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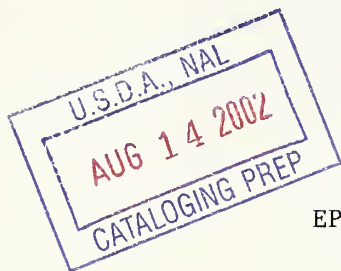
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FINAL REPORT

ULTRAVIOLET EFFECTS OF PHYSIOLOGICAL  
ACTIVITIES OF BLUE-GREEN ALGAE

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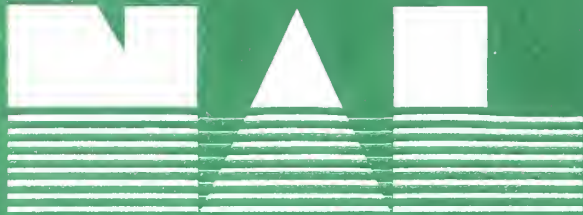
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## Introduction

The blue-green algae (Cyanobacteria) are found widespread in nature, in soil, water, and in association with a variety of plant and marine life (2). Various species can tolerate a variety of climatic conditions and are found even in hot springs and arctic regions. These cells lack differentiated chloroplasts and contain chlorophyll in membranous structures; consequently, they have recently been classified as blue-green bacteria, analogous to photosynthetic bacteria. The cyanobacteria carry out a typical plant-type photosynthesis, however, with water photolysis and oxygen evolution as major features. Consequently, these ubiquitous organisms constitute a particularly useful microbial system for monitoring worldwide environmental effects on plants as might result from enhanced solar UV-B (280-320 nm) irradiation due to depletion of stratospheric ozone (10).

We have evaluated both Anabaena flos-aquae and the water fern Azolla as laboratory test systems for environmental studies. Azolla is an aquatic nitrogen-fixing plant which contains a symbiotic cyanobacterium, Anabaena, within its leaf cavity (4). This fern is also found worldwide, but is particularly important for its use as a green manure in rice paddies in the Orient. Many species of cyanobacteria fix atmospheric nitrogen and contribute to nitrogen input into soils in a variety of ways. Both systems appear to be particularly important contributors of nitrogen to rice culture.



Our studies show that the nitrogen-fixing enzyme system in cyanobacteria is particularly sensitive to UV-B damage. Furthermore, inhibition of nitrogenase activity (measured as acetylene reduction) takes place in the absence of any nucleic acid damage or lethal effects on the cells. These studies indicate, therefore, that measurement of acetylene reduction activity in nitrogen-fixing systems may provide a simple biochemical assay for assessing the effects of UV-B on plants.

### Materials and Methods

Azolla caroliniana, a nitrogen-fixing water fern, was obtained from Dr. S. A. Peters, C. F. Kettering Foundation Laboratories, Yellow Springs, Ohio, and was grown on modified Hoaglands salts as described by Peters and Mayne (6). Anabaena flos-aquae (Lyngle.) Breb. ATCC 22664 was grown on nitrogen-free BG-11 medium (8). Cultures of plants and cyanobacteria were grown at 25°C in light chambers under cool white fluorescent lamps at light intensity of 10-20 watts/M<sup>2</sup>. Measurements of total light intensity were made with a Yellow Springs Instrument Co. (Yellow Springs, Ohio) model 65A Radiometer equipped with a 6551 Radiometer probe having a constant wavelength response from 0.28 to 2.6 microns (reduced to 65% at 0.21 microns).

UV-B irradiation of samples was obtained using a bank of six 8-watt RPR 3000 A Rayonet photochemical reactor lamps (Southern New England Ultraviolet Co., 954 Newfield St., Middletown, Conn.) placed above cyanobacterial and plant material at 25°C in flat dishes covered with 5 mil cellulose acetate films. The unfiltered RPR 3000A lamp has, in addition to UV-B, a strong emission in the short wavelength region ( $\lambda_{\max} \sim 254$  nm). Such lamps were used either singly or in multiples to increase irradiation.





(We are grateful to Drs. K. Eskins and H. J. Dutton of this Center for suggesting the use of these lamps as a source of UV-B radiation.) The lamps were aged 100 hours and did not significantly decrease in irradiance levels during prolonged use thereafter. As recommended by the Agricultural Equipment Laboratory of the Beltsville Agricultural Research Center (BARC), 5 or 10 mil cellulose acetate (CA) film was used to filter out low wavelength UV radiation from the lamps (5). The CA was pre-irradiated 6 hours and discarded after 30-40 hours of use. Since we have no knowledge of the actual targets involved, other than to exclude DNA, our data are reported as total incident UV-B light over the range indicated and does not assume any biological effectiveness of a particular wavelength.

UV-B irradiance levels in  $W/m^2$  were measured with an Optronics Laboratories, Inc. Model 725 UV-B Radiometer (7). We calibrated this instrument against a Rayonet lamp which had been scanned at distances of 13 and 20 cm (5 mil CA filter) with the Instrument Research Laboratory, BARC, spectroradiometer over the 250-400 nm region. Integrated  $W/m^2$  over the range of 280-320 nm at these distances were taken as reference points (0.44 and 0.82  $W/m^2$ , respectively) and linearly extrapolated to provide estimates of higher UV-B irradiances.

Cyanobacterial suspensions of 40 ml were stirred during irradiation. Aliquots were removed, rapidly agitated to separate clumped cells, plated on BG-11 (N free) medium, and assayed for nitrogenase, fixation of  $C^{14}O_2$  and hydrogen evolution. The data reported are typical examples selected from many experiments which all gave consistent results.



Acetylene reduction and hydrogen evolution were measured gas chromatographically on cyanobacterial and fern preparations incubated in light in screw-capped vials containing argon-acetylene or argon atmospheres. Samples of the gas phase were periodically withdrawn with gas-sampling syringes. The ethylene formed from acetylene was separated on columns of Poropak R (9) and hydrogen measured using a molecular sieve 5A column (1).

$C^{14}O_2$  fixation was measured on aliquots of either A. flos-aquae or fern fronds in growth media containing  $Na_2HC^{14}O_3$ . Samples were collected on glass fiber papers, rinsed with 6N HCl, and the incorporated  $C^{14}$  determined in a liquid scintillation counter using a water-miscible scintillation fluid.

Concentrations of A. flos-aquae in irradiated suspensions, determined by measurement of optical densities at 650 nm, were correlated with protein content (3). With our cultures, an optical density of 1.0 at 650 nm corresponded to approximately 200  $\mu$ grams algal protein per milliliter.

## Results

Because of their extensive pigment system, cyanobacteria are known to be fairly resistant to short wavelength UV irradiation and to possess an active photoreactivation system (11). In our early studies, we confirmed both of these effects and determined killing curves for our strains using an unfiltered Rayonet UV lamp (Figure 1). Comparison of

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Fig. 1

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killing curves obtained by plating cell aliquots on plates which were immediately incubated in the light with those allowed to incubate in the dark 24 hours before illumination showed an active photoreactivation of UV killing.

Figure 2 shows that when CA is used as a filter to remove short

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Fig. 2

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wavelength UV, the killing effect is virtually eliminated, even though the measured UV-B radiation intensity has now been increased fivefold to approximately  $2.1 \text{ W/m}^2$ . Note also that although the time scale has changed from minutes to hours of irradiation, no lethal effect can be observed.

We attempted to increase the UV-B irradiation by using a curved bank of six lamps with a reflector to impinge the light more directly on the reaction vessel. Figure 3 illustrates the results of such an

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Fig. 3

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experiment in which the UV-B intensity has been approximately doubled to  $5.2 \text{ W/m}^2$ . These data indicate some killing; however, there was only a slow decline in the population of viable cells which suggests that only a fraction of the cells may be sensitive to high intensity UV-B. It would be of interest to use this approach as a means of selecting strains with either enhanced resistance or sensitivity to UV-B.



Two biosynthetic activities of A. flos-aquae were examined after exposure to sub-lethal doses of UV-B: fixation of  $C^{14}O_2$  and nitrogen fixation (measured by acetylene reduction and hydrogen evolution).

Table 1 lists the effects of total UV irradiation and UV-B on acetylene

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Table 1

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reduction by Anabaena and indicates a decline in activity of algae irradiated with UV-B in the absence of a lethal effect. For physiological studies, concentrations of suspensions of A. flos-aquae were increased tenfold. Plate counts of these suspensions indicated that, over the range of 6-80  $\mu$ g protein/ml, identical survival curves were obtained allowing direct comparison of the results of viable cell count and physiological activity of the suspensions.

Data in Table 2 show that, under similar conditions of irradiation,

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Table 2

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effects of UV-B on  $CO_2$  fixation were slight. From these results, it appears that the nitrogenase system is a more specific and sensitive target for UV-B damage in A. flos-aquae.

Experiments were performed to gain some insight into the nature of the nitrogenase inhibition by UV-B. Since nitrogenase is a multienzyme complex which can be assayed for in a variety of ways, we have also measured the effect of UV-B on the ability of the complex to photoevolve





molecular hydrogen. As can be seen in Table 3, the effect of UV-B on

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Table 3

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nitrogenase is negligible when this assay is used. Apparently, the activity of nitrogenase measured specifically by the acetylene reduction assay is the most sensitive indicator of UV-B damage.

Visible photobleaching/suspensions of occurred after 6 hours irradiation with UV-B. However, no destruction of a specific pigment could be detected by examination of difference spectra of acetone extracts from irradiated and unirradiated cells.

Discussion

From a practical standpoint, it is obvious that assessment of the environmental effects of enhanced UV-B irradiation on biological material is going to require development of simple assay procedures with wide applicability. Our studies have consistently revealed a surprising sensitivity of the nitrogenase complex to UV-B irradiation. The UV-B irradiation level (ca.  $3 \text{ W/m}^2$ ), which we find inhibitory to nitrogenase, is approximately the same as that of noon sunlight in the 280-330 nm region. The main drawback to this approach to this means of assessment of environmental damage is that it requires the use of those limited systems which possess nitrogenase activity.

It should be emphasized that, by performing direct microbiological plate counts on a large population of irradiated cells, we have ruled out the possibility that the UV-B effect observed on nitrogenase is due to nucleic acid damage. This finding suggests that the cellular target



may be another pigment associated with the nitrogenase complex or its electron transport system. Further studies on the action spectrum of this effect may help to reveal the cellular component involved as UV-B receptor.

The Azolla system provides an opportunity to examine the effect of UV-B on a plant and, simultaneously, its symbiont. Since nitrogenase activity (acetylene reduction) is exclusively a property of the symbiont, this specific physiological activity can be measured after irradiation of the fern. Measurement of fixation of  $C^{14}O_2$  by the symbiosis serves as a general index of the physiological activity of the system. Data in Table 4 summarize such an experiment, in which  $C^{14}O_2$  fixation and

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Table 4

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acetylene reduction are measured in UV-B-irradiated plants. Although there was a slow decline in general physiological activity of the plants as the culture aged, the nitrogenase activity of irradiated plants showed a significant decrease over control plants.

Information now available (12) on the effects of short wavelength UV irradiation on biological material has come virtually exclusively from studies of microorganisms. It seems likely, therefore, that microorganisms may again prove to be the material of choice to study biological UV-B effects. Nitrogen fixation consumes a substantial fraction of the energy of a cell in which it occurs; consequently, it is possible that a minor physiological disturbance would be expressed more readily in such a system. Furthermore, this assay (acetylene reduction) is readily adaptable to field studies and could serve as a convenient assay for a variety of environmental studies.



There seems little doubt that the green and blue-green algae will be organism of choice to study large populations of plant material under controlled conditions. Furthermore, since algal nitrogen fixation is confined to blue-green algae (cyanobacteria), we seem to have selected an ideal class of microorganism for evaluation of UV-B effects on plant material. Worldwide distribution of these organisms suggests that they might, in this way, serve as a convenient indicator of the extent of stratospheric ozone depletion.

### Abstract

The effect of UV-B (280-320 nm) irradiation on physiological activities of Anabaena flos-aquae and the water fern Azolla caroliniana has been studied where lethal effects of irradiation are known to be absent. Nitrogenase activity specifically declined at low levels of UV-B, under conditions which had little effect on general physiological activity of the irradiated cells. These findings indicate that measurement of acetylene reduction (nitrogenase assay) may serve as a simple biochemical assay to assess environmental UV-B damage to plants due to depletions of stratospheric ozone.



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Table 1  
Effect of UV-B on nitrogenase activity  
of A. flos-aquae

Irradiation time <sup>a</sup>	Acetylene reduction <sup>b</sup>		
	Control	UV-B	UV <sup>c</sup>
h	nmol/h/mg protein		
0	1,490	1,490	945
0.5	---	---	370
1	---	---	105
2	---	---	10
3	1,300	840	---
6	1,350	340	---

<sup>a</sup>UV-B, 2.1 W/m<sup>2</sup>, cell suspension, 40 ml; protein 65 µg/ml.

<sup>b</sup>Aliquots, 5 ml of suspensions incubated in light in atmosphere of argon-90%, acetylene 10% for assay.

<sup>c</sup>Rayonet lamps without cellulose acetate filter, 10 W/m<sup>2</sup> separate experiment, 40 µg/ml algal protein.



Table 2  
 Effect of UV-B on fixation of  $^{14}\text{CO}_2$  by  
A. flos-aquae<sup>a</sup>

Irradiation time <sup>a</sup> h	$^{14}\text{CO}_2$ fixed		
	Control	UV-B	UV <sup>b</sup>
	cpm/mg protein/min in light		
0	9,300	9,200	8,800
2	9,800	7,400	70
4	9,200	5,700	
6	7,800	5,500	

<sup>a</sup>Cell suspension, 37 ml; protein, 50  $\mu\text{g}/\text{ml}$ .

UV-B, 2.1  $\text{W}/\text{m}^2$ .

<sup>b</sup>Rayonet lamps without cellulose acetate filter,  
 10  $\text{W}/\text{m}^2$ .



Table 3

Effect of UV-B on photoevolution of H<sub>2</sub> byA. flos-aquae

Irradiation time <sup>a</sup> h	H <sub>2</sub> evolution <sup>b</sup>	
	Control	UV-B
	nmol/h/mg protein	
0	460	460
3	350	343
6	265	215

<sup>a</sup>Cell suspensions, 40 ml, 80 µg protein/ml, exposed to 2.1 W/m<sup>2</sup> UV-B.

<sup>b</sup>Aliquots, 5 ml, of suspension incubated anaerobically (argon atm.); 30 W/m<sup>2</sup> white light for assay.





Table 4

Effect of enhanced irradiation with UV-B on  $^{14}\text{CO}_2$  fixation  
and acetylene reduction by Azolla

Irradiation time <sup>a</sup> days	Control		UV-B enhanced	
	$^{14}\text{CO}_2$ fixed <sup>b</sup>	Acetylene reduced <sup>c</sup>	$^{14}\text{CO}_2$ fixed <sup>b</sup>	Acetylene reduced <sup>c</sup>
1	24,000	450	20,200	300
2	17,800	380	15,200	100
4	7,200	320	5,900	130
6	4,350	350	4,700	100

<sup>a</sup>Visible light,  $10 \text{ W/m}^2$ , supplemented with UV-B,  $2 \text{ W/m}^2$ .

<sup>b</sup>cpm/g plants (wet)/min in visible light,  $30 \text{ W/m}^2$ .

<sup>c</sup>nmol/g plants (wet)/h; argon atm., visible light,  $30 \text{ W/m}^2$ .



### Figure Legends

Fig. 1. UV killing and photoreactivation of A. flos-aquae. Single, unfiltered, 8W Rayonet lamps, 15 cm from surface of stirred cell suspension. Algal protein, 6  $\mu\text{g/ml}$ ; total light,  $2.7 \text{ W/m}^2$ .

Fig. 2. UV-B irradiation of A. flos-aquae. Six Rayonet lamps in flat bank array held 17 cm from surface of stirred cell suspension (40 ml, 6  $\mu\text{g protein/ml}$ ). Total light,  $5 \text{ W/m}^2$ ; UV-B,  $2.1 \text{ W/m}^2$ . Cellulose acetate filter (CA), 10 mil.

Fig. 3. Effect of higher UV-B intensity on A. flos-aquae. Six Rayonet lamps in curved reflector fixture held 17 cm from surface of stirred cell suspension (40 ml, 7.6  $\mu\text{g protein/ml}$ ). Total light,  $12.5 \text{ W/m}^2$ ; UV-B,  $5.2 \text{ W/m}^2$ . Cellulose acetate filter, 10 mil.



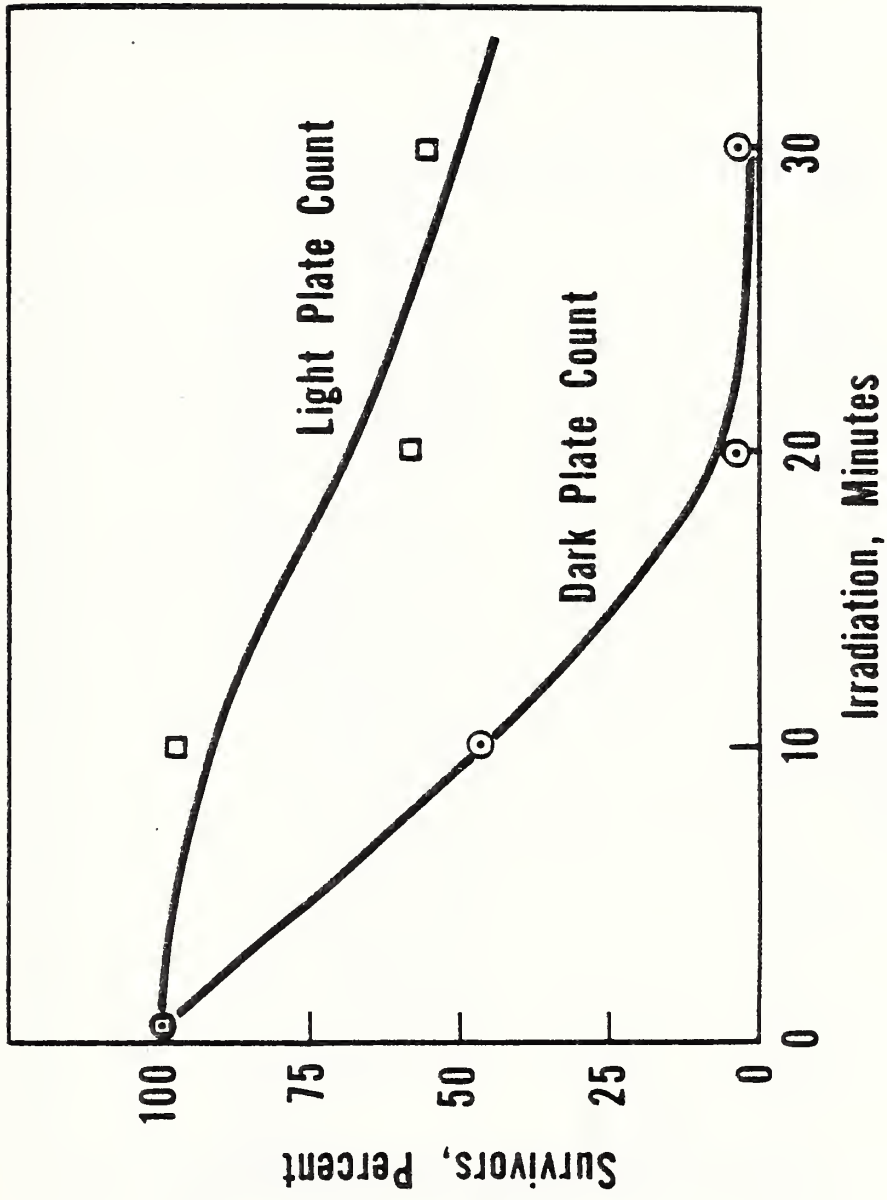


Figure 1



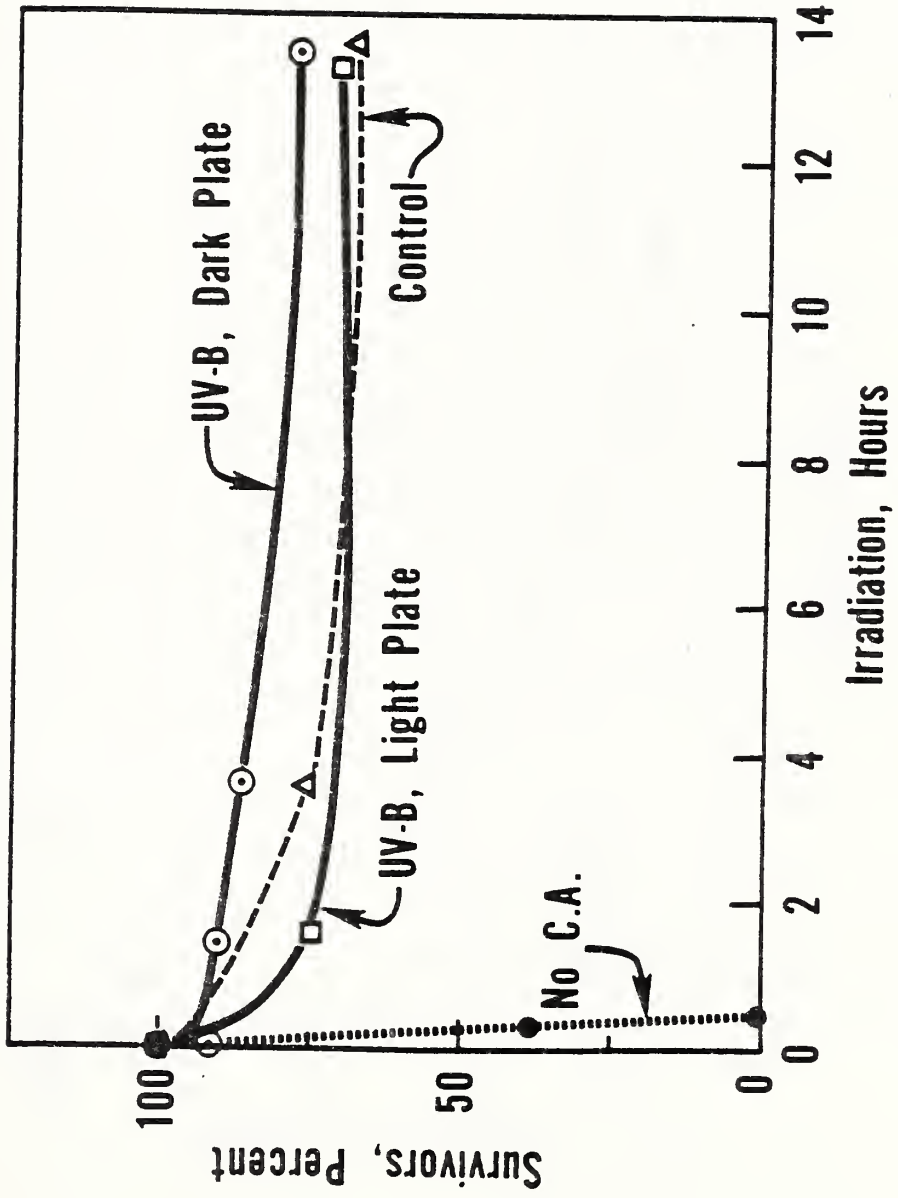


Figure 2





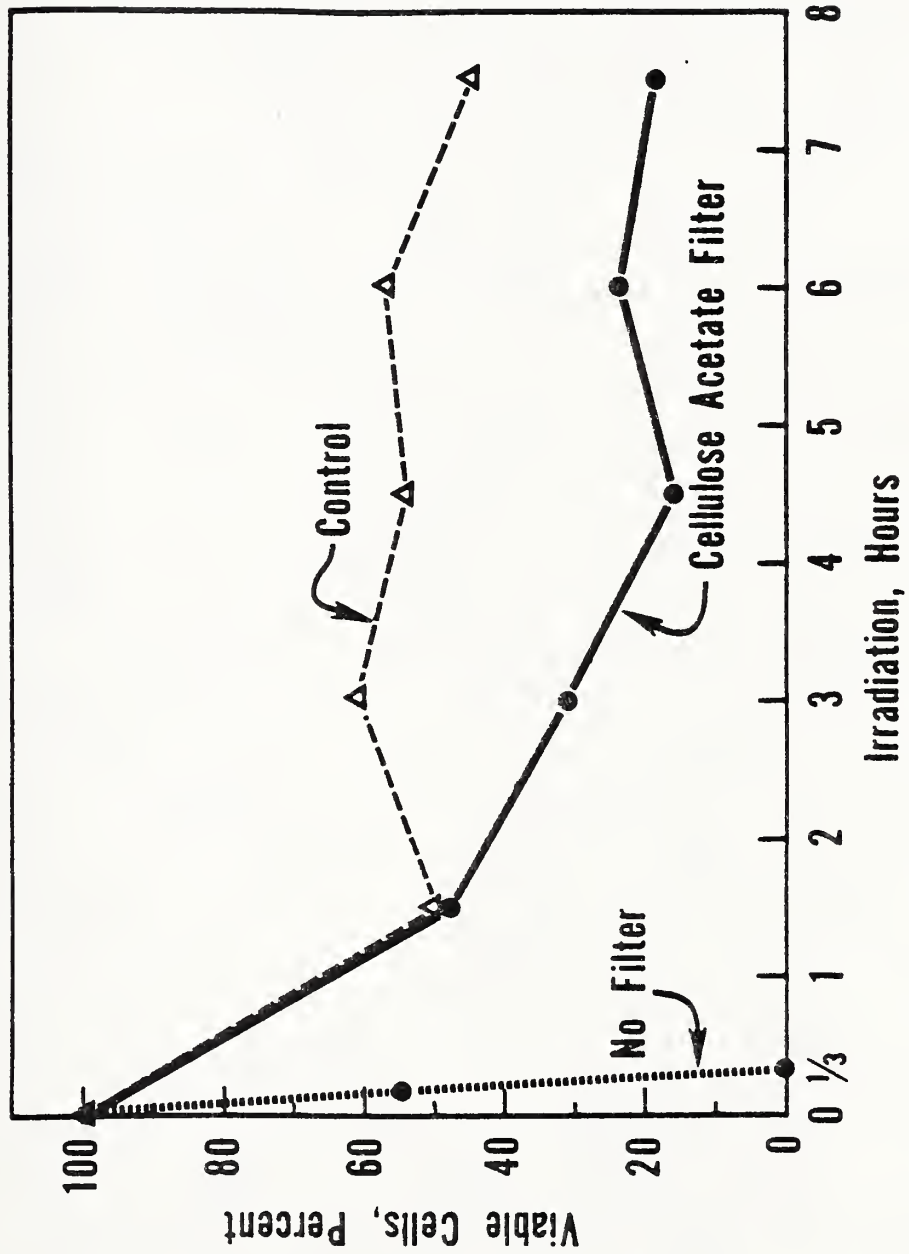


Figure 3

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