

# Influence of Bleaching Herbicides on Chlorophyll and Carotenoids

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Over 24 and 48 hour cultivation periods the influence of SAN 9789 (norflurazon), EMD-IT 5914 (difunon) and fluridone on growth, photosynthetic oxygen evolution and pigment content of the green alga *Scenedesmus acutus* was determined. Four effects were observed:

- Both carotenoid and chlorophyll formation were inhibited.
- Carotenoids were destroyed in the presence of air, but not nitrogen. The level of chlorophyll, however, did not change.
- $\beta$ - (and  $\alpha$ -)carotene was markedly decreased in the presence of oxygen.
- Photosynthetic oxygen evolution was decreased with the disappearance of carotenoids.

These effects, which are accompanied by reduced growth, are believed to represent primary herbicidal modes of action. The decrease of oxygen evolution is not due to a direct inhibition of photosynthetic electron transport by the herbicides applied.

## Introduction

Some pyridazinone derivatives like SAN 9789 or SAN 6706 are effective bleaching agents which interfere with the desaturation processes during biosynthesis of carotenoids. This has been shown with higher plants [1–4], algae [5, 6], and a mycobacterium [7]. Some of them also inhibit photosynthetic electron transport to some extent [8–10]. Under the influence of the bleaching pyridazinones colourless polyenes, namely phytoene and phytofluene [6, 11] are accumulated with a concurrent decrease of coloured carotenoids. However, a decrease of already synthesized pigments or a preferential destruction of *e. g.*  $\alpha$ , $\beta$ -carotene was not observed [12]. Simultaneously with the inhibition of carotenoid formation, chlorophyll content is lowered [1, 6, 10, 12]. This lowering is dependent on the light intensity used [11].

It is assumed that a decreased chlorophyll content of a tissue is a secondary process caused by photo-oxidation due to the missing protection by  $\beta$ -carotene [13, 14]. Further, some authors discuss the possibility that by reduction of the plastidic ribosomes [1, 4, 11] due to the presence of herbicides general metabolic changes may be caused which

eventually affect all constituents of the cell. Ribulose-1,5-biphosphate carboxylase and enzymes of leaf microbodies were found to be absent or present in very low amounts after treatment of seedlings with SAN 6706 or EMD-IT 5914 [15].

This paper reports four effects which are observed during the first hours after bleaching herbicides have been added to the green alga *Scenedesmus acutus* under different cultivation conditions. *Scenedesmus* has been found to be particularly useful for this line of herbicide experiments [16, 17]. Emphasis is put upon the correlation between chlorophyll and carotenoid content and on whether a decreased and changed inventory of carotenoids of the cell has an influence on light-induced destruction of chlorophyll under air or nitrogen.

## Materials and Methods

*Scenedesmus acutus* [= *Sc. obliquus* (Turp.) Kütz, strain 276-3 a of the Algae Culture Collection, University of Göttingen]. The unicellular green alga, was grown autotrophically in sterile liquid medium under continuous light [18] in a special growth apparatus of Kniese Comp. (Marburg, Germany). Cell density at start of the culture was 0.2  $\mu$ l packed cell volume (pcv) per ml cell suspension which was determined in graduated microcentrifuge tubes (80  $\mu$ l volume). Illumination was achieved by fluorescent light (2 tubes of Osram type 32/1 and

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Abbreviations: See legend of Fig. 1.

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one of type 25/1) with an intensity of 8000 lux (approx.  $30 \text{ W/m}^2$ ) at the surface of the culture vessels containing 250 ml medium. The pure herbicides were dissolved in methanol and added to the sterile medium, the final concentration of methanol kept below 0.1%. Chlorophyll content of the cells was determined by methanol extraction [19]. Total carotenoids were determined after the method of Goodwin [20]: 10 ml algae suspension was centrifuged, extracted with methanol including 1% NaOH for 30 min and then extracted with petroleum ether (b.p.  $40-60^\circ\text{C}$ ). The yellow extract was measured photometrically at 440 nm. An extinction coefficient of 2500 (1 cm light path) for a 1% carotenoid solution was used. Particular carotenoids or precursors were determined after a modified method of Hager and Meyer-Bertenrath [21]: 0.3 to 0.5 g of wet algal paste was homogenized in cold acetone for 1 min using 25 ml glass beads (0.5 mm diameter) and a homogenizer type Merkenschlager (Braun, Melsungen, Germany). The extract was separated with a glass filter funnel (G 1, Schott) and — after addition of 10 ml of water — extracted with petroleum ether (b.p.  $40-60^\circ\text{C}$ ) and dried over  $\text{Na}_2\text{SO}_4$  for 1 hour. Then, most of the petroleum ether was removed by a rotary evaporator and the remaining 2–3 ml of extract used for thin-layer chromatography according to [21], the concentration of the single carotenoids determined using the extinction coefficient given in ref. [22].

Photosynthetic activity of intact cells was determined in the culture medium using the Clark electrode.

## Results

Determination of the growth parameters of pigments in algae cells under the conditions applied included an average error of about  $\pm 15\%$ . Therefore, the data given in this paper are means of determinations of at least 3 consecutive growth periods. It should be noted that algae cultures have the advantage that the data can be referred to a constant culture volume and not only to cell mass like packed cell volume [17]. BAS 13033 and SAN 9774 with the  $10^{-6} \text{ M}$  concentration used exhibited little influence on the cells. Only the herbicides Nos. 3, 4, 5 of Fig. 1 exhibited a strong bleaching effect concomitant with a decrease of photosynthetic oxygen evolution. Cultivation of *Scenedesmus* in the

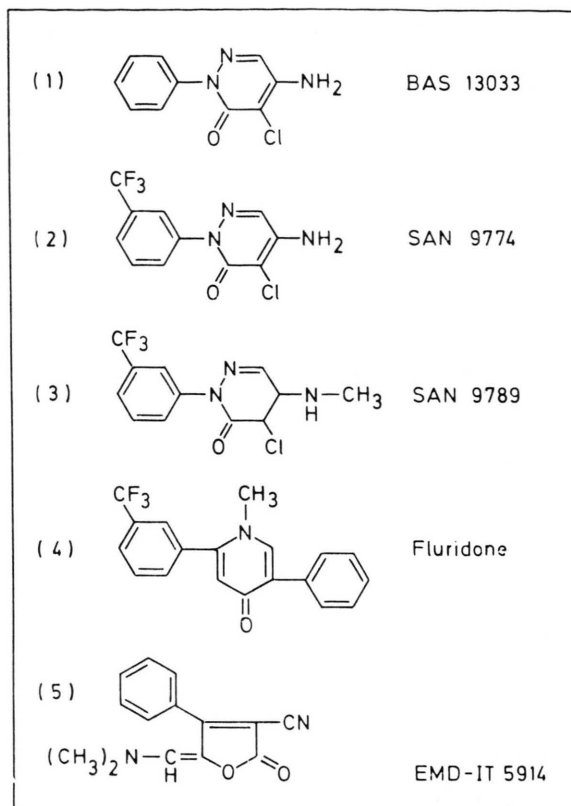


Fig. 1. Structures of herbicides assayed. (1) BAS 13033 4-chloro-5-amino-2-phenyl-pyridazin-3-(2H)-one [from BASF AG]. (2) SAN 9774: 4-chloro-5-amino-2-(3-trifluoromethylphenyl)pyridazin-3(2H)-one; [from BASF AG]. (3) SAN 9789 (norflurazone): 4-chloro-5-methylamino-2-(3-trifluorophenyl)pyridazin-3(2H)-one; [from Sandoz AG]. (4) Fluridone: 1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone; [from Elli Lilly Comp.]. (5) EMD-IT 5914 (difunon): 5-dimethylamino-methylene-2-oxo-4-phenyl-2,5-dihydrofuran-carbonitrile(3); [from Celamerck]. Diuron (DCMU) is N-(3,4-dichlorophenyl)-N',N'-dimethylurea [from Riedel de Haen].

presence of  $10^{-5} \text{ M}$  SAN 9789 for 24 h leads to a 50% decrease in growth rate as compared to the control. The content of chlorophyll is more affected; chlorophyll is only 12% of the control. Total carotenoid content and net photosynthetic oxygen evolution decrease 30–40% below the value at start of the culture, both parameters being apparently correlated. Further, an increased respiration is observed in norflurazon-treated cultures. The same effects are observed with fluridone and EMD-IT 5914. They all become stronger using a smaller inoculum and a 48 h cultivation period (Table I).

Table II demonstrates the carotenoid inventory of the cells in some detail. The absolute content of

Table I. Influence of herbicides on *Scenedesmus acutus*

Herbicide (1 $\mu\text{M}$ ) added	$\mu\text{l}$ packed cell volume (pcv)	$\mu\text{g}$ Chlorophyll	$\mu\text{g}$ Carotenoids	$\mu\text{mol}$ Oxygen evolution/h
(-) Herbicide	3.80 (4.18)	64.60 (63.4)	2.20 (2.86)	6.06 (4.88)
(+) BAS 13033	3.14	59.60	2.10	4.90
(+) SAN 9774	2.78	57.0	1.95	4.60
(+) SAN 9789	1.06 (2.99)	3.18 (23.4)	0.05 (0.70)	0.20 (1.20)
(+) Fluridone	0.80	4.20	0.02	0.27
(+) EMD-IT 5914	0.97	4.40	0.04	0.28

Cultures kept under air, measurements at harvest after 48 h and 24 h (these latter data in brackets). In the long-term experiment, the packed cell volume at start was 0.2  $\mu\text{l}$ , the pigment content 3.3  $\mu\text{g}$  of chlorophyll and 0.09  $\mu\text{g}$  of carotenoids; oxygen evolution 0.4  $\mu\text{mol/h}$ ; in the 24 h experiment herbicide concentration was 10  $\mu\text{M}$  and the cells were pre-cultured for 24 h. pvc at start was 1.65  $\mu\text{l}$ , chlorophyll 17.9  $\mu\text{g}$ , carotenoids 1.0  $\mu\text{g}$ , oxygen evolution 2.1  $\mu\text{mol/h}$ . All data refer to ml culture suspension. Details for measurement see Methods.

Table II. Influence of 10  $\mu\text{M}$  SAN 9789 on *Scenedesmus acutus*.

	Control at start	Control at harvest	Control +10 $\mu\text{M}$ SAN 9789 at harvest
under air + 4% $\text{CO}_2$			
(1) nmol $\alpha$ -carotene	0.18	0.75	0.02
(2) nmol $\beta$ -carotene	0.49	1.90	0.18
(3) nmol lutein + zeaxanthin	1.36	4.32	1.57
(4) nmol phytofluene	none	none	0.007
under nitrogen			
(5) nmol $\alpha$ -carotene	0.18	0.12	0.09
(6) nmol $\beta$ -carotene	0.49	0.53	0.39
(7) nmol lutein + zeaxanthin	1.36	1.36	1.42

Experiment conducted over 24 h under air or nitrogen in the light. Data refer to ml culture suspension. Details for measurement see Methods.

$\alpha$ - and  $\beta$ -carotenes in the algae culture strongly decreases under the influence of the herbicide (lines 1, 2). Concurrently, the xanthophylls lutein and zeaxanthin, increase *vs.* the content at the start of the experiment. Also neoxanthin exhibits a slight increase, whereas violaxanthin decreases by approx. 20% (data not shown). The precursors phytoene and phytofluene could barely be detected during the experimental time used here. When gassing the culture with nitrogen instead of an air/ $\text{CO}_2$  mixture, chlorophyll content with SAN 9789 present was found to be identical to the control culture, the total carotenoid content was only 10% less. Under these conditions, the carotenes decreased somewhat while no substantial increase in xanthophyll was measured. Referred to a molar basis, the ratio lutein

+ zeaxanthin/ $\alpha$  +  $\beta$ -carotene was found to be 7.9 under air *vs.* 2.9 under nitrogen. It should be noted that the disappearance of  $\alpha$ - and  $\beta$ -carotene occurs during the first 24 h after herbicide application, while the photooxidative decrease of xanthophylls continues for the next 24 and 48 h. This latter effect apparently is not correlated to the first one.

Fig. 2 A–D demonstrates in some detail the changes of packed cell volume, pigments and photosynthetic oxygen evolution of the *Scenedesmus* cul-

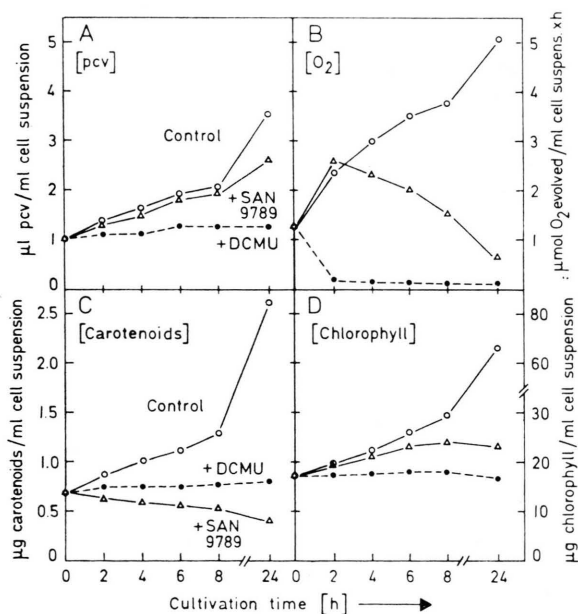


Fig. 2. A–D. Influence of  $10^{-5}$  M SAN 9789 ( $\Delta$ — $\Delta$ ) and DCMU ( $\square$ — $\square$ ) [control  $\circ$ — $\circ$ ] on packed cell volume (A), photosynthetic oxygen evolution (B), carotenoids (C) and chlorophyll content (D) of *Scenedesmus acutus* over 24 hours in the light. Details for measurements see Methods. The culture was precultivated for one day.

ture during the first 8 hours after addition of either SAN 9789 or diuron (DCMU). With the concentration used, both herbicides still allow some increase of packed cell volume (Fig. 2 A). Oxygen evolution in the presence of DCMU immediately decreases to 5–10% of the control and remains inhibited during the experimental time. In the culture treated with SAN 9789 the oxygen-evolving activity is the same as in the control for the first 2 h, but then decreases during the next 22 h to 40% of the control (Fig. 2 B, Table II). The total carotenoid content decreases by 30% during 24 h (Fig. 2 C, Table I). As shown by Fig. 2 C and D, a correlation between chlorophyll and carotenoid content is not observed in the cells treated with SAN 9789. Until 6 h after addition of the herbicide the chlorophyll content of the culture increases almost as much as that of the control and then remains approximately the same. A bleaching of either chlorophyll or carotenoids in the presence of DCMU is not observed. The carotenoid content apparently increases somewhat during the first 2 h, a small decrease of chlorophyll could be observed between the 8th and the 24th hour.

## Discussion

Bleaching may be expressed as an absolute decrease of pigments due to their herbicide-induced destruction and/or as an inhibition of pigment formation, leaving constant the absolute amount once formed in the culture. The first case is observed here with respect to carotenoid content, the second with chlorophylls. However, both effects are seen as a decrease of pigments when referred to packed cell volume, since growth, although lowered, leads to "dilution" of the pigments. It is the algae culture described here which advantageously allows the discrimination between these two effects. During the experimental time used in this study, the decrease of the carotenoid amount is not correlated with disappearance of chlorophyll. During preparation of this manuscript, the same finding in *Hordeum vulgare* was published by [23]. This is in contrast to the report with *Euglena* [12] where a direct correlation between carotenoid and chlorophyll was observed, and the authors postulate regulation of chlorophyll biosynthesis and membrane structure by carotenoids. In our experiments, the

decrease in carotenoids is accompanied by a disappearance of  $\alpha$ - and  $\beta$ -carotene which occurs under air in the light (Table II, [24]). Furthermore, there is a striking inhibition of photosynthetic oxygen evolution. It has to be shown whether there is a direct correlation to carotenoids or not.

We believe that the decrease in carotenoids, particularly that of  $\alpha$ - and  $\beta$ -carotene, and the strong inhibition of photosynthetic oxygen evolution are primary effects of bleaching herbicides such as SAN 9789, difunon and others. Apparently, their primary mode of action is not the inhibition of biosynthesis alone. The disappearance of carotenes, which are the natural quenchers of activated singlet oxygen [13] may allow oxidative processes that affect the membrane lipids which may have a direct bearing on photosynthetic electron transport. Galactolipid fatty acid composition can be changed by substituted pyridazinones [25]. We also have findings with paraquat (methylviologen) showing that a rather small destruction of chlorophylls is accompanied by a strong inactivation of the photosystem II region [26].

It still remains to be shown whether xanthophylls are able to take over the role as quenchers of activated singlet oxygen or how high the level of carotenes has to be to protect the chlorophylls against light-induced photooxidative destruction. Our data suggest that carotenoids themselves are destroyed by photooxidation. Such a photooxidation is possibly induced by the particular herbicides used here. Apparently, this process is different from the bleaching effect reported for DCMU [27, 28], since the curves of Fig. 2 C have a different course as compared to those with SAN 9789 (and EMD-IT 5914 [16] or fluridone, data not shown). Further, inhibition of electron transport within the cells in the presence of 10  $\mu$ M SAN 9789 is only about 10% vs. 90% inhibition by DCMU.

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