

Chlorophyll Fluorescence Imaging of Individual Algal Cells: Effects of Herbicide on *Spirogyra distenta* at Different Growth Stages

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Serious environmental degradation of aquatic ecosystems has been caused by eutrophication and by pollutants such as herbicides. Therefore, measurement of in situ algal photosynthetic activity is important for environmental monitoring. With ordinary nonimaging fluorometers, algal chlorophyll fluorescence can be measured easily, but heterogeneity within samples cannot be detected. Effects of a herbicide preparation containing 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on photosynthetic activity at different growth stages of *Spirogyra distenta* were investigated by using a computer-aided microscopic imaging system for chlorophyll *a* fluorescence. Photosystem II photochemical yield (ϕ_{PSII}) images were used to diagnose photosynthetic activity of spiral filate chloroplasts in algal cells. The herbicide treatment caused a stronger decline in ϕ_{PSII} values in younger than in mature algae cells. This result indicated that heterogeneity within algal samples should be considered when algae are used for environmental monitoring. Thus, measurement of chlorophyll fluorescence from young and mature chloroplasts with a microscopic imaging system makes it possible to improve the sensitivity for monitoring the environmental degradation of aquatic ecosystems.

Introduction

In recent years, serious environmental degradation of aquatic ecosystems in lakes, ponds, and rivers has been caused by human activities (1). Such ecosystems are greatly eutrophied by high rates of nitrogen and phosphorus release from agricultural fields (2). Furthermore, aquatic pollution arising from applications of agrochemicals to fields also severely affects aquatic ecosystems (3). Because green algae act as producers in aquatic ecosystems, the ecological balance is easily lost if algal photosynthesis is affected by environmental degradation. Hence, measurement of in situ algal photosynthetic activity is important for monitoring environmental degradation of aquatic ecosystems (4).

For nondestructive assessment of photosynthetic activity, chlorophyll *a* fluorescence measurement is very effective (5–8). Various instrumentations have been developed for analyzing chlorophyll fluorescence of aquatic plants and algae (9–11). Recently, for highly sensitive water toxicity biotests, a new type of dual-channel pulse-amplitude modulation (PAM) chlorophyll fluorometer has become available (10).

Many results pertaining to aquatic degradation caused by eutrophication and herbicides have been obtained with this type of instrument (1, 4, 12, 13). Haynes et al. (1) assessed the impact of diuron pollution on photosynthesis of three species of tropical seagrasses and pointed out that diuron is a potential risk to photosynthetic activity by seagrass in nearshore sediments. Frickelf and Senger (14) reported about changes in diuron sensibility during the life cycle of trichome. Schreiber et al. (12) showed that measurement of chlorophyll fluorescence in outdoor freshwater mesocosms was a reliable and effective method for monitoring the effects of atrazine pollution.

Nevertheless, the above-mentioned results were all achieved with nonimaging instruments; consequently, heterogeneity within samples was not taken into account. The application of imaging techniques makes the spatial analysis of chlorophyll fluorescence possible (15–20). Recently, microscopic fluorescence imaging systems have been developed for cellular-level, and even chloroplast-level, analyses (21–24). Oxborough and Baker (21) observed chlorophyll fluorescence images of individual stomata of intact leaves and calculated the photosystem II (PSII) photochemical yield, ϕ_{PSII} , which is a measure of linear electron transport activity through PSII. Additionally, Oxborough et al. (24) used microscopic fluorescence and ϕ_{PSII} images to examine individual and interspecific differences in photosynthetic activity of benthic phytoplankton, caused by an increase of light intensity. Confocal microscopic imaging of chlorophyll fluorescence has also been used to analyze the phylogeny of green algae and the origin of grana (25, 26).

Endo and Omasa (23) recently applied a microscopic fluorescence imaging technique to observe the effects of the herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on photosynthetic activity of mature algae. However, to date no one has investigated the effect of any herbicide on photosynthetic activities of algae at different growth stages. We report the effect of the herbicide containing DCMU as its main component on photosynthetic activity in individual algae at different growth stages. Microscopic imaging data of chlorophyll fluorescence were measured with our computer-aided microscopic imaging system, which is capable of resolving cell organelles. Inhibition of photosynthetic activities by the herbicide was compared in young and mature algae by calculating their ϕ_{PSII} image values.

Experimental Section

Culture Conditions and Herbicide Treatment. *Spirogyra distenta* (green microalga) was cultured at the agricultural field of the University of Tokyo. Algae were incubated in a test tube at 24 °C/22 °C (day/night) air temperature in a controlled environment incubator (Eyela, Eyclatron FLI-301N) under fluorescent lights with a photosynthetic photon flux (PPF) of 180 μmol of photons $\text{m}^{-2} \text{s}^{-1}$. The photoperiod was 12 h/12 h (day/night).

The herbicide preparation Nekosogi-ace (Rainbow Yaku-hin, Japan) was used in the experiment. Nekosogi-ace contains 6.0% DCMU, which inhibits photosynthetic electron transport from PSII to photosystem I (PSI) at the thylakoid membrane in the chloroplast.

Computer-Aided Microscopic Imaging System for Chlorophyll *a* Fluorescence. The computer-aided microscopic imaging system for analyzing chlorophyll *a* fluorescence (Figure 1) uses an optical microscope with a 20 \times objective lens (Mitutoyo, BD Plan Apo 20), which has a long working distance and a high numerical aperture. A halogen lamp (Nikon, PSM-1520) with a 620-nm cutoff filter (Corning, 4-96)

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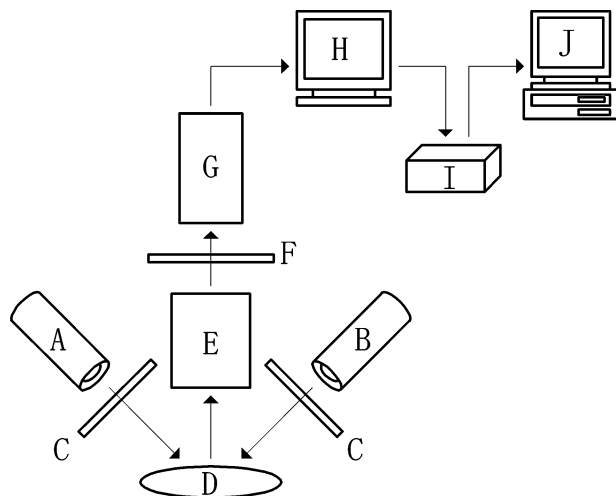


FIGURE 1. Computer-aided microscopic imaging system for chlorophyll *a* fluorescence: (A) actinic light source; (B) light source for saturation pulse; (C) short-pass filter ($\lambda \geq 620$ nm); (D) culture solution containing *Spirogyra distenta*; (E) objective lens (20 \times); (F) long-pass filter ($\lambda \geq 640$ nm) for imaging only chlorophyll fluorescence; the reflection image is taken by removing F filter; (G) cooled charge-coupled device (CCD) camera; (H) monitor; (I) digital video recorder; (J) computer.

and heat-absorbing filters provides $200 \mu\text{mol}$ of photons $\text{m}^{-2} \text{s}^{-1}$ actinic light. A metal halide lamp (Sumita, LS-M180), filtered as described above, provides a 2-s saturating light pulse of $2000 \mu\text{mol}$ of photons $\text{m}^{-2} \text{s}^{-1}$, which causes a transient saturation of PSII photochemistry. The light pulse is controlled by an electric shutter (EMS, shutter controller). Chlorophyll *a* fluorescence excited by these lights is imaged by a cooled charge-coupled device (CCD) camera (Hamamatsu Photonics, C5985-02) through an optical filter (Corning 2-64, wavelength $\lambda \geq 640$ nm) in the lens barrel of the microscope. Subsequently, fluorescence images are recorded by a digital video recorder (Sony, DSR-V10) and converted into 8-bit gray scale images with a resolution of 640×480 pixels. A fluorescent card that has a constant fluorescence yield at any light intensity was used for confirming the relationship between fluorescence intensity and the analog-to-digital conversion level of the obtained images. The result showed good linearity.

Analysis of Effects of the Herbicide on *Spirogyra distenta*.

A drop of culture solution containing *S. distenta* was placed in the hollow of a depression slide. Then the preparation was set on the microscope stage, where it was exposed to a PPF of $200 \mu\text{mol}$ of photons $\text{m}^{-2} \text{s}^{-1}$ actinic light. Air temperature was kept at 24°C . After 10 min of adaptation time on the microscope stage, a fluorescence intensity image *F* was measured under the actinic light. Just after that measurement, a fluorescence intensity image F_m' was measured during a 2-s saturation light pulse during steady-state photosynthesis. Each intensity image (*F* and F_m') was divided by the average value of fluorescence emitted from a fluorescent standard induced by the corresponding light intensity to convert it into a relative yield image. Finally, the ϕ_{PSII} was calculated (27, 28):

$$\Phi_{\text{PSII}} = \frac{\frac{F_m'}{R_{\text{SL}}} - \frac{F}{R_{\text{AL}}}}{\frac{F_m'}{R_{\text{SL}}}}$$

where R_{SL} and R_{AL} are values of fluorescence emitted by the fluorescent standard induced by the saturation light pulse and the actinic light, respectively.

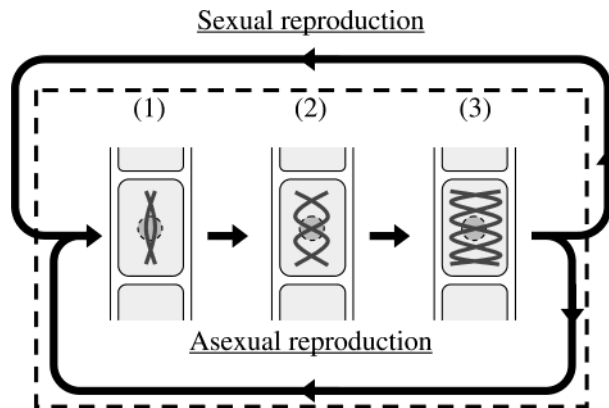


FIGURE 2. Schematic diagram of the life cycle of *Spirogyra distenta*. *S. distenta* has two reproductive modes: sexual, in which a diploid zygote divides by meiosis, and asexual, in which haploid cells divide by mitosis. In the asexual mode, two small chloroplasts located centrally extend to both ends of the cell, creating a double spiral shape (stages 1–3). Strands grow in length by somatic cell division and then break, thus forming more individuals.

Subsequently, the Nekosogi-ace solution was prepared and then a drop was added to the *S. distenta* solution on the preparation. The DCMU concentration in the final *S. distenta* solution was approximately 3×10^{-6} M. After a 15-min adaptation period, the measurement and analysis sequence described above was repeated.

Results and Discussion

A schematic diagram of the life cycle of an individual cell of *Spirogyra distenta* is shown in Figure 2. *S. distenta* has two reproductive modes: sexual, in which a diploid zygote divides by meiosis, and asexual, in which haploid cells divide by mitosis. Each haploid *S. distenta* cell has one centrally located nucleus and one to three spiral filate chloroplasts, and each cell is part of a continuous line of numerous cells. The spiral chloroplasts increase in length and density as they grow (Figure 2, stages 1–3). Reproduction in this species is usually asexual. Therefore, the effects of DCMU on *S. distenta* were examined at different growth stages of haploid cells.

Figure 3A shows a reflection image of two *S. distenta* filaments observed at a resolution of $0.5 \mu\text{m}$ under actinic light at $200 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}$ PPF. The spiral ribbonlike organelles are algal chloroplasts at two different haploid growth stages (see Figure 2). Other kinds of cell organelles without pigmentation could not be observed. The algae on the right side of Figure 3 are mature (stage 3 in Figure 2) and those on the left are younger (stage 1 in Figure 2), as determined from the shapes of their chloroplasts.

The distribution of ϕ_{PSII} values in control algal chloroplasts, before the herbicide treatment, was calculated from the F_m' and *F* images (Figure 3B). The microscope background, slide, and aquatic solution did not fluoresce in the experiment. The ϕ_{PSII} images contained heterogeneity caused by various factors. Defocus and nonuniform absorption of light caused by the spiral and uneven shapes of the chloroplasts in the F_m' and *F* images might lead to inaccurate values of ϕ_{PSII} at some sites. However, these sites were omitted by comparing the F_m' and *F* images with the reflection image shown in Figure 3A. An example of a possible physiological factor was an area with low ϕ_{PSII} values, indicated by the dotted arrow in Figure 3B, where the reflection image showed no difference. This phenomenon was temporary; it disappeared after less than a minute (data not shown).

Two areas (area a within a young chloroplast and area b within a mature chloroplast; Figure 3B) showed in-focus and uniform reflection (see Figure 3A). The ϕ_{PSII} values in those areas ranged from 0.39 to 0.46 in the mature alga (site b in

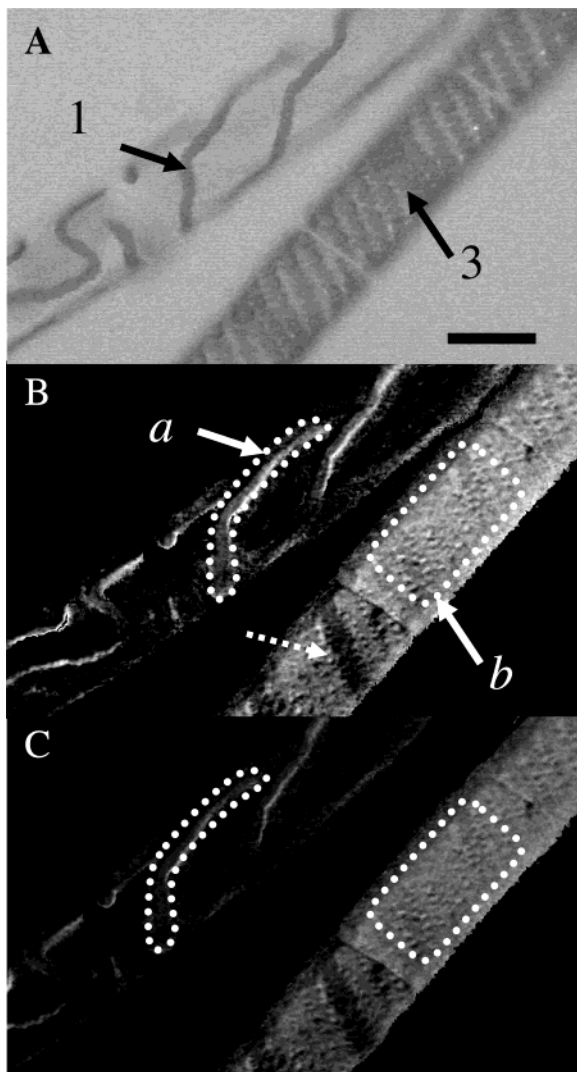


FIGURE 3. Microscopic reflection image (A) and ϕ_{PSII} images just before DCMU (3×10^{-6} M) treatment (B) and 15 min after treatment (C) of *Spirogyra distenta* at different growth stages. Haploid stages correspond to stages 1 and 3 in Figure 2. (a, b) Areas with in-focus and uniform reflectance. Images were taken at PPFs of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ actinic light and during a saturating light pulse of $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$. Scale bar = $50 \mu\text{m}$.

Figure 3B) and from 0.35 to 0.44 in the younger alga (site a in Figure 3B). The difference in ϕ_{PSII} values between chloroplasts of site a and those of site b was small. This result indicates that in the younger chloroplast the electron transport activity from PSII to PSI was already similar to that in the mature chloroplast under ordinary conditions. Moreover, that activity was nearly uniform within the algal chloroplast.

After a 15-min herbicide treatment of these algae (Figure 3C), the ϕ_{PSII} values decreased. A different response was observed, however, between mature and younger chloroplasts. The ϕ_{PSII} values decreased to 0.28–0.35 at sites in area b, in the mature alga, and to 0.13–0.23 at sites in area a, in the younger alga. Thus, after the treatment, the ϕ_{PSII} values of the younger chloroplast decreased more than those of the mature chloroplast. Incidentally, no treatment effect was observed in the reflection image.

Figure 4 shows the effects of the herbicide treatment on the ϕ_{PSII} values of chloroplasts in *S. distenta* cells at different growth stages (stages 1–3 in Figure 2). The ϕ_{PSII} values were

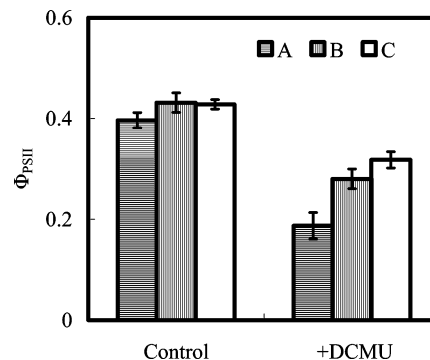


FIGURE 4. Effects of DCMU (3×10^{-6} M) treatment on ϕ_{PSII} values of *Spirogyra distenta* at different growth stages (stages 1–3 in Figure 2; see Figure 3). The ϕ_{PSII} values were obtained at sites with in-focus and uniform reflectance, such as a and b in Figure 3. Experimental conditions were the same as those in Figure 3.

obtained at sites with in-focus and uniform reflectance. Before the treatment, no difference in ϕ_{PSII} values was found among the three stages (control in Figure 4). However, differences in the amount of decrease of ϕ_{PSII} values were found between these stages after the herbicide treatment; in younger algae, the decrease was higher than in mature algae. This result indicates that the photosynthetic response of the algae to this herbicide depends on their growth stage. The difference in the effect may indicate differences in permeability of the cell wall or the cell membrane to the herbicide solution. It would be expected that younger chloroplasts would be more permeable and therefore would take up more DCMU. This scenario would result in a higher concentration of DCMU in younger algae cells than the mature algae and would cause a larger difference in ϕ_{PSII} values in the younger algae cells. Because the surface area and volume of chloroplasts differ at each stage, uptake of the herbicide into the chloroplast may also differ, leading to varying concentrations within the chloroplast. However, to determine the effect of the herbicide on photosynthetic PSII activity per unit chloroplast at different growth stages, an in-depth physiological analysis would be required.

Algal chlorophyll fluorescence can be measured easily with ordinary nonimaging fluorometers. However, photosynthetic heterogeneity within samples is not detectable by that technique. Our results indicated that young chloroplasts are more sensitive to a herbicide solution than mature chloroplasts, indicating that heterogeneity within algal samples should be taken into account when the algae are used for environmental monitoring. Thus, measurement of chlorophyll fluorescence from young chloroplasts with a microscopic imaging system makes it possible to improve sensitivity when monitoring environmental degradation of aquatic ecosystems.

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