

Flavobacterial Community Structure in a Hardwater Rivulet and Adjacent Forest Soil, Harz Mountain, Germany

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Abstract The great increase in the abundance and phylogenetic diversity of *Flavobacterium* spp. within a few hundred meters downstream of the discharge site of the Westerhöfer Bach, a hardwater rivulet, raised the question whether adjacent soil may serve as a reservoir of bacteria not detected in discharge water. To address this question, denaturing gradient gel electrophoresis (DGGE) analyses of the V3 region of *Flavobacterium* 16S rRNA genes were performed on DNA from nine soil samples and five rivulet sites. The resulting patterns were tested for the significance of differences between the sampling habitats using the nonparametric analysis of similarities and multidimensional scaling procedures. Even though both habitats were sampled in two consecutive years DGGE patterns of soil and downstream water samples showed significant overlap ($R = 0.614$). Sequencing of 57 DGGE bands resulted in 30 different sequences, which, on the basis of BLAST analyses, were between 96% and 100% similar to published clone, DGGE, and strain sequences from a wide range of different habitats. Forty-five percent of the highly similar sequences included those of isolates from the Westerhöfer Bach, while the other sequences were more closely related to clones and cultures from other habitats, especially agricultural soil. Based on these results we suggest that the increase in flavobacterial strain diversity and abundance in the rivulet may originate from soil microflora.

Introduction

In contrast to molecular approaches, isolation studies are not at the leading front of diversity studies because of the recognized large discrepancy between the number of bacterial colonies that form on solid media when soil is used as an inoculum and the total number of bacterial cells actually present in the same soil [2, 10]. The metabolic versatility let flavobacteria often appear to be abundant in culture-dependent studies [5, 9], as their fast growth suppresses the growth of fastidious members of the community. The R2A medium is often used for isolation, e.g., in studies on sea ice and marine salinity lake samples [12], Antarctic lake mats [18], and greenhouse soil [20]. The same medium was used in a recent study on the elucidation of the culturable bacterial diversity in a small German hardwater creek [5], showing significant deposition of calcium carbonate tufa about 50 m downstream of the discharge site [15]. While the spring water carried only a small number of colony-forming units (CFUs; 3×10^2), the number of CFUs and the phylogenetic diversity of aerobic and heterotrophic bacteria increased significantly: 50 m downstream of the spring the CFUs reached 6×10^3 , while they were determined to be 2×10^4 about 380 m downstream of the discharge site. Representatives of 34 bacterial genera were identified, 28 of which were not isolated from spring water. As the water flow of the rivulet is high ($15\text{--}20 \text{ cm s}^{-1}$), it can be excluded that culturable organisms multiply to reach significant cell densities at the sampling sites. To test the hypotheses that flavobacteria from soil contribute to the diversity of rivulet bacteria, the denaturing gradient gel electrophoresis (DGGE) technique was used to assess the degree of phylogenetic overlap between these two habitats and a tufa biofilm sample and between the molecular and the cultivation approaches.

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Materials and Methods

Processing of Water, Soil, and Biofilm Samples

About 3 liters of rivulet water was collected from stations 1 to 4 in the summer of 2005 and transported to the laboratory under cooled conditions. In the same period the cultures were isolated from the same rivulet [3]. Filtration of 2 liters was done first through a 1.2- μm filter to remove debris and then through a 0.45- μm filter connecting the glass filter holder to a vacuum pump. Filters were stored at -80°C . In September 2007 one-quarter of the 0.45- μm filter was submitted to a phenol-chloroform treatment concomitant with the bead beating extraction method [21], using glass beads (0.2 mm) and a bead beater (Braun, Melsungen, Germany). DNA was purified using the JETquick kit (Genomed, Bad Oeynhausen, Germany) following the manufacturer's recommendation. Soil samples were collected May 23, 2006, in close proximity (10 to 50 cm) to the rivulet (samples S1 to S4, S6, S8, and S9) and 50 m away from the water on the south side (S5). Sample S7 (Fig. 1) was a sediment sample. About 500 mg of soil from the A horizon and biofilm material were used for extraction of DNA. Samples were extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals LLC, USA) according to the manufacturer's instructions using a bead beater. The yield of DNA was determined electrophoretically and subsequently the S1 DNA sample was extracted-concentrated four times to ensure a sufficient DNA concentration.

Polymerase Chain Reaction (PCR)-DGGE

DNA amplification was carried out in 0.5-ml polypropylene tubes (Eppendorf, Hamburg, Germany) using a PTC-100 thermocycler (MJ Research, Inc., Waltham, MA, USA), according to Van der Gucht et al. [17]; Taq polymerase was from Roche (Mannheim, Germany). Specific *Flavobacterium* primers were designed manually from a

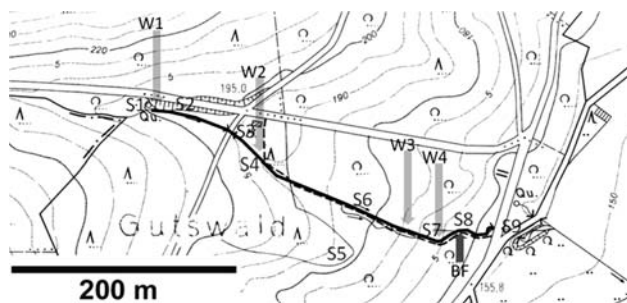


Fig. 1 Westerhöfer Bach sampling sites. Downward arrows: spring (W1), hut (W2), waterfall (W3), and trench (W4). Upward arrows: biofilm sampling site (BF). Soil sampling sites S1 to S9 follow the water flow from left to right

sequence alignment, generated by AE2 editor [5], of type strains of all Bacteroidetes genera described until 2006; Flavo-153F (5'-GGATAGCCCAGAGAAATTTGG-3') and Flavo-1141R (5'-GGCAGTCTYGTAGAG-TTCCC-3') were used in the first round of a nested PCR reaction. The mix contained 1 \times PCR buffer, 1 U of Taq DNA polymerase, and a 200 μM concentration of each deoxynucleoside triphosphate (Roche), 0.25 μM primers, 100 ng bovine serum albumin, and 0.5 μl of DNA, the final volume being adjusted to 50 μl . Reaction mixtures were incubated at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 62°C for 1 min, and 72°C for 2 min, then a final extension at 72°C for 10 min. This was followed by a second PCR [17], using primers spanning the V3 region of the 16S rRNA gene (between position 357 and position 518). PCR products (5 μl) were electrophorized in ethidium bromide-containing agarose, and the bands visualized by UV fluorescence and compared with a molecular weight Smart ladder (Eurogentec, Belgium). PCR products were run for 16 h at 75 V on a DGGE gel of 35–70% denaturants as described [16]. A standard, applied every fourth lane, was made of *Flavobacterium* strains isolated from the rivulet [3]. Gels were stained using 20 μl SYBR Gold (10,000 \times in DMSO; Invitrogen, Karlsruhe, Germany) in 200 ml TAE for 1 h, and then transferred to a Dark Reader (Clare Chemical Research, Inc., Dolores, CO, USA). A DC290 Kodak camera (Rochester, NY, USA) with an integrated SYBR Gold filter was used to digitalize the images. DGGE gel banding pattern analysis was done as described [17], using Bionumerics (Applied Maths, Kortrijk, Belgium), a band presence-absence matrix was established, and a dendrogram calculated using the Dice similarity coefficient.

Band pieces were cut from the middle of each band using 1-ml tips (Eppendorf, Hamburg, Germany). The gel pieces were transferred into 30 μl TE allowing the DNA to diffuse into the buffer overnight at 8°C . DNA extracted from bands was reamplified [16] and sequenced using the CEQ Dye Terminator Cycle Sequencing kit and a CEQ 8000 Genetic Analysis System. Sequence chromatograms were checked manually using Chromas 2.31 (Technelysium Pty., Ltd., Tewantin, Australia). In a few cases chromatograms indicated the presence of accompanying rDNA, causing the appearance of some double-peak positions. However, as the sequenced DNA contained a single variable region only, the dominating compensatory nucleotides of the helical region were selected for analysis after a manual check for secondary structure. The DGGE band sequences were analyzed using BLAST [1] to determine the degree of similarity to published clone and DGGE sequences. The acquired sequences have been deposited at EMBL under accession numbers AM493312–AM493365, AM999988–AM999998, and FM160744.

Statistical Analysis of DGGE Fingerprints

Normalization of DGGE banding profiles by Bionumerics allows generation of the absolute intensity value of the bands, which was further imported into the PRIMER 5 software package. A similarity matrix of Bray-Curtis coefficients was generated based on the presence-absence (ignoring band intensities) transformations of the bands (bands designation as variables) [4]. The similarity matrix was used to build a nonmetric multidimensional scaling (NMDS) analysis. The resulting plot represents similarities among the communities of different sampling sites; the stress value 0.14, indicated in the two-dimensional plot, indicates the faithfulness of relationships between the sites [4]. In addition, a cluster analysis was done on the basis of the similarity matrix using the group average clustering method. The resulting dendrogram, with its superimposed threshold lines, permits a better illustration of flavobacterial community composition between sample sites. To determine the statistical significance of the observed groups, an analysis of similarities (ANOSIM) was performed. ANOSIM is a nonparametric permutation procedure, based on the similarity matrix underlying the ordination of the samples. It allows a statistical test of the null hypothesis that there are no differences among the water, soil, and biofilm samples (one-way layout). ANOSIM calculates the R test statistic, which ranges from $R = 0$ (completely random pattern) to $R = 1$ (completely separated). A p -value is then computed by randomizing the band patterns to determine whether the R value generated from user-defined groups is higher than any randomly generated R value [4].

Results and Discussion

Despite reported pitfalls in the PCR-based assessment of microbial diversity [19], the DGGE approach is one of the most widely used technologies in the determination of population richness and diversity. The increase in both CFU on R2A agar and the phylogenetic diversity of bacterial isolates downstream of the discharge site may be largely influenced by strains entering the rivulet from adjacent soil sites. To test this hypothesis a nested PCR approach was used to generate *Flavobacterium*-specific V3 regions of 16S rRNA gene sequences, which were separated by DGGE. As shown in Fig. 2, the discharge (W1) and the biofilm site (BF) show the lowest number of bands (11 and 10, respectively), while most of the other samples ranged between 12 and 20 bands; among the water samples, site 4 was more complex (W4; 19 bands). While only a single band (at the top of the gel) was present in all samples, one common band was present in soil and water

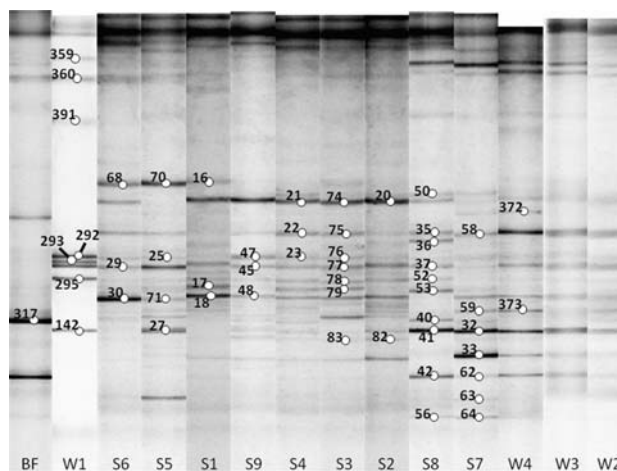


Fig. 2 Composite DGGE of 16S rRNA gene fragments of the *Flavobacterium* population of the soil (S1 to S9), water (W1 to W4), and Biofilm (BF) of the Westerhöfer Bach analysis using Bionumerics. The white dots represent bands cut out for DNA sequencing, with their identifier numbers at the left

samples (W1-295), according to Bionumerics. Several common bands, however, were found in the majority of samples, including the biofilm site.

The flavobacterial community was analyzed by means of NMDS (Fig. 3) and ANOSIM, both nonparametric multivariate tests for determining changes in community structure [4]. Presence-absence transformation of bands was used to minimize the bias created by the methodological approach (extraction of DNA, PCR).

The stress value of the NMDS was 0.14, which, according to Clarke and Gorley [4], suggested that the observed patterns are close to reality. An ANOSIM value of $R > 0.75$ indicates a substantial difference in overall

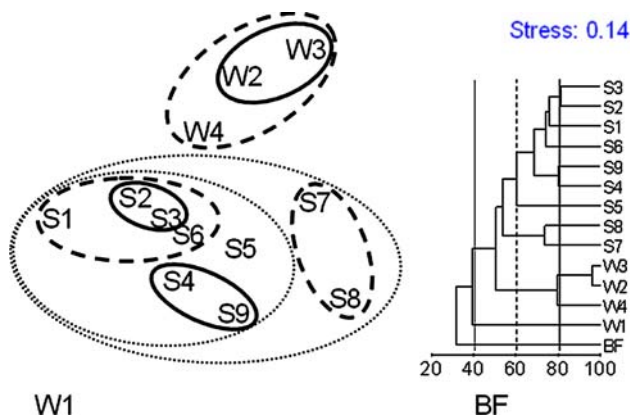


Fig. 3 Nonmetric multidimensional scaling ordination plot based on the presence-absence of the DGGE profile bands. The group average clustering at 80, 60, and 40% levels of similarity are superimposed on the right side of the plot (vertical lines). The line format refers to cluster similarity (ellipse). S soil, W water, BF biofilm from the rivulet bottom. Numbers refer to sites (see map, Fig. 1)

community structure [4], while $R > 0.5$ is interpreted as being separated but overlapping, and $R < 0.25$ as being barely separated. The global result of the one-way ANOSIM in the present study indeed indicated significant differences in flavobacterial composition among soils, waters, and biofilm sites (global $R = 0.7$, $p = 0.002$). The analysis also showed the significance of the community similarity between the soil and the water sites ($R = 0.614$, $p = 0.001$) while the water and the biofilm communities were completely different ($R = 1$, $p = 0.2$). Also the biofilm community showed a strong difference from the soil samples ($R = 0.963$, $p = 0.1$).

Both NMDS and cluster analyses showed similarities among flavobacterial communities of all soil samples (S1–S9) and the three downstream water sites (W2–W4). Clustering apart are the communities of the discharge site (W1) and the biofilm site (BF). Within soil samples some communities from adjacent sites were similar (S1–S3 and S7/S8) but similar communities were also detected from locations S4 and S9 (Fig. 1). The community diversity of soil samples is greater than that of the three downstream water sites, which are highly similar. This agrees with the finding of culture studies [3, 5], which demonstrated not only a similar high percentage of *Flavobacterium* isolates from these three sites (40–59%) but also high phylogenetic similarities (16S rRNA gene similarities $> 98.5\%$) among many of these isolates. Also not unexpected from culture studies is the isolated position of the population of flavobacteria from the discharge site (W1; Fig. 2). The total number of bacterial isolates at the discharge site (W1) was an order of magnitude lower than that at the other rivulet sites and dominated by mainly one novel *Flavobacterium* species, i.e., *Flavobacterium aquidurensense* [6], not found at downstream sites. As judged from the sparse banding profile, the biofilm community (BF) contains only a few dominant flavobacterial organisms. This, too, is in accord with cultivation studies which, compared to the water sample analyzed at the site of the biofilm, demonstrated the marked depletion of flavobacterial strains (unpublished).

The specificity of the 16S rRNA gene amplification primers originally selected in silico on the basis of almost-complete sequences of type strains of the family Flavobacteriaceae was confirmed by the affiliation of DGGE band sequences of the V3 region to entries in public sequence databases. Of the 57 sequences obtained, only 2 identical sequences were more similar (98–99% BLAST identity) to sequences of members of the Flavobacteriaceae genera *Gelidibacter*, *Subsaxibacter*, and *Gaetbulibacter* strains and clones than to those of the genus *Flavobacterium*. All other sequences showed the highest BLAST hits (96–100%) to a wide range of *Flavobacterium*-type strains and to uncharacterized and uncultured *Flavobacterium* strains (Table 1). The taxonomic potential of the V3 region

was not sufficiently high enough to distinguish between the type strains of all *Flavobacterium* species, i.e., the sequences of bands 16 and 70 did not discriminate between *F. hercynium* DSM 18282^T and *F. frigidimaris* DSM 15937^T. As BLAST analysis resulted in the listing of many strains or clones of different origin with identical high scores, only one entry (rarely two) is indicated in Table 1. As two study sites repeatedly emerged with high scores from the BLAST hit match list, they are indicated preferably: the Westerhöfer rivulet [3] and an agricultural soil in the United States [11]. The origins of other high-scoring strains and clones span a wide range of habitats and geographic areas.

Thirteen different sequences representing 29 DGGE bands obtained from DNA samples from soil, water, and biofilm show between 100 and 97% match with isolates from the Westerhöfer rivulet. Among the 99–100% matches, soil (e.g., S8-40, S8-50, S1-16, S6-68), water (e.g., W1-391, W4-372), and biofilm (e.g., BF-317) samples are represented. A similar relationship emerged between the band sequences and the sequences originating from an unpublished study [11] on conventional agricultural soil and successional community soils. The matches between soil and water DGGE sequences showing 98% similarity with public database entries are too numerous to be listed here. Among those, some soil sequences display similarities to those of described species, e.g., S8-50 with *F. gelidilacus*, S8-53 with *F. aquatile*, S8-36 with *F. xinjiangense*, and S2-20 with *F. xanthum*. These organisms are typical freshwater and soil flavobacteria.

It is obvious from Fig. 2 and Table 1 that a few DGGE band sequences from the soil samples share a high similarity to the sequences from rivulet isolates. Several soil sequences are highly related to more than a single isolate from one rivulet sampling site and to isolates from more than a single site. Noticeable is the absence of *F. aquidurensense*-specific DGGE sequences from the discharge site, though this organism is the most abundantly cultured organism from this site. The sequence of *F. hercynium*, on the other hand, abundant in water samples 2 to 4, is matched by two DGGE band sequences from soil. *F. frigidimaris*, an isolate from Antarctic seawater, shows the same sequence variation in the V3 region as *F. hercynium* but has not been isolated from the rivulet [5]. The only sequence of the biofilm DGGE sample generated (BF-317) shows a 99% match to some sequences from soil, water, and an isolate. The moderate degree of overlap should take into account that several DGGE band sequences show mismatches in only one or two nucleotides (99% identity). As the DGGE approach may differentiate between rrn operons with sequence microheterogeneity [15], it cannot be excluded that sequences with 1–2% difference from the sequence of a given isolate indeed originate from the same

Table 1 Compilation of accession numbers of band sequences (see Fig. 2), BLAST results of comparison of 178 bp, and accession numbers of selected closest relatives and their origins; each band class is separated by a semicolon

DGGE band class and representative accession no. ^a	Highest BLAST similarity to	BLAST similarity (%)	Accession no.	Origin/habitat of closest relative determined by BLAST
S8-50 (AM493326);	Isolate WB4.3-86	100	AM934673	Westerhöfer Bach, Germany ^b
S1-16 (AM493314), S5-70;	<i>Flavobacterium hercynium</i> ^c WB4.2-33 ^T (DSM 18292 ^T) group	100	AM265623	Westerhöfer Bach, Germany
S6-68 (AM493312) = S6-28;	WB4.4-89	100	AM934675	Westerhöfer Bach, Germany
S8-41 (AM493322);	WB 3.4-22	100	AM934663	Westerhöfer Bach, Germany
S5-27 (AM493361);	WB2.3-13	100	AM934645	Westerhöfer Bach, Germany
W1-391 (AM999988);	Isolate WB2.1-49 & clone MA73_2004T8d_G01	100	AM934636 EF378574	Westerhöfer Bach, Germany Agricultural soil, USA [11]
S4-21 (AM493338);	Isolate WB2.3-15	100	AM934646	Westerhöfer Bach, Germany
W4-372 (AM999989);	Isolate WB2.3-38	99	AM934641	Westerhöfer Bach, Germany
S8-40 (AM493363) = S8-54, BF-17;	Clone MA33_2003T8a_D08 & isolate WB2.6-68	99	EF378104 AM934651	Agricultural soil, USA Westerhöfer Bach, Germany
W1-142 (AM999990);	Isolate WB3.4-22 group	98	AM934663	Westerhöfer Bach, Germany
S8-52 (AM493329), S3-78, W1-295;	Isolate WB4.3-36 group	98	AM934669	Westerhöfer Bach, Germany
S5-25 (AM493337), S3-76, S4-23, S9-47;	Isolate WB2.3-15	98	AM934646	Westerhöfer Bach, Germany
S3-74 (AM493333), S2-20;	Isolate WB2.3-15	98	AM934646	Westerhöfer Bach, Germany
W1-359 (AM999992);	Isolate WB3.2-63	97	AM934660	Westerhöfer Bach, Germany
S7-59 (AM493340), W4-373;	Clone MA72_2004T1d_C01	100	EF378452	Agricultural soil, USA
S2-82 (AM493347), S3-83;	Clone MA34_2003DFa_H06	100	EF378270	Agricultural soil, USA
S7-63 (AM493319);	Clone MA73_2004T8d_D12	100	EF378553	Agricultural soil, USA
W1-292 (AM999994);	Clone MA34_2003DFa_F08	99	EF378285	Agricultural soil, USA
S6-30 (AM493343), S9-48, S5-71, S1-18;	Clone MA72_2004T1d_C01	98	EF378452	Agricultural soil, USA
W1-360 (AM999995);	Clone MA32_2003T1a_F12	98	EF377779	Agricultural soil, USA
W1-293 (FM160744);	Clone MA72_2004T1d_F11	97	EF378485	Agricultural soil, USA
S3-79 (AM493356);	Clone MA42_2003T8b_C06	96	EF378201	Agricultural soil, USA
S4-22 (AM493327), S7-58, S3-75, S8-35;	<i>Flavobacterium</i> sp. W5	100	EU794397	gastrointestinal tract of Atlantic salmon
S7-32 (AM999998);	Clone S003D	100	AM158330	Rhizosphere, Spain
S7-62 (AM493351), S8-42 = S8-55;	Clone N06Jun-34	100	EU442924	Water of Nam Co, Tibet, China
S1-17 (AM999997), S8-53 (AM493354);	DGGE b & J10-2	100, 99	EU399730	Manganese removal filter, China
S7-33 (AM493318) = S7-61;	Isolate WB3.4-76	99	AM494664	Westerhöfer Bach, Germany
S6-29 (AM493348), S9-45, S8-37, S3-77;	Clone c1LKS42	98	AM086084	Sediment of Lake Kinneret, Israel [14]
S8-36 (AM493331) = S8-51;	Isolate XJ49	98	EF648127	Aerobic activated sludge, China
S7-64 (AM493315), S8-56;	<i>Gelidibacter</i> strain BSD S2 05	99	AY259512	Salt marsh sediment, USA

DGGE denaturing gradient gel electrophoresis

^a (=) The bands (S6-68 and S6-28) were cut two times from different gels as a control

^b From Refs. [6] and [8]

^c Also *Flavobacterium frigidimaris* DSM 15937^T (AB183888)

genome of this isolate. For example, *F. hercynium* and *F. aquidurensis* differ in a single nucleotide in the V3 region.

The present study compared the results of two different approaches: while freshly isolated DNA was obtained from

soil, frozen cells of water and biofilm samples were used. In contrast to what has been pointed out for a possible effect of freezing on the diminished survival of strains, freezing should have little effect on the availability of DNA

as long as the sample is frozen immediately after sampling, as was the case in the present study. Even if some members of the microbial community died or lysed due to the freeze/thaw processes [8], the DNA should be still suitable for PCR amplification. It is therefore assumed that the DGGE patterns indeed reflect, by and large, the different phylogenetic identities of the flavobacteria present. As judged from the dendrogram of DGGE pattern relationships (Fig. 3), the diversity of soil flavobacteria is greater than that of rivulet flavobacteria. This difference can be explained by the uneven distribution of microbial composition in the habitat [7, 13], by the finding that flavobacterial isolates from the Westerhöfer Bach, including *F. aquidurens* strains, are able to pass through 0.45- μ m filters (S. Gronow, personal communication), and one can speculate that runoff from soil is not a continuous and evenly distributed process. The second approach compares the non-culture-based DGGE study with the culture-based isolation approach. The well-documented cultivation bias, e.g., cultivation on R2A medium at two different temperatures, will by no means unravel the full depth and breadth of the flavobacterial communities. Differences in flavobacterial community complexity observed between the discharge site and the downstream water samples as well as between the W4 sample and the biofilm sample, with its different microenvironment, are even more supportive arguments for the hypothesis that soil indeed contributes significantly to the flavobacterial richness and abundance in rivulet water. The high similarities between a large fraction of the sequences from these environments and, for example, the agricultural soil in the United States [19] let us assume that these flavobacterial populations are universal populations, spreading from soil to aquatic environments. It is not known at this time whether the flavobacteria of soil origin continue to grow and multiply in the water habitat. The metabolic functions of the rivulet bacteria have not yet been evaluated, but experiments are designed to evaluate their presence in tufa biofilms and their possible contribution to limestone precipitation.

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