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A photographic method for estimating chlorophyll in periphyton on artificial substrata

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Abstract

The collection of time-series of periphyton biomass is a difficult task due to the destructive nature of the standard methods. A non-destructive method based on photography and digitalization, for the estimation of Chla of periphyton colonizing artificial substrata is presented. The standard spectrophotometric method was used to obtain a calibration curve. The relative errors of the proposed method were similar to those of other published methods. The photographic method should be used when a large quantity of samples from the same community is needed and a high precision on the individual measurement is not required.

Introduction

The spectrophotometric methods to determine Chla as indicator of producer biomass are very useful techniques in limnological research (Wetzel, 1964). These methods have been widely used with different types of algae (phytoplankton, periphyton, marine or freshwater algae, etc.) and a variety of slightly different techniques were developed (Nusch, 1980). However, they do not allow the repeated measurement in time of the same sampling unit, because of their destructive nature. To follow the temporal dynamics of a periphyton community using these methods, the mean value of biomass must be estimated from a relatively large number of artificial or natural substrata samples at different times (Morin & Cattaneo, 1992). This requires the processing of a large amount of samples sometimes using complex or time consuming laboratory procedures. Additionally, this method of obtaining temporal series adds another source of variability. The stochastic nature of the colonization process can lead to differences in the species composition and in the quantity of algal cells on each sampling unit. As the colonization develops, minimal differences can be amplified becoming an important factor in the variability of periphyton samples (Lowe et al., 1996). Thus, a method that allows the repeated measurement in the same unit will reduce this source of variability and will increase the number of replicates used in the study.

A non-destructive photographic method to assess Chla was developed by Thieberger et al. (1995) for the diagnosis of stress in corals. This method is based on photography of a substratum colonized with periphyton and can be readily used for the acquisition of temporal series. The Chla is measured by analyzing a close-up slide with a spectrophotometer as it was an algal extract. The build-up of a special support for the slide inside the spectrophotometer is required, and each reading corresponds to a point on the slide. Obtaining an average measure of the entire slide requires the determination of a large sample points. So the method can be very time consuming and thus is inadequate for long time series where a large number of colonized samples must be analyzed.

Another non-destructive method that permits *in vivo* measurement of Chla uses an epifluorescence microscope equipped with a photometer (Becker et al., 1997). This combination allows the recording of flu-

orescence of the periphyton growing on natural substrata for Chla estimation. But as in the previous method, a large number of point measurements is necessary to obtain a good overall estimation.

The goal of the present study was to develop a nondestructive, fast, and cheap method for Chla estimation in periphyton. We calibrated and compared the precision and usefulness of this photographic method against the standard spectrophotometric determination.

Materials and methods

The periphyton colonization was carried out on squares of 5 × 5 × 0.1 cm made of high impact polystyrene. Every week, 10 squares were added to the bottom of a 60 × 60 cm glass aquarium for six weeks. This artificial substrate was convenient because its white, not glossy, colour provides good contrast photographs and the surface is not completely smooth. The experimental device was kept in a controlled environment with a light period of 12 h and a temperature of 20 °C. Initially, the aquarium was filled with water filtered through a 30 μM pore mesh. To compensate for evaporation and accelerate the colonization rate, twice a week, water with periphytic algae from *Egeria densa* was added. In the seventh week, all artificial substrates were removed for photographic recording and spectrophotometric Chla measurement. In this experimental setting each group of 10 squares could be exposed to different initial conditions because the external addition of algae and water change the aquarium environment over the weeks. This is not a problem for this study because we only need to obtain a series of different values of Chla to calibrate the method.

After the first experiment, a second colonization run was performed to obtain a wider range of Chla. Three squares were left in the aquarium and the same procedures followed in the previous colonization study were used. No new squares were added and all the squares were removed after 9 weeks. In this case each 5 × 5 cm square was cut in ca. 20 smaller pieces of 0.36 cm^2, allowing us to evaluate the performance of the method at two different scales.

With these two runs, of periphyton samples ranging from one to nine weeks of colonization were collected, encompassing low and progressively higher densities of algae respectively. This wide range of algal densities (biomass) was necessary to obtain a reliable calibration curve of the photographic method against the standard spectrophotometric one. Color slides (Fuji, ASA 100) of the colonized periphyton squares were taken using a CANON camera with a 50 mm lens and 1.5 cm extension tube to get a close-up shot. The photograph area was illuminated with two 500-Watts tungsten lamps of 3000 K color temperature. A metallic ruler was included in each image as a control surface and to make a more precise measurement of the colonized area.

After being photographed, each square was placed in methanol, which proved to be a better extraction solvent than acetone in freshwater samples (Sartory & Grobbelaar, 1984). The modified Lorenzen monochromatic and trichromatic methods were used to estimate Chla (Aminot, 1983).

The color slides were digitalized with a Kodak RFS 2035 film scanner with a 1000 dpi resolution. The scanning process is based in an array of thousands of electronic sensors that capture the light which pass through the slide. At each point (called pixels) a group of three sensors measure the light in a different range of wavelengths, corresponding to blue, green and red. The resulting images have three intensity values for each original point in the slide, corresponding to red, green and blue basic colors. The set of all blue intensity values is called the blue channel or the blue band, and the same applies to red and green.

The intensity values captured photographing and scanning depends on the reflectivity of the surface. For the periphytic algae, the reflectivity is mainly determined by the light absorption of pigments and the scattering produced by the algal cells. The absorption of the water film that covers the periphyton when the shot was done could be important too.

The mean intensity in the red, green and blue channels was recorded, using image analysis software, selecting only the colonized area of each image. At the same time, the program calculates the dimensions in pixels of colonized squares. The length in pixels of 5 cm was measured over the ruler for each photograph to convert the pixels to cm, for a more precise calculation of colonized area. We recorded the control values for each image selecting only the yellow portion of the ruler to calculate its mean intensity.

A correction factor (CF_i) was built dividing a randomly chosen slide (R) by the mean control intensity (M_i) of each square:

$$CF_i = \frac{R}{M_i}.$$

The mean intensity values (B_i) of the colonized area of each square were then corrected by multiplying it by the CF_i.

$$C_i = B_i \times CF_i,$$

C being the corrected mean intensity. The Chla values were used to calibrate the method against the C_i values obtained by photography:

$$Y = e^{(C_i - \alpha)/\beta},$$

where Y is the Chla value. The use of an exponential function is based in a highly simplified absorption model: the Lambert–Beer's law. To estimate the values of α and β, we performed non-linear regressions for each band and for both methods of Chla estimation. As a measure of goodness of fit for each case, we used the coefficient of determination (R^2). To evaluate the performance of the method, the weekly average and standard deviation were calculated for the estimated Chla using both methods. In order to compare the two scales used (25 cm^2 vs 0.36 cm^2), the relative error was estimated taking the square root of the residuals average and dividing it by the average Chla for each week.

Results

The R^2 values of the nonlinear regression between each band of the photographic method and both spectrophotometric methods (trichromatic and monochromatic) were similar (Figures 1, 2). The trichromatic ones were higher mainly in the blue channel, and so the calibration for this method was better (Figure 2c). This effect could be explained by the greater spread of the low intensity values in the monochromatic method (Figure 1). Thiebeger et al. (1995) performed a spectrophotometric analysis of slides with different algal colonization. They also found that a wavelength in the blue zone of the spectrum gives the best fit for the Chla estimation. In a study of the optical properties of high-density productive algal ponds Gitelson et al. (1995) also found that one of the most sensible spectral areas for Chla estimation was in the blue band. Additionally, we explored other basic combinations of the three bands, like the ratio of green to blue (Peñuelas et al., 1993) suggested by remote sensing studies in oceanic waters (Lawrence et al., 1994). None of these combinations gave better results than the blue band alone.

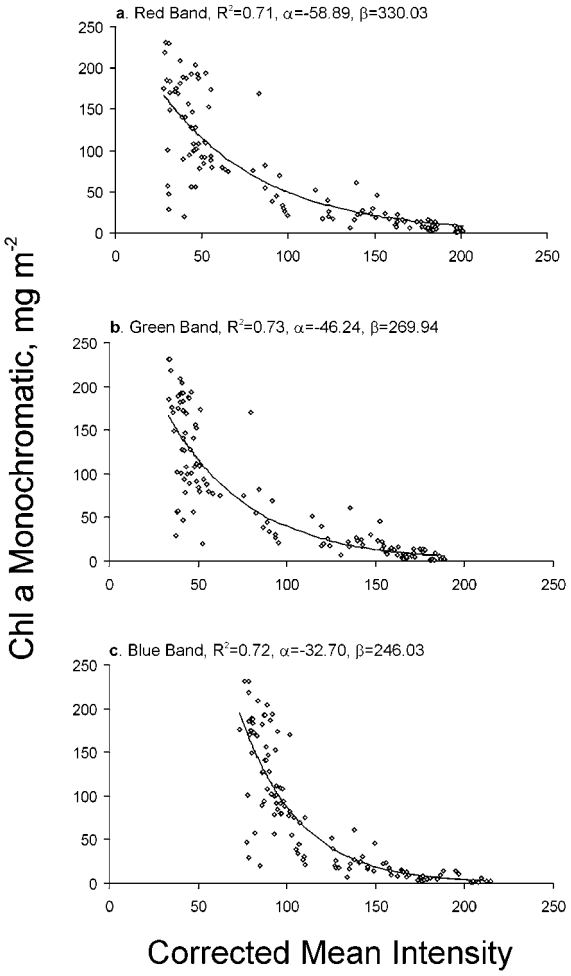

Figure 1. Chla estimated by the monochromatic method vs. corrected mean intensity for the three bands of the digitalized photographs. The line is the fitted equation, α and β are the parameters (see text). The number of points used was 116 in all the cases.

The biomass increase of periphytic community is shown in Figure 3. The predicted values of Chla, obtained from the calibration, were very similar to the spectrophotometric measurement and a lower standard deviation.

In Figure 4 the relative errors for both spectrophotometric methods are shown. Weeks one to six represent the bigger scale (25 cm^2) and week nine the smaller one (0.36 cm^2). The first week, the monochromatic method presented the highest relative error. In the rest of the weeks for the bigger scale the relative errors varied between 0.3 and 0.7, the errors of mono chromatic method tended to be higher. In the ninth week the relative error for the trichromatic method was lower than the lowest from the other scale (week 3).

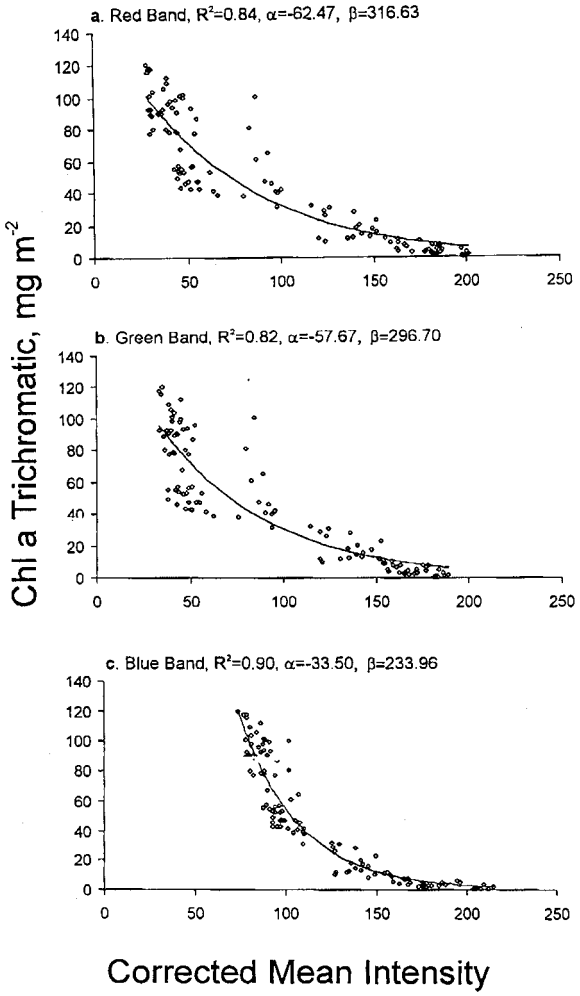

Figure 2. Chla estimated by the trichromatic method vs. corrected mean intensity for the three bands of the digitalized photographs. The line is the fitted equation, α and β are the parameters (see text). The number of points used were 116 in all the cases.

However, the monochromatic error was in the same range as that of the other scale. There are no important differences between these two scales so the method could be used at any of them.

Discussion

The total amount of artificial substrata available for colonization is often limited in periphyton experiments. The non-destructive nature of the photographic method permits a greater replication without increasing the surface devoted to colonization. The method demands the building up of a calibration curve for the particular community to be analyzed, this will reduce

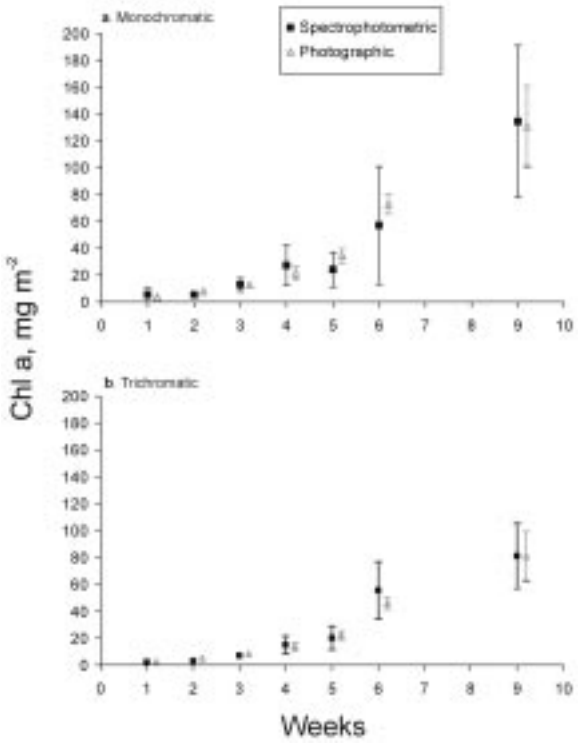

Figure 3. Chla vs. Time. The development of the colonization is showed. Squares: week averages of Chla values measured with the spectrophotometric method (mg m^{-2}). Triangles: week averages of the predicted Chla values using the photographic method. Bars represent standard deviation in both cases. (a) Results based on the modified Lorenzen spectrophotometric method. (b) Results based on the trichromatic method.

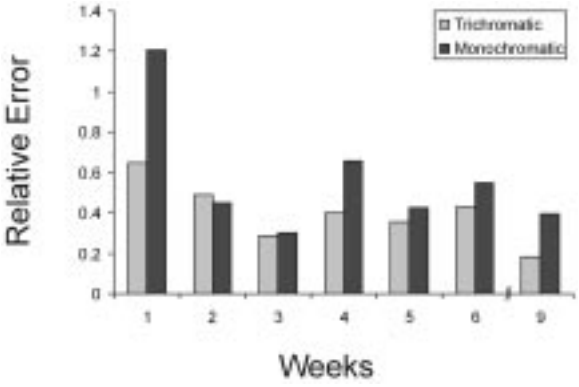

Figure 4. Relative error of the photographic estimation for both spectrophotometric methods. The relative error was calculated based on week averages of the residuals (RESS) obtained in the nonlinear regression. The square root of RESS was taken, and after that was divided by the week average of Chla to make it independent of the Chla level. Weeks one to six corresponds to estimations in areas of 25 cm^2 and week nine to areas of 0.36 cm^2.

the advantages of fast processing if a large number of measurements is not needed. Another drawback of the method is that for the individual estimations the relative errors are generally high (Figure 4), but the average Chla contents of the community can be predicted with relatively high precision (Figure 3). Thus, the photographic method allows for the repeated measurements on the same sampling unit, but the errors in the estimation prevent the possibility of obtaining more precise time series than with the spectrophotometric method.

The non-destructive methods developed for periphyton that we found in the literature have in common the necessity of a relatively large quantity of point measurements to obtain the Chla estimation of a given area. All the three methods require a calibration step to be used in a particular community. The photographic method can make areal Chla estimation in one step, and permits the study of the spatial pattern of the periphyton community with less effort. The relative errors of the other methods were not specified by the authors, but a rough estimation made using the figures of the published papers (Figure 3 A, B and C in Thieberger et al., 1995; Figure 5 a in Becker et al., 1997) showed that all the methods have similar error levels. The higher sensitivity of the equipment used in the other methods does not render better results. The fact that the three methods use the information carried by reflected light (re-emitted in the case of the fluorescence) from the periphyton, could explain this. Thus, the relatively low quality of this signal for Chla estimation preclude an accurate Chla estimation even with high precision equipment.

The method could be used with natural substrata with the same simple image procedures (Thieberger et al., 1995) or more advanced digital analysis can be applied (Hänninen et al., 1993). In both cases the substrata used must maintain a similar texture and color across sampling units and the calibration performed can only be applied with this particular substratum.

Thieberger et al.(1995) suggested that for highly colonized samples the illumination should be increased to improve the estimation. They also suggested another improvement that could render a more precise correction, taking into account the spatial heterogeneity of light in the photograph. They measured the intensity by mean of a two dimensional array of points over a photograph of a control surface. Then the respective correction is applied only to points with similar spatial coordinates.

The use of a digital camera could prevent all the problems related to differences between films and development procedures. Some new point and shoot digital cameras have a close-up capability and provide enough spatial resolution to be used with this method. Also, this kind of cameras can make the procedure even faster because they can be directly attached to a computer, making the process almost instantaneous if a calibration run has already been done.

The inclusion of a standard color control patch (Kodak Q-13 for example) in each photography should permit a better correction so that changes in the color balance, exposure or illumination can be controlled. This should allow to make the calibration in laboratory conditions, and take estimation shots in the field under a different light environment. Another improvement should be taking sub-aquatic photographs. In this way, almost all the disturbance produced when the substrata is taken away from its environment to be photographed should be removed.

The photographic method presented in this paper is non-destructive, faster, permits a greater replication, can be used at different scales and the relatives errors are not higher than the other similar methods found in the literature. The disadvantage of this method is the need of a calibration curve.

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