Circadian Rhythms of Resistance to UV-C and UV-B Radiation in *Euglena* as Related to 'Escape from Light' and 'Resistance to Light'

Aoen Bolige[1], Maki Kiyota[2], and Ken Goto[1]

[1]Laboratory of Biological Rhythms, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan 080-8555

[2]Department of Food Science and Nutrition, Sagami Women's University, Sagamihara, Japan 228-8533

Corresponding author:
Ken Goto
Address: Laboratory of Biological Rhythms, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan 080-8555
Phone & fax: +81-155-49-5612
Email: kgotoken@obihiro.ac.jp

Key words: antioxidant, L-ascorbic acid, circadian rhythms, dimethylsulfoxide, *Euglena gracilis*, photo-oxidation, UV-sensitivity

Abbreviations: Asc, L-ascorbic acid; CPD, cyclobutane pyrimidine dimer; CT, circadian time; DD, continuous darkness; DMSO, dimethylsulfoxide; LD: x, y, a 24-h light/dark cycle where x hours of light exposure are followed by y hours of darkness; LL, continuous light; NR, neutral red; PET, photosynthetic electron transport; ROS, reactive oxygen species; TB, trypan blue

Abstract

Radiation-induced stress, either from visible or UV light, is strongest at midday. We found that, in the absence of stress or time cues, *Euglena gracilis Z* was the most resistant to UV-C and UV-B at subjective midday, whether judged from immediate or reproductive survival. The circadian UV-resistance rhythms were free-running in stationary cultures under 1-h light/1-h dark cycles or continuous darkness, indicating that cell-cycle dependent DNA susceptibility to UV was not involved. We moreover examined what was the primary cause of the circadian UV resistance, estimated as the immediate cell survival. The half-maximal lethal dose (LD_{50}) of UV-C at subjective midday (the most resistant phase) was 156 J/m^2, which is ~3-fold that at subjective midnight. The same was true for UV-B, except the LD_{50} was ~13-fold that of UV-C. Temperature during UV irradiation had little effect, indicating that survival was not mediated via enzymatic reactions. Non-enzymatic antioxidants were added 5 min before UV irradiation. Dimethylsulfoxide (a hydroxyl radical scavenger) increased survival after UV-B, but had little effect after UV-C; conversely, sodium ascorbate increased survival after UV-C, but not after UV-B. These findings suggest that circadian rhythms of resistance to UVs involve a common mechanism for maximizing non-enzymatic antioxidative capacity at subjective midday, but the specific antioxidants differ.

1. Introduction

Photosynthetic organisms cannot escape solar radiation because they use solar energy for photosynthetic electron transport (PET). Major cellular compounds, such as nucleic acids, proteins, and lipids, absorb, and are thereby damaged by, solar UV radiation, which is most intense at midday when plants must photosynthesize. Even visible light can bring about photosensitization, the process whereby a molecule excited by light excites another molecule that does not absorb light, that results in photo-oxidative damage [1, 2, 3, 4], which is also greatest at midday.

Despite these dangers, many photosynthetic organisms, including the algal flagellate *Euglena gracilis*, display a circadian rhythm of PET capacity with a maximum at subjective midday [5, 6, 7, 8]. This endogenous state occurs naturally at midday and recurs with a circadian period, which is ~26 h in this alga, in an environment without external time cues. The circadian enhancement of PET capacity would amplify the photo-oxidative stress at midday because PET activity itself generates reactive oxygen species (ROS) [1, 2, 3, 4]

Midday is the most challenging time for photosynthetic organisms; radiation-induced stress is greatest at midday, whether the radiation is visible or UV and whether it attacks cellular constituents directly or indirectly through photosensitization. Therefore, we propose the hypothesis we call 'resistance to light'. Photosynthetic organisms may display a circadian rhythm that maximizes protection from the radiation-induced stress at subjective midday, so that their opportunity to live and reproduce may increase..

This hypothesis complements and does not contradict Pittendrigh's 'escape from light' hypothesis [9, 10, 11], which is the prevailing opinion on the evolutionary origins of circadian rhythm. Without resistance to light-induced damage, cells would have been killed by photo-oxidation; only cells that had endogenously allocated light-sensitive reactions to night intervals would have survived. Undoubtedly, the allocation would be minimally essential, at least for organisms that thrive in the euphotic zone. However, we think it would have been difficult for photosynthetic organisms to survive the damaging effects of sunlight by that mechanism alone. Therefore, the 'resistance to light' hypothesis predicts that mechanisms to defend against radiation-induced stress might be allocated to day intervals, becoming maximal at subjective midday.

This study tested this prediction in *E. gracilis*, which has been studied previously with respect to protection from oxidative damage [12, 13, 14]. Moreover, numerous aspects of the circadian rhythm in this alga have been studied [6, 7], including the biochemical mechanisms for autonomous oscillation [15], physiological mechanisms for 'gated' cell population growth [16] and photoperiodic induction of cell reproduction [17].

2. Materials and Methods

2.1. Organisms and chemicals

The algal flagellate *Euglena gracilis* Klebs (Z) was cultured photoautotrophically and axenically at 25°C. Cultures were irradiated unilaterally by an array of daylight-type white fluorescent lamps (Toshiba Mellow-White, Tokyo) at a light intensity of 84 μmol m^{-2} s^{-1} (6 klx), unless otherwise stated [17, 18]. In order to reduce opportunities for contamination, Cramer's medium [19] was slightly modified, such that the metal solution containing sodium citrate was replaced with Hutner's metal solution [20]. This made it much easier to handle the cultures. The alga was first grown under continuous light (LL) and was then transferred

to either the cycles of 1-h light and 1-h dark (LD: 1, 1) or continuous dark (DD). Approximately 7 ml of *Euglena* culture were withdrawn automatically and fixed with 0.5 ml of 20% neutral formalin containing 20% NaCl every 2 h. The cell number was counted with a Coulter Electronic Particle Counter.

All the chemicals were the highest grade commercially available. Dimethylsulfoxide (DMSO), sodium ascorbate (Asc) and trypan blue (TB) were purchased from Wako Chemicals Inc. (Tokyo) and neutral red (NR) from Merck Japan (Tokyo).

2.2. Definition of CT in non-dividing cultures

The time of the transfer from LL to DD is defined as CT12 [6, 7]. Our previous study indicated that the period of the circadian rhythm of the photo-induction of the commitment to cell division is approximately 26 h under DD [17]. Therefore, CT12 was considered to occur at the 0^{th}, 26^{th}, 52^{nd}, 78^{th}, and 104^{th} h in DD. When the culture was transferred from LL to LD: 1, 1, the onset of cell population growth, or CT12, occurs on average at the 22^{nd}, 48^{th}, 74^{th}, and 100^{th} h [16]. We assumed that CT12 in non-dividing cultures also occurs at these times in LD: 1, 1.

2.3. Survival of UV irradiation

The effects of UV-C and UV-B radiation on cell survival were examined in stationary (non-dividing) cultures. The stationary cultures were obtained either by inducing dark-arrest of cell cycle progression by transferring the cultures to DD [18] or by bringing about population-induced cell cycle arrest by culturing to the highest possible cell density at ~2.0×10^5 cells/ml in LL; the overcrowded cultures were transferred to LD: 1, 1 to be UV-irradiated.

UV-C from a germicidal lamp (peak at 254 nm; Hitachi, GL-15W, Tokyo) was used at an intensity of 1.3 W/m^2 for 30 s (giving 40 J/m^2), unless otherwise stated. UV-B radiation from a Philips UV-B lamp (peak at 315 nm with λ between 300 and 320 nm; Philips, TL20W/01, Tokyo) was used at 10 W/m^2 for 180 s (giving 1.8 kJ/m^2), unless otherwise stated. A different Philips UV-B lamp (TL20W/12RS) was used in one experiment only.

The fluence rate was measured with UV Meter model UVC-254 (Custom, Tokyo) for UV-C with UV Light Meter model UV-340 (Lutron, Tokyo) for UV-B. The spectral properties of these three lamps was measured with EPP2000C (StellarNet Inc., USA) and shown in Figure 1. UV irradiation was carried out against a dark background at 25°C, except for one experiment carried out at 0°C. Cell survival was evaluated as the percentage of cells that excluded the dyes trypan blue (TB) or neutral red (NR); surviving cells capable of reproducing were estimated by colony-forming activity.

2.4. Colony forming activity

About 300 cells (0.1 ml) were spread on each triplicate plate (9 cm diameter) of agar (1.5%) containing the medium above; the plates were immediately placed on a turntable that rotated 60 cm below a germicidal lamp to give an even exposure to the UV-C light. Immediately, they were then transferred to LL at ~42-84 μmol m^{-2} s^{-1}; another triplicate set of plates was treated similarly but UV-irradiation as controls.

2.5. Viable cell counts

UV irradiation was essentially the same as 2.4, but cell suspension (1.0 ml) with no

dilution was placed in a Petri dish (3.7 cm diameter) and a UV-B lamp was usually located ~ 20 cm above the table, unless otherwise stated. The percent survival was examined either immediately after UV-C or UV-B radiation or one day later. About 300 cells were counted three times on a hemocytometer.

Trypan blue (TB) (0.4%) stained all the dead cells fixed with 80% ethanol, but did not stain any dead cells fixed with formalin (1 to 10%). Obviously, TB-stain depended on the way cells died. , When almost all the cells were killed with UV-C at 1.3 W/m^2 for 10 min (UV-C dose at 780 J/s) as judged by microscopic observation with neutral red stains (see below), TB stained only 20 to 30% of the total cells. Since the percentage was positively related to the number of dead cells, as judged by the dose-response curve of the percentage stained versus UV-C radiation, it was used to assess UV-C sensitivity.

Neutral red (NR) stained dead but not viable *E. gracilis* cells. Unlike TB, NR (0.03 %) stained all the dead cells evenly within a few minutes of death brought about by ethanol, formalin, UV-C, or UV-B; cell body, i.e., all the matrix space of both the cytoplasm and the organelles like the nucleus and the chloroplasts. Viable cells were not stained immediately after UV irradiation. After some time, viable cells took up the dye, perhaps into food vacuoles; no other parts of the viable cells were stained. Thus, both types of cells can be easily distinguished, and all the experiments except Fig. 2B used NR for examining the viability. When either Asc or DMSO was added to cell suspension, it was done 5 min before UV irradiation.

3. Results

Cell cycle phases have different sensitivities to UV and ionizing radiation. In yeast [21], G2 phase is the least sensitive; late-S phase is the next least sensitive; and mitosis is the most sensitive phase. Mammalian cells display a similar tendency [22, 23]. Circadian rhythms regulate the timing of cell cycle transitions; in *E. gracilis*, G1-to-S, S-to-G2, and G2-to-M transitions are gated to occur only between CT03 and 23, CT12 and 24 (= 00), and CT08 and 20, respectively [16]. In order to determine the circadian effects, if any, on UV-C and UV-B resistance, experiments were performed using stationary, non-dividing cultures.

3.1. Circadian rhythm of UV-C resistance under LD: 1, 1

Two independent cultures in which cell cycle progression was arrested by overcrowding at ~2 × 10^5 cells/ml were transferred from LL to LD: 1, 1. Cells, either on solid agar or in liquid suspension, were irradiated with UV-C at 1.3 W/m^2 for 30 s, which gives a dose of 40 J/m^2. Unpublished results (Goto, K.) show that the cells are arrested primarily in either G1 or G2 phase and not in S or M phase.

The irradiated plates were immediately placed in LL at ~42-84 μmol m^{-2} s^{-1} and ~25°C. Six to seven days later, the number of colonies that had survived the UV-C irradiation were counted. Only reproductive cells, and not all viable cells, were counted with this method. As shown in Figure 2A, the reproductive survival of UV-C irradiation clearly displays a circadian rhythm with the minimum and maximum occurring at the 58^{th} h (subjective midnight; see below) and the 66 to 70^{th} h (subjective midday), respectively, in LD: 1, 1. Only ~50% of the cells survived at around subjective midnight; nearly 90% of the cells survived at around subjective midday.

The suspension cultures that were irradiated with UV-C were immediately mixed with TB,

and the viability, but not the reproducibility, was examined. As seen in Figure 2B, the survival after UV-C irradiation displays a circadian rhythm with the same phasing as in Figure 2A. When the viability of the irradiated cell suspension kept in DD was examined the next day, neither the total cell count nor the percentage survival had changed significantly from the values shown in Figure 2B; because darkness cannot support cell reproduction [17, 18], the result indicates that no further cell death took place after irradiation.

Note that TB did not stain all of the dead cells, as described in Materials and Methods, and the percentage survival depicted in Figure 2B should have been much higher than measured. Therefore, the rhythm shown in Figure 2B accurately reflected the phasing of the resistance rhythm, but it did not accurately reflect the amplitude.

When the data in Figure 2A were averaged, the minimum survival occurred at the 58^{th} h and the maximum at the 68^{th} h in LD: 1, 1; these times are 8 and 18 h, respectively, after the 50^{th} h, which is assumed to be CT12 (see Materials and Methods). Therefore, given a period of 26 h, the minimum and maximum occurred at CT19 and C05, respectively; *i.e.*, 18 h after the 50^{th} h (CT12) becomes CT12 + CT(18 × 24/26) = CT29 = CT05.

3.2. Circadian rhythm of UV-C resistance under DD

In subsequent experiments, NR that stained only and all the dead cells in *E. gracilis* cultures was used (cf. 2.5), and DD was used to obtain stationary cultures for three reasons: cell-cycle progression is arrested by darkness within ~10 h [17, 18]; overcrowded cell suspensions in LD: 1, 1 contained dirty materials, such as cell debris; and DD is much simpler than LD: 1, 1. Cells can be arrested at G1, S, or G2 phases, but not at M phase; in the culture conditions used, cells arrested at G1, S, and G2 comprised 51, 28, and 21% of the total cells, respectively [17, 18]. Two anti-parallel cultures, each covering the time range of either the maximum or minimum survival time, were used as in Figure 2. UV-C irradiation at 1.3 W/m^2 was continued for either 30 or 60 s providing a dose of 40 or 80 J/m^2, respectively. Figure 3 shows clearly that the circadian rhythm of survival after UV-C irradiation persisted in DD.

The maximum survival occurred at the 18^{th} h and again at the 42^{nd} to 44^{th} h in DD. Since the 26^{th} h in DD can be considered CT12 and the period was approximately 26 h, the maximum survival times corresponded to CT05 and CT03 to 05, respectively; for example, 16 h after the 26^{th} h (CT12) becomes CT12 + CT(16 x 24/26) = CT27 = CT03. Therefore, on average, the maximum survival occurred at CT05. Similarly, the minimum survival occurring at the 32^{nd} h and the 56^{th} to 58^{th} h in DD corresponded to CT17, on average. Combined with the results for LD: 1, 1, these findings suggest that the circadian rhythm of survival after UV-C irradiation attained its maximum and minimum at CT05 (around subjective midday) and CT18 (just midnight), respectively.

3.3. Circadian rhythm of UV-B resistance under DD

Stationary cultures in DD were obtained as described for the UV-C experiments. UV-B at 10 W/m^2 was used to irradiate cultures for 120 s, giving a dose of 1.2 kJ/m^2, which was 10-20 times as strong as the UV-C irradiation. As shown in Figure 3, the most resistant phase occurred near subjective midday, or the 44^{th} h, in DD, which corresponded to CT05. Likewise, the most sensitive phase occurred near subjective midnight, or the 32^{nd} h, in DD, which corresponded to CT18. Therefore, the circadian rhythm of UV-B resistance ran in phase with that of UV-C resistance, indicating that both rhythms shared a common mechanism, which maximizes the resistance at subjective midday.

3.4. LD_{50} of UV-B and UV-C

The dose responses of lethality to UV-C and UV-B were examined for cells in DD at both the most resistant (subjective midday) and most sensitive (subjective midnight) phases. Cultures were transferred to DD from LL; different doses were obtained by changing the exposure times to UV-C (1.3 W/m^2) and UV-B (10 W/m^2). As shown in Figure 4A, the half-maximal doses of lethality (LD_{50}) of UV-C were ~156 J/m^2 near subjective midday (44^{th} h in DD; CT05) and 52 J/m^2 at midnight (58^{th} h in DD; CT18). The LD_{50} of UV-B was 2070 J/m^2 near subjective midday (44^{th} h in DD; CT05) and 620 J/m^2 at midnight (58^{th} h in DD; CT18), which is 12-13 times the LD_{50} of UV-C (Fig. 4B).

Combined with the fact that in some systems UV-C has been shown to cause more than ~100 times as much direct DNA damage as UV-B [24, 25, 26], these results suggest that the circadian rhythms of resistance to UV-C and UV-B could not be fully attributed to the direct UV-damage of DNA.

3.5. One-day incubationafter UV irradiation

For the data in Figures 2B to 4, viable cells were counted immediately after UV irradiation. Figure 5 shows that the survival percentage was little changed by an additional one-day incubation in DD or LL (84 $\mu mol\ m^{-2}\ s^{-1}$) at 25°C, whether the irradiation was performed at the most (44^{th} h in DD) or least (58^{th} h in DD) resistant phase; total cell counts remained essentially constant. Therefore, UV-induced killing was completed within an irradiation period shorter than 10 min. The lack of changes in total cell counts means that no cells were fragmented or lysed after the immediate count; cell did not divide even in LL. Therefore, the percentage of survivors, estimated as the ratio of viable cells to the total cell count, represented the actual percentage of cells that remained alive. The total cell count immediately after UV irradiation was essentially the same as that of non-irradiated controls (data not shown).

3.6. UV irradiation at 0°C

UV irradiation was also carried out at 0°C to compare the survival with that of UV irradiation at 25°C using the NR exclusion test. As shown in Figure 6, the percentage of survivors changed little at the lower temperature during UV-C or UV-B irradiation whether the experiments were performed at subjective midday (44^{th} h in DD) or midnight (34^{th} or 58^{th} h in DD). Therefore, UV resistance did not involve any enzymatic reactions and was characterized as a non-enzymatic capacity. A distinction should be made between UV resistance and its circadian change; the former is an instantaneous state of the latter process, which of course involves enzyme reactions.

Moreover, the results revealed that cell death in this study could not be regarded as programmed cell death because programmed cell death involves enzymatic reactions [27]. Cell death was regarded as accidental and passive, proceeding without energy expenditure [27]. Nevertheless, the dead cells did not rupture, as exemplified by the constant cell number at the beginning of UV irradiation and one day later. UV light did not result in necrosis, but caused cells to look as if they had been fixed.

3.7. Effect of DMSO on UV-induced cell death

The results described above are compatible with the hypothesis that the circadian rhythms of UV resistance did not involve enzymatic reactions. The intrinsic vulnerability to UV light,

which is dependent on the cell cycle phase, was not responsible for our rhythms either. Therefore, we examined whether these rhythms are related to a non-enzymatic capacity for antioxidation.

Dimethylsulfoxide (DMSO) is a potent scavenger of hydroxyl radicals [28, 29, 30, 31, 32]. At concentrations above 2%, DMSO killed *Euglena* (data not shown); DMSO concentrations up to 2% were used in the experiments. Either UV-C or UV-B was used to irradiate cultures 5 min after the addition of DMSO. As shown in Figure 7A, DMSO hardly increased UV-C resistance, if any. Conversely, it increased UV-B resistance remarkably (Fig. 7B).

DMSO at ~0.5% increased the UV-B resistance level of cells at the least resistant phase (subjective midnight) to the physiological UV-B resistance level of cells at the most resistant phase (subjective midday). DMSO at 1 to 2% diminished the difference between UV-B resistance of cells at the least and most resistant phases by increasing cell viability to ~85%. Collectively, these results suggest that UV-B (but not UV-C) resistance is due primarily to the capacity of scavenging hydroxyl radicals.

3.8. Effect of Asc on UV-induced cell death

Asc is a physiological and potent antioxidant [2, 3, 12, 14]. In this experiment, UV-C was used to irradiate cultures at 1.3 W/m^2 for 30 s as before. Opposite to the effects of DMSO, the universal antioxidant ASC produced a greater resistance to UV-C than to UV-B irradiation, as shown in Figure 8A. Although Asc enhanced the survival after UV-C by ~10%, it did not increase the UV-C resistance level of cells at the least resistant phase (subjective midnight) to the level achieved physiologically by the cells at the most resistant phase (subjective midday).

Thinking that the UV-C intensity might have been too strong for Asc to fully overcome the UV-C stress, we halved the intensity to 0.68 W/m^2; the difference in UV-C resistance between the most and least resistant phases was still obvious at this intensity, as shown in Figure 8B. Under these conditions, Asc at 25 to 50 mM increased the UV-C resistance level of the least resistant phase to the UV-C resistance level physiologically achieved at the most resistant phase, whether the dose was 27 or 40 J/m^2 (Fig. 8B).

Since Asc combats ROS, such as singlet oxygen, superoxide radicals, and hydroxyl radicals [33], its effects on UV-C resistance may involve its antioxidant capacity for scavenging various ROS. Another aspect of the results was that, at the same dose of 40 J/m^2, the survival after UV-C irradiation was greater at a fluence rate of 0.68 W/m^2 (Fig. 8B) than at 1.3 W/m^2 (Fig. 8A), in the presence or absence of Asc. This intensity effect was more pronounced for cells at the least resistant phase (subjective midnight; 44^{th} h in DD); the Bunsen-Roscoe reciprocity law [34] was not followed.

As noted above, Asc increased the UV-C resistance level of cells at the least resistant phase to the physiological, *i.e.*, Asc-untreated, UV-C resistance level of cells at the most resistant phase (Fig. 8B). Exogenous Asc also enhanced the resistance of cells at the most resistant phase (Fig. 8B). Since the physiological level of resistance was already high at a UV-C dose of 27 J/m^2, it was not dramatically increased by Asc. As a result, 50 to 100 mM Asc almost fully alleviated the effect of UV-C irradiation at 27 J/m^2 (0.68 W/m^2, 40 s), with a resultant survival of ~95% at both the most and least resistant phases.

By contrast, at the higher UV-C dose of 40 J/m^2 (0.68 W/m^2, 60 s), Asc did not diminish the difference between the UV-C resistance of cells at the most and least sensitive phases

(Fig. 8B). One possible explanation for this is that more antioxidants are present in cells at the most resistant phase and they act differently than does Asc to detoxify ROS as the UV-C dose increased.

As noted above, the 1.3 W/m^2 fluence rate was more dangerous than was the 0.68 W/m^2 rate, although the same UV-C dose of 40 J/m^2 was given. The dose response to UV-C at 0.68 W/m^2 was determined by varying the exposure time in the presence or absence of Asc (Fig. 9). Exogenous ASC enhanced survival at all UV-C doses. Nevertheless, it could not fully overcome the killing by UV-C at doses exceeding 27 J/m^2 (0.68 W/m^2 for 40 s). UV-C-induced killing was accelerated by doses exceeding 27 J/m^2, but this acceleration was not as evident in Asc-treated cells at the most resistant phase (subjective midday; 58^{th} h in DD).

4. Discussion

This study revealed that both UV-C and UV-B brought about accidental and passive cell death in *E. gracilis*, which could be overcome by the antioxidants Asc and DMSO, respectively. Moreover, we found that circadian rhythms regulated resistance to both types of UV light; the alga was most resistant near subjective midday and least resistant at midnight, independent of cell-cycle phase. The evidence strongly suggests the presence of a circadian rhythm of the capacity for non-enzymatic antioxidation, which generates the circadian rhythms of UV-resistance. A parallel rhythm was found in the resistance to UV-C as judged from surviving cells capable of reproducing (Fig. 2A), suggesting that the subjective midday is the most resistant phase to photo-oxidative stress in general. In the following, we mainly concern with the UV-resistance as judged from survival immediately after UV irradiation (Figs. 2B to 9).

4.1. What produces UV resistance rhythms?

The pathways by which UV-C and UV-B caused cell death differed. The primary target of each type of UV light differs; DMSO enhanced the resistance to UV-B, but not to UV-C (Fig. 7), while Asc did the opposite (Fig. 8). Nevertheless, both pathways shared common mechanisms. First, the circadian rhythms of both resistivities paralleled each other, suggesting a common circadian signal that afforded the alga the highest resistance at subjective midday. Second, cell death in both cases was categorized as accidental cell death and not programmed cell death because it did not involve an enzymatic reaction (Fig. 6). Third, resistance to UV-C and UV-B were both enhanced by the application of exogenous non-enzymatic antioxidants (Figs. 7 to 9).

4.2. Independence of cell cycle phases

Sensitivity to UV irradiation is dependent on the cell cycle phase in yeast [21] and mammalian cells [22, 23]. However, the circadian resistance rhythms presented here represent only the CT-dependent differences in resistance to UV irradiation because the survival studies were performed with stationary cultures in which there were constant numbers of cells arrested in G1, S, and G2 phases, but not in M phase, throughout the experiments. Moreover, the intrinsic susceptibility (vulnerability *per se*) of chromosomes or DNA to UV irradiation is reported to change only in a cell cycle phase-dependent manner [21, 22, 23]. The persistent nature of the circadian resistance rhythm in stationary cultures suggests two possibilities, which are not mutually exclusive. First, the intrinsic vulnerability of DNA might also change in a manner dependent on circadian phase. Second, the rhythm

might result from factors outside of the chromosome or DNA.

Circadian transcription might be relevant to the first possibility. Bulk RNA synthesis in the duckweed *Lemna gibba* G3 is shown to follow a circadian rhythm with its maximum and minimum occurring respectively at subjective midnight and midday [35], and 36 % genome of *Arabidopsis* is found to be potentially under transcriptional control by the circadian clock with the similar phasing [36]. It is likely that transcribed DNA is more vulnerable to UV stress, which may partly explain a circadian rhythm of the UV-resistance as judged from reproductive survival (Fig. 2A).

Nikaido and Johnson [37] reported that, for the green alga *Chlamydomonas reinhardtii*, reproductive survival after UV-C irradiation (40 J/m^2) followed a circadian rhythm in cultures under dim LL at an intensity of 0.2 $\mu mol/m^2/s$ (~14 lx); we think that this intensity is too low to support cell cycle progression because the light compensation point for photosynthesis of this alga is much higher [38, 39]. Unlike their proposal for the involvement of the circadian timing of DNA synthesis or mitosis, we suggest that our conclusion for *E. gracilis* also applies to *C. reinhardtii*. In addition, note that, in the dim LL, *C. reinhardtii* was most resistant near subjective midday.

4.3. Photoreactivation or dark repair?

Neither the circadian UV-C- (except for Fig. 2A) nor the UV-B-resistance rhythm involved photoreactivation or dark-repair based on our findings. First, lowering the temperature at the time of UV irradiation did not significantly alter the survival percentage (Fig. 6), indicating that no enzymatic processes were involved in the rhythms. Second, a one-day incubation in either LL or DD did not alter the survival percentage (Fig. 5). These results not only excluded the involvement of repair mechanisms, but also revealed that the process that led to cell death was completed within the irradiation period.

This conclusion does not necessarily mean that there are no mechanisms for photoreactivation or dark-repair [40, 41] in this alga, but rather that the way in which we measured survival could not detect them. We counted dead cells immediately after UV irradiation (Figs. 2B to 9), and dead cells could not of course be revived. Nevertheless, the UV resistance as judged from reproductive survival (Fig. 2A) may partly reflect photoreactivation, because UV-irradiated cells were immediately placed under white light at 42-84 $\mu mol\ m^{-2}\ s^{-1}$; our preliminary data indicated that one-day incubations in LL immediately after UV irradiation gave higher colony-counts than did incubation in DD, supporting the presence of photoreactivation in this alga [42, 43].

4.4. Direct DNA damage caused by UV light

The action spectrum for the loss of cell reproducibility closely resembles the absorption spectrum of DNA molecules, although the former is red-shifted by ~5 nm relative to the latter [44]. Therefore, it is widely believed that UV light damages DNA directly, resulting in the loss of cell reproducibility. The loss of cell reproducibility that leads to clonal death is different from the immediate, accidental, and passive cell death that we primarily studied, except for the experiment in Figure 2A.

Evidence supports the idea that UV-induced direct DNA damage and UV-induced accidental cell death are not so intimately related, even with the loss of cell reproducibility. For example, a comparative study [45] revealed that the differences in sensitivity, judged

from clonal death, to UV-B among different species of marine bacteria do not necessarily reflect the differences in the degree of UV-B-generated cyclobutane pyrimidine dimer (CPD). Since DNA repair mechanisms did not operate in that condition, the result suggests that the UV-B-induced clonal death results from a mechanism other than UV-B-induced direct DNA damage. This notion is consistent with another result in the same study [45]; the removal of nearly all UV-B-generated CPD did not cause any cells to become reproducible in one species, whereas the removal of one-half of the UV-B-generated CPD resulted in only a 5% increase in the number of surviving reproductive cells in another species.

Little is known about whether UV- caused direct DNA damage results in accidental and passive cell death, or, if so, how the DNA damage leads to death, despite numerous studies of DNA damage-induced cell cycle arrest and apoptotic cell death [27]. Dose-response curves revealed that UV-C was ~13 times as effective as UV-B at killing *E. gracilis* (Fig. 4), while UV-C is ~100-1000 times as effective as UV-B at directly damaging DNA (or generating CPD) [25, 26, 27]. Therefore, although we cannot rule out the possibility that the cell death may be due, at least partly, to UV-caused direct DNA damage, such a mechanism could not fully explain our results. Since one possible mechanism for UV-induced accidental cell death involves UV-generated ROS, we examined whether antioxidants can rescue *Euglena* from UV-induced accidental cell death.

4.5. Defense from ROS stress

Since UV-related cell death was found to be independent of temperature (Fig. 6), any involvement of antioxidation must be in a non-enzymatic capacity. DMSO had a greater impact on increasing the UV-B-resistance of cells, whereas Asc had a greater effect on the UV-C resistance, although within a certain limit of the dose and intensity of UV-C irradiation. Therefore, we concluded that the circadian rhythms of UV-C and UV-B resistance might involve different non-enzymic antioxidants with intracellular levels that follow the same circadian rhythm with a peak at subjective midday.

DMSO is an amphiphilic scavenger of hydroxyl radicals [28, 29, 30, 31, 32]. Therefore, UV-B resistance may involve defenses against the hydroxyl radicals produced in both membranes and the cytosol. In contrast, Asc is a hydrophilic scavenger of various ROS, such as singlet oxygen species, super oxide radicals, and hydroxyl radicals [2, 3, 12, 14, 33]. Therefore, within certain limits of UV-C intensity and dose, UV-C resistance could be fully afforded by aqueous antioxidants acting universally like Asc; beyond these limits, UV-C resistance might also involve other antioxidants that act quite differently from Asc, perhaps a lipophilic quencher of singlet oxygen.

Our purpose was not to identify the specific antioxidants involved in UV resistance, but to identify the type of ROS, *e.g.*, lipophilic, hydrophilic, singlet oxygen, or hydroxyl radical, against which the cells were protected. A more thorough study along this line is currently underway. We also found, as will be published elsewhere, a circadian rhythm of the intracellular level of Asc in this alga, as well as in spinach, with the same phasing as that of the UV resistance rhythm. Interestingly, DMSO is also synthesized intracellularly and can act as both a cryoprotectant and an antioxidant in some marine phytoplankton [30, 31], although we do not know whether DMSO is a physiological substance in *E. gracilis*.

4.6. ‘Resistance to light’ vs. ‘escape from light’

Aerobes respond to and activate defenses against oxidative stress, but the circadian

rhythms of the resistance to UV-C or UV-B are not a direct response to UV irradiation and can be manifested in the absence of an external time-cue or UV-stress. It is interesting to consider the ultimate reason for this behavior.

UV-B radiation is the solar radiation most harmful to organisms because UV-C is blocked by the ozone layer [46]. In the ancient ocean, however, damage by UV-C was significant [47]. Moreover, visible light would also have burned the organisms [1, 2, 3, 4, 48]. In all cases, radiation-induced stress has always been most severe at midday.

With repeated natural selection, the organisms that allocated radiation-sensitive reactions (or biomolecules) to the night would have better survived, so that circadian rhythms would have evolved. This traditional hypothesis, known as the 'escape from light' hypothesis, was originally proposed by Pittendrigh [9, 10, 11]. In accordance with this hypothesis, most, if not all, unicellular organisms undergo mitosis, the most radiation-sensitive phase in the cell cycle [21, 22, 23], only during subjective night [6,7]. As to a possible mechanism, we recently found that a circadian rhythm in *E. gracilis* prevents G2-phase cells from progressing to mitosis during CT20 to 08 [16].

However, photosynthetic organisms must inevitably be exposed to solar radiation. Therefore, they have had to activate defenses against radiation-induced stress that are maximal at midday; this strategy might represent the evolutionary origin of circadian rhythms. Therefore, our hypothesis is that the 'resistance to light' or the 'defense against radiation-induced stress' drove the evolution of circadian rhythm. Both mechanisms, 'escape from light' and 'resistance to light', probably exerted simultaneous pressures on photosynthetic organisms to evolve a circadian rhythm; 'escape from light' compelled the allocation of light-sensitive reactions to night intervals, while 'resistance to light' promoted the allocation of light-protective defenses to day intervals. Alternatively, perhaps a circadian rhythm first evolved for other reasons and then subsequently regulated UV resistance. In either case, the UV resistance rhythm is an important mechanism for an environmental adaptation.

Other studies are also consistent with the 'resistance to light' hypothesis: a circadian rhythm of superoxide dismutase activity is found in a marine dinoflagellate [49], and a diurnal rhythm of the activity of CPD-specific DNA photolyase with a circumstantial evidence for its endogeneity in cucumber [50]; the phasing consistent with the hypothesis.

The hypothesis also suggests that the UV-resistance rhythms found here most likely represent only the tip of an iceberg of circadian rhythms of a whole system of antioxidative capacity with the same phasings. Finally, the robust persistence of the circadian UV-resistance rhythms, which did not wane for as long as ~62 h in DD, is surprising when we consider that *E. gracilis* should be severely starved in DD, in which there are no external sources of energy or materials.

Acknowledgments

We thank Ms. Naoko Toda for her preliminary study that helped us to carry out the experiments shown in Figure 2A.

References

[1] K. L. Steffen, Avoidance of photooxidative stress: Balancing energy flux within the chloroplast, In: E.J. Pell, K.L. Steffen, Active Oxygen/ Oxidative Stress and Plant Metabolism, Amer. Soc. Plant Physiol., Maryland. 1991, pp. 119-130.

[2] C.H. Foyer, M. Lelandais, E. Anne-Edwards, P. M. Mullineaux, The role of ascorbate in plants, interactions with photosynthesis, and regulatory significance, in; E. J. Pell, K. L. Steffen , Active Oxygen/ Oxidative Stress and Plant Metabolism, Amer. Soc. Plant Physiol., Maryland. 1991, pp. 131-144.

[3] K. Asada, The water-water cycle as alternative photon and electron sinks, Philos. Trans. R. Soc. Lond. B Biol. Sci. 355 (2000) 1419-1431.

[4] U. Heber, N. G. Bukhov, V. A. Shuvalov, Y. Kobayashi, O. L. Lange, Protection of the photosynthetic apparatus against damage by excessive illumination in homoiohydric leaves and poikilohydric mosses and lichens, J. Exp. Bot. 52 (2001) 1999-2006.

[5] T. A. Lonergan, M. L. Sargent, Regulation of the photosynthesis rhythm in *Euglena gracilis*, II. Involvement of electron flow through both photosystems, Plant Physiol. 64 (1979) 99-103.

[6] B. M. Sweeney, Rhythmic Phenomena in Plants, Acad. Press, San Diego., 1987, p. 172.

[7] L. N. Jr. Edmunds, Cellular and Molecular Bases of Biological Clocks, Springer-Verlag New York, New York, 1988, p. 497.

[8] K. Goto, Toward a molecular mechanism for circadian clock(s) in Euglena gracilis., in: T. Hiroshige, K. Honma, Circadian Clocks and Ecology, Hokkaido Univ. Press, Sapporo, 1989, pp. 61-81.

[9] C. S. Pittendrigh, The functions, ancient and modern, of circadian oscillations, In Science and the Sixties. Proc. Cloudcroft Symp. Air Force Office of Scientific Research, (1965) 96-111.

[10] J. Paietta, Photooxidation and the evolution of circadian rhythmicity, J. theor. Biol. (1982) 77-82.

[11] C. S. Pittendrigh, Temporal organization: Reflections of a Darwinian clock-watcher, Annu. Rev. Physiol. 55 (1993) 17-54.

[12] S. Shigeoka, A. Yokota, Y. Nakano, S. Kitaoka, The effect of illumination on the L-ascorbic acid content in *Euglena gracilis Z*., Agric. Biol. Chem. 43 (1979) 2053-2058.

[13] S. Shigeoka, Y. Nakano, S. Kitaoka, Metabolism of hydrogen peroxide in *Euglena gracilis Z* by ascorbate peroxidase, Biochem. J. 186 (1980) 377-380.

[14] S. Shigeoka, T. Onishi, Y. Nakano, S. Kitaoka, Change of L-ascorbic acid content in synchronized cultures of *Euglena gracilis*, J. General Microbiol. 133 (1987) 221-225.

[15] K. Goto, D. L. Laval-Martin, L. N. Jr. Edmunds, Biochemical modeling of an autonomously oscillatory circadian clock in *Euglena*, Science 228 (1985) 1284-1288.

[16] A. Bolige, S. Hagiwara, K. Goto, Circadian G_2-arrest as related to circadian gating of cell population growth in *Euglena*, Plant Cell Physiol. 46 (2005) yyy-zzz (in press).

[17] S. Hagiwara, A. Bolige, Y. Zhang, M.Takahashi, A. Yamagishi, K. Goto, Circadian gating of photoinduction of commitment to cell cycle transitions in relation to photoperiodic control of cell reproduction in *Euglena*, Photochem. Photobiol. 76 (2002) 105-115.

[18] S. Hagiwara, M. Takahashi, A. Yamagishi, Y. Zhang, K. Goto, Novel findings regarding photoinduced commitments of G1-, S- and G2-phase cells to cell-cycle transitions in darkness and dark-induced G1-, S- and G2-phase arrests in *Euglena*, Photochem. Photobiol. 74 (2001) 726-733.

[19] L. N. Jr. Edmunds, Studies on synchronously dividing cultures of *Euglena gracilis* Klebs (strain Z). III. Circadian components of cell division, J. Cell Physiol. 67 (1966) 35-44.
[20] S. H. Hutner, L. Provasoli, A. Schatz , C. P. Haskins, Some approaches to the study of the role of metals in the metabolism of microorganisms, Proc. Am. Philos. Soc. 94 (1950) 152-170.

[21] W. Siede, E. C. Friedberg, Influence of DNA repair deficiencies on the UV sensitivity of yeast cells in different cell cycle stages, Mutat. Res. 245 (1990) 287-292.
[22] L. N. Petersen, D. K. Orren, V. A. Bohr, Gene-specific and strand-specific DNA repair in the G1 and G2 phases of the cell cycle, Mol. Cell Biol. 15 (1995) 3731-3737.
[23] T. M. Pawlik, K. Keyomarsi, Role of cell cycle in mediating sensitivity to radiotherapy, Int. J. Radiat. Oncol. Biol. Phys. 59 (2004) 928-942.
[24] H. Z. Hill, G. J. Hill, K. Cieszka, P. M. Plonka, D. L. Mitchell, M. F. Meyenhofer, P. Xin, R. E. Boissy, Comparative action spectrum for ultraviolet light killing of mouse melanocytes from different genetic coat color backgrounds, Photochem. Photobiol. 65 (1997) 983-989.
[25] T. Douki, D. Perdiz, P. Grof, Z. Kuluncsics, E. Moustacchi, J. Cadet, E. Sage, Oxidation of guanine in cellular DNA by solar UV radiation: biological role, Photochem. Photobiol. 70 (1999) 184-190.
[26] Z. Kuluncsics, D. Perdiz, E. Brulay, B. Muel, E. Sage, Wavelength dependence of ultraviolet-induced DNA damage distribution: involvement of direct or indirect mechanisms and possible artefacts, J. Photochem. Photobiol. B. 49 (1999) 71-80.
[27] A. Haunstetter, S. Izumo, Apoptosis: Basic mechanisms and implications for cardiovascular disease, Circ. Res. 82 (1998) 1111-1129.
[28] C. F. Babbs, M. G. Steiner, Detection and quantitation of hydroxyl radical using dimethyl sulfoxide as molecular probe, Methods Enzymol.186 (1990) 137-147.
[29] P. Landgraf, E. Ohmann, H. Tschiersch, Light induced oxidative stress in *Euglena gracilis*, Photosynthetica 33 (1997) 433-442.
[30] P. A. Lee, S. J. de Mora, (1999) Intracellular dimethylsulfoxide (DMSO) in unicellular marine alage: speculation on its origin and possible biological role, J. Phycol. 35 (1999) 8-18.
[31] R. A. Fleck, E. E. Benson, D. H. Bremner, J. G. Day, Studies of free radical-mediated cryoinjury in the unicellular green alga *Euglena gracilis* using a non-destructive hydroxyl radical assay: a novel approach for developing protistan cryopreservation strategies, Free Radic Res. 32 (2000) 157-170.
[32] W. Sunda, D. J. Kiebe, R. P. Kiene,S. Huntsman, An antioxidant function for DMSP and DMS in marine algae, Nature 418 (2002) 317-20.
[33] B. Halliwell, Vitamin C: poison, prophylactic or panacea? Trends Biochem. Sci. 24 (1999) 255-259.
[34] R. Sommer, T. Haider, A. Cabaj, E. Heidenreich, M. Kundi, Increased inactivation of *Saccharomyces cerevisiae* by protraction of UV irradiation, Appl. Environ. Microbiol. 62 (1996) 1977-1983.
[35] H. Nakashima, Diurnal rhythm of uridine incorporation into RNA regulated by two-perceiving systems in a long-day duckweed, *Lemna gibba* G3, Plant Cell Physiol. 17 (1976) 209-217.
[36] T. P. Michael, C. R. McClung, Enhancer trapping reveals widespread circadian clock transcriptional control in *Arabidopsis*, Plant Physiol. 132 (2003) 629-639.

[37] S. S. Nikaido, C. H. Johnson, Daily and circadian variation in survival from ultraviolet radiation in *Chlamydomonas reinhardtii*, Photochem. Photobiol. 71 (2000) 758-765.

[38] R. A. Danilov, N. G. Ekelund, Effects of increasing doses of UV-B radiation on photosynthesis and motility in *Chlamydomonas reinhardtii*, Folia Microbiol. (Praha) 45 (2000) 41-44.

[39] R. A. Danilov, N. G. A. Ekelund, Effects of Cu^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} and pentachlorophenol on photosynthesis and motility in *Chlamydomonas reinhardtii* in short-term exposure experiments, BMC Ecol. 1 (2001) 1.

[40] D. Perdiz, P. Gróf, M. Mezzina, O. Nikaido, E. Moustacchi, E. Sage, Distribution and repair of bipyrimidine photoproducts in solar UV-irradiated mammalian cells, J. Biol. Chem. 275 (2000) 26732-26742.

[41] E. Cleaver, Classics in DNA Repair: Photoreactivation. DNA Repair 2 (2003) 629-638.

[42] H. Lyman, H. T. Epstein, J. A. Schiff, Studies of chloroplast development in *Euglena*. I. Inactivation of green colony formation by U. V. light, Biochim. Biophys. Acta 50 (1961) 301-309.

[43] H. Z. Hill, H. T. Epstein, J. A. Schiff, Studies of chloroplast development in *Euglena*. XIV. Sequential interactions of ultraviolet light and photoreactivating light in green colony formation, Biophys. J. 6 (1966) 135-144.

[44] H. B. Wright, W. L. Cairns, Ultraviolet light, Trojan Technologies Inc. Technical Bulletin #52. 1998.

[45] F. Joux, W. H. Jeffrey, P. Lebaron, D. Mitchell, Marine bacterial isolates display diverse responses to UV-B radiation, Appl. Environ. Microbiol. 65 (1999) 3820-3827.

[46] S. Madronich, R. L. McKenzie, L. O. Björn, M. M. Caldwell, Changes in biologically active ultraviolet radiation reaching the Earth's surface, J. Photochem. Photobiol. B: 46 (1998) 5-19.

[47] D. D. Wynn-Williams, H. G. M. Edwards, E. M. Newton, J. M. Holder, Pigmentation as a survival strategy for ancient and modern photosynthetic microbes under high ultraviolet stress on planetary surfaces, Intl. J. Astrobiol. 1 (2002) 39-49.

[48] A. M. Edwards, E. Silva, Effect of visible light on selected enzymes, vitamins and amino acids, J. Photochem. Photobiol. B. 63 (2001) 126-131.

[49] P. Colepicolo, V.C.P.C. Camarero, J. W. Hastings, A circadian rhythm in the activity of superoxide dismutase in the photosynthetic alga *Gonyaulax polyedra*, Chronobiol. Int. 9 (1992) 266-268.

[50] S. Takahashi, N. Nakajima, H. Saji, N. Kondo, Diurnal change of cucumber CPD photolyase gene (CsPHR) expression and its physiological role in growth under UV-B irradiation, Plant Cell Physiol. 43 (2002) 342-349.

Figure Legends

Figure 1. Spectral properties of the three UV lamps.

Irradiance specra were measured at the fluence rates used in most experiments.
(A) Hitachi germicidal lamp (UV-C; GL-15W), (B) Philips UV-B lamp (TL20W/01), (C) Philips UV-B lamp (TL20W/12RS).

Figure 2. Circadian rhythm of UV-C-survival in stationary cultures under LD: 1, 1.

Two independent stationary cultures in LL (closed and open circles) were transferred to LD: 1, 1 at times 12 h apart. At the times indicated on the ordinates, cell suspensions were withdrawn at the beginning of the 1-h light-interval and subjected to UV-C irradiation at 1.3 W/m^2 for 30 s, which provided a dose of 40 J/m^2. The vertical bar crossing each symbol represents the standard error of the mean (SEM). The data from different cultures were plotted using different symbols. Some data points were obtained from both cultures to see the culture-dependent differences, if any. (A) At the indicated times, 0.1-ml aliquots of each culture (~300 cells) were spread on six agar plates, three of which were irradiated with UV-C. Immediately after irradiation, all the plates were placed in LL at ~42-84 $\mu mol\ m^{-2}\ s^{-1}$ to form colonies for 6-7 d. The percentage survival was calculated against the average number of colonies formed on the three control plates. (B) At each time point, a 10-ml cell suspension was placed on a Petri dish (9 cm diameter) and irradiated. Immediately, a viability test was carried out in duplicate using TB.

Figure 3. Circadian rhythm of survival after UV-C or UV-B in stationary cultures under DD.

The experimental protocol was essentially the same as in Fig. 2B, but stationary cells were obtained by DD transfer of the culture in LL, and the viability test was carried out immediately after UV irradiation using NR instead of TB. Closed and open symbols represent different cultures. (A) UV-C at 1.3 W/m^2 for 30 s (circles; 40 J/m^2) or 60 s (triangles; 80 J/m^2) against a dark background. (B) UV-B at 10 W/m^2 for 90 s (circles; 900 J/m^2) or 180 s (triangles; 1800 J/m^2).

Figure 4. LD_{50} of UV-C and UV-B at subjective midday and midnight.

The viability test was the same as in Fig. 3. Cells were taken from the cultures in DD that had been transferred from LL. Different doses were obtained by changing the time of exposure to UV-C at 1.3 W/m^2 and to UV-B at 10 W/m^2. Open and closed symbols represent the results with cells at the 44^{th} h (subjective midday) and 58^{th} h (subjective midnight) in DD, respectively. Data connected by solid and dotted lines represent measurements respectively made immediately and one day after irradiation. (A) Dose-response to UV-C; (B) Dose-response to UV-B. Circles and triangles represent data obtained using the TL20W/01 and 20W/12RS UV-B-lamp, respectively; the former lamp was standard in this paper.

Figure 5. Effect of a one-day incubation on survival after UV.

Cells at the 44th h (subjective midday) or 58th h (subjective midnight) in DD were irradiated with either UV-C (40 J/m^2) or UV-B (1.8 kJ/m^2). Viability was examined using NR either immediately after UV irradiation or after one day in LL at 84 μmol m^{-2} s^{-1} or DD.

Figure 6. Effect of UV irradiation at 0°C.

Either UV-C (40 J/m^2) or UV-B (1.8 kJ/m^2) was used to irradiate cells at the 44th h (subjective midday) or 58th h (subjective midnight) at 25 or 0°C in DD. Irradiation at 0°C was performed by placing ice around the Petri dish containing the cell suspension. Viability was examined using NR immediately after UV irradiation.

Figure 7. Effect of DMSO on survival after UV-C or UV-B irradiation.

DMSO was added to cells at the 44th h (subjective midday; open circles) or 58th h (subjective midnight; closed circles) in DD, and the cells were UV-irradiated 5 min later. Viability was examined using NR immediately after UV irradiation. (A) UV-C (1.3 W/m^2; 40 J/m^2). (B) UV-B (10 W/m^2; 1.8 kJ/m^2)

Figure 8. Effect of Asc on survival after UV-C or UV-B irradiation.

Asc at pH 7.2 was added to cells at the 44th h (subjective midday; open circles) or 58th h (subjective midnight; closed circles) in DD, and cells were UV-irradiated 5 min later. Viability was examined using NR immediately after UV irradiation. (A) UV-C (solid lines) at 1.3 W/m^2 for 30 s (40 J/m^2); UV-B (dotted lines) at 10 W/m^2 for 3 min (1.8 kJ/m^2). (B) UV-C at 0.68 W/m^2 for either 40 s (27 J/m^2; smaller circles) or 60 s (41 J/m^2; larger circles).

Figure 9. Dose-response to UV-C and the effect of Asc.

Cells were irradiated with UV-C at the 44th h (subjective midday; open circles) or 58th h (subjective midnight; closed circles) in DD at 0.68 W/m^2 for 20, 30, 40, 50, or 60 s in the absence of ASC. The same procedure was performed in the presence of 50 mM Asc with cells at the 44th h (subjective midday; open triangles) or 100 mM Asc with cells at the 58th h (subjective midnight; closed triangles).

Appendix figure 1. Discriminating dead or viable cells by NR

A microscope (x 100 objective) photograph of cells treated with NR was taken within 5 min after cells were irradiated with UV-C at 1.3 W/m^2 for 3 min. A scale bar represents 10 μm.

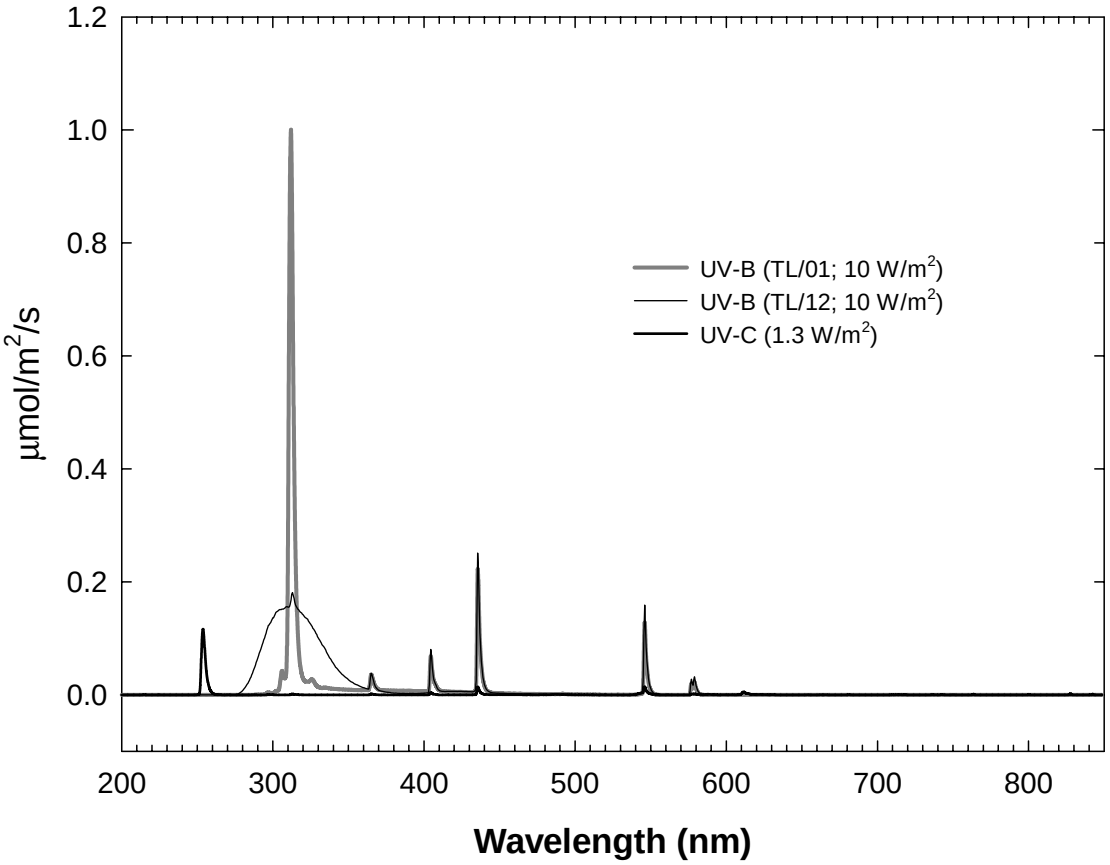

1.2
1.0
0.8
0.6
0.4
0.2
0.0
µmol/m²/s
200
300
400
500
600
700
800
Wavelength (nm)
UV-B (TL/01; 10 W/m²)
UV-B (TL/12; 10 W/m²)
UV-C (1.3 W/m²)

figure 1

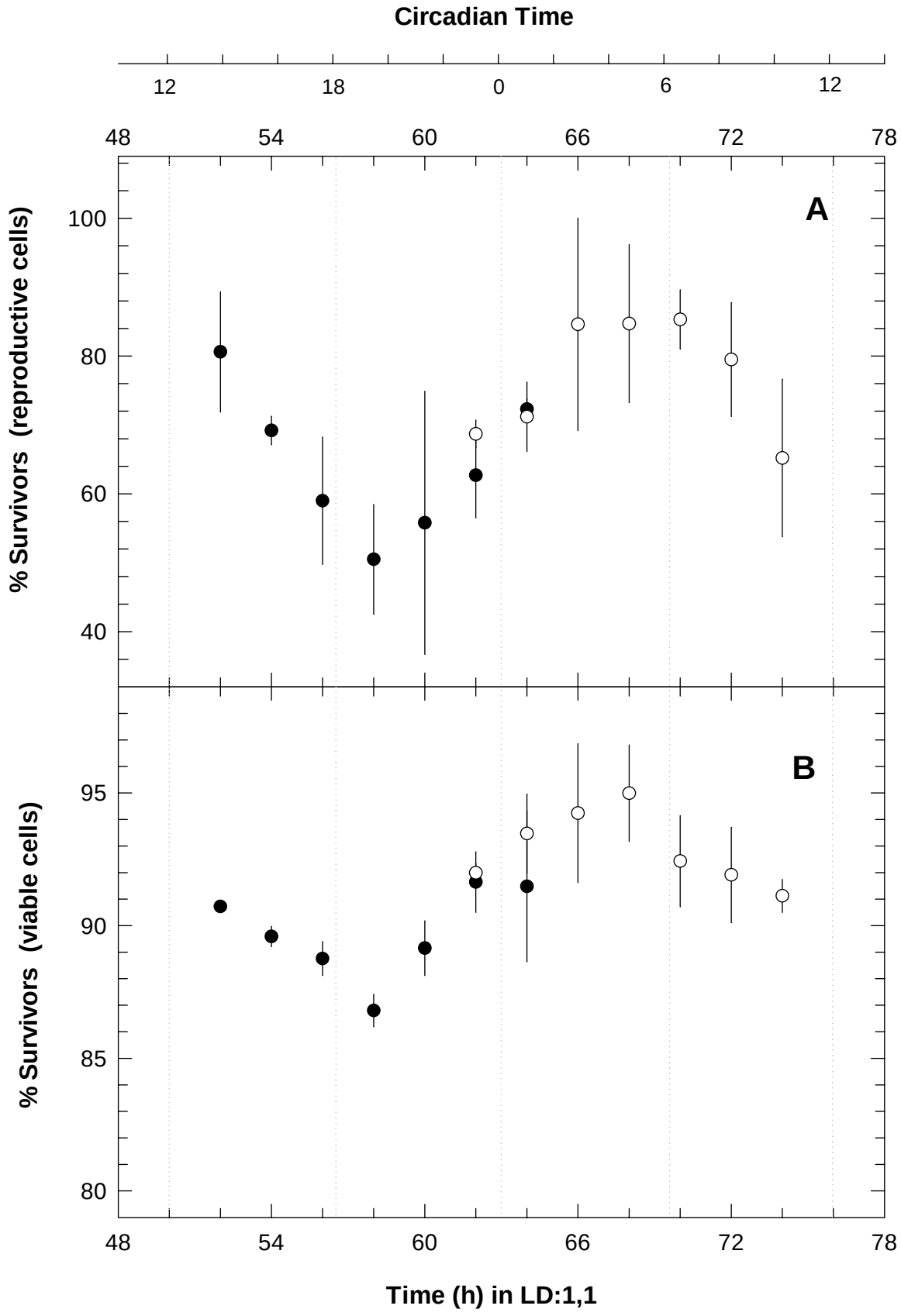

Circadian Time
12
18
0
6
12
48
54
60
66
72
78
A
100
80
60
40
% Survivors (reproductive cells)
B
95
90
85
80
% Survivors (viable cells)
48
54
60
66
72
78
Time (h) in LD:1,1

figure 2

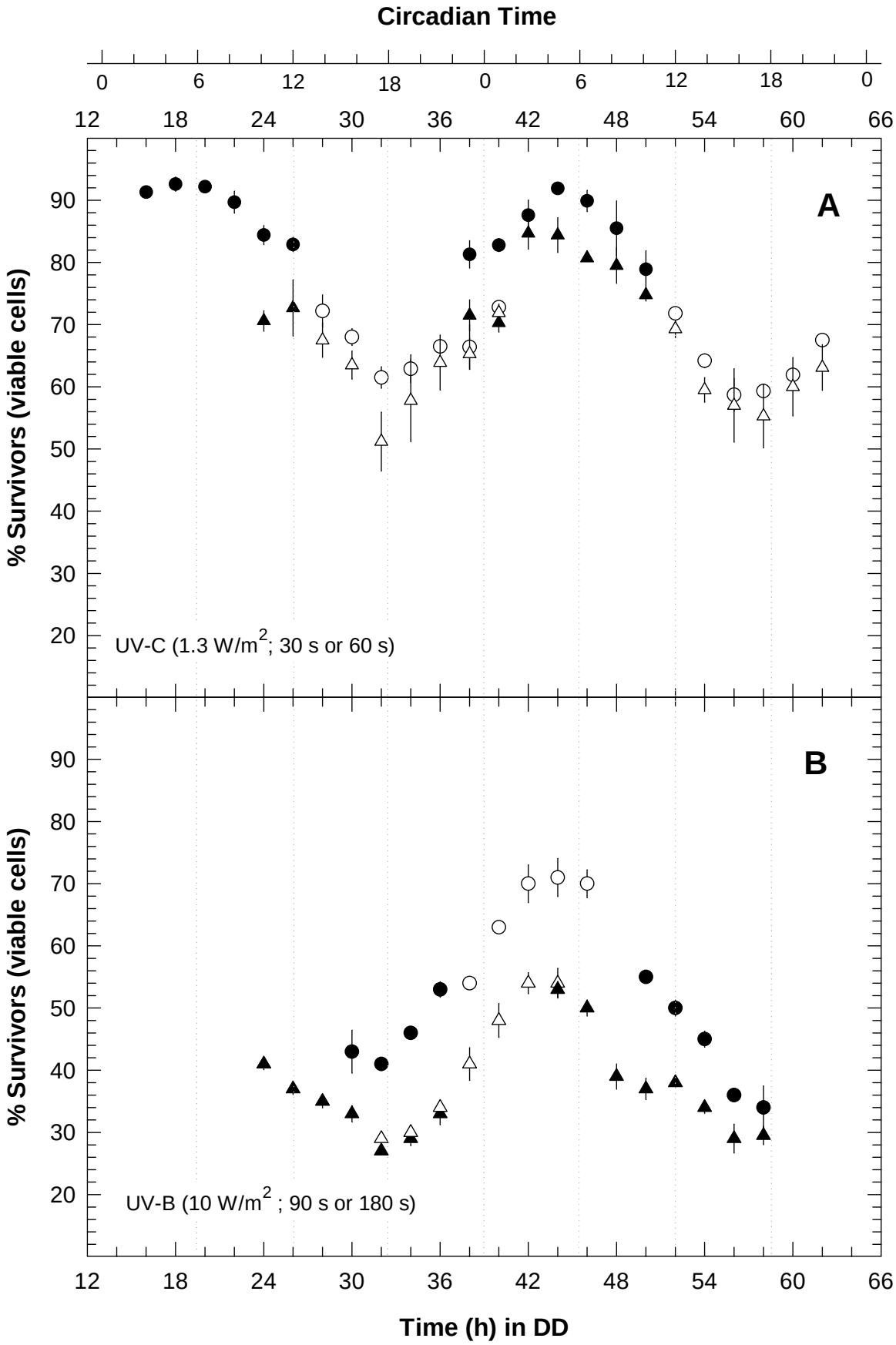

Circadian Time
0
6
12
18
0
6
12
18
0
12
18
24
30
36
42
48
54
60
66
A
% Survivors (viable cells)
90
80
70
60
50
40
30
20
UV-C (1.3 W/m2; 30 s or 60 s)
B
% Survivors (viable cells)
UV-B (10 W/m2 ; 90 s or 180 s)
Time (h) in DD

figure 3

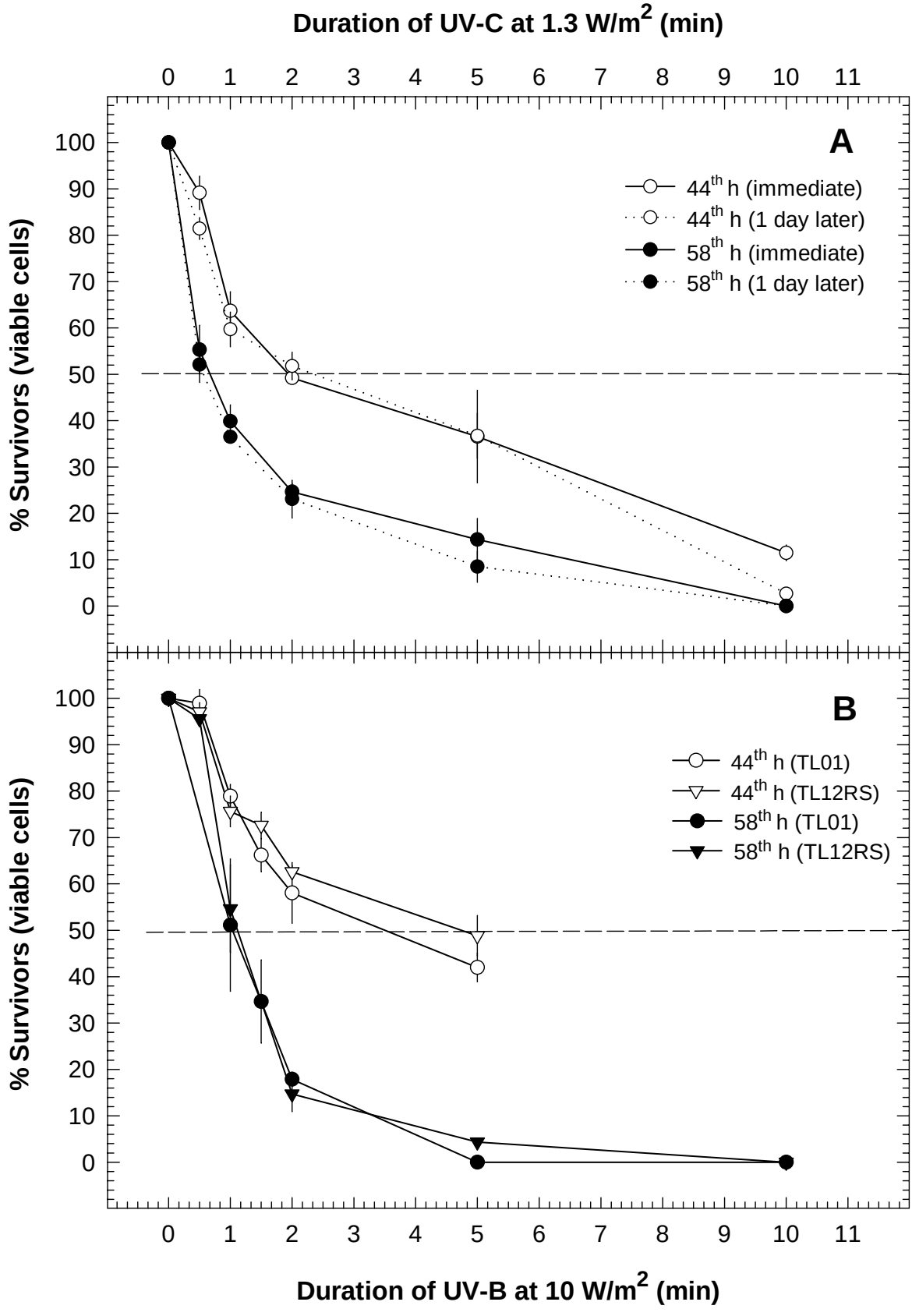

Duration of UV-C at 1.3 W/m2 (min)
0 1 2 3 4 5 6 7 8 9 10 11
A
44th h (immediate)
44th h (1 day later)
58th h (immediate)
58th h (1 day later)
% Survivors (viable cells)
100 90 80 70 60 50 40 30 20 10 0
B
44th h (TL01)
44th h (TL12RS)
58th h (TL01)
58th h (TL12RS)
% Survivors (viable cells)
100 90 80 70 60 50 40 30 20 10 0
0 1 2 3 4 5 6 7 8 9 10 11
Duration of UV-B at 10 W/m2 (min)

figure 4

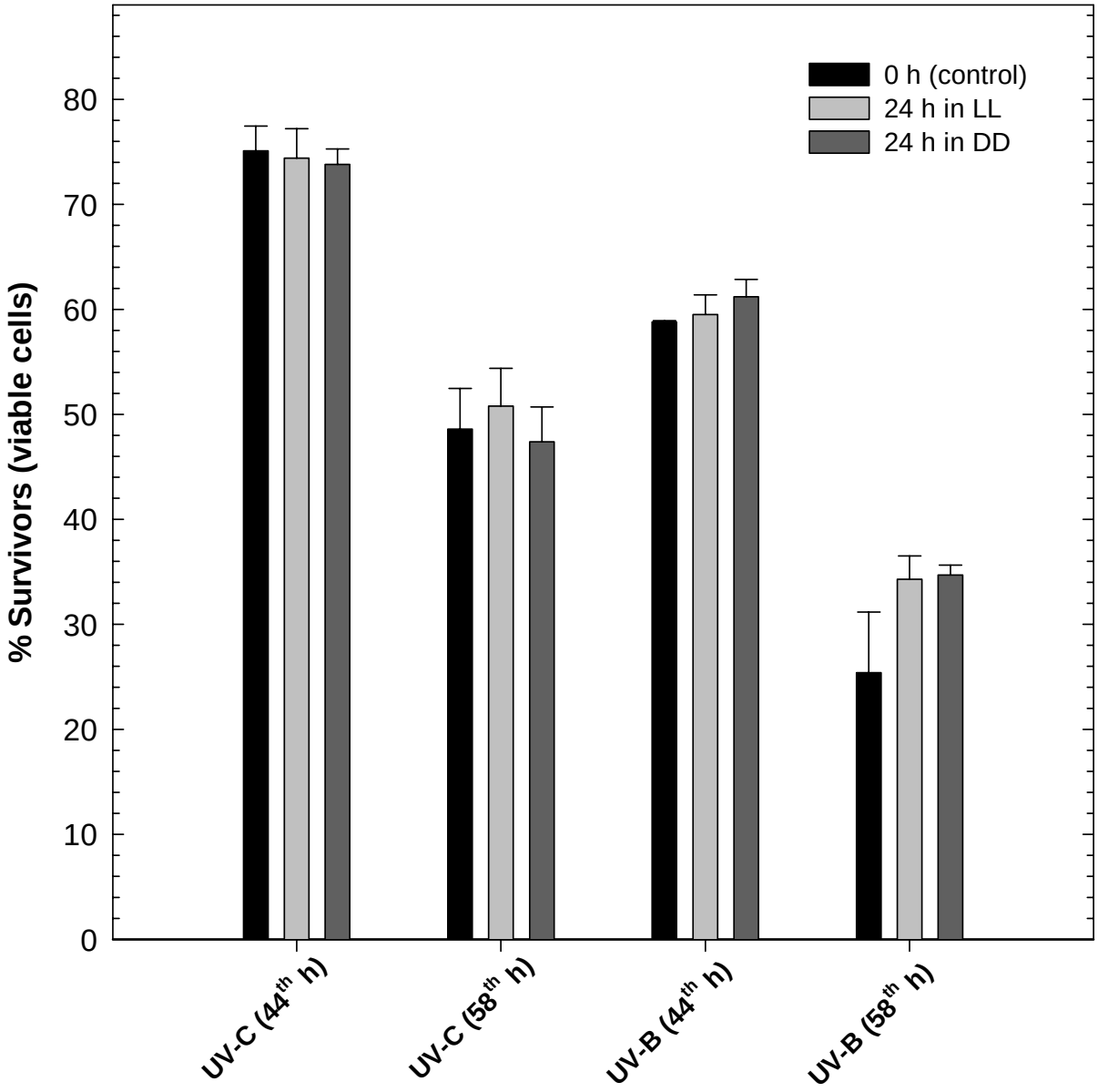

0 h (control)
24 h in LL
24 h in DD
% Survivors (viable cells)
0
10
20
30
40
50
60
70
80
UV-C (44th h)
UV-C (58th h)
UV-B (44th h)
UV-B (58th h)

figure 5

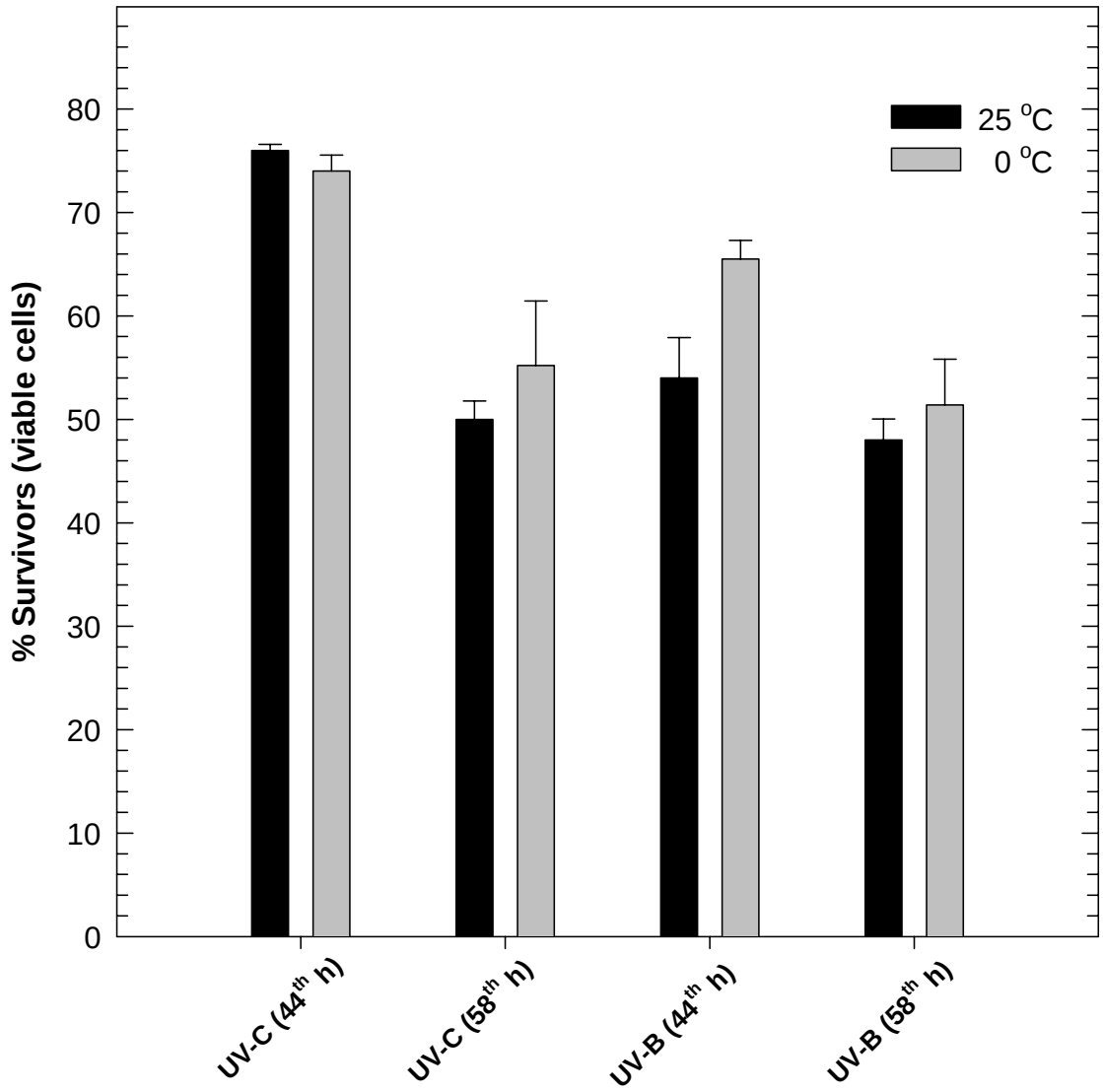

% Survivors (viable cells)
0
10
20
30
40
50
60
70
80
25 °C
0 °C
UV-C (44th h)
UV-C (58th h)
UV-B (44th h)
UV-B (58th h)

figure 6

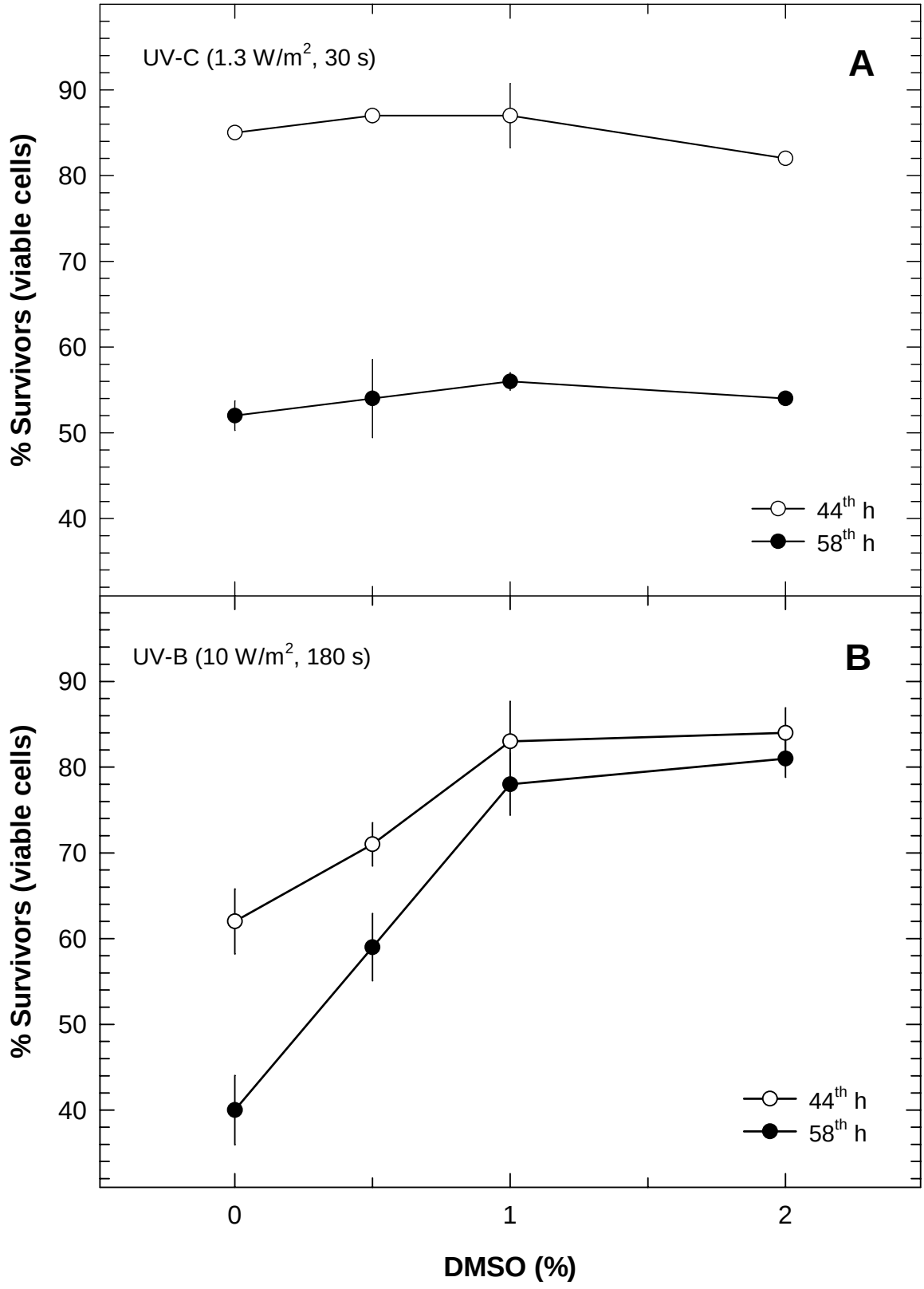

UV-C (1.3 W/m2, 30 s)
A
% Survivors (viable cells)
90
80
70
60
50
40
44th h
58th h
UV-B (10 W/m2, 180 s)
B
% Survivors (viable cells)
90
80
70
60
50
40
44th h
58th h
0
1
2
DMSO (%)

figure 7

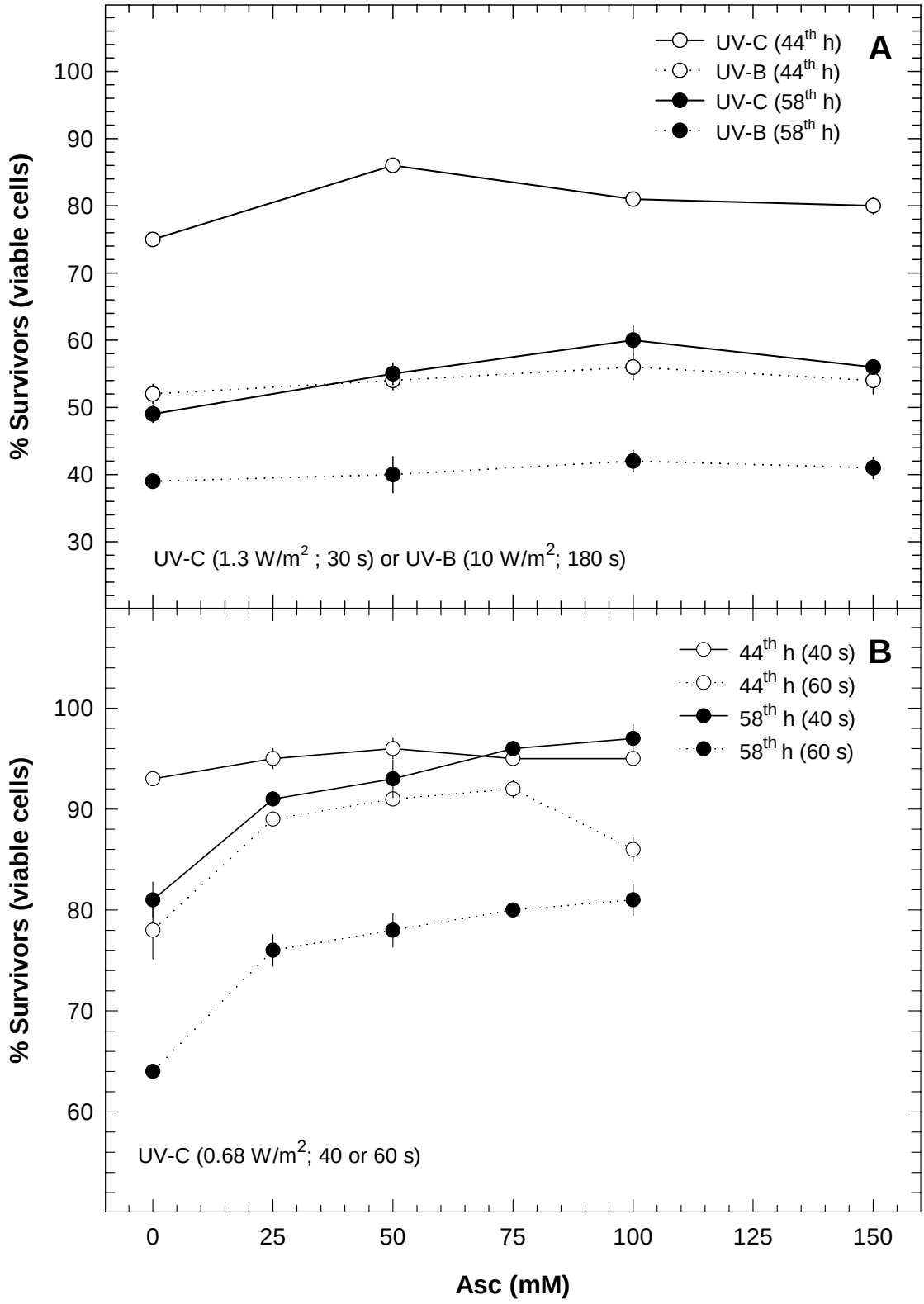

A
UV-C (44th h)
UV-B (44th h)
UV-C (58th h)
UV-B (58th h)
% Survivors (viable cells)
UV-C (1.3 W/m2 ; 30 s) or UV-B (10 W/m2; 180 s)
B
44th h (40 s)
44th h (60 s)
58th h (40 s)
58th h (60 s)
% Survivors (viable cells)
UV-C (0.68 W/m2; 40 or 60 s)
Asc (mM)

figure 8

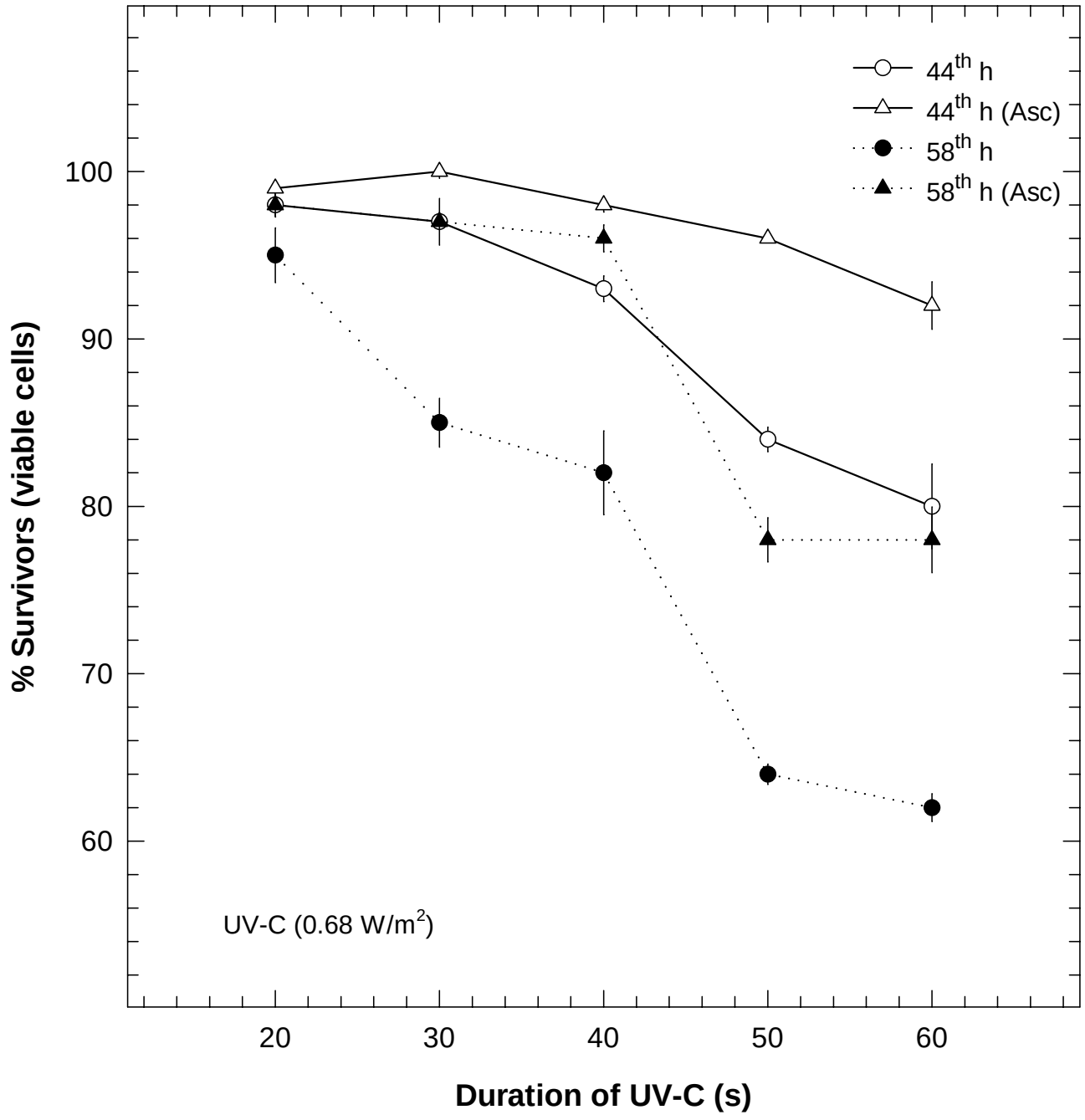
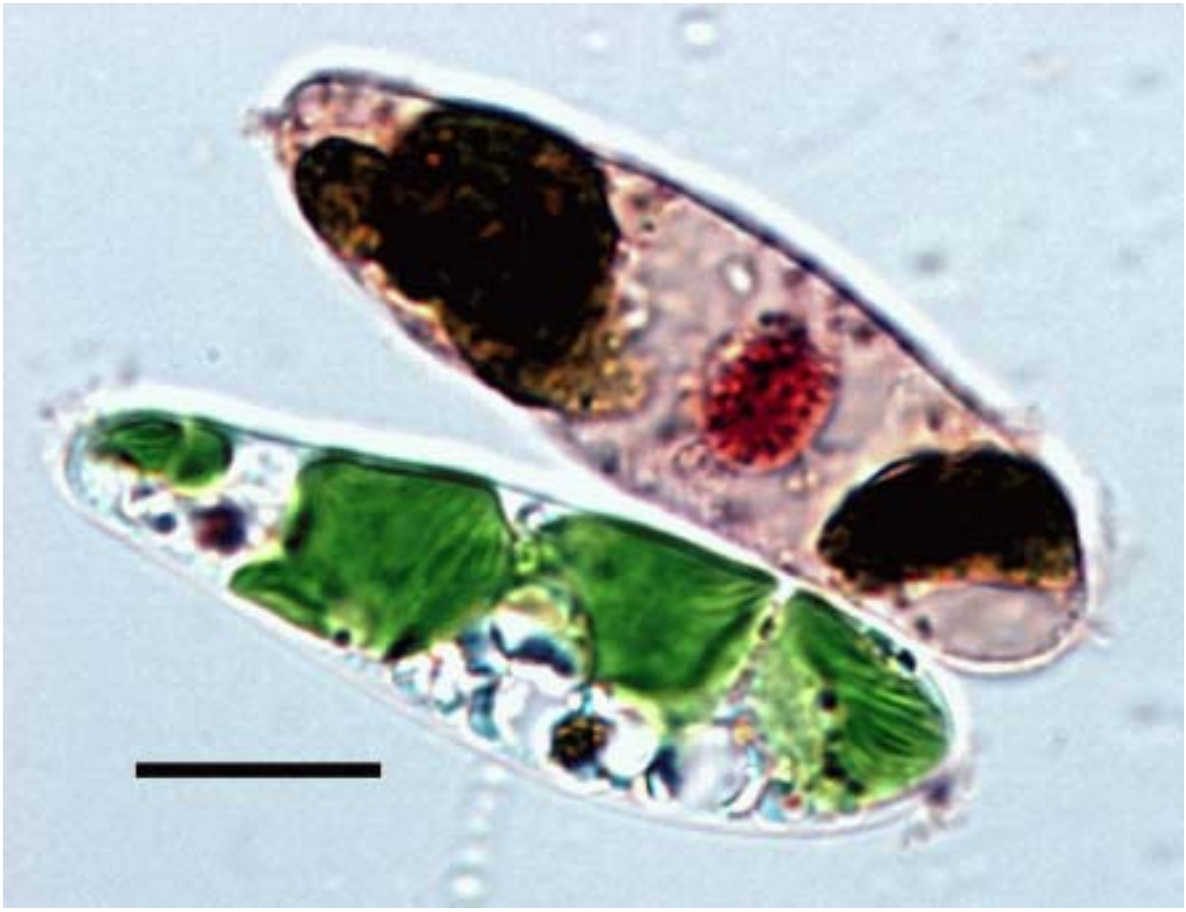

44th h
44th h (Asc)
58th h
58th h (Asc)
100
90
80
70
60
% Survivors (viable cells)
UV-C (0.68 W/m2)
20
30
40
50
60
Duration of UV-C (s)

figure 9



Appendix figure 1