Video Article

Characterization of Aquatic Biofilms with Flow Cytometry

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Abstract

Biofilms are dynamic consortia of microorganism that play a key role in freshwater ecosystems. By changing their community structure, biofilms respond quickly to environmental changes and can be thus used as indicators of water quality. Currently, biofilm assessment is mostly based on integrative and functional endpoints, such as photosynthetic or respiratory activity, which do not provide information on the biofilm community structure. Flow cytometry and computational visualization offer an alternative, sensitive, and easy-to-use method for assessment of the community composition, particularly of the photoautotrophic part of freshwater biofilms. It requires only basic sample preparation, after which the entire sample is run through the flow cytometer. The single-cell optical and fluorescent information is used for computational visualization and biological interpretation. Its main advantages over other methods are the speed of analysis and the high-information-content nature. Flow cytometry provides information on several cellular and biofilm traits in a single measurement: particle size, density, pigment content, abiotic content in the biofilm, and coarse taxonomic information. However, it does not provide information on biofilm composition on the species level. We see high potential in the use of the method for environmental monitoring of aquatic ecosystems and as an initial biofilm evaluation step that informs downstream detailed investigations by complementary and more detailed methods.

Video Link

The video component of this article can be found at https://www.jove.com/video/57655/

Introduction

Biofilms are dynamic consortia of microorganism that play a key role in freshwater ecosystems, ranging from primary production, nutrient cycling, and water purification to influencing the distribution of microorganisms and their biodiversity in the ecosystem[1]. When biofilms are exposed to changing environmental conditions or to stressors, such as chemicals, their community structure quickly shifts towards more tolerant species[2,3]. Their high sensitivity turns biofilms into attractive model systems for environmental monitoring[4], however none of the current methods is perfectly suited to actually trace the dynamic of a biofilm community in a fast and easy way.

The commonly used set of methods to characterize biofilms consists of the measurement of functional and structural endpoints. At the level of the entire community, photosynthetic and respiratory activity as well as the activity of extracellular enzymes provides information on the functional state of the biofilm[5,6,7,8,9]. Biomass accrual is used as an indicator for overall biofilm growth. Structural changes are currently measured either by using traditional species identification with light microscopy or with nucleotide-based techniques (*e.g.*, denaturing gradient gel electrophoresis (DGGE), automated ribosomal intergenic spacer analysis (ARISA), metagenomics)[10,11,12]. These methods provide information but are time-consuming to perform, or require specific knowledge, or are still under development. Finally, new methods for the evaluation of the extracellular polymeric substances (EPS)[13,14] and the biofilm architecture[1], while sensitive, are low-throughput and have not yet been developed towards monitoring purposes.

It is evident that for a complete characterization of freshwater biofilms, it is necessary to combine several different methods, which provide insight into the biofilm function, composition and architecture. For environmental monitoring, on the other hand, a fast and sensitive method that is able to detect changes in the biofilm and enable basic biological interpretation of the shifts at the functional and structural level is required.

We have developed a new method for microbial community characterization of the phototrophic component of stream biofilms (periphyton), which is fast enough for monitoring purposes, and at the same time provides sufficient information on the biofilm community structure to allow biological interpretation[15]. It is based on single-cell flow cytometry (FC) of biofilm samples and coupled with computational visualization and provides information on optical and fluorescent properties of the biofilm on the single cell level.

The workflow after biofilm sampling consists of sample preparation in the form of sonication, fixation and size-based filtering of the samples followed by assessing the sample by flow cytometry. The acquired data provide information on several cellular traits in a single measurement: particle size, density, pigment content, abiotic content (*e.g.* microplastics) in the biofilm. This set of data is than analyzed via computational visualization using visual stochastic neighbor embedding (viSNE)[16], which enables fast and easy interpretation of the data. Although a few weeks

are required to set up and optimize the method, once set up, it takes only a few hours from collecting the biofilm samples to interpreting the results.

The main advantages of the presented method over others are the speed of analysis and high-information-content. Moreover, the samples can be stored for several weeks after collection without loss of their optical and fluorescence properties. This can be very useful when characterization of a large number of samples is required, such as large sampling studies or biomonitoring programs, but can also provide a substantial amount of information in smaller exploratory studies.

The presented protocol is based on flow-cytometric analysis of phototrophic biofilms (periphyton) collected from different locations of a stream. Many steps of the protocol, *e.g.* the selection of sites appropriate for monitoring purposes, depend on the goals of the research and therefore cannot be prescribed. Others allow less freedom and require that the protocol is followed closely, this is made clear in the detailed protocol below.

The protocol starts with the selection of sites for biofilm-based environmental monitoring. The next step is to set-up the flow-cytometer (FC) so that its measurements enable discrimination between different phototrophic organisms living in biofilms in the monitored environment. This is performed by collecting biofilm samples from the sites, identifying the fluorescent properties of different species present in the biofilm and setting up the flow-cytometer with lasers and filters that enable measurement at the appropriate optical wavelengths. Once the FC has been set-up, biofilms can be collected from the sites periodically, the optical and fluorescent properties of the single particles present in the biofilm measured by flow-cytometry, and the data analyzed by visual clustering. For better interpretation of the results, it is possible to build a FC reference database of local biofilm-living species and their phenotypes and use the database to identify different taxonomies in the FC data. Validation is possible through fluorescence-based sorting of the identified clusters of single cells using FACS and microscopy-based taxonomic identification of the present species. A schematic of the protocol is given in **Figure 1**.

Protocol

1. Ecosystem Selection and Biofilm Sampling

1. Select an aquatic ecosystem of interest and find sampling sites where biofilms grow. Shallow parts of a stream with slow to medium water flow and a stony streambed for biofilm attachment are appropriate[17,18].
2. Optional: If the site does not have enough surfaces for biofilm attachment, or to decrease the biofilms variability within a site, place artificial substrates for biofilm attachment (*e.g.* glass slides, ceramic tiles) into the site, while making sure that they are placed in the same direction with respect to the water flow and at similar depth and light intensity[19].
3. To determine the environmental conditions at the sampling site, use portable instruments to measure light intensity, water temperature, conductivity, oxygen and pH right above the surface biofilm is to be sampled from. Over the course of the sampling, take repeated measures (3–5) in order to calculate mean values.
4. Optional: Take water samples in order to determine relevant water chemistry parameters (dissolved organic carbon (DOC), K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, F-, SO_4^{2-}, PO_4^{2-}, NO_3^{2-}, SiO_4^{4-}).
5. Using protective gloves, collect comparable stones (in size, shape, and type) from each site (or artificial substrates). The stones should be exposed to similar flow and light conditions[18].
6. Filter enough stream water from the site through 0.22 µm filters, so that it is ready for all collected samples (*e.g.,* 15 mL per sample).
7. Use a soft toothbrush to remove the biofilm from the substrate into a flask (brush until all biofilm is removed) with 15 mL of filtered stream water[20].
8. Fix the samples in 0.01% paraformaldehyde and 0.1% glutaraldehyde[21].

2. Determination of Optimal Flow-cytometric (FC) Parameters

1. Transfer subsamples to a light microscope (using a 40X/0.75 objective; if necessary use a 100 × 1.3 Oil objective) and identify the species present in the samples[22,23].
2. Order identified single species from appropriate culture collections (*e.g.* the Experimental Phycology and Culture Collection of Algae at the University of Goettingen [EPSAG] or Culture Collection of Algae at the University of Cologne [CCAC] if monitoring is performed in central European ecosystems).
3. Grow the single species continuously in similar environmental conditions (*e.g.,* light, temperature, pH) to the environment the biofilm samples were taken from. Staying within 1 °C and 0.5 pH point from the environmental samples is recommended.
4. To detach/disperse the cells, put 15 mL of single species samples into centrifuge tubes and put the tubes in the center of a water bath and submerge, so that the liquid surface of the sample is at the same level as the surface of the bath. Sonicate the samples for 1 min at 45 kHz[24,25].
5. Record the absorbance and fluorescence spectra of the single species using a plate reader. Please refer to plate reader of choice for instructions. In this example, 96-well flat bottom transparent polystyrol plates were loaded with 200 µL sample volume and absorbance was measured. (Settings used: (a) fluorescence scan mode; emission wavelength step size 10 nm, emission scan number 30, bandwidth 20 nm, gain 100, 25 flashes, 20 us integration time or (b) absorbance scan mode; 25 flashes, bandwidth 9 nm).
6. Set up the flow cytometer with lasers, dichroic splitters and filters that in combination cover all those fluorescent ranges in which different species have specific properties. For central European streams, a 405 nm, 488 nm and 638 nm laser produce useful results.
7. Adjust the voltage setting of the photomultipliers (not applicable to all flow cytometers) and the range of the measured fluorescences to include at least 99% of all events of the samples.
8. Alternatively, if knowledge on species present in the biofilms is available, start with step 2.2. If fluorescent properties are known, start with Step 2.6.

3. Optional: Preparation of a Flow-cytometric Reference Database

NOTE: FC does not allow taxonomic identification of cells present in the biofilm. A reference database of FC measurements of single species known to be present in the biofilms, grown in similar conditions as the biofilms, can help with the interpretation of the data.

1. **Reference: single species**
    1. Using the optimized FC parameters set up in 2.5–7, measure the optical and fluorescent properties of single species (fixed in paraformaldehyde and glutaraldehyde and filtered through filters of appropriate size for the flow cytometer).
    2. Normalize the data from single species in the same way as the data from the biofilms (see step 6.1.2).

2. **Reference: damaged and decaying cells**
   One of the most important indicators of biofilm health is the fraction of damaged/dying cells present in the biofilm. To quantify these cells, a reference of decaying cells can be prepared.
    1. Take 1 mL subsamples of diluted biofilms and centrifuge them at room temperature at 8,000 x *g* for 10 minutes in a tabletop centrifuge. Suspend the resulting pellet in 1 mL 90% ethanol and store the suspension overnight at 4 °C. Repeat on the next day.
    2. Using the optimized FC settings, measure the optical and fluorescent properties of the damaged and decaying cells (fixed and filtered).

3. **Reference: microplastics**
   NOTE**:** If interested in monitoring microplastic particles in the biofilms, check whether they differ enough from biotic particles in optical and fluorescence properties for identification.
    1. Suspend the microplastic particles according to the instructions of the microplastics producer and measure their optical and fluorescent properties (using FC parameters from 2.7).
    2. Add the microplastic particles to the biofilm sample and leave over night at 4 °C.
    3. Filter the suspension and measure with the same FC parameters as above.

4. Sample Preparation and Storage

1. Once the FC has been set up and the FC reference database is ready, one can proceed with the evaluation of fresh biofilm samples, collected according to Steps 1.1–7. To detach/disperse the biofilm, transfer 15 mL of the samples from the flasks (see Step 1.7) into centrifuge tubes.
2. Put each tube in the center of a water bath and submerge so that the liquid surface of the sample is at the same level as the surface of the bath. Sonicate the samples for 1 min at 45 kHz.
3. Fix the samples in 0.01% paraformaldehyde and 0.1% glutaraldehyde (stock in nanopure water). Store at 4 °C until ready for analysis.
4. Important: Use half of the samples for flow-cytometry analysis and keep half in storage for safety and for optional validation with fluorescence-activated cell sorting and/or light microscopy (see Section 6).
   NOTE**:** Stored in the fridge, the fixed biofilm samples should keep their optical and fluorescent properties for up to three weeks.

5. Running the Sample Through the FC

1. If the samples were stored in the fridge, sonicate them quickly or pipette several times to separate the particles. Filter the subsamples with 50 µm filters (filter size depends on the width of the FC capillary) before measuring.
2. Load the individual samples (1–2 mL, this depends on the flow cytometer used) into the flow cytometer and measure the optical and fluorescent properties of each particle. Repeat the measurement three times per each biological replicate (three technical replicates).
3. Export the data from the FC in .csv format.

6. Data Preparation and Correlation Analysis

FC can analyze several parameters (*e.g.*, signal area, height, width) for each measured optical property and fluorescence range. Run a correlation analysis on the measured variables and only keep those measurements that are not highly correlated. This usually removes all but one FC parameter (*e.g.*, signal area) and can also remove highly correlated fluorescent measurements (usually stemming from the same laser and neighboring filters).

1. **Run CYT as a toolbox in Matlab:**
    1. Import the reduced FC data in .csv format from all the samples that are to be analyzed into the CYT software[16], including all technical and biological replicates. (Click +, File filter *.csv, select files for import).
    2. Transform the fluorescent channels of all the samples using the hyperbolic arcsine transformation. The value of the cofactor needs to be selected; 150 works for most phototrophic biofilms and flow cytometers, but optimization might be necessary for particular experimental setups and heterotrophic biofilms. (Right-Click/Transform/Enter Cofactor 150)
    3. For quality control, visually compare (in CYT) the histograms of individual samples and individual optical and fluorescent channels to make sure there are no outliers at the technical and biological level of replication. Outliers will manifest in significantly shifted fluorescent/optical distributions between the replicates. (Select Data, Select Channel, Plot)
        1. Alternative: run a principal component analysis (PCA) on the median values of fluorescent distributions and make sure that technical and biological replicates cluster together.
    4. Merge the technical replicates in each biological replicate, and subsample the biological replicates, so that each is represented by the same number of particles (cells, cell fragment and abiotic particles) and so that the total number of particles analyzed by Barnes-

Hut stochastic neighbor embedding (bh-SNE) is around 150,000 (for best visualization results[26]). (Select Data, Right-Click, Merge/ Subsample)
5. For comparison between samples, it is important to select only those samples that are to be compared. Select the samples and run bh-SNE[27]. Once the algorithm is finished, new channels, named bh-SNE1 and bh-SNE2 appear. These are the SNE coordinates of all analyzed particles, which can be used to visualize the data in a scatter plot (viSNE map). (Select Data, Select Channels, Right-Click, bh-SNE, Yes normalized data)
6. In the viSNE map, particles are positioned according to similarity and grouped into visually separable clusters. Inspect the optical/ fluorescent properties of the clusters and mark and name those that are well separated and have different optical/fluorescence properties by using the drawing tools available in CYT. (Select Channel bh-SNE1, bh-SNE2)
7. Optional: If a reference database of single microbial species (measured with the same flow-cytometry setting and grown under similar conditions!) is available, export the viSNE maps into Matlab and project the reference database onto the maps. In this way, particular clusters can be connected to particular species/taxonomic groups (scripts available for download at https://github.com/anzezupanic/FC_analysis). (Session/Save)
8. Troubleshooting: If the bh-SNE analysis does not return visually separable clusters, but a single large one, too many particles were used in the bh-SNE. Try again with fewer particles per sample. If there are no clusters, but rather sparse individual points, increase the number of particles used.

2. Optional: Instead of relying on bh-SNE for the clustering, other clustering methods (*e.g.*, hierarchical, centroid-based or density based clustering) can be used on the normalized data and then superimposed on the viSNE map (https://github.com/anzezupanic/FC_analysis).
3. Once the clusters have been identified, count the number of particles in each cluster belonging to each biological replicate in each sample. Assess differences between the samples, using ANOVA and Tukey's test with appropriate (*e.g.*, Holmes) correction for multiple comparison.

7. Optional: Validation of Cluster Interpretation Using Fluorescence-activated Cell Sorting

1. Once the clusters have been identified, use fluorescence-activated cell sorting (FACS) to sort them out of the samples if desired, provided that the FACS has a similar (or identical) configuration of lasers and fluorescent filters.
2. For each identified cluster, CYT provides optical and fluorescent gates that define the cluster. Use these gates to sort out the particles in each cluster with the FACS and then identify the sorted cells using light microscopy.

Representative Results

Using the procedure presented here (**Figure 1**), samples taken from several sites of a local stream in Switzerland were analyzed. At each site, three similar sized stones (10–12 cm) were taken, and biofilms were brushed off the stones. The samples were then sonicated, fixed, filtered and later analyzed by flow cytometry. The setup of the flow cytometer and the reference database used were the same as described in a previous paper[15]: three lasers were used (405, 488 and 638 nm), seven dichroic splitters and ten filters that covered fluorescence emissions from 425 to >755 nm, and the reference database including >30 species covering cyanobacteria, green algae, diatoms and red algae that were found in biofilms in Swiss stream. The total number of particles used for creation of the viSNE maps was approximately 150,000 (**Figure 2A**). Using the described approach, it is possible to distinguish between the different sites (**Figure 2B**), categorize different parts of the viSNE map in subpopulation of cells with coarse taxonomic information (**Figure 3**) and establish which subpopulations were different between the samples (**Figure 4**).

A similar approach was used to quantify microplastic particles in a biofilm. Pure samples of microplastic particles of similar size to cells in freshwater biofilms (ca. 10 µm), biofilms samples grown in the laboratory in the presence or absence of microplastic particles (using biofilm growth protocols described in Tlili *et al.* 2011[5]) were measured and compared (**Figure 5**). It was determined that microplastic particles with known optical and fluorescent properties can be robustly detected at concentrations above 1,000 ppm.

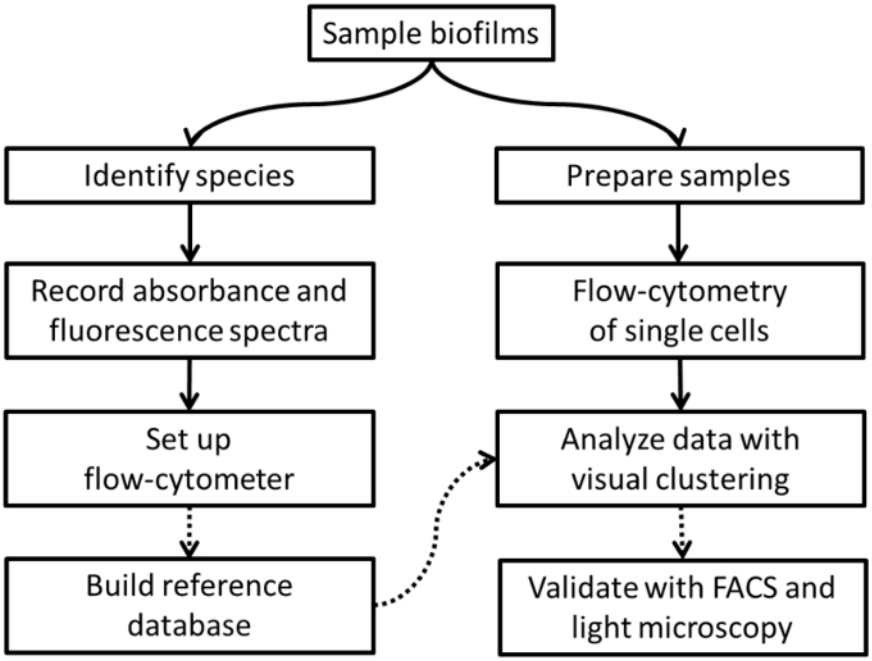

Figure 1: Schema of the experimental protocol. Collection of the environmental biofilms samples is followed by identification of species present in the biofilm, determination of their optical and fluorescent properties and fitting the flow-cytometer with appropriate lasers and filters to measure these properties. For flow-cytometric measurements, the samples are sonicated, fix and stored. When enough samples have been gathered they are measured by flow cytometry where for each measured cell, a number of optical and fluorescence properties are obtained and analyzed using visual clustering. Optionally (dotted arrows), it is possible to build an FC database of species present in monitored biofilms and use the database for interpretation of the results. It is also possible to use further validation methods, *e.g.* fluorescence-based cell sorting and taxonomic analysis using light microscopy Please click here to view a larger version of this figure.

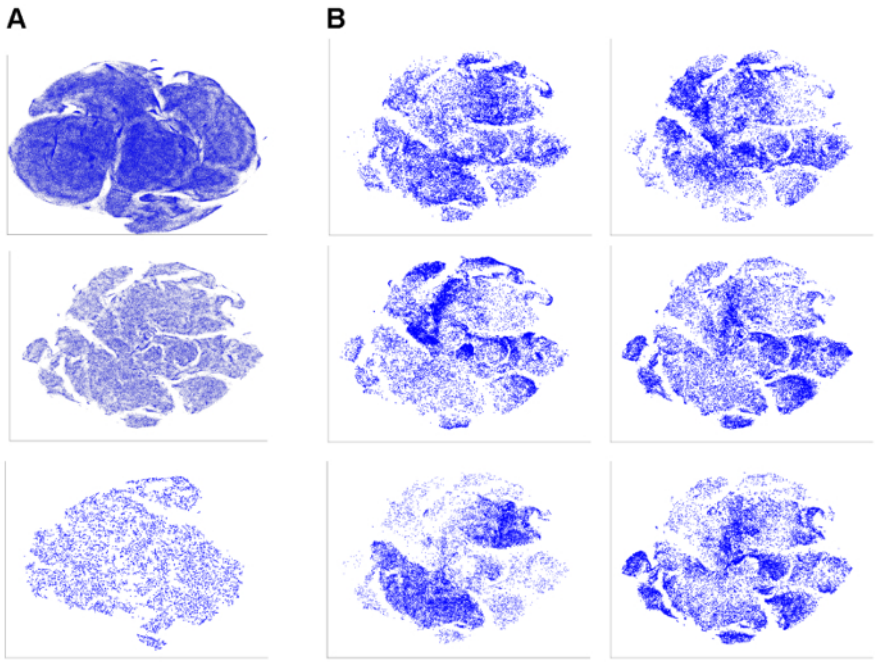

Figure 2: Comparing environmental biofilms using viSNE maps. (A) viSNE maps obtained from too many cells (300,000) have a single large cluster (top), too few (10,000) cells do not form clusters at all (bottom), while an optimal number of cell (ca. 150,000) form clearly visually separable clusters (middle). **(B)** Subsets of the viSNE map that belong to six different samples feature different densities in different part of the viSNE map. Please click here to view a larger version of this figure.

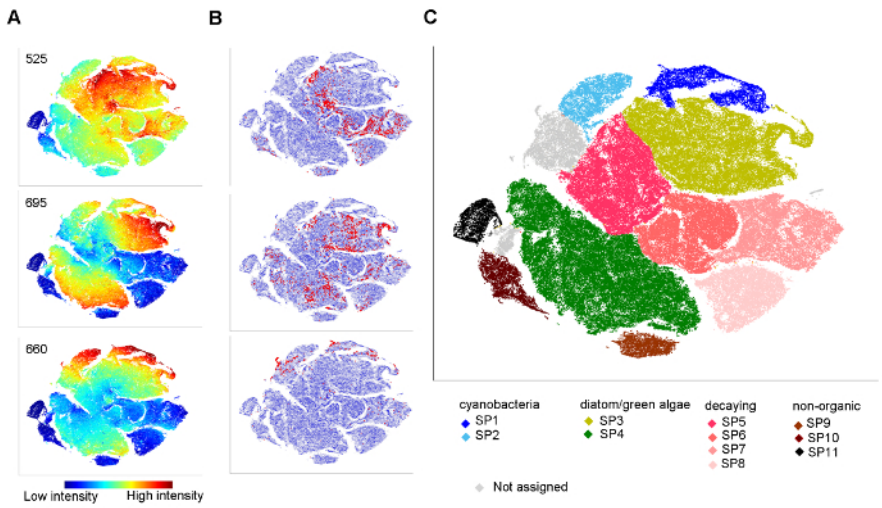

Figure 3: Clustering of the subpopulations of particles in biofilms. (A) Different parts of the viSNE map are populated by particles with different optical and fluorescence properties that give clues to the biotic/abiotic origin of the particles and to which taxonomic group the biotic particles (cells) belong to (wavelengths from top to bottom: 525 nm, 695 nm, 660 nm). For each wavelength, the color scale is normalized from highest measured fluorescence (red) to lowest measured (blue). **(B)** Projection of the reference database (red dots) onto the viSNE map can help interpret which parts of the map are populated by which taxa and/or abiotic particles: decaying cells (Top), diatoms (Middle), cyanobacteria (Bottom). **(C)** Based on information in (A) and (B) and the visually separable clusters of the viSNE map, it is possible assign different parts of the viSNE map to different clusters (SP1-11) and categorize the clusters accordingly. Please click here to view a larger version of this figure.

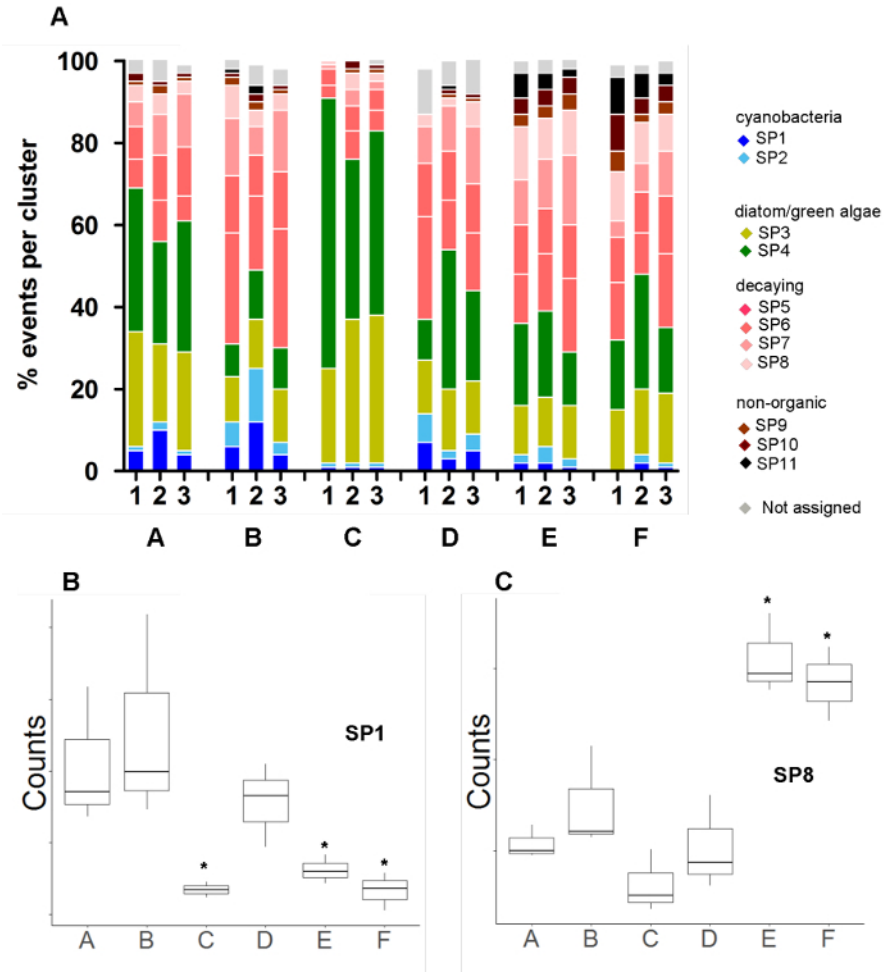

Figure 4: Statistical analysis of the biofilm samples. (A) Quantification is performed by counting the number of particles of each subpopulation belonging to different biological replicates. A–F represent six different locations along a local stream, from which the biofilm samples were taken. **(B)** ANOVA can be used to evaluate statistical difference between the samples, with appropriate multiple comparison correction (p <0.05, pairwise Tukey HSD all sites vs site A). Please click here to view a larger version of this figure.

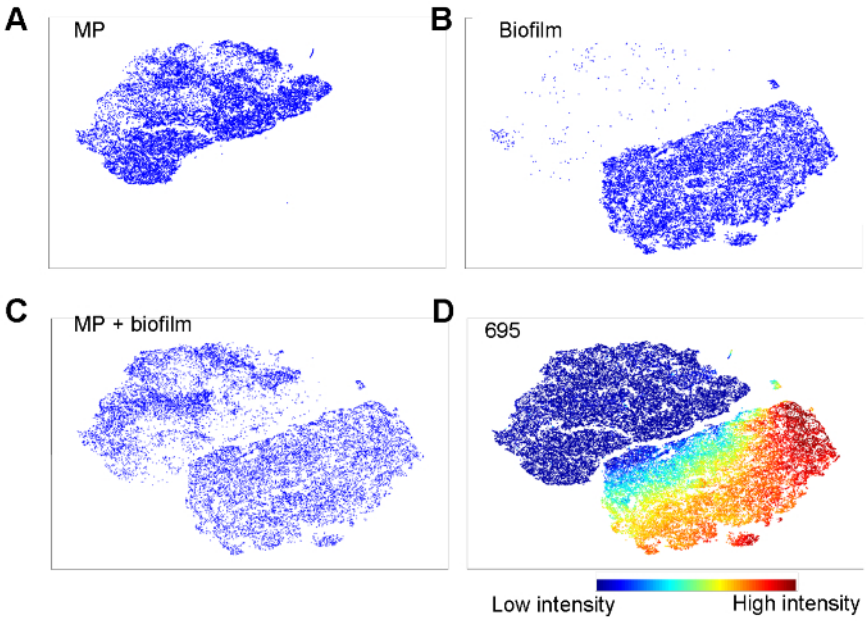

Figure 5: Tracking microplastic particles with viSNE. (A) Microplastic particles, **(B)** freshwater biofilms, **(C)** freshwater biofilm mixed with microplastic particles, **(D)** fluorescence of the particles measured by FC at 695 nm. The color scale is normalized from highest measured fluorescence (red) to lowest measured fluorescence (blue) at 695 nm. The ratio between microplastic particles and biofilm particles is approximately 1:1. Please click here to view a larger version of this figure.

Discussion

The protocol described above is relatively simple to implement. However, while the presented default settings have been shown to be suitable for all phototrophic biofilm tested thus far, optimization (as described in the protocol) is necessary to maximize the information obtained from the method. Indeed, the optical and fluorescent properties of biofilms can vary, depending on the environmental conditions (season, temperature, chemical composition of the water)[1]. Therefore, it is important to take into account this variability when setting up the FC analyses. One way to do this is by taking representative samples of biofilms from the sampling sites and/or taking single species and growing them in different conditions and measure them using different setups. Growing single species is also useful to construct a database of fluorescent properties that can be used to interpret the FC results. However, to do this, it is important that the single species have been measured in similar conditions to those under which the biofilms are going to be sampled. For example, a database of FC measurements of single species growing at 25 °C will tell very little about the composition of a biofilm growing at 15 °C. It is, however, not absolutely necessary to build a reference database to use this method effectively. Already with using the basic FC protocol, it is possible to get information on the size, granularity and presence of different pigment in the cells present in the biofilm. Visual clustering allows classification of cells into clusters that are similar in the measured properties and determination of which property has changed the most between samples. The reference database, however, becomes important when assigning taxonomic identities to the different clusters. Once changes in biofilm properties have been detected, it is possible to correlate them with physical and chemical measurements of the water at the sampling sites. This allows determining which environmental factor has the strongest influence over the biofilm community in the monitored ecosystem.

There are also some limitations to the method. For instance, FC does not allow the identification of the biofilm composition at the species level. Moreover, when changes in biofilm properties are detected, FC cannot ascribe responsible causes: a shift towards more tolerant species or towards more tolerant phenotypes (species remaining the same). Other, complementary methods, such as metagenomics, are needed to address this question. Another limitation of the method is that only the phototrophic component of biofilms, rich with pigments with different fluorescent properties, is analyzed. While it is certainly possible to use the method for heterotrophic biofilm characterization, it is not clear whether enough information can be obtained with stain-free analysis. Due to the low natural fluorescence of bacteria and fungi, different settings have to be used, and it is therefore not possible to evaluate the phototrophic and heterotrophic components of the biofilm together in a single FC run. Rather, separating the sample and evaluating one part with phototrophic and one part with the heterotrophic setting would be necessary. Finally, there is a limitation with using the viSNE for biofilm visualization. The best results are achieved with ca. 150,000 cells imaged together. This sets a limit for the number of samples that can be analyzed and compared with the method without computationally diluting the samples too much. For example, if one wanted to compare 150 samples, then each sample would only be represented by ca. 1,000 cells. One way around this limitation is to analyze the samples using a "moving window" approach in which overlapping groups of samples can be analyzed separately using viSNE and then the results pooled together, such a solution, however, has not yet been implemented.

We believe that the flow-cytometry based evaluation of biofilms can be efficiently applied to monitoring of aquatic ecosystems. It can be used to compare time-series samples or samples taken at different locations, and, with the exception of the sampling itself, has potential for complete automation. The method can be considered as an initial biofilm evaluation step that provides information for downstream detailed investigations with complementary and more detailed methods. Finally, it can be extended to more specific applications, such as detection of abiotic contamination of biofilms, as we have shown for the case of microplastic.

Disclosures

The authors have nothing to disclose.

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