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SHORT COMMUNICATION

Architectural differentiation reflects bacterial community structure in stream biofilms

Katharina Besemer[1], Iris Hödl[1], Gabriel Singer[1] and Tom J Battin[1,2]
[1]*Department of Freshwater Ecology, University of Vienna, Vienna, Austria and* [2]*WasserCluster Lunz, Dr Carl Kupelwieser Promenade 5, Lunz am See, Austria*

Laboratory studies have documented the extensive architectural differentiation of biofilms into complex structures, including filamentous streamers generated by turbulent flow. Still, it remains elusive whether this spatial organization of natural biofilms is reflected in the community structure. We analyzed bacterial community differentiation between the base and streamers (filamentous structures floating in the water) of stream biofilms under various flow conditions using denaturing gradient gel electrophoresis (DGGE) and sequencing. Fourth-corner analysis showed pronounced deviation from random community structure suggesting that streamers constitute a more competitive zone within the biofilm than its base. The same analysis also showed members of the *α-Proteobacteria* and *Gemmatimonadetes* to preferentially colonize the biofilm base, whereas *β-Proteobacteria* and *Bacteroidetes* were comparatively strong competitors in the streamers. We suggest this micro-scale differentiation as a response to the environmental dynamics in natural ecosystems.
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An important part of prokaryotic biodiversity and biomass is contained in sedimentary and interfacial environments (Whitman *et al.*, 1998). Here microbes form attached and matrix-enclosed biofilms with extensive architectures, including mushroom-like structures, ripples and streamers (Costerton *et al.*, 1995; Hall-Stoodley *et al.*, 2004). Fossil evidence shows that architectural differentiation is an ancient and integral characteristic of biofilms (Hall-Stoodley *et al.*, 2004). However, it remains elusive whether this spatial organization is reflected in the community structure. Illuminating this link is essential to better understand biofilm architectural differentiation as a possible adaptation to the environment.

Laboratory-based studies have extensively documented the differentiation of biofilms into complex structures and morphologies (Costerton *et al.*, 1995; Stoodley *et al.*, 1999; Hall-Stoodley *et al.*, 2004). A remarkable degree of phenotypic diversity can underlie the development of such structures now increasingly considered as a response to stress and as a dispersal strategy (for example, Koh *et al.*, 2007). Filamentous streamers were repeatedly reported from monospecies or mixed bacterial biofilms and seem largely generated by turbulence-induced shear (Stoodley *et al.*, 1999). They can form biofilms themselves, develop either directly from the biofilm base or from its canopy, and float in the bulk liquid. Such streamers also form frequently in benthic stream biofilms (Figures 1a and b). They can associate with diatoms (Figure 1c) and other algae to develop extraordinarily long filaments in circumneutral streams (Besemer *et al.*, 2007) and contribute most biomass to acidophilic species-poor biofilms (Hallberg *et al.*, 2006).

In this study, we analyzed bacterial community differentiation between the base (attached to the substratum) and streamers (floating in the water) of biofilms. To capture a large variation of biofilms, we sampled them from various hydrodynamic environments and growth stages. In fact, headwater streams, where biofilms dominate microbial life, are characterized by varying streambed geomorphologies and associated flow conditions. Therefore, we studied biofilm differentiation under controlled laminar, transitional and turbulent flow in laboratory flumes (Singer *et al.*, 2006; Besemer *et al.*, 2007) and in large-scale streamside flumes along triangular

Correspondence: TJ Battin, Department of Freshwater Ecology, University of Vienna, Althanstrasse 14, Vienna, A-1090, Austria.
E-mail: tom.battin@univie.ac.at
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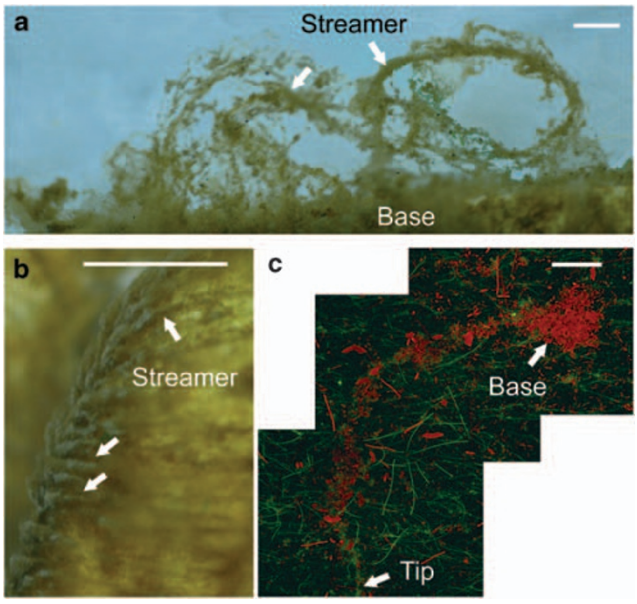

Figure 1 (**a**) Representative stream biofilm (day 61) from the streamside flumes showing differentiation into the base and streamers; lateral view. The scale bar refers to 5 mm. (**b**) Macroscopic streamers (day 17) at the crest of a stone on the streambed. Scale bar refers to 5 mm. (**c**) Streamer (day 8) as seen by epifluorescence microscopy. Bacteria appear green (stained with SYTO 13, Invitrogen) and algae red (auto-fluorescence of chlorophyll *a*). Scale bar refers to 250 μm.

bedforms inducing gradients of flow velocity and turbulence (Supplementary Figure 1). Though we did not explicitly study the temporal development of streamers, we sampled biofilms repeatedly depending on streamer development, which differed in both experimental setups (days 50, 62 and 83 of biofilm growth in the laboratory flumes; days 17 and 61 in the streamside flumes; see Supplementary information for details). Macroscopic streamers (minimum a few mm long; Figure 1a) were carefully separated from the biofilm base in the laboratory using sterile (ethanol-flamed) tweezers and dissection microscopy. In total, this resulted in 45 paired base and streamer samples from five growth stages and eight different hydrodynamic conditions (Supplementary Table 1). Denaturing gradient gel electrophoresis (DGGE) was carried out on all biofilm samples. In all, 54 clearly visible DGGE-bands were excised, the DNA fragment was reamplified and sequenced (see Supplementary information and Supplementary Figure 2). Sequences were analyzed using the software of the Ribosomal Database Project (Wang *et al.*, 2007; Cole *et al.*, 2009).

The community 'fingerprints' (from DGGE) were transferred into a band presence-absence matrix. DGGE-bands appearing at the same position on a gel were regarded as the same operational taxonomic unit (OTU). The similarity between the base and streamer of a given biofilm was calculated as the Sørensen's Index, $SI = 2S/(A_1 + A_2)$, where S is the number of shared OTUs, and A_1 and A_2 are the total numbers of OTUs in the respective samples. We carried out a fourth-corner analysis to test for differences in the distribution of phylogenetic groups between the biofilm base and streamers (Supplementary information). This analysis relates the biological attributes (in our case the phylogenetic affiliation) of organisms to the environmental conditions of their habitats (Legendre *et al.*, 1997). Several permutation models have been proposed to test the overall null hypothesis that the attributes of an organism are unrelated to the environmental conditions. Permutation model 1 tests the hypothesis that species are found in locations where they encounter optimal living conditions versus the null hypothesis that all parts of the environment are equally suitable for all species (environmental control model). The number of locations occupied by a given species remains fixed and is considered to reflect characteristics of the species, such as abundance, intraspecific competition and ecological plasticity. Permutation model 2 does the same at the level of species assemblages, assuming strong biotic ties among the members of the community. Permutation model 3 tests the hypothesis that species have a competitive advantage in the location where they are found versus the null hypothesis that the identity (species) of an individual settling is a chance event (lottery model). In spite of holding the number of occurrences of a given species constant, it assumes a fixed number of niches available in a given location. Permutation model 4 differs from model 3 in the way that it tests the relevance of the biological attributes for given preferences of organisms for environmental conditions (Legendre *et al.*, 1997; Dray and Legendre, 2008). We chose model 1 and 3 because they test two contrasting hypotheses and are less restrictive than model 2 and 4, which take the link between species and their traits (model 2) or the species preferences for sites (model 4) for granted (Legendre *et al.*, 1997; Dray and Legendre, 2008). It should be noticed, however, that the fourth-corner analysis does not reveal the nature of the competition (for example, for resources).

We generally observed streamer development starting from several single filaments that merged to a broad 'head' and successively elongated into a 'tail', as also described from monospecies biofilms (for example, Stoodley *et al.*, 1999). In nascent streamers, bacterial cells were initially mixed with small diatoms (Figure 1c) and the inclusion of further algae allowed streamers to develop remarkable length (up to several centimetres in the streamside flumes; Figures 1a and b). It should be noted that *Diatoma* microcolonies, but also filamentous algae, can form networks that provide stability and putatively elasticity to streamers; they may also provide exudates that support microbial heterotrophs within these complex assemblages.

Average α-diversity (that is, richness of OTUs as derived from DGGE) was significantly higher in the biofilm base than in the streamers ($P<0.001$) when all the dates from both setups were pooled. No significant trend emerged across biofilm age (Figure 2a) or hydrodynamic treatment (data not shown). γ-diversity (that is, the total number of OTUs in base or streamers at a given date) and the numbers of OTUs specific for either base or streamers were also higher in the base (Figures 2b and c) at four of the five sampling occasions. Collectively, these patterns point to a community shift between architectural features, such as streamers and the base, and support recent findings from an acidophilic biofilm (Wilmes *et al.*, 2009).

The Sørensen's Index indicates that the community structure of the biofilm base and the streamers was generally more similar in the laboratory than in the streamside flumes (Figure 2d), which we tentatively attribute to scaling effects. Biofilms in the streamside flumes had more physical space available to develop long streamers often well aloof from the base. They were continuously exposed to fresh inoculum also including various algae as potential building blocks. The Sørensen's Index increased significantly ($P<0.05$ for the laboratory flumes, $P<0.01$ for the streamside flumes) with biofilm development in both systems (Figure 2d), suggesting successive convergence of the base and the streamer communities. Biofilm maturation could induce structural homogeneity due to the increased presence of larger building blocks (for example, algae) that shape the overall architecture (Besemer *et al.*, 2007), and repeatedly observed sloughing may induce a certain turnover and loss of stratification of the community. In addition, protozoan grazing is known to affect bacterial growth and survival in biofilms (Matz *et al.*, 2005) and may differentially shape community structure in the base and streamer.

The 54 prominent DGGE-bands sequenced for phylogenetic analysis yielded sequences of 29 OTUs—some of them repeatedly detected in the various samples (Figure 3 and Table 1). Sequences

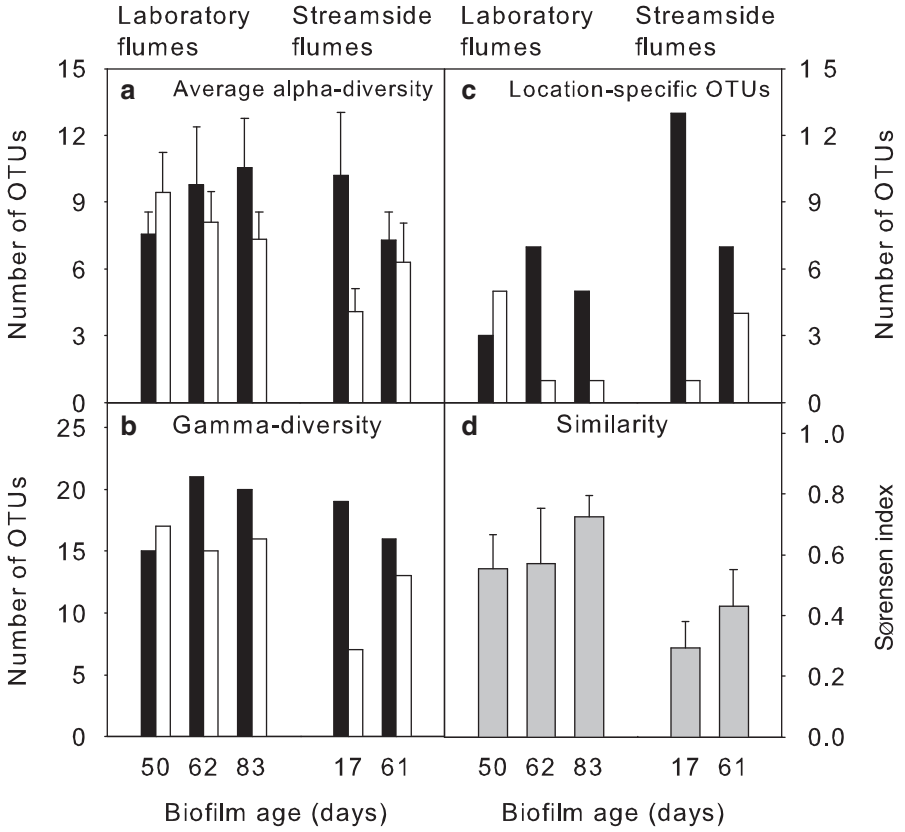

Figure 2 Numbers of operational taxonomic units (OTUs) in the biofilm base (black bars) and streamers (white bars) at different growth states. (**a**) Average α-diversity (as richness). Error bars indicate the standard deviation ($n=9$ for the laboratory flumes and $n=10$ for the streamside flumes experiment). (**b**) γ-diversity, as the total number of OTUs found in base (black bars) and streamers (white bars). (**c**) Number of OTUs specific for either the biofilm base (black bars) or streamers (white bars) for the respective sampling occasion. (**d**) Average similarity (as the Sørensen Index) between the biofilm base and the respective streamers of each sample. Error bars indicate the standard deviation ($n=9$ for the laboratory flumes and $n=10$ for the streamside flumes experiment). The mean Sørensen Index was significantly higher at day 83 than at day 50 in the laboratory flumes (Kruskal–Wallis analysis of variance and Tamhane's test, $P<0.05$) and significantly higher at day 61 than at day 17 in the streamside flumes (Kruskal–Wallis analysis of variance $P<0.01$).

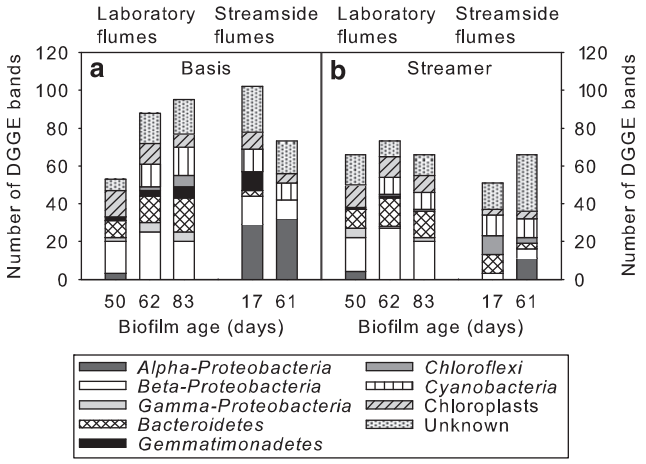

Figure 3 Frequency of operational taxonomic units related to phylogenetic groups in (**a**) the base and (**b**) the streamers of biofilms at different growth states.

were generally more closely related to published environmental sequences than to cultured bacteria. Most OTUs were classified as α- and *β-Proteobacteria*, members of the *Bacteroidetes* phylum, *Cyanobacteria* and algal chloroplasts. Several OTUs belonged to the *γ-Proteobacteria* and the *Gemmatimonadetes* group; one OTU was classified as a member of the *Chloroflexi* phylum, but was not closely related to any published sequence. The fourth-corner analysis on these phylogenetic groups showed significant differences in the distribution of phylogenetic groups between the biofilm base and the streamers (Table 2). The environmental control model (permutation model 1) showed that *α-Proteobacteria* and *Gemmatimonadetes* members were preferentially found in the biofilm base compared with the streamers. The lottery model (permutation model 3) revealed *α-Proteobacteria* and *Gemmatimonadetes* as comparatively weak competitors and *β-Proteobacteria* and *Bacteroidetes* as comparatively strong competitors in the streamers; the community structure in the biofilm base showed no significant deviation from randomness. Together with the lower richness observed in the streamers, these results might indicate that streamers constitute a more competitive biofilm zone than the biofilm base.

Similar to the fourth-corner analysis, OTU frequencies were related to both biofilm zones using G-statistics and permutations to acquire better resolution on the shifts in the community structure (Supplementary Table 2). More than 50% of the identified OTUs deviated significantly from random distribution. Not unexpectedly, the behavior of OTUs differed remarkably within a given phylogenetic group. Only one *α-Proteobacterium* (OTU 8), likely an endosymbiont of *Acanthamoeba* (Table 1), was under-represented in the biofilm. The other *α-Proteobacteria* were affiliated to the *Sphingomonadaceae* family, members of which were reported to pioneer biofilm formation (for example, Pang and Liu, 2006; Zhang *et al.*, 2006). Further, members of this group were shown to copiously produce exopolymers (Venugopalan *et al.*, 2005) and to coaggregate with other biofilm members (Rickard *et al.*, 2002), abilities that may be advantageous to colonization and early biofilm formation. We suggest that these OTUs were among the initial biofilm formers and that their populations persisted in the base, whereas stronger competitors built-up the biofilm canopy, including streamers.

Several OTUs affiliated to the *β-Proteobacteria, Bacteroidetes* and *Cyanobacteria* identified in this study are related to genera possessing filamentous morphology (for example, *Lewinella*, *Haliscomenobacter* and *Tychonema bourellyi*; Schauer and Hahn, 2005) or which form chains (for example, *Arcicella*; Manz *et al.*, 1999; Nikitin *et al.*, 2004). Though it is certainly difficult to draw conclusions about bacterial phenotypes from their phylogenetic affiliation, we hypothesize that filamentous or filament-forming cells may be advantageous for life in streamers. For instance, Kindaichi *et al.* (2004) predominantly observed filamentous *Bacteroidetes* in the canopy of autotrophic biofilms, whereas single *Proteobacteria* were present throughout the whole biofilm. Motility by gliding or flagella may also have a role for the successful colonization of streamers and contribute to the diversification of base and streamer communities. For instance, in *Pseudomonas aeruginosa* biofilms, migrating cells climb the stalks and form the mushroom caps—a process driven by type-IV pili and putatively induced by the search for nutrients (Klausen *et al.*, 2003).

We do recognize the potential limitations of DGGE and sequencing. For instance, it does not show the exact spatial organization of the various bacterial species as would have been shown by other techniques (for example, Wilmes *et al.*, 2009). However, largely used in ecological studies on community structure (for example, Jackson *et al.*, 2001), DGGE enables the throughput and data acquisition required by fourth-corner analysis, for instance, to address ecological questions.

In summary, our results indicate pattern congruency between architectural differentiation and community structure in stream biofilms. We suggest this micro-scale differentiation as a potentially important adaptation to the dynamics (for example, flow, resource availability) of natural ecosystems—comparable to the phenotypic diversification in bacterial biofilms as a survival strategy under adverse conditions (Koh *et al.*, 2007). Flow and resources are highly heterogeneous in sedimentary ecosystems, thereby creating divers microhabitats with ample opportunities for biofilms to differentiate into complex architecture and accompanying community structures. Eventually, this may contribute to the high prokaryotic biodiversity in sedimentary ecosystems.

Table 1 Phylogenetic affiliation of sequences obtained for dominant DGGE-bands

OTU no.[a]	*Phylogenetic group*[b]	*Closest relative (accession number)*[c]	*Similarity (%)*	*Closest cultured relative (accession number)*[c,d]	*Similarity (%)*
OTU 8 (EF188846)	*α-Proteobacteria*	Uncultured bacterium (AJ965830)	0.943	*Candidatus Odyssella thessalonicensis* (AF069496)	0.694
OTU 9 (FJ796385)	*α-Proteobacteria*	*Sphingomonas sp.* (AY584572)	1.000	*Novosphingobium hassiacum* (AJ416411)	0.805
OTU 10 (FJ796386)	*α-Proteobacteria*	Uncultured bacterium (FM872965)	0.981	*Novosphingobium lentum* (AJ303009)	0.826
OTU 11 (FJ796387)	*α-Proteobacteria*	Alpha proteobacterium (AF235997)	1.000	*Novosphingobium lentum* (AJ303009)	0.847
OTU 12 (FJ796395)	*α-Proteobacteria*	Uncultured alpha proteobacterium (AJ867917)	1.000	*Sphingomonas yabuuchiae* (AB071955)	0.784
OTU 1 (EF396239)	*β-Proteobacteria*	Hydrogenophaga atypical (AJ585992)	0.963	Hydrogenophaga atypical (AJ585992)	0.963
OTU 2 (EF396241)	*β-Proteobacteria*	Uncultured bacterium (EU491796)	0.986	*Herbaspirillum seropedicae* (Y10146)	0.766
OTU 4 (EF396242)	*β-Proteobacteria*	Uncultured bacterium (DQ337076)	0.990	*Azoarcus evansii* (X77679)	0.790
OTU 13 (FJ796388)	*β-Proteobacteria*	Uncultured bacterium (EU443091)	0.986	*Mitsuaria chitosanitabida* (AB006851)	0.901
OTU 14 (FJ796389)	*β-Proteobacteria*	Uncultured hydrogenophaga sp. (AF523009)	0.986	Hydrogenophaga atypical (AJ585992)	0.942
OTU 15 (FJ796392)	*β-Proteobacteria*	Uncultured organism (AY707574)	1.000	*Leptothrix mobilis* (X97071)	0.907
OTU 16 (FJ796403)	*β-Proteobacteria*	Commamonadaceae bacterium (AJ556799)	0.990	*Aquabacterium parvum* (AF035052)	0.875
OTU 7 (EF451827)	*γ-Proteobacteria*	Uncultured bacterium (AM158337)	0.992	*Lysobacter koreensis* (AB166878)	0.857
OTU 6 (EF451826)	*Bacteroidetes*	Uncultured bacterium (AY863079)	0.644	*Lewinella cohaerens* (AF039292)	0.537
OTU 17 (FJ796383)	*Bacteroidetes*	*Flectobacillus sp.* (AY584583)	0.971	*Arcicella aquatica* (AJ535729)	0.883
OTU 18 (FJ796394)	*Bacteroidetes*	Uncultured bacterium (EU104120)	0.985	*Haliscomenobacter hydrossis* (M58790)	0.836
OTU 19 (FJ796398)	*Bacteroidetes*	Uncultured bacterium (EU101256)	0.772	*Lewinella persicus* (AF039295)	0.475
OTU 20 (FJ796400)	*Chloroflexi*	Uncultured bacterium (EF208597)	0.771	*Thermoanaerobacter thermohydrosulfuricus* (L09161)	0.385
OTU 21 (FJ796390)	*Gemmatimonadetes*	Uncultured bacterium (DQ521491)	0.951	*Gemmatimonas aurantiaca* (AB072735)	0.699
OTU 22 (FJ796401)	*Gemmatimonadetes*	Uncultured bacterium (FJ392347)	0.970	*Gemmatimonas aurantiaca* (AB072735)	0.711
OTU 23 (FJ796382)	*Cyanobacteria*	*Tychonema bourrellyi* (AB045897)	0.988	*Nostoc punctiforme* (AF027655)	0.634
OTU 24 (FJ796396)	*Cyanobacteria*	Uncultured bacterium (EF451617)	0.899	*Oscillatoria acuminata* (AB039014)	0.564
OTU 25 (FJ796397)	*Cyanobacteria*	Uncultured cyanobacterium (DQ181677)	0.870	*Chroococcidiopsis thermalis* (AB039005)	0.598
OTU 26 (FJ796399)	*Cyanobacteria*	Uncultured cyanobacterium (DQ531865)	0.991	*Chroococcidiopsis thermalis* (AB039005)	0.549
OTU 5 (EF396243)	Streptophyta chloroplast	*Zygnema circum-carinatum* (AY958086)	0.573	*Nodularia spumigena* (AB039002)	0.449
OTU 27 (FJ796384)	Bacillariophyta chloroplast	Uncultured cyanobacterium (DQ130046)	1.000	*Nodularia spumigena* (AB039002)	0.477
OTU 28 (FJ796391)	Bacillariophyta chloroplast	Uncultured bacterium (EU376191)	0.881	*Chroococcidiopsis thermalis* (AB039005)	0.484
OTU 29 (FJ796393)	Chlorophyta chloroplast	Uncultured cyanobacterium (DQ366074)	0.956	*Nodularia spumigena* (AB039002)	0.423
OTU 30 (FJ796402)	Chlorophyta chloroplast	*Scenedesmus obliquus* (DQ396875)	0.945	*Nodularia spumigena* (AB039002)	0.374

Abbreviation: DGGE, denaturing gradient gel electrophoresis and OTU, operational taxonomic unit.
[a]OTU 1-2 and OTU 4-8 were also found in samples from a different study performed in the laboratory flumes experimental setup (Besemer *et al.*, 2007). To avoid ambiguities, the numbers of these OTUs were retained and the novel OTUs were numbered continuously OTU 9 to OTU 30.
[b]Classified using the RDP Naive Bayesian Classifier (Wang *et al.*, 2007; Cole *et al.*, 2009).
[c]Estimated by comparison to the Ribosomal Database Project II database (February 2009).
[d]Species type strains.

Table 2 Frequency of occurrences and results of the fourth-corner analysis of the distribution of phylogenetic groups classified by the Naïve Bayesian rRNA Classifier Version 2.0 provided by the Ribosomal Database Project (Wang *et al.*, 2007) in biofilm basis and streamer

	Occurrence basis/canopy	*Env. control model*		*Lottery model*	
		Basis	*Canopy*	*Basis*	*Canopy*
Global test (G-statistics)		$P<0.001$		$P<0.001$	
α-Proteobacteria	64/15	overrep. $P<0.01$	underrep. $P<0.01$	—	underrep. $P<0.01$
β-Proteobacteria	87/73	—	—	—	overrep. $P<0.01$
γ-Proteobacteria	12/8	—	—	—	—
Bacteroidetes	44/52	—	—	—	overrep. $P<0.01$
Cloroflexi	8/2	—	—	—	—
Gemmatimonadetes	21/2	overrep. $P<0.01$	underrep. $P<0.01$	—	underrep. $P<0.01$
Cyanobacteria	48/39	—	—	—	—
Chloroplasts	46/39	—	—	—	—

Abbreviations: env., environmental; overrep., overrepresented; underrep., underrepresented.
P-values were estimated using G-statistics.

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