Use of a Microscope Photometer To Analyze In Vivo Fluorescence Intensity of Epilithic Microalgae Grown on Artificial Substrata

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An epifluorescence microscope photometer was used to develop a new, in vivo fluorimetric method for analyzing fluorescence intensities of epilithic microalgae grown on clay tiles in the field. This enabled a nondestructive, direct quantification of algal biomass on the substratum surface. Measurements of a chlorophyll *a* standard in ethanol (90%) with our fluorimetric method (excitation at 546 nm; emission, >590 nm) correlated well with those from conventional spectrofluorimetric and spectrophotometric methods. Biofilms were analyzed with the microscope photometer by measuring the in vivo fluorescence intensity of 70 spots distributed randomly over the tile surface. They were then analyzed by the two in vitro methods after photopigment extraction. Chlorophyll *a* content and in vivo fluorescence intensity correlated well. The regression curves were linear up to $6 \mu\text{g cm}^{-2}$ but were quadratic or hyperbolic at higher concentrations of up to $28 \mu\text{g cm}^{-2}$. The degree of scatter among individual measurements was higher in biofilms than chlorophyll *a* standards. This in vivo analysis is well suited to ecological experiments and has the advantage of measuring on an extremely small scale, which enables direct analysis of the microdistribution of epilithic microalgae in live biofilms. We demonstrated this by comparing fluorescence intensities of the grazing tracks of the snail *Ancylus fluviatilis* with those of ungrazed areas. Our in vivo analysis is also unique in enabling biofilms on artificial substrata to be removed, analyzed, and then returned intact in field or laboratory experiments.

In vitro estimates of algal biomass are based on quantification of chlorophyll *a* by photometric, fluorimetric, or high-performance liquid chromatography (HPLC) analyses. HPLC has the highest precision but is time consuming (20, 35, 43). Common to all these techniques is the need to extract photosynthetic pigments prior to their analysis.

Filtration provides a simple means of sampling and concentrating phytoplankton. By contrast, algae attached to coarse substrata first have to be removed before extraction. The efficiency of this removal step varies according to the procedure used (12).

Flow cytometry coupled with fluorimetry has been used for quantifying algal biomass in plankton studies (e.g., see references 3, 6, 11, 22, and 48), but this approach is generally not suitable for attached communities. The problems associated with current techniques available for nondestructive monitoring of microbial biofilms, which increase with the biofilms' complexity and variability, have been summarized by Nivens et al. (36). A recently published in vivo technique enables the growth of attached phototrophic microorganisms to be traced (24). This involves isolating cyanobacteria from microbial mats and measuring their fluorescence intensity while they are growing in petri dishes.

Here we report on the development of a new approach to the fluorimetric, in vivo quantification of photosynthetic pigments and, hence, algal biomass, in biofilms grown on artificial substrata in the field. The biofilms need not be removed from the substrata, which conserves their three-dimensional structure. Jones (21) reported a method for observing and counting

algal cells directly on the surface of natural substrata with an epifluorescence microscope. This had the advantage of avoiding losses due to removing and transferring the algal material. We developed this approach further and used an epifluorescence microscope equipped with a photometer to determine the in vivo fluorescence intensity of epilithic biofilms. These were grown on artificial substrata in the Breitenbach, a first-order stream in central Germany. We purposely did not use the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea to optimize and stabilize the fluorescence (41, 44, 46, 50), so as to leave the biofilms intact. This left us the option of conducting time series analyses on the biofilms, which could then be reinstalled in an ongoing experiment.

In developing this method, we addressed the following questions. How well do the data from our in vivo method correlate with data obtained from commonly used in vitro methods? Can our in vivo method be used in place of these in vitro methods? For which algal densities is our in vivo method suitable? Is this method suitable for detecting small-scale differences in algal densities, e.g., those resulting from grazing?

MATERIALS AND METHODS

Initially, the fluorescence intensity of pure chlorophyll *a* (extracted from *Anacystis nidulans*; Sigma) dissolved in ethanol was analyzed. This enabled an evaluation of the performance of the microscope photometer (MICP) as well as a comparison of these data with other, more conventional assays for chlorophyll *a* under well-defined conditions. Then we conducted comparative analyses on biofilms grown in the Breitenbach. First, the in vivo fluorescence intensity of the biofilms was measured with the MICP. The biofilms were then removed from the tiles, and the photopigments were extracted prior to in vitro analyses with a spectrofluorimeter (FLSP) and an absorption and spectrophotometer (ABSP). Biofilms taken from the Breitenbach were analyzed in two series in the spring and autumn of 1995. In both seasons, the biofilms were grown on smooth unglazed clay tiles (5 by 5 cm) incubated in the Breitenbach in batches of 12 for various lengths of time.

MICP. In vivo measurements were made with a fluorescence microscope (Axiophot; Zeiss) with attached photometer (MPM 200; Zeiss) and system processor (MSP 21; Zeiss). The use of epifluorescence enabled analyses of

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biofilms grown on nontransparent substrata such as clay tiles. The excitation wavelength was 546 nm, and the fluorescence intensity emitted at wavelengths greater than 590 nm was recorded (filter combination: BG 546, FT 580, LP 590). Each value reported for fluorescence is derived from several single measurements taken at intervals of 2.5 ms. Thus, the time required for obtaining one value depends on the number of single measurements made. In this study, a mean of 150 single measurements was chosen in order to minimize background noise. The total exposure time for each value was less than 1 s.

The area of biofilm analyzed in each measurement could be varied according to the choice of lens, optovar factor (an additional feature between the ocular and the objective of the Zeiss microscope for increasing the magnification by a factor of up to 2.5), and 10 different exposure apertures (diameters, 0.05 to 3.0 mm). A 10 $\times$ lens was used for all measurements, which maintained a large distance between the biofilms and the lens, while achieving a relatively large measurement area. This resulted in effective measurement areas of between 3.14 μm^2 and 11.31 mm^2 .

As the pure chlorophyll *a* standard was not always available, a plastic fluorescence standard (Plexiglas orange 478 [spectral range, 510 to 800 nm]; Zeiss) was used to calibrate the MICP. This fluorescence standard was also used to assess the correlation between measurement area and fluorescence intensity. The high correlation coefficient of this linear relationship ($r^2 = 0.99$) meant that a range of apertures could be used in the various series of measurements.

(i) Measurement of pure chlorophyll *a* with the MICP. The fluorescence intensity of pure chlorophyll *a* dissolved in ethanol (90%) was measured with a plankton counting chamber (diameter, 26 mm; height, 4 mm; volume, 2 ml). After calibrating the system with the Plexiglas standard and correcting for any background fluorescence, the chamber was filled with 1 ml of chlorophyll *a* solution to a depth of 2 mm. A small glass splinter was placed on the bottom of the chamber and used to standardize focusing. The microscope stage was moved at random to select a total of 10 positions per aperture for measuring fluorescence intensity. Measurements were expressed per unit area so as to be able to compare measurements of pure chlorophyll *a* with biofilm concentrations of chlorophyll *a*.

In an additional series of measurements, the amount of chlorophyll *a* in the chamber was increased incrementally from 0.4 to 2.0 ml. This tested the relationship between the volume of standard solution and fluorescence intensity, especially whether there was any shading effect as the depth of the standard solution increased (from 0.8 to 4.0 mm).

(ii) In vivo measurements of biofilms with the MICP. In addition to the system calibration, a correction was made for background fluorescence with a combusted tile. This had only a low level of fluorescence. Measurements were then made on experimental tiles colonized by biofilms in the Breitenbach. These tiles were placed on the microscope stage, and the microscope was focused on the biofilm surface in order to measure the fluorescence intensity. The biofilm surface was kept moist in order to prevent desiccation.

An important concern was the appropriate measuring strategy and the number of measurements necessary for obtaining a representative mean fluorescence intensity for the entire tile surface. We used a random nested analysis of variance (ANOVA) (47) to measure the fluorescence distribution of the biofilms on three randomly chosen tiles. Ten areas at the 0.3-cm scale were nested within three areas at the 0.5-cm scale, and in turn, these were nested in three areas at the 1-cm scale. Using ANOVA, we could not detect any significant difference at the 1- and 0.5-cm scales, and at least 99% of the total variation was due to variability at the 0.3-cm scale. Hence, a simpler method was used thereafter. For each single tile, we measured 70 individual spot values (each, 3.22 mm^2 ; total, 225 mm^2 , corresponding to 9% of the tile surface) distributed at random over the surface. Focusing was checked regularly. The median was used for correlations with ABSP and FLSP measurements.

As fluorescence and photochemistry are competing processes (8, 37, 44, 46), measurements were conducted in the dark in order to prevent photochemical and nonphotochemical quenching.

ABSP. Ethanol (90%) was chosen for extracting chlorophyll *a* because of its high efficiency (20, 38). Before removing biofilms from the tile surface, the edges and undersides of the tiles were carefully freed of biofilms with steel wool and a stream of water. The tiles were then placed in a polyvinyl chloride container (inner dimensions, 53 by 53 by 39 mm) and just covered with ethanol. This minimized the volume of the extraction medium. The biofilms were removed from the tiles with a dental ultrasonic probe (Piezon Master 400; Electro Medical System) (12), altered according to the method of Werneke (50a) by scraping with the metal tip of the handpiece and applying simultaneous ultrasonic vibration. The extraction mixture was then transferred to a graduated centrifuge tube (15 ml) and stored at 6°C for 17 to 24 h in order to promote chlorophyll extraction. This was followed by centrifugation at 2,700 $\times g$ for 15 min prior to spectrophotometric and fluorimetric assays. The efficiencies of chlorophyll *a* removal from tiles of differing surface roughness (unglazed clay tiles with smooth or rougher surfaces) were compared with the ultrasonic probe and by brushing the surfaces with a toothbrush.

Absorption was measured before and after acidification (10 μl of 0.6-mol liter^{-1} HCl, to correct for phaeophytin) at 665 nm with a Kontron Uvikon 810 spectrophotometer. Corrections for turbidity were made by taking additional measurements at 750 nm. Concentrations of chlorophyll *a* and phaeophytin were calculated according to the methods of Lorenzen (31) and Nusch (38), expressed

per 25 cm^2 of tile surface. Concentrations of the chlorophyll *a* standards were also determined photometrically in the same manner.

FLSP. The fluorescence intensity of the chlorophyll *a* standard and the algal extract from the first set of biofilm samples was also determined with a spectrofluorimeter (Kontron SFM 25). At an excitation wavelength of 546 nm, the maximum emission was determined by scanning between 650 and 700 nm. Chlorophyll *a* and the biofilm extracts had maximum emission intensities at 676 nm. However, at chlorophyll *a* concentrations above 10.3 $\mu\text{g liter}^{-1}$, this maximum shifted to a higher wavelength, which necessitated dilution of these samples. Correction for phaeophytin content by acidification was not possible in the in vivo analysis, so this step was also omitted in the fluorimetric analyses of biofilm extracts.

Exemplary application of the in vivo analysis. The potential of the in vivo analysis of photosynthetic pigments was explored with clay tiles which had been incubated for various lengths of time in the Breitenbach. Two individuals of the snail *Ancylus fluviatilis* (Gastropoda: Ancylidae), with shell lengths of 6 to 8 mm, were placed on each tile and left to graze upon these biofilms for 3 h. The fluorescence intensities of the biofilms in the grazing tracks and on the ungrazed areas next to these were then measured with 35-spot measurements. These data were compared with a Mann-Whitney U test. The total chlorophyll *a* content of the biofilms on the tiles was then determined with the spectrophotometric analysis (ABSP), followed by determination of the biofilm ash-free dry mass after drying and combustion of the biofilm extract (50a).

Statistical analysis. The random nested ANOVA was carried out with SAS software. Statistical tests of significance were conducted with SPSS software. Regression curves were calculated and plotted with SPSS and Sigmaplot graphics software.

RESULTS

Epilithic algae in the Breitenbach are dominated by diatoms, and cyanobacteria are more common than green algae (2, 4). Thus, an emission wavelength suitable for detecting the in vivo fluorescence of a mixture of diatoms and cyanobacteria was chosen. Cyanobacteria exhibit only a weak emission at 680 nm when excited at 440 nm (14, 23, 28, 30, 45). An excitation wavelength of 546 nm which yielded a substantial emission at 680 nm was used. In contrast to the Chlorophyceae, which are not prominent on Breitenbach substrata, diatoms also emit strongly at 680 nm when excited at 546 nm (19, 28).

Efficiency of removal of chlorophyll *a* from colonized surfaces. A comparative analysis showed that the ultrasonic probe was more effective at removing chlorophyll *a* from colonized surfaces than brushing alone. Eighteen unglazed clay tiles, nine each with smooth and rough surfaces, were colonized in the Breitenbach. First, the tiles were brushed intensively and rinsed with water. The brushed tiles were then treated with the ultrasonic probe, using only one extraction volume of ethanol for three of each type of tile in order to concentrate the chlorophyll *a* for the ABSP assay. Chlorophyll *a* concentrations on both types of tile ranged between 0.6 and 2.0 $\mu\text{g cm}^{-2}$. On the smooth tiles, $5.0\% \pm 0.9\%$ (mean $\pm$ standard deviation) of the chlorophyll *a* failed to be recovered by brushing, while $17.6\% \pm 1.2\%$ was not recovered from the rough tiles.

The efficiency of chlorophyll *a* removal with the ultrasonic probe was tested with sequential analyses on five smooth and five rough tiles. After the initial extraction, as described above, the tiles were then treated in a similar manner for a second time. In the second analysis, 1 extraction volume of ethanol was used for all five tiles of each type. A mean of 2% of chlorophyll *a* on the smooth tiles and 3.4% on the rough tiles was not recovered with a single extraction with the ultrasonic probe. The sum of chlorophyll *a* concentrations from both extractions ranged between 0.6 and 1.2 $\mu\text{g cm}^{-2}$ on smooth tiles and between 1.1 and 5.1 $\mu\text{g cm}^{-2}$ on rough tiles.

Influence of the solution depth on fluorescence intensity. Figure 1 shows the relationship between fluorescence intensity of the chlorophyll *a* standard (5.9 $\mu\text{g ml}^{-1}$) and the volume of standard in the measuring chamber, using a range of measuring apertures. Fluorescence intensity did not increase linearly with solution depth (volume), and the resultant curves corre-

FIG. 1. Correlation between the volume of chlorophyll *a* standard and its fluorescence intensity (median values, $n = 10$) measured at various apertures (AP).

sponded to a quadratic regression. As the microscope was always focused on the base of the chamber (glass splinter), only a proportion of the fluorescence intensity between the lens and the plane of focus was registered by the photometer.

Comparative analyses of chlorophyll *a* standards. Figure 2a shows the correlation between the concentration of chlorophyll *a* in ethanol, measured with the ABSP, and the corresponding fluorescence intensity measured with the MICP. There was only a low degree of scatter among the individual measurements. The relationship was linear up to a concentration of about $6 \mu\text{g cm}^{-2}$ but then levelled off, so that the entire curve corresponded to a quadratic regression. In contrast, the curve increased up to a concentration of only about $4 \mu\text{g cm}^{-2}$ when chlorophyll *a* was measured fluorimetrically. Again, this corresponded to a quadratic regression, with a high correlation coefficient and a low degree of scatter among the individual measurements (Fig. 2b). At higher concentrations, the fluorescence intensity was reduced markedly, and this was accompanied by a shift in the maximum emission wavelength (see above). Thus, these data were not included in the regression. While the fluorescence intensity of chlorophyll *a* at $7.7 \mu\text{g cm}^{-2}$ was 120%, this was reduced to 100% at a concentration of $15.5 \mu\text{g cm}^{-2}$. This shows that the FLSP method could only be used for chlorophyll *a* concentrations of up to $4 \mu\text{g cm}^{-2}$ and that more concentrated extracts need to be diluted. Up to this concentration, the fluorescence intensities measured by the MICP and FLSP methods correlated well (quadratic regression), with a high correlation coefficient and a low degree of scatter (Fig. 2c).

Comparative analyses of biofilms. The comparative analyses of chlorophyll *a* concentrations in biofilms from the spring, 1995, series, with concentrations of up to $6.3 \mu\text{g cm}^{-2}$, also correlated significantly with each other ($P < 0.001$). There was a higher degree of scatter in the correlation between chlorophyll *a* concentrations (ABSP) and the median in vivo fluorescence intensities of the biofilms (MICP) (Fig. 3a) than in the correlation between chlorophyll *a* concentrations (ABSP) and in vitro fluorescence intensities of the algal extracts (FLSP) (Fig. 3b). The in vivo and in vitro fluorescence intensities also correlated well (Fig. 3c), although there was a higher degree of scatter among individual measurements than was witnessed in the analyses of pure chlorophyll *a* (Fig. 2c).

When plotted against chlorophyll *a* concentration, the bio-

FIG. 2. Comparison of methods for analyzing chlorophyll *a* standards. Shown is the correlation between chlorophyll *a* concentration and fluorescence intensity measured with the MICP (a) or the FLSP (b). Also shown is the correlation between fluorescence intensities measured with the MICP and the FLSP (c). MICP data are median values, $n = 10$ measurements per tile, and all other data are single values from individual tiles.

films had a greater fluorescence intensity than the chlorophyll *a* standard but also a higher degree of scatter among the individual measurements (Fig. 4).

We investigated whether the in vivo analysis could also be used for thicker biofilms by analyzing a second set of biofilms from autumn, 1995, with chlorophyll *a* concentrations ranging between 0.2 and $28 \mu\text{g cm}^{-2}$ (measured by ABSP). Figure 5 shows the raw data fitted with the Michaelis-Menten equation along with the same data after linearization. There was a significant correlation ($P < 0.001$) between chlorophyll *a* concentration (ABSP) and the median, in vivo fluorescence intensity (MICP). This correlation continued beyond the range of concentrations in the biofilms from the first series, and these data can be used as a calibration curve for concentrations of up to $20 \mu\text{g cm}^{-2}$. However, there was a high degree of scatter in the data, caused in particular by the biofilms from six tiles at two sites exposed to currents of less than 12 cm s^{-1} . These biofilms

FIG. 3. Comparison of methods for analyzing natural biofilms. Shown is the correlation between chlorophyll *a* concentration and in vivo fluorescence intensity (a) and in vitro fluorescence intensity (b) of the respective biofilm extracts. Also shown is the correlation between the respective in vivo and in vitro fluorescence intensities (c). MICP data are median values, $n = 70$ measurements per tile, and all other data are single values from individual tiles.

had chlorophyll *a* concentrations of about $4 \mu\text{g cm}^{-2}$ but had markedly higher fluorescence intensities than comparable biofilms used in this analysis.

Comparative analysis of grazed and ungrazed biofilms. One useful application for the in vivo analysis of photosynthetic pigments lies in estimating the efficiency with which biofilms are removed by grazers such as the snail *A. fluviatilis* (Fig. 6). After grazing, chlorophyll *a* concentrations on the tiles ranged between 1.6 and $2.9 \mu\text{g cm}^{-2}$, and the ash-free dry mass ranged between 0.13 and 0.34 mg cm^{-2} . The difference between fluorescence intensities of the grazed and ungrazed biofilms was significant (U test, $P < 0.001$) (Fig. 7). The photosynthetic biomass of the biofilms was reduced significantly by the grazing action of the snails, irrespective of differences in the amount of material present at the start of the experiment.

DISCUSSION

Our data demonstrate the efficiency with which the ultrasonic probe removed chlorophyll *a* from colonized tile surfaces. Only 2% of the total chlorophyll *a* on smooth tiles and

3.4% of that on rough tiles were recovered in the repeat analysis. In contrast, brushing alone failed to recover 5% of chlorophyll *a* on smooth tiles and 17% on rough tiles. Gale (12) demonstrated the inefficiency of scraping and brushing for the removing of epilithic algae from granite stones. Up to 62% of algal cells were missed by scraping and brushing compared to the amount removed with the additional use of ultrasonic vibration.

In vivo fluorescence intensity of biofilms measured with the MICP generally correlated well with in vitro measurements of fluorescence intensity (FLSP) and chlorophyll *a* absorption (ABSP) of the corresponding biofilm extracts. However, the range of individual measurements was higher in the in vivo analysis than in the in vitro analyses of the biofilm extracts and the chlorophyll *a* standard. These observations concur with several studies on marine and freshwater phytoplankton, which have reported good correlations between in vivo fluorescence and chlorophyll *a* concentrations in field samples and laboratory cultures, and also with broad ranges of individual measurements (7, 10, 15, 26, 34, 39, 42, 46, 48, 50, 51, 52). This was because the fluorescence of living algal cells is influenced by numerous factors and is dependent directly on the physiological state of the photosynthetic system. Thus, photochemical and nonphotochemical quenching of chlorophyll *a* fluorescence is a direct consequence of photosynthetic control processes and adaptation to external factors (27). Apart from quenching, species specificity (15, 46, 48), nutrient availability (7, 26, 46, 52), and light intensity and quality (34, 39, 42, 48) can also have a marked influence on pigment composition and, hence, the fluorescence intensity. In addition, high densities of photopigment can lead to reabsorption and self-shading of the excitation energy (28, 33). The fluorescence intensity of the biofilm extracts also had a higher degree of scatter than values for the chlorophyll *a* standard, which is probably due in part to the lack of acidification and determination of phaeophytin in the in vivo analyses. A high degree of scatter has also been reported in other studies (13, 25, 35, 43).

Heaney (15) and Vyhnaek et al. (50) noted that the correlation between in vivo fluorescence intensity and chlorophyll *a* concentration in phytoplankton was poor when significant numbers of cyanobacteria were present. For this reason, we

FIG. 4. Comparison of the fluorescence intensities of chlorophyll *a* standards ($n = 10$ measurements per standard) and biofilm extracts ($n = 70$ measurements per tile) over a comparable range of chlorophyll *a* concentrations (single values from individual samples), measured with the MICP. Data are median values. CHL *a*, chlorophyll *a*.

FIG. 5. Correlation between chlorophyll *a* concentrations (single values from individual tiles) and the in vivo fluorescence intensity of biofilms (median values; $n = 70$ measurements per tile). (a) Data with a hyperbolic regression curve fitted with the Michaelis-Menten equation. (b) The same data presented as a Hanes-Woolf plot (chlorophyll *a*/in vivo fluorescence intensity [CHLa/IVFI] versus chlorophyll *a*), which linearized the data to yield a more suitable calibration curve. Dashed lines, 95% confidence intervals; dotted lines, 95% prediction intervals.

used an excitation wavelength of 546 nm, which does not yield the optimal fluorescence intensity for chlorophyll *a* but, in the presence of cyanobacteria, does yield a much more intense emission at 680 nm than when excited at 440 nm. In addition to the energy emitted from chlorophyll *a*, this in vivo analysis also measures the energy from accessory pigments (phycobilins) which is transferred to chlorophyll *a* (19, 34, 40, 42). With diatoms, energy absorbed by carotenoids, which themselves do not fluoresce, is also transferred to chlorophyll *a* and emitted. This might be one explanation for our observation that the in vivo fluorescence intensity of the biofilms was markedly higher than that of comparable concentrations of the chlorophyll *a* standard. Photochemical quenching occurs mainly at higher light intensities, on account of photochemical processes as well as fluorescence emission being competing processes (16, 44), but this was probably not a significant factor in our study because the analysis was conducted in the dark (15). Noack (37) and Kolber et al. (26) showed that 10 to 30 min of adaptation in the dark was sufficient for all quenchers to decay. A further explanation for the higher fluorescence intensity in the in vivo measurements may lie with our choice of cutoff filter

(LP 590), which resulted in the emission of all wavelengths greater than 590 nm being recorded. In addition to fluorescence from chlorophyll *a* (maximum at 676 nm), the fluorescence from other substances such as phycoerythrin (40), an accessory pigment in cyanobacteria, was also measured. Several authors have reported high background fluorescence due to unknown substances (10, 20, 50). It is also possible that other fluorescing substances such as humic acids (43, 49) might have been registered in our in vivo analyses.

In contrast to our results, Nusch (38) found that live phytoplankton had a 10-fold-lower fluorescence intensity than the corresponding cell extract. In our analysis, it is difficult to make a direct comparison between the fluorescence of pure chlorophyll *a* and the in vivo fluorescence of biofilms, because of the fundamental difference in approach. Pure chlorophyll *a* was dissolved in ethanol and measured in a 2-mm column in a plankton chamber, while the biofilm photopigments were concentrated directly on the tile surface in a layer of less than 2 mm. In both cases, the chlorophyll *a* concentration was measured photometrically (ABSP), but the proportion of the actual fluorescence registered when focusing on the base of the chamber or on the tile surface and the influence of the sample depth are not known. However, by varying the depth of the chlorophyll *a* standard, we do know that fluorescence intensity decreases at higher depths. Possible reasons for part of the intensity not being measured are shading effects and an increasing proportion of the liquid column lying beyond the plane of focus.

The linear correlation between chlorophyll *a* concentration (ABSP) and in vivo fluorescence intensity (MICP) up to around 6 µg cm⁻² shows that our in vivo analysis is most appropriate for analyzing thin biofilms. Thicker biofilms with higher densities of photopigments are more difficult to quantify with our technique, on account of the unavoidable shading effects and reabsorption of emitted light, as discussed. These impair measurements of fluorescence intensity, resulting in a quadratic or, at higher chlorophyll *a* concentrations, a hyperbolic relationship between chlorophyll *a* concentration and fluorescence intensity. For our purposes, this is not a problem, because the linear region, up to 6 µg cm⁻², is directly relevant for many stream studies. For example, chlorophyll *a* concentrations are usually below 5 µg cm⁻² on stones in the Breitenbach (5). Detailed studies on feeding areas of grazers in the Breitenbach have shown that chlorophyll *a* concentrations were usually far less than 6 µg cm⁻² in the presence of high grazer densities (2a). Marker (32) reported chlorophyll *a* concentrations of up to 5 µg cm⁻² in biofilms of soft-water streams. High grazing pressure due to trichopteran scrapers (9, 17, 18, 29) and mayflies (1) can reduce chlorophyll *a* concentrations to below 1 µg cm⁻². Hence, the in vivo analysis of biofilms is well suited to the substrata of smaller running waters sustaining high densities of grazers. When we tested the in vivo analysis on biofilms with chlorophyll *a* concentrations of up to 28 µg cm⁻², we found that this technique also yielded a useful analysis of these higher concentrations after the data had been linearized (Fig. 5).

A further difficulty with the in vivo measurement of fluorescence intensity in live biofilms is the large variability in the microdistribution of algal cells, arising from the three-dimensional structure of biofilms (see the results of the ANOVA and Fig. 7). The variance of the fluorescence intensity increased with biofilm thickness. This makes it more difficult to determine an exact mean value with our subsampling method (70 spot measurements per tile) than with the integrative photometric (ABSP) or fluorimetric analyses of biofilm extracts, which evaluate the absorption of fluorescence of an entire tile.

FIG. 6. Grazing tracks of *A. fluviatilis* on a clay tile. The darker areas are the ungrazed biofilms. Magnifications, $\times 8$ (a) and $\times 20$ (b).

Our in vivo method is therefore not suitable as a rapid method for analyzing the chlorophyll *a* content of larger areas.

Instead, one strength of our in vivo method is its ability to address numerous ecological questions by analyzing chlorophyll *a* concentrations on an extremely small scale with a high degree of resolution. We demonstrated this by comparing the

fluorescence intensity of a grazing track of *A. fluviatilis* with that of the surrounding, ungrazed area of biofilm. This enabled us to assess the grazing efficiency of this organism. There were significant differences between the grazed and ungrazed areas, irrespective of the original biofilm biomass. This small-scale evaluation of algal biomass is possible only with an in vivo

FIG. 7. Comparison of the in vivo fluorescence intensity of grazed (*A. fluviatilis*) and ungrazed biofilms. The black boxes are outliers.

method combining the use of a microscope and a photometer, since algae cannot be removed from substratum surfaces with this fine degree of resolution.

Our in vivo analysis of biofilm fluorescence has two further advantages. First, the algal biomass of natural biofilms can be determined directly on artificial hard substrata, enabling an evaluation of the microdistribution of phototrophic biofilm organisms. This avoids the need to remove the biofilms from their substrata, which always leads to a loss of material (see above). This problem is greatest with thin biofilms. Removal of the biofilms also renders an analysis of their three-dimensional structure impossible. Second, our in vivo analysis is unique in enabling biofilms to be evaluated in a nondestructive manner during the course of a field or laboratory experiment. The artificial substrata can be removed, the fluorescence intensity can be measured, and the substrata can then be returned to the experiment with the biofilms still intact. This enables densities of biofilm algae to be observed on the same substratum over a longer period of time, which can reduce variance in the data obtained. The nondestructive analysis also means that the substratum area needed for a given experiment can be reduced, thus, for example, enabling the use of smaller laboratory systems. In field experiments, this is an advantage, because there can be a high degree of variability in factors such as current and light intensity over a small area, particularly in small streams. Reducing the substratum requirement should mean a reduction in the variance of factors influencing the composition, development, and growth of biofilms, resulting in a more uniform colonization of the substrata.

Our new in vivo method is well suited to ecological experiments where the three-dimensional structure of biofilms must remain intact and where the emphasis is less on absolute values of chlorophyll *a* than on relative measures of substratum colonization in relation to factors such as grazing pressure, current, nutrient availability, and light conditions. Fluorescence intensity is influenced directly by the species composition of

algae in the biofilm, depending on the season and type of running water as well as the physiological status of the algae, as discussed above. These factors make it imperative that the in vivo analysis of fluorescence intensity in biofilms be calibrated with a conventional analysis of chlorophyll *a*.

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