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Fiber-Optic Fluorometer for Microscale Mapping of Photosynthetic Pigments in Microbial Communities

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Microscale fluorescence measurements were performed in photosynthetic biofilms at a spatial resolution of 100 to 200 μm with a new fiber-optic fluorometer which allowed four different excitation and emission wavelengths and was configured for measuring phycobiliproteins, chlorophylls, and bacteriochlorophylls. We present details of the measuring system and describe examples of applications in different microbial communities.

Microbial phototrophic communities in sediments and biofilms are often used as small-scale model systems in studies of microbial interaction (15). In the absence of bioturbation and grazing, such systems exhibit characteristic stratification of both microbial processes and different photosynthetic populations over micrometer to submillimeter distances. Microsensor techniques (8, 14) allow repetitive and nondestructive high-resolution measurement of physical parameters (light and temperature), chemical parameters (e.g., pH and O_2, CO_2, and H_2S contents), and metabolic rates (photosynthesis and respiration) in photosynthetic sediments and biofilms (5, 16). For the most part, analysis of the microdistribution of phototrophic microorganisms has relied on determining characteristic depth-dependent photopigment signatures by destructive techniques, such as horizontal slicing of a sample and subsequent pigment extraction and analysis (1, 10, 13), or by spectral absorbance and fluorescence microscopy of vertical thin sections obtained by freeze sectioning (4).

Nondestructive techniques have involved laser scanning confocal microscopy and reflectance spectroscopy of translucent biofilms, but the generally high optical densities of most sediments or microbial mats limit this approach to the upper 100 to 200 μm of samples (9, 17). Another approach is based on spectral measurement of the internal light fields of biofilms and sediments with fiber-optic microprobes, in which photopigment distributions are inferred from depth-dependent changes in spectral signatures for characteristic pigments (7, 11, 13). Besides reflectance and transmission spectroscopy, fiber-optic microprobes can also be used for microscale fluorescence analysis of biofilms and sediments (4). Here we describe a new fiber-optic fluorometer which can detect photopigments by their characteristic fluorescence as sampled via a fiber-optic microprobe.

Fiber-optic fluorometer. The technical concept of the new fluorometer is shown in Fig. 1. Ultrabright light-emitting diodes (LEDs) equipped with short-pass filters are used as excitation light sources. The excitation light is coupled into a single-strand step index silica glass fiber (core/cladding diameter, 100/140 μm) via a standard ST connector. The fiber is connected to a multimode 2×2 fused fiber coupler (Gould Inc.) (i.e., a fiber-optic beam splitter). One of the coupler branches is connected to the fiber-optic microprobe (see below). The excitation light illuminates the sample around the microprobe tip and excites the fluorophores present. A fraction of the induced diffuse fluorescent light reenters the glass fiber through the microprobe tip and is guided via the fiber coupler to a photomultiplier tube (PMT) (H5702-50; Hamamatsu). Removable light filters are positioned between the fiber end face and the PMT detector. The PMT provides a voltage signal proportional to the measured light intensity.

The light intensity of the LEDs is modulated at a frequency of 750 Hz. Subsequently, the fluorescence signal detected by the PMT is also modulated at 750 Hz. A lock-in amplifier selectively amplifies only signals at this frequency in order to minimize the influence of ambient light or electronic noise. The electronics is controlled by a built-in microcontroller (V25; GME GmbH) that reads the fluorescence signal measured by the lock-in amplifier via an analogue-to-digital converter. A reference zero value obtained with the microprobe immersed in pure water is subtracted from each measurement. The results of the measurements are shown in digits on a small text display. Alternatively, a personal computer can be connected via a serial (RS 232) interface in order to control the measuring procedure by custom-made programs.

Fiber-optic microprobe. Fiber-optic microprobes were prepared as described by Kühl and Jørgensen (6) from a 100/140 μm (core/cladding ratio) step index fused silica fiber cable (numerical aperture, 0.22) with a standard ST plug at one end. The polyvinyl chloride tubing and protective plastic coating of the glass fiber were stripped for a length of approximately 40 mm, and the bare fiber was tapered in a small acetylene-oxygen flame to obtain a tip diameter of 5 to 10 μm. The taper was cut to obtain a fiber tip diameter of 50 μm. The tip was heated in a hot flame, which resulted in a rounded tip without sharp edges. For better handling the fiber-optic microprobe was fixed with epoxy resin in a hypodermic needle mounted on a syringe,

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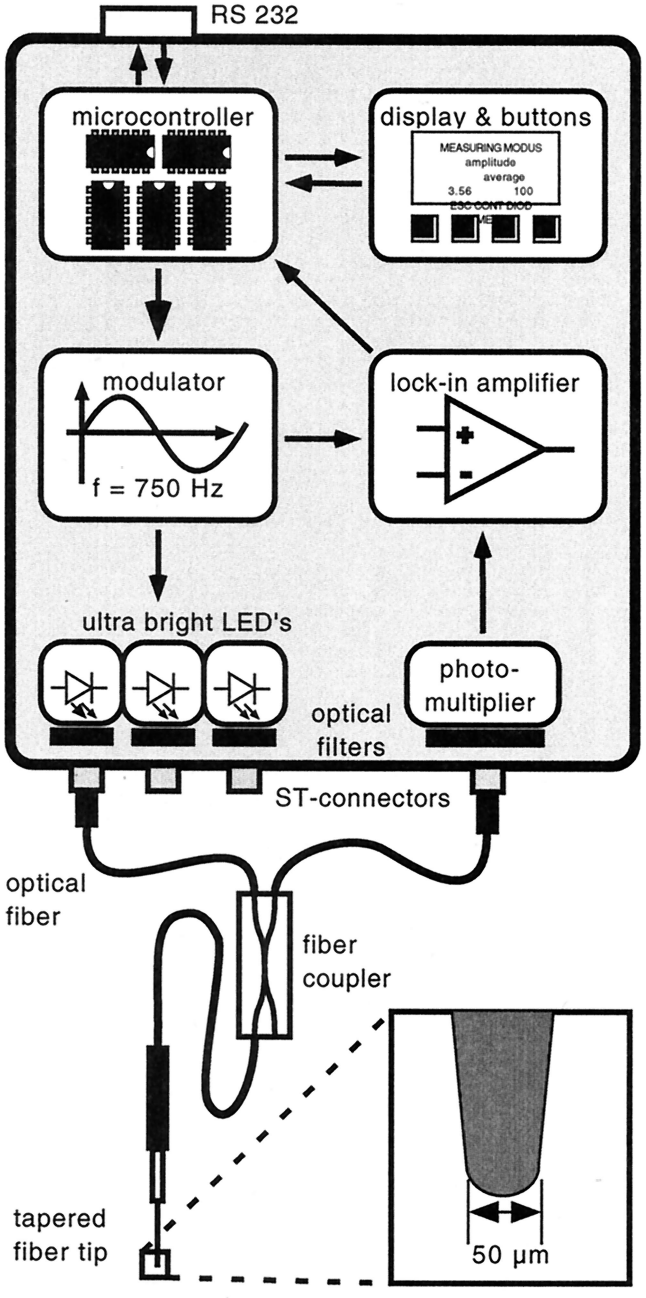

FIG. 1. Technical concept of the fiber-optic fluorometer.

which then was attached to a micromanipulator (Märtzhäuser GmbH).

Light sources and filter combinations. The fluorometer was configured with four ultrabright LEDs with the following peak wavelengths and half band widths, respectively: 470 and 20 nm (BP280CWPB1K; DCL Components), 515 and 35 nm (NSPE 510S; Nichia, Inc.), 590 nm (TLHE 5800; VISHAY), and 610 and 20 nm (L-513TUEC; DCL Components). The LEDs were combined with short-pass filters KP 480 and KP 540 (OIB GmbH), SP 600 (CVI), and KP 620 (OIB) with cutoff wavelengths of 480, 540, 600, and 620 nm, respectively. Below these filtered excitation light sources are referred to as blue, green, yellow, and orange, respectively.

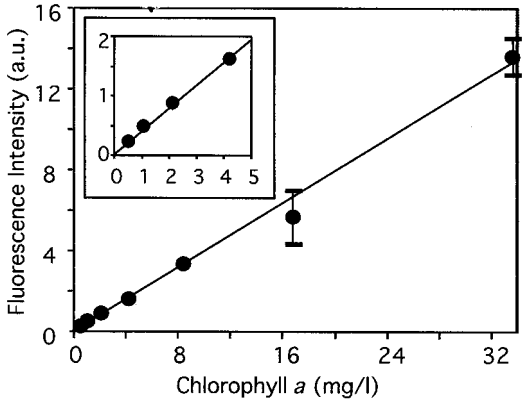

FIG. 2. Chlorophyll *a* fluorescence in a liquid culture of *Nannochloris* sp. The error bars indicate standard deviations ($n = 4$). a. u., arbitrary units.

The fluorescence light was detected in three different wavelength regions: orange, red plus near-infrared (NIR), and NIR. The orange region was cut out by a filter combination consisting of a 600-nm long-pass filter and a 650-nm short-pass filter (LP 600 and SP 650; CVI). For the red plus NIR region and the NIR region 645- and 780-nm long-pass glass filters (RG 645 and RG 780; Schott GmbH), respectively, were used. As the glass filters themselves exhibited weak fluorescence if they were excited with blue light, an additional 570-nm long-pass filter (Deep Golden Amber; LEE Filters Ltd.) was placed in front of them. Detection of red plus NIR light and NIR light was limited at longer wavelengths by the spectral sensitivity of the PMT; at 800 nm the sensitivity of the PMT was only 50% of the maximal sensitivity, and then it decreased significantly at longer wavelengths up to 900 nm. However, new PMT detectors with higher NIR sensitivity have recently become available (Hamamatsu). A more detailed description of the measuring techniques involved has been given by Holst et al. (2).

Chlorophyll in liquid media. A pure liquid culture of the marine green alga *Nannochloris* sp. was diluted in seven steps by a factor two. At each dilution step the chlorophyll *a* fluorescence was measured with the blue → red+NIR (excitation → emission) combination. The chlorophyll *a* content of the undiluted culture was measured by a standard ethanol extraction method (12). A strong linear correlation ($r^2 > 0.998$) was found between the fluorescence signal and the chlorophyll concentration (Fig. 2). Even though the system was optimized for obtaining measurements in optically dense biofilms and sediments, it can also be used for direct quantitative measurement of chlorophyll in liquid media with reasonable sensitivity. Greater sensitivity in liquid media can be obtained, but this would involve modification of the sampling geometry at the fiber tip.

Fluorescence fingerprints. Fluorescence fingerprints of phototrophic microorganisms were obtained by placing the sensor tip in samples of five different species that were representative of different types of phototrophs (Fig. 3). Pure cultures of *Euglena gracilis, Chromatium gracile* (recently reclassified as *Marichromatium gracile* comb. nov. [3]), and *Synechococcus* sp. were centrifuged in a test tube ($150 \times g$, 4 min). Subsequently, the fluorescence fingerprints inside the pellets obtained were

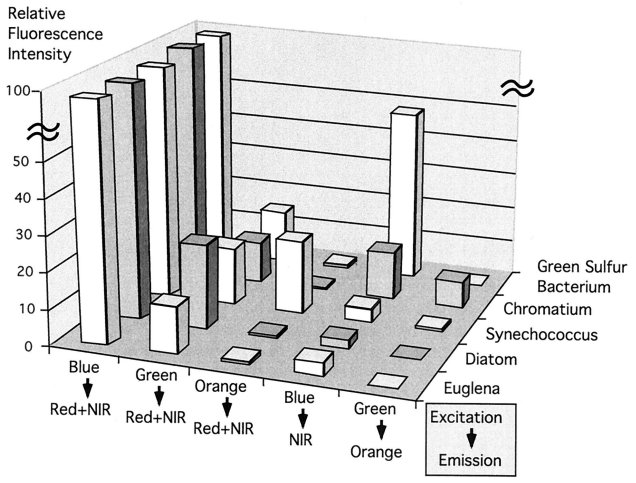

FIG. 3. Fluorescence fingerprints of different phototroph species.

determined. A sample of green sulfur bacteria was obtained from an enrichment culture in a Winogradsky column incubated for several months at 20°C in dim daylight. An in vivo absorption spectrum of the enrichment culture revealed that bacteriochlorophyll *c* was the dominant pigment. Pelagic diatoms were obtained from Øresund (Denmark) with a plankton net (mesh size, 10 μm). In order to avoid any influence of spatial heterogeneity, all excitation → emission values were determined in each sample without moving the sensor tip. The fluorescence signals were expressed as percentages of the blue → red+NIR fluorescence, which detected fluorescence from both chlorophylls and bacteriochlorophylls.

The microalgal phototrophs *E. gracilis* and diatoms exhibited for the green → red+NIR combination values of 10 and 20%, respectively. This combination targeted the carotenoids. The higher value for the diatom sample could be anticipated by its brownish color compared to the bright green of the *E. gracilis* sample. The other fluorescence combinations did not produce significant values.

The fluorescence fingerprint of the cyanobacteria (*Synechococcus*) resembled that of the microalgae with one exception for the orange → red+NIR combination. For this combination a signal of about 20% was detected, whereas no other sample produced significant signals. The strong fluorescence was due to phycobiliproteins, which are characteristic of cyanobacteria. Unicellular red algae like *Cyanidium* sp. also contain phycobiliproteins, but these organisms are found mainly in acidic springs and soils. Thus, the orange → red+NIR combination can be used as a sensitive indicator for cyanobacteria in most benthic systems.

The green sulfur bacteria exhibited a fluorescence fingerprint similar to that of oxygenic phototrophs except for the blue → NIR combination; for this combination bacteriochlorophyll *c* produced a peak of almost 50%. The same peak, but less pronounced, was obtained with the *Chromatium* sample, which contained mainly bacteriochlorophyll *a*. The weaker signal in the *Chromatium* sample can be explained by the longer wavelength for maximum fluorescence of bacteriochlorophyll *a* (~870 nm) compared to that of bacteriochlorophyll *c* (~750 nm). The sensitivity of the PMT at 870 nm is about 10 times less than that at 750 nm. In addition, the *Chromatium* sample exhibited a peak for the green → orange combination, which may have been due to characteristic carotenoids.

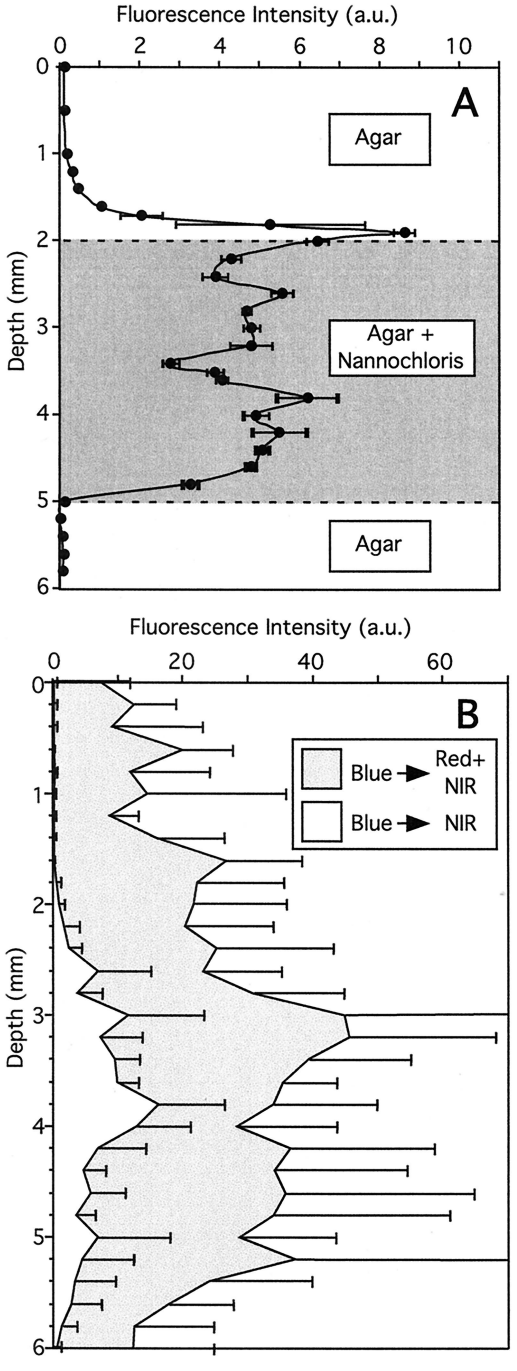

FIG. 4. Fluorescence microprofiles in artificial biofilms. (A) *Nannochloris* sp. embedded in an agar layer. (B) Multilayer system containing diatoms (depth, 0 to 2.3 mm) and green sulfur bacteria (depth, 2.3 to 5.5 mm). a.u., arbitrary units.

In summary, the data above show that fluorescence fingerprints allow discrimination of the dominant photopigments characteristic of benthic microalgae, cyanobacteria, and anoxygenic phototrophs.

Measurements in artificial biofilms. In order to investigate the spatial resolution of the fiber-optic microprobe, we obtained measurements in defined biofilms of known composition and thickness. Three stratified layers consisting of 2% agar were prepared in a test tube. A homogenized suspension of *Nannochloris* sp. was added to the semisolid (~45°C) agar used for the middle layer. Fluorescence was measured in 200-μm vertical resolution steps with the blue → red+NIR combination (Fig. 4A). As the fiber tip approached the upper surface of the *Nannochloris* layer, the fluorescence signal increased exponentially from a distance 1 mm above that surface. About 100 μm above the interface the signal exhibited a maximum value, which was about two times higher than the average signal value inside the *Nannochloris* layer. When the fiber tip approached the lower limit of the *Nannochloris* layer at a depth of 5 mm, the signal decreased to one-half of the initial value within ~200 μm. The spatial resolution of a microscale fluorescence measurement is very dependent on the optical density of the sample in front of the fiber tip. At a low optical density (e.g., when measurements are obtained in agar), a large volume in front of the fiber tip is excited, which results in low spatial resolution, as shown by the increase in fluorescence 1 mm above the *Nannochloris* layer (Fig. 4B). At a higher optical density, a smaller volume is excited, which results in greater spatial resolution, as shown by the rapid decrease in the fluorescence signal at the lowermost boundary of the *Nannochloris* layer. It is important to keep these limitations of the technique in mind when fluorescence profiles are determined for natural samples like stratified microbial communities in sediments or microbial mats. However, in many cases the optical density is much greater than that of the *Nannochloris* layer described here and the spatial resolution is <100 to 200 μm (4). It is important to consider spatial resolution in samples with gelatinous layers of exopolymers with a low optical density comparable to that of agar.

A defined two-layer system was prepared by sequentially centrifuging a different cell suspension in a test tube (150 × *g*, 4 min). The lower 30 mm of the test tube was filled with 1% agar. A 3- to 3.5-mm-thick layer of green sulfur bacteria was added, which was covered by a 2- to 2.5-mm-thick layer of diatoms. The thicknesses of the various layers were determined after the experiment by freezing the sample at −80°C and subsequent vertical sectioning. Fluorescence profiles for the blue → red+NIR and blue → NIR combinations were determined at 200-μm depth intervals, as shown in Fig. 4B. As the same excitation light was used for both fluorescence measurements, the difference between the two profiles represented the red fluorescence in the visible spectrum. Two layers could be distinguished: (i) an upper layer showing only fluorescence for the blue → red+NIR combination and (ii) a lower layer in which a signal for the blue → NIR combination was also detected. Thus, the fluorescence measurements showed that bacteriochlorophyll was abundant only in the lower layer. The thicknesses of the layers in the fluorescence profile correlated well with the actual layer thicknesses in the sample. The results show that the fiber-optic fluorometer can resolve the zonation of different phototrophs in biofilms.

Application in a photosynthetic sediment. The system was used to study cyanobacterial distribution in a marine sulfidic sediment sample obtained with a core tube (diameter, 45 mm)

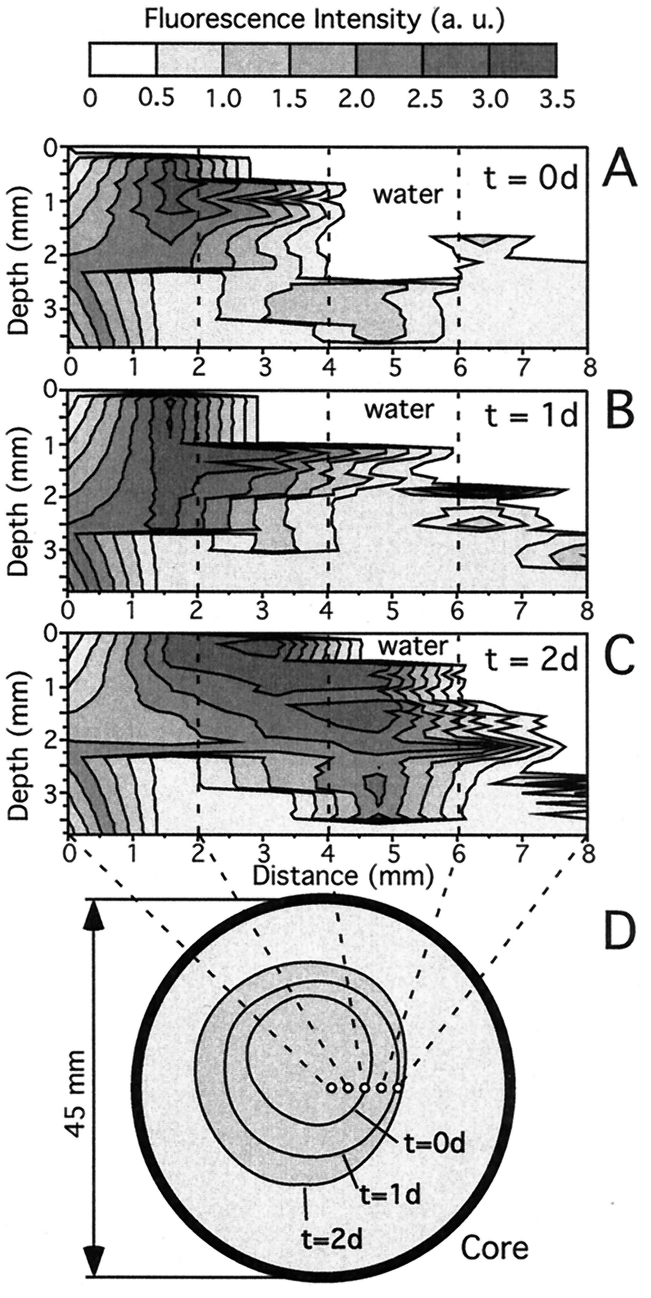

FIG. 5. Distribution of *Oscillatoria* sp. in a marine sulfidic sediment core for three consecutive days. Additional illumination was applied at zero time. (A to C) Transects of chlorophyll *a* fluorescence. (D) Sediment surface area covered by *Oscillatoria* sp. (top view). The positions of the profile sites are indicated by open circles. a.u., arbitrary units; t, time; d, day.

in Øresund (Denmark) at a water depth of 6 m. The overlaying water in the core had a salinity of 22 ppt and was constantly aerated. The core was kept at 20°C in dim daylight before the experiment. Most of the sediment surface was covered with *Beggiatoa* sp. In the center of the surface layer, a circular area (diameter, ~15 mm) was covered by a dense 2-mm-thick layer of motile, large, filamentous cyanobacteria (*Oscillatoria* sp.). At the beginning of the experiment, additional illumination was applied with a halogen bulb (Philips 6423; 15 V; 150 W;

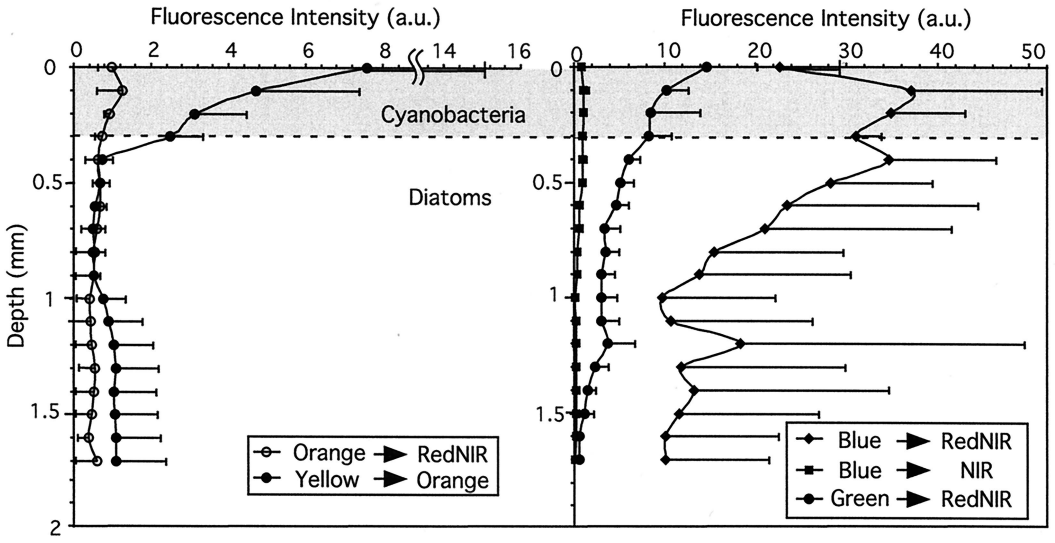

FIG. 6. Fluorescence microprofiles for different excitation → emission combinations in an intertidal sediment from the Rhone delta (Camargue, France). The error bars indicate standard deviations ($n = 5$). a.u., arbitrary units.

distance, 20 cm), which resulted in an increase in the surface area covered by the cyanobacteria (Fig. 5D). The chlorophyll fluorescence was monitored over a transect in the upper 3 mm of the sediment. The transect data included five fluorescence profiles for the blue → red+NIR combination (200-μm depth intervals) for points that were 2 mm from each other. The transect data, which were acquired just before the illumination was switched on, are shown as a contour plot in Fig. 5A. The chlorophyll distribution showed that only the leftmost 4 mm of the sediment transect was covered by cyanobacteria. The transect values for the next 2 days (Fig. 5B and C) show the increase in the area covered by cyanobacteria. The measurements obtained show the potential of the new system for monitoring dynamic changes in the distribution of benthic phototrophic microorganisms due to migration.

Application in intertidal sediment. Zonation of diatoms and cyanobacteria was investigated with intertidal sediments from the Rhone delta (Camargue, France). The sediment was dominated by diatoms overgrown by a thin layer of filamentous cyanobacteria. Numerous fluorescence microprofiles were obtained in a 100-mm^2 area. Figure 6 shows profiles obtained for all excitation → emission combinations. Vertical freeze sectioning and subsequent epifluorescence microscopy revealed two distinct layers: an upper layer ~300 μm thick dominated by filamentous cyanobacteria and a second layer 1.3 to 1.7 mm thick dominated by diatoms. The combined biomass of phototrophs was much greater in the upper layer than in the lower layer. Anoxygenic phototrophic bacteria were not detected by microscopy, and consequently the blue → NIR combination resulted in no significant fluorescence signal in the sediment. The yellow → orange and orange → red+NIR combinations revealed a high level of phycobilins over a depth interval which was similar to the measured thickness of the cyanobacterial top layer. The profiles obtained with the blue → red+NIR and green → red+NIR combinations also showed peaks in the top layer, but high signal values were also obtained for deeper layers where diatoms dominated.

Summary. It is now possible to obtain microscale fluorescence measurements with fiber-optic microprobes for nondestructive mapping of photopigments in biofilms, sediments, and other surface-associated microbial communities. Microscale fluorescence measurements can also be used for quantitative measurement of pigment concentrations in liquid cultures. However, quantitative measurements cannot be obtained for optically dense samples, like sediments or microbial mats, without knowledge of the spatially heterogeneous absorption and scattering properties of the samples.

In the present study we used fiber-optic microprobes with a tip diameter of 50 μm, but both smaller and larger tip diameters can be obtained easily by using different optical fibers and varying the tapering process. Smaller tip diameters should increase spatial resolution. With a fiber tip diameter of 10 μm fast signal changes were detected due to movement of filamentous cyanobacteria in a biofilm. Larger tip diameters allow integration of heterogeneity in pigment distribution, but this leads to greater disturbance of the sample. Thus, larger fiber diameters are useful mostly for monitoring fluorescence patterns at the surfaces of biofilms or sediments.

The new device described here is useful for a wide range of biological applications where fast fluorescence measurement at a high spatial resolution is needed. In addition to the distribution of photopigments, the distribution of other fluorescent dyes can also be mapped (e.g., in studies of mass transfer within biofilms or sediments). Other possible applications are to combine microscale fluorescence measurements with molecular techniques to map the distribution of specific fluorescently labeled microbes and to monitor expression of specific genes in genetically modified microbes (e.g., via green fluorescent proteins).

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