison for subsurface matrix material, cell counts range from 10^4 to 10^9 cells/g sediment (Turco and Sadowsky 1995; Balkwill and Boone 1997; Griebler and Lueders 2009). These ranges typically vary with depth (Lin et al. 2012a), pH (Fierer and Jackson 2006), soil and sediment texture and porosity (Schwoerbel 1961; Balkwill and Ghiorse 1985; Strayer 1994; Hahn 2006), redox conditions, dissolved oxygen, mineral content and moisture content (Sirisena et al. 2014). More recently, the distribution of viruses within subsurface groundwaters and the impact on microbial abundance has been studied (Pan et al. 2017). Thorough reviews on cell count abundances within different areas of the subsurface have been synthesized (Goldscheider, Hunkeler and Rossi 2006; Akob and Küsel 2011) and cell count surveys have consistently shown differences in the abundances between attached and free-living phases (Griebler and Lueders 2009) dependent upon variations in physicochemical parameters (Sinclair and Ghiorse 1989). For example, microbial population density estimates correlated positively with sand content and pore-water pH and declined with clay content and pore-water heavy metals (Sinclair and Ghiorse 1989).

In laboratory experiments, biofilm growth within granular/porous reactor systems has been shown to reduce pore spaces that leads to the blockage of pores and flow (Taylor and Jaffé 1990a,b; Cunningham et al. 1991), alteration of water retention (Or et al. 2007) and significantly reduces hydraulic conductivity (Rodriguez-Escales et al. 2016). Biofilms can further reduce permeability by the entrapment of fine grained or colloidal materials that block flow (Hama 1997; Hama et al. 2001). It has also been shown that fine textured materials have higher occurrences of clogging compared to coarse textured materials (Vandevivere et al. 1995). Undoubtedly, biofilms have significant impacts on the porosity and permeability in natural porous aquifer systems; however, it is likely that biofilm heterogeneity and distribution *in situ* will be different than observed in reactor and consolidated aquifer studies. Environmental biofilms *in situ* have been shown to be patchy rather than uniform in distribution and thickness, and conceptual models have been applied to microbial growth and transport in subsurface habitats (Vandevivere et al. 1995; Clement, Hooker and Skeen 1996; Ebigo et al. 2010). Historically, different models have been used to estimate unsaturated (Farthing and Ogden 2017) and saturated (Molnar et al. 2015) water flow in porous media relevant to the shallow subsurface; however, the lack of data and understanding of microbial processes in the shallow subsurface challenges the incorporation of microbial ecology and physiology into these models. Hopefully in the future, drivers of microbial biofilm assembly and maintenance (e.g. selection, dispersal and drift) can be investigated and modeled with respect to hydrological parameters (e.g. porosity, permeability and mixing).

Laboratory approaches to the study of subsurface biofilms

While not a focus of this mini-review, there are numerous examples of using laboratory experiments to study subsurface microbial transport under unsaturated and saturated conditions (Tufenkji, Redman and Elimelech 2003; Jordan et al. 2004;

Gargiulo *et al.* 2007; Harvey, Harms and Landkamer 2007; Bradford, Schijven and Harter 2015). Laboratory experiments are routinely used to mimic and investigate environmental subsurface processes (*i.e.* grain size distribution, biofilm thickness/diffusion, biodegradation, pore clogging, flow, mass transport and hydraulic conductivity), and different reactor and incubation conditions (*e.g.* column, flat plate and serum bottles) and surrogate sediments (*e.g.* silica beads/sand or collected sediment core material) are used in various combinations depending on the process being investigated. When sediment cores are taken for laboratory studies, the pore structure may be altered by packing and repacking that results in porous media flow and transport properties significantly different from *in situ* conditions. Thus, an iterative approach combining field and laboratory studies is beneficial for ensuring laboratory findings that hold relevance to the natural system while maintaining controlled laboratory conditions necessary for developing and testing predictive models. For example, with column reactors filled with different-sized silica beads (coarse and fine), coarse sediments had higher biofilm biomass and activity although overall functionality was impaired (activity and diversity) (Perujo *et al.* 2017). Fine beads constrained biofilm activity and biomass while bead-size transitions promoted increased OM degradation and biomass at the interface (Perujo *et al.* 2017). The results corroborate the notion that particle size impacts interstitial fluxes and mixing, and thereby biofilm growth and activity for sandy sediment. Future work is needed to further elucidate these relationships under various conditions of flow, substrate flux and biofilm accumulation/activity. The hydrological impacts in the capillary fringe and water table boundary could affect biofilm dynamics in different ways that result in varying levels of biofilm diversity and activity (Fig. 2).

Hydrogeochemical mixing

As stated above, mixing can impact taxonomic diversity in groundwater communities and recent studies suggest it also affects sediment associated communities in similar ways (Ebrahimi and Or 2016, 2018). However, due to chemical (mineralogy) and physical (*e.g.* size, arrangement) heterogeneity of the sediment matrix, niche partitioning and species filtering are likely additional factors that impact the composition of attached communities. In addition, mixing in the shallow subsurface due to faster and shorter local flow paths could impact local hydrodynamic dispersion and thus biofilms. Therefore, sediment biofilms likely have distinct zone-specific responses (*e.g.* vadose to capillary fringe to saturated zones) (Fig. 1). Also, the subsurface sediment zones likely experience different degrees and rates of flow that impact the formation and stability (chemical, physical, and biological) of sediment-associated biofilms, and the biofilms are impacted by fluctuations of the water table and associated re-distribution in the capillary fringe (Moser *et al.* 2003; Stegen *et al.* 2016).

Based on the available information from diversity-based studies, bulk activities and biofilm studies, hydrodynamics likely affects biofilm structure, function and dispersal in shallow subsurface aquifers both by vertical and horizontal mixing. In high shear environments (*e.g.* water distribution lines), high shear stress is observed to decrease biofilm diversity and thickness (Rochex *et al.* 2008); however, the relationship between mixing and biofilm diversity is not known for low-shear conditions analogous to shallow subsurface environments that can have unsteady groundwater flow (Sposito 2006). If required resource ratios are not available, microbial activity cannot be

sustained and continued non-growth could promote dispersal and/or death. Some work has attempted to explain the occurrence of microbial populations in terms of a resource ratio theory, where a given level of resource is needed to sustain a population (*i.e.* a consumption rate that is greater than a death rate at a given resource concentration) (Smith 1993; De Mazancourt and Schwartz 2010). However, this relationship does not account for varying substrate affinities, interacting populations or different behavior across phase boundaries under mixing conditions. Recently, it has been hypothesized that biofilm cells with restricted growth can outcompete populations with faster growth in the bulk-phase based upon a laboratory model (Coyte *et al.* 2016). This is an interesting hypothesis to test relevant conditions for the shallow subsurface that includes different populations, interactions and/or activities in a porous medium with dynamic mixing in the shallow and deep saturated zone (Fig. 2).

Microbial interactions within biofilms

Many shallow subsurface biofilms are likely comprised of multiple species as in numerous other environments; however, few studies have delineated the spatial arrangement of microbial cells on particles from the shallow subsurface nor have deciphered potential metabolic interactions *in situ*. Certainly, laboratory studies of multispecies biofilms observe that populations are not always randomly distributed but organized based on needs (Møller *et al.* 1998; Watnick and Kolter 2000). Conducting such studies with native material under *in situ* conditions are challenging. Recent work with upper layer soil/sediment particles have shown particle-specific communities (Jackson and Weeks 2008; Hemkemeyer *et al.* 2015, 2018), but similar work for shallow subsurface sediments (>1 m depth) is sparse. From cell counts, one cannot determine whether attached populations reside as individual cells separated by micrometers of space, as clonal microcolonies or as multispecies biofilms over preferred locations (*e.g.* nutrient/mineral availability). This is also an issue with community analyses via amplicon or shotgun sequencing. Samples large enough to yield sufficient DNA quantities often encompass too much physical space to confidently infer representative microscale interactions, although progress has been made with upper layer soil particles. Future work is needed to elucidate whether sediments from the shallow subsurface are amenable to the same methods and if microscopy methods can be used with intact sediment samples in order to retain inherent physical structure.

FUTURE DIRECTIONS

It has become increasingly apparent that free-living and biofilm associated cells have distinct physiologies and function but the potential impacts on shallow subsurface systems is not well understood (Hall-Stoodley, Costerton and Stoodley 2004; Anneser *et al.* 2010). Many questions remain regarding the biofilm 'life-cycle' including attachment transitions, the distribution and rate of specialized and general activity, cooperative/competitive interactions, and mechanisms of dispersal in the shallow subsurface mixing zones (Fig. 2). Due to sampling challenges and the complexity of the heterogeneous subsurface matrix that ranges across the vadose, capillary fringe and saturated zones, few field sites have been comprehensively described and studied despite the important ecosystem services associated with shallow subsurface systems. The shallow subsurface has historically been considered a stable environment,

but it is now clear that temporal and seasonal dynamics influence hydrological mixing, particularly between and within the saturated and capillary fringe zones. Aquifer recharge and fluctuating water table can occur via seasonal patterns, and not surprisingly, the transition zones between the variably saturated and saturated zones has been shown to be an important ecotone for microbial diversity and activity. Due to the complexity of the system and logistical challenges in sampling, there is much yet to be learned about the distribution of shallow subsurface biofilms, the physiological activities/responses to environmental disturbances related to geochemical cycling and the roles these systems play in groundwater maintenance and stability. Technological advances for sample retrieval and fine-scale analyses (spatial, temporal, cellular) of subsurface samples are needed, including samplers that can retrieve intact porous media and associated biofilms. Similarly, engineered reactor systems need to be modified to accurately simulate subsurface environmental conditions and address inconsistencies in reproducibility that currently exist.

In order to understand and predict the role of microorganisms accurately within an environmental context, it is essential to distinguish between active and inactive organisms. The majority of studies on activity in subsurface porous aquifers are from the 1980–1990's and very few recent studies incorporate activity measurements with sequencing technologies. While the more recently adopted metagenomics-based sequencing approaches have opened new windows as to the functional diversity present within porous aquifers, activity is seldom linked to phylogeny. Of the open reading frames recovered with metagenomic sequencing, typically the functions from a small fraction can be linked to known genes and only a few of these genes have been studied in depth (Ferrer et al. 2016). Currently, untargeted metagenomic sequencing predominantly retrieves genomic sequences from dominant organisms and does not allow active organisms to be differentiated from inactive. While this is more suitable for low diversity habitats (Tyson et al. 2004; Woyke et al. 2006), subsurface environments can be highly diverse (Hug et al. 2015). Therefore, in order to accurately capture rare, underexplored and possibly environmentally significant metabolic processes, it will be imperative to apply functional/targeted metagenomic approaches. While the use of sequencing technologies has allowed microbial ecologists to glean the taxonomic compositions of microbial communities as never before, it is important to note that such methodologies contain inherent problems (Wintzingerode, Göbel and Stackebrandt 1997; Bent and Forney 2008; Fraser et al. 2009). Recently, Carini et al. (2016) showed that extracellular, or 'relic', DNA was abundant in a variety of soil and sediment types and that this DNA can skew diversity measurements.

Due to the rapid evolution of technologies capable of working with small DNA and transcript quantities, the application of targeted strategies (i.e. sequencing data within a functional context) has increased for environmental studies (Lueders et al. 2016). Approaches already exist that target active fractions of microbial communities (e.g. bioorthogonal non-canonical amino acid tagging (BONCAT) (Hatzepichler and Orphan 2015), DNA and RNA stable isotope probing (SIP) (Lueders et al. 2016) and propidium monoazide (PMA)-Seq (Carini et al. 2016)) which can then be combined with metagenomic or rRNA sequencing strategies. In the near future, these methods will be combined with microscopic/spectroscopic techniques that allow physical structure to be maintained and the proper scale for sediment-associated biofilms to be determined.

The ability to infer subsurface-specific functional capabilities from genetic information, as well as the generation of testable hypotheses that can be confirmed at ecologically relevant microscales, is limited by the current lack of subsurface-specific reference sequences. In addition, laboratory studies are needed at the microscale with field-relevant isolates to confirm hypotheses generated from sequencing data. Of the currently available subsurface isolates, many have slow growth rates and/or most likely use forms of C, N and P associated with sediments not typically used in cultivation/microcosms. It is becoming increasingly crucial to frame *in situ* experimentation to ecological questions and conditions pertinent to the respective environment (e.g. subsurface transport through porous media with intermittent inputs of temporally- and spatially-relevant OM). As noted by Prosser (2012), many microbial ecology questions require studies that focus on smaller spatial scale, phenotypic diversity, temporality and activity/rates. Through these combined approaches, a more complete understanding of shallow subsurface ecosystems will be gained that includes biofilm dynamics at zone interfaces.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](http://FEMSEC.org) online.

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