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How Chlorella Species Isolated from Contrasting Habitats Respond to UV-B Induced Stress?

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Abstract. We hypothesize that algal species isolated from habitats with extreme environmental conditions would differ in their susceptibility to UV-B irradiation having better survival strategies. The aim of this study was to evaluate UV-B induced stress response of *Chlorella* species, isolated from different habitats: *Chlorella vulgaris* (Antarctic) - from soil in island Livingston, Antarctic, *Chlorella vulgaris 8/*1 (Thermophilic) - from thermal springs in region of Rupite, Bulgaria and *Chlorella kessleri* (Mesophilic) - from the Trebon collection. Unicellular green algae were chosen as a model organism because they are a robust model in genotoxicology due to the following reasons: photosynthetic eukaryotic organisms with typical for plants cell structure and genome organization; cell - organism with short life cycle - the response of a single cell is equivalent to the response of an individual organism; not expensive microbiological and molecular methods. *Chlorella* species were cultivated on TAP medium under standard conditions 23 °C ± 0.1° and 60 µmol m−2 s^{-1} in a growth chamber Phytotron GC 40. Cell suspensions in the end of the exponential/ beginning of the stationary phase were used. Cells were irradiated in BLX-254 (Life Technology, UV crosslinker, λ= 312nm). Cell response of *Chlorella* species was examined based on spot-test, micro-colonies assay, growth rate and DSB induction. The results demonstrated strong bioactivity of UV-B doses equal to or higher than 250 J/m^2. The magnitude of photoreactivation sectors revealed that *Chlorella* species are photoreactivation and dark-repair proficient but differ in their capacity to overcome damages induced by UV-B light. New data were provided concerning UV-B capacity to induce DNA double-strand breaks in *Chlorella* species. Based on the complex of methods used, it was established that according to their resistance to UV-B induced stress, the different species can be arranged in the following order: *Chlorella vulgaris* Antarctic > *Chlorella vulgaris 8/1* > *Chlorella kesseri*.

Key words: UV-B irradiation, *Chlorella*, DSBs, photo-reactivation.

Introduction

In recent decades, the sun's ultraviolet-B (280-315 nm) reaching the earth's surface has increased due to ozone depletion (Kshama & Agrawal, 2017). Ultraviolet-B (UV-B), being high energy can impact the biota at different levels causing various biological damage including sunburn, skin cancer, inhibition of immune responses etc (Gill et al., 2015; Rigo et al., 2015).

The bioactivity of UV-B irradiation results from both direct and indirect mechanisms involving endogenous sensitizers and the generation of active oxygen species. Primary radicals formed as a result of UV irradiation lead to the formation

of lipid radicals, which react with oxygen to produce lipid peroxyl radicals (Pouneva & Minkova, 2010; Medeiros et al., 2015).

Due to the potential impact of UV-B on human health, extensive research has been carried out in determining the mechanisms of UV-B induced damage in mammalian systems (Rigo et al., 2015). DNA is one of the key targets for UV-induced damage in a variety of organisms ranging from bacteria to humans (Sinha & Hader, 2002).

Although plants, as photosynthesizing organisms, have an absolute need for sunlight and are particularly sensitive to occurring changes, modern knowledge of the mechanisms of UV-B effects on plants is not as satisfactory as in mammals. At present state of knowledge, UV-B can affect many processes in plants - inhibition of photosynthesis, inactivation of enzymes, damaging DNA etc. (Wong et al., 2015).

Organisms respond to environmental impact by developing a series of physiological, biochemical and molecular strategies.

Previously, we have described that strains more resistant to various inducers of oxidative stress or isolated from contrasting habitats differ in their DSBs repair capacity, cells membrane stability, activation of antioxidant and/or chaperone systems (Chankova et al., 2002; 2013; 2014; Chankova & Yurina, 2012; 2016).

Following these findings here we hypothesize that algal species isolated from habitats with extreme environmental conditions would differ in their susceptibility to UV-B irradiation having better survival strategies. *Chlorella* species have been chosen because they are widely spread photosynthesizing unicellular eukaryotes with a short life cycle, cell structure and genome organization typical for plants so the results could be extrapolated to higher plants, with haploid genome - recessive mutations could be revealed at the next generation, routine inexpensive laboratory cultivation techniques could be applied, very suitable organism for molecular studies.

The aim of this study was to compare UV-B susceptibility of *Chlorella* species, isolated from different contrasting habitats at different levels: cellular and molecular.

Materials and Methods

Species and cultivation: Chlorella vulgaris Antarctic – isolated from soil in island Livingston, Antarctic, *Chlorella vulgaris 8/*1 - from thermal springs in the region of Rupite, Bulgaria, cultivated since 1975 in our lab, and *Chlorella kessleri* Mesophilic - from the Trebon collection.

Cell suspensions were cultivated on TAP (Tris Acetate Phosphate) medium (Harris, 1989) under continuous light of 60 µmol m−2 s^{-1} and a temperature 23 °C ± 0.1° in a Phytotron GC 40 growth chamber. Cell suspensions in the end of exponential and the beginning of the stationary phase (5-days old) were used.

UV-B Irradiation: Five-days old cell suspensions with a density $1x10^6$ cell/ml were irradiated with UV-B (λ= 312 nm) in BLX-254, Life Technology, UV crosslinker. The irradiation was done with doses in the range 50, 100, 250, 500 and 1000 J/m^2 in a dark or yellow light to prevent photo-reactivation. After the irradiation, samples were split in two- samples: "with" photo-reactivation (kept at continuous light of 60 µmol m^{-2} s^{-1} for 24 h) and "without" photo-reactivation (kept in a dark for 24h). After that, Petri dishes were cultivated in a Phytotron GC 40 growth chamber at temperature 23 °C ± 0.1° at continuous light of 60 µmol m^{-2} s^{-1}.

Genotypes resistance of species to UV-B was assessed based on several endpoints:

Spot-test (Harris, 1989) - 10µl of cell suspension irradiated with appropriate UV-B doses were pipetted on solid TAP medium in Petri dishes to form drops. Every drop contained 1000 cells. The Petri dishes were kept in the growth chamber at "with" and "without" photo-reactivation conditions.

Micro-colonies survival assay (Vlcek et al., 1987) - very rapid method allowing to obtain information concerning strains survival in a

next 72 hours after the UV-B irradiation. Observations were made under a microscope Amplival at a magnification of 16/40. The method is based on counting of the survived microcolonies and single none divided cells vs dead cells and microcolonies 72 hours after the irradiation with appropriate doses of UV-B.

Growth rate (Harris, 1989; Shevchenko, 1979) **-** this method provides useful information concerning population potential to restore its reproductivity and the rate of growth after provocative exposure (in our case UV-B irradiation. Observation was made microscopically using Amplival microscope at a magnification of 16/40. Cell density was counted microscopically on a hemocytometer (Burker) every 24 hr for 72 hours

Doses determining three levels of lethality - LD_{20}, LD_{50} and LD_{80} were calculated in order to compare species sensitivity to UV-B irradiation (Lidanski, 1988)

Photo-reactivation sectors (Harm, 1968, Serafin et al., 2003; Kiefer, 2012). The magnitude of photo-reactivation sectors (PRS) provides information about photo-reactivation proficiency or photo-reactivation deficiency of species.

DSBs induction and repair capacity by Constant field gel electrophoresis (CFGE)

Chlamydomonas reinhardtii protocol (Chankova and Bryant, 2002; Chankova et al., 2005) was optimized for *Chlorella* species. Additional step for cell wall disruption with sonicator BANDELIN Sonopuls HD 2070 was added due to the differences in the cell wall composition of *Chlamydomonas reinhardtii* and *Chlorella*.

Parameters for cell wall degradation were: *Chlorella vulgaris* (Antarctic species) - 3 minutes 4 cycles, 75% strength; *Chlorella kessleri* (mesophilic species) - 3 minutes, 2 cycles, 25% strength; *Chlorella vulgaris* 8/1 (thermophilic species) - 3 minutes 4 cycles, 25% strength.

Data analysis. The **e**xperiments were repeated at least three times using independently grown algal cultures. One-way ANOVA with Tukey multiple comparison test was performed to compare mean differences among genotypes (GraphPad Prism 6.04).

Results

Survival after UV-B irradiation

Spot-test was used for initial evaluation of the bioactivity of UV-B irradiation depending on the dose-range and the genotype. Reducing the intensity of spots was read at irradiation doses equal to or greater than 250J/m^2 in photo-reactivation conditions for both species – *Chlorella vulgaris* 8/1 and *Chlorella kessleri.* Only irradiation with two-fold higher dose - 500 J/m^2 resulted in slight reduction of spots intensity in *Chlorella vulgaris* Antarctic that is informative for highly expressed capacity of Antarctic *Chlorella vulgaris* to repair at light conditions damages induced by UV-B.

More clear differences in species response were expressed in samples "without" photo-reactivation. The most sensitive was *Chlorella kessleri*>*Chlorella vulgaris* 8/1>*Chlorella vulgaris* Antarctic (data not shown).

As a next step, the micro-colonies survival assay was performed in order to obtain more detailed information concerning species sensitivity to UV-B irradiation.

Cell survival data of samples with photo-reactivation presented in Fig. 1A show that this parameter was significantly reduced after UV-B irradiation with doses equal to or higher than 500J/m^2 in all three species. Comparing the slope of curves, it is evident that both species *Chlorella vulgaris* 8/1 and *Chlorella kessleri* follow the same trend. Spots test information was confirmed by those of micro-colonies survival assay – dose 1000 J/m^2 can induce around 100% lethality for *Chlorella vulgaris* 8/1 and *Chlorella kessleri*. Even at this high dose, *Chlorella vulgaris* Antarctic has survived, albeit with low frequency.

Further, we have evaluated species cell survival at conditions preventing photo-reactivation.

Cell survival of *Chlorella kessleri* was dramatically decreased comparing with those

of *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 in samples "without" photo-reactivation after 250 J/m^2 UV-B irradiation (Fig. 1B). Our data illustrate that *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 probably have a better dark-repair potential depending on the dose comparing with *Chlorella kessleri*. This trend is the same at higher doses only for *Chlorella vulgaris* Antarctic. The most sensitive was *Chlorella kessleri*>*Chlorella vulgaris* 8/1>*Chlorella vulgaris* Antarctic.

Based on our cell survival data after UV-B irradiation, three levels of lethality ($LD_{20,}$ LD_{50} and LD_{80}) were calculated. These criteria are good tools to compare genotypes resistance to different mutagenic factors, in our case UV-B irradiation. Looking at the Table 1 it is obvious that doses for Antarctic chlorella are significantly higher than those causing the same level of lethality, for *Chlorella vulgaris* 8/1 and *Chlorella kessleri,* following dose-dependent relationships. In samples "with" photo-reactivation, doses that can induce these three levels of lethality in both species *Chlorella vulgaris* 8/1 and *Chlorella kessleri* were approximately similar (Table 1).

Data presented in the same table for samples kept in a dark for 24 h ("without" photo-reactivation) demonstrate approximately similar doses determining LD_{20} and LD_{50} for both species *Chlorella vulgaris* 8/1 and *Chlorella kessleri*.

Based on our data we can speculate that *Chlorella vulgaris* Antarctic was less susceptible to UV-B radiation comparing with *Chlorella vulgaris* 8/1 and *Chlorella kessleri*.

Further, sectors of photo-reactivation (PRSs) calculated by three methods (Harm, 1968; Serafin et al., 2003; Kiefer, 2012) have revealed similar tendencies:

Comparison of the results following the procedure described by Kiefer (2012) reveals that *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 are characterized with dose-dependent decrease in the magnitude of PRSs. No such trend was found for *Chlorella kessleri*. As it is seen in a Table 1 the differences among average PRSs are not large but they could be a good reason to assume that *Chlorella kessleri* probably has a less pronounced dark repair and "relies" mainly on its photo-enzyme repair.

Similar trend (Table 2) was found using one more method (Harm, 1968). Results revealed dose-dependent decrease in the photo-enzymatic repair capacity of both species, isolated from habitats with extreme environment - *Chlorella vulgaris* Antarctic and Chlorella vulgaris 8/1 and approximately similar sectors for all the doses in *Chlorella kessleri* (Table 2).

Data obtained according to Serafin et al. (2003) are in a Fig. 2. The bars show the magnitude of area between curves "with" and "without" photo-reactivation.

We have calculated again slightly increased PRS for *Chlorella kessleri* and approximately similar PRSs for *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1.

Growth rate after UV-B irradiation

Our results show that in both *Chlorella* species - *Chlorella vulgaris* 8/1 and *Chlorella kessleri*, doses of 500 and 1000 J/m^2 can induce damages with lethal effect or such leading to full blocking of cell division - no doubling of cells was scored (Fig. 3A). Statistically significant correlation between both cell survival and growth rate was obtained for *Chlorella vulgaris* 8/1 and *Chlorella kessleri* - 0.895 and 0.912, respectively. In *Chlorella vulgaris* Antarctic at photo-reactivation conditions, cell division at doses of 500 and 1000J/m^2 was severely delayed.

In samples "without" photo-reactivation, cell division was completely inhibited. Single dead cells as well as dead micro-colonies were found after the irradiation with doses equal to or higher than 500 J/m^2. Strong decrease of doubling capacity was read in samples irradiated with 250 J/m^2 for *Chlorella kessleri*. The curves at Fig. 3B demonstrate that both species *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 isolated from extreme habitats, can overcome the UV-B induced stress to

some extent. Some doubling potential of cells in *Chlorella vulgaris* Antarctic population was obtained even after the irradiation with 500J/m^2.

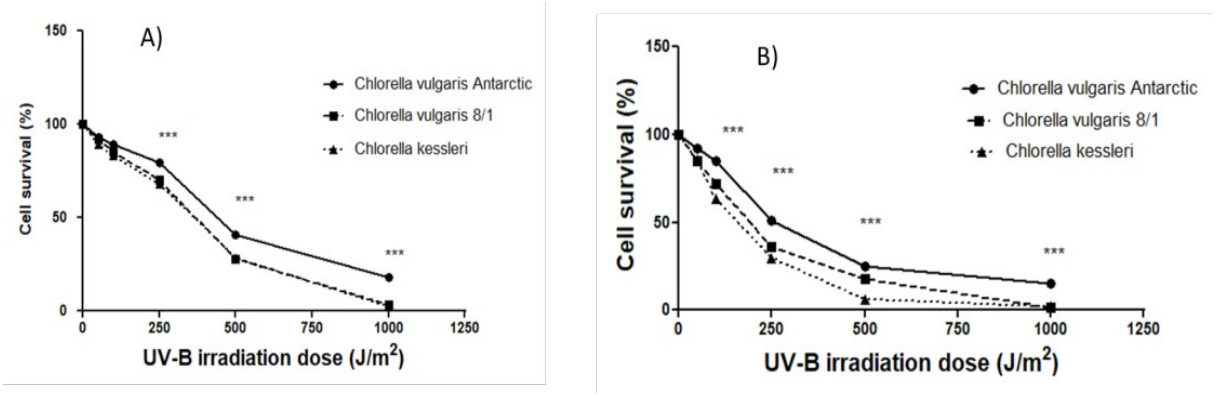

Fig. 1. Cell survival after UV-B irradiation at photo-reactivation conditions (A) and "without" photo-reactivation (B) of *Chlorella vulgaris* Antarctic, *Chlorella vulgaris* 8/1, *Chlorella kessleri* from 3 independently grown cell cultures. Where standard errors are not visible, they are equal to or less than the symbols on the graphs. The differences between *Chlorella vulgaris* Antarctic and the other two species are statistically significant *** p <0.001.

Table 1. UV-B doses determining three levels of lethality of Chlorella species, DMF and PRS. *Legend:* Data are averages from 3 independently grown cultures; PHR (+) represents samples grown at light - "with" photo-reactivation; PHR (-) - samples "without" photo-reactivation conditions; DMF – dose-modifying factor; PRS – photo-reactivation sector.

		Chlorella vulgaris Antarctic			*Chlorella vulgaris* 8/1			*Chlorella kessleri*		
		J/m$_2$	DMF	PRS	J/m$_2$	DMF	PRS	J/m$_2$	DMF	PRS
LD$_{20}$	(+) PR	239	2.07	0.52	138	2.12	0.53	120	2.03	0.51
	(-) PR	113			65			59		
LD$_{50}$	(+) PR	425	1.65	0.40	348	1.98	0.50	343	2.38	0.58
	(-) PR	257			175			144		
LD$_{80}$	(+) PR	940	1.32	0.24	627	1.37	0.27	626	1.88	0.47
	(-) PR	710			458			333		
∑ PRS			1.16			1.29			1.56	
Average PRS			0.39			0.43			0.52	

Table 2. Photo-reactivation sectors according to Harm (1968).

	LD_{20}	LD_{50}	LD_{80}
***Chlorella vulgaris* Antarctic**	0.590	0.393	0.252
***Chlorella vulgaris* 8/1**	0.540	0.493	0.275
Chlorella kessleri	0.525	0.575	0.472

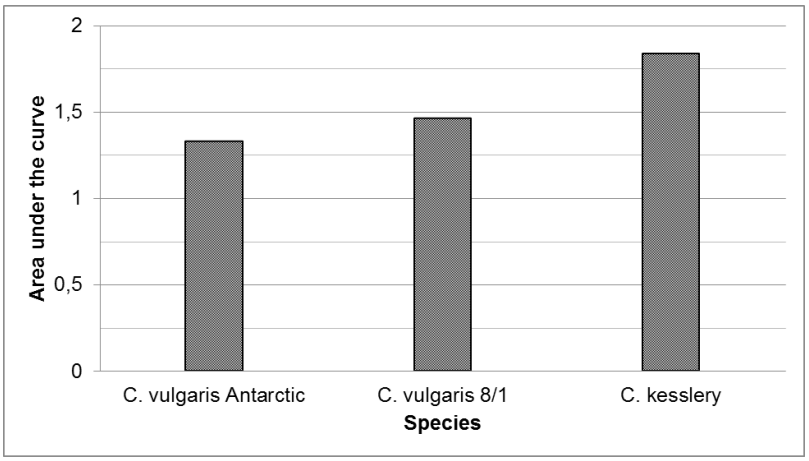

Fig. 2. Photo-reactivation sectors presented as Area under the curve based on Serafin et al. (2003).

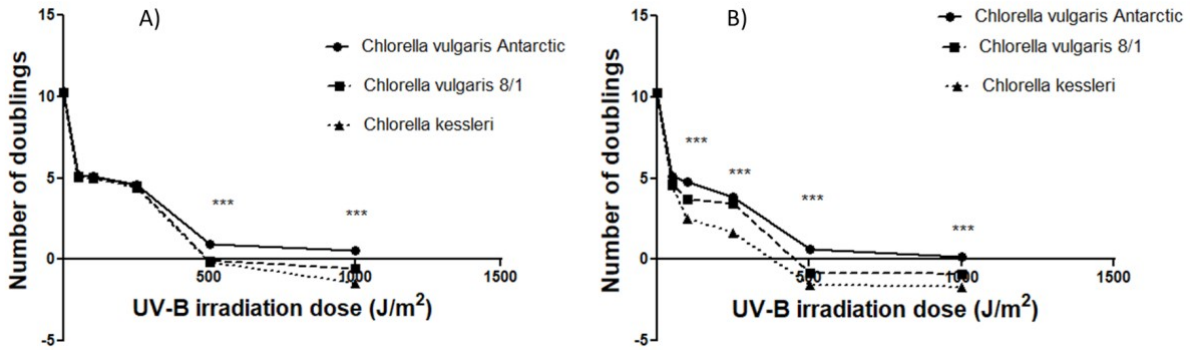

Fig. 3. Growth rate measured as number of doubled cells in samples "with" (A) and "without" (B) photo-reactivation. Data are averages from 3 independently grown cell cultures. Where standard errors are not visible, they are equal to or less than the symbols of the graphs. The differences between *Chlorella vulgaris* Antarctic and the other two species are statistically significant *** $p < 0.001$.

Again, in conditions without photo-reactivation species UV-B resistance could be arranged as follows: *Chlorella vulgaris* Antarctic > *Chlorella vulgaris* 8/1> *Chlorella kessleri*.

Statistically significant correlation between the cell survival and the growth rate was obtained for *Chlorella kessleri* – 0.914.

The information presented above was also confirmed using another method (Shevchenko, 1979). Data presented in Table 3 illustrate the same tendency described above.

Up to 250J/m^2, the three algal species have a similar capacity to cope with the harmful action of UV-B. Over 500 J/m^2 the difference is noticeable. Some potential to

recover cell population was found again for *Chlorella vulgaris* Antarctic.

In samples "without" photo-reactivation inhibition of cell division dependent on both the dose and genotype was revealed. The effect was most pronounced for *Chlorella kessleri*. Again some cell division was obtained for *Chlorella vulgaris* Antarctic.

These data have confirmed those obtained for cell survival. Again, *Chlorella vulgaris* Antarctic was shown as the most resistant and *Chlorella kessleri* as the most susceptible to UV-B irradiation.

Induction of DSB after UV-B irradiation

No statistically, significant differences were calculated among spontaneously occurred DSBs. DSBs, induced by UV-B in a dose range 50- 500 J/m^2 are presented in a Fig.4. All three *Chlorella* species respond to UV-B irradiation in a similar, dose dependent way up to a dose 250 J/m2. Doses higher 250 J/m^2 resulted to the formation of plateau for all three *Chlorella* species.

Chlorella vulgaris Antarctic and *Chlorella vulgaris* 8/1 isolated from habitats with extreme environment respond to UV-B irradiation in a similar way – approximately the same, lower DSBs levels from those measured in *Chlorella kessleri*.

Next, species DSBs repair capacity was evaluated when 24 h recovery time was given. Results revealed that when the recovery time is at optimal for cell growth conditions (Fig. 5A), the three species repair the UV-B induced DSBs in a similar manner.

Interestingly, in unfavorable conditions (Fig. 5B) while *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 follow the same trend of similar DSB repair capacity, *Chlorella kessleri* showed very low levels of DSB. Based on a correlation analysis, we can suppose that the low DSB levels measured after 24 h recovery time in unfavorable conditions are the result of huge DNA fragmentation rather than higher repair capacity.

Discussion

Climate changes and anthropogenesis contribute for increased levels of UV-B light that has an adverse impact on the biota, including microalgae. Microalgae are very important from ecological point of view as primary producers, as well as economically - as main sources of health supplements and pigments (Lai et al., 2019). Until now very reliable, information has been provided mainly on the negative effects of UV/UV-B on growth and development as well as on the photosynthetic apparatus of microalgae and plants (Pessoa, 2012; discussed in Apostolova et al., 2014; Ganapathy et al., 2017). A few studies report differences in the response of microalgae depending both on natural UV-irradiance of the environment (Pessoa, 2012) and specificity of habitats (discussed in Apostolova et al., 2014).

Table 3. Growth rate in samples "with" (+) and "without" (-) photo-reactivation. Legend: Data are averages from 3 independently cell cultures. Where standard errors are not visible, they are equal to or less than the symbols of the graphs. The differences are statistically significant *** p <0.001.

Doses	***Chlorella vulgaris* Antarctic**	***Chlorella vulgaris* 8/1**	***Chlorella kessleri***
	PHR (+) PHR(-)	**PHR (+) PHR(-)**	**PHR (+) PHR(-)**
control	2.38 2.37	2.30 2.29	2.28 2.20
50J/m^2	1.19 1.18	1.18 1.08	1.16 1.05
100J/m^2	1.18 1.10	1.16 0.85	1.14 0.58
250J/m^2	1.06 0.89	1.05 0.80	1.03 0.39
500J/m^2	0.22 0.15	-0.02 -0.19	-0.04 -0.36
1000J/m^2	0.13 0.03	-0.13 -0.21	-0.34 -0.39

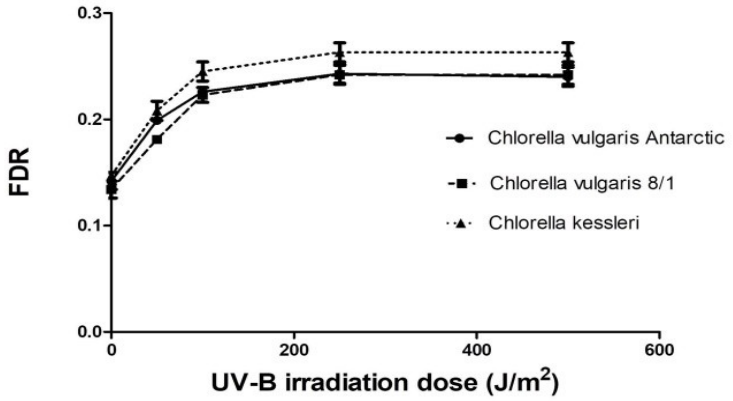

Fig. 4. DSB induction after UV-B irradiation with doses in the range 50 - 500 J/m^2. Data are averages from at least three independent experiments. Where standard errors are not visible, they are equal to or less than the symbols of the graphs.

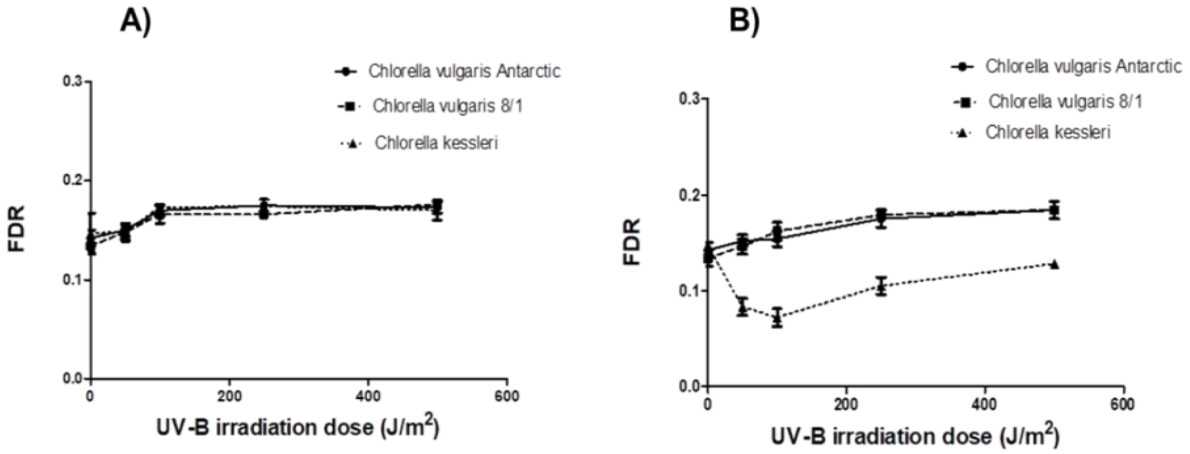

Fig. 5. DSB repair capacity after 24 h recovery time at: (A) optimal conditions (light, room temperature); (B) unfavorable conditions (dark, on ice). Data are averages from at least three independent experiments. Where standard errors are not visible, they are equal to or less than the symbols of the graphs.

UV-B irradiation directly or indirectly via generation of ROS may induce different types of DNA lesions - cyclobutane pyrimidine dimmers (CPD) and 6-4-photoproduct (6-4PP), DNA/DNA and DNA protein cross-links, double-strand breaks (DSB) and single-strand breaks (SSBs) leading to disruption both DNA structure and the processes of replication and transcription (Rastogi et al., 2010; He et al., 2002; Lesser, 2008; Rastogi et al., 2020). It has been supposed that DNA DSB and SSBs are formed not because of the direct absorption of UV radiation but rather as the consequence of the attempted repair of UV radiation-induced base damage in DNA – NER dependent manner (Wakasugi et al. 2014). Photolesions induced by UV-B irradiation could be overcome by a number of DNA repair mechanisms, including photo-reactivation, nucleotide excision repair (NER), base excision repair (BER), recombinational repair and post replication repair (Smith & Mpoloka, 2008; Jones & Baxter, 2017; Gill et al., 2015; Yin et al., 2017).

In the present work, we have attempted to broaden our understanding of the variety

of types of damage induced by UV-B and capacity of *Chlorella* species, isolated from contrasting habitats to repair these damages.

The first step of our investigation was to compare cell survival and growth rate of species at photo-reactivation and none photo-reactivation conditions. Photo-reactivation (PHR), the so called "light repair," is a very old, evolutionary developed mechanism to overcome harmful effect of solar radiation using blue to near-UV light energy to repair UV-induced lesions - CPDs or (6-4) PPs, by directly rearranging bonds (Jones and Baxter, 2017). Comparing UV-B resistance based on doses determining three levels of lethality at conditions "with" and "without" photo-reactivation *Chlorella* species were arrange in the following way: *Chlorella vulgaris* Antarctic > *Chlorella vulgaris* 8/1~ *Chlorella kessleri*. *Chlorella vulgaris* Antarctic was more resistant to UV-B irradiation comparing with *Chlorella vulgaris* 8/1 and *Chlorella kessleri*. These results are in a good agreement with those of Pessoa (2012) where isolates of the marine microalga *Chattonella marina* (Raphidophyte) from Australia exhibits higher tolerance to high intensities of visible light than *C. marina* collected from Japan waters.

Looking at the slopes of survival curves and LD levels we can say that the three species are photo-reactivation and dark-repair proficient with the most pronounced capacity for *Chlorella vulgaris* Antarctic. Analysing the magnitude of photo-reactivation sectors (PRS) we have found the same trend using three methods good enough for such purpose. The magnitude of ∑PRS expressing the space between both survival curves - "with"and "without" photo-reactivation slightly increases from *Chlorella vulgaris* Antarctic to *Chlorella vulgaris* 8/1 and is higher for *Chlorella kessleri*. This finding show better expressed dark repair capacity of *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1. Probably mesophilic *Chlorella kessleri* is mostly dependent on the photo-reactivation and probably with impaired dark repair.

The last step of our investigation was to evaluate DSBs repair capacity of species because it is known that the generation of SSBs and DSBs in UV-B irradiated cells, is observed extensively as a result of transcription/replication blockage (Rastogi et al, 2010; Marabini et al., 2020). We have measured similar quantities of spontaneously arisen DSBs for the species investigated by us. In the levels of DSBs induced by different UV-B doses, we did not find any differences that mean similar DNA susceptibility. Having in mind the present state of knowledge that genotypes resistance is rather related to repair capacity than to primary induced damages we were interested to compare the repair capacity of species. At conditions, not preventing DSBs repair no statistically significant differences among the species were found. In the case when post irradiation conditions prevent DSBs repair approximately similar levels of DSBs were measured for *Chlorella vulgaris* Antarctic and *Chlorella vulgaris* 8/1 and strong DNA degradation for *Chlorella kessleri*.

Conclusion

Our finding provides additional information concerning cellular and molecular differences of *Chlorella* species, isolated from contrasting habitats. Species investigated by us differ in their cell survival, growth rate, photoreactivation, dark and DNA double-strand breaks repair capacity. Both species isolated from extreme habitats are photo and dark repair proficient, while *Chlorella kessleri* is probably with impaired dark repair. UV-B induction of DSBs was confirmed.

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