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Non-random processes determine the colonization of groundwater sediments by microbial communities in a pristine porous aquifer

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Originality-significance statement

Groundwater bodies are the largest terrestrial habitat for microorganisms on Earth, where the majority of the microbial biomass lives attached to sediment surfaces. In these unique, low-productivity environments, microbial communities are the drivers of key biogeochemical processes and furthermore provide important services to society like maintaining groundwater quality as one of the most important sources of freshwater and drinking water worldwide. Over the past years, studies on sediment-attached communities in groundwater-surface water mixing zones as well as surface-attached biofilms in other, non-subsurface habitats have provided important insights regarding the ecological processes that drive the assembly of these microbial communities. Compared to most of these environments, however, pristine groundwater is characterized by significantly lower levels of energy and productivity as well as comparatively more stable environmental conditions, which may promote the effect of stochastic processes on community assembly. Moreover, the microbial communities that colonize subsurface sediments typically exhibit much lower cell densities and occur as small, spatially separated micro-colonies rather than dense, coherent biofilms as they are found in other non-subsurface environments. Therefore, our study was motivated by the question whether findings on the processes that govern microbial community assembly and succession of surface-attached communities in those other more dynamic and nutrient-rich environments also apply to sediment-attached microbial communities in pristine groundwater environments. Our study shows intriguing similarities between the community succession on newly-colonized sediments in our investigated porous, pristine aquifer and succession patterns observed for biofilms in other more dynamic aquatic environments, indicating that the assembly of microbial communities on surfaces may be governed by similar underlying mechanisms across a wide range of different habitats. Our results indicate that differences between planktonic and sediment-attached communities often reported for groundwater environments are not the result of purely stochastic events, but that sediment surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way. Furthermore, our data suggest that specific genera, especially within the *Comamonadaceae* and *Oxalobacteraceae*, played a particularly important role in this process.

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Summary

Sediments accommodate the dominating share of groundwater microbiomes, however the processes that govern the assembly and succession of sediment-attached microbial communities in groundwater aquifers are not well understood. To elucidate these processes, we followed the microbial colonization of sterile sediments in *in situ* microcosms that were exposed to groundwater for almost one year at two distant but hydrologically connected sites of a pristine, shallow, porous aquifer. Our results revealed intriguing similarities between the community succession on the newly-colonized sediments and succession patterns previously observed for biofilms in other more dynamic aquatic environments, indicating that the assembly of microbial communities on surfaces may be governed by similar underlying mechanisms across a wide range of different habitats. Null model simulations on spatiotemporally resolved 16S rRNA amplicon sequencing data further indicated selection of specific OTUs rather than random colonization as the main driver of community assembly. A small fraction of persistent OTUs that had established on the sediments during the first 115 days dominated the final communities (68%-85%), suggesting a key role of these early-colonizing organisms, in particular specific genera within the *Comamonadaceae* and *Oxalobacteraceae,* for community assembly and succession during the colonization of the sediments. Overall, our study suggests that differences between planktonic and sediment-attached communities often reported for groundwater environments are not the result of purely stochastic events, but that sediment

surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way.

Introduction

The groundwater-saturated zones of the terrestrial subsurface are one of the largest habitats for microorganisms on Earth (Griebler and Lueders, 2009; McMahon and Parnell, 2013). In these unique, low-productivity environments, microbial communities lie at the heart of key biogeochemical processes like the turnover of carbon and other nutrients, mineral cycling, or pollutant degradation (Griebler et al., 2014; Griebler and Avramov, 2015). Sediment-attached communities play a particularly important role in these ecosystems as they represent the bulk of the microbial biomass and activity (Lehman et al., 2001; Griebler et al., 2002; Zhou et al., 2012; McMahon and Parnell, 2013). Previous studies have repeatedly shown that the composition of sediment-attached communities can differ substantially from planktonic communities suspended in the surrounding groundwater (Zhou et al., 2012; Flynn et al., 2013; Hug et al., 2015). However, the ecological processes that give rise to these differences during community assembly and succession are not well understood. Recent studies have suggested a strong link between biogeochemical functions and microbial community composition as well as the underlying ecological assembly processes (Graham et al., 2016a; Graham et al., 2016b; Graham and Stegen, 2017). Therefore, a better understanding of the processes that drive the assembly of sediment-attached microbial communities in groundwater environments is a key step towards a better understanding of the functioning of these ecosystems ~~functioning~~.

The question of the influence of deterministic (or niche-based) versus stochastic (or neutral) processes on the assembly, succession, and diversity of microbial communities has increasingly sparked the curiosity of microbial ecologists over the past years (for reviews see Nemergut et al. (2013); Zhou and Ning (2017)). Deterministic theory assumes that environmental factors, both biotic and abiotic, determine the composition and structure of a community by selecting for species with certain traits that enable them to thrive and compete in a given environment (also known as species sorting or environmental filtering). Accordingly, under similar environmental conditions, communities at different locations or points in time are expected to be composed of species with similar traits. In contrast, the stochastic point of view holds

that communities are assembled randomly from species with equivalent trait spectra, and that differences in community composition and structure are the result of random events, for example caused by species dispersal or drift due to stochastic birth-death events (Chase and Myers, 2011). Although purely neutral models have been shown to explain observed diversity patterns of microbial communities with surprising accuracy (e.g. Woodcock et al. (2007); Ofiţeru et al. (2010); Woodcock and Sloan (2017)), there has been growing consent that both deterministic and stochastic processes can act simultaneously and that the contribution of either process can shift over time ~~and/~~or with changing environmental conditions (Dumbrell et al., 2009; Ofiţeru et al., 2010; Chase and Myers, 2011; Langenheder and Székely, 2011; Ferrenberg et al., 2013; Stegen et al., 2013; Wang et al., 2013; Zhou et al., 2014; Dini-Andreote et al., 2015; Stegen et al., 2015; Stegen et al., 2016b; Veach et al., 2016; Graham et al., 2017).

One aspect where the interaction between deterministic and stochastic processes comes into play is the initial assembly of communities and the following species succession in newly-colonized environments (Tilman, 2004; Langenheder and Székely, 2011), for instance during the development of biofilms on initially empty surfaces (Jackson, 2003; Battin et al., 2007). At the initial stage of colonization, the arrival of species in a new environment is often driven by stochastic dispersal (Tilman, 2004; Ferrenberg et al., 2013; Dini-Andreote et al., 2015), which can overrule deterministic effects in homogeneous environments ~~and/~~or if environmental filtering between the environment of the source community and the newly colonized environment is weak (Stegen et al., 2012; Wang et al., 2013; Battin et al., 2016). However, once established, resident species can affect the establishment of newly-arriving species (positively or negatively) during the subsequent succession directly via species interactions or indirectly by modification of their environment (Fukami, 2015). Thus the order and timing of species arrival, although initially stochastic, can determine the composition and functioning of the final community, known as priority effect (Fargione et al., 2003; Tilman, 2004; Fukami et al., 2010; Peay et al., 2012; Tan et al., 2012; Nemergut et al., 2013; Rummens et al., 2018; Svoboda et al., 2018).

A general, conceptual model that summarizes the successional stages during biofilm development has been described by Jackson (2003). According to this model, initially empty surfaces offer ample space

and resources to allow for the establishment of diverse microorganisms resulting in a rapid increase in species richness and diversity that is fueled by the dispersal of newly-arriving species from a regional species pool like overlaying water during initial biofilm assembly. The steady arrival of new species eventually leads to niche depletion and growing competition between established and newly-arriving species, which more and more suppresses the increase in species richness. As the competition intensifies, less competitive species are lost from the community, which leads to a decline in species richness after the initial phase of community assembly. However, as the biofilm matures further and becomes more heterogeneous, new niches are created that enable specialized species to establish, leading again to an increase in richness and diversity in the mature biofilm. Although Woodcock and Sloan (2017) have demonstrated using a neutral modeling approach that these patterns can be explained based on stochastic processes only, empirical evidence suggests that the assembly of biofilm communities is in fact characterized by a shift from initially stochastic community assembly towards deterministically driven succession at the later stages, for instance caused by species interactions or growing niche space due to increasing spatial and chemical heterogeneity (Martiny et al., 2003; Lyautey et al., 2005; Battin et al., 2016; Veach et al., 2016; Brislawn et al., 2018).

To date, most of the studies on ecological processes behind the assembly of microbial communities in groundwater environments have focused on planktonic communities suspended in the groundwater (Stegen et al., 2012; Stegen et al., 2013; Beaton et al., 2016; Danczak et al., 2018), while studies on sediment-attached communities are scarce (Stegen et al., 2016a). In contrast, much insight has been gained over the past years into the assembly of sediment-attached communities in groundwater-surface water mixing zones (hyporheic zone). In these studies, the assembly of planktonic communities generally tended to be more subject to stochastic effects and shifts in assembly processes related to changes in water chemistry, whereas selection had a relatively more pronounced effect on the assembly of sediment-attached communities which, at the same time, was less affected by hydrochemical changes (Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017; Stegen et al., 2018). Compared to the hyporheic zone, pristine groundwater environments (in the absence of surface water impacts) are more

stable and only experience little environmental changes (Griebler and Lueders, 2009), which may promote the effect of stochastic processes on community assembly compared to more dynamic environments (Ofiţeru et al., 2010; Stegen et al., 2012; Wang et al., 2013; Zhou et al., 2013). Moreover, in contrast to the typically studied biofilms in other environments like surface waters, which form dense, spatially coherent, heterogeneous structures that can reach a thickness in the range of several hundred micrometers (Battin et al., 2016), sediment-attached microbial communities in groundwater aquifers occur as small, patchily distributed micro-colonies that consist of only a few cells (Schmidt et al., 2017), which may be hypothesized to be more prone to stochastic effects than their biofilm counterparts in other environments.

In this study we set out to 1) investigate whether the assembly of sediment-attached microbial communities in pristine groundwater environments can be explained by the general patterns observed for surface-attached biofilms in other environments; 2) study the importance of early colonizers for community succession during the colonization of the sediments; and 3) see if the dominating role of selection on community assembly that has been observed for sediment-attached microbial communities in the hyporheic zone also drives community assembly in comparatively stable, pristine groundwater environments. To tackle these goals, we incubated *in situ* microcosms filled with sterilized sediment in monitoring wells at two distant but hydrologically connected sites of a pristine, porous aquifer (Zhou et al., 2012), and followed the succession of the microbial communities as they colonized the sediments over a period of almost one year. We used 16S rRNA amplicon sequencing data to study changes in alpha and beta diversity of the sediment-attached communities incubated at the two sites as well as differences in community composition between sediment-attached and planktonic communities in the surrounding groundwater over the course of the succession. To explore the influence of deterministic and stochastic processes on microbial community assembly and succession, we applied the null model approach developed by Stegen et al. (2012; 2013), which has previously also been used in studies on community assembly in the hyporheic zone (Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017; Stegen et al., 2018) as well as biofilms in other environments (Langenheder et al., 2017; Brislawn et al., 2018), and thus allows us to compare our results to those previous findings.

Results

Site description

The field experiment was conducted over a period of 347 days from March 2010 until February 2011, with intermediate sampling campaigns in May (day 49), July (day 115), and December (day 263). The area with the two monitoring wells used for the incubation of the *in situ* microcosms was located at the foothills of the Bavarian Alps in the upper Isar River valley close to the village of Mittenwald, Germany (Fig. 1). The wells were installed in a pristine, shallow, porous aquifer composed of quaternary sediment mainly consisting of gravel and coarse sand. Well MIT052 was located on a mountain pasture in the forested Riedboden nature reserve 400 m away from the nearby river; well MIT039 was located approximately 2 km away from MIT052 in proximity to the village with a distance of 240 m to the river (for a detailed site description, see Zhou et al. (2012)). Over the course of the experiment, we observed only small fluctuations in physicochemical conditions (Table 1; the temporal dynamics of the individual parameters are shown in Fig. S1).

Temporal dynamics of microbial biomass and alpha diversity of sediment-attached and planktonic microbial communities

Despite the spatial distance between the two sites, the microbial communities that developed on the initially sterile sediments followed identical trends in alpha diversity and biomass patterns (Fig. 2). Already after the first 49 days, the microbial biomass (measured as prokaryotic cell counts) of attached microbial communities at both sites had reached a plateau of ~10^7 cells cm^{-3} of sediment followed by a slight decline for the remaining time of the experiment. Although the biomass of sediment-attached microbial communities stayed more or less constant, noticeable changes in the communities still occurred as indicated by OTU richness and diversity which steadily increased by about 50% and 25%, respectively, from May until December, followed by a decline of both parameters in February. Over the same period, community evenness remained relatively high and only changed moderately. The changes in biomass and

alpha diversity observed for the newly colonized sediments appeared to be independent from the changes that occurred in the planktonic microbial communities, which were more variable over time and less comparable between the two sites. Microbial biomass was about one to two orders of magnitude lower for the planktonic communities compared to the sediments.

Establishment and persistence of newly-arriving OTUs in sediment-attached microbial communities

To assess the impact of early colonizers on microbial community succession, we looked at the number of newly-arriving OTUs that entered the developing sediment-attached communities at each time point over the course of the experiment (Fig. 3). Newly-arriving OTUs are defined here as OTUs that showed an abundance > 0% in the community for the first time at a given time point. At both sites, the number of newly-arriving OTUs declined over time showing that the majority of OTUs had established during the initial phase of the incubation. Despite this declining trend, the fraction of newly-arriving OTUs relative to the total OTU richness at the end of the incubation was still noticeable with ~15-20%. However, looking at the changes in the cumulative relative abundances of the newly-arriving OTUs over time, we saw that the OTUs that had arrived towards the later stages only accounted for a relatively small fraction of the final communities. Even though the cumulative relative abundance of OTUs that had established in the communities within the first 49 days steadily declined, these OTUs still made up 36% and 47% of the final communities at MIT052 and MIT039, respectively. At MIT052, these OTUs together with those that emerged at the following time point in July comprised the majority of the final community at the end of the incubation (together 85%), while OTUs that arrived at the final time point accounted for only 5%. At MIT039, OTUs that had arrived at the first two time points made up for 68% of the final community, whereas OTUs that had arrived in December and February comprised 12% and 20%, respectively. Although these results clearly show the dominance of early-colonizer OTUs in the final communities, a closer look at how many of these OTUs actually persisted until the final time point showed that only ~12% of newly-arriving OTUs from each time point were still present in the final communities (data not shown). Looking at the taxonomies of these newly-arriving OTUs that persisted until the end of the

incubation, we again found highly similar patterns for both sites (Fig. S2). At each time point, the most dominant groups were OTUs affiliated to *Comamonadaceae*, mainly *Aquabacterium* and *Polaromonas* spp., in addition to *Oxalobacteraceae*, mainly consisting of *Duganella*, *Massilia*, and *Undibacterium* spp., as well as *Pseudomonas* spp. and diverse *Caulobacteraceae* and *Sphingomonadaceae*.

Microbial community composition and beta diversity

Similar to the alpha diversity patterns, the microbial communities on the newly colonized sediments displayed comparable compositions at the two sites (Fig. 4). Especially during the initial phase of the incubation in May, sediment-attached communities at both sites were dominated by *Oxalobacteraceae* in addition to *Comamonadaceae* and smaller fractions of *Flavobacteriaceae* and *Caulobacteraceae*. Over the further course of the incubation, these taxonomic groups gradually receded and were in part replaced mainly by increasing numbers of *Comamonadaceae* (mainly *Aquabacterium* spp.), *Pseudomonadaceae*, *Nocardiaceae*, and *Rhodocyclaceae* especially at MIT052, in addition to *Sphingomonadaceae*, uncultured *Deltaproteobacteria*, and *Moraxellaceae* at MIT039. Moreover, OTUs affiliated with diverse low-abundant families (with an abundance <10% in the entire dataset; mean = 0.1%; max. = 4.7%) gradually increased in abundance. In contrast, planktonic communities were mainly dominated by members of the *Rhodocyclaceae*, *Comamonadaceae* (mainly *Curvibacter*, *Simplicispira*, and *Rhodoferax* spp.), and *Leptospiraceae*.

To get a better picture of the organisms that were responsible for differences between sediment-attached and planktonic communities, we performed similarity percentage (SIMPER) analysis across all samples on relative abundances of OTUs grouped at genus level. Interestingly, we found high agreement between the genera that significantly contributed to the observed dissimilarities between the two types of communities and the taxa identified as successful, persistent colonizers in the previous analysis (Fig. S2). *Aquabacterium*, *Massilia*, and *Duganella* spp. ranked among the genera that contributed most to the dissimilarity (together > 15%; all $p < 0.002$) and were highly differentially abundant in the sediment-attached communities, next to *Flavobacteria* and uncultured members of the *Oxalobacteraceae* (Fig. S3).

The changes in microbial community composition over time as well as differences between sediment-attached and planktonic communities in the groundwater were revealed by non-metric multidimensional scaling (NMDS) performed on abundance-weighted β-mean nearest taxon distance (β-MNTD) between communities (Fig. 4). At all measured time points, sediment-attached and planktonic communities clustered separately from each other as reflected by the distinct separation of the two types of communities along the first NMDS axis. Changes in microbial community composition over time were reflected by the separation of data points along the second NMDS axis. Permutational analysis of variance (PERMANOVA) revealed that community type (i.e. sediment-attached vs. planktonic) explained most of the variance in β-MNTD between communities (R^2 = 0.626; p = 0.001), followed by sampling time point (R^2 = 0.104; p = 0.001), while site location was not significant (R^2 = 0; p = 1), showing that communities across sites were similar within each community type and time point. Moreover, fitting environmental variables to the NMDS ordination with stratification of permutations within the community types did not reveal significant correlations between changes in community composition and any of the measured physicochemical parameters (Table 1 and Figure S1) (all R^2 < 0.32; p > 0.1).

Since community type explained most of the variance in beta diversity, we applied partitioning of beta diversity to identify the underlying causes of the differences between sediment-attached and planktonic communities within sites and sampling time points according to Baselga (2012). This approach is based on the additive partitioning of incidence-based Jaccard dissimilarity between two communities into a nestedness and a turnover component. A high contribution of nestedness to the total dissimilarity indicates that two communities are subsets of each other and that differences are caused by differences in species richness (i.e. gain or loss of species). On the other hand, a high contribution of turnover indicates little overlap in species composition, i.e. species in one community have been replaced by other species in the other community. The analysis showed that turnover was the dominating process behind the differences between the two types of communities at each time point (for all time points > 97%) (Fig. S4), showing that both community types were composed of distinct sets of OTUs.

We applied the same approach to the dissimilarity between sediment-attached communities at different time points within sites to investigate the degree to which nestedness and turnover contributed to changes in community composition over the course of the sediment colonization. Also in this case turnover dominated over nestedness in all comparisons, especially over long time scales (i.e. comparing communities between May and February; 95-97%), and with a slightly weaker effect over short time scales of the succession (i.e. comparing communities between consecutive time points; 74-90%) (Fig. S5).

Impact of stochastic and deterministic processes on community assembly and succession inferred from null models

To infer the impact of deterministic and stochastic processes on community assembly, we applied the two-tiered null model approach developed by Stegen et al. (2012; 2013). Briefly, under the assumption that phylogenetically closely related species occupy more similar ecological niches than less closely related species, the strength of deterministic processes is evaluated in tier one based on the β-nearest taxon index (β-NTI). β-NTI < -2 and > +2 indicates that two communities are phylogenetically significantly more or less similar to each other than expected by chance, which is interpreted as homogeneous selection (i.e. selection of similar OTUs) or variable selection (i.e. selection of dissimilar OTUs) in the two communities, respectively. |β-NTI| < 2 indicates that two communities are as dissimilar as expected by chance, hinting at stochastic community assembly. In this case, the RC_{bray} index is used in tier two to evaluate the effect of stochastic dispersal. RC_{bray} < -0.95 and > +0.95 means that two communities share significantly more or less OTUs than expected by chance, indicating that communities are assembled by homogenizing dispersal or dispersal limitation in combination with drift, respectively. $|RC_{bray}| < 0.95$ indicates that differences between two communities are due to random drift acting alone. We applied this approach to study the role of deterministic and stochastic processes on three levels (Fig. 5): 1) spatial community turnover between the two sites within community types and time points; 2) turnover between sediment-attached and planktonic communities within sites and time points; 3) temporal turnover between communities at consecutive time points within community types and sites.

Deterministic and stochastic processes had different influences on the spatial community turnover for sediment-attached and planktonic communities, respectively. In case of planktonic communities, pairwise comparisons between sites for each time point resulted in values for β-NTI that were not significantly different from the null expectation, indicating that differences between the planktonic communities at the two sites were caused by stochastic processes. Pairwise comparisons based on the RC_{bray} index identified homogenizing dispersal as the dominating process (all RC_{bray} = -1; the only exception was observed for December: RC_{bray} = +0.99, indicating dispersal limitation together with drift; data not shown). In contrast to the predominantly stochastic exchange of OTUs between the two sites via dispersal through the groundwater, pairwise comparisons of sediment-attached communities clearly tended towards homogenous selection as main cause of the similarities between the sediment-attached communities (with one exception observed for July where β-NTI was not significant, but a slightly significant RC_{bray} = 0.97 hinted at dispersal limitation together with drift).

Since the sediments had to be colonized by microorganisms that were recruited from the surrounding groundwater, even though beta diversity partitioning revealed little overlap between these two communities as discussed above, we tested for the effect of selection processes on the assembly of sediment-attached communities from planktonic microorganisms suspended in the groundwater. The differences between the communities on the newly colonized sediments and the planktonic communities at each site were caused by stochastic process during the first 115 days of the incubation. Pairwise comparisons based on RC_{bray} hinted at dispersal limitation in combination with drift as the processes responsible for these differences (all RC_{bray} = +1; data not shown). This trend changed at the later stage in December after 263 days; at this point significantly positive values for β-NTI hinted at variable selection of phylogenetically distinct OTUs in sediment-attached communities compared to the microorganisms in the surrounding groundwater.

Unlike the trends observed for the spatial community turnover, the influence of deterministic and stochastic effects on changes in community composition that occurred over time was much more variable and no clear trends could be observed. Although selection effects appeared to have played a role (both

286 homogenous and variable selection), they mostly did not occur consistently at both sites for neither
287 sediment-attached nor planktonic communities.

288 We used Mantel correlation analysis to investigate whether changes in individual physicochemical
289 parameters in the groundwater had an effect on the changes in assembly processes (based on β-NTI).
290 Similar to the lack of correlations between environmental variables and differences in community
291 composition mentioned above, we did not find significant effects of changes in environmental conditions
292 in this analysis for neither planktonic (all |Spearman's rho| < 0.34; p > 0.08) nor sediment-attached
293 communities (all |Spearman's rho| < 0. 27; p > 0.1).

294

295 **Discussion**

296 The alpha diversity patterns for the newly-colonized sediments at both sites followed identical trends that
297 closely matched the conceptual model for the formation of biofilms on empty surfaces outlined by Jackson
298 (2003), which describes changes in alpha diversity over three main stages of biofilm development. At the
299 early stage, the large niche space of an initially empty surface allows for the establishment of diverse
300 microorganisms, resulting in a steady increase in alpha diversity, which subsequently levels off and
301 eventually declines due to niche depletion and the loss of less competitive species as the biofilm grows
302 over the course of the succession. However, at the final stage, the mature biofilm becomes increasingly
303 spatially and chemically heterogeneous, which opens new niches for specialized species to thrive and
304 thereby fuels a renewed increase in alpha diversity. Our results only deviated from this model at the final
305 stage of the incubation, where we did not see an increase in species diversity and richness~~, which~~
306 ~~according to the model should occur due to the presence of specialized niches that are brought about by~~
307 ~~the spatial and chemical heterogeneity in mature biofilms~~. However, this framework was conceptualized
308 for biofilms in resource-rich, high-productivity environments like activated sludge, wetlands, and lakes
309 (Jackson, 2003). Although we cannot exclude that alpha diversity may have increased again with a
310 prolonged incubation time, we may argue that diverse, specialized niches that develop in mature, spatially
311 heterogeneous biofilms might not form to such an extent in the small, patchily distributed micro-colonies

that typically colonize groundwater sediments (Schmidt et al., 2017). Hence, the total niche space in such micro-colonies may be smaller compared to mature biofilms in other environments, similar to what Graham et al. (2016a) have proposed for sediments in the hyporheic zone. Moreover, although the general pattern of decreasing fractions of newly-arriving OTUs was also apparent in our experiment, reflecting the saturation of niche space according to Jackson's biofilm model (Jackson, 2003), we noticed that the fraction of these OTUs at the end of the incubation was still 5-10 times higher compared to findings on biofilms in other environments (e.g. Brislawn et al. (2018)). These deviations of our results from assembly patterns of biofilms, together with the findings made for hyporheic zone sediments (Graham et al., 2016a), might point towards important differences in ecological niche structures between biofilms in resource-rich surface environments and sediment-attached microbial communities in the typically more energy-poor and less productive subsurface.

Looking at the abundance changes of newly-arriving OTUs over time, we saw that OTUs colonizing the sediments during the early stage of community assembly (i.e. the first 49 to 115 days) largely dominated the final communities at the end of the experiment. However, at the same time, these dominant OTUs represented only a small fraction of newly-arriving OTUs found at each time point. This was further reflected by the large dominance of OTU turnover over nestedness between successional stages in the sediment-attached communities inferred from beta diversity partitioning, showing that the majority of OTUs that had established at a given time point were in fact replaced by others over the course of the succession. Therefore, in agreement with the findings by Brislawn et al. (2018), the mere timing of OTU arrival did not seem to be a determining factor for the final community structure. Rather, the consistent dominance of specific taxa among these persistent OTUs (mainly genera belonging to the *Oxalobacteraceae*, *Comamonadaceae*, *Caulobacteraceae*, *Sphingomonadaceae*, in addition to *Pseudomonas* spp.) suggests the involvement of certain traits that enable these taxa to sustainably colonize and thrive on sediment surfaces. Interestingly, we also found the same genera among the most important contributors to differences between sediment-attached and planktonic communities and to be highly differentially abundant in the former. The association of these taxa with biofilms and traits that facilitate

surface colonization like motility or production of extracellular polysaccharides have been reported before for other environments (Kalmbach et al., 2000; Baldani et al., 2014; Bižić-Ionescu et al., 2014; Niederdorfer et al., 2016; Niederdorfer et al., 2017), supporting the hypothesis about their importance for the development of sediment-attached communities in our study. Over the course of the succession, these dominant OTUs may have facilitated the recruitment of other more diverse taxa that were observed at the later stages of the colonization (Battin et al., 2007; Nemergut et al., 2013; Fukami, 2015).

Comparisons of beta diversity patterns revealed that sediment-attached and planktonic communities, respectively, were similar at each time point across the two sampling locations. Using the null model approach developed by Stegen et al. (2012; 2013) revealed that different processes were responsible for the observed similarities. Whereas the spatial turnover of planktonic microbial communities was driven by stochastic processes, mostly homogenizing dispersal (75%), the high similarities between the sediment-attached communities at the two sites were mostly caused by homogenous selection (75%). We are aware that our study consists of only a relatively limited number of observations and therefore the results should be interpreted with the necessary caution. Nevertheless, our results fit observations on assembly processes for communities in the hyporheic zone (Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017; Stegen et al., 2018) as well as ~~for~~ biofilms in surface water streams (Besemer et al., 2012; Veach et al., 2016), suggesting that selection not only plays a determining role in the assembly of surface-attached microbial communities in those dynamic environments but also in pristine groundwater aquifers, despite the comparatively more stable environmental conditions, which have been shown to promote the effect of stochastic over deterministic processes in other environments (Ofiţeru et al., 2010; Stegen et al., 2012; Wang et al., 2013; Zhou et al., 2013). Mineral composition has previously been demonstrated to be a driving factor for microbial community composition and assembly (Grösbacher et al., 2016; Stegen et al., 2016a; Jones and Bennett, 2017). Since the *in situ* microcosms that we incubated at the two sites in our study were filled with sediment that originated from the same source, it is likely that identical sediment properties selected for the highly similar microbial communities at the two sites.

Given the high similarities between the sediment-attached communities at both sites throughout the experiment, we would have expected to also find similar patterns regarding the processes that drove the temporal microbial community turnover. However, contrary to this expectation, this was not fully the case as assembly was highly variable without a clearly discernable trend in favor of a single process. Changes in environmental conditions such as nutrient inputs, fluctuating water tables, or surface water-groundwater mixing have been observed to not only affect the composition of (groundwater) microbial communities, but also influence the ecological assembly processes that determine those changes (Lyautey et al., 2005; Stegen et al., 2012; Stegen et al., 2013; Zhou et al., 2014; Dini-Andreote et al., 2015; Stegen et al., 2015; Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017). However, in our case, we did not find indications that changes in physicochemical conditions of the groundwater were related to changes in community composition or shifts in ecological community assembly processes. This could suggest that the changes in community composition over time and the influence of deterministic versus stochastic effects were determined by changes in unmeasured environmental variables (Stegen et al., 2013). Alternatively, the observed lack of correlations between changes in environmental conditions and the processes that determined community assembly can also hint at the impact of endogenous factors like species interactions (Konopka et al., 2015; Battin et al., 2016; Cordero and Datta, 2016). Recently, Danczak et al. (2018) could show that interaction network structures can affect assembly processes of planktonic microbial communities in pristine aquifers. Although our results show that the assembly of sediment-attached communities was mainly deterministic, and that the succession of OTUs was highly reproducible between the two sites, the compositions of the two communities at each time point, and therefore possibly interaction networks, were not totally identical. Hence, the variable patterns of processes that determined the community turnover between successional stages at each site might, at least in part, be attributed to possible differences in interaction networks within the communities between the two sites.

An additionally important factor in the assembly and succession of surface-attached communities in aquatic environments is the invasion by species from the surrounding water phase (Battin et al., 2016).

The establishment of invading species in a biofilm community depends on both stochastic dispersal as well as interactions with already established species (Battin et al., 2007; Battin et al., 2016). Beta diversity partitioning showed that sediment-attached and planktonic communities were composed of distinct sets of OTUs. We again used the null model approach to test in how far deterministic and stochastic processes contributed to these differences. We found that over the first successional stages the turnover between sediment-attached and planktonic communities was caused by dispersal limitation acting alongside drift and later on shifted towards variable selection. The latter observation could be explained in the light of previous studies which suggested that species with similar ecological niches as resident species have a lower chance of successfully invading a community than species that have less niche overlap with already established species (Fargione et al., 2003; Tilman, 2004; Peay et al., 2012; Tan et al., 2012).

The processes that were indicated to have driven community turnover between groundwater and sediment at the earlier stages were however counterintuitive. Unexpectedly, significantly positive values for RC_{bray} suggested dispersal limitation acting alongside drift to have been responsible for the observed differences in community composition, rather than the intuitively more expected scenario of random drift acting alone. Multiple causes could explain these unexpected findings. It has to be noted that the sediment microcosms were incubated in groundwater monitoring wells. It is known that communities found inside monitoring wells may differ from the communities that are actually present in the surrounding groundwater of an aquifer (Griebler et al., 2002; Korbel et al., 2017). In fact, previous analyses of our samples by T-RFLP fingerprinting did indeed reveal some differences between groundwater and well water microbial communities (Zhou et al., 2012). However, Langenheder et al. (2017) have reported identical results for differences between lake biofilms and microbial communities in the overlaying water column, which were not separated by any barrier that could have limited OTU dispersal. They argued in the light of these findings, and based on the arguments provided by Chase et al. (2011), that significantly positive deviations of RC_{bray} from the null expectation may also be caused by strong biotic factors such as competition between species. As niches become more crowded over time, some organisms may try to avoid competition by occupying non-optimal niches, which would not necessarily result in a deviation

from the null expectation in phylogenetic null models. Moreover, even though the assumption underlying the β-NTI-based approach about the link between phylogenetic relatedness and niche similarity of microbial species is supported by empirical evidence (Peay et al., 2012; Stegen et al., 2012; Tan et al., 2012; Wang et al., 2013; Dini-Andreote et al., 2015; Martiny et al., 2015), and was also confirmed in our system by a significant phylogenetic signal (Fig. S6), it is known that some species traits are phylogenetically more conserved than others (Martiny et al., 2015). Hence, we may speculate that the significantly positive deviation of RC_{bray} from the null exception at the early stage of the colonization might indicate the involvement of traits that are important for the colonization of sediment surfaces, but which are phylogenetically not well conserved and therefore did not result in a significant signal of β-NTI. Only at the later stage, when the communities on the sediments had matured further, phylogenetically more conserved traits may have gained importance in the turnover between planktonic and attached microbial communities.

Conclusion

We have shown that the microbial colonization of sediments in a pristine groundwater aquifer in several aspects follows the general patterns that have also been described for the development of biofilms in other more energy-rich, non-subsurface, aquatic environments (Jackson, 2003), as well as the assembly of sediment-attached communities in highly dynamic hyporheic zones, suggesting that the assembly of microbial communities on surfaces might be governed by similar underlying mechanisms across a wide range of different habitats. Our results indicate that differences between planktonic and sediment-attached communities often reported for groundwater environments are not the result of purely stochastic events, but that sediment surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way, probably determined by sediment properties rather than hydrochemistry. Although we found that early-colonizing OTUs dominated the final communities on the sediments, mere timing OTU of arrival during the succession was likely not a determining factor, as the majority of these early-colonizers were not very persistent. Rather, traits associated with identified key taxa, especially

within the *Comamonadaceae* and *Oxalobacteraceae*, seemed to have been a more decisive factor for the persistence of these OTUs. However, the ecological processes behind the temporal succession of OTUs during the colonization still remain unclear and might be influenced by species interaction network structures at a given time point. Moreover, we found indications that different traits with different degrees of phylogenetic conservation may have determined the establishment of OTUs in the developing sediment-attached communities from the surrounding groundwater at different stages of community development. A better understanding of these traits and how they may integrate into species interaction networks will be an important aspect for future research. Computational modelling of microbial communities based on metaomics data, albeit still in its infancy, offers a promising tool to elucidate complex species interactions within microbial communities (Faust and Raes, 2012; Hanemaaijer et al., 2015). If successful, the extra in depth insight gained from such models could be a valuable addition to current approaches that strive for a better understanding of the links between microbial community composition, assembly, and biogeochemical functions (Graham et al., 2016b; Graham and Stegen, 2017) .

Experimental procedures

Experimental setup and sampling

To study the assembly and succession of sediment-attached microbial communities, fresh sediments were taken from the Isar River that drains the investigated aquifer. Sediments were sieved (0.2-0.63 mm) and packed into perforated polyethylene columns with a mesh size of 1-2 mm. Sediment columns were submerged in deionized water and sterilized by autoclaving five times at 121°C for 30 min; after each step, the sediments were rinsed with and again submerged in fresh deionized water. The columns were stored at 4°C submerged in sterile water until the start of the experiment. Replicate sediment columns were incubated in each well; duplicate columns were sampled destructively at each sampling campaign. Samples for DNA extraction were put on dry ice for transport to the lab and were stored at -20°C until DNA extraction according to the method described by Anneser et al. (2010). For the comparison of attached versus planktonic microbial communities, cells from 5 L groundwater were collected on a 0.2 µm

polycarbonate filter (Merck Millipore, Darmstadt, Germany) on-site. Filters were shock-frozen on dry ice and stored at -20°C until extraction using the same method as for the sediment samples. For cell counting, 0.5 mL groundwater (or 0.5 cm^3 sediment) was fixed on-site with glutardialdehyde at a final concentration of 2.5% v/v; samples were stored in the dark at 4°C until further processing according to Bayer et al. (2016). Cells were stained with SYBR-Green I (Invitrogen, Karlsruhe, Germany) at a ratio of 1:10,000 and subsequently counted using a LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany). For a description of measurements of physicochemical parameters listed in Table 1 the reader is referred to Zhou et al. (2012).

16S rRNA amplicon sequencing

PCR amplification (28 cycles) and subsequent bidirectional 454-pyrosequencing of 16S rRNA gene fragments was done according to Pilloni et al. (2011) using the primer pair Ba27f-Ba519r extended with sequencing adapters and multiplex barcodes. Each of the sample duplicates was amplified again in duplicate; after amplification, all replicates of a given sample were combined before purification using magnetic beads (AMPure-XP; Beckmann Coulter, Brea, CA, USA) according to the manufacturer's instructions. After purification, DNA concentrations were determined using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK). Barcoded amplicons from all samples were pooled in equimolar amounts before sequencing on a 454 GS FLX pyrosequencer using Titanium chemistry (Roche, Penzberg, Germany). The sequence data was processed with QIIME (version 1.9.0) (Caporaso et al., 2010). Demultiplexing and quality filtering (min./max. sequence length: 250/600 bp; primer mismatches and barcode errors: 0; min. quality score: 25; quality score window size: 50 bp) was done using the 'split_libraries.py' command. Chimera filtering was done by mapping reads against the SILVA SSU reference database (release 128) (Quast et al., 2013) using 'identify_chimeric_seqs.py' with usearch61 as detection method. After quality and chimera filtering, the average number of combined forward and reverse reads per sample was 5,709 with an average length of 388 bp. OTUs were clustered by uclust against the SILVA SSU reference database at 97% similarity using the 'pick_open_reference_otus.py'

494 command. After removing low-confidence OTUs (combined abundance of < 0.01% across all samples)
495 and OTUs classified as chloroplasts, a total of 910 OTUs remained in the final OTU table. The total
496 number of reads per sample was rarefied to 2,045 which was the lowest number of reads observed for a
497 single sample. A midpoint-rooted phyologenetic tree was constructed from the alignment of OTU
498 reference sequences using FastTree (Price et al., 2009). Sequence data have been deposited in the NCBI
499 Sequence Read Archive under accession number SRP139256.
500
501 **Data analysis**
502 All analyses were done in R (version 3.5.0) (R Core Team, 2018). Alpha diversity (OTU richness (*S*),
503 Shannon diversity (*H'*), Pilou's evenness (*J'*)) was calculated using the vegan package (version 2.5-2)
504 (Oksanen et al., 2018). The number of newly-arriving OTUs (*Sn*) in sediment samples at a given time
505 point was defined as the number of OTUs that displayed an abundance > 0% for the first time at that time
506 point. Phylogenetic beta diversity was assessed based on β-MNTD that was calculated using the
507 'comdistnt' function of the picante package (version 1.7) (Kembel et al., 2010). Differences in microbial
508 community composition between samples across time, space, and community type were illustrated by
509 NMDS performed on the β-MNTD matrix using the 'metaMDS' function of the vegan package with 40
510 iterations. To test for the effect of physicochemical variables (Table 1) on changes in community
511 composition for sediment-attached and planktonic communities, respectively, variables were standardized
512 to z-scores before fitting to the NDMS ordination using the 'envfit' function of the vegan package with
513 10,000 permutations stratified within community types. PERMANOVA was used to estimate the marginal
514 effects of each of the three categorical variables community type, sampling time point, and site location,
515 respectively, while holding the other two constant using the 'adonis2' function in vegan package with
516 10,000 permutations. For the identification of key organisms that were responsible for the differences
517 between community types, relative OTU abundances were summarized at genus level before SIMPER
518 analysis using the 'simper' function in vegan with 1,000 permutations for significance testing. Beta

519 diversity partitioning based on Jaccard dissimilarity was done using the 'betapart' package (Baselga and
520 Orme, 2012).

521 To study the effect of deterministic versus stochastic processes on microbial community assembly,
522 we used the null model approach developed by Stegen et al. (2012; 2013). β-NTI compares the mean
523 phylogenetic distance of OTUs based on β-MNTD between two communities against the distribution of β-
524 MNTD values expected for randomly assembled communities. This distribution is obtained from repeated
525 randomizations in which the OTUs observed in the two communities and their relative abundances are
526 shuffled across the tips of the according phylogenetic tree. The value of β-NTI indicates by how many
527 standard deviations the observed β-MNTD deviates from the mean of the null expectation with $|\beta\text{-NTI}| > 2$
528 indicating significant deviations. β-NTI was calculated with abundance-weighting and 999 randomizations
529 for each pairwise comparison. The assumption of a significant phylogenetic signal was verified using
530 Mantel correlograms as in Dini-Andreote et al. (2015) (see SI and Fig. S6). The RC_{bray} index measures
531 how much the observed Bray-Curtis dissimilarity between two communities differs from the distribution
532 of dissimilarities between probabilistically assembled communities for which the probability of OTUs
533 being drawn is proportional to their respective abundances in the two compared communities and their
534 occurrence frequencies in the regional species pool, while keeping local community richness and the
535 number of individuals constant. RC_{bray} takes values from -1 to +1 where absolute values > 0.95 indicate
536 significant deviations from the null expectation. RC_{bray} was calculated with 999 iterations for each
537 pairwise comparison. Regional species pools for null model simulations were constructed from all OTUs
538 in the full dataset over space and time as in Veach et al. (2016), because we expected that regional species
539 pools constructed separately for each time point from OTUs at the two sites that only spanned a relatively
540 short transect would have been too conservative to estimate the total regional diversity in the aquifer. To
541 evaluate in how far this large regional species pool may have led to an overestimation of the effects of
542 selection and/or dispersal, we compared these results to simulations where regional species pools were
543 constructed for individual time points for which paired samples of sediment-attached and planktonic
544 communities were available. The outcomes of the null models in both situations were in high agreement

545 with each other, indicating that using the full dataset to construct the regional species pool did not
546 introduce a substantial bias in our analyses (see SI and Fig. S7). To test for the effect of changes in
547 physicochemical conditions on community assembly processes, Mantel tests (Spearman's rank
548 correlation, 10,000 permutations, function 'mantel' in vegan) were performed on the β-NTI matrix and
549 individual Euclidean distance matrices that were calculated for each physicochemical variable separately
550 after standardization.
551

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561

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Figure legends

Figure 1: Schematic illustration of the main geographical features of the study site, the locations of the two monitoring wells, and general groundwater flow directions.

Figure 2: Temporal dynamics of biomass and alpha diversity for sediment-attached communities and planktonic communities in the groundwater at MIT052 and MIT039, respectively. Biomass is given as the number of prokaryotic cells per mL groundwater or the equivalent volume (cm^3) of sediment. The time in days for each time point is given in parentheses.

Figure 3: (A) Changes over time in the fraction of newly-arriving OTUs (*Sn*) relative to the total number of OTUs (*S*) in sediment-attached communities. (B) Changes over time in the cumulative relative abundance of newly-arriving OTUs that first occurred at the time point indicated in the upper right corner of each graph.

Figure 4: (A) Taxonomic microbial community composition of sediment-attached communities and planktonic communities in the groundwater per time point. Taxonomic groups are summarized at family

867 level. Families with an abundance < 10% in the entire dataset were grouped as 'Diverse others' for clarity
868 of display (mean abundance: 0.1%; max: 4.7%). (B) NMDS plot showing differences in microbial
869 community composition based on abundance-weighted β-MNTD (stress: 0.09).
870
871 **Figure 5:** Values for β-NTI from pairwise microbial community comparisons. The range of β-NTI
872 indicating stochastic community turnover is shaded in grey. (A) Spatial community turnover of planktonic
873 communities in the groundwater and sediment-attached communities, respectively, between MIT052 and
874 MIT039 per time point. (B) Community turnover between planktonic and sediment-attached communities
875 within sites per time point (note: the bar corresponding to the comparison of communities at MIT052 in
876 July is not visible; β-NTI = 0.04). (C) Temporal community turnover of planktonic and sediment-attached
877 communities, respectively, between consecutive time points within sites (note: bar for the comparison of
878 July vs. December for planktonic communities at MIT052 is not visible; β-NTI = -0.005). NA: data not
879 available.
880
881 **Figure S1:** Changes in physicochemical parameters over time measured in the groundwater at the two
882 sites. DOC: dissolved organic carbon; AOC: assimilable organic carbon; DO: dissolved oxygen.
883
884 **Figure S2:** Occurrence frequencies of the most dominant families and most dominant single genera within
885 those families among newly-arriving OTUs (*Sn*) in the sediment-attached communities that first occurred
886 at the indicated time point and were still detected in the final communities at MIT052 and MIT039,
887 respectively. Families with an individual occurrence frequency < 3% are grouped as 'Diverse others' for
888 clarity of display. Bars representing each family are ordered by occurrence frequency in descending order
889 from top to bottom.
890
891 **Figure S3:** Differential abundances of genera that contributed most to the dissimilarity between sediment-
892 attached communities and planktonic communities in the groundwater identified by SIMPER analysis.

Only the genera with the highest significant contribution to the dissimilarity are shown (> 0.1%; $p < 0.05$); the average contribution of each displayed genus is indicated by the color intensity of the bars. (A) Log_{10}-ratios of differential average abundances in planktonic communities over sediment-attached communities for genera found in both community types. (B) Average relative abundances of genera exclusively found in one community type.

Figure S4: Relative contributions of turnover and nestedness to the total Jaccard dissimilarity between sediment-attached and planktonic communities at each site per time point inferred from beta diversity partitioning.

Figure S5: Relative contributions of turnover and nestedness to the total Jaccard dissimilarity between sediment-attached communities within sites across time points inferred from beta diversity partitioning.

Figure S6: Phylogenetic signal inferred from Mantel correlograms showing Pearson correlation between phylogenetic distances and differences in environmental optima between OTUs within phylogenetic distance classes evaluated at distance steps of 0.01 for (A) sediment-attached and (B) planktonic communities. Filled symbols indicate significant correlations ($p < 0.05$).

Figure S7: Comparison of the outcomes of null model simulations to estimate (A) β-NTI and (B) RC_{bray} based on different regional species pools for pairwise community comparisons shown in Figure 5 and discussed in the main text. The horizontal axes represent results based on regional species pools constructed from OTUs found in the full data set; vertical axes show results based on regional species pools constructed from OTUs found in subsets of samples within time points, or in case of comparisons to estimate temporal community turnover, from two consecutive time points (only for β-NTI). Colors represent the different investigated turnover processes shown in Figure 5 (see main text). Dashed lines mark significance thresholds for each index (see main text). Linear regression slopes of the straight line ±

919 0.95 confidence intervals and Pearson correlation coefficients are indicated in the figures. Flags indicate
920 pairwise comparisons for which the outcomes of the null models did not agree between the two strategies
921 for constructing regional species pools.
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Tables

Table 1: Mean values and standard deviation (SD) of physicochemical parameters averaged over the two investigated sites and all time points (n = 10). Individual values for each site and time point are shown in Figure S1.

	Water table (m bs)[a]	DOC[b] (mg L^{-1})	AOC[c] (µg L^{-1})	pH	Temperature (°C)	Electrical conductivity (µS cm^{-1})	DO[d] (mg L^{-1})	PO_4^{3-} (mg L^{-1})	SO_4^{2-} (mg L^{-1})	NO_3^- (mg L^{-1})	Cl^- (mg L^{-1})	K^+ (mg L^{-1})	Na^+ (mg L^{-1})	Ca^{2+} (mg L^{-1})	Mg^{2+} (mg L^{-1})
Mean	8.9	1.10	11.4	7.81	6.88	297	10.21	0.011	14.5	2.49	1.80	0.30	1.03	46.1	12.1
SD	5.0	0.37	14.7	0.10	0.59	41	0.82	0.015	8.8	0.78	1.13	0.07	0.62	5.5	1.0

[a] meter below surface
[b] dissolved organic carbon
[c] assimilable organic carbon
[d] dissolved oxygen

Non-random processes determine the colonization of groundwater sediments by microbial communities in a pristine porous aquifer

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Originality-significance statement

Groundwater bodies are the largest terrestrial habitat for microorganisms on Earth, where the majority of the microbial biomass lives attached to sediment surfaces. In these unique, low-productivity environments, microbial communities are the drivers of key biogeochemical processes and furthermore provide important services to society like maintaining groundwater quality as one of the most important sources of freshwater and drinking water worldwide. Over the past years, studies on sediment-attached communities in groundwater-surface water mixing zones as well as surface-attached biofilms in other, non-subsurface habitats have provided important insights regarding the ecological processes that drive the assembly of these microbial communities. Compared to most of these environments, however, pristine groundwater is characterized by significantly lower levels of energy and productivity as well as comparatively more stable environmental conditions, which may promote the effect of stochastic processes on community assembly. Moreover, the microbial communities that colonize subsurface sediments typically exhibit much lower cell densities and occur as small, spatially separated micro-colonies rather than dense, coherent biofilms as they are found in other non-subsurface environments. Therefore, our study was motivated by the question whether findings on the processes that govern microbial community assembly and succession of surface-attached communities in those other more dynamic and nutrient-rich environments also apply to sediment-attached microbial communities in pristine groundwater environments. Our study shows intriguing similarities between the community succession on newly-colonized sediments in our investigated porous, pristine aquifer and succession patterns observed for biofilms in other more dynamic aquatic environments, indicating that the assembly of microbial communities on surfaces may be governed by similar underlying mechanisms across a wide range of different habitats. Our results indicate that differences between planktonic and sediment-attached communities often reported for groundwater environments are not the result of purely stochastic events, but that sediment surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way. Furthermore, our data suggest that specific genera, especially within the *Comamonadaceae* and *Oxalobacteraceae*, played a particularly important role in this process.

Non-random processes determine the colonization of groundwater sediments by microbial communities in a pristine porous aquifer

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Summary

Sediments accommodate the dominating share of groundwater microbiomes, however the processes that govern the assembly and succession of sediment-attached microbial communities in groundwater aquifers are not well understood. To elucidate these processes, we followed the microbial colonization of sterile sediments in *in situ* microcosms that were exposed to groundwater for almost one year at two distant but hydrologically connected sites of a pristine, shallow, porous aquifer. Our results revealed intriguing similarities between the community succession on the newly-colonized sediments and succession patterns previously observed for biofilms in other more dynamic aquatic environments, indicating that the assembly of microbial communities on surfaces may be governed by similar underlying mechanisms across a wide range of different habitats. Null model simulations on spatiotemporally resolved 16S rRNA amplicon sequencing data further indicated selection of specific OTUs rather than random colonization as the main driver of community assembly. A small fraction of persistent OTUs that had established on the sediments during the first 115 days dominated the final communities (68%-85%), suggesting a key role of these early-colonizing organisms, in particular specific genera within the *Comamonadaceae* and *Oxalobacteraceae,* for community assembly and succession during the colonization of the sediments. Overall, our study suggests that differences between planktonic and sediment-attached communities often reported for groundwater environments are not the result of purely stochastic events, but that sediment

surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way.

Introduction

The groundwater-saturated zones of the terrestrial subsurface are one of the largest habitats for microorganisms on Earth (Griebler and Lueders, 2009; McMahon and Parnell, 2013). In these unique, low-productivity environments, microbial communities lie at the heart of key biogeochemical processes like the turnover of carbon and other nutrients, mineral cycling, or pollutant degradation (Griebler et al., 2014; Griebler and Avramov, 2015). Sediment-attached communities play a particularly important role in these ecosystems as they represent the bulk of the microbial biomass and activity (Lehman et al., 2001; Griebler et al., 2002; Zhou et al., 2012; McMahon and Parnell, 2013). Previous studies have repeatedly shown that the composition of sediment-attached communities can differ substantially from planktonic communities suspended in the surrounding groundwater (Zhou et al., 2012; Flynn et al., 2013; Hug et al., 2015). However, the ecological processes that give rise to these differences during community assembly and succession are not well understood. Recent studies have suggested a strong link between biogeochemical functions and microbial community composition as well as the underlying ecological assembly processes (Graham et al., 2016a; Graham et al., 2016b; Graham and Stegen, 2017). Therefore, a better understanding of the processes that drive the assembly of sediment-attached microbial communities in groundwater environments is a key step towards a better understanding of the functioning of these ecosystems.

The question of the influence of deterministic (or niche-based) versus stochastic (or neutral) processes on the assembly, succession, and diversity of microbial communities has increasingly sparked the curiosity of microbial ecologists over the past years (for reviews see Nemergut et al. (2013); Zhou and Ning (2017)). Deterministic theory assumes that environmental factors, both biotic and abiotic, determine the composition and structure of a community by selecting for species with certain traits that enable them to thrive and compete in a given environment (also known as species sorting or environmental filtering). Accordingly, under similar environmental conditions, communities at different locations or points in time are expected to be composed of species with similar traits. In contrast, the stochastic point of view holds

that communities are assembled randomly from species with equivalent trait spectra, and that differences in community composition and structure are the result of random events, for example caused by species dispersal or drift due to stochastic birth-death events (Chase and Myers, 2011). Although purely neutral models have been shown to explain observed diversity patterns of microbial communities with surprising accuracy (e.g. Woodcock et al. (2007); Ofiţeru et al. (2010); Woodcock and Sloan (2017)), there has been growing consent that both deterministic and stochastic processes can act simultaneously and that the contribution of either process can shift over time or with changing environmental conditions (Dumbrell et al., 2009; Ofiţeru et al., 2010; Chase and Myers, 2011; Langenheder and Székely, 2011; Ferrenberg et al., 2013; Stegen et al., 2013; Wang et al., 2013; Zhou et al., 2014; Dini-Andreote et al., 2015; Stegen et al., 2015; Stegen et al., 2016b; Veach et al., 2016; Graham et al., 2017).

One aspect where the interaction between deterministic and stochastic processes comes into play is the initial assembly of communities and the following species succession in newly-colonized environments (Tilman, 2004; Langenheder and Székely, 2011), for instance during the development of biofilms on initially empty surfaces (Jackson, 2003; Battin et al., 2007). At the initial stage of colonization, the arrival of species in a new environment is often driven by stochastic dispersal (Tilman, 2004; Ferrenberg et al., 2013; Dini-Andreote et al., 2015), which can overrule deterministic effects in homogeneous environments or if environmental filtering between the environment of the source community and the newly colonized environment is weak (Stegen et al., 2012; Wang et al., 2013; Battin et al., 2016). However, once established, resident species can affect the establishment of newly-arriving species (positively or negatively) during the subsequent succession directly via species interactions or indirectly by modification of their environment (Fukami, 2015). Thus the order and timing of species arrival, although initially stochastic, can determine the composition and functioning of the final community, known as priority effect (Fargione et al., 2003; Tilman, 2004; Fukami et al., 2010; Peay et al., 2012; Tan et al., 2012; Nemergut et al., 2013; Rummens et al., 2018; Svoboda et al., 2018).

A general, conceptual model that summarizes the successional stages during biofilm development has been described by Jackson (2003). According to this model, initially empty surfaces offer ample space

and resources to allow for the establishment of diverse microorganisms resulting in a rapid increase in species richness and diversity that is fueled by the dispersal of newly-arriving species from a regional species pool like overlaying water during initial biofilm assembly. The steady arrival of new species eventually leads to niche depletion and growing competition between established and newly-arriving species, which more and more suppresses the increase in species richness. As the competition intensifies, less competitive species are lost from the community, which leads to a decline in species richness after the initial phase of community assembly. However, as the biofilm matures further and becomes more heterogeneous, new niches are created that enable specialized species to establish, leading again to an increase in richness and diversity in the mature biofilm. Although Woodcock and Sloan (2017) have demonstrated using a neutral modeling approach that these patterns can be explained based on stochastic processes only, empirical evidence suggests that the assembly of biofilm communities is in fact characterized by a shift from initially stochastic community assembly towards deterministically driven succession at the later stages, for instance caused by species interactions or growing niche space due to increasing spatial and chemical heterogeneity (Martiny et al., 2003; Lyautey et al., 2005; Battin et al., 2016; Veach et al., 2016; Brislawn et al., 2018).

To date, most of the studies on ecological processes behind the assembly of microbial communities in groundwater environments have focused on planktonic communities suspended in the groundwater (Stegen et al., 2012; Stegen et al., 2013; Beaton et al., 2016; Danczak et al., 2018), while studies on sediment-attached communities are scarce (Stegen et al., 2016a). In contrast, much insight has been gained over the past years into the assembly of sediment-attached communities in groundwater-surface water mixing zones (hyporheic zone). In these studies, the assembly of planktonic communities generally tended to be more subject to stochastic effects and shifts in assembly processes related to changes in water chemistry, whereas selection had a relatively more pronounced effect on the assembly of sediment-attached communities which, at the same time, was less affected by hydrochemical changes (Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017; Stegen et al., 2018). Compared to the hyporheic zone, pristine groundwater environments (in the absence of surface water impacts) are more

stable and only experience little environmental changes (Griebler and Lueders, 2009), which may promote the effect of stochastic processes on community assembly compared to more dynamic environments (Ofiţeru et al., 2010; Stegen et al., 2012; Wang et al., 2013; Zhou et al., 2013). Moreover, in contrast to the typically studied biofilms in other environments like surface waters, which form dense, spatially coherent, heterogeneous structures that can reach a thickness in the range of several hundred micrometers (Battin et al., 2016), sediment-attached microbial communities in groundwater aquifers occur as small, patchily distributed micro-colonies that consist of only a few cells (Schmidt et al., 2017), which may be hypothesized to be more prone to stochastic effects than their biofilm counterparts in other environments.

In this study we set out to 1) investigate whether the assembly of sediment-attached microbial communities in pristine groundwater environments can be explained by the general patterns observed for surface-attached biofilms in other environments; 2) study the importance of early colonizers for community succession during the colonization of the sediments; and 3) see if the dominating role of selection on community assembly that has been observed for sediment-attached microbial communities in the hyporheic zone also drives community assembly in comparatively stable, pristine groundwater environments. To tackle these goals, we incubated *in situ* microcosms filled with sterilized sediment in monitoring wells at two distant but hydrologically connected sites of a pristine, porous aquifer (Zhou et al., 2012), and followed the succession of the microbial communities as they colonized the sediments over a period of almost one year. We used 16S rRNA amplicon sequencing data to study changes in alpha and beta diversity of the sediment-attached communities incubated at the two sites as well as differences in community composition between sediment-attached and planktonic communities in the surrounding groundwater over the course of the succession. To explore the influence of deterministic and stochastic processes on microbial community assembly and succession, we applied the null model approach developed by Stegen et al. (2012; 2013), which has previously also been used in studies on community assembly in the hyporheic zone (Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017; Stegen et al., 2018) as well as biofilms in other environments (Langenheder et al., 2017; Brislawn et al., 2018), and thus allows us to compare our results to those previous findings.

Results

Site description

The field experiment was conducted over a period of 347 days from March 2010 until February 2011, with intermediate sampling campaigns in May (day 49), July (day 115), and December (day 263). The area with the two monitoring wells used for the incubation of the *in situ* microcosms was located at the foothills of the Bavarian Alps in the upper Isar River valley close to the village of Mittenwald, Germany (Fig. 1). The wells were installed in a pristine, shallow, porous aquifer composed of quaternary sediment mainly consisting of gravel and coarse sand. Well MIT052 was located on a mountain pasture in the forested Riedboden nature reserve 400 m away from the nearby river; well MIT039 was located approximately 2 km away from MIT052 in proximity to the village with a distance of 240 m to the river (for a detailed site description, see Zhou et al. (2012)). Over the course of the experiment, we observed only small fluctuations in physicochemical conditions (Table 1; the temporal dynamics of the individual parameters are shown in Fig. S1).

Temporal dynamics of microbial biomass and alpha diversity of sediment-attached and planktonic microbial communities

Despite the spatial distance between the two sites, the microbial communities that developed on the initially sterile sediments followed identical trends in alpha diversity and biomass patterns (Fig. 2). Already after the first 49 days, the microbial biomass (measured as prokaryotic cell counts) of attached microbial communities at both sites had reached a plateau of ~10^7 cells cm^{-3} of sediment followed by a slight decline for the remaining time of the experiment. Although the biomass of sediment-attached microbial communities stayed more or less constant, noticeable changes in the communities still occurred as indicated by OTU richness and diversity which steadily increased by about 50% and 25%, respectively, from May until December, followed by a decline of both parameters in February. Over the same period, community evenness remained relatively high and only changed moderately. The changes in biomass and

alpha diversity observed for the newly colonized sediments appeared to be independent from the changes that occurred in the planktonic microbial communities, which were more variable over time and less comparable between the two sites. Microbial biomass was about one to two orders of magnitude lower for the planktonic communities compared to the sediments.

Establishment and persistence of newly-arriving OTUs in sediment-attached microbial communities

To assess the impact of early colonizers on microbial community succession, we looked at the number of newly-arriving OTUs that entered the developing sediment-attached communities at each time point over the course of the experiment (Fig. 3). Newly-arriving OTUs are defined here as OTUs that showed an abundance > 0% in the community for the first time at a given time point. At both sites, the number of newly-arriving OTUs declined over time showing that the majority of OTUs had established during the initial phase of the incubation. Despite this declining trend, the fraction of newly-arriving OTUs relative to the total OTU richness at the end of the incubation was still noticeable with ~15-20%. However, looking at the changes in the cumulative relative abundances of the newly-arriving OTUs over time, we saw that the OTUs that had arrived towards the later stages only accounted for a relatively small fraction of the final communities. Even though the cumulative relative abundance of OTUs that had established in the communities within the first 49 days steadily declined, these OTUs still made up 36% and 47% of the final communities at MIT052 and MIT039, respectively. At MIT052, these OTUs together with those that emerged at the following time point in July comprised the majority of the final community at the end of the incubation (together 85%), while OTUs that arrived at the final time point accounted for only 5%. At MIT039, OTUs that had arrived at the first two time points made up for 68% of the final community, whereas OTUs that had arrived in December and February comprised 12% and 20%, respectively. Although these results clearly show the dominance of early-colonizer OTUs in the final communities, a closer look at how many of these OTUs actually persisted until the final time point showed that only ~12% of newly-arriving OTUs from each time point were still present in the final communities (data not shown). Looking at the taxonomies of these newly-arriving OTUs that persisted until the end of the

incubation, we again found highly similar patterns for both sites (Fig. S2). At each time point, the most dominant groups were OTUs affiliated to *Comamonadaceae*, mainly *Aquabacterium* and *Polaromonas* spp., in addition to *Oxalobacteraceae*, mainly consisting of *Duganella*, *Massilia*, and *Undibacterium* spp., as well as *Pseudomonas* spp. and diverse *Caulobacteraceae* and *Sphingomonadaceae*.

Microbial community composition and beta diversity

Similar to the alpha diversity patterns, the microbial communities on the newly colonized sediments displayed comparable compositions at the two sites (Fig. 4). Especially during the initial phase of the incubation in May, sediment-attached communities at both sites were dominated by *Oxalobacteraceae* in addition to *Comamonadaceae* and smaller fractions of *Flavobacteriaceae* and *Caulobacteraceae*. Over the further course of the incubation, these taxonomic groups gradually receded and were in part replaced mainly by increasing numbers of *Comamonadaceae* (mainly *Aquabacterium* spp.), *Pseudomonadaceae*, *Nocardiaceae*, and *Rhodocyclaceae* especially at MIT052, in addition to *Sphingomonadaceae*, uncultured *Deltaproteobacteria*, and *Moraxellaceae* at MIT039. Moreover, OTUs affiliated with diverse low-abundant families (with an abundance <10% in the entire dataset; mean = 0.1%; max. = 4.7%) gradually increased in abundance. In contrast, planktonic communities were mainly dominated by members of the *Rhodocyclaceae*, *Comamonadaceae* (mainly *Curvibacter*, *Simplicispira*, and *Rhodoferax* spp.), and *Leptospiraceae*.

To get a better picture of the organisms that were responsible for differences between sediment-attached and planktonic communities, we performed similarity percentage (SIMPER) analysis across all samples on relative abundances of OTUs grouped at genus level. Interestingly, we found high agreement between the genera that significantly contributed to the observed dissimilarities between the two types of communities and the taxa identified as successful, persistent colonizers in the previous analysis (Fig. S2). *Aquabacterium*, *Massilia*, and *Duganella* spp. ranked among the genera that contributed most to the dissimilarity (together > 15%; all $p < 0.002$) and were highly differentially abundant in the sediment-attached communities, next to *Flavobacteria* and uncultured members of the *Oxalobacteraceae* (Fig. S3).

The changes in microbial community composition over time as well as differences between sediment-attached and planktonic communities in the groundwater were revealed by non-metric multidimensional scaling (NMDS) performed on abundance-weighted β-mean nearest taxon distance (β-MNTD) between communities (Fig. 4). At all measured time points, sediment-attached and planktonic communities clustered separately from each other as reflected by the distinct separation of the two types of communities along the first NMDS axis. Changes in microbial community composition over time were reflected by the separation of data points along the second NMDS axis. Permutational analysis of variance (PERMANOVA) revealed that community type (i.e. sediment-attached vs. planktonic) explained most of the variance in β-MNTD between communities (R^2 = 0.626; p = 0.001), followed by sampling time point (R^2 = 0.104; p = 0.001), while site location was not significant (R^2 = 0; p = 1), showing that communities across sites were similar within each community type and time point. Moreover, fitting environmental variables to the NMDS ordination with stratification of permutations within the community types did not reveal significant correlations between changes in community composition and any of the measured physicochemical parameters (Table 1 and Figure S1) (all R^2 < 0.32; p > 0.1).

Since community type explained most of the variance in beta diversity, we applied partitioning of beta diversity to identify the underlying causes of the differences between sediment-attached and planktonic communities within sites and sampling time points according to Baselga (2012). This approach is based on the additive partitioning of incidence-based Jaccard dissimilarity between two communities into a nestedness and a turnover component. A high contribution of nestedness to the total dissimilarity indicates that two communities are subsets of each other and that differences are caused by differences in species richness (i.e. gain or loss of species). On the other hand, a high contribution of turnover indicates little overlap in species composition, i.e. species in one community have been replaced by other species in the other community. The analysis showed that turnover was the dominating process behind the differences between the two types of communities at each time point (for all time points > 97%) (Fig. S4), showing that both community types were composed of distinct sets of OTUs.

We applied the same approach to the dissimilarity between sediment-attached communities at different time points within sites to investigate the degree to which nestedness and turnover contributed to changes in community composition over the course of the sediment colonization. Also in this case turnover dominated over nestedness in all comparisons, especially over long time scales (i.e. comparing communities between May and February; 95-97%), and with a slightly weaker effect over short time scales of the succession (i.e. comparing communities between consecutive time points; 74-90%) (Fig. S5).

Impact of stochastic and deterministic processes on community assembly and succession inferred from null models

To infer the impact of deterministic and stochastic processes on community assembly, we applied the two-tiered null model approach developed by Stegen et al. (2012; 2013). Briefly, under the assumption that phylogenetically closely related species occupy more similar ecological niches than less closely related species, the strength of deterministic processes is evaluated in tier one based on the β-nearest taxon index (β-NTI). β-NTI < -2 and > +2 indicates that two communities are phylogenetically significantly more or less similar to each other than expected by chance, which is interpreted as homogeneous selection (i.e. selection of similar OTUs) or variable selection (i.e. selection of dissimilar OTUs) in the two communities, respectively. |β-NTI| < 2 indicates that two communities are as dissimilar as expected by chance, hinting at stochastic community assembly. In this case, the RC_{bray} index is used in tier two to evaluate the effect of stochastic dispersal. RC_{bray} < -0.95 and > +0.95 means that two communities share significantly more or less OTUs than expected by chance, indicating that communities are assembled by homogenizing dispersal or dispersal limitation in combination with drift, respectively. $|RC_{bray}| < 0.95$ indicates that differences between two communities are due to random drift acting alone. We applied this approach to study the role of deterministic and stochastic processes on three levels (Fig. 5): 1) spatial community turnover between the two sites within community types and time points; 2) turnover between sediment-attached and planktonic communities within sites and time points; 3) temporal turnover between communities at consecutive time points within community types and sites.

Deterministic and stochastic processes had different influences on the spatial community turnover for sediment-attached and planktonic communities, respectively. In case of planktonic communities, pairwise comparisons between sites for each time point resulted in values for β-NTI that were not significantly different from the null expectation, indicating that differences between the planktonic communities at the two sites were caused by stochastic processes. Pairwise comparisons based on the RC_{bray} index identified homogenizing dispersal as the dominating process (all RC_{bray} = -1; the only exception was observed for December: RC_{bray} = +0.99, indicating dispersal limitation together with drift; data not shown). In contrast to the predominantly stochastic exchange of OTUs between the two sites via dispersal through the groundwater, pairwise comparisons of sediment-attached communities clearly tended towards homogenous selection as main cause of the similarities between the sediment-attached communities (with one exception observed for July where β-NTI was not significant, but a slightly significant RC_{bray} = 0.97 hinted at dispersal limitation together with drift).

Since the sediments had to be colonized by microorganisms that were recruited from the surrounding groundwater, even though beta diversity partitioning revealed little overlap between these two communities as discussed above, we tested for the effect of selection processes on the assembly of sediment-attached communities from planktonic microorganisms suspended in the groundwater. The differences between the communities on the newly colonized sediments and the planktonic communities at each site were caused by stochastic process during the first 115 days of the incubation. Pairwise comparisons based on RC_{bray} hinted at dispersal limitation in combination with drift as the processes responsible for these differences (all RC_{bray} = +1; data not shown). This trend changed at the later stage in December after 263 days; at this point significantly positive values for β-NTI hinted at variable selection of phylogenetically distinct OTUs in sediment-attached communities compared to the microorganisms in the surrounding groundwater.

Unlike the trends observed for the spatial community turnover, the influence of deterministic and stochastic effects on changes in community composition that occurred over time was much more variable and no clear trends could be observed. Although selection effects appeared to have played a role (both

homogenous and variable selection), they mostly did not occur consistently at both sites for neither sediment-attached nor planktonic communities.

We used Mantel correlation analysis to investigate whether changes in individual physicochemical parameters in the groundwater had an effect on the changes in assembly processes (based on β-NTI). Similar to the lack of correlations between environmental variables and differences in community composition mentioned above, we did not find significant effects of changes in environmental conditions in this analysis for neither planktonic (all |Spearman's rho| < 0.34; $p > 0.08$) nor sediment-attached communities (all |Spearman's rho| < 0. 27; $p > 0.1$).

Discussion

The alpha diversity patterns for the newly-colonized sediments at both sites followed identical trends that closely matched the conceptual model for the formation of biofilms on empty surfaces outlined by Jackson (2003), which describes changes in alpha diversity over three main stages of biofilm development. At the early stage, the large niche space of an initially empty surface allows for the establishment of diverse microorganisms, resulting in a steady increase in alpha diversity, which subsequently levels off and eventually declines due to niche depletion and the loss of less competitive species as the biofilm grows over the course of the succession. However, at the final stage, the mature biofilm becomes increasingly spatially and chemically heterogeneous, which opens new niches for specialized species to thrive and thereby fuels a renewed increase in alpha diversity. Our results only deviated from this model at the final stage of the incubation, where we did not see an increase in species diversity and richness. However, this framework was conceptualized for biofilms in resource-rich, high-productivity environments like activated sludge, wetlands, and lakes (Jackson, 2003). Although we cannot exclude that alpha diversity may have increased again with a prolonged incubation time, we may argue that diverse, specialized niches that develop in mature, spatially heterogeneous biofilms might not form to such an extent in the small, patchily distributed micro-colonies that typically colonize groundwater sediments (Schmidt et al., 2017). Hence, the total niche space in such micro-colonies may be smaller compared to mature biofilms in other

environments, similar to what Graham et al. (2016a) have proposed for sediments in the hyporheic zone. Moreover, although the general pattern of decreasing fractions of newly-arriving OTUs was also apparent in our experiment, reflecting the saturation of niche space according to Jackson's biofilm model (Jackson, 2003), we noticed that the fraction of these OTUs at the end of the incubation was still 5-10 times higher compared to findings on biofilms in other environments (e.g. Brislawn et al. (2018)). These deviations of our results from assembly patterns of biofilms, together with the findings made for hyporheic zone sediments (Graham et al., 2016a), might point towards important differences in ecological niche structures between biofilms in resource-rich surface environments and sediment-attached microbial communities in the typically more energy-poor and less productive subsurface.

Looking at the abundance changes of newly-arriving OTUs over time, we saw that OTUs colonizing the sediments during the early stage of community assembly (i.e. the first 49 to 115 days) largely dominated the final communities at the end of the experiment. However, at the same time, these dominant OTUs represented only a small fraction of newly-arriving OTUs found at each time point. This was further reflected by the large dominance of OTU turnover over nestedness between successional stages in the sediment-attached communities inferred from beta diversity partitioning, showing that the majority of OTUs that had established at a given time point were in fact replaced by others over the course of the succession. Therefore, in agreement with the findings by Brislawn et al. (2018), the mere timing of OTU arrival did not seem to be a determining factor for the final community structure. Rather, the consistent dominance of specific taxa among these persistent OTUs (mainly genera belonging to the *Oxalobacteraceae*, *Comamonadaceae*, *Caulobacteraceae*, *Sphingomonadaceae*, in addition to *Pseudomonas* spp.) suggests the involvement of certain traits that enable these taxa to sustainably colonize and thrive on sediment surfaces. Interestingly, we also found the same genera among the most important contributors to differences between sediment-attached and planktonic communities and to be highly differentially abundant in the former. The association of these taxa with biofilms and traits that facilitate surface colonization like motility or production of extracellular polysaccharides have been reported before for other environments (Kalmbach et al., 2000; Baldani et al., 2014; Bižić-Ionescu et al., 2014;

Niederdorfer et al., 2016; Niederdorfer et al., 2017), supporting the hypothesis about their importance for the development of sediment-attached communities in our study. Over the course of the succession, these dominant OTUs may have facilitated the recruitment of other more diverse taxa that were observed at the later stages of the colonization (Battin et al., 2007; Nemergut et al., 2013; Fukami, 2015).

Comparisons of beta diversity patterns revealed that sediment-attached and planktonic communities, respectively, were similar at each time point across the two sampling locations. Using the null model approach developed by Stegen et al. (2012; 2013) revealed that different processes were responsible for the observed similarities. Whereas the spatial turnover of planktonic microbial communities was driven by stochastic processes, mostly homogenizing dispersal (75%), the high similarities between the sediment-attached communities at the two sites were mostly caused by homogenous selection (75%). We are aware that our study consists of only a relatively limited number of observations and therefore the results should be interpreted with the necessary caution. Nevertheless, our results fit observations on assembly processes for communities in the hyporheic zone (Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017; Stegen et al., 2018) as well as biofilms in surface water streams (Besemer et al., 2012; Veach et al., 2016), suggesting that selection not only plays a determining role in the assembly of surface-attached microbial communities in those dynamic environments but also in pristine groundwater aquifers, despite the comparatively more stable environmental conditions, which have been shown to promote the effect of stochastic over deterministic processes in other environments (Ofiţeru et al., 2010; Stegen et al., 2012; Wang et al., 2013; Zhou et al., 2013). Mineral composition has previously been demonstrated to be a driving factor for microbial community composition and assembly (Grösbacher et al., 2016; Stegen et al., 2016a; Jones and Bennett, 2017). Since the *in situ* microcosms that we incubated at the two sites in our study were filled with sediment that originated from the same source, it is likely that identical sediment properties selected for the highly similar microbial communities at the two sites.

Given the high similarities between the sediment-attached communities at both sites throughout the experiment, we would have expected to also find similar patterns regarding the processes that drove

the temporal microbial community turnover. However, contrary to this expectation, this was not fully the case as assembly was highly variable without a clearly discernable trend in favor of a single process. Changes in environmental conditions such as nutrient inputs, fluctuating water tables, or surface water-groundwater mixing have been observed to not only affect the composition of (groundwater) microbial communities, but also influence the ecological assembly processes that determine those changes (Lyautey et al., 2005; Stegen et al., 2012; Stegen et al., 2013; Zhou et al., 2014; Dini-Andreote et al., 2015; Stegen et al., 2015; Graham et al., 2016a; Stegen et al., 2016b; Graham et al., 2017). However, in our case, we did not find indications that changes in physicochemical conditions of the groundwater were related to changes in community composition or shifts in ecological community assembly processes. This could suggest that the changes in community composition over time and the influence of deterministic versus stochastic effects were determined by changes in unmeasured environmental variables (Stegen et al., 2013). Alternatively, the observed lack of correlations between changes in environmental conditions and the processes that determined community assembly can also hint at the impact of endogenous factors like species interactions (Konopka et al., 2015; Battin et al., 2016; Cordero and Datta, 2016). Recently, Danczak et al. (2018) could show that interaction network structures can affect assembly processes of planktonic microbial communities in pristine aquifers. Although our results show that the assembly of sediment-attached communities was mainly deterministic, and that the succession of OTUs was highly reproducible between the two sites, the compositions of the two communities at each time point, and therefore possibly interaction networks, were not totally identical. Hence, the variable patterns of processes that determined the community turnover between successional stages at each site might, at least in part, be attributed to possible differences in interaction networks within the communities between the two sites.

An additionally important factor in the assembly and succession of surface-attached communities in aquatic environments is the invasion by species from the surrounding water phase (Battin et al., 2016). The establishment of invading species in a biofilm community depends on both stochastic dispersal as well as interactions with already established species (Battin et al., 2007; Battin et al., 2016). Beta diversity

partitioning showed that sediment-attached and planktonic communities were composed of distinct sets of OTUs. We again used the null model approach to test in how far deterministic and stochastic processes contributed to these differences. We found that over the first successional stages the turnover between sediment-attached and planktonic communities was caused by dispersal limitation acting alongside drift and later on shifted towards variable selection. The latter observation could be explained in the light of previous studies which suggested that species with similar ecological niches as resident species have a lower chance of successfully invading a community than species that have less niche overlap with already established species (Fargione et al., 2003; Tilman, 2004; Peay et al., 2012; Tan et al., 2012).

The processes that were indicated to have driven community turnover between groundwater and sediment at the earlier stages were however counterintuitive. Unexpectedly, significantly positive values for RC_{bray} suggested dispersal limitation acting alongside drift to have been responsible for the observed differences in community composition, rather than the intuitively more expected scenario of random drift acting alone. Multiple causes could explain these unexpected findings. It has to be noted that the sediment microcosms were incubated in groundwater monitoring wells. It is known that communities found inside monitoring wells may differ from the communities that are actually present in the surrounding groundwater of an aquifer (Griebler et al., 2002; Korbel et al., 2017). In fact, previous analyses of our samples by T-RFLP fingerprinting did indeed reveal some differences between groundwater and well water microbial communities (Zhou et al., 2012). However, Langenheder et al. (2017) have reported identical results for differences between lake biofilms and microbial communities in the overlaying water column, which were not separated by any barrier that could have limited OTU dispersal. They argued in the light of these findings, and based on the arguments provided by Chase et al. (2011), that significantly positive deviations of RC_{bray} from the null expectation may also be caused by strong biotic factors such as competition between species. As niches become more crowded over time, some organisms may try to avoid competition by occupying non-optimal niches, which would not necessarily result in a deviation from the null expectation in phylogenetic null models. Moreover, even though the assumption underlying the β-NTI-based approach about the link between phylogenetic relatedness and niche similarity of

microbial species is supported by empirical evidence (Peay et al., 2012; Stegen et al., 2012; Tan et al., 2012; Wang et al., 2013; Dini-Andreote et al., 2015; Martiny et al., 2015), and was also confirmed in our system by a significant phylogenetic signal (Fig. S6), it is known that some species traits are phylogenetically more conserved than others (Martiny et al., 2015). Hence, we may speculate that the significantly positive deviation of RC_{bray} from the null exception at the early stage of the colonization might indicate the involvement of traits that are important for the colonization of sediment surfaces, but which are phylogenetically not well conserved and therefore did not result in a significant signal of β-NTI. Only at the later stage, when the communities on the sediments had matured further, phylogenetically more conserved traits may have gained importance in the turnover between planktonic and attached microbial communities.

Conclusion

We have shown that the microbial colonization of sediments in a pristine groundwater aquifer in several aspects follows the general patterns that have also been described for the development of biofilms in other more energy-rich, non-subsurface, aquatic environments (Jackson, 2003), as well as the assembly of sediment-attached communities in highly dynamic hyporheic zones, suggesting that the assembly of microbial communities on surfaces might be governed by similar underlying mechanisms across a wide range of different habitats. Our results indicate that differences between planktonic and sediment-attached communities often reported for groundwater environments are not the result of purely stochastic events, but that sediment surfaces select for specific groups of microorganisms that assemble over time in a reproducible, non-random way, probably determined by sediment properties rather than hydrochemistry. Although we found that early-colonizing OTUs dominated the final communities on the sediments, mere timing OTU of arrival during the succession was likely not a determining factor, as the majority of these early-colonizers were not very persistent. Rather, traits associated with identified key taxa, especially within the *Comamonadaceae* and *Oxalobacteraceae*, seemed to have been a more decisive factor for the persistence of these OTUs. However, the ecological processes behind the temporal succession of OTUs

during the colonization still remain unclear and might be influenced by species interaction network structures at a given time point. Moreover, we found indications that different traits with different degrees of phylogenetic conservation may have determined the establishment of OTUs in the developing sediment-attached communities from the surrounding groundwater at different stages of community development. A better understanding of these traits and how they may integrate into species interaction networks will be an important aspect for future research. Computational modelling of microbial communities based on metaomics data, albeit still in its infancy, offers a promising tool to elucidate complex species interactions within microbial communities (Faust and Raes, 2012; Hanemaaijer et al., 2015). If successful, the extra in depth insight gained from such models could be a valuable addition to current approaches that strive for a better understanding of the links between microbial community composition, assembly, and biogeochemical functions (Graham et al., 2016b; Graham and Stegen, 2017) .

Experimental procedures

Experimental setup and sampling

To study the assembly and succession of sediment-attached microbial communities, fresh sediments were taken from the Isar River that drains the investigated aquifer. Sediments were sieved (0.2-0.63 mm) and packed into perforated polyethylene columns with a mesh size of 1-2 mm. Sediment columns were submerged in deionized water and sterilized by autoclaving five times at 121°C for 30 min; after each step, the sediments were rinsed with and again submerged in fresh deionized water. The columns were stored at 4°C submerged in sterile water until the start of the experiment. Replicate sediment columns were incubated in each well; duplicate columns were sampled destructively at each sampling campaign. Samples for DNA extraction were put on dry ice for transport to the lab and were stored at -20°C until DNA extraction according to the method described by Anneser et al. (2010). For the comparison of attached versus planktonic microbial communities, cells from 5 L groundwater were collected on a 0.2 µm polycarbonate filter (Merck Millipore, Darmstadt, Germany) on-site. Filters were shock-frozen on dry ice and stored at -20°C until extraction using the same method as for the sediment samples. For cell counting,

468 0.5 mL groundwater (or 0.5 cm^3 sediment) was fixed on-site with glutardialdehyde at a final concentration
469 of 2.5% v/v; samples were stored in the dark at 4°C until further processing according to Bayer et al.
470 (2016). Cells were stained with SYBR-Green I (Invitrogen, Karlsruhe, Germany) at a ratio of 1:10,000
471 and subsequently counted using a LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany). For a
472 description of measurements of physicochemical parameters listed in Table 1 the reader is referred to
473 Zhou et al. (2012).
474
475 **16S rRNA amplicon sequencing**
476 PCR amplification (28 cycles) and subsequent bidirectional 454-pyrosequencing of 16S rRNA gene
477 fragments was done according to Pilloni et al. (2011) using the primer pair Ba27f-Ba519r extended with
478 sequencing adapters and multiplex barcodes. Each of the sample duplicates was amplified again in
479 duplicate; after amplification, all replicates of a given sample were combined before purification using
480 magnetic beads (AMPure-XP; Beckmann Coulter, Brea, CA, USA) according to the manufacturer's
481 instructions. After purification, DNA concentrations were determined using the Quant-iT PicoGreen
482 dsDNA Assay Kit (Invitrogen, Paisley, UK). Barcoded amplicons from all samples were pooled in
483 equimolar amounts before sequencing on a 454 GS FLX pyrosequencer using Titanium chemistry (Roche,
484 Penzberg, Germany). The sequence data was processed with QIIME (version 1.9.0) (Caporaso et al.,
485 2010). Demultiplexing and quality filtering (min./max. sequence length: 250/600 bp; primer mismatches
486 and barcode errors: 0; min. quality score: 25; quality score window size: 50 bp) was done using the
487 'split_libraries.py' command. Chimera filtering was done by mapping reads against the SILVA SSU
488 reference database (release 128) (Quast et al., 2013) using 'identify_chimeric_seqs.py' with usearch61 as
489 detection method. After quality and chimera filtering, the average number of combined forward and
490 reverse reads per sample was 5,709 with an average length of 388 bp. OTUs were clustered by uclust
491 against the SILVA SSU reference database at 97% similarity using the 'pick_open_reference_otus.py'
492 command. After removing low-confidence OTUs (combined abundance of < 0.01% across all samples)
493 and OTUs classified as chloroplasts, a total of 910 OTUs remained in the final OTU table. The total

number of reads per sample was rarefied to 2,045 which was the lowest number of reads observed for a single sample. A midpoint-rooted phyologenetic tree was constructed from the alignment of OTU reference sequences using FastTree (Price et al., 2009). Sequence data have been deposited in the NCBI Sequence Read Archive under accession number SRP139256.

Data analysis

All analyses were done in R (version 3.5.0) (R Core Team, 2018). Alpha diversity (OTU richness (*S*), Shannon diversity (*H'*), Pilou's evenness (*J'*)) was calculated using the vegan package (version 2.5-2) (Oksanen et al., 2018). The number of newly-arriving OTUs (*Sn*) in sediment samples at a given time point was defined as the number of OTUs that displayed an abundance > 0% for the first time at that time point. Phylogenetic beta diversity was assessed based on β-MNTD that was calculated using the 'comdistnt' function of the picante package (version 1.7) (Kembel et al., 2010). Differences in microbial community composition between samples across time, space, and community type were illustrated by NMDS performed on the β-MNTD matrix using the 'metaMDS' function of the vegan package with 40 iterations. To test for the effect of physicochemical variables (Table 1) on changes in community composition for sediment-attached and planktonic communities, respectively, variables were standardized to z-scores before fitting to the NDMS ordination using the 'envfit' function of the vegan package with 10,000 permutations stratified within community types. PERMANOVA was used to estimate the marginal effects of each of the three categorical variables community type, sampling time point, and site location, respectively, while holding the other two constant using the 'adonis2' function in vegan package with 10,000 permutations. For the identification of key organisms that were responsible for the differences between community types, relative OTU abundances were summarized at genus level before SIMPER analysis using the 'simper' function in vegan with 1,000 permutations for significance testing. Beta diversity partitioning based on Jaccard dissimilarity was done using the 'betapart' package (Baselga and Orme, 2012).

519 To study the effect of deterministic versus stochastic processes on microbial community assembly,
520 we used the null model approach developed by Stegen et al. (2012; 2013). β-NTI compares the mean
521 phylogenetic distance of OTUs based on β-MNTD between two communities against the distribution of β-
522 MNTD values expected for randomly assembled communities. This distribution is obtained from repeated
523 randomizations in which the OTUs observed in the two communities and their relative abundances are
524 shuffled across the tips of the according phylogenetic tree. The value of β-NTI indicates by how many
525 standard deviations the observed β-MNTD deviates from the mean of the null expectation with $|\beta\text{-NTI}| > 2$
526 indicating significant deviations. β-NTI was calculated with abundance-weighting and 999 randomizations
527 for each pairwise comparison. The assumption of a significant phylogenetic signal was verified using
528 Mantel correlograms as in Dini-Andreote et al. (2015) (see SI and Fig. S6). The RC_{bray} index measures
529 how much the observed Bray-Curtis dissimilarity between two communities differs from the distribution
530 of dissimilarities between probabilistically assembled communities for which the probability of OTUs
531 being drawn is proportional to their respective abundances in the two compared communities and their
532 occurrence frequencies in the regional species pool, while keeping local community richness and the
533 number of individuals constant. RC_{bray} takes values from -1 to +1 where absolute values > 0.95 indicate
534 significant deviations from the null expectation. RC_{bray} was calculated with 999 iterations for each
535 pairwise comparison. Regional species pools for null model simulations were constructed from all OTUs
536 in the full dataset over space and time as in Veach et al. (2016), because we expected that regional species
537 pools constructed separately for each time point from OTUs at the two sites that only spanned a relatively
538 short transect would have been too conservative to estimate the total regional diversity in the aquifer. To
539 evaluate in how far this large regional species pool may have led to an overestimation of the effects of
540 selection and/or dispersal, we compared these results to simulations where regional species pools were
541 constructed for individual time points for which paired samples of sediment-attached and planktonic
542 communities were available. The outcomes of the null models in both situations were in high agreement
543 with each other, indicating that using the full dataset to construct the regional species pool did not
544 introduce a substantial bias in our analyses (see SI and Fig. S7). To test for the effect of changes in

physicochemical conditions on community assembly processes, Mantel tests (Spearman's rank correlation, 10,000 permutations, function 'mantel' in vegan) were performed on the β-NTI matrix and individual Euclidean distance matrices that were calculated for each physicochemical variable separately after standardization.

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Figure legends

Figure 1: Schematic illustration of the main geographical features of the study site, the locations of the two monitoring wells, and general groundwater flow directions.

Figure 2: Temporal dynamics of biomass and alpha diversity for sediment-attached communities and planktonic communities in the groundwater at MIT052 and MIT039, respectively. Biomass is given as the number of prokaryotic cells per mL groundwater or the equivalent volume (cm^3) of sediment. The time in days for each time point is given in parentheses.

839

840 **Figure 3:** (A) Changes over time in the fraction of newly-arriving OTUs (*Sn*) relative to the total number
841 of OTUs (*S*) in sediment-attached communities. (B) Changes over time in the cumulative relative
842 abundance of newly-arriving OTUs that first occurred at the time point indicated in the upper right corner
843 of each graph.

844

845 **Figure 4:** (A) Taxonomic microbial community composition of sediment-attached communities and
846 planktonic communities in the groundwater per time point. Taxonomic groups are summarized at family
847 level. Families with an abundance < 10% in the entire dataset were grouped as 'Diverse others' for clarity
848 of display (mean abundance: 0.1%; max: 4.7%). (B) NMDS plot showing differences in microbial
849 community composition based on abundance-weighted β-MNTD (stress: 0.09).

850

851 **Figure 5:** Values for β-NTI from pairwise microbial community comparisons. The range of β-NTI
852 indicating stochastic community turnover is shaded in grey. (A) Spatial community turnover of planktonic
853 communities in the groundwater and sediment-attached communities, respectively, between MIT052 and
854 MIT039 per time point. (B) Community turnover between planktonic and sediment-attached communities
855 within sites per time point (note: the bar corresponding to the comparison of communities at MIT052 in
856 July is not visible; β-NTI = 0.04). (C) Temporal community turnover of planktonic and sediment-attached
857 communities, respectively, between consecutive time points within sites (note: bar for the comparison of
858 July vs. December for planktonic communities at MIT052 is not visible; β-NTI = -0.005). NA: data not
859 available.

860

861 **Figure S1:** Changes in physicochemical parameters over time measured in the groundwater at the two
862 sites. DOC: dissolved organic carbon; AOC: assimilable organic carbon; DO: dissolved oxygen.

863

Figure S2: Occurrence frequencies of the most dominant families and most dominant single genera within those families among newly-arriving OTUs (*Sn*) in the sediment-attached communities that first occurred at the indicated time point and were still detected in the final communities at MIT052 and MIT039, respectively. Families with an individual occurrence frequency < 3% are grouped as 'Diverse others' for clarity of display. Bars representing each family are ordered by occurrence frequency in descending order from top to bottom.

Figure S3: Differential abundances of genera that contributed most to the dissimilarity between sediment-attached communities and planktonic communities in the groundwater identified by SIMPER analysis. Only the genera with the highest significant contribution to the dissimilarity are shown (> 0.1%; $p < 0.05$); the average contribution of each displayed genus is indicated by the color intensity of the bars. (A) Log_{10}-ratios of differential average abundances in planktonic communities over sediment-attached communities for genera found in both community types. (B) Average relative abundances of genera exclusively found in one community type.

Figure S4: Relative contributions of turnover and nestedness to the total Jaccard dissimilarity between sediment-attached and planktonic communities at each site per time point inferred from beta diversity partitioning.

Figure S5: Relative contributions of turnover and nestedness to the total Jaccard dissimilarity between sediment-attached communities within sites across time points inferred from beta diversity partitioning.

Figure S6: Phylogenetic signal inferred from Mantel correlograms showing Pearson correlation between phylogenetic distances and differences in environmental optima between OTUs within phylogenetic distance classes evaluated at distance steps of 0.01 for (A) sediment-attached and (B) planktonic communities. Filled symbols indicate significant correlations ($p < 0.05$).

890

891 **Figure S7:** Comparison of the outcomes of null model simulations to estimate (A) β-NTI and (B) RC_{bray}
892 based on different regional species pools for pairwise community comparisons shown in Figure 5 and
893 discussed in the main text. The horizontal axes represent results based on regional species pools
894 constructed from OTUs found in the full data set; vertical axes show results based on regional species
895 pools constructed from OTUs found in subsets of samples within time points, or in case of comparisons to
896 estimate temporal community turnover, from two consecutive time points (only for β-NTI). Colors
897 represent the different investigated turnover processes shown in Figure 5 (see main text). Dashed lines
898 mark significance thresholds for each index (see main text). Linear regression slopes of the straight line ±
899 0.95 confidence intervals and Pearson correlation coefficients are indicated in the figures. Flags indicate
900 pairwise comparisons for which the outcomes of the null models did not agree between the two strategies
901 for constructing regional species pools.

902

Tables

Table 1: Mean values and standard deviation (SD) of physicochemical parameters averaged over the two investigated sites and all time points (n = 10). Individual values for each site and time point are shown in Figure S1.

	Water table (m bs)[a]	DOC[b] (mg L^{-1})	AOC[c] (µg L^{-1})	pH	Temperature (°C)	Electrical conductivity (µS cm^{-1})	DO[d] (mg L^{-1})	PO_4^{3-} (mg L^{-1})	SO_4^{2-} (mg L^{-1})	NO_3^- (mg L^{-1})	Cl^- (mg L^{-1})	K^+ (mg L^{-1})	Na^+ (mg L^{-1})	Ca^{2+} (mg L^{-1})	Mg^{2+} (mg L^{-1})
Mean	8.9	1.10	11.4	7.81	6.88	297	10.21	0.011	14.5	2.49	1.80	0.30	1.03	46.1	12.1
SD	5.0	0.37	14.7	0.10	0.59	41	0.82	0.015	8.8	0.78	1.13	0.07	0.62	5.5	1.0

[a] meter below surface
[b] dissolved organic carbon
[c] assimilable organic carbon
[d] dissolved oxygen

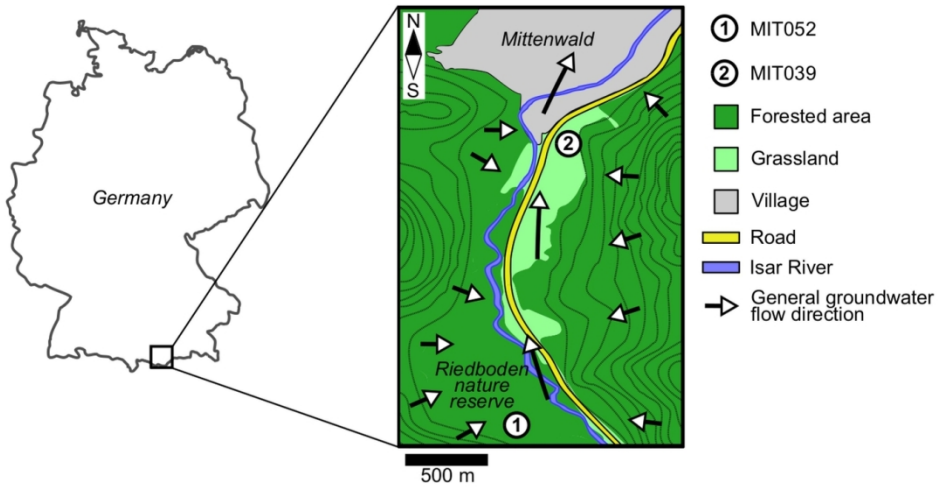

Figure 1: Schematic illustration of the main geographical features of the study site, the locations of the two monitoring wells, and general groundwater flow directions.

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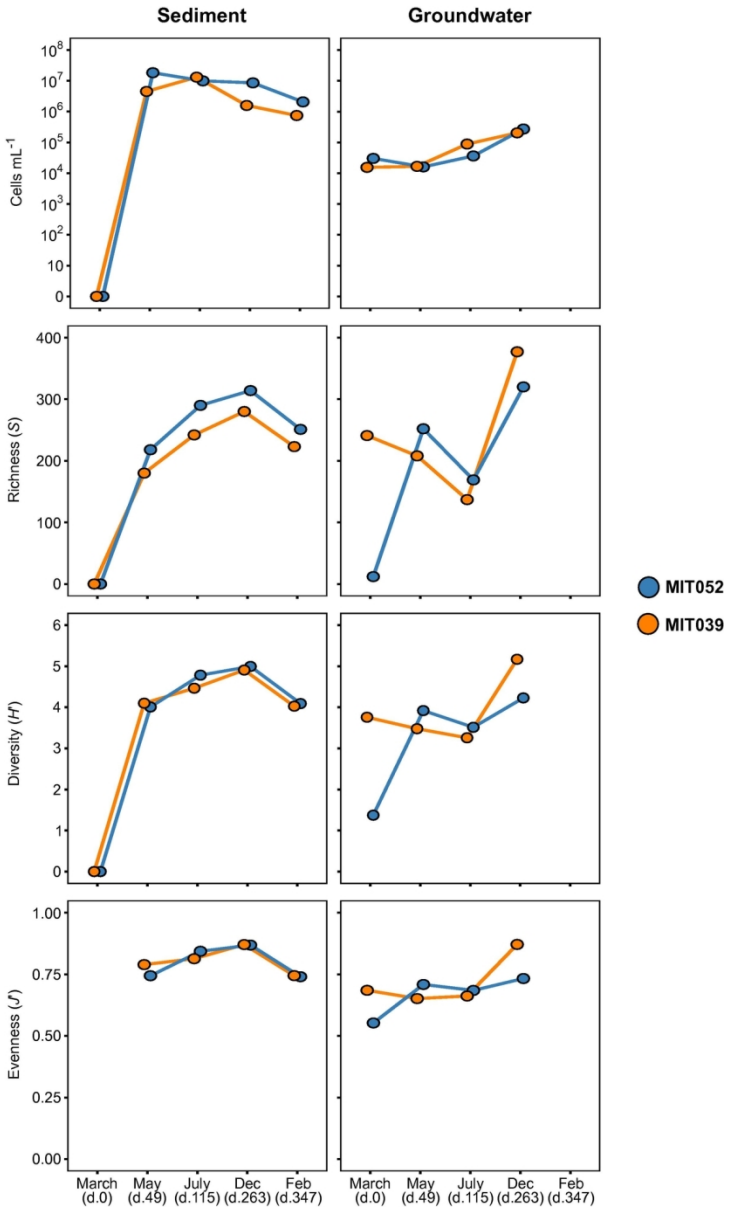

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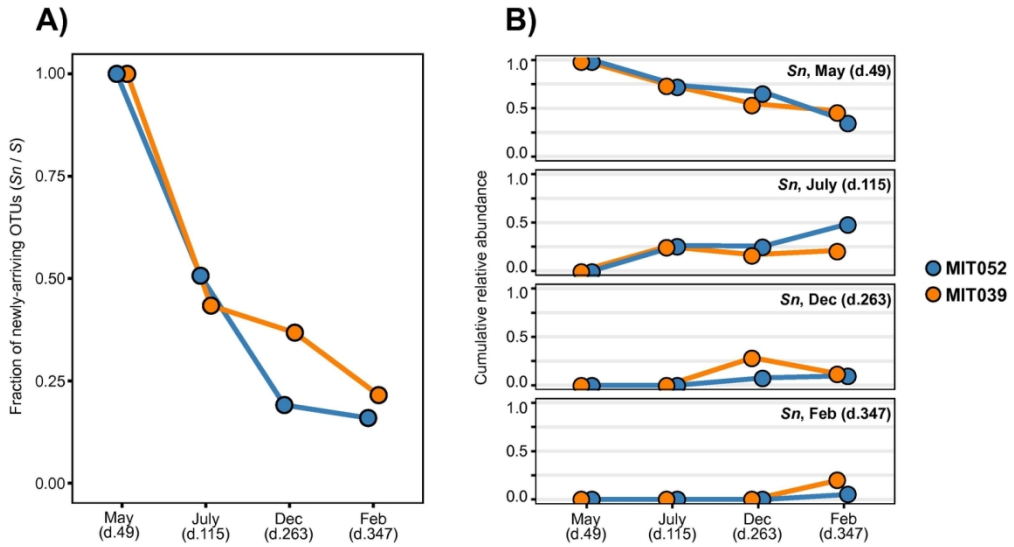

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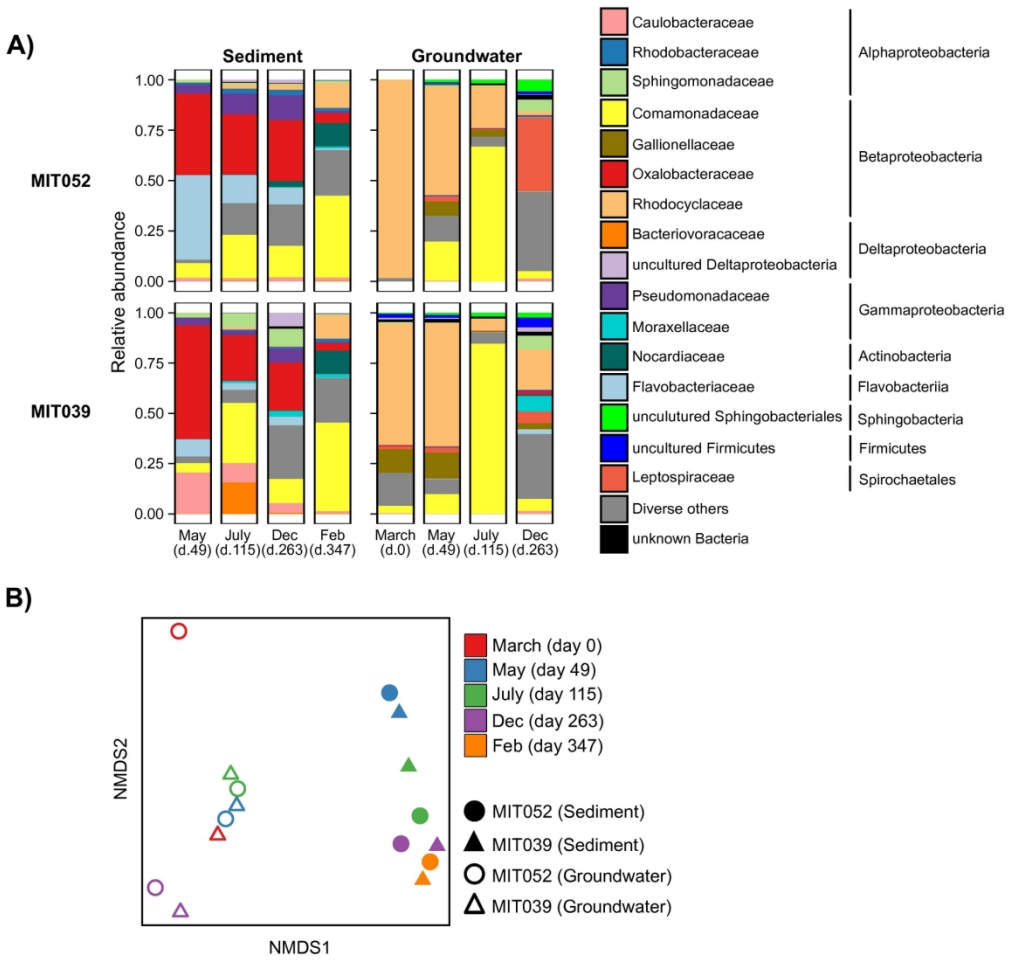

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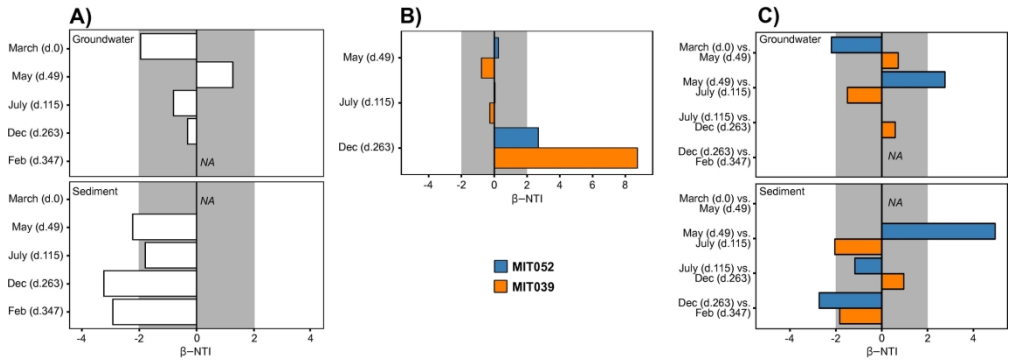

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