Long-term dynamic changes in attached and planktonic microbial communities in a contaminated aquifer

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**Abstract**

Biodegradation is responsible for most contaminant removal in plumes of organic compounds and is fastest at the plume fringe where microbial cell numbers and activity are highest. As the plume migrates from the source, groundwater containing the contaminants and planktonic microbial community encounters uncontaminated substrata on which an attached community subsequently develops. While attached microbial communities are important for biodegradation, the time needed for their establishment, their relationship with the planktonic community and the processes controlling their development are not well understood. We compare the dynamics of development of attached microbial communities on sterile substrata in the field and laboratory microcosms, sampled simultaneously at intervals over two years. We show that attached microbial cell numbers increased rapidly and stabilised after similar periods of incubation (~100 days) in both field and microcosm experiments. These timescales were similar even though variation in the contaminant source evident in the field was absent in microcosm studies, implying that this period was an emergent property of the attached microbial community. 16S rRNA gene sequencing showed that attached and planktonic communities differed markedly, with many attached organisms strongly preferring attachment. Successional processes were evident, both in community diversity indices and from community network analysis. Community development was governed by both deterministic and stochastic processes and was related to the predilection of community members for different lifestyles and the geochemical environment.

**Main findings:**

Attached microbial communities take ~100 days to develop when a substrate is exposed to a contaminant plume in both field and laboratory microcosm experiments. The attached and planktonic communities differ markedly, with community structure driven by a combination of deterministic and stochastic processes, with networks of microbes showing strong preference for attached growth.

**Keywords:** attachment, microbial dynamics, community preferences

**Introduction**

Groundwater contamination represents a major threat to the environment and human health (Schwarzenbach et al., 2010) and its remediation requires large economic and technical investments (Wilson et al., 2007). Of the numerous remediation techniques available, Monitored Natural Attenuation (MNA) has emerged as a cost effective, risk-based strategy to manage groundwater contaminated with petroleum hydrocarbons, chlorinated solvents and aromatic compounds (McGuire et al., 2004; Rivett and Thornton, 2008; Thornton et al., 2001a). MNA relies on an understanding of the physical, chemical and biological processes that reduce the toxicity, mobility and concentration of contaminants (Carey et al., 2000). Of these processes, only biodegradation can reduce the pollutant mass. In contaminated aquifers, biodegradation is naturally enhanced at the chemical interface between the background groundwater and the contaminant plume, known as the *plume fringe*, where cell numbers rise and the activity of microorganisms is high compared to surrounding locations (Lerner et al., 2000; Pickup et al., 2001; Thornton et al., 2001b; Tuxen et al., 2006a; Tuxen et al., 2006b). This zone of enhanced microbial activity is supported by dissolved electron acceptors supplied from the background groundwater by dispersion, which mix with electron donors from the core of the plume (Rees et al., 2007; Thornton et al., 2001a; Thornton et al., 2001b; Wilson et al., 2004)

The biodegradation activity of a microbial community results from contributions from both planktonic and attached members. Attached and planktonic communities often differ in abundance, membership and function. For example, Rizoulis *et al* (Rizoulis et al., 2013) found that the composition of attached and microbial communities differed at 30 metres below ground level (mbgl) in a phenol-contaminated aquifer. In a creosote-contaminated sandy aquifer, the majority of microbes were bound to particles (Godsy et al., 1992) with denitrifiers showing a strong preference for attachment (84-97%). In pristine aquifers, archaeal communities were found to be predominantly planktonic (Flynn et al., 2013; Gregory et al., 2014). Despite their acknowledged importance, the role of attached microbial communities that develop on aquifer substrata is poorly understood (Gregory et al., 2014; Griebler et al., 2002). This lack of knowledge arises from the inherent difficultly in sampling attached communities. Aquifer cores provide access to attached communities but only at a single time point. To overcome these limitations, material can be incubated in laboratory microcosms (Elliott et al., 2010; Rivett and Thornton, 2008; Tuxen et al., 2006a) or sterile substrates can be incubated *in situ* (Converse et al., 2015; Silver et al., 2010)to allow attached communities to develop. These two approaches complement each other, but there have been few systematic, long-duration studies of the time required for attached communities to develop, the changes in microbial composition that occur during this time and comparisons of field and microcosm samples in contaminated aquifers. Developing this understanding is important in contaminated aquifers, given the key role of biodegradation processes in reducing the environmental risk of pollutant releases to groundwater.

Microbial successions during biofilm formation have been described in other environments, from oligotrophic systems such as lakes (Brislawn et al., 2019; Jackson et al., 2001), model water distribution systems (Martiny et al., 2003), substrates exposed by receding glaciers (Zumsteg et al., 2012) and streams (Besemer et al., 2012) to carbon-enriched environments such as greywater (Truu et al., 2019) and waste water distribution systems (Zhang et al., 2019), coal seams (Beckmann et al., 2019; Vick et al., 2019) and bioreactors (Liebana et al., 2019). The time-scales and trajectories of these successions are governed by stochastic and/or deterministic processes that are often highly dependent on the environment (Fierer et al., 2010). Understanding these processes provides insight into the factors that control community assembly (Stegen et al., 2012). Interactions between organisms are also important drivers of biofilm community assemblage and can be uncovered by network analysis, identifying microbes that co-occur and show a preference for attached growth. Biofilm formation is a complex and dynamic multi-stage process. Primary colonizers attach to substrates and produce a variety of extracellular polymeric substances (EPS) that strengthen binding, adhesion and cohesion of cells (Costerton et al., 1995; Stoodley et al., 2002; Wingender et al., 1999). These early colonisers can proliferate *in situ* but also facilitate the attachment of late colonizers by co-adhesion. The resulting cell aggregates form micro-colonies eventually leading to the development of multi-species biofilms in which diverse physical and metabolic interactions can occur (Hall-Stoodley et al., 2004; Rickard et al., 2003).

Cell numbers in uncontaminated aquifers are typically very low, reflecting the oligotrophic environment. Microbial numbers typically increase 100-1000 fold at a contaminant plume fringe due to the increased availability of carbon and energetically favourable electron acceptors, with a concomitant increase in biodegradation rates (Thornton et al., 2001a; Tuxen et al., 2006a; Tuxen et al., 2006b). As plumes advance, previously uncontaminated aquifer substrate encounters the mobile aqueous phase, but the sequence and timing of microbial development on the substratum in response to these incursions is poorly understood. Given the relative importance of the attached and planktonic microbial communities in biodegradation, understanding these processes is vital for parameterising models that underpin MNA implementation and, more generally, understanding the microbial ecology of natural environments.

The aim of this study was to examine how attached microbial communities developed at the anaerobic fringe of a plume of phenolic compounds in a sandstone aquifer (Lerner et al., 2000; Thornton et al., 2001b). This plume is migrating slowly (1-10 m year-1) and biodegradation is also slow, with only 4% of the original contaminant input consumed over 47 years (Thornton et al., 2001a). At the plume fringe cell numbers increased by several orders of magnitude and conditions quickly become anaerobic as oxygen in the groundwater was consumed by respiration (Pickup et al., 2001; Thornton et al., 2001b). We incubated substratum (of the same mineralogy as the aquifer) *in situ* at the plume fringe and concurrently in laboratory microcosms, using groundwater from the plume fringe as inoculum. Measurements were made over an extended period (days-years) allowing us to compare, directly, community development *in situ* and in microcosms. Specifically, we wanted to determine how quickly attached communities developed when exposed to a contaminant plume and its associated planktonic microbial community, the relationship between attached and planktonic communities, how community structure changed over time and the mechanisms that controlled these processes. We hypothesized that some microbial groups from the planktonic community would preferentially attach to the substrate, thus dominating early stages of attached communities then, as the biofilm matured, other groups would increase in relative abundance. We also explored the processes that governed community assemblage and identified networks of organisms that developed in attached communities.

**Results**

Groundwater was sampled at the fringe (30 metres below ground level - mbgl) of a phenol plume in a sandstone aquifer using a multilevel groundwater sampler (MLS). This region of the plume is contaminated with a mixture of phenolic compounds (phenol, cresols, xylenols) and represents the most active area of biodegradation (Thornton et al., 2001b). Large numbers of planktonic cells are present (1010 planktoniccells L-1) compared to the surrounding uncontaminated groundwater where cell numbers are ~1000-fold lower (Pickup et al., 2001). This groundwater was used to establish microcosms which contained sterilised sand of the same composition as the aquifer matrix. At the same time, small bags of the sand substratum were introduced into the aquifer (suspended within the 0.5m screen of the MLS used to collect the groundwater). Groundwater and sand bags were then collected from the field site, and aqueous phase and sand were sampled from laboratory microcosms, to determine how attached and planktonic communities developed over the 780 days of the experiment. Sampling was completed at the same time intervals and analysis was destructive, so each sample represented an independent biological replicate.

**Geochemistry and microbial abundance**

Selected geochemical measurements of groundwater samples from the field and the aqueous phase of microcosms are shown in Fig. 1. In the field there were marked changes in groundwater chemistry during the experiment (Fig. 1A). At the start of the experiment the phenol concentration was 308 mg L-1, m,p-cresols 146 mg L-1, o-cresols 12 mg L-1 and xylenols 67 mg L-1. During the first 100 days, the concentrations of these organic compounds varied markedly, decreasing to approximately half the starting concentrations within 7 days and then rising to values approximately double the starting concentrations by 100 days. Concentrations then fell and, by the end of the experiment (780 days), were much lower (~4.0 mg L-1). These changes arose from natural variation in the contaminant plume source term and were accompanied by changes in the concentration of soluble electron acceptors. The concentrations of the reduced electron acceptors Fe2+ and Mn2+ followed the same pattern as the phenolics, indicative of anaerobic respiratory processes deeper within the plume (9). The concentration of nitrate showed the opposite pattern, falling from a starting concentration of 30 mg L-1 to values between 5-10 mg L-1. Sulphate remained relatively constant, between 10 and 30 mg L-1.

As expected, variation in the geochemistry of the laboratory microcosms was much lower, as source term variation was eliminated due to the static nature of these systems. Concentrations of phenol, cresols and xylenols remained relatively constant during the experiment, with <8% consumed, consistent with the slow rates of biodegradation seen at this site. However, limited biodegradation of organic compounds in the live microcosms occurred, as shown by the accumulation of the metabolites 4-hydroxybenzoic acid and 4-hydroxy-3-methyl benzoic acid at concentrations of ~0.1 mg L-1 after 47 days. These metabolites persisted during the experiment (Fig. S1). There was greater variation in the concentrations of electron acceptors (or their reduced products). Soluble Fe2+ decreased from 1.3 mg L-1 to 0.3 mg L-1 in the live microcosms. Soluble Mn2+ increased from 0.9 mg L-1 to 3.5 mg L-1, similar changes occurred in both live and abiotic systems, indicating a geochemical interaction between the groundwater and introduced substrate, with differences between the abiotic and biotic samples only evident at later time-points, after 400 and 780 days. The nitrate concentration decreased rapidly from 20.7 mg L-1 to 0.3 mg L-1 and continued to decline, likely due to consumption by denitrification. Nitrate could not be measured in the abiotic controls as the sterilizing agent sodium azide interfered with the assay. The sulphate concentration remained stable during the experiment.

The number of attached and planktonic microbial cells in field and microcosm samples are shown in Fig. 2. In the field, planktonic cell numbers declined from an initial value of 1.8 x 1010 cells L-1 to ~ 1 x 109 cells L-1. Attached cell numbers rose rapidly over the first 100 days. After 1 week ~2 x 107 cells L-1 had attached, increasing to ~2 x 109 cells L-1 by 104 days and increasing slowly thereafter. In microcosms, planktonic cell numbers fell to ~ 7 x 108 cells L-1 but then recovered to ~1 x 1010 cells L-1. Attached cell numbers in microcosms followed a similar pattern to that seen in the field, with numbers rising over the first 100 days to reach ~2 x 109 cells L-1. Statistical analysis using ANCOVA of log-transformed cell counts showed that in all communities there were significant differences over time. There was no significant difference between field and microcosm attached cell numbers (*p* = 0.99), but planktonic samples differed at later timepoints (*p* =0.0125 for the interaction ‘sample x day’). Microscopic analysis of the sediments showed that the attached cells were widely separated and formed only small aggregates (Fig. S2).

**Microbial community structure and diversity**

The structure and diversity of the planktonic and attached microbial communities were assessed by extraction of total DNA followed by amplification and high-throughput sequencing of 16S rRNA genes. A mean of 77,292 sequences per sample were obtained from 69 independent samples (See Table S1 for details). A total of 14,892 Operational Taxonomic Units (OTUs) were identified at 97% sequence similarity. After removing taxa that were poorly represented (less than 30 reads across all samples) and those that did not appear at least 3 times in 20% of the samples, 786 unique OTUs were identified of which 780 were classified as bacteria, and only 6 as Archaea. The composition of the microbial communities was broadly similar at the Class taxonomic level (Fig. 3 A) with major contributions from Proteobacteria (particularly α, β and γ), Actinobacteria, Bacteriodia, Bacilli and Clostridia.

Principal components analysis using the phylogenetically-aware Unifrac distance measure based on OTU presence/absence (Fig. 3B) showed clear separation of attached and planktonic samples in the field and microcosms. In an analysis weighted for OTU abundance (Fig. 3C) differences between systems were less evident. Permutational multivariate analysis of variance (PERMANOVA) analysis showed that there was a significant effect of time (p = 0.008) for each community in the field and in microcosms.

Richness and Inverse Simpson indices are shown in Fig. 3 D, E. In field samples, the richness of the planktonic community was relatively constant over time whereas the richness of the attached community was initially lower than that of the planktonic community (p = 0.014) and increased over time. In microcosm samples, the richness of attached and planktonic samples was similar (p = 0.903) and remained constant over time. The richness of attached samples was similar between field and microcosm samples, but the richness of planktonic samples was greater in field samples compared to planktonic samples. The Inverse Simpson indices of planktonic samples was relatively constant during the experiment, but marked changes were evident in both field and microcosm attached communities. In both communities the Inverse Simpson index was greatest at the earliest timepoint measured (7 days) and then decreased markedly over the following 40 days. The index then increased again over the following year.

**Differences between attached and planktonic microbial communities.**

A generalized linear model implemented in DESeq2 was used to identify microbes in field or microcosm communities that showed a preference for the attached or planktonic phase. Comparisons were made for each time point separately. Of the 786 unique OTUs in the filtered data set, 110 OTUs showed a preference for the attached phase and 267 showed a preference for the planktonic phase. Significant overlap was evident between field and microcosm samples, with 30 OTUs common in the attached communities and 94 in the planktonic communities.

Fig. 4 shows the relative abundance of these OTUs grouped by Class. In both field and microcosm samples, members of the α-, β- and γ-Proteobacteria showed preferential attachment. The α-Proteobacteria included Sphingomonads and Methylobacteria, the β-Proteobacteria included Oxalobacteria and the γ -Proteobacteria included Alkanindiges and Pseudomonads. In microcosms members of the Bacilli and Actinobacteria also showed preferential attachment (although the latter were only significant at later time points).

The preferentially planktonic communities in the field and microcosms included members of the Bacteroidia (Bacteroidales sp, Paludibacter and Porphyromonadaceae sp), Clostridia (Anaerococcus, Desulfosporosinus, Sedimentibacter and Veillonellaceae) and diverse β-Proteobacteria (Azoarcus, Comamonadaceae sp, Gallionella, Rhodocyclaceae sp , Simplicispira, Sulfuritalea, Thiobacillus). In the field, preferentially planktonic communities also included members of the δ-Proteobacteria (Desulfobacteraceae sp, Desulfobulbaceae sp, Desulfovibrio, Geobacter, Syntrophorhabdaceae sp) and Synergistia.

A network analysis was performed on the microbes that showed preferential attachment in the microcosms (Fig. 5a). Of the 82 OTUs that were preferential attachers, 61 were members of the network and formed 10 submodules. The largest submodule (I) contained 11 members, including Moraxellaceae (*Alkanindiges spp*) and Oxalobacteraceae. Other large submodules were (II) including Rhodospirillaceae (*Magnetospirillum spp.*) (III) including *Corynebacteriaceae* (IV) including *Pseudomonadaceae* (XIII) including *Methylobacteriaceae* (IX) which contained *Pseudomonadaceae* and *Sphingomonadaceae.* Figure 5b shows the relative abundance of the major submodules and their change over time. In most cases initial high values measured 7 days after incubation fell and then recovered. The recovery in submodule II was maximal at 101 days, in submodules III, IV, V and IX was maximal at 174-400 days and in submodule IIX was maximal at 780 days.

**Processes controlling community assembly**

Clear phylogenetic patterns were evident within these data, with some classes of OTUs showing marked preferences for attached or planktonic growth (e.g. the majority of γ -Proteobacteria showed preferential attachment whilst Bacteroidia, Clostridia and δ-Proteobacteria wee preferentially planktonic). To determine the potential ecological processes driving microbial community development in these different samples, ecological models were analysed using the approach described in (Stegen et al., 2012). β-mean taxon distances were calculated for each pairwise comparison. The deviation of this value from that expected by chance is known as the β-nearest taxon index (βNTI). Values of 2 or greater indicate that communities differ more than expected by chance and values less than -2 indicate more similarity than by chance. Values of βNTI between -2 and 2 are indicative of stochastic processes. Stochastic processes were analysed using Raup-Crick measurements. After scaling, BCRC values greater than 0.95 indicate dispersal limitation, whereas values less than -0.95 indicate homogenising dispersal - intermediate values indicate that multiple processes are operating.

Calculations were performed for both individual replicates and the means of replicates for all samples (field and microcosm). The results obtained were for individual samples and aggregated means were similar, so only results from mean values are shown for clarity. Fig. 6 shows the βNTI and BCRC values calculated for field and microcosm, attached and planktonic samples. Values for BCRC are only informative for stochastic processes, so are shown only when βNTI values were between -2 and 2.

Overall, a mixture of deterministic and stochastic processes was operating. For those comparisons found to be deterministic all βNTI values were >2 (red squares in Fig. 6), indicative of variable selection where communities differ more than expected by chance. Where stochastic processes were indicated, the majority were either mixed or had values of BCRC >0.95, indicating that turnover between communities was greater than expected by chance. The exception to this were later timepoints within planktonic microcosm communities with values of BCRC < -0.95, found when communities can freely move within environments.

Within a single community (e.g. comparisons made within attached field samples at different times) this mixture of deterministic and attached processes operated over time (highlighted with dotted lines in Fig. 6). In contrast, comparisons between attached field and planktonic field samples were dominated by deterministic responses which was true, albeit to a lesser extent, of comparisons between attached microcosm and planktonic microcosm communities.

**Discussion**

The aim of this study was to investigate microbial community development as biofilms formed at a chemical interface in a contaminated aquifer, in this case, the fringe of a phenol plume. Such locations are typically characterised by steep gradients in geochemical conditions such as contaminants, their metabolites and terminal electron acceptors. Despite the very high carbon availability at these sites, microbial numbers are relatively low as other factors (typically N and P) limit microbial growth (Demoling et al., 2007), in both planktonic and attached phases. Even after extended periods of incubation, biofilms are sparse and not limited by the availability of attachment sites.

**Geochemical variation at the plume fringe and microcosms**

In the field chemical concentrations at the lower plume fringe of this site are known to vary markedly over time (Lerner et al., 2000; Pickup et al., 2001; Spence et al., 2001; Thornton et al., 2001b) as a consequence of source term variation and vertical transverse dispersion (Thornton et al., 2001a; Thornton et al., 2001b). These processes support the enhanced microbial activity and rates of biodegradation at the fringe due to constant re-supply of dissolved electron acceptors (e.g. nitrate and sulphate) in background groundwater to the plume (Baker et al., 2012; Kummel et al., 2015; Lerner et al., 2000; Meckenstock et al., 2015; Thornton et al., 2001b; Tuxen et al., 2006a). In the microcosms these processes did not occur and hence contaminant concentrations remained relatively stable. Phenolic compounds were biodegraded, as evident from the accumulation of organic metabolites 4-hydroxybenzoic acid and 4-hydroxy-3-methyl benzoic acid which originate from metabolism of phenol, *o-*cresol and *p-*cresol via para-carboxylation and anaerobic oxidation (Bisaillon et al., 1991; Boll and Fuchs, 2005; Bossert and Young, 1986). These organic metabolites also accumulated in the anaerobic anode chamber of microbial biofuel cells established using groundwater from the upper plume fringe (12 mbgl) from this field site (Hedbavna et al., 2016).

Changes in concentrations of dissolved electron acceptors in the field were masked by source term variation but were evident in the static microcosms, where source term variation was eliminated. As expected, nitrate, an energetically favourable electron acceptor, was utilised quickly in the anaerobic biodegradation of the phenolic compounds (Gonzalez et al., 2006). This confirms the presence of either nitrate reducers or denitrifying activity, which have been observed in groundwater monitoring wells at the field site and in microcosms constructed with this groundwater as inoculum (Spence et al., 2001; Williams et al., 2001). The increased concentration of dissolved manganese most likely resulted from the microbial reduction of manganese oxide coatings on the aquifer material (Baker et al., 2012; Lerner, 2002; Lerner et al., 2000; Thornton et al., 2001b). The difference between live and abiotic microcosms at the later stages of the experiment may be related to the activity of manganese reducers, which have been described in contaminant plumes in groundwater (Christensen et al., 2000; Gounot, 1994) and observed in the phenolic plume in this aquifer (Pickup et al., 2001; Rizoulis et al., 2013; Thornton et al., 2001a). Although sulphate reduction was not evident, sulphate reducing bacteria in the Class *delta-proteobacteria* (e.g. *Desulfobacterales*, *Desulfovibrionales*, *Desulfomonadales*) were identified in the attached and planktonic communities of microcosms. These taxa can use sulphate as an electron acceptor in different anaerobic environments (Kuever, 2014; Song et al., 2015; Varon-Lopez et al., 2014; Zapata-Peñasco et al., 2016).

**Timescales for development of attached microbial communities and phylogenetic differences between attached and planktonic communities**

Cell numbers in the planktonic and attached microbial communities in the aquifer and microcosms were consistent with previous studies at this site (Elliott et al., 2010; Pickup et al., 2001; Rizoulis et al., 2013). Despite the differences in geochemistry and planktonic communities between field and microcosm experiments, the numbers of attached cells increased at similar rates in both environments, reaching a maximum value after ~100 days. The time needed for attached cell numbers to stabilise, despite variation in the stability of the geochemistry of the systems, can therefore be considered as an emergent property of the microbial community (Casadevall et al., 2011). Phylogenetic analysis of microbial 16S rRNA genes indicated that planktonic and attached communities differed from each other, whether considering presence/absence, relative abundance or phylogenetic relatedness. Rizolulis *et al* (Rizoulis et al., 2013) also found that these communities differed in samples collected from this same site (30 mbgl), but studied only a single time point. Differences in abundance and community composition have also been observed in other aquifers (Flynn et al., 2013; Godsy et al., 1992; Gregory et al., 2014; Griebler et al., 2002; Lehman et al., 2001a; Lehman et al., 2001b).

The differences in attached and planktonic communities that we observed were sustained over time in both field and microcosm samples. In biofilms that develop in nutrient-rich environments, space for attachment is often a limiting factor and competition for space a major driver of community composition (Ellis et al., 2015; Ghoul and Mitri, 2016). However, in many natural environments, nutrients (in this case, particularly P and, to a lesser extent, N) are limiting, such that biofilms are relatively sparse. Microscopic analysis of grains showed that space was not a limiting factor, even after extended incubation periods. For the planktonic field samples, richness (community membership) and diversity (as assessed by the Inverse Simpson index) were relatively high during the experiment but varied as a consequence of changing field conditions. In the microcosms planktonic richness and diversity were somewhat lower, with a marked fall in diversity at the outset of the experiment, accompanied by a transient fall in planktonic cell numbers. This can be attributed to a ‘bottle effect’ (Holm et al., 1992), where a proportion of microbes from the field environment were not able to establish in the microcosms whilst others grew preferentially, reducing overall diversity. The attached field and microcosm communities responded in a similar manner to each other. Initial cell numbers were low, but diversity was high, indicating that diverse planktonic cells had attached to the uncolonized grains. As the incubation continued, attached cell numbers increased rapidly whilst diversity decreased, which we attribute to proliferation of a subset of the attached community with a preference for this growth phase. Diversity then increased (up to 200 days), consistent with recruitment of additional community members, either as a consequence of their ability to attach to existing biofilm members and/or metabolic interactions due to metabolite availability. Once attached community numbers had stabilised (100 days+) diversity fell, which we attribute to competition within the attached community and selection of preferential attachers.

These patterns were reflected in the relative abundance of OTUs that formed submodules in the network of preferentially attached microbes. Their relative abundance tended to fall, then recover, with different submodules showing maximum relative abundance at different times, indicative of successional processes within the attached community. These continued even after extended periods of incubation (780 days), showing that the composition of the attached community was dynamic.

Dynamic changes in attached communities have been observed during the development of multi-species biofilms in drinking water distribution systems, human dental plaque and colon (Jackson, 2003; Marsh, 2004; Martiny et al., 2003; O'Toole et al., 2000; O'Toole and Kolter, 1998; Rickard et al., 2003; Wingender et al., 1999). These dynamic changes during the development of attached communities are consistent with microbial ecological successions expected to occur when new substrates become available and begin to be colonised by different members of the planktonic microbial community, and by different interactions (physical and metabolic) between the attached community members. Successions have been described in biofilms of oligotrophic environments (streams, rivers, and simulated-marine systems) where interactions between attachers occur as biofilms develop (Besemer et al., 2012; Brislawn et al., 2019; Lyautey et al., 2005; Moura et al., 2018; Poltak and Cooper, 2011; Woodcock and Sloan, 2017). Similar dynamic changes also occur in carbon-enriched environments (i.e. wastewater bioreactors and distribution systems, coal seams and filters used in greywater treatment), with cell numbers increasing during biofilm development and changes in the relative abundances of different microbial groups (Beckmann et al., 2019; Lequette et al., 2019; Liebana et al., 2019; Truu et al., 2019; Vick et al., 2019; Zhang et al., 2019).

**Processes controlling community assembly**

Both deterministic and stochastic processes (or mixtures of the two) have been proposed to explain the trajectory and time scales of microbial successions. For example, it has been suggested that stochastic processes (e.g. stochastic attachment, reproduction and death) are the major drivers of successions at early stages during biofilm formation, especially in rapidly changing communities, in which early recruitment is largely dependent on the source planktonic community (Besemer et al., 2007; Fierer et al., 2010; Martiny et al., 2003). In contrast, other studies point to deterministic processes (e.g. selection) having a greater influence during the early stages of microbial succession, mostly driven by the ability of some microbes to overcome the new environmental conditions imposed by disturbances (Liebana et al., 2019). Different processes also influence the medium and late stages of biofilm formation. For example, stochastic processes are proposed to be more important in biofilm formation in hyper-saline lakes (Brislawn et al., 2019) whilst deterministic processes, such as competition, influence biofilm development in rivers (Besemer et al., 2012). Despite this variation in mechanisms, time-dependency is a common factor which is influenced by the type of community (planktonic or attached) or disturbances (Zhou et al., 2014).

In the current study, analysis of the processes that controlled microbial community structure demonstrated that both deterministic and stochastic processes were important. Where deterministic processes operated, βNTI values were always positive indicating that communities were more different than expected by chance. Changes in field, planktonic samples over time were dominated by source-term variation. During the first 100 days of sampling, total phenolics were high and variable but were lower and more stable thereafter. Field planktonic samples represent ‘volumes’ of groundwater flowing past the sampling well. High phenolic concentrations have a strong selective effect on the microbial community leading to high, positive βNTI values if the environment differs between sampling points. After 100 days, when conditions were less variable, βNTI values were lower and stochastic processes became important, indicative of dispersal limitation. In field, attached communities, a mixture of deterministic and stochastic processes operated. Strong deterministic processes were evident in comparisons between field, attached and planktonic communities, supporting the view that organisms are adapted to the different growth phases.

Broadly similar responses were seen in microcosm samples although deterministic processes, whilst evident, were less strong. At later timepoints (100 days+) planktonic, microcosm communities were driven by stochastic processes with high dispersal, i.e. organisms within these systems could mix freely. This is expected as the planktonic community had adapted to the stable microcosm environment and differences between samples occurred large by chance. Deterministic processes were still evident in comparisons between microcosm, attached and planktonic communities.

High throughput sequencing of 16S rRNA genes allowed us to identify OTUs that showed a preference for attached or planktonic growth phases. These preferences were evident in a large proportion of the total community. Whilst organisms that showed a preference for growth in the planktonic phase were still present in the attached phase, albeit at lower abundance, preferential attachers showed a much more polarised response, being largely absent from the planktonic phase. This preference could be related to the advantages associated with living within biofilms in chemically changeable environments (Hall-Stoodley et al., 2004; Watnick and Kolter, 2000), or close physical association facilitating metabolic interactions. The ability to resist environmental disturbances (e.g. changes in temperature, pH, aqueous geochemistry) can be tolerated more successfully when microbes are associated in heterogeneous environments, and protected with EPS (Jefferson, 2004; Stewart and Franklin, 2008). Other advantages include the transfer of advantageous genetic material (Angles et al., 1993; Hausner and Wuertz, 1999; Watnick and Kolter, 2000) and the recycling of electron acceptors in environments where these molecules are limited (Hernandez and Newman, 2001). Although a ‘bottle effect’ is evident in the microcosm samples, our data show that preferences for attachment are also seen in samples incubated in the field, with some of the same OTUs found in both field and microcosm samples. For example, preferential attachers included members of the *Alkanindiges* in both field microcosm and field samples, which are known hydrocarbon degraders.

The preference of specific OTUs for the aquifer sediment could be explained by different physical and physiological properties (e.g. presence of extracellular motile organelles and production of EPS) aiding their rapid attachment. Network analysis identified 10 main submodules within the preferential attachers, which included many OTUs with known abilities to degrade organic compounds. Module I included *Alkanindiges spp.* (Family Moraxellaceae), whilst module II contained *Magnetospirillum* *spp*. These are able to degrade aromatic compounds, particularly *p*-cresol using iron as an electron acceptor and have been identified in biofilms of pump and treat systems treating gasoline-contaminated groundwater (Benedek et al., 2016; Shinoda et al., 2005). Module III contained several *Cornybacterium* spp. which are able to use manganese as an electron acceptor and are proposed to be present in aquifer sediments (Di-Ruggiero and Gounot, 1990; Du et al., 2010). Some organisms found to be preferential attachers included those first identified as human or animal associated (e.g. members of the *Neisseria* or *Staphylococcaceae*), (module VIII), but many of these have subsequently been identified in environmental samples and associated with biofilms. *Staphylococcus* spp. have been detected in groundwater from contaminated aquifers (Grisey et al., 2010; Krapac et al., 2002; Ozler and Aydin, 2008) and is known to form biofilms on environmental surfaces (Leroy et al., 2010). *Neisseria spp.* are commonly found associated with animal hosts but also in contaminated environments and can degrade pollutants (Liu et al., 2015). In the field, late colonisers included *Ellin6607 sp., Dokdonella sp*., which has been described in biofilms used to treat wastewater (Jiang et al., 2018; Xia et al., 2005) and *Rhodococcus sp*. reported in biofilters treating naphthenic acids (Zhang et al., 2019). Other modules (IV and IX) contained other common degraders such as *Pseudomonas* and *Sphingomonas* spp. These have been described as early colonisers and EPS producers during biofilm formation on reverse osmosis membranes, and facilitating the attachment of other microbes (Bereschenko et al., 2010; Vu et al., 2009).

Preferential planktonic OTUs were more flexible, in that they were also represented in the attached community at moderate relative abundances. This could reflect their ability to express different properties or physiological states according to their lifestyle (Costerton et al., 2003; Mah and O'Toole, 2001; Moreno-Paz et al., 2010). This is a common strategy used by microbial community members during their lifecycle, which can include attachment and detachment events during the biofilm formation (Watnick and Kolter, 2000).

The microbial dynamics (i.e. time scale of microbial attachment, progressive changes in diversity indices and preferences for different growth phases) seen in this study are likely to be relevant to other systems where space for attachment is not limited, but other limitations (e.g. micronutrients) restrict microbial growth, leading to the formation of sparse biofilms. The microcosm samples mirrored many aspects of the *in situ* field samples, demonstrating that microcosm studies can replicate many aspects of attached community development *in situ.* This has significant implications for studies seeking to parameterise models underpinning the application of bioremediation or MNA for groundwater treatment, as microcosm studies are amenable to manipulation, thus allowing different field and intervention scenarios to be investigated.

**Conclusions**

This study has shown that numerically-stable attached microbial communities developed after ~100 days when sterile aquifer material was introduced into a complex planktonic community in a contaminated aquifer. This interval is therefore likely an emergent property of the attached microbial communities, as *in situ* and laboratory microcosms required a remarkably similar period for cell numbers to stabilise. However, community membership continued to change over longer periods. We provide evidence that significant numbers of community members showed preferences for different lifestyles. Attached members showed a strong predilection for attachment to substrates and co-occurred with microbes with similar preferences. These findings are of relevance to environments where the carbon supply is not limited and the attached community develops as sparse biofilms. In such systems, the time scale of microbial attachment is often assumed, but here we offer evidence of the periods required for different aspects of the community to become apparent. In terms of plume management and hydrogeological modelling, our study explores microbial dynamics and community membership that is often put into a “black-box”, by management or engineered interventions that seek to remediate groundwater contamination. These data will help parameterise models underpinning the application of bioremediation and MNA for the treatment and management of contaminated groundwater in a more realistic manner.

**Methods**

**Substrate preparation**

Permo-Triassic red sandstone with similar mineralogy to the original aquifer material, as described by Harrison *et al* (Harrison et al., 2001), was obtained from Penkridge Pottal Pool (Hanson Aggregates, Penkridge, UK). The sandstone was crushed and size-fractionated using 150 and 300 μm sieves (Endecotts Ltd, London, UK) to remove coarse components. This fraction is representative of the *in situ* aquifer material. The prepared material was sterilized by autoclaving at 121°C for 20 min.

**Microcosm design and inoculum**

The microcosms were assembled under aseptic and anaerobic conditions using an anaerobic chamber (PlasLabs 815-Glove Box, PlasLabs Inc, USA). Each microcosm consisted of a 0.125 L Wheaton glass serum bottles washed with Decon 90® and 0.1 M HNO3. All bottles were sterilized by autoclaving at 121°C for 20 min. Groundwater from the contaminant plume fringe previously characterized (Pickup et al., 2001; Thornton et al., 2001b; Williams et al., 2001) and containing a mixture of phenol, cresols and xylenols was collected from the 0.5m screen of a MLS 30 mbgl at the study site using a peristaltic pump and transferred via a closed system into sterile, N2-filled amber glass bottles (Elliott et al., 2010). Each microcosm consisted of 100 g of sterilized aquifer material and 0.1 L of groundwater. Microcosms were sealed with an aluminium crimp cap and butyl rubber stopper (Fisherbrand, UK). Abiotic controls were constructed in a similar way and included sodium azide (NaN3) at a final concentration of 2 g L-1 to inhibit microbial activity. The microcosms were incubated at 10°C in the dark.

**Incubation *in situ***

Sterile nylon mesh (100 μm pore diameter) (Plastok, Ltd., Birkenhead, UK) was used to construct 10 cm long bags filled with 75 g of prepared sand grains. Sandbags were placed in a 316 grade stainless steel cage (50 cm length x 4 cm diameter) which was suspended on May 29th 2014 in the middle of a 0.5m screen at 30 mbgl within the MLS (BH59) used for the incubation experiment at the field site (Thornton et al., 2001b). A schematic of the experimental design is shown in Figure S3.

**Microcosm sampling**

Triplicate microcosms were sampled destructively at 7, 23, 47, 104, 174, 400 and 780 days after inoculation. Triplicate abiotic controls were also sampled. The supernatant was removed with a sterile pipette and 30 mL filtered through a sterile 0.22 µm filter (Whatman® polycarbonate membrane, 25 mm diameter), and stored at -80°C prior to DNA extraction. A 5 mL sample of the supernatant was fixed with 4% (v/v) formaldehyde and stored at 4°C for cell counts. The remaining supernatant was filtered with a 0.22 μm filter and stored at 4°C for chemical analysis. Two aliquots were fixed using HNO3 at a final concentration of either 1 or 10% for metal analysis. The sediments were collected and homogenized using a sterile spatula. The homogenized sediments were separated into two aliquots, one stored at -80°C for DNA extraction and the other fixed with 4% formaldehyde and stored at 4°C for cell counts.

**Fieldsampling**

For sand bag collection the well head casing above ground was covered with a clear polythene bag and a hose introduced into the monitoring well below the water table (approximately 4 mbgl) to purge the standing water and overlying head space with N2 within the casing, to ensure anaerobic conditions for sample retrieval. The metal cage containing the sand bags was then brought to the surface inside the polythene bag under a steady flow of N2 and one sand bag removed and placed in an N2-filled container (sterile Anaerocult jar). All manipulations of the sand bags at the field site were undertaken inside the polythene bag under an N2 atmosphere. The metal cage was then lowered back into position within the well screen for future sampling. Groundwater from 30 mbgl depth was collected at the same time for hydrochemical and microbiological analysis using sampling tubes attached to the MLS (Thornton et al., 2001b). Samples were taken after 30 min purging of the sample tubes with a peristaltic pump at a rate of ~0.1 L min-1 for 20 minutes, filtered onsite and preserved as per samples collected from the microcosm experiments.

**Cell counts**

Groundwater and supernatant from microcosm samples was filtered through black 0.22 µm filters (Whatman® polycarbonate membranes, 25 mm diameter). Sediments samples were placed on a Cover Well™ Imaging Chamber (300 µL volume and 0.9 mm depth) (Science services, UK). Sand grains were washed with filter-sterilised 0.15 M NaCl. Samples were stained with 200 μL of SYTO® 9 (Green Fluorescent Nucleic Acid Stain; Dilution 1:800) for 15 minutes. At least 10 randomly distributed fields of view were visualized with a RT-KE Slider 7.4 digital camera (Diagnostic Instruments, USA) and epifluorescence microscope (BX50, Olympus Optical Co., London, UK) using excitation at 470 nm and emission at 510 nm. Fluorescent microbial cells were counted using Image Pro Plus version 4.5 (MediaCybernetics, UK) (Rizoulis et al., 2013). For the grains multiple images were taken at different focal planes under both bright field and fluorescent light, and an extended depth-of-field image produced. To calculate cell numbers in the attached community, the average grain size diameter was measured (215.6 µm) and the volume of 1 grain was estimated using Vgrain = 4/3πr3, where r is the radius. The volume was used to estimate the number of grains contained in 1 mL, assuming an aquifer porosity of 26% (7). Using the average number of bacteria attached to 1 grain and considering only one side of the grain could be imaged (Gough and Stahl, 2003), the cell densities of the biofilm were calculated for 1 mL of water-saturated sand.

**DNA extraction**

DNA from the planktonic community was extracted using the UltraClean® Microbial DNA Isolation kit (MoBio, UK). The polycarbonate membrane used to collect the planktonic community was placed into the microbead tube and then processed according to the manufacturer’s instructions. DNA from the attached community was extracted using the PowerSoil® DNA Isolation Kit (Qiagen, UK). DNA from sediments was concentrated using the DNA obtained from 6 independent extractions per sample, plus 1200 µL of pure ethanol and a final concentration of 11 mM of NaCl. The sample was centrifuged at 10000 *g* for 5 min and dried in a vacuum centrifuge before resuspension in 50 µL of ultrapure water.

**16S rRNA gene fragment library preparation**

To remove inhibitors from DNA extracted from sediment, 30 µL of DNA solution from each sediment sample was diluted with 470 µL ultrapure water and re-concentrated using a Microcon device (DNA Fast Flow PCR Grade with Ultracel membrane, Merck) according to the manufacturer's instructions. Three aliquots from each sample were amplified in separate PCR reactions using the primer pair Illumina-341F (5`- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3`) and Illumina-805R (5`- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3`) (Klindworth et al., 2013). The PCR conditions were 95 °C for 5 minutes, 30 cycles of 95°C for 40 s, 55.0°C for 60 s, and 72°C for 60 s and then a final step of 72°C for 7 min. 2% (w/v) agarose gels were used to visualize the amplicons. Amplification conditions were chosen to produce bands of low-to-mid intensity (Ihrmark et al., 2012). If high band intensities were obtained, input DNA samples were diluted until the desired intensity was obtained. The PCR products from each amplification were pooled and purified using AMPure XP PCR purification beads in ratio of 1:0.95 (sample:beads) according to the manufacturer instructions (Beckman Coulter). A 5 µL volume of purified amplicons was indexed using Illumina Nextera® XT DNA Library Preparation Kit (Illumina). The Index-PCR conditions were 95 °C for 3 minutes, 12 cycles of 95°C for 30 s, 55.0°C for 30 s, and 72°C for 30 s, and then a final step of 72°C for 5 min. The AMPure purification step was repeated. Quantification of the final Indexed samples was made using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermoscientific, UK). Samples were diluted as required to a final concentration of 16 ng DNA µL-1 to produce a library for sequencing. Samples were sequenced by synthesis using the IlluminaR MiSeq instrument, on a V2 chemistry flow cell with 250bp paired end reads at the Earlham Institute (Norwich Research Park Innovation Centre, Colney Ln, Norwich,UK, NR4 7UZ).

**Bioinformatic analysis**

Demultiplexed 16S rRNA sequences were provided by The Earlham Institute as forward (R1) and reverse (R2) reads in FASTQ format. For quality checks Usearch 8.1 (Edgar, 2010) was used. For data processing Quantitative Insights into Microbial Ecology (QIIME) was used (Caporaso et al., 2010). Sequences with greater than 1 in 1000 errors and short reads below 350 bp were removed. The forward and reverse reads were merged and converted to FASTA format. The primer sequences (17 bp from 5’ and 21 bp from 3’ ends respectively) were removed. Chimeras were identified and removed using *de novo* abundances and comparing with the RDP gold data base (Wang et al., 2007). A biom table was generated using Greengenes data base (DeSantis et al., 2006; McDonald et al., 2012) at 97% similarity. Further processing was performed using the phyloseq package (McMurdie and Holmes, 2013) in R (R Core Team, 2020) . Where needed, samples were rarefied to the same sequencing depth (20,000 reads). Principal components analysis using weighted and unweighted Unifrac distances was used to visualise distances between samples and statistical analysis of samples performed by Permanova using the ‘adonis’ package (Lozupone et al., 2011). Differential OTU abundance was determined with DESeq2 using unrarefied data and parametric estimation of the count-variance relationship (Love et al., 2014). Network analysis used the ‘spiecEasi’ package (Kurtz et al., 2015) with submodules identified using the ‘cluster\_fast\_greedy’ algorithm (Clauset et al., 2004) implemented in the package ‘igraph’ (Csardi and Nepusz, 2006). Ecological models described in (Stegen et al., 2012) were implemented using code adapted from (Danczak et al., 2018)

**Chemical analysis**

Chemical analysis of groundwater samples was performed as described in [18, 48]. Phenol, o-cresol, m/p-cresol, xylenols (2,3-xylenol, 2,4/2,5-xylenol, 2,6-xylenol, 3,5-xylenol, 3,6-xylenol), 4-hydroxybenzoic acid and 4-hydroxy-3-methyl benzoic acid were analysed by high-pressure liquid chromatography (HPLC). The stationary phase was an Hypersil C18 column, 250 mm length, 4.6mm diameter and 5 µm particle size (Thermo Scientific, UK). The mobile phase consisted of 20% pure Acetonitrile HPLC grade (Merck, UK) and 80% of a 1% (v/v) acetic acid solution. The instrument used was a Perkin-Elmer Series 200 (Perkin Elmer, UK) system with a UV/VIS detector calibrated with standards and analytical quality controls. The method detection limit and precision were 1mg L-1 and ± 5%, respectively. A 1 mL sample volume was put into a 2 mL glass vial (Restek, UK) and 100 µL of sample was injected into the instrument. For the mass spectrometry analysis different fractions of the HPLC eluent were collected in 2 mL glass vials filled with nitrogen. The samples were manually injected into a QStar Elite (ABI Sciex, UK) mass spectrometer with an ion spray injector. Samples were run in negative mode; the ion spray voltage was -38000 V and the source temperature was 100⁰C. Major ions (including NO3-, NO2- and SO42-) were determined by ion chromatography using a Dionex 3000 (Thermo Scientific, UK) system with a detection limit of 1 mg L-1 and precision of ± 3%. Samples were diluted 10 times and put in 2 mL glass vials. Metal (Mn 2+, Fe2+) concentrations were determined by inductively coupled plasma spectrophotometry using a Spectro Flame M120 instrument with a detection limit of 0.01 mg L-1 and precision of ± 2%. Samples were serially diluted to 10, 20, 50 and 100 times and acidified at 1% using trace metal grade nitric acid (Fischer Chemical, UK.

**Data availability**

The 16S rRNA gene sequences are available through the European Nucleotide Archive (ENA) at the European Bioinformatics Institute (EBI) under accession number PRJEB34776.

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A close up of a map

Description automatically generated

Figure 1

Concentration of phenolic compounds (phenol, m/p-cresols, o-cresols and xylenols), soluble electron acceptors (nitrate and sulfate) and metals (Fe2+ and Mn2+) in field and microcosm samples. Single measurements were made for field samples. Microcosm samples are means +/- SD for triplicate samples. Live/field samples are shown as open circles and abiotic controls are shown as closed circles.

A close up of a map

Description automatically generated

Figure 2.

Attached (closed circles) and planktonic (open circles) cells in (A) field and (B) microcosm samples. Results are means +/- SD.

Chart, bar chart

Description automatically generated

Figure 3

Analysis of community composition. (A) Composition of planktonic and attached microbial communities at the Class level. Average values are shown for microcosm samples, field samples are a single measurement. Classes that contribute <3% of the total are shown as ‘Other’ for clarity. Principal components analysis using the phylogenetically-aware distance measure Unifrac, calculated on (B) presence/absence (unweighted) or (C) weighted for OTU abundance. Attached (circles) and planktonic (squares) are coloured according to time after the start of incubation. The ellipses indicate the 95% confidence intervals of attached or planktonic samples in field and microcosm experiment for all timepoints. (D) Richness and (E) Inverse Simpson values for these microbial communities. Individual values for each sample are shown as points (filled circles - attached, open circles -planktonic) with the solid line connecting means +/- SE (grey).

Diagram

Description automatically generated

Figure 4.

Relative abundance of OTUs grouped by Class that exhibit a preference for attached or planktonic growth. The relative abundance of OTUs that were significantly different on the specified days are shown as coloured bars. White bars show the relative abundance of the same OTUs on days where the differences were not statistically significantly different. Grey bars show the total relative abundance of all OTUs in the Class. Only Classes where the relative abundance exceeded 3% are shown for clarity. Values below the axis show relative abundance in the attached phase, values above the axis in the planktonic phase. Each OTU is separated by black lines. Results are shown as the mean of 3 individual replicates.

A close up of a map

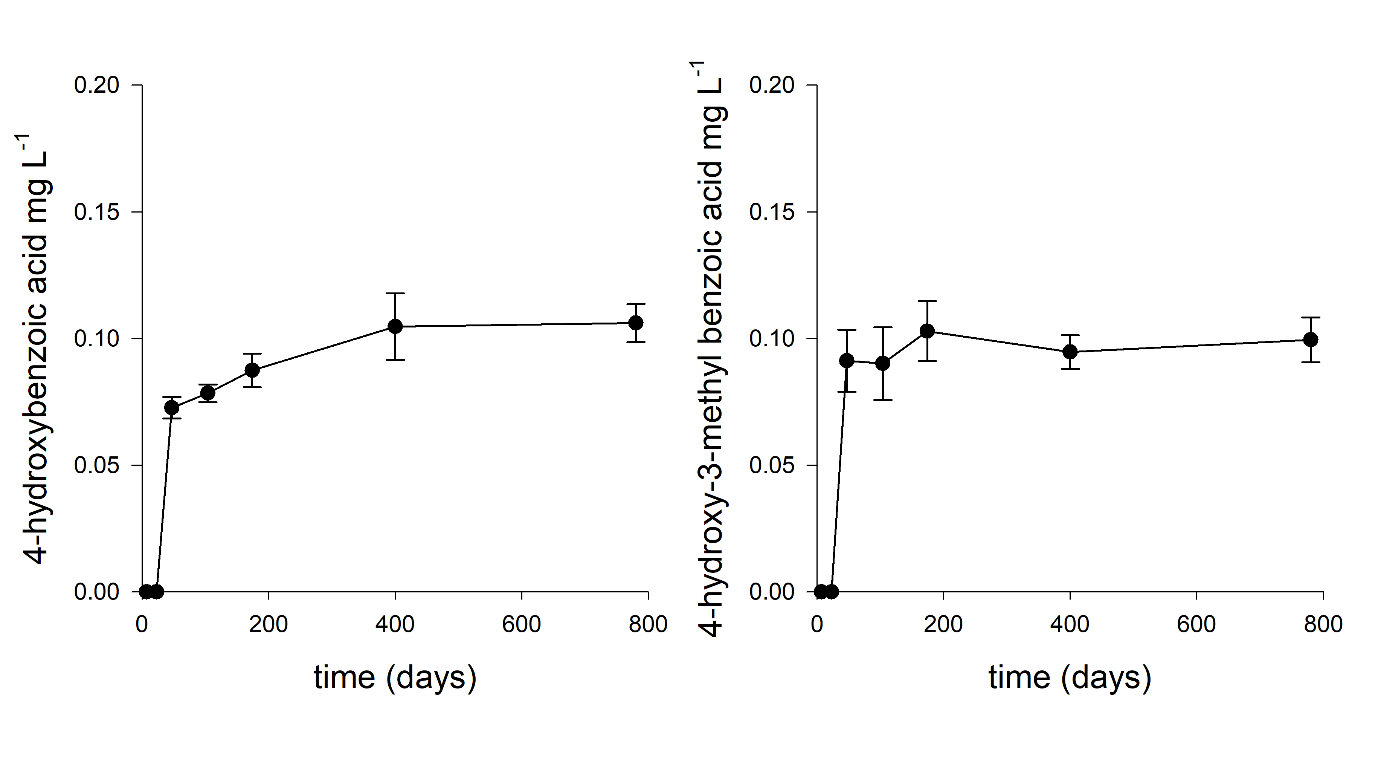
Description automatically generated

Figure 5. Network analysis of OTUs that showed preferential attachment in microcosms. (A) Networks of OTUs that show preferential attachment coloured at the Family taxonomic level. Submodules are labelled I-X. (B) shows the relative abundance of major submodules in microcosm samples. Values in the planktonic phase are shown above the x axis, values in the attached phase, below the x axis. Values are the means of three independent replicate microcosms.

A picture containing drawing

Description automatically generatedFigure 6. βNTI (upper, right) and BCRP (lower, left) values for (A) field and (B) microcosm samples. βNTI values are shown in red for deterministic processes (>2) and yellow/grey for stochastic processes (between -2 and 2). No values were below -2. BCRP values are only shown where stochastic processes are prevalent. BCRC values above 0.95 (green) and below -0.95 (blue) indicative of single stochastic processes are shown as bold colours. Intermediate values, indicative of multiple processes, are shown as pale colours.

**Supplementary Figures**



**B**

**A**

Figure S1.

Detection of metabolic intermediates in the microcosm. (A) 4-hydroxybenzoic acid, (B) 4-hydroxy-3-methyl benzoic acid. Results are the mean +/- SE of 3 independent replicates.

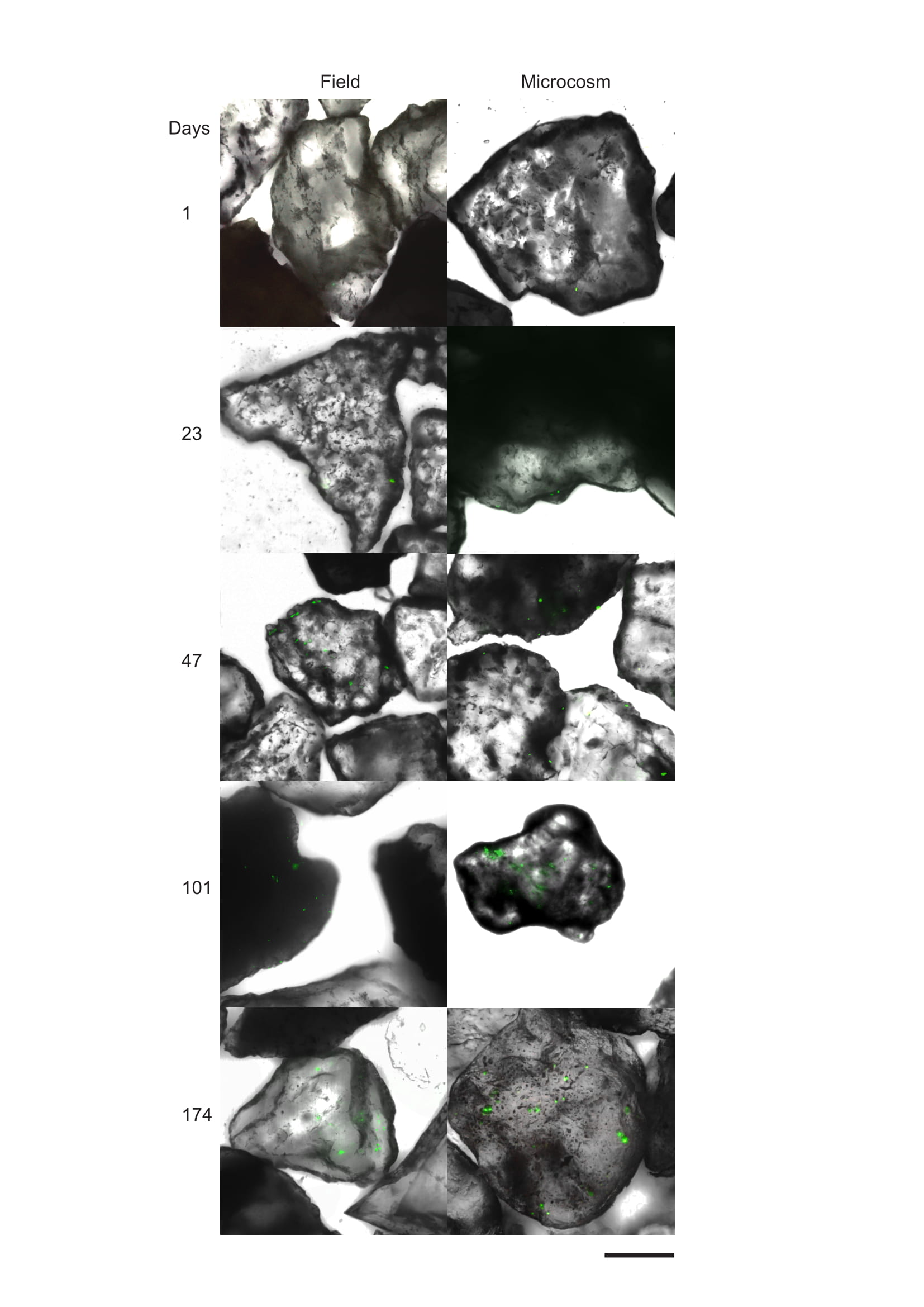
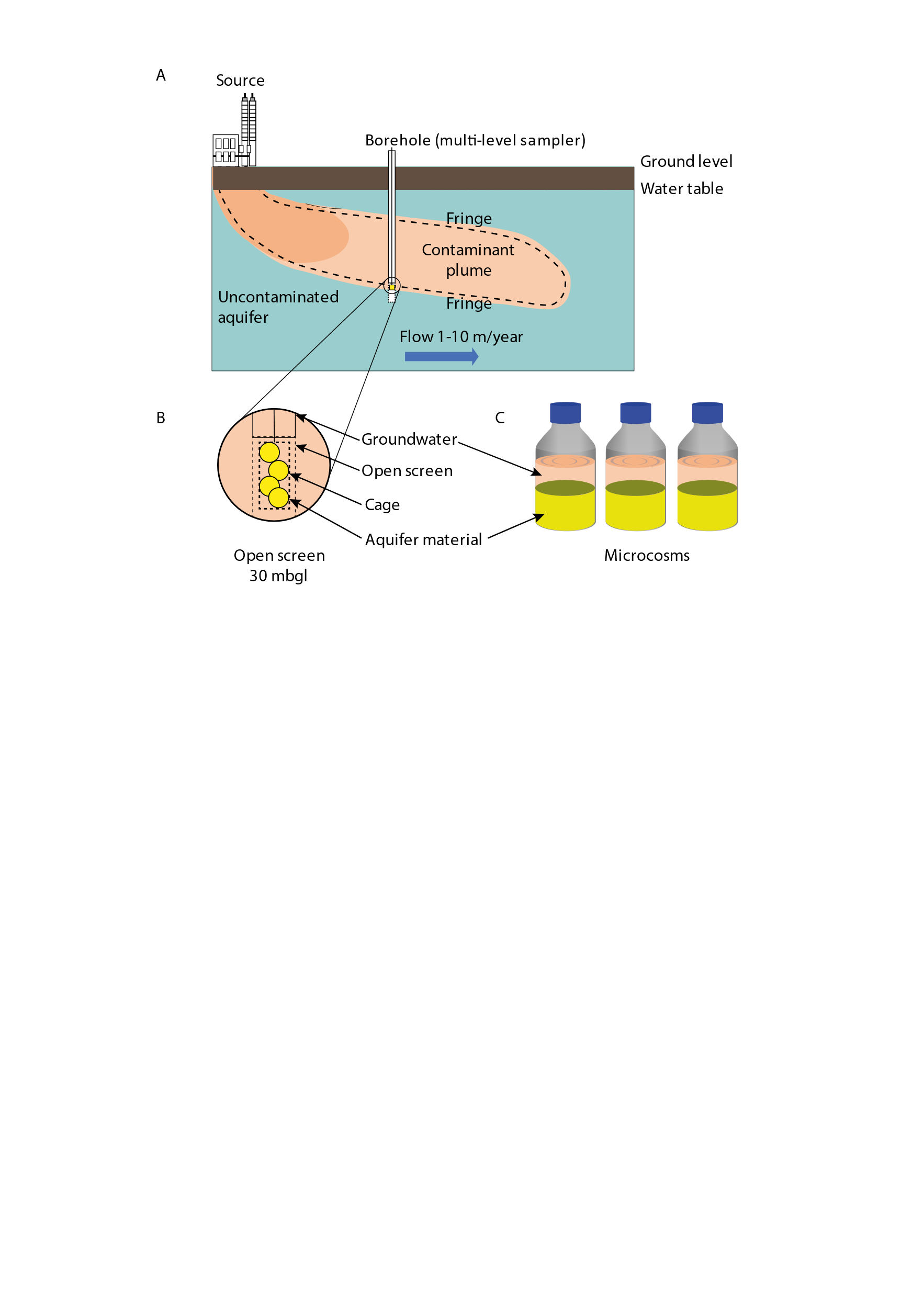


Figure S2.

Sand grains from field and microcosm samples harvested at different time intervals. Each picture is a composite image generated by a combination of bright field and fluorescence images. Scale bar = 120 µm.

Figure S3



A schematic representation of the experimental design (not to scale). (A) the contaminated plume extends from the source into an uncontaminated sandstone aquifer. Microbial activity is greatest in the plume fringe. The borehole contains a multi-level sampler and an open screen. (B) A cage containing bags of aquifer material was suspended at 30 mbgl in the lower plume fringe. Bags were removed at intervals for sampling. (C) Microcosms were established containing aquifer material and groundwater pumped from 30 mbgl (containing planktonic microbial communities) and sampled destructively at intervals.

Table S1.

Number of raw reads obtained from Illumina sequencing of each sample.

| Experiment | Community | Days | Replicate | Number of reads |
| --- | --- | --- | --- | --- |
| Field | planktonic | 1 | 1 | 55105 |
| Field | planktonic | 1 | 2 | 73617 |
| Field | planktonic | 1 | 3 | 183761 |
| Field | planktonic | 7 | 1 | 59842 |
| Field | planktonic | 7 | 2 | 72127 |
| Field | planktonic | 7 | 3 | 59432 |
| Field | planktonic | 23 | 1 | 99547 |
| Field | planktonic | 23 | 2 | 1765343 |
| Field | planktonic | 23 | 3 | 1418557 |
| Field | planktonic | 47 | 1 | 63590 |
| Field | planktonic | 104 | 1 | 171336 |
| Field | planktonic | 104 | 2 | 111678 |
| Field | planktonic | 104 | 3 | 380181 |
| Field | planktonic | 174 | 1 | 109356 |
| Field | planktonic | 174 | 2 | 221472 |
| Field | planktonic | 174 | 3 | 70912 |
| Field | planktonic | 400 | 1 | 118496 |
| Field | planktonic | 780 | 1 | 94094 |
| Field | planktonic | 780 | 2 | 214858 |
| Field | planktonic | 780 | 3 | 328323 |
| Field | attached | 7 | 1 | 135412 |
| Field | attached | 23 | 1 | 174019 |
| Field | attached | 47 | 1 | 148332 |
| Field | attached | 104 | 1 | 141337 |
| Field | attached | 174 | 1 | 136631 |
| Field | attached | 400 | 1 | 199871 |
| Field | attached | 780 | 1 | 111830 |
| Field | attached | 780 | 2 | 215380 |
| microcosm | planktonic | 7 | 1 | 102863 |
| microcosm | planktonic | 7 | 2 | 137868 |
| microcosm | planktonic | 7 | 3 | 109377 |
| microcosm | planktonic | 23 | 1 | 42961 |
| microcosm | planktonic | 23 | 2 | 73554 |
| microcosm | planktonic | 23 | 3 | 89403 |
| microcosm | planktonic | 47 | 1 | 97212 |
| microcosm | planktonic | 47 | 2 | 97847 |
| microcosm | planktonic | 47 | 3 | 40338 |
| microcosm | planktonic | 104 | 1 | 188413 |
| microcosm | planktonic | 104 | 2 | 178445 |
| microcosm | planktonic | 104 | 3 | 202026 |
| microcosm | planktonic | 174 | 1 | 126224 |
| microcosm | planktonic | 174 | 2 | 257205 |
| microcosm | planktonic | 174 | 3 | 106340 |
| microcosm | planktonic | 400 | 1 | 173618 |
| microcosm | planktonic | 400 | 2 | 107969 |
| microcosm | planktonic | 780 | 1 | 120948 |
| microcosm | planktonic | 780 | 2 | 207263 |
| microcosm | planktonic | 780 | 3 | 119448 |
| microcosm | attached | 7 | 1 | 46479 |
| microcosm | attached | 7 | 2 | 123521 |
| microcosm | attached | 7 | 3 | 183626 |
| microcosm | attached | 23 | 1 | 71008 |
| microcosm | attached | 23 | 2 | 141528 |
| microcosm | attached | 23 | 3 | 140979 |
| microcosm | attached | 47 | 1 | 69046 |
| microcosm | attached | 47 | 2 | 123380 |
| microcosm | attached | 47 | 3 | 314380 |
| microcosm | attached | 104 | 1 | 261587 |
| microcosm | attached | 104 | 2 | 275096 |
| microcosm | attached | 104 | 3 | 80089 |
| microcosm | attached | 174 | 1 | 316528 |
| microcosm | attached | 174 | 2 | 92254 |
| microcosm | attached | 174 | 3 | 163186 |
| microcosm | attached | 400 | 1 | 173335 |
| microcosm | attached | 400 | 2 | 135966 |
| microcosm | attached | 400 | 3 | 153211 |
| microcosm | attached | 780 | 1 | 134676 |
| microcosm | attached | 780 | 2 | 164373 |
| microcosm | attached | 780 | 3 | 115956 |

**Abbreviations**

**MNA:** Monitored Natural Attenuation

**EPS:** Extracellular polymeric substances

**MLS**: Multilevel groundwater sampler

**mbgl:** Metres below ground level

**OTUs:** Operational taxonomic units

**PERMANOVA:** Permutational multivariate analysis of variance

**ANCOVA:** Analyis of covariance

**βNTI:** β-nearest taxon index

**BCRC**: Raup-Crick (Bray Curtis)

**rRNA:** ribosomal RNA

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