

Variable Fluorescence and Fluorescence Spectra of Algae after Herbicide-Induced Pigment Bleaching

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Herbicides like norflurazon, oxadiazon, or oxyfluorfen affect the pigment apparatus of green algae. Their influence on variable ($F_{\max} - F_0$) and initial fluorescence (F_0) as well as on the state of chlorophylls were investigated. Two main modes of action of these herbicides on the photosynthetic apparatus during growth have been found:

First: *Scenedesmus* cells grown with norflurazon, a carotene-biosynthesis inhibitor, show chlorophyll bleaching concurrently with decrease or inactivation of photosystem-II reaction centers, with small changes in fluorescence spectra. Further, electron transport is blocked at the acceptor side of photosystem II. Disappearance or inactivation of photosystem-II reaction centers after a 50% loss of chlorophyll is accompanied by a sharp increase of the F_0 yield, with small changes in the chlorophyll state. The slow-decay phase in fluorescence induction of these cells, enhanced by dithionite, is ascribed to a reversible photobleaching of chlorophyll during the measurement.

Second: Oxadiazon and oxyfluorfen alter substantially the state of chlorophylls and cause strong bleaching, but reaction centers of photosystem II, although less than in control cells, are active even after an 80% disappearance of chlorophyll. The yields of F_0 and ($F_{\max} - F_0$) in treated cells are independent of the extent of chlorophyll bleaching. In contrast to norflurazon, the latter two herbicides do not inhibit carotenogenesis. Decrease of photosystem-II reaction centers may be due to non-specific pigment destruction (oxyfluorfen) or interference with chlorophyll biosynthesis.

Introduction

Certain 2-phenylpyridazinones like norflurazon* [1–3] or difunon [4] are powerful inhibitors of carotene biosynthesis. Lack of carotenes, in turn, induces photobleaching of chlorophylls [5, 6]. Furthermore, they cause inhibition of the electron-acceptor side of photosystem II [6]. *p*-Nitrodiphenyl ethers like oxyfluorfen also result in strong pigment bleaching, due to pigment breakdown showing up in the course of peroxidation of unsaturated fatty acids of thylakoid lipids [7, 8]. Again, photosynthetic electron transport is affected. The mode of action of a third herbicide class represented by oxadiazon is

unknown although – by our preliminary experiments – evidence was presented that it acts differently from the two classes indicated above [9].

This paper presents a comparison between three herbicides based on fluorescence induction at room and low temperature [6, 10, 11], thereby extending previous investigations [12]. Fluorescence spectra were taken as indicators of the state of chlorophyll in the pigment apparatus. It became evident that – although pigment bleaching can be achieved with all three herbicides mentioned – the changes with respect to photosystem-II reaction centers or fluorescence yield were different.

Materials and Methods

Scenedesmus acutus (276-3a, Algae Culture Collection, University of Göttingen, Germany) and *Bumilleriopsis filiformis* (stock at Konstanz) were grown autotrophically in 200-ml batches in a thermo-controlled water bath (built by Kniese-Edwards, Marburg, Germany) at 23 °C, and gassed with CO₂-enriched air (3–4% v/v); light intensity from fluorescent lamps was 18 W/m²; cell density at

* Chemical names of herbicides used herein: Norflurazon (SAN 9788), 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2H)-one. Difunon (EMD-IT 5914), 5-(dimethylaminomethylene)-2-oxo-4-phenyl-2,5-dihydrofurane-carbonitrile-(3). Oxyfluorfen (RH 2915), 2-chloro-4-trifluoromethylphenyl-3'-ethoxy-4'-nitrophenyl ether. Oxadiazon, 2-*tert*-butyl-4-(2,4-dichloro-5-isopropoxyphenyl)-1,3,4-oxadiazolin-5-one. DCMU (diuron), 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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start was 0.5 to 0.6 μl packed cell volume (= pcv) per ml culture suspension. Algae were taken from a continuously growing stock culture which had 4 to 6 μl pcv/ml suspension. The media are given in [13] for *Scenedesmus* and [14] for *Bumilleriopsis*.

The three herbicides used were present during growth in concentrations as indicated in Table I unless mentioned otherwise, to obtain loss of chlorophyll of about 30–40% after one day of growth and 60–90% two days. Intermediate chlorophyll levels (see e.g. Fig. 4) were obtained by extending or shortening the 1- or 2-day cultivation period.

Herbicides were added from a 10^{-1}M methanolic stock solution; the final methanol concentration was kept below 0.1% (v/v). When necessary, the suspension was diluted after harvest with nutrient medium to measure oxygen evolution, or with Tris-HCl buffer [tris-(hydroxymethyl)-aminomethane] (pH 7.5; 0.1 M final concentration) for fluorescence induction and yield of maximum fluorescence (F_{max}) in the presence of DCMU and sodium dithionite (see legend of Figure 1).

Fluorescence induction at room temperature (20 to 22 °C) was measured with a one-beam instrument. Exciting light from a He–Ne laser (632.8 nm; 30 W/m²) was guided through a light pipe to a 1-cm square cuvette; fluorescent light was collected by a second light guide mounted to the cuvette at a 90° angle. The signal was registered by a photomultiplier, amplified, and stored in a transient recorder (Datalab DL901). The time course of variable fluorescence was observed by an oscilloscope (Tektronix, mod. 5102 D10). An exciting-light shutter allowed to resolve kinetics down to 5 ms and to determine the yield of initial fluorescence (F_0). Fluorescence was measured above 715 nm using an RG715 cut-off glass filter (Schott, Mainz). – Chlorophyll concentration in the assays was adjusted to 1 to 2 $\mu\text{g}/\text{ml}$ or as indicated.

Since the ratio $(F_{\text{max}} - F_0) \times 1/F_0$ is a parameter of photosystem-II activity and the difference $F_{\text{max}} - F_0$ is indicative of active photosystem-II reaction centers, we tried to accurately determine the value of F_{max} by adding 0.1 mM DCMU and 10 mM dithionite to the cells, just before the measurement using a 2-s time scale. Smaller DCMU concentrations gave lower yields. Before the fluorescence-induction measurement, diluted cell suspensions were incubated in the dark for at least 5 min without additions.

Fluorescence induction and fluorescence spectra at low temperature (–196 °C) in the region of 650 to 800 nm were measured as described [15, 16]. A laser beam of 632.8 nm was used as actinic light. Absorption spectra in the region of 600 to 750 nm were recorded with a Shimadzu spectrophotometer, model 300. Oxygen evolution of suspension aliquots was traced by a Clark-type electrode [17] with saturating red light (> 610 nm) immediately after removal from the culture thermostat. Chlorophyll (Chl) concentration was determined after a 1-h extraction with 80% (v/v) methanol at 65 °C. Chlorophyll bleaching was referred to packed cell volume of cells grown with herbicides present [18]. All values of bleaching given in the table and figures are expressed as percent of the remaining chlorophyll vs. the untreated control, calculated as Chl/pcv.

Results and Discussions

Room-temperature fluorescence

The (absolute) amount of chlorophyll in the cultures increased with moderate concentrations of norflurazon present, remained about the same with oxadiazon, and showed a strong decrease with oxyfluorfen (Table I, col. 1). Some growth was still possible (col. 2). In all three cases, a strong bleaching was observed (col. 3) which had a different influence on fluorescence induction.

Oxyfluorfen and oxadiazon caused a decrease of variable fluorescence ($F_{\text{max}} - F_0$) and diminished the transient phenomena (Figure 1). Time courses were found to be similar with cells having lost 30 to 40% of their pigments (after a 1-day growth period) or 70 to 80% after 48 h of cultivation. Small transient phenomena were observed even after strong bleaching (Fig. 1, c–f), but the value of variable fluorescence was decreased. Because fluorescence yield of bleached cells was the same in the presence of DCMU (and DCMU plus dithionite), the small transient phenomena are due to decrease (or inactivation) of photosystem-II reaction centers. Oxyfluorfen had a stronger influence than oxadiazon. The initial fluorescence of cells grown with these two herbicides present did not change substantially by bleaching. The initial phase of fluorescence induction is demonstrated by a 100-ms time scale in Fig. 1, parts b, d, f.

Table I. Chlorophyll content, initial (F_0) and variable fluorescence ($F_{\max} - F_0$), and rate of oxygen evolution by *Scenedesmus* grown in the presence of herbicides.

| Growth conditions ^a | (1) $\frac{\mu\text{g Chl}}{\text{ml}}$ | (2) $\frac{\mu\text{l pcv}}{\text{ml}}$ | (3) $\frac{\text{mg Chl}}{\text{ml pcv}}$ | (4) F_0 | (5) $F_{\max} - F_0$ | (6) $\frac{F_{\max} - F_0}{F_0}$ | (7) $\frac{\mu\text{mol O}_2 \text{ evolved}}{\text{ml pcv}}$ |
|---------------------------------|--|--|--|--------------|-------------------------|-------------------------------------|--|
| A) 21-h growth period | | | | | | | |
| Control | 21.1 | 2.2 | 9.6 0 | 1.0 | 2.8 | 2.8 | 2280 (100%) |
| +Oxyfluorfen, 1 μM | 5.1 | 0.9 | 5.5 (43%) | 0.9 | 0.6 | 0.7 | 200 (9%) |
| +Norflurazon, 5 μM | 10.6 | 1.5 | 7.0 (27%) | 3.5 | 1.8 | 0.5 | 430 (19%) |
| +Oxadiazon, 3 μM | 6.5 | 1.1 | 6.0 (38%) | 0.8 | 0.8 | 1.0 | 900 (40%) |
| B) 44-h growth period | | | | | | | |
| Control | 67.8 | 5.9 | 11.5 0 | 0.9 | 2.1 | 2.3 | 2366 (100%) |
| +Oxyfluorfen, 0.5 μM | 1.5 | 1.1 | 1.4 (88%) | 0.8 | 0.2 | 0.2 | 144 (6%) |
| +Norflurazon, 1 μM | 9.5 | 2.4 | 3.9 (66%) | 7.2 | 0 | 0 | 150 (6%) |
| +Oxadiazon, 1 μM | 6.0 | 2.1 | 2.9 (75%) | 1.2 | 1.2 | 1.0 | 296 (13%) |

^a Algae were grown autotrophically in the presence of herbicides as indicated. The values for F_0 and $F_{\max} - F_0$ (cols. 4 to 6) were referred to 1 μg chlorophyll/ml assay. Chlorophyll concentration of the culture was 7.6 $\mu\text{g}/\text{ml}$ suspension at start. In cols. 1, 2 Chl and pcv is referred to ml algae culture. Loss of chlorophyll/packed cell volume (col. 3) or decrease of photosynthetic activity (col. 7), both in percent of control, is given in parentheses.

The bleaching induced by norflurazon resulted in a different response by changing both time course of variable fluorescence and yields of $F_{\max} - F_0$ as well as F_0 . In accordance with [12], the time course of treated cells is characterized by a rapid initial fluorescence increase followed by a slow decay phase. Furthermore, based on the same chlorophyll content, the fluorescence yield of norflurazon-treated

cells was much higher than that of the control (Table I, col. 5, 6).

The variable part of initial fluorescence still present in moderately bleached cells (Fig. 2b) indicates that photosystem II is active. However, the variable part was less than that of the control due to a decrease of (active) photosystem-II reaction centers and increase of F_0 . Addition of DCMU and

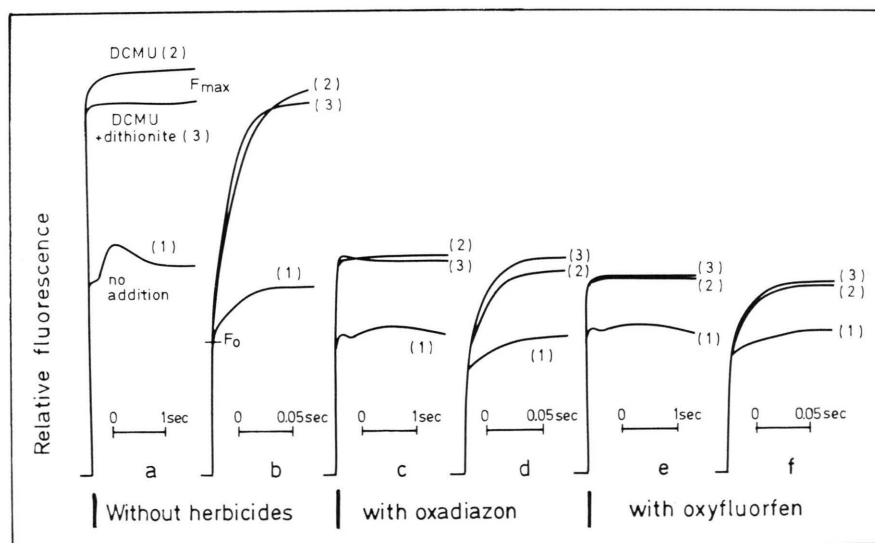


Fig. 1. Fluorescence induction in *Scenedesmus* cells grown for 42 h in the presence of 1.1 μM oxadiazon (c, d) or 0.5 μM oxyfluorfen (e, f). Traces a, b are controls; traces (1): without further additions. Traces (2) were obtained with 100 μM DCMU added to the fluorescence assay cuvette; traces (3) with 100 μM DCMU + 10 mM sodium dithionite. Time scale as indicated. Loss of chlorophyll (bleaching; see Methods) was 70% (c, d) and 80% (e, f) of control. Chlorophyll content of the cuvette for fluorescence measurement was 1.7 $\mu\text{g}/\text{ml}$ in (1), 1.4 and 1.2 $\mu\text{g}/\text{ml}$ in (2) and (3), respectively.

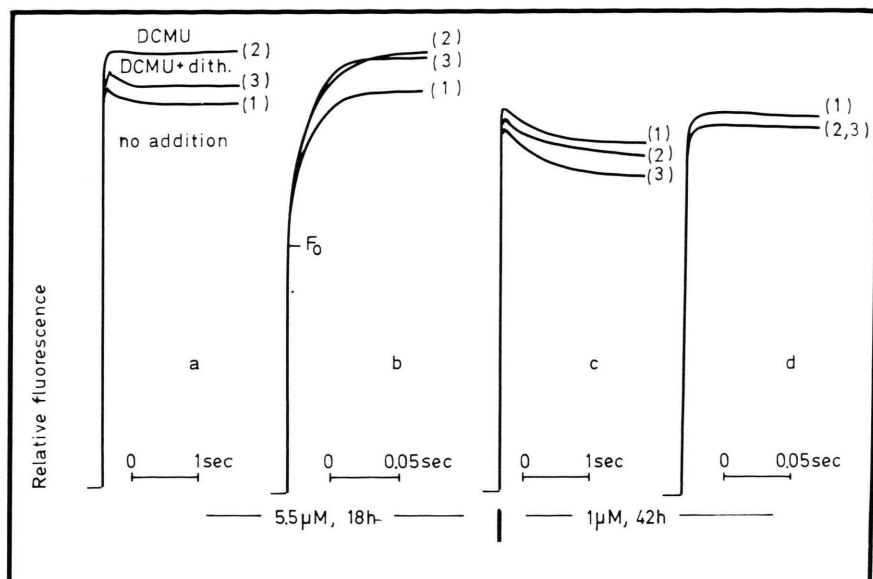


Fig. 2. Fluorescence induction in *Scenedesmus* cells grown in the presence of norflurazon for 18 h (a, b) or for 42 h (c, d) with the concentrations indicated. Additions were like in Fig. 1 and refer to the same numbers noted at the tracings. Chlorophyll bleaching was 33% (a, b) or 67% (c, d) vs. control [comp. legend of Figure 1]. Chlorophyll content of the assay: 1 $\mu\text{g}/\text{ml}$ (a, b); 0.6 $\mu\text{g}/\text{ml}$ (c, d).

dithionite induced a small increase of F_{max} . The high fluorescence yield apparently is due to lowered electron-transport capacity at the reducing side of photosystem II, which has to await a detailed investigation. DCMU partially inhibited the slow-decay phase, indicating that this phase is not strongly dependent on electron transport between the photosystems.

Extended norflurazon treatment of *Scenedesmus* (with a bleaching of more than 50% after a 2-day cultivation) resulted in a rapid initial phase followed by a more pronounced slow decay of fluorescence yield. This decay is not influenced by DCMU, but somewhat increased by dithionite (Fig. 2c), indicating that the decay is not due to oxidation of the primary acceptor Q. No transient phenomena were observed (Fig. 2d). This finding and the abolished oxygen evolution (Table I, part B, col. 7) suggest that strongly bleached cells have no active photosystem-II reaction centers.

The slow-decay phase accelerated by dithionite is due to reversible photobleaching of chlorophylls (photosystem-II antenna) as a result of photosystem-II inactivation, as was reported earlier for isolated chloroplasts of norflurazon-treated leaves

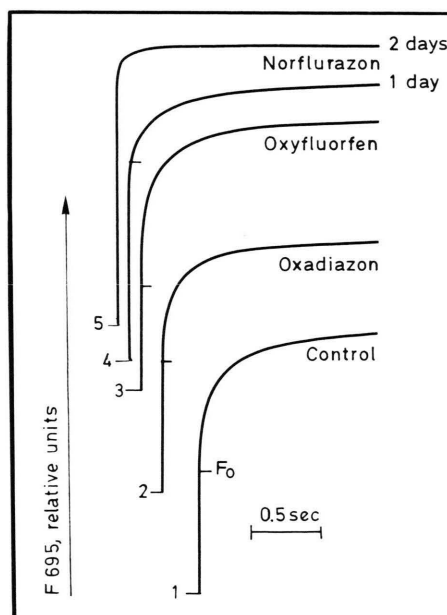


Fig. 3. Low-temperature fluorescence induction of *Scenedesmus* at 695 nm, grown for 2 days without herbicides present (trace 1) or with oxadiazon (63% bleaching, trace 2), oxyfluorfen (80% bleaching, trace 3) present. Traces 4, 5 represent norflurazon samples treated for 1 day (30% bleaching, 4) or for 2 days (60% bleaching, 5).

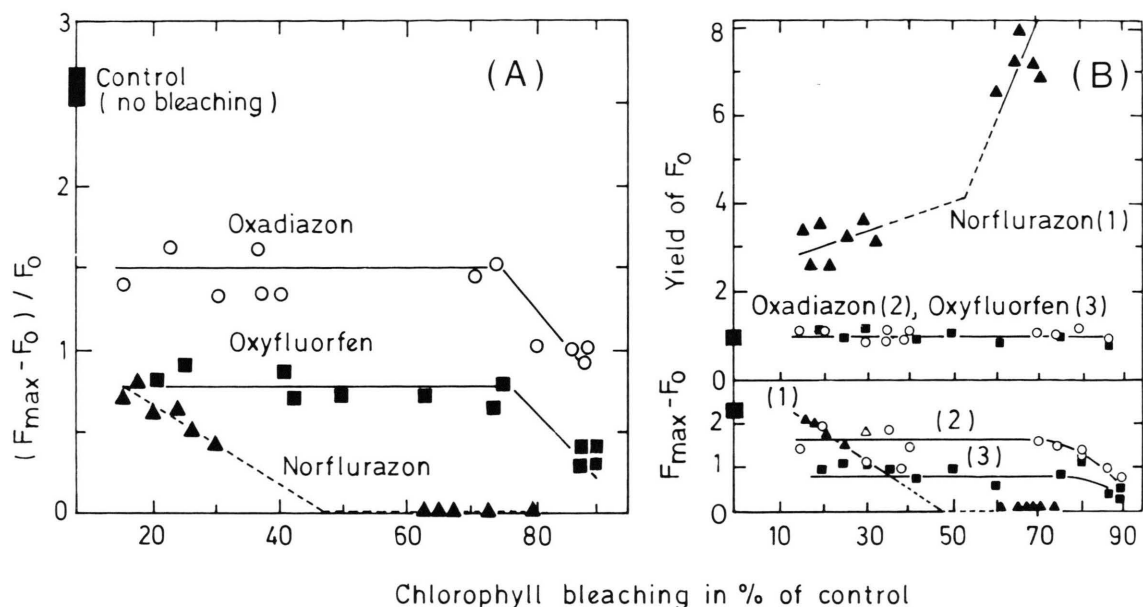


Fig. 4. Various degrees of bleaching induced by three herbicides are related to $(F_{\max} - F_0) \times 1/F_0$ (part A), F_0 yields (B, upper part) or $F_{\max} - F_0$, respectively (B, bottom). The filled squares on the ordinate indicate the figures for zero bleaching (*i.e.* the control). In part (B), all fluorescence values are calculated for 1 μg of chlorophyll per ml assay.

[6, 19]. — In a concentration range of 1 to 10 μM , the herbicides used in this study did not inhibit photosynthetic electron transport directly and immediately, neither that of *Scenedesmus* cells nor of isolated spinach chloroplast material (see [4] for methods).

Low-temperature fluorescence

The absence of photosystem-II reaction centers in strongly norflurazon-bleached *Scenedesmus* cells is corroborated by the absence of low-temperature fluorescence induction (Fig. 3, curve 5). In moderately bleached norflurazon-treated cells, however, fluorescence induction at -196°C was observed (Fig. 3, curve 4) indicative of active photosystem-II reaction centers still being present (comp. Table I, A, col. 7). The same holds for strongly bleached oxadiazon- and oxyfluorfen-treated samples (cells having lost 60 to 70% of their chlorophyll, curves 2, 3). Noteworthy, fluorescence induction of these oxadiazon-treated cells was similar to the control*,

that means reaction centers of photosystem II and their pigment environment have not been changed.

Photosystem-II reaction center activity; initial fluorescence

Data on fluorescence induction are summarized in Figure 4. As shown in part A, norflurazon-treated cells exhibited a decrease of photosystem-II reaction-center activity, *i.e.* the $(F_{\max} - F_0) \times 1/F_0$ ratio with increasing bleaching attained zero above 50% loss of chlorophyll per cell. For oxadiazon- or oxyfluorfen-bleached cells this ratio remained constant between 20 and 80% bleaching.

In Fig. 4B, $F_{\max} - F_0$ as well as F_0 are calculated on a chlorophyll basis and again plotted *vs.* degree of bleaching. As demonstrated by the bottom part levels of variable fluorescence ($F_{\max} - F_0$) of oxyfluorfen- or oxadiazon-treated cells are lowered *vs.* control, while those of norflurazon-bleached cells are similar to the control during the early stages of (moderate) bleaching. We take this as an evidence that norflurazon has no direct influence on photosystem-II reaction centers themselves, and that their decrease or inactivation, eventually observed after

* This observation also holds for fluorescence induction at room temperature (Dr. G. Sandmann, Konstanz 1981, unpubl.).

stronger bleaching, is an indirect result of inhibited carotenogenesis.

The mode of action of the other two herbicides on chlorophylls is not yet clear. They do not substantially affect carotenogenesis [9]. At start of the bleaching process, oxadiazon and particularly oxyfluorfen led to a decrease or inactivation of photosystem-II reaction centers. Nevertheless, the amount of (active) reaction centers, as well as their accompanying chlorophylls (as indicated by F_0 in the upper part of Fig. 4B) were found quite independent from various degrees of bleaching induced by these two herbicides. In norflurazon-bleached cells, however, the F_0 yield increased slowly with stronger bleaching. It rose sharply when bleaching exceeded a 50% chlorophyll loss, that is to say, after loss or inactivation of reaction centers had become complete.

Spectra. Additional information on the different modes of action of the three herbicides is contributed by absorption and fluorescence spectra. Weak chlorophyll bleaching decreased the 680-nm band with similar intensities at 680 and 670 nm. Strong bleaching led to an increase of the 670-nm absorption band (data not shown). Unfortunately, the distribution of chlorophylls between the antenna of the *Scenedesmus* photosystems is not known. However, spectral data clearly indicated differences showing up due to the action of the herbicides.

Low-temperature fluorescence spectra from *Scenedesmus* (though less informative than from chloroplasts of leaves because strong overlapping of fluorescence bands is evident) had a main band at 720 nm and two shoulders at 687 and 700 nm (Fig. 5, curve 1). Norflurazon-bleached cells (after a 1-day growth period, 30% bleaching) were similar to controls although variable fluorescence of these cells had decreased (curve 2, left). However, oxyfluorfen- and oxadiazon-treated cells with about the same bleaching degree as norflurazon-treated ones exhibited a new band at 685 to 687 nm and 700 to 705 nm (curves 3, 4, left part). After stronger bleaching (right part, curves 3, 4), the fluorescence bands became broader and a decrease at 687 nm was observed. In strongly bleached norflurazon cells, only a small change at 687 nm was found, with no effect in the main band (curve 2, right). Again, we interpret these findings as bleaching of the pigment apparatus by oxyfluorfen and oxadiazon, which is not accompanied by loss or inactivation of photo-

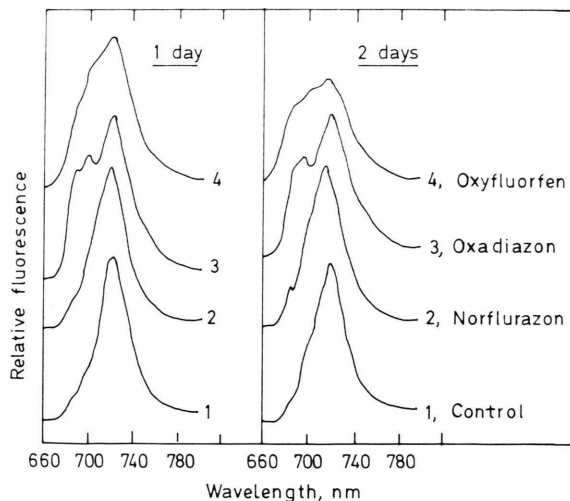


Fig. 5. Low-temperature fluorescence spectra of *Scenedesmus* cells grown for one (A) or two (B) days in the presence of three herbicides as indicated. Degrees of bleaching vs. control: left: 30% (2); 35% (3); 46% (4); right: 80% (2); 64% (3); 94% (4).

system-II reaction centers. In contrast, norflurazon-induced loss or inactivation of reaction centers had little effect on antenna chlorophylls.

Difunon gave results similar to norflurazon, but its bleaching activity was less powerful.

Bumilleriopsis (Xanthophyceae)

This alga has a different pigment inventory. Generally its sensitivity against the herbicides applied here was smaller. Again, norflurazon decreased the reaction centers proportional to bleaching, but even at higher bleaching (60%) photosystem-II reaction centers were still found active, F_0 yield was only slightly increased. With oxadiazon- and oxyfluorfen-treated cells no effect on reaction centers was observed even after a 60% decrease of the cellular chlorophyll content. No substantial changes of the shape and position of absorption bands were noticed after herbicide treatment, but a small change between the 677- and 625-nm absorption. More pronounced was the bleaching effect on the low-temperature fluorescence spectrum: With all herbicides assayed herein, the long-wavelength band at 707 nm was shifted to about 696 nm. A bleaching up to a 60% loss of chlorophyll had no substantial effect on photosynthesis. These comparative results indicate that the reactive part of the pigment apparatus of *Bumilleri-*

opsis apparently has a different susceptibility to herbicide attack as compared to that of *Scenedesmus*.

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