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ORIGINAL PAPER

Visualizing Acidophilic Microorganisms in Biofilm Communities Using Acid Stable Fluorescence Dyes

Sina Brockmann · Thuro Arnold · Bernd Schweder · Gert Bernhard

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Abstract Bacteria in acidophilic biofilm communities, i.e. acid streamers and snottites, obtained from a subsurface mine in Königstein were visualized by fluorescence microscopy using four new fluorescent dyes (DY-601XL, V07-04118, V07-04146, DY-613). The pH of the bulk solution in which these bacteria thrive was pH 2.6 to 2.9. The new fluorescent dyes were all able to clearly stain and microscopically visualize in-situ the bacteria within the biofilm community without changing pH or background ion concentration. The commonly used fluorescent dyes DAPI and SYTO 59 were also applied for comparison. Both dyes, however, were not able to visualize any bacteria in-situ, since they were not stable under the very acid conditions. In addition, dye V07-04118 and dye DY-613 also possess the ability to stain larger cells which were presumably eukaryotic origin and may be attributed to yeast cells or amoeba-like cells. PCR analyses have shown that the dominant bacterial species in these acidophilic biofilm communities was a gram negative bacterium of the species *Ferrovum myxofaciens*. The presented four new dyes are ideal for in-situ investigations of microorganisms occurring in very acid conditions, e.g. in acidophilic biofilm communities when in parallel information on pH sensitive incorporated fluorescent heavy metals should be acquired.

Keywords Acidophilic macroscopic streamer · Snottites · Acid mine drainage · Acid stable fluorescence dyes

S. Brockmann · G. Bernhard
Radiochemistry, Technical University Dresden,
01062 Dresden, Germany

S. Brockmann (✉) · T. Arnold · G. Bernhard
Institute of Radiochemistry, FZ Dresden-Rossendorf e. V.,
P.O.Box 510119, 01314 Dresden, Germany
e-mail: s.brockmann@fzd.de

B. Schweder
Dyomics GmbH,
07745 Jena, Germany

Introduction

Biofilms are complex microbial communities that are attached to interfaces [1, 2] and occur ubiquitously in nature. They consist of microorganisms such as bacteria, archaea, fungi, algae, and protozoa which are embedded in a gelatinous matrix of extracellular polymeric substances (EPS) which are able to incorporate inorganic materials, such as washed in sediments and dissolved substances. EPS make up 60–90% of the biofilm dry weight [2]. However the main fraction of the biofilm consist of water (between 50–95%) [2]. Biofilms have a very complex and heterogeneous structure consisting of cell clusters and voids which form a network of water channels [3].

Biofilms are able to grow under extreme conditions, like high salinity, high temperatures, and very low pH conditions. Some of these extreme habitats, e.g. acid mine drainage (AMD) environments, are characterized by very acid conditions, high concentration of toxic heavy metals and a limited microbial diversity. Microorganisms in such heavy metal contaminated environments have been intensively studied, e.g. in [4, 5] and in the extreme acidic river of Rio Tinto (in southwest Spain) [6, 7]. Most of these studies try to give an insight in the microbial diversity to get a comprehensive understanding of extremophile live-communities. Other studies focus on the influence of such acidophilic biofilms on the transport and immobilization behavior of heavy metals in acid environments [8, 9].

Fluorescent dyes have become commonly used tools in combination with Confocal laser scanning microscopy (CLSM) or other fluorescence microscopy techniques to stain and visualize non-fluorescent bacterial cells in complex mixed microbial populations [10]. The use of fluorescent dyes is essential to get an insight into the complex biofilm structure and thus into the metabolic processes occurring within biofilms. However, many commonly used fluorescence dyes cannot be directly applied to biological samples from extreme pH environments, in particular biofilms growing under acid conditions, since most fluorescence dyes decompose at pH values below 3 and thus no fluorescence occurs. In an attempt to overcome this drawback it is essential to increase the pH of the sample and adjust the pH to at least pH 3 or above, e.g. by adding NaOH as described by Baffico et al. [11]. Another approach is to dilute the dyes in buffer solutions before they were added to the sample. Conclusively, both the pH and the ionic strength within the biofilm in the above described methods were changed during the staining procedure. This change can lead to undesirable effects in the microbial metabolism and in particular to the geochemistry of pH sensitive heavy metals within the biofilm. Such an increase in pH during sample processing for microscopic investigations causes changes in solubility and speciation of in biofilms incorporated dissolved metals. This is true, especially in acidophilic biofilms, thriving in uranium contaminated waters. At acid pH values of pH <4 in oxic environments uranium occurs predominantly as mobile dissolved free uranyl(VI) species (UO_2^{2+}) [12], whereas at circumneutral pH uranium has its solubility minimum and might precipitate as uranium hydroxide or uranium silicate, depending on the presence of available ligands. This may result in the formation of artifacts in form of metal precipitates, occurring as particulates in the microscopically observed biofilm sample.

Another method applied to acidic biofilms is fluorescent in situ hybridization (FISH) [13–16]. In rare cases the investigation of FISH-stained biofilm sections with the CLSM technique was used to generate three-dimensional images of dominating microbial species within biofilm structures [4]. However, the preparation of FISH-samples requires a number of fixation and washing steps, which inevitably induces changes in chemical composition and pH-value of the incorporated biofilm water. Hence, FISH is only applicable to obtain information on the microbial diversity of biofilm samples. FISH, however, is not suited to get in parallel information on the metal speciation within biofilms, since it may create artifacts in form of precipitated heavy metal particulates. Conclusively, biofilms that were stained in this ways are not useful to study the interactions of biofilms with heavy metals, e.g. uranium. Thus, to investigate the migration and sorption behavior of heavy metals by fluorescence microscopy in situ it became necessary to develop and test new fluorescent dyes which are acid-stable and would be able to stain, i.e. to visualize microorganisms under extreme low ambient pH-values and high ionic strengths. To our knowledge no acid-stable fluorescent dyes have been reported in the literature and were applied to acidophilic microorganisms in biofilm communities.

In this study a number of fluorescent dyes were tested for their ability to in-situ stain microorganisms in acidophilic biofilm communities. The emission maxima of the selected fluorescent dyes occur in the near red wavelength region. This is important for further investigations on the interactions of fluorescent heavy metals, in particular uranium, with biofilm components in which information on visualization of bacteria and fluorescent heavy metals are recorded in parallel. For such investigation a superposition of the emission of the fluorescent dyes with the fluorescence of incorporated uranium species (fluorescence emission range: 415–560 nm [17]) has to be avoided. Such acid stable dyes are urgently required to study the interactions of pH sensitive metals with biofilm components and would help to develop a better understanding of the immobilization behavior of migrating heavy metals in such acidophilic biofilm communities.

Materials and methods

Sample site and biofilm samples

The biofilm samples were collected in August 2009 in a gallery of pit 390 on level +50 m above sea level of the former uranium mine of the WISMUT GmbH near Königstein (Saxony, Germany). Thick biofilms in form of filamentous and gelatinous streamers, also denoted as acid streamers, thrive in the acid mine drainage channels. In another parallel gallery dripstone-like biofilms, so-called snottites are hanging from the ceiling with a solid mineral basis and a soft slimy whitish ending. Such biofilms have been described by Hose and Pisarowicz [18] and Bond et al. [19]. The biofilm samples were collected in sterile vials and were subsequently transported to the laboratory and there stored at 4°C until examination or further sample processing. The chemical composition of both waters, i.e. the water that drops down from the ceiling and the drainage water that flows in the AMD channels were analyzed by ICP-MS (ELAN 9000, Perkin Elmer) and ion chromatography (IC-system 732/733, Metrohm). The chemical analyses of the drainage water and the water dripping down the snottites are summarized in Table 1. pH values of 2.9 in the flowing drainage channels and 2.5 in the dripping waters of the snottites, respectively were determined and represent an extreme low pH habitat for microorganisms. The measured low pH values are related

Table 1 Water chemistry at the biofilm sampling sites

	AMD biofilms	dripstone-like biofilms
pH-value	2,9	2,5
Fe (ppm)	47,7	316,8
U (ppm)	9,3	59,0
SO_4^{2-} (ppm)	836,0	2460,0

to the former mining activity, occurring in the years between 1984 and 1990, in which the sandstone host rock was leached in-situ with sulfuric acid solution [20]. As a consequence, the drainage water is still very acid and contains high concentrations of iron and sulfate.

Bacterial diversity

The bacterial diversity of the biofilm samples was analyzed by extracting the total DNA using the Fast DNA Spin for Soil Kit (MP Biomedicals LLC, Ohio) followed by 16 S-rDNA-PCR using the primer TPU1 (5'-AGAGTTTGAT CMTGGCTCAG-3′) and 1387r (5′-GGGCGGWGTGTAC AAGGC-3′). Amplification was performed for 30 cycles with an annealing temperature of 55°C using the Hotmastermix from Eppendorf.

The purified amplified 16S rDNA fragments were cloned into *E. coli* (chemical competently TOP 10 F′) using the pMBL T/A Cloning Kit (Genaxxon BioScience), following the manufacturers recommendations. The recombinant clones (84 clones of AMD biofilm samples and 17 clones of dripstone-like biofilm sample) were selected by blue-white colony selection. Representative clones were picked for sequencing to enable the phylogenetic classification of the predominant bacterial populations. The high-quality sequences were compared to sequences available in the non-redundant nucleotide database of the National Center of Biotechnology using BLASTN.

Confocal laser scanning microscopy (CLSM)

A TCS-SP2 CLSM (Leica Microsystem, Heidelberg) was used to obtain structural information of the biofilms. The CLSM system was equipped with a Helium-Neon Laser with a wavelength of 633 nm, which is a component of the standard equipment of the microscope system. A further laser (Argon UV-Laser 351/364 LP5, Europe, Coherent Inc., Santa Clara, USA) with a wavelength of 351 nm was coupled into the CLSM via the UV port and used for excitation of the DAPI-fluorescence. The biofilm samples were observed with a 100.0 × 1.40 oil-immersible objective (Leica HCX PL APO CS 100.0x1.40 OIL BD). All micrographs were further image processed with Jasc Paint Shop Pro 8 software (Jasc Software, Inc.).

Fluorescent dyes

Several new fluorescent dyes (DYOMICS, Jena, Germany) were applied to bacteria in acidophilic macroscopic streamer and snottite biofilms and tested for their ability to stain bacteria, and therefore enable the in-situ visualization of microorganisms within the biofilms. In this paper we present results of the four most suitable dyes which were so far commercially not available. These dyes are: DY-601XL, V07-04118, V07-04146, and DY-613.

Dye DY-601XL is a hydroxy-cumarin-derivative with a hydrophobe hydroxyl-cumarin-part (with diethyl-amino-group) and a hydrophile substituted molecule part (Fischer Base with two sulphonates). At extreme acidic conditions, i.e. < pH 3, this dye forms a hydroxyl-benzopyrylium-compound, which is very acid-stable. In addition, this dye shows distinct properties as protein-stain [21]. The other three dyes, i.e. V07-04118, V07-04146, and DY-613, are all non-polar derivatives of 1-benzopyrylium-compounds with substituents as tert-butyl-, diethylamino- or ethyl-(3-hydroxypropyl)amino-, and methyl-groups. As shown in Fig. 1 dye DY-613 possesses a combination of a 1-benzopyrylium-part with a Fischer-base as basic structure. The other two dyes V07-04118 and V07-04146 are symmetrical monomethines of 1-benzopyrylium-compounds. The stability at extreme acidic pH-conditions is a typical property of 1-benzopyrylium-compounds [22]. This property is additionally increased by the steric shield of the tert-butyl-group in 2-position. Due to the positive charge of these three dyes (V07-04118, V07-04146, and DY-613) it is likely that they all act as intercalator. However, there are also other possible types of bonds. Since all four dyes described above are

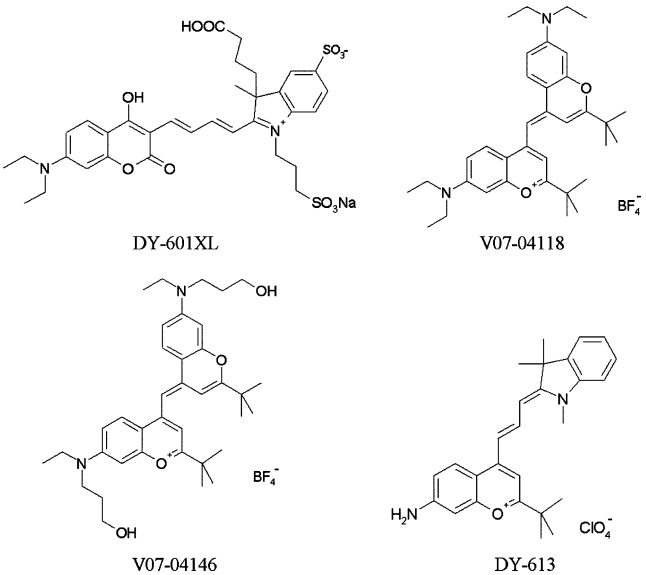

Fig. 1 The structures of the fluorescent dyes: DY-601XL, V07-04118, V07-04146, and DY-613

Table 2 Properties of the four fluorescent dyes

Dye	Molecular formula	Molecular mass [g/mol]	Absorption maximum[1] λ_{abs} [nm]	Emission maximum[1] λ_{em} [nm]	Molar absorbance[1] ε [l/mol·cm]
DY-601XL	$C_{33}H_{37}N_2O_{11}S_2 \cdot Na$	724.79	604	641	130.000
V07-04118	$C_{35}H_{47}N_2O_2 \cdot BF_4$	614.58	616	650	138.000
V07-04146	$C_{37}H_{51}N_2O_4 \cdot BF_4$	674.63	618	649	125.000
DY-613	$C_{27}H_{31}N_2O \cdot ClO_4$	499.01	614	639	200.000

[1] measured in methanol

cationic dyes they should probably not penetrate the intact cell wall and stain the cell membrane. The main properties of the four new dyes are listed in Table 2.

Two well-known nucleic acid stains, i.e. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) and SYTO 59 (both from Molecular Probes, Inc.) were used in comparison with the four new dyes to stain microorganisms in acidophilic biofilms.

Staining procedure

Stock solutions of 1 mg/mL were prepared of all new fluorescent dyes. The solid dyes were dissolved in deionized water (for dye DY-601XL) or in methanol (for dyes V07-04118, V07-04146, and DY-613). The common dyes SYTO 59 and DAPI were already at hand in form of stock solutions which were frozen at −25°C. Thus, these two dyes only had to be defrosted.

Subsequently, all fluorescent dyes were diluted to their final concentration of 10 µmol/L with sterile filtrated water from the acid mine drainage (AMD) channel of the mine site, except for DAPI which was diluted to a final concentration of 1.14 µmol/L. This was necessary because the addition of the fluorescent dye to the biofilm should not change ionic strength or pH-value. The biofilms were cut with a razor blade into small pieces and subsequently placed into a reservoir of a 24-well cell culture plate. To each biofilm specimen 300 µl of the respective fluorescent dye were added. The samples were incubated and kept for 30 min in the dark. After this, the overlaying fluorescence dyes were removed with pipettes from the biofilm samples and eventually the stained samples were washed twice to remove unattached dye. This was achieved by adding sterile filtrated water from the AMD and next placing the stained samples on a micro plate shaker (PMS-1000, Grant) for 10 min. The washing solution was then removed with a pipette. This procedure was repeated and the stained biofilm sample remained in the washing solution until it was used for microscopic observation. For this the sample was placed on a clean microscopic slide and a cover slip was placed on top of the biofilm specimen. The edges of the cover slip were sealed with nail polish (01 transparent, express finish, Maybelline Jade) to avoid dewatering of the biofilm specimens which would lead to artifacts, e.g. shrinking of the sample.

Absorption and emission- spectra

Absorption and emission spectra of the dyes were measured as methanolic solution with an optical density smaller than 0.5 and a pathway of 1 cm. Absorption spectra were recorded on a double-beam spectrophotometer (Specord 205, Analytikjena, Jena, Germany). For recording emission spectra a fluorescence spectrometer (Jasco Spectrofluorometer FP-6600, Bouguenais, France) was used with 3 mm slit for excitation and 6 mm slit for emission respectively.

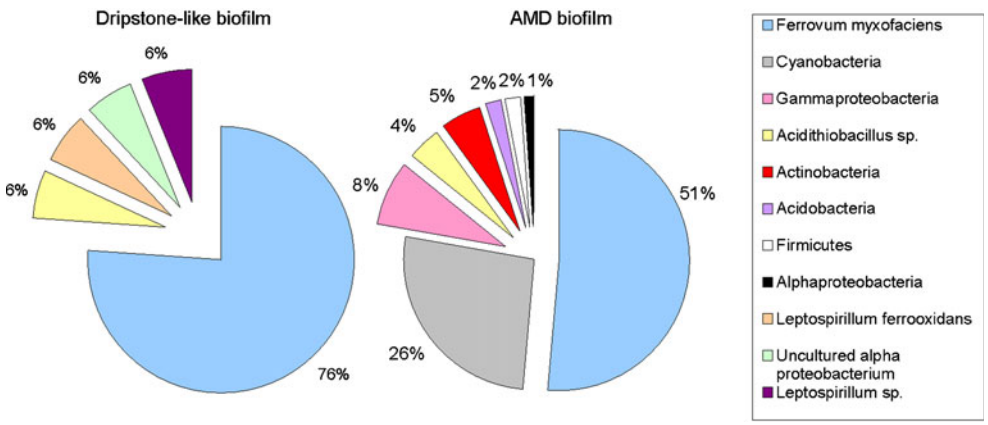

Fig. 2 Bacterial composition of the biofilms on basis of the analysed 16 S rDNA-sequences. Left side: composition of the dripstone-like biofilms; Right side: the composition of the biofilm from the AMD

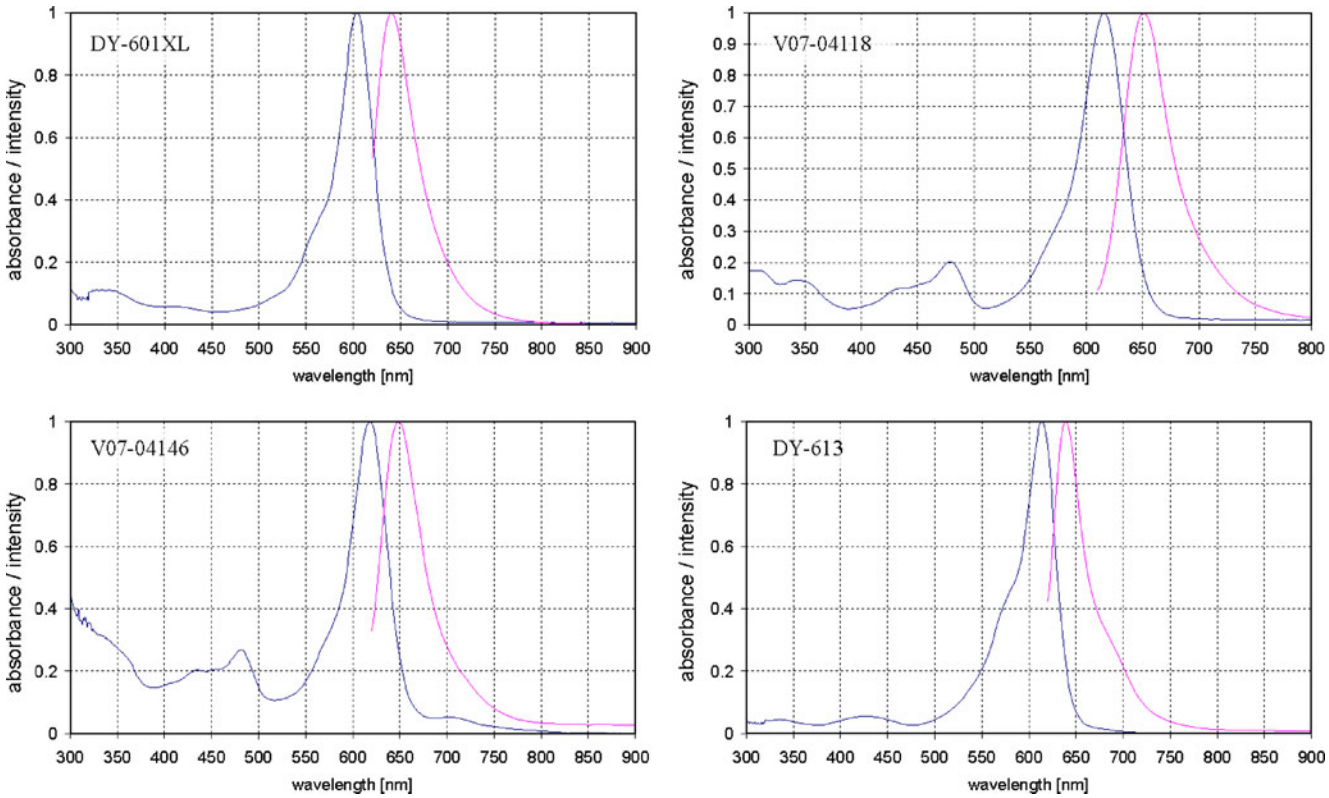

Fig. 3 Absorption- and emission- spectra of the new fluorescent dyes

Results and discussion

The bacterial diversity of the two types of biofilm samples, i.e. the acidophilic macroscopic streamers collected in the drainage channels and the snottites was investigated by constructing 16S rDNA clone libraries. As shown in Fig. 2 the bacterial community in both biofilms is dominated by *Ferrovum myxofaciens*. This bacterium was described for the first time in an acid mine drainage environment in Wales by Hallberg et al. (2006) [13] and later by Heinzel et al. (2009) [23, 24]. In the snotite biofilm additional clones, however of minor importance, were attributed to members of the *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans*, an unculturable *Alphaproteobacterium*, and a *Leptospirillum* species. In the biofilm samples from the acid mine drainage channels surprisingly additional clones of the species *Cyanobacteria* were observed and in addition of much less importance species of *Gammaproteobacteria, Acidithiobacillus* species*, Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Alphaproteobacteria* were found. Currently it is unclear if the *Cyanobacteria* are metabolically active and thrive under the dim light of the mine gallery illumination or if the observed species are washed in from the surface.

Absorption- and emission- spectra of the four new fluorescent dyes were recorded and are shown in Fig. 3. Table 3 shows the applied excitation wavelengths together with applied emission ranges for the respective fluorescent dyes. The ability of these four fluorescence dyes to stain microorganisms in acidophilic biofilms was checked and compared with the efficiency of two common nucleic acid stains, i.e. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) and SYTO 59 (both from Molecular Probes, Inc.).

These four new fluorescent dyes were tested for their applicability and suitability to stain in-situ microorganisms in living acidophilic biofilms at pH <3. For comparison, two commonly used nucleic acid dyes DAPI and SYTO 59 were also employed and tested of how they perform on staining microorganisms under extreme acid conditions.

Table 3 applied excitation (ex) and emission (em) wavelength of the used fluorescent dyes

Fluorescent Dye	applied ex [nm]	applied em range [nm]
DY-601XL	633	645–750
V07-04118	633	645–750
V07-04146	633	645–750
DY-613	633	645–750
DAPI	351	400–531
SYTO 59	633	645–750

The new four dyes and the common dyes DAPI and SYTO were tested in parallel at the same natural biofilm samples. For this the macroscopic streamer biofilm from the acid mine drainage channels and the snotite biofilm sample was cut into several pieces and subsequently the procedures for the respective fluorescent dyes were applied to different "aliquots" of the respective biofilm samples. This was done to ensure very similar conditions in each sample and for each fluorescent dye. However, it has to be mentioned that biofilms exhibit a natural heterogeneity. Geochemical parameters and species distribution may change on a µm scale distance within the same biofilm. In addition, microorganisms within biofilms are heterogeneously distributed, due to their tendency to form micro-colonies [25], i.e. they are locally concentrated and show areas of high cell numbers and areas which are almost devoid of microorganisms.

The recorded fluorescent images of the stained biofilm specimen are displayed in Fig. 4 for the macroscopic streamer biofilm and in Fig. 5 for the snottite biofilm. Both pictures impressively show that the four new fluorescent

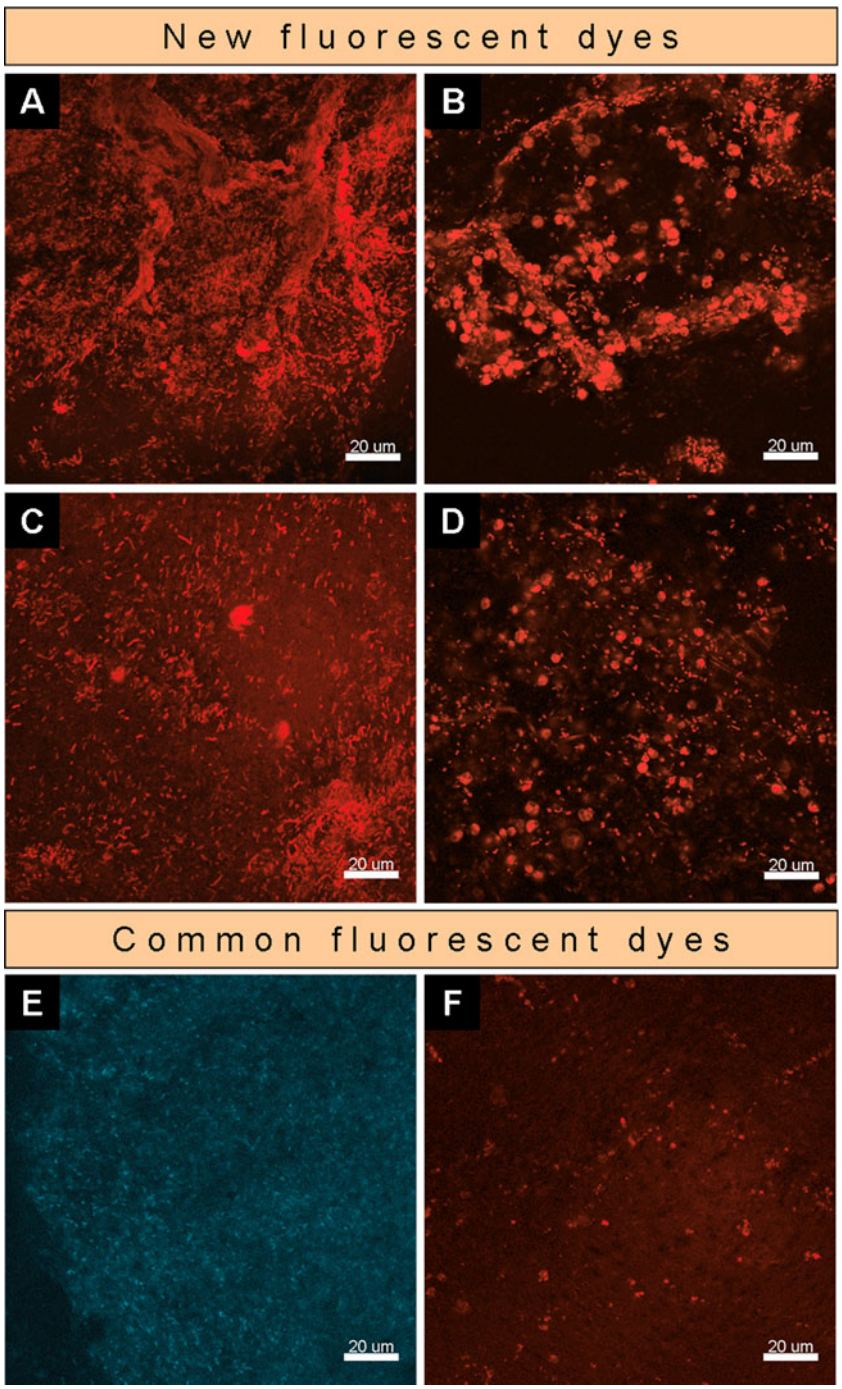

Fig. 4 Overview of the staining results of the biofilm from the AMD with the new fluorescent dyes in comparison to the common dyes DAPI and SYTO 59. **a** Dye DY-601XL; **b** Dye V07-04118; **c** Dye V07-04146; **d** DY-613; **e** DAPI; **f** SYTO 59

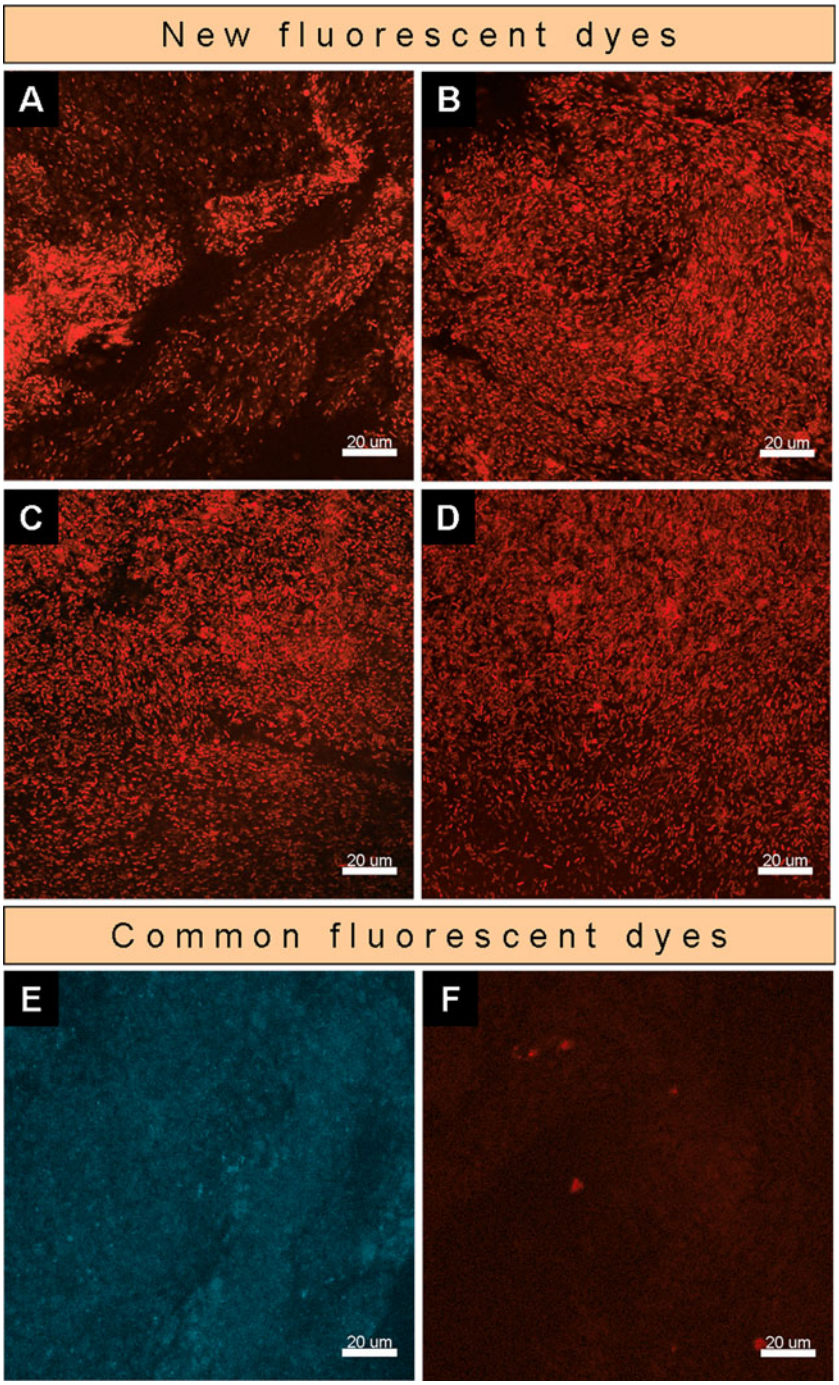

Fig. 5 Overview of the staining results of the dripstone-like biofilm from pit 390 with the new fluorescent dyes in comparison to the common dyes DAPI and SYTO 59. **a** Dye DY-601XL; **b** Dye V07-04118; **c** Dye V07-04146; **d** Dye DY-613; **e** DAPI; **f** SYTO 59

dyes DY-601XL, V07-04118, V07-04146, and DY-613 clearly stain the microorganisms in-situ in the living acidophilic biofilm samples. DAPI and SYTO 59, in contrast, showed only a very weak fluorescence signal which made the visualization of microorganisms quite doubtful or even impossible. The optical resolution of the images A, B, C, and D in Figs. 4 and 5, respectively, allows distinguishing single microorganisms. However, the images E (DAPI) and F (SYTO 59) are characterized by a poor quality and show no adequate resolution, so that single microorganisms are not detectable in these images. The DAPI-images show some minor fluorescence, but it has to be mentioned that the bacteria of these biofilms show slight auto-fluorescence behavior when excited in the UV-wavelength-range in which there is also the excitation of DAPI. From this we conclude that the fluorescence in these images E is not caused by the dye itself but by the weak autofluorescence of the bacterial cells itself as additional microscopic investigations showed in which no fluorescence dyes were used.

The images F, representing staining with SYTO 59, in Figs. 4 and 5 show only some punctiform fluorescence appearance. These could be related to possible precipitations as a consequence of the decomposition of the fluorescent dye. As a result, the biofilm samples stained with SYTO showed no stained microorganism at all.

To exclude the possibility that the common dyes DAPI and SYTO 59 became degenerated while stored in frozen state and lost their ability to stain nucleic acids of microorganisms further biofilm specimens from the acidophilic dripstone-like biofilm was stained with DAPI and SYTO 59. However, this time the biofilm was washed two times with tap water to increase the pH in the biofilm sample and to dilute the high concentrations of ions. For this test, the common dyes were also previously diluted in tap water. All other steps of the staining procedure were the same as mentioned before. The fluorescent images of this staining procedure are shown in Fig. 6. The images show a good resolution and the bacteria are very well stained and easy to visualize. This test clearly shows the dependency of the staining result on the pH-value. It documents the experimental problem by using common fluorescent dyes which are not acid-stable and the necessity to use acid-stable fluorescent dyes for microscopic in-situ examinations of living biofilms from extreme low pH environments.

All images where staining was positive showed predominantly rod shaped bacterial cells. These bacterial cells are most likely attributed to *Ferrovum myxofaciens*, the dominant bacterial species in both biofilm samples as identified by 16S rDNA-PCR. Especially the images B and D in Fig. 4 show also some bigger and roundish cells. Based on their size it was assumed that these cells are eukaryotic origin. These roundish cells are regularly found in the acidophilic macroscopic streamer biofilms collected in the acid mine drainage channels from the Königstein mine and were particularly well stained by dye V07-04118 and dye DY-613. These two fluorescent dyes seem to be suited to stain bacteria as well as eukaryotic cells. Unfortunately, we were so far not able to identify these cells. These big cells with diameters of approximately 3 to 5 µm showed some similarity to yeast cells or to "Vahlkampfia-like-cells" as described by Baker et al. [5] in biofilms obtained from another AMD environment. Further studies on the identification of the eukaryotic cells are required to identify without doubt these roundish cells. The roundish cells are also found in the snotite samples, however not as prevalent. They are not homogeneously distributed in the biofilm samples, but rather appear heterogeneously and are locally concentrated in the biofilms. Conclusively, the observation that these cells are only visible in images B and D [in Fig. 4] does not mean that only these two dyes stain these presumably eukaryotic cells. Rather, it can be suspected that there were locally none of these seemingly eukaryotic cells present in the investigated biofilm specimen despite the fact they were from exactly the same biofilm sample.

Conclusions

There are many investigations of biofilms from AMD environments where FISH-analyses were used to get an insight in the microbial composition and the biofilm structure. In this context, DAPI-stained fluorescence images are often shown. However, it has to be clearly pointed out that all FISH samples have to be intensively pretreated. The pH-conditions within the biofilm samples were changed by fixation and washing steps, so that DAPI can bind to the nucleic acids and stain the bacteria. Yet such staining procedures on acidophilic biofilm samples are not suitable when in parallel information on pH sensitive incorporated fluorescent heavy metals will be acquired. To prevent artifacts it is very important to keep the original pH value when chemical information, e.g. metal speciation, is combined with visual information obtained with fluorescence microscopic methods.

The four new fluorescent dyes, DY-601XL, V07-04118, V07-04146, and DY-613, show superior features for in-situ

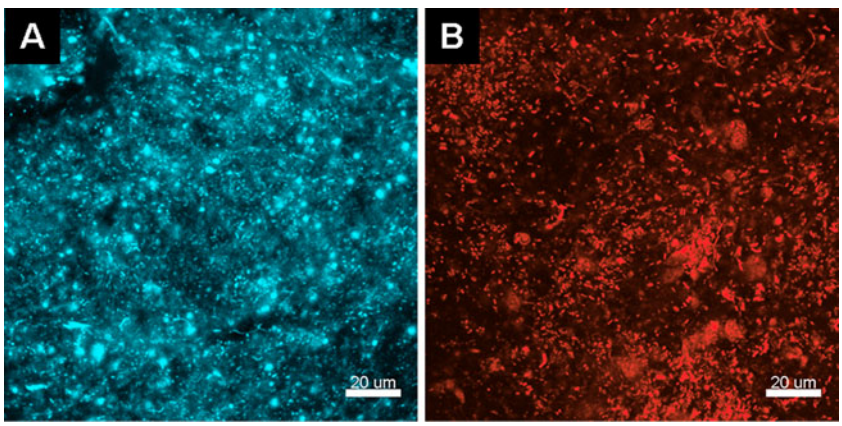

Fig. 6 CLSM image of the dripstone-like biofilm stained with DAPI (**a**: ex=351 nm) and SYTO 59 (**b**: ex=633 nm) after washing with tap water

staining of microorganisms in living biofilms which grow in very acid environments (pH <3), since the respective staining procedure does not require to increase the pH-value. In addition, at least V07-04118 und DY-613 also possess the ability to stain additional larger cells which are presumably eukaryotic origin. The presented new four fluorescent dyes allow investigating in situ acidophilic microorganisms in biofilm communities.

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