PHOTOSYNTHESIC PERFORMANCE AND DNA MUTATION OF TROPICAL, TEMPERATE AND POLAR *Chlorella* SPECIES (CHLOROPHYTA) SUBJECTED TO SHORT-TERM UVR STRESS

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a

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ABSTRACT

The increase in ground ultraviolet radiation (UVR) levels due to ozone depletion is one of the seriously debated issues among environmental scientists. UVR can cause detrimental biological impacts to photosynthetic organisms. The current research focused on the effects of short-term UVR stress on the (1) photosynthetic responses and the (2) DNA mutation in *Chlorella* species from tropical, temperate, Antarctic and Arctic regions. In this study, photosynthetic performance of *Chlorella* post UVR treatment was determined using Pulse-Amplitude-Modulation (PAM) fluorometry, which is based on the determination of chlorophyll fluorescence. During photosynthesis, a small amount of the solar energy is absorbed and transformed into chemical energy while the rest are dissipated as heat and released as fluorescence. An increase in one of the process will result in a decrease in the other two as these three rate constants are in equilibrium. Thus, changes in photosynthesis efficiency and heat dissipation can be detected by measuring the yield of chlorophyll fluorescence, and this can also be used as an early indicator of stress. The photosynthetic parameters determined in this study by using PAM flurometry are: maximum quantum yield (Fv/Fm), photosynthetic efficiency (α), maximum electron transport rate (rETRm) and photoadaptive index (Ek). On the other hand, DNA mutation can be assessed via the Random Amplified Polymorphic DNA (RAPD) analysis which detects genetic variation ranging from changes in a single nucleotide to complex chromosomal rearrangements, by comparing the amplification products generated from the control (untreated sample) and treated samples. Three light treatments: (i) control (Photosynthetic active radiation, PAR), (ii) PAR+UVA (UVA stress) and (iii) PAR+UVA+UVB (UVB stress) were conducted over a continuous, five-hour duration in this study. *Chlorella* species subjected to PAR (control) treatment did not show significant photosynthetic dysfunction, as reflected by the insignificant change in the values of Fv/Fm, α, rETRm and Ek. However, UVB radiation has an apparent negative impact on the mentioned

photosynthetic parameters in all strains whereas only tropical isolate showed significant photosynthetic effect under UVA treatment. In addition, no significant changes were observed for the photosynthetic pigment contents (chlorophyll *a* and carotenoids) and chl *a*: carotenoids ratio in all treatments. Although no significant photosynthetic dysfunction was detected in all *Chlorella* strains subjected to UVA treatment, RAPD analysis revealed DNA mutation caused by both UVA and UVB treatments in all strains. In addition, the photosynthetic performance of *Chlorella* spp. appeared origin-dependent, which could be important in ensuring optimal survivability in different habitats.

ABSTRAK

Peningkatan radiasi ultraungu (UVR) akibat penipisan lapisan stratosferik ozon merupakan salah satu isu yang dibincang hangat oleh penyelidik-penyelidik alam sekitar. UVR mendatangkan impak biologi yang besar kepada organisma yang fotosintetik. Kajian ini memfokus pada kesan-kesan tekanan UVR jangka pendek ke atas: I) tindakbalas fotosintesis dan 2) mutasi DNA pada mikroalga *Chlorella* yang diperoleh dari kawasan tropika, kawasan beriklim sederhana, Antartik and Artik. Dalam kajian ini, prestasi fotosintesis *Chlorella* terhadap pendedahan pada UVR dinilai menggunakan kaedah *Pulse-Amplitude-Modulation* (PAM) *fluorometry* yang berdasarkan floresens klorofil. Semasa fotosintesis, sejumlah kecil tenaga suria yang diserap akan ditukar menjadi tenaga kimia manakala selebihnya akan dibebaskan sebagai haba dan floresens. Disebabkan oleh keseimbangan antara ketiga-tiga proses tersebut, peningkatan dalam salah satunya akan menyebabkan penurunan dalam kedua-dua proses yang lain. Maka, perubahan dalam prestasi fotosintesis dan pembebasan haba boleh dikesan melalui kuantifikasi floresens klorofil dan dijadikan indikasi tekanan yang awal. Dalam kajian ini, parameter fotosintesis yang dikaji menggunakan PAM *fluorometry* ialah: *Maximum quantum yield* (Fv/Fm), *photosynthetic efficiency* (α), *maximum electron transport rate* (rETRm) dan *photoadaptive index* (Ek). Manakala, mutasi DNA boleh dikesan melalui analisis *Random Amplified Polymorphic DNA* (RAPD). RAPD dapat mengesan variasi genetik dari perubahan satu nekleotit sehingga penyusunan semula kompleks kromosom melalui perbandingan produk amplifikasi dari sampel kontrol dan sampel yang didedahkan pada tekanan. Kultur-kultur dalam kajian ini telah didedahkan kepada tiga jenis eskperimen cahaya secara berterusan selama lima jam, iaitu: (i) kontrol (radiasi aktif fotosintesis, PAR), (ii) PAR+UVA (tekanan UVA) and (iii) PAR+UVA+UVB (tekanan UVB). Prestasi fotosintesis (Fv/Fm, α, rETRm dan Ek) tidak terjejas secara ketara akibat pendedahan pada PAR (kontrol). Sebaliknya, prestasi fotosintesis menurun setelah pendedahan pada UVB. Bagi dedahan

UVA, prestasi fotosintesis tidak menurun pada *Chlorella* dari tropika sahaja. Selain itu, tiada penyusutan komposisi pigmen (klorofil *a*, karotenoid dan nisbah klorofil *a*:karotenoid) diperhati berikutan rangsangan UVR. Walaupun pendedahan pada UVA tidak menjejaskan prestasi fotosintesis pada keempat-empat *Chlorella* tetapi analisis RAPD menunjukkan mutasi DNA yang disebabkan oleh UVA dan UVB atas kesemua *Chlorella*. Keputusan menunjukkan prestasi fotosintesis *Chlorella* adalah bergantung kepada tempat asalnya, dan aspek ini adalah penting untuk kemandirian optima di habitat berlainan.

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LIST OF SYMBOLS AND ABBREVIATIONS

ATP	:	Adenosine triphosphate
BBM	:	Bold's Basal Medium
Chl	:	Chlorophyll
Chl a	:	Chlorophyll a
DNA	:	Deoxyribonucleic acid
Ek	:	Photoadaptive index
ETR	:	Electron transport chain
Fv/Fm	:	Maximum quantum yield
mg	:	Milligram
mg/L	:	Milligram per liter
min	:	Minutes
mL	:	Milliliter
nm	:	Nanometers
NPQ	:	Non-photochemical quenching
OD_{620nm}	:	Optical density at 620nm
PAM	:	Pulse amplitude modulation
PAR	:	Photosynthetic active radiation
PCR	:	Polymerase Chain Reaction
PSI	:	Photosystem I
PSII	:	Photosystem II
RAPD	:	Random Amplified Polymorphic DNA
*rbc*L	:	Large subunit of the ribulose-biphosphate carboxylase
rETRm	:	Maximum electron transport rate
ROS	:	Reactive oxygen species
rpm	:	Rotation per minute
TAE	:	Tris-Acetate-EDTA
UMACC	:	University of Malaya Algae Culture Collection
UVA	:	Ultraviolet radiation-A
UVB	:	Ultraviolet radiation-B
UVC	:	Ultraviolet radiation-C
UVR	:	Ultraviolet radiation
%	:	Percent
µL	:	Microliter
$\mu molphotons^{-1}m^{-2}$	:	Micro mole per meter square per second
^{o}C	:	Degree celcius
w/v	:	Weight per volume percent
Wm^{-2}	:	Watt per meter square
α	:	Photosynthetic efficiency
18S rDNA	:	Small subunit of rDNA gene

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Discovery of spring time ozone depletion over Antarctica by the British Antarctic Survey (BAS) Scientists in 1984 (Farman et al., 1985; Stolarski et al., 1986) brought ultraviolet radiation (UVR) studies to a new milestone. Müller et al. (1997) reported the first clear occurrence of an "Arctic ozone hole" during the winter of 1995 to 1996 whereas the reduction of the ozone layer in mid-latitude was observed since the late 1970s (Antón et al., 2011). The depletion of the ozone layer is one of the major causes of increased ultraviolet radiation (UVR) arriving at the Earth's surface (Bhattacharya et al., 2012).

UVR consists of UVA, UVB and UVC (Han et al., 1998). UVC is absorbed by the stratosphere and thus does not reach the Earth's surface. However, UVA and UVB could result in numerous biological impacts to animals and plants. In plants, this includes failure in photoprotection, damages to the photosystem II, decrease in growth, fatty acid profile and superoxidese dismutase activity (Heraud and Beardall, 2000; Turcsanyi and Vass, 2000; Wong et al., 2007; Wong et al., 2011). Pyrimidine (6-4) pyrimidine dimers and (5-6) cyclobutyl pyrimidine dimers are common products caused by UV-induced DNA damage (Friedberg et al., 1995). These damages may be sensitive parameters to monitor UV effects even when the UVR-induced photoinhibition is not detected (Lud et al., 2001). UVB also causes cell-related damages, that is, the degradation of D1 or D2 heterodimer, direct molecular damage and induction of reactive oxygen species (ROS) (Meador et al., 2009; Hideg et. al., 2013; Marija and Dieter, 2014). Occasionally, UVA does exert positive effects on organisms, including the induction of photorepair and photoreactivation (Dionisio-Sese, 2010) as well as the stimulation of photosynthesis and growth in algae (Xu and Gao, 2010).

The regulation of the photosynthetic apparatus is for protecting cells against damaging radiation, as well as maximizing the light-harvesting ability for optimum photosynthesis (Goncalves et al, 2001). The deleterious effects of elevated UVB on photosynthetic activities in microalgae has been documented in several publications, including photoinhibition (Villafañe et al. 2008), degradation of photosystem II, D1 and D2 proteins (Bouchard et al. 2006, Vass, 2012) and the reduction in Rubisco activity (Bouchard et al. 2008). After being absorbed by chlorophyll molecules, radiant energy is either: (i) utilized for photosynthesis, (ii) re-emitted as chlorophyll fluorescence, or (iii) dissipated as heat (Maxwell and Johnson, 2000). Hence, by measuring the yield of chlorophyll fluorescence, initial photosynthesis-related stress can be indicated via the changes in photosynthetic efficiency and heat dissipation (Pedrós et al., 2008). The Pulse-Amplitude Modulated Fluorometer (PAM) is a useful device to measure the photosynthetic efficiency of organisms such as macroalgae (Bischof et al., 2000; Lud et al., 2001, Runcie and Riddle 2006), diatoms (Petrou et al., 2011; Reeves et al., 2011; Boelen et al., 2011) as well as green microalgae i.e. *Nannochloropsis oculata* Hibberd, *Chlorella vulgaris* Beijerinck, *Dunaliella salina* Teodoresco, *Chlamydomonas eugametos* Moewus and *Tisochrysis lutea* Bendif & Probert (Heraud and Beardall, 2000; Herlory et al., 2013; Chazalon et al., 2014; Ng et al., 2014; Szabó et al., 2015) .

Individuals from different habitats are known to display varying degrees of sensitivity and response towards UVR. Cells that are fit to withstand high UV radiation posses several key protective mechanisms, including the limiting of light absorption to minimize damage, activation of non-photochemical quenching (NPQ) mechanisms, stimulation of non-assimilatory electron flow and increased enzymatic scavenging of oxygen radicals (Anderson et al., 1995; Eggert et al., 2006). Several studies reported that light and UVR sensitivity of microalgae are strain-dependent, or vary biogeographically (Wong et al., 2007; Reeves et al., 2011; Ng et al., 2014). Marshall

and Newmann (2002) postulated that UVR sensitivity is related to the natural UV-irradiance of its original habitat whereby isolates from the tropics that are normally exposed to high intensity of visible light and UVR would exhibit higher light tolerance.

Although the effect of UVR on the photosynthetic performance of microalgae has been extensively studied (Heraud and Beardall 2000; Gao et al. 2008; Pedrós et al. 2008; Reeves et al. 2011), few studies comparing the response of microalgae from different geographical regions to UVR stress have been reported. In the present study, the effect of UVR stress on the photosynthesis of tropical, temperate, Antarctic and Arctic *Chlorella* species was assessed by Pulse Amplitude Modulation (PAM) fluorometry in addition to photosynthetic pigment profiles. Molecular analysis was used to confirm the species of *Chlorella* strains used in this study. Random Amplified Polymorphism DNA (RAPD) assay was used to detect the DNA mutation caused by UVR exposure.

The objectives of this study are:

1. To investigate the photosynthetic performance of *Chlorella* sp. from different geographical regions (tropical, temperate, Antarctica and Arctic) when exposed to short-term ultraviolet radiation (UVR) stress.
2. To determine the changes in photosynthetic pigments of these *Chlorella* sp. in response to short-term UVR stress.
3. To study the DNA mutation of these *Chlorella* sp. after short-term UVR radiation.

The research approach of the present study is as follows:

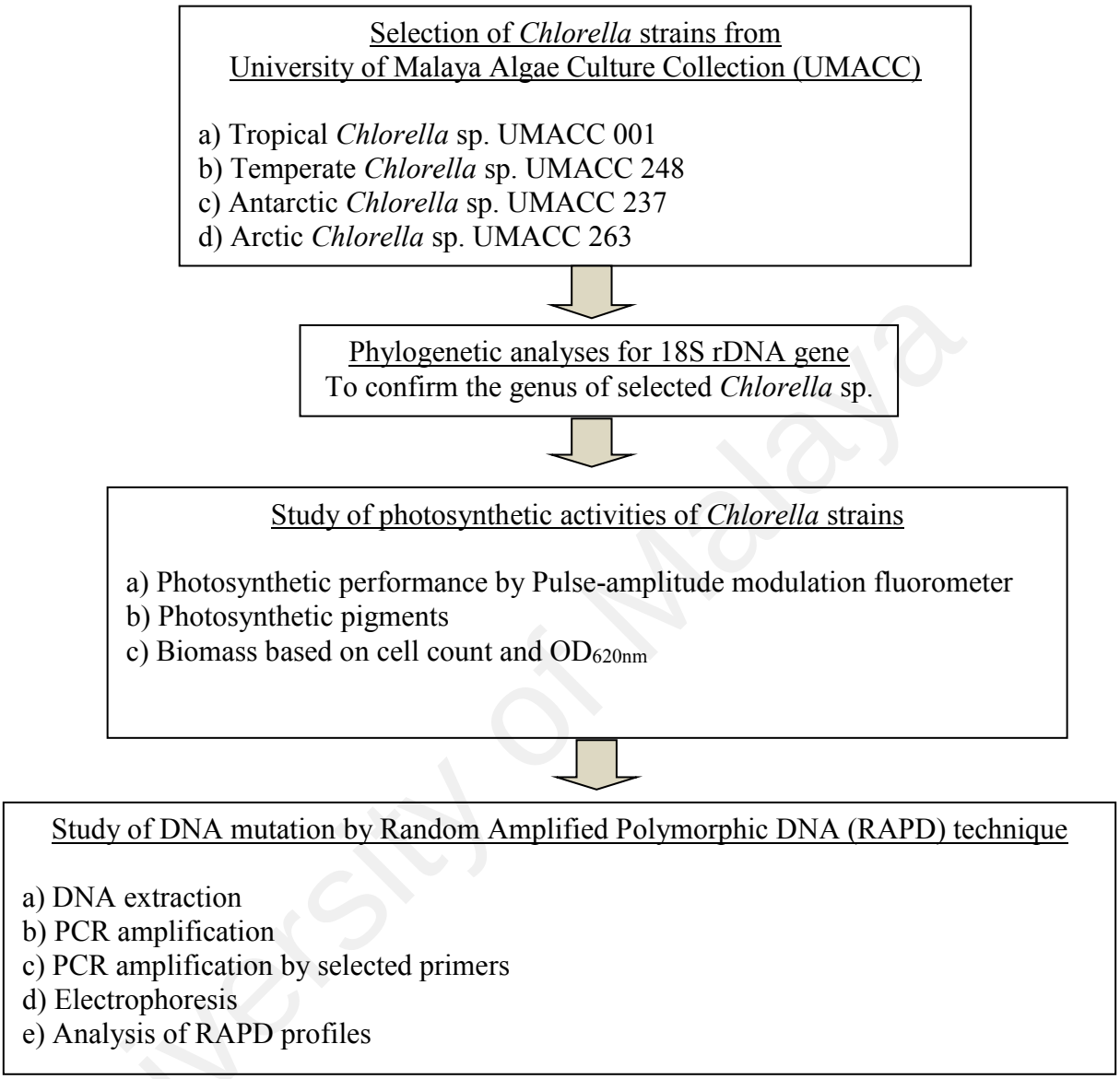


Figure 1.1: Outline of Research Approach

CHAPTER 2: LITERATURE REVIEW

2.1 *Chlorella* species

2.1.1 Introduction to *Chlorella* sp.

Chlorella vulgaris Beijerinck is a genus of single-celled green algae that are commonly found in freshwater and marine habitats (Guiry and Guiry, 2015). According to Hoek et al. (1995), *Chlorella* is classified in the kingdom of Chlorobionta, division Chlorophyta, subdivision Chlorophyceae, class Chlorococcales and order Chlorellales. Based on molecular data corroborated by biochemical evidences, Huss and Sogin (1990) and Huss et al. (1999) showed that *Chlorella* was split into numerous groups spreading across the classes Chlorophyceae and Trebouxiophyceae.

Serving as the model organisms, members of the genus *Chlorella* are among the most studied unicellular green algae (Huss and Sogin, 1990). Photosynthesis studies using *Chlorella* was started in the 1940's, when Melvin Calvin used *Chlorella* strains to elucidate light-independent reactions of photosynthesis, which is now known as the Calvin Cycle (Benson and Calvin, 1948).

Chlorella is an important oxygenic phototrophic green alga in the ecosystem which serves as a primary producer as well as contribues to inorganic carbon fixation and autotrophic energy production. This allows development of food webs (Morgan-Kiss et al. 2006). Green algae and dinoflagellates constitute 12% of the community abundance in the Arctic (He et al., 2005). Kim et al. (2011) reported 26 taxa in 23 genera of freshwater and terrestrial algae at Ny-Alesund in the Arctic, with *Chlorella* sp. as one of the most common algae. The richness of microalgal biodiversity contributes significantly to the energy flow in cold polar ecosystems (Cao et al., 2015). Teoh et al. (2004) and Ahn et al. (2012) reported that Antarctic and Arctic *Chlorella* species are eurythermal organisms which exhibit high primary production rates.

2.1.2 Phylogenetic and Taxonomy Studies of *Chlorella* sp.

Species identification, classification and delineation of phylogenetic relationships is more accurate if a higher number of gene copies, variable and conserved regions are used (Mullis et al., 1986; Mullis and Faloona, 1987; Saiki et al., 1988). As algae cells harbor chloroplasts, they contain chloroplast-encoded small subunit (SSU) rDNA in addition to the commonly used, nuclear-encoded rDNA. These characteristics help in algae taxonomy and phylogenetic relationships as they give us a better representative of the actual evolution path of algae (Huss and Sogin, 1990).

18S rDNA is the primary phylogenetic marker which contains highly conserved regions that favors the development of universal primers for the broad sampling of individual lineages within Trebouxiophyceae (Lelieart et al., 2012). According to Nestupa et al. (2013), 18S rDNA is useful for the recognition of individual genus-level taxa. On the other hand, *rbc*L is a standard second marker which allows a straightforward alignment, and its phylogenies are generally concurred with the 18S rDNA-based tree topologies (Novis et al., 2009).

The taxonomy of *Chlorella* remains unresolved due to the lack of conspicuous morphological characters and the simple, asexual reproductive cycle by means of autospores (Kessler and Huss, 1992). In the past, numerous methods have been applied to study the taxonomy of *Chlorella* sp., including chemotaxonomical classification (Kessler and Soeder 1962), nutritional requirements in terms of autotrophic or heterotrophic cultivation (Shrift and Sproul, 1963; Shihira and Krauss, 1965), morphological, structural and physiological states (Fott and Novakova, 1969), the serological cross-reactions (Sanders et al., 1971) as well as the ultrastructure and chemical composition of the cell wall (Atkinson et al., 1972).

Ever since the description of the first *Chlorella vulgaris* Beijerinck (Beijerinck, 1890), around 1,000 orbicular-shaped '*Chlorella'* species have subsequently been described (Luo et al., 2006). Fott & Nováková (1969) revised and reduced the number of *Chlorella* species to nine and 11, based respectively on the comparative morphology and reproduction method. Only four *Chlorella* species remain within *Chlorella* based on biochemical and SSU sequence data evaluation, i.e. *Chlorella vulgaris, Chlorella kessleri* Fott et Nováková, *Chlorella lobophora* Andreyeva and *Chlorella sorokiniana* Shihira et Krauss (Huss et al., 1999). Krienitz et al. (2004) reported the reduction of "true" *Chlorella* within Chlorellaceae into three spherical taxa based on the SSU rDNA sequences, in which *Chlorella kessleri* was transferred to the genus *Parachlorella* Krienitz. The study showed that *Chlorella*-related coccoid green algae evolved in two different sister clades of Chlorellaceae: *Chlorella* and *Parachlorella*.

The latest revision of the genus *Chlorella* was based on a combination of biochemical and molecular data, and this showed that there are only five "true" *Chlorella* species, i.e. *Chlorella vulgaris* Beyerinck, *Chlorella lobophora* Andreyeva, *Chlorella sorokiniana* Shihira et Krauss, *Chlorella heliozoae* Proschold et Darienko and *Chlorella variabilis* Shihira et Krauss (Huss et al., 1999; Krienitz et al., 2004; Proschold et al., 2011). The identification of *Chlorella* species is one of the most difficult tasks in the systematics of coccoid green algae and the current challenges of *Chlorella* taxonomy studies are to distinguish individual species among the genetically diverse groups of *Chlorella* (Ettl and Gartner, 1995; Neustupa et al., 2009).

2.2 Ozone thinning and increase in the ultraviolet radiation

In recent years, ground-based measurements and satellite-estimated data provided evidence that the penetration of ultraviolet radiation has increased over large

geographical regions (from the northern to the southern hemisphere) as a result of ozone depletion (WMO, 2010). Frederick et al. (1989) postulated a 5 and 100% increase in surface UVA and UVB radiation, respectively, due to 10% decline in the ozone column. The discovery of spring time ozone depletion over Antarctica by the British Antarctic Survey Scientists in 1984 (Farman et al., 1985; Stolarski et al., 1986) have brought ultraviolet radiation studies to a new milestone. Ozonesonde observations at the South Pole consistently indicate that more than 90 % of the ozone in Antarctica was removed each winter since the mid-1990s (Hofmann et al., 2009). The first clear occurrence of an "Arctic ozone hole" was observed during the winter of 1995 to 1996 (Müller et al., 1997) while the reduction of ozone layer in mid-latitude was observed since the end of the 1970s (Antón et al., 2011). Ozone loss over the Arctic is less serious than that of Antarctica due to the difference in land and sea topologies in the Northern Hemisphere which allows only weak vortexes over the Arctic (Blumthaler and Webb, 2003). It is known that the level of UVR is higher in the tropics as compared to polar and temperate regions, as UVR decreases with increasing latitude (Madronich et al., 1995).

In polar regions, the melting of ice layers and snow by ozone depletion were associated with the increase of UVB levels (Snell et al., 2007; Scinocca et al., 2009). Simulation models suggested that the summer ice cover in the Arctic will disappear within the next few decades (Comiso et al., 2008; Overland et al., 2008). The largest ozone depletion in Arctic was observed in 2011 as a result of few global effects such as increase in temperature and persistence strong and cold stratospheric polar vortex (Manney et al., 2011). Model calculations estimated that by 2050 UVB will increase by up to 20% at northern high latitudes as compared to levels during the 1980s (Williamson et al., 2014). Moreover, Petkov et al. (2014) discovered a reduction of the ozone column in mid-latitudes due to the strong ozone depletion in the Arctic during the

spring of 2011. McKenzie et al. (2011) reported a 5% rise of UVB in temperate regions as compared to that in 1980.

There are three types of UVR, namely ultraviolet-A radiation (UVA, 320-400 nm), ultraviolet-B radiation (UVB, 280-320 nm) and ultraviolet-C radiation (UVC, 200-280 nm) (Han et al., 1998). Table 1 shows the irradiance range of PAR, UVA and UVB in tropical, temperate and polar regions during summer, noon or sunny days. PAR is an abbreviation of photosynthetic active radiation with a spectral range from 400 to 700 nm that enables photosynthesis by phototrophs (Pessoa, 2012). PAR that is normally expressed in μmol photons m^{-2} s^{-1} was converted to unweighted energy unit at Wm^{-2} according to McCree (1981) in order to obtain the PAR : UVA : UVB ratio.

Table 2.1: Irradiance range of PAR, UVA and UVB in tropical, temperate and polar regions

Regions	Irradiance range					References
	PAR (μmol photons m^{-2} s^{-1})	PAR (Wm^{-2})	UVA (Wm^{-2})	UVB (Wm^{-2})	Estimated ratio (PAR:UVA:UVB)	
Tropical						
Kuala Lumpur, Malaysia (daily mean)	992.10	215.28	14.11	0.90	239.2 : 15.7 : 1	Jeannette et. al. (unpublished)
Belize (Noon)	2012	436.60	59	0.24	1819.2 : 245.8 : 1	Hanelt and Roleda (2009)
Temperate						
Northern mid-latitude	400 - 1400	86.8 - 303.8	45 - 50	0.09 - 0.32	964.4 : 500: 1 to 949.4 : 156.3 : 1	Pankaew et al. (2014) Castenholz and Garcia-Pichel (2000) Bais et al. (2015)
New Zealand (January)	2031	441	37.1	2.49	177.1 : 14.9 : 1	Hanelt et al. (2006)
Antarctica						
Casey Station	1.35 - 14.80	0.29 - 3.21	2.50 - 24.30	0.60 – 13.00	0.5 : 4.2: 1 to 0.2 : 1.9 : 1	Wong (2011)
Arctic						
Ny-Alesund	30 - 38	6.51 - 8.25	6.7 - 8	0.25 - 0.36	26 : 26.8 : 1 to 22.9 : 22.2 : 1	Aguilera et al. (2002) Karsten et al. (2003)
Spitsbergen (June)	1300	282.10	19	1.1	256.5 : 17.3 : 1	Bischof et al. (1998)

2.3 Photosynthesis

Photosynthesis is a process that takes places in the chloroplast which converts light energy into chemical energy (Berg et al., 2002). The process is divided into two parts: (i) a light reaction, which happens in the thylakoid membranes transforming light energy into reducing power and ATP; (ii) a dark reaction, which occurs in the stroma driving carbon dioxide reduction and glucose conversion. Photosynthesis is mediated by two light-sensitive complexes: Photosystem I (PSI) and Photosystem II (PSII), where electron flows from PSII to PSI through the cytochrome *bf* complex in order to generate a proton gradient that synthesizes ATP. PSI is composed of *psa*A and *psa*B subunits whereas PSII consists of D1 and D2 proteins subunits. During the process of photosynthesis, energy transfer occurs from a donor in the excited state to an acceptor of equal or lower energy. This allows the utilization of light energy to boost electrons to a high-energy state. The photosynthesis apparatus maximizes the photo-induced charge separation and minimizes any unproductive return of electrons to their ground states. The site at which charge separation takes place is the reaction center known as P680 in PSII and P700 in PSI.

An imbalance between the light energy absorbed through photochemistry and energy utilization through photosynthetic electron transport will ultimately lead to photosynthesis inhibition, which is also known as photoinhibition (Powles, 1984; Long et al., 1994). PSII is the primary target of photoinhibition (Morgan-Kiss et al., 2006), as recovery from photoinhibition in most green algae and plants involves the PSII repair cycle, in which the photodamaged D1 in the thylakoid membrane is degraded and replaced by a newly synthesized one in order to form a photochemically functional PSII reaction center (Aro et al., 1993; Melis, 1999). Karen and Ohad (1998) and Melis (1999)

reported the rate of repair in photoinhibition was relative to the rate of photodamage to D1 protein.

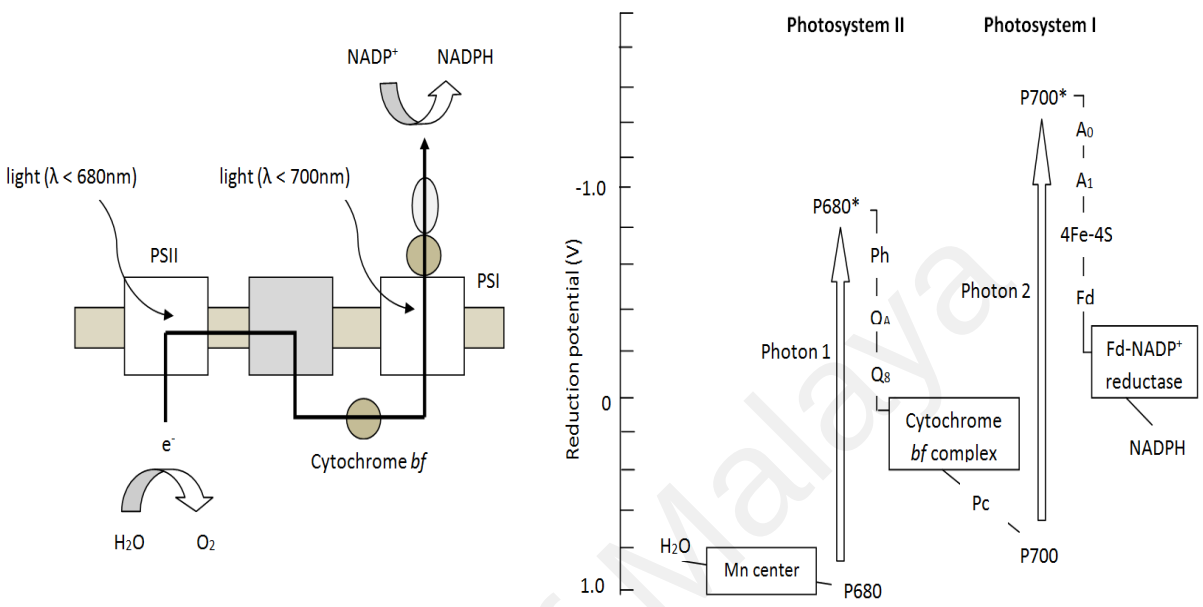


Figure 2.1: The pathway of electron flow from water to $NADP^+$ in photosynthesis. (Left) The absorption of photons by photosystems via cytochrome *bf*. (Right) The pathway of electron flow from water to $NADP^+$ in photosynthesis. (Adapted from Berg et al., 2002).

2.4 Photosynthetic Pigments

The trapping of light energy is the key to photosynthesis. Substances that are involved in light absorption are called pigments, and different pigments absorb light at different wavelengths. Chlorophylls (Chl) are the primary photosynthetic pigments synthesized in the chloroplast of higher plants, algae and photosynthetic bacteria. Chl biosynthesis is a sophisticated process as pigment accumulation are associated with thylakoid development and photosynthetic activity. According to Kirk and Tilney-Bassett (1967), the biosynthesis of Chl is sensitive to inhibitors of both cytoplasmic (cycloheximide) and organelle (chloraamphenicol) ribosomes. There are four major Chl in algae: Chl *a*, Chl *b*, Chl *c* and Chl *d* (Meeks, 1974). Chl *a* is found in reaction centers

I and II, as well as in the light-harvesting complexes (Brown, 1988). Chl *a* shows two main absorption bands *in vitro* at the red light region near 660nm and 665nm, and a region at around 430nm (Meeks, 1974).

Carotenoids are phytonutrients that play a role as photoreceptors and photoprotectors by acting as physical quenchers of electronically excited molecules (Krinsky, 1989; Tian and Yu, 2009). They are also accessory photosynthetic pigments that transfer absorbed quanta to chlorophyll for photosynthesis (Hall and Rao, 1994). An increase in carotenoid level is an essential mechanism for acclimation to high irradiance environment to prevent phtooxidative injury to chloroplast pigments (Goncalves et al., 2001). Huner et al. (1998) suggested the increase in carotenoid-binding protein, *Cbr*, is one of the low-temperature acclimatization strategies for unicellular green algae like *Chlorella vulgaris*. Carotenoids absorb light most strongly in the blue region within wavelengths from about 400 to 550nm.

Chl *a* and carotenoids can be extracted in 80-90 % acetone or 90 % methanol, and their concentration can be estimated fluorometrically or spectrophotometrically (Meeks, 1974). Studies have reported that environmental stress like limitations in light or nutrients will affect the Chl and carotenoid content in cells (Goericke and Montoya, 1998; Steiger et al., 1999; Goncalves et al., 2001). The relationship between Chl *a* and accessory pigments in response to light stress, i.e. differential pigment ratio for chl *a*:carotenoids, was reported by Lichtenthaler et al. (2000), Ralph (2000) and Marschall and Proctor (2004). At high irradiance, chlorophyll tends to become photooxidized (degraded), whereas carotenoids prevent photooxidation. Thus, the chl *a*-carotenoid ratio is a sensitive indicator of photooxidative damages (Hendry and Price, 1993).

2.5 Consequences of UVR in Photosynthesis Mechanisms

Photosynthesis is a sensitive metabolic process. The photosynthetic organelle, chloroplast, acts as an "environmental sensor" that responds to the changes in environmental parameters such as light changes (Villarejo et al. 2002). Chloroplasts contains highly organized thylakoid membrane systems that optimizes light harvesting (Allen and Forsberg, 2001). Holzinger et al. (2006) postulated that UV is one of the major destructive factors on exposed green algae, including those at tropical, temperate and polar regions (Helbling et al., 1992; Smith et al., 1992; Villafañe et al., 1999; Villafañe et al., 2004). Reports have also shown that UVB and UVA are more damaging on PSII as compared to PAR (Turcsanyi and Vass, 2000). UVA generally exerts positive effects in plant physiology by inducing photorepair and photoreactivation (Dionisio-Sese, 2010), or stimulating photosynthesis and growth in algae (Xu and Gao, 2010). On the other hand, UVB is an environmental stressor which hampers primary production (Gartia et al., 2003, Mengelt and Prézelin, 2005).

Phototrophs face a dilemma in that they require sunlight to drive photosynthesis but they are simultaneously exposed to UVR which is biologically harmful. Exposure to low UVR may have significant benefits on living organisms. However, increased or excessive UVR levels can seriously damage photosynthetic organisms (Heraud and Beardall 2000; Wong et al., 2007; Wong et al., 2011). Tolerance against enhanced UVB levels in the aquatic and terrestrial, polar plants and algae were reported. This ability was a result of the Earth being exposed to high ambient UVB levels approximately 500 million years ago (Rozema and Bjorn, 2002).

PSII is the primary target of UVB whereas PSI is relatively insensitive to UVB damage (Bouchard et al., 2008). The deleterious effects of elevated UVB on photosynthetic activities in microalgae were documented, including photoinhibition

(Villafañe et al., 2008); the degradation of PSII, D1 and D2 proteins (Bouchard et al., 2006, Vass, 2012); the reduction in RuBisco activity (Bouchard et al. 2008); decreased in chlorophyll and carotenoid contents (Surabhi et al., 2009; Wong et al., 2011), as well as the generation of reactive oxygen species (ROS) (Hideg et. al., 2013).

Several protective and acclimation strategies which limits photodamage were reported, i.e. the reduction in light absorption to limit damage, activation of non-photochemical quenching (NPQ) mechanisms, stimulation of non-assimilatory electron flow and increased enzymatic scavenging of oxygen radicals (Anderson et al., 1995; Eggert et al., 2006). Cells that are tolerant of UV radiation poss several key protective mechanisms, including the development of effective adaptations to shield against high UV (Holzinger et al., 2006), production of mucilage (Oertel et al., 2004), additional carotenoid formation (Gorton and Vogelmann, 2003), production of astaxanthin (Hagen et al., 1993), self-shading by surrounding cells or formation of multilayed mat-like structure on top of soil (Gray et al., 2007; Karsten et al., 2010).

2.6 Adaptive Strategies in Microalgae Living in Cold Environments

Polar microalgae have to evolve adaptative mechanisms in order to overcome the inhibiting effects of a low kinetic environment (Lyon and Mock, 2014). Gorton and Vogelmann (2003) reported that phototrophic microorganisms growing in glacial environments are exposed to high irradiance as well as UVR.

Snow green algae have a higher ability to develop increased tolerance towards UV, as their habitats are normally exposed to unfavorably high irradiance as a result of high albedo, which is higher reflectivity of sunlight hitting the surface and bouncing back towards space (Seidlitz et al., 2001). Studies have also reported the development of adaptive mechanisms to chilling in photosynthesis (Hawes, 1990; Anning et al., 2001)

and in biochemical composition (Sang et al., 2012). However, increased UVB may induce rapid photochemical damage among Arctic vegetation as the enzymatic repair processes are much slower due to the low ambient temperature (Bjorn et al., 1997). Berry and Bjorkman (1980) stated that plants from habitats with larger temperature variations, especially during the summer daylight and winter cold, tend to possess a greater acclimation potential of photosynthesis. Table 2 shows a summary of reports on the adaptive strategies of microalgae from cold habitats.

Table 2.2: Research on cold tolerance mechanisms in polar microalgae

Organisms	Adaptive Strategies	Functions	References
Chlamydomonas sp. *Chlorella* sp. *Navicula* sp. *Amphiprora* sp.	Increased concentration of polyunsaturated fatty acids (PUFAs)	-To maintain high membrane fluidity for efficient transportation of nutrients and metabolic waste -To maintain integrity to electron transport chains of cellular metabolism	Teoh et al. (2012)
Fragilariopsis curta *Navicula* sp. *Nitzschia medioconstricta*	Increased concentration in specific chloroplast PUFAs	To enhance fluidity of thylakoid membranes for better electron flow	Mock et al. (2002)
Natural assemblages of Antarctic sea-ice microalgae	Optimal freezing temperature in metabolic enzymes (such as NADPH and NADH)	To enhance metabolic production per energy units	Priscu et al. (1989)
Chlamydomonas subcaudata	Increased concentration of ATP synthase proteins within the chloroplast	To increase ATP production	Morgan et al. (1998)
Thalassiosira pseudonana *Fragilariopsis cylindrus*	Abundance of ribosomal proteins	To maintain adequate translation at low kinetic environment	Toseland et al. (2013)
Fragilariopsis cylindru	Synthesise of fucoxanthin-chlorophyll binding proteins (FCPs)	To enhance thylakoid fluidity for enabling high photosynthetic efficiency	

Table 2.2: Cont.

Organisms	Adaptive Strategies	Functions	References
Microorganisms	Enrichment and storage of cellular-compatible solutes (such as sugars, amino acids, DMSP)	-To reduce intracellular freezing point -To maintain enzyme hydration spheres to stabilize catalytic activities -Promote enhance nutrients uptake during extended darkness periods	Welsh (2000)
Psychrophiles	Synthesis of cold-shock proteins	To minimize cold denaturation	Casanueva et al. (2010)
Chlamydomonas subcaudata	High stoichiometry of PSII and PSI	To maximize blue-light harvesting	Morgan-Kiss et al. (2006)
Chlorella sp.	State transitions ability in PSII and PSI	-To balance light absorption in PSII and PSI -To maintain optimal photosynthetic activity	Morgan-Kiss et al. (2008)
Xanthonema sp.	Ability to disassemble PSII but keep LHC proteins intact	To maintain thylakoid structural proteins primed to reassembly PS quickly in order to utilize the first short irradiance period after extended darkness	Baldisserotto et al. (2005)
Surface sea ice algae	Utilization of non-photosynthetic quenching (NPQ) mechanisms (such as diatoxanthin-diadinoxanthin xanthophyll cycle)	To dissipate excess heat energy to prevent photoinhibition and cellular damage	Robinson et al. (1997)
Chlamydomonas raudensis	Energy redistribution between PSI and PSII	To balance the changes in excitation pressures of two photosystems	Takizawa et al. (2009)
Chlorella sp.	Metabolic shifts between autotrophic growth and heterotrophic growth during different seasons	To achieve highest growth rates	Morgan-Kiss et al. (2008)

2.7 Chlorophyll Fluorescence

Application of chlorophyll fluorescence is a widely used technique for plant ecophysiological studies among the plant physiologists and ecophysiologists. The basic principal of chlorophyll fluorescence analysis is rather simple. Under normal physiological conditions, radiant energy undergoes three fates after being absorbed by chlorophyll molecules: (i) utilized for photosynthesis (photochemistry), (ii) re-emitted as chlorophyll fluorescence and, (iii) dissipated as heat (Maxwell and Johnson, 2000). The rate constants in these three major dissipation mechanisms are in equilibrium, thus an increase in the efficiency in one of the processes will result in a decrease in the yield of the other two (Krause and Weis, 1991; Govindjee, 1995). Hence, changes in the efficiency of photochemistry and heat dissipation can be detected by measuring the yield of chlorophyll fluorescence, which can also be used as an early stress indicator (Pedrós et al., 2008).

The association of fluorescence with photosynthesis was first described by Kautsky and Hirsch (1931) with the discovery of Kautsky's effect. Upon transferring photosynthetic material from the dark into the light condition, Kautsky and coworkers discovered the reduction of electron acceptors in the photosynthetic pathway (PSII and plastoquinone Q_A) would result in fluorescence induction within a time period of around one second. This is because when the PSII absorbs light and Q_A has accepted an electron, the PSII reaction center is considered to be "closed" and eventually leads to an overall reduction in photochemistry efficiency and so to a corresponding increase in fluorescence yield, until the electron has passed onto a subsequent electron carrier (Q_B). When the latter happened, the fluorescence level typically starts to fall again over a time-scale about 15 to 20 minutes dependent on plant species (Johnson et al., 1990), and this process is known as fluorescence quenching. Fluorescence quenching is explained

in two ways: (i) photochemical quenching (ii) non- photochemical quenching (NPQ). During the photochemical quenching, light-induced activation of the enzymes that are involved in carbon metabolism and the opening of stomata would increase the rate at which electrons are transported away from PSII. At the same time, there is an increase in the efficiency of heat dissipation, and this process is termed as non-photochemical quenching. Table 2.3 shows the commonly used fluorescence parameters.

Table 2.3: Two general types of fluorescence parameters: The photochemical, and non-photochemical quenching parameters. Adapted from Maxwell and Johnson (2000).

Photochemical quenching parameters	
Φ_{PSII}	Quantum yield of PSII
qP	Proportion of open PSII
Fv/Fm	Maximum quantum yield of PSII
Non-photochemical quenching parameters	
NPQ	Non-photochemical quenching
NPQ_F	Fast relaxing NPQ
NPQ_S	Slowly relaxing NPQ

Chlorophyll fluorescence analysis is useful in giving information about the state of photosystem II (PSII) i.e. the extent to which PSII is using the energy absorbed by chlorophyll, or the extent to which PSII is being damaged by excess light (Maxwell and Johnson, 2000). This application is powerful if it combines with other techniques, i.e. the gas exchange measurements, nutrient uptake activities and enzymatic test, in order to obtain a full picture of the response of plants to their environment (Rai and Rai, 1997; Bischof et al., 2000).

2.8 Measurement of Photosynthetic Efficiency using Pulse Amplitude Modulated (PAM) Fluorometry

The use of a modulated measuring system has been considered as a revolutionizing system in the application of chlorophyll fluorescence (Quick and Horton,

1984). In the modulated measuring system, the light source used to measure fluorescence is modulated (switched on and off at high frequency) and the detector is tuned in order to detect only fluorescence excited by the measuring light. Thus, the relative yield of fluorescence can be measured in the presence of background illumination under laboratory condition, or under the presence of full sunlight in the field (Quick and Horton, 1984; Schreiber, 2004). Most of the modern fluorometers, for example the PhytoFlash Submersible Active Fluorometer (Turner Designs, Sunnyvale, CA, USA), Fluorescence Induction and Relaxation System (FIRe) (Gorbunov and Falkowsi, 2004) and Fast Repetition Rate Fluorometer (FRRF) (Kolber and Falkowski, 1992), are using the modulated measuring system to investigate the plant ecophysiological condition.

The world's first Pulse-Amplitude-Modulation (PAM) Fluorometer was designed by Ulrich Schreiber and built by Walz (Walz GambH, Effeltrich, Germany) and is able to detect the chlorophyll fluorescence that provides information on the efficiency of primary energy conversion (Schreiber, 1986). Numerous literature showed that PAM is a suitable tool in studying the photosynthetic efficiency of microalgae in response to stress i.e. light and UVR (Heraud and Beardall, 2000; Runcie and Riddle, 2006; Petrou et al., 2011). The background theory of the application of PAM is similar to what have discussed in Section 2.7. Figure 2.2 shows a typical PAM fluorescence measurement.

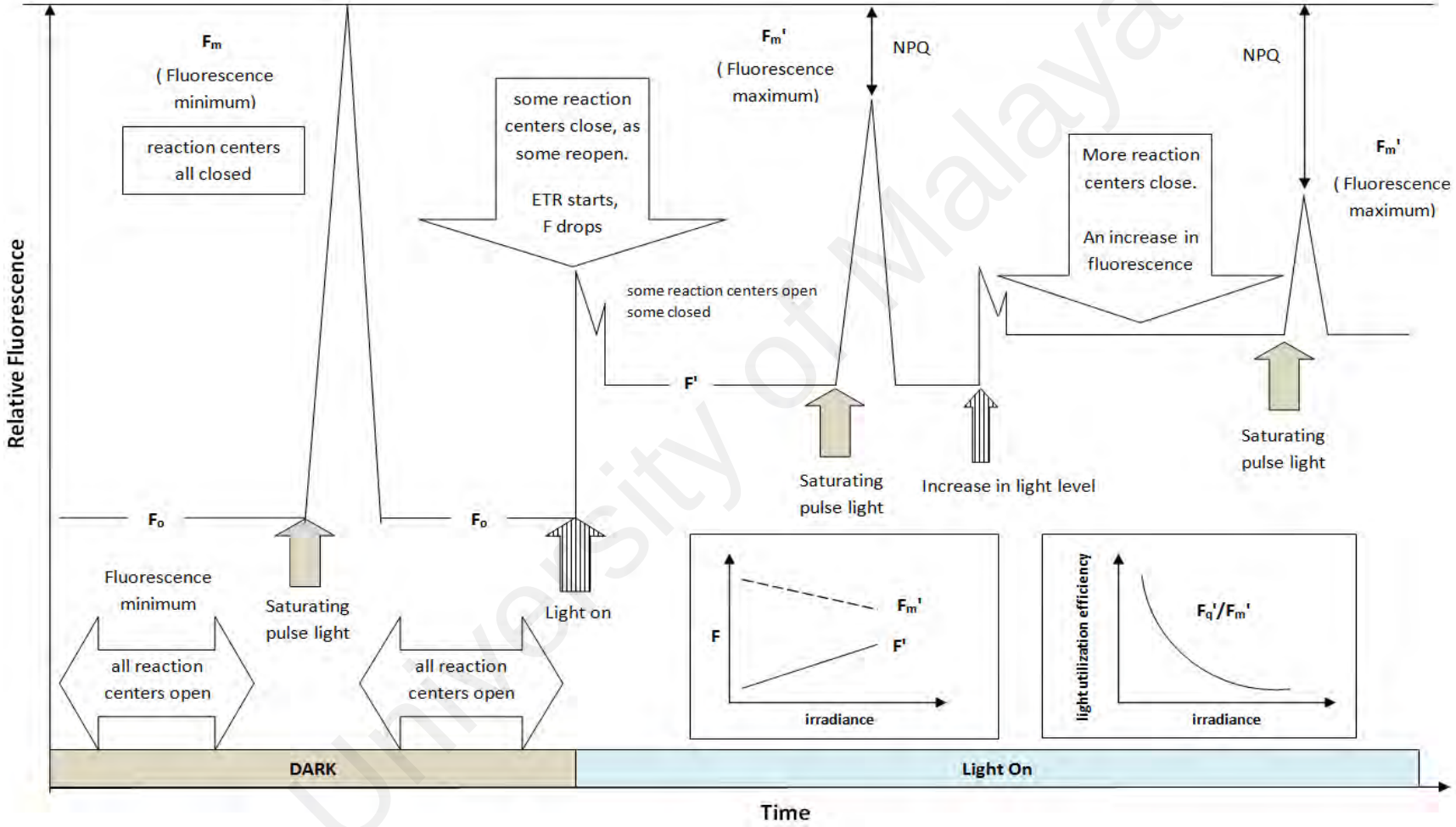


Figure 2.2: A typical PAM fluorescence measurement. Adapted from Consalvey et al. (2005).

There are a few common parameters normally measured using PAM, i.e. the maximum quantum yield (Fv/Fm), photosynthetic efficiency (α), maximum electron transport rate (rETRm) and photoadaptive index (Ek) (Schreiber,2004). Fv/Fm is a common parameter used to examine the photosynthetic health of a phototroph, by estimating its maximal photosynthetic efficiency (Bischof et al., 2000; Reeves et al., 2011). Many environmental factors that affect PSII, such as irradiance and temperature, would cause direct or indirect impacts on Fv/Fm (Wozniak et al., 2002). Rapid light curves (RLCs) on the basics of a photosynthesis-irradiance (P-E) curve is used to examine the relationship between photosynthesis and light intensity, as well as to define photosynthetic parameters of rETRm (or known as P_{max}), α and Ek (Figure 2.3). The α is referred as the electron transport rate per photon (Eggert et al., 2006). The rETRm refers to the capacity of the cell to tolerate short-term changes in irradiance (Ralph and Gademann 2005) whereas Ek is a light adaptation index used to examine the photoacclimation status (Sakshaug et al., 1997). Fv/Fm is dimensionless while the unit of α and E_k are μmol photons m^{-2} s^{-1} and that for rETRm is μmol electrons m^{-2} s^{-1} .

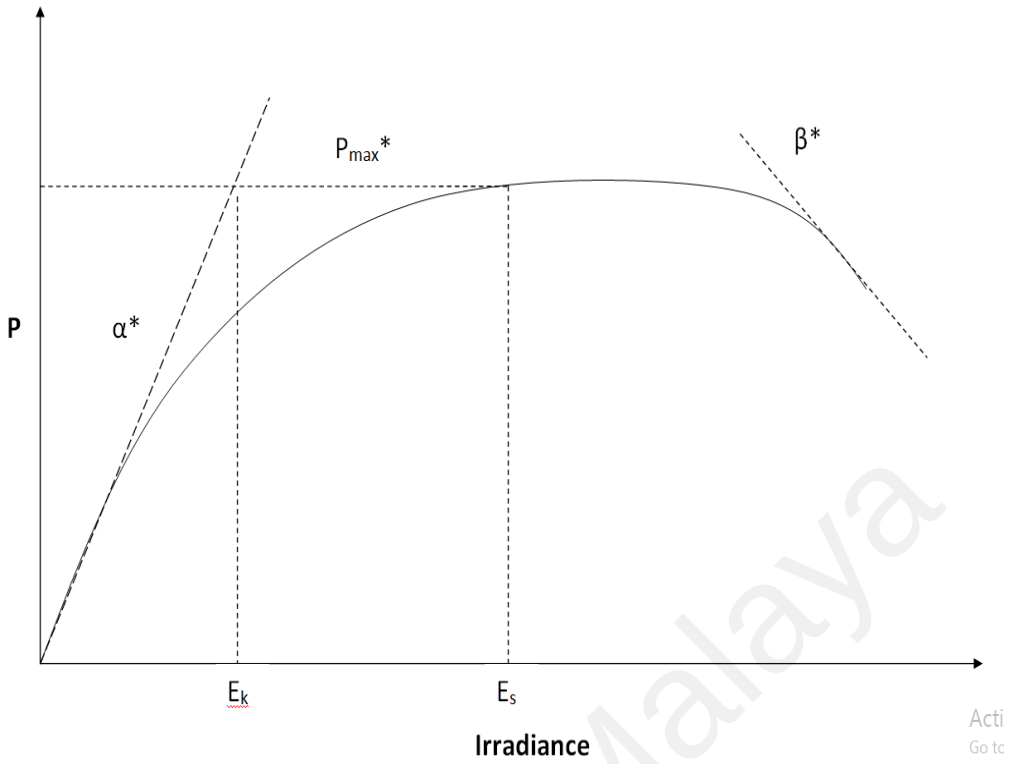


Figure 2.3: P-E curve shows the response of photosynthetic parameter to light. Adapted from Consalvey et al. (2005).

2.9 Random Amplified Polymorphic DNA (RAPD) Assay

The random amplified polymorphic DNA (RAPD) technique was first described by Williams et al. (1990). In a RAPD reaction, anonymous DNA regions of a given genome are amplified using single, short and arbitrary oligonucleotide primers (Bardakci, 2001). The oligonucleotide primers of that random sequence bind to several priming sites on the complementary sequences in the template DNA and produce discrete amplified products (Welsh and McClelland, 1994) which are then resolved electrophoretically (Power, 1996).

RAPD is a sensitive assay in detecting genetic variation in laboratory-treated organisms by comparing of amplification products generated by control (untreated sample) and treated samples (Atienzar et al., 2000; Castano et al., 2002). The

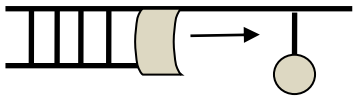
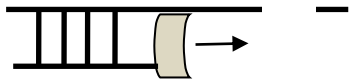
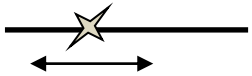
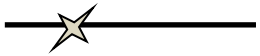
alterations may range from single bases (point mutations) to complex chromosomal rearrangements i.e. insertion or deletions (Paran and Michelmore, 1993; Atienzar, 1999; Bardakci and Skibinski, 1999). The decrease in genomic template stability is another useful evaluation in RAPD assay to study the mutagenic effects on treated samples (Liu et al., 2008; Sarkar et al., 2015). Figure 2.4 shows the direct effects of DNA damage and mutation on RAPD profiles by different DNA alterations events.

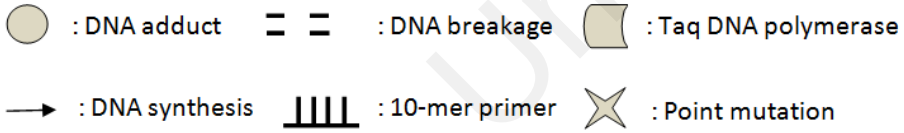
The RAPD analysis is capable of detecting DNA changes at lower concentrations of mutants that may not manifest themselves as mutations (Liu et al., 2005; Atienzar and Jha, 2006) as the detection limit of DNA damage in RAPD was as low as 2% (Jones and Kortenkamp, 2000). Bands could be amplified in an RAPD assay even if most of the primer binding sites were damaged (Theodorakis et. al., 1999).

Although generation of RAPD profiles had been criticized as unreliable due to its typeability, reproducibility, discrimination and its low stringency conditions (Ellsworth et al., 1993; Power, 1996), the RAPD method was capable of successfully detecting DNA damage induced by UV, X-ray and gamma radiation in plants, algae and human (Kuroda et al., 1999; Atienzer et al., 2000; Jones and Kortenkamp, 2004; Atak et al., 2004). DNA profiles were also reproducible from a range of organisms and have been successfully used to detect genotoxins induced DNA damage (Atienzer and Jha, 2006).

Cost and time effectiveness are the main advantages in RAPD assay at detecting DNA damage or genetic changes in cells exposed to stress. Omitting the need for cloning, sequencing or other DNA-based techniques, only small amounts of sample DNA are required to gain large number of genetic profiles (Bardakci, 2001).

Table 2.4: Consequences on RAPD profiles by several DNA alterations. Adapted from Atienzer and Jha (2006).

DNA alterations	Consequences	Effects on RAPD profiles	Probability to detect the lesion by RAPD
DNA adduct 	By pass → processivity affected	Decrease in band intensity	Medium
	By pass → processivity unaffected	No effect	-
	Block → dissociation enzyme adduct	Band loss	Low
	Block → dissociation → more free Taq	Increase in band intensity	Medium
	Block → no dissociation	Band loss	Low
	No primer / DNA association (adduct within the priming site)	Band loss	Low
DNA breakage 	Block → dissociation enzyme DNA	Band loss	Low
Point mutation 	No primer / DNA association	Band loss	Low
	Creation of a new annealing site	Appearance of band	Low
Rearrangement 	Loss of pre-existing annealing site	Band loss	Low
	New priming site	Appearance of band	High



2.10 DNA damage as a result of UVR exposure

The primary consequences of UVB radiation at the cellular level are structural changes and damages to DNA (Karentz et al., 1991a; Buma et al., 1995). UVB-induced DNA damage was largely reported in algae and phytoplankton (van Rijssel and Buma, 2002; Gao et al., 2007; Meador et al., 2009). Lud et al. (2001) suggested that DNA damage was shown to be a sensitive parameter to monitor UVB effects even though photosynthetic activity was unaffected. Short wavelengths of UVB and UVC radiation, especially in the region of 260 nm, were strongly absorbed by DNA and were detrimental to microbes due to cytotoxic lesions (Horneck, 1988).

Pyrimidine (6-4) pyrimidine dimers (6-4 PPs) and (5-6) cyclobutyl pyrimidine dimers (CPDs) are common UV photoproducts caused by DNA damage (Friedberg et al., 1995). 6-4 PPs often results in long term DNA damage as they cannot be excised and repaired by normal cellular photoreactivation mechanisms (Brash et al., 1985; Franklin and Haseltine, 1986). CPDs that block DNA replication, RNA transcription and translation which causes cell death (Karentz et al., 1991a; Buma et al., 1995), on the other hand, are repairable by excision or photodependent repair (Sancar and Sancar, 1988).

Thymine dimers formed as a result of strong UV-B radiation affect normal cell metabolism and division (Yarosh, 2002). Down-regulation of plastid-localized proteins, down-regulation in photosynthetic genes expression and the up-regulation of the defense genes are the most fundamental alternation of environmental stresses on gene expression of prototroph (Strid, 1993). Change in gene expression e.g. increased transcription of defense genes and reduced transcription of normal chloroplast genes were observed upon exposure to UVB (Jordan et al., 1991; Brosche and Strid, 1999).

It is well established that high ambient UVB is deleterious towards most photosynthetic mechanisms but Singh et al. (2014) reported that a photoreceptor named UV Resistance Locus 8 (UVR8) produced under low UVB was capable of mediating photoprotection and photoacclimation by regulating the synthesis of secondary metabolites and photomorphogenesis. UVB-induced photoprotection such as enhancement in protein repair capacity in intact cells was reported when subjected to moderate UVB radiation accompanied by a moderate intensity of visible light (Teramura et al., 1980; Dring et al., 2001). Positive effects (eustress) of low UVB dose such as increased accumulation of UV-screening pigments, changes in phytochemical content and alteration of UVB-specific gene expression were observed (Hideg et al., 2013).

CHAPTER 3: METHODOLOGY

3.1 Microalgae cultures

Four *Chlorella* strains from different origins were obtained from the University of Malaya Algae Culture Collection (UMACC) for this study (Table 3.1). The identification of the *Chlorella* spp. in UMACC was done previously based on morphological studies. The molecular analysis of selected *Chlorella* strains was done to confirm their identification, as discussed in Chapter 3.4.

Table 3.1: *Chlorella* isolates used in this study

Chlorella isolates	Origin	References
UMACC 001 *Chlorella* sp.	Experimental Farm, University of Malaya	Phang and Chu (1999)
UMACC 248 *Chlorella vulgaris*	Freshwater lake in the Netherlands	UTEX Culture Collection (original code LB2046)
UMACC 237 *Chlorella* sp.	Casey Station, Antarctica	Chu et al. (2002)
UMACC 263 *Chlorella vulgaris*	Rock-tunnel, Ny-Alesund, Arctic	Chu et al. (2002)

All *Chlorella* cultures were grown in Bold's Basal Medium (BBM) (Nichol, 1973) and maintained in a controlled-environment incubator (EnviroMaker PQS FS-9383, Malaysia) at their optimum growth temperature. An incubator temperature of 4 ^{o}C was used for Antarctic and Arctic algae, 18 ^{o}C and 28 ^{o}C for temperate and tropical *Chlorella* respectively. The cultures were grown in 5 litre conical flasks in a shaking incubator at 150 rpm, and were illuminated with 42 µmol photons m^{-2} s^{-2} cool white fluorescent lamps (36W Philips TLD 36W/54-765) on a 12:12 light-dark cycle. Cultures at exponential phase (OD_{620} = 0.2) as determined by the UV-Vis spectrophotometer (Shimadzu UV-2450, Fisher Scientific USA), were transferred into Whirl-Pack^{R} bags before being subjected to light treatments.

3.2 UVR treatments

The *Chlorella* cultures were exposed to three light treatments: PAR (photosynthetic active radiation), PAR+UVA (UVA stress) and PAR+UVA+UVB (UVB stress) in a controlled-environment incubator set.

The cultures were irradiated with a combination of three types of lamps: two tubes of day-light fluorescent lamps providing 42 μmol photons m^{-2} s^{-1} for PAR, two tubes of UVA lamps (18W Phillips TLK 40W/10Z) providing irradiance of 8.54 Wm^{-2}, and a tube of UVB lamp (38W Phillips TL 20W/12RS) providing irradiance of 1.17 Wm^{-2} (Wong et al. 2015). The irradiance was measured using SpectroSense2 Meter (Skye, United Kingdom) directly under each of the cut-off filters.

Various cut-off filters were used to obtain the different light treatments. Whirl-Pack^{R} bags (Nasco, United States) that allow light spectrum above 280 nm to pass through were used for the PAR+UVA+UVB treatment. For PAR alone, the cultures were placed in Whirl-Pack^{R} bags that were then covered by a clear Lexan^{R} polycarbonate sheet (Sabic, Netherlands) to eliminate UVA and UVB while an OHP Film (Faber Castell, Germany) that excludes UVB radiation was used to cover bags containing cultures which were subjected to PAR+UVA treatment. Table 3.2 summarizes the cut-off filter and lamps used to obtain the above mentioned light treatments.

All cultures were exposed to the same UVR intensity, for five hours continuously. Measurement of photosynthetic parameters and determination of pigment contents were done at 0 hour (before the exposure) and at every hour interval along the treatment, until the fifth hour of light treatment. Independent samples were run thrice in triplicates to confirm the reproducibility of the results.

Table 3.2: Summary of cut-off filters for each type of light treatment and their respective irradiance range

Light treatment	Cut-off filter	Light spectrum that pass through (type of light)	Irradiance range				
			PAR (µmol photons m^{-2} s^{-1})	PAR (Wm^{-2})	UVA (Wm^{-2})	UVB (Wm^{-2})	Ratio (PAR:UVA:UVB)
PAR (control)	Clear Lexan^{R} polycarbonate sheet (Sabic, Netherlands)	above 400 nm (PAR)	42	9.114	-	-	-
UVA	OHP Film (Faber Castell, Germany)	above 320 nm (PAR+UVA)	42	9.114	8.54	-	-
UVB	Whirl-Pack^{R} bags (Nasco, United States)	above 280 nm (PAR+UVA+UVB)	42	9.114	8.54	1.17	7.8 : 7.3 : 1

3.3 Measurement of photosynthetic performance

3.3.1 Measurement of photosynthesis efficiency

Photosynthetic activity was determined by measuring chlorophyll fluorescence using a Water-Pulse Amplitude Modulated fluorometer (Water-PAM; Walz GmbH, Effeltrich, Germany) as described by Ralph and Gademann (2005).

One mL aliquot of sample from respective treatments was transferred into a customized cuvette containing 3 mL BBM and dark-acclimatized (15 minutes) in an enclosed box to allow complete oxidation of PSII reaction centers (Heraud and Beardall 2000). After that, the cuvette was immediately inserted into the Emitter-detector (ED) unit and the stirrer unit was fitted (in a darkened room). The stirrer (Water-S device, Walz GmbH, Effeltrich, Germany) was switched on for 5 seconds prior to initiation of chlorophyll fluorescence measurement, to avoid cell settlement to the bottom of the cuvette (Cosgrove and Borowitzka 2006).

A five second far-red pulse (FR) (30 µmol m^{-2} s^{-1} at 735 nm) was applied to ensure full oxidation of the electron transport chain (Q_A). At the same time, the minimum fluorescence yield (F_o) was detected. Once the fluorescence becomes stabilized after applying FR, the rapid light curve (RLC) was produced by initiating the RLC programe (WinzControl Sofware, Walz). The RLC is generated by exposure to a series of incremental actinic irradiance (saturation pulse) at every 10 second interval (48, 70, 105, 158, 233, 358, 530, 812 and 1216 µmol photons m^{-2} s^{-1}). Upon application of the saturation pulse, the maximum fluorescence yield (F_m) as well as the maximum quantum yield (F_v/F_m) were measured (Schreiber 2004).

The relative electron transport rate (rETR) was calculated by multiplying quantum yield by PAR at each light interval (Schreiber 1998). Photosynthetic parameters such as the photosynthetic efficiency (α) and maximum relative electron transport rate (rETRm) were determined using equation described by Ralph et al. (2005),

where the Ek was calculated by Ek = rETRm/α (Serodio et al, 2006). Fv/Fm is dimensionless while the unit of α and E_k are μmol photons m^{-2} s^{-1} and that of rETRm μmol electrons m^{-2} s^{-1}.

Inhibition in photosynthetic parameter was calculated by:

Percentage of inhibition (%)

$$= \frac{\text{initial value of photosynthetic parameter - final value of photosynthetic parameter}}{\text{starting value of photosynthetic parameter}} \times 100\%$$

3.3.2 Pigment determination

Thirty mL of algae cultures were filtered under low vacuum (≤ 20 mm Hg, Millipore) onto GF/C filter paper (47 mm, Whatman, Göttingen, Germany). The pigments were extracted in 90 % acetone (Friendemann Schmidt) overnight in darkness at 4 ^{o}C. Chlorophyll *a* and total carotenoid contents were measured and calculated spectrophotometrically according to the colorimetric method (Strickland and Parson 1968; Vonshak and Borowitzka 1991). The ratio between the chlorophyll *a* and total carotenoids was calculated (Lichtenthaler et al. 2000; Marschall and Proctor 2004).

A cell count was measured using a counting device (Improved Double-Neubauer Haemocytometer, Germany). Appropriate dilution and homogenization of the algal culture was performed prior to counting in order to limit the cell number to less than 100 cells per field counted. Chlorophyll *a*, total carotenoid contents and ratio of chl *a* : carotenoids per cell was calculated.

3.3.3 Data analyses

Data were presented as mean ± standard deviation. Kolmogorov-Smirnov test was done to test the normality prior to all statistical data analyses. Three-way ANOVA (level of significant $P < 0.05$) was applied to observe statistical differences due to the following factors: algae strain, light treatment and duration of treatment. One-way ANOVA (P = 0.05) was used to detect the differences between strain or type of treatments with photosynthetic performances. Strain and type of treatments were used as factors. Tukey's post hoc test was used to locate the significant differences (P = 0.05). All analyses were done using Statistica 8.0 software (Statsoft).

3.4 Molecular identification and phylogeny

3.4.1 DNA extraction

DNA extraction for cultures was performed using *i*-genomic plant DNA Extraction Kit (iNtRON Biotechnology Inc., South Korea) according to the manufacturer's instructions.

Ten mL of the liquid cell suspension from flask was pipette into a 15 mL Falcon tube in a laminar flow cabinet. Then, the tube was centrifuged at 4000 rpm for 10 minutes to obtain the cell pellet. The supernatant was then removed, leaving approximately 50 µL. The cell pellet was resuspended in the remaining supernatant and was transferred to a 1.5 mL eppendorf tube. The sample was snap frozen for approximately 60 seconds using liquid nitrogen and the sample was vortexed quickly. This step was repeated 3 to 4 times, in order to lyse the cell.

i-genomic Plant DNA Extraction Mini Kit was then used to extract the DNA. 390 µL of buffer PG, 7 µL of enhancer solution, 20 µL of Proteinese K and 5 µL of RNase A solution were added to the lysate and vortexed vigorously. Then, the mixture

was incubated for 30 minutes at 65˚C. Mixing was done occasionally during incubation by inverting the tube. After incubation, 100 µL of Buffer PPT was added to the lysate, mixed and incubated for 5 minutes on ice. Lysate was then centrifuged at 13,000 rpm for 5 minutes. This is the precipitation step.

For DNA binding step, 200 µL of supernatant was transferred into a 1.5 mL tube. 650 µL of Buffer PB was added to the lysate and mixed well by pipetting but not vortex. Then 650 µL of mixture was pipetted into the spin column which inserted in a 2.0 mL collection tube. The spin column was centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. The remaining mixture was pipette into the spin column and centrifuged again.

A new 2.0 mL collection tube is placed into the spin column. 700 µL of Buffer PWA was added and centrifuged at 13,000 rpm for 1 minute. The flow-through was discarded. Next, 700 µL Buffer PWB was added to spin column and centrifuged at 13,000 rpm for 1 minute. The spin column was centrifuged again to dry the membrane completely. This is the washing step.

For the elution step, a new 1.5 mL collection tube is placed into the spin column. 100 µL Buffer PE is placed onto the membrane and incubated at room temperature for 1 minute. Lastly, the column is centrifuged at 13,000 rpm for 1 minute.

3.4.2 Polymerase Chain Reaction (PCR)

PCR amplification for the 18S rRNA gene was performed using a MultiGene^{TM} Gradient Thermacycler (Labnet, USA) with the following profile: an initial denaturation at 94 ^{o}C for 10 minutes; followed by 35 cycles of denaturation at 94 ^{o}C for 1 minutes, annealing at 50 ^{o}C for 0.5 minutes and extension at 72 ^{o}C for 2 minutes; with a final

extension at 72 °C for 10 minutes, and the PCR products stored at 10 °C. The primers used for the amplification of the 18S rDNA were NS1 and NS2 (Wu et al, 2001). PCR products were purified using the LaboPass PCR Kit (Cosmo Genetech, South Korea) and sent for sequencing (First Base Laboratories Sdn Bhd, Selangor).

Table 3.3: Primers used for PCR amplification of 18S rDNA region (primer names are universal).

Primer	Direction	Source	Sequence	Product size (bp)
NS1	Forward	Nuclear SSU	5'-GTAGTCATATGCTTGTCTC-3'	550
NS2	Reverse	Nuclear SSU	5'-GGCTGCTGGCACCAGACTTGC-3'	550

3.4.3 Gel electrophoresis

Successful amplification was visualized by gel electrophoresis using 1% (w/v) agarose gel with 100 volt for 20 minutes in a Tris-Acetate-EDTA (1 × TAE) buffer system.

A known weight of agarose was dissolved in 1 × TAE buffer by heated in microwave oven for about 1 minute. The dissolved agarose mixed with SYBR Safe (Invitrogen, NY, USA) was poured into an agarose gel electrophoresis container (Major Science, CA, USA) with a comb immersed into the gel solution. The agarose solution was solidified at room temperature and the comb was taken out slowly after the gel solidification.

The solidified agarose gel was placed in an electrophoresis system (Major Science, CA, USA) with the gel covered by 1 × TAE buffer. PCR products mixed with gel loading dye (analytical grade water containing 40% sucrose and 0.2% of bromophenol blue) was loaded onto the agarose gel. A 1 kb DNA molecular weight

marker (Bioron) was run for each agarose gel. Visualization and documentation of gel were done with Alpha Imager TM2200 (Alpha Innotech, USA).

3.4.4 Bioinformatics

Resulting sequences were manually edited and assembled using ChromasPro v1.5 (Technelysium Pty. Ltd., Australia). DNA sequences of relevant taxa were downloaded from the Genbank and subjected aligned using ClustalX version 2.0 (Larkin et al. 2007). The multiple sequence block was checked by eye and trimmed using Bioedit v.7.0.8.0 (Hall 1999). Aligned sequences were then subjected to phylogenetic analysis using Bayesian inference in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). *Chlamydomonas* spp. were selected as the outgroup in the analysis.

The best fitting substitution model with parameters for the dataset was deduced from the Bayesian Information Criterion (BIC) implemented in Modeltest v.3.7 (Posada and Crandall 1998). The K80+I+G model was selected for the dataset. Bayesian analyses were initiated with a random starting tree and two parallel runs of Markov Chain Monte Carlo (MCMC) iterations for 2 x 10^6 generations. The trees in each chain were sampled every 200^{th} generation. The convergence of the two MCMC runs to the stationary distribution was determined by looking at the standard deviation of split frequencies (always less than 0.01) and by the convergence of parameter values in the two independent runs. The first 200 trees were discarded as burn-in, and the remaining trees were used to generate a 50% majority rule tree and to determine the posterior probabilities for the dataset.

3.5 Study of DNA mutation by Random Amplified Polymorphic DNA (RAPD)

3.5.1 DNA extraction

DNA extraction for cultures subjected to 5 h of stress was performed using *i*-genomic plant DNA Extraction Kit (iNtRON Biotechnology Inc., South Korea) as mentioned in Chapter 3.4.1.

3.5.2 Polymerase Chain Reaction (PCR)

Random Amplified Polymorphic DNA (RAPD) analysis was carried out as an assessment to detect UVR-induced mutation on DNA of *Chlorella* sp. from different geographical regions (tropical, temperate, Antarctica and Arctic).

DNA amplification was performed in a 20 µL reaction containing 1× PCR buffer, 10 pmol primer, 200 µM dNTPs, 1 U *i*-Taq plus DNA polymerase (iNtRON Biotechnology Inc., South Korea) and 2 µL of genomic DNA. Amplification was performed using a Labnet Thermocycler (Labnet International Inc., North America) with the profile shown in Table 3.3. A negative control consisted of all PCR mixture excluded DNA was done for every batch of PCR amplification.

Table 3.4: Amplification profile

Steps	**Temperature / Time**
Denaturation	94°C, 2 min
Denaturation	94°C, 1 min
Annealing	32 - 36°C depending on primer, 1 min } 45 cycles
Extension	72°C, 2 min
Final Extension	72°C, 5 min
Storage	4°C, ∞

3.5.3 Gel electrophoresis

Gel electrophoresis was performed as discussed in Section 3.4.2, using 2% (w/v) agarose gel with 50 volt for 150 minutes in a Tris-Acetate-EDTA (1 × TAE) buffer system.

3.5.4 Screening and Optimization of Suitable Primers for RAPD Analysis

A total of 60 primers (Integrated DNA Technologies, Singapore) were screened in each sample. Primers which can amplify the most number of clear bands were selected for further studies. Table 3.4 shows the list of primers used in this study.

Once the suitable primers were selected, optimization of PCR conditions was done individually in order to determine the suitable annealing temperature for optimum amplification for each primer. Three different annealing temperatures (32, 34 and 36 ^{o}C) were tested. The best annealing temperatures for each selected primer will be used for RAPD analysis in microalgae exposed to UVR stress. All RAPD analysis of selected primers were carried out triplicates to make sure its reproducibility.

Table 3.5: RAPD primers and their sequences

No	Primers	Sequences (5' - 3')	No	Primers	Sequences (5' - 3')	No	Primers	Sequences (5' - 3')
1	OPA 10	GTGATCGCAG	21	S 19	ACCCCCGAAG	41	S 37	GACCGCTTGT
2	OPK 7	AGCGAGCAAG	22	S 20	GGACCCTTAC	42	S 38	AGGTGACCGT
3	S 1	GTTTCGCTCC	23	S 21	CAGGCCCTTC	43	S 39	CAAACGTCGG
4	S 2	TGATCCCTGG	24	S 22	TGCCGAGCTG	44	S 40	GTTGCGATCC
5	S 3	CATCCCCCTG	25	S 21	CAGGCCCTTC	45	S 41	ACCGCGAAGC
6	S 4	GGACTGGAG	26	S 22	TGCCGAGCTG	46	S 42	GGACCCAACC
7	S 5	TGCGCCCTTC	27	S 23	AGTCAGCCAC	47	S 43	GTCGCCGTCA
8	S 6	TGCTCTGCCC	28	S 24	AATCGGGCTG	48	S 44	TCTGGTGAGG
9	S 7	GGTGACGCAG	29	S 25	AGGGGTCTTG	49	S 45	TGAGCGGACA
10	S 8	GTCCACACGG	30	S 26	GGTCCCTGAC	50	S 46	ACCTGAACGG
11	S 9	TGGGGGACTC	31	S 27	GAAACGGGTG	51	S 47	TTGGCACGGG
12	S 10	CTGCTGGGAC	32	S 28	GTGACGTAGG	52	S 48	GTGTGCCCCA
13	S 11	GTAGACCCGT	33	S 29	GGGTAACGCC	53	S 49	CTCTCCAGAC
14	S 12	CCTTGACGCA	34	S 30	GTGATCGCAG	54	S 50	GGTCTACACC
15	S 13	TTCCCCCGCT	35	S 31	CAATCGCCGT	55	S 62	GTGAGGCGTC
16	S 14	TCCGCTCTGG	36	S 32	TCGGCGATAG	56	S 64	CCGCATCTAC
17	S 15	GGAGGGTGTT	37	S 33	CAGCACCCAC	57	S 76	CACACTCCAG
18	S 16	TTTGCCCGGA	38	S 34	TCTGTGCTGG	58	S 88	TCACGTCCAC
19	S 17	AGGGAACGAG	39	S 35	TTCCGAACCC	59	S 90	AGGGCCGTCT
20	S 18	CCACAGCAGT	40	S 36	AGCCAGCGAA	60	S 93	CTCTCCGCCA

3.5.5 Analysis of RAPD profiles

3.5.5.1 Appearance of new bands, disappearance of bands and similarity of the band

Change observed in the RAPD profiles in comparison to control group in the RAPD profiles was measured by given arbitrary score of:

Appearance of new band = +1

Disappearance of band = -1

The average was then calculated for each experimental group. All the date were recorded and transformed into percentage (%) using the following formula:

i) Percentage of new band appearance (%)

$$= \frac{\text{number of new band appeared in treated culture}}{\text{number of bands in control culture}} \times 100\%$$

ii) Percentage of band disappearance (%)

$$= \frac{\text{number of bands disappeared in treatment culture}}{\text{number of bands in control culture}} \times 100\%$$

iii) Percentage of band similarity (%)

$$= \frac{\text{number of bands in treated culture similar to control}}{\text{number of bands in control culture}} \times 100\%$$

3.5.5.2 Estimation of genomic template stability (GTS)

Genomic template stability (GTS) is a qualitative measurement reflecting the changes in RAPD pattern due to level of DNA damage. It was calculated using the following formula described by Atienzar et al. (2000):

Genomic template stability (%) = 100 - (100a/n)

where a = the average number of changes in a DNA profiles

n = the number of bands selected in a control DNA profiles

CHAPTER 4: RESULTS

4.1 Pulse Amplitude Modulated (PAM) Fluorometry

4.1.1 Maximum Quantum Yield (Fv/Fm)

Figures 4.1, 4.2 and 4.3 show the Fv/Fm values in all strains of *Chlorella* exposed to UVB, UVA and control treatment, respectively.

In Figure 4.1, Fv/Fm in all *Chlorella* strains dropped dramatically after the first hour of UVB exposure. Fv/Fm values in temperate and Antarctic *Chlorella* experienced a drop to nearly zero after two hours of UVB treatment, while Fv/Fm of the tropical and Arctic strains decreased to less than 0.10 on the third, and fourth hour of UVB exposure, respectively. There was a reduction in Fv/Fm due to UVA (Figure 4.2) of about 25% after five hours of treatment ($P > 0.05$) in the tropical *Chlorella*.

Fv/Fm under the PAR treatment (control) did not vary throughout the experimental period, with Fv/Fm values ranging from 0.50 to 0.70 in all *Chlorella* strains (Figure 4.3). The temperate *Chlorella* UMACC 248 exhibited a lower initial Fv/Fm value (Fv/Fm around 0.50) compared to tropical and polar strains with an initial Fv/Fm of approximately 0.70.

Figure 4.4 shows the overall percentage inhibition of Fv/Fm in all *Chlorella* strains under both UV treatments. UVB dropped 98.68, 99.54, 97.09 and 97.49 % in tropical, temperate, Antarctic and Arctic strains, respectively. Under UVA treatment, the lowest percentage inhibition, 22.34 % ($P < 0.05$), was detected in the tropical *Chlorella* whereas other strains recorded inhibition of about 50 % ($P < 0.05$).

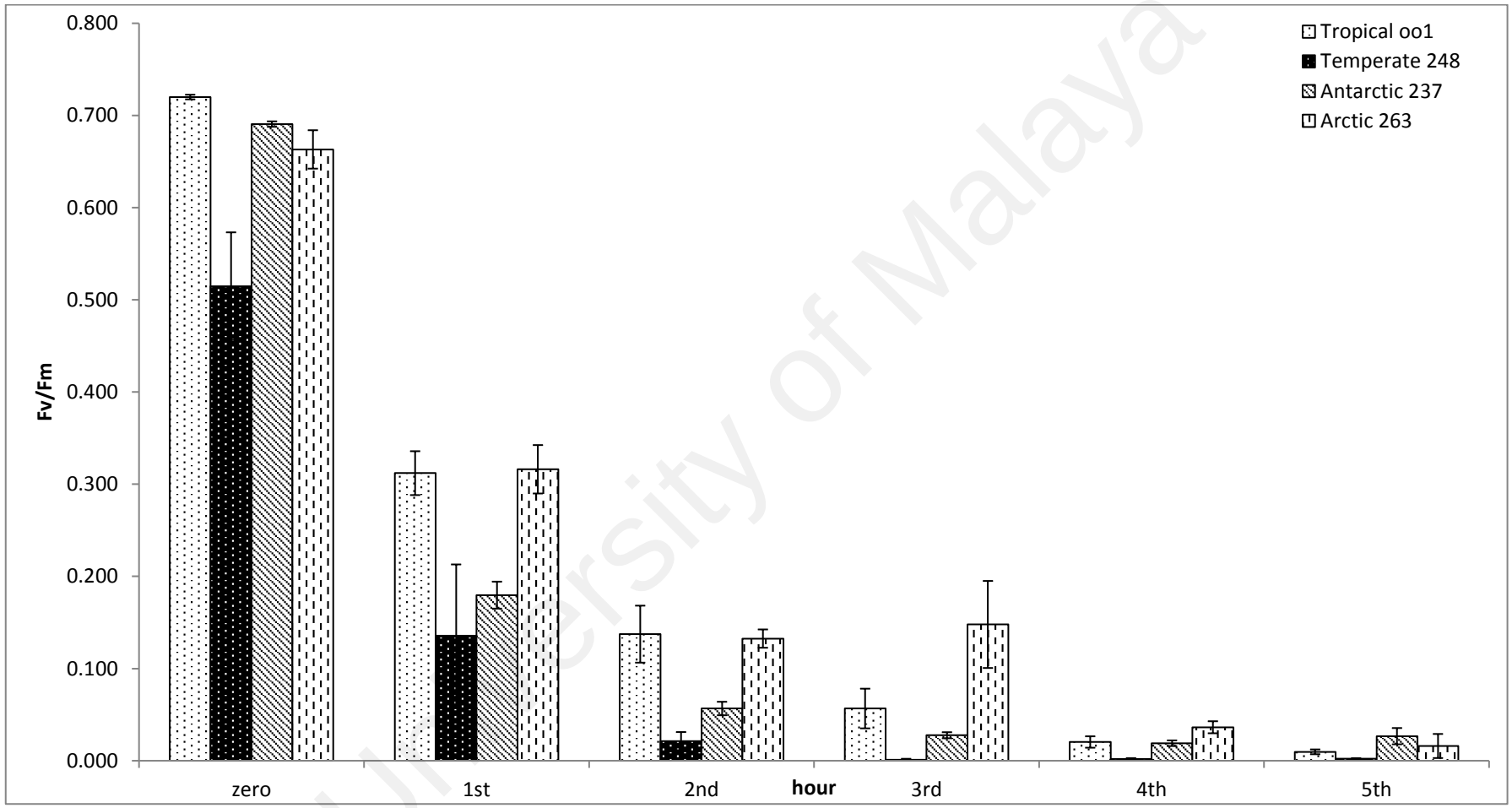


Figure 4.1: Maximum quantum yield (Fv/Fm) of all *Chlorella* strains subjected to a five hours UVB treatment. Data as means ± S.D. (n=3).

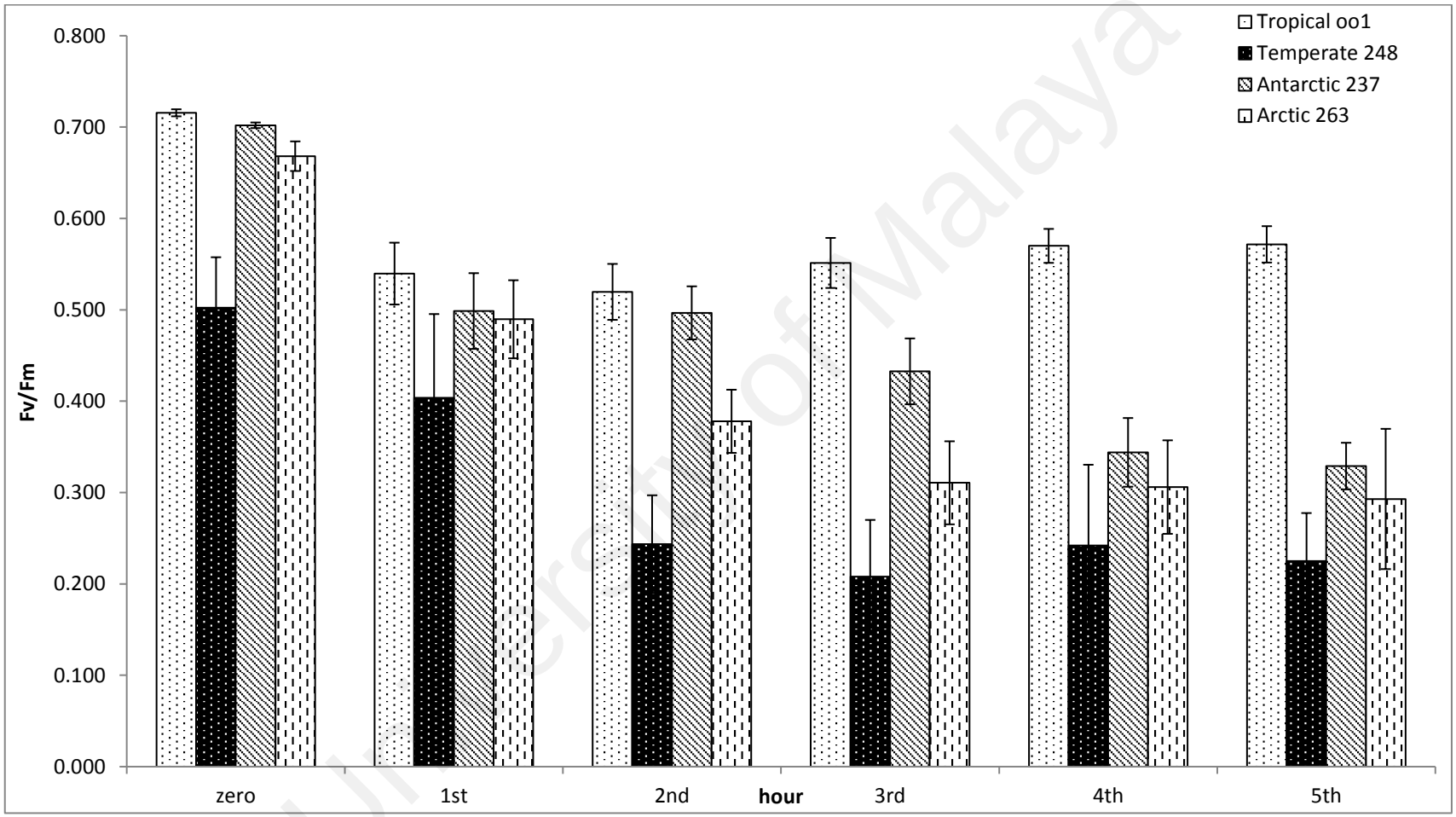


Figure 4.2: Maximum quantum yield (Fv/Fm) of all *Chlorella* strains subjected to a five hours UVA treatment. Data as means ± S.D. (n=3).

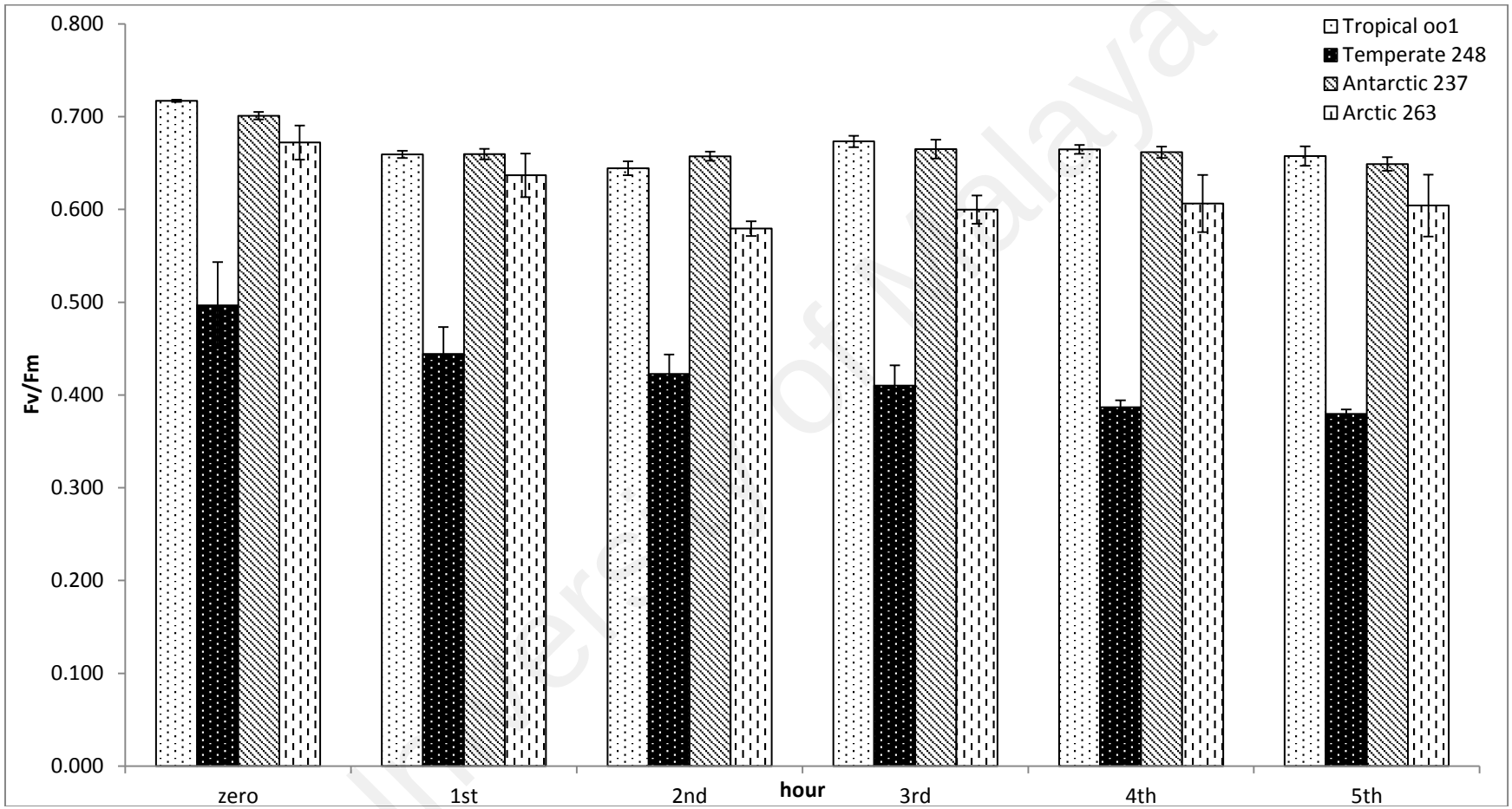


Figure 4.3: Maximum quantum yield (Fv/Fm) of all *Chlorella* strains subjected to a five hours PAR light treatment (control). Data as means ± S.D. (n=3).

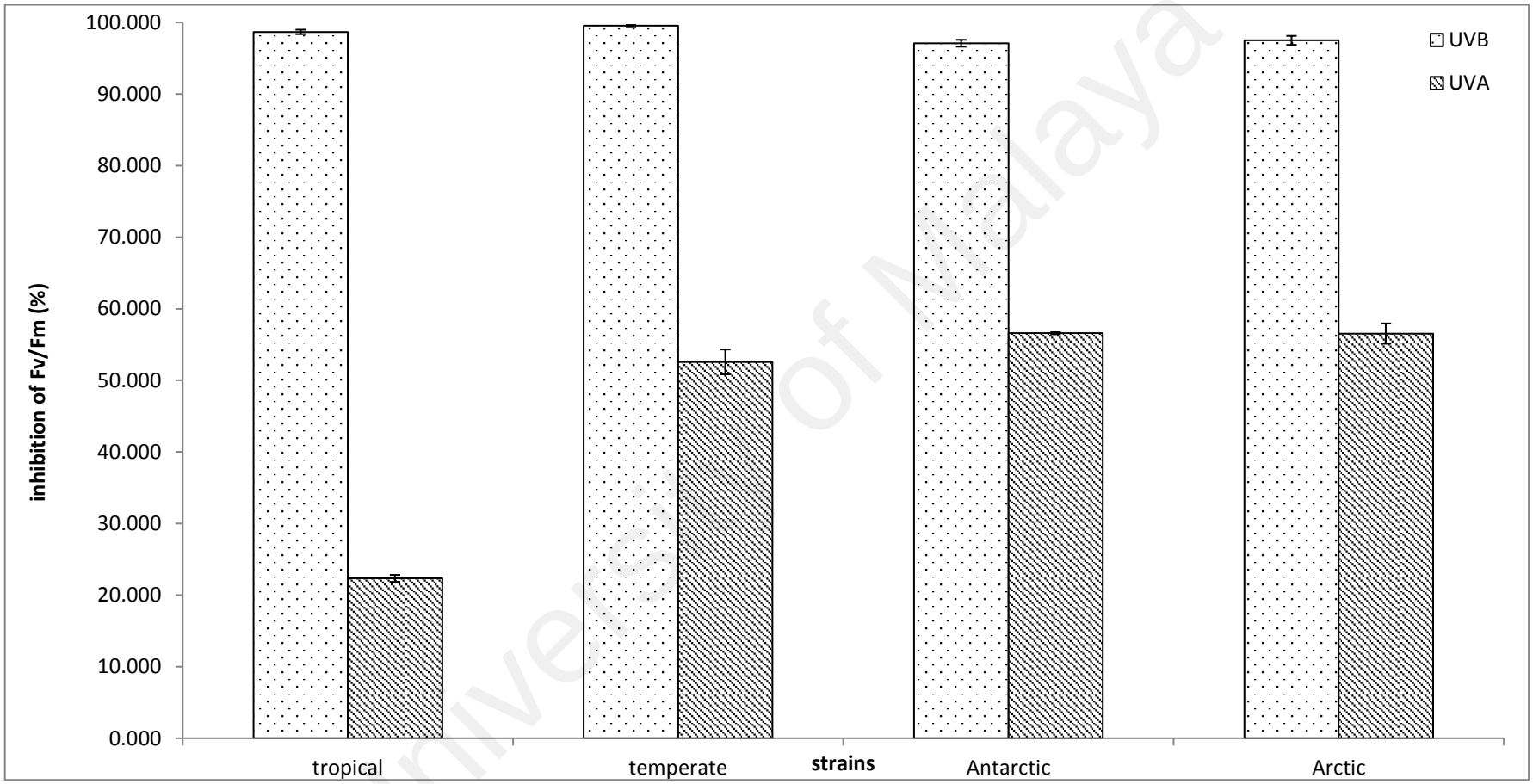


Figure 4.4: Inhibition of maximum quantum yield (Fv/Fm) of all *Chlorella* strains subjected to a five hours UV treatment. Data as means ± S.D. (n=3).

4.1.2 Photosynthetic efficiency (α)

Figure 4.5 shows a significant decrease in α value throughout the five hour UVB treatment ($P < 0.05$) in all *Chlorella* strains. After five hours of UVB exposure, the α value in all *Chlorella* strains were recorded at less than 0.30 μmol photons m^{-2} s^{-1}. In Figure 4.6, all *Chlorella* strains exhibited a gradual decline in α values when subjected to UVA radiation but the reductions were not significant ($P > 0.05$).

During PAR exposure, the α value remained approximately constant throughout the experimental period (Figure 4.7). The initial α value in the temperate *Chlorella* UMACC 248 was lower (α = 0.28 μmol photons m^{-2} s^{-1}) as compared to the tropical and polar strains with initial α values ranging from 0.45 to 0.60 μmol photons m^{-2} s^{-1}.

The inhibition of α caused by UVB stress is higher compared to that caused by UVA (Figure 4.8). UVB caused significant inhibition in α ($P < 0.05$), with an overall reduction of 98.69, 99.75, 74.57 and 92.85 % in tropical, temperate, Antarctic and Arctic strains, respectively. There was about 50 % decrease in α value in all treated samples after UVA exposure, where the inhibition was insignificant ($P > 0.05$).

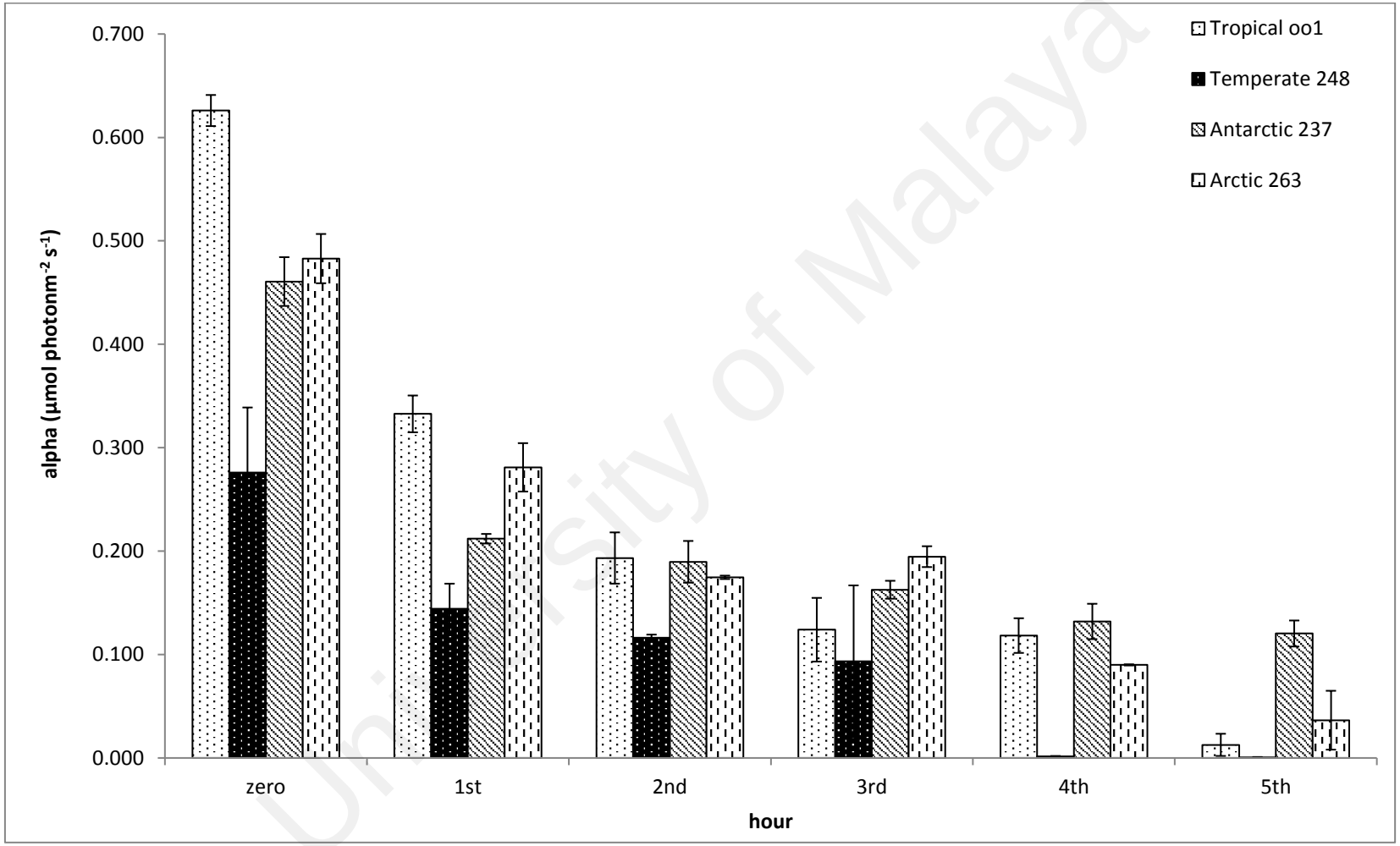


Figure 4.5: Photosynthetic efficiency (α) of all *Chlorella* subjected to a five hours UVB treatment. Data as means ± S.D. (n=3).

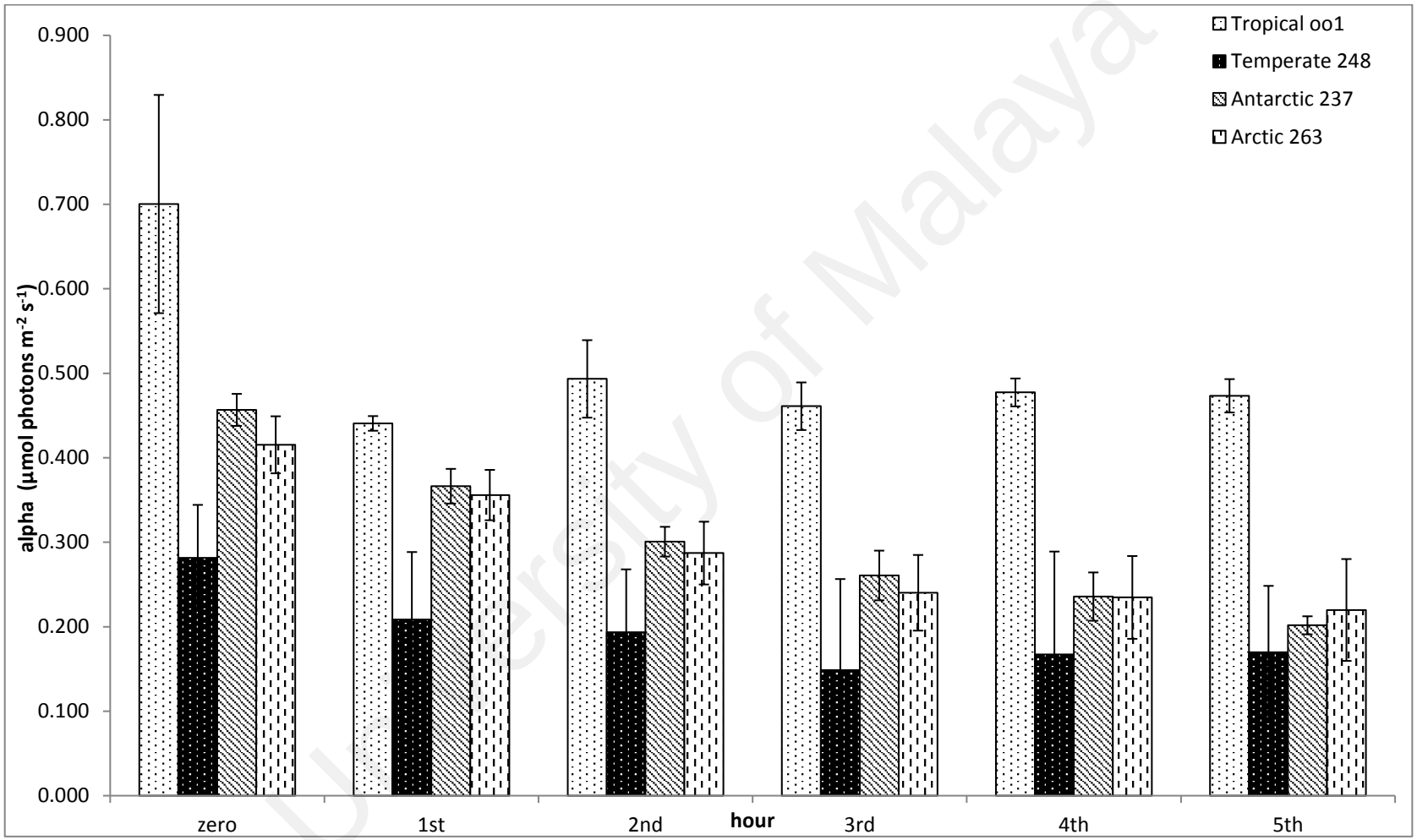


Figure 4.6: Photosynthetic efficiency (α) of all *Chlorella* subjected to a five hours UVA treatment. Data as means ± S.D. (n=3).

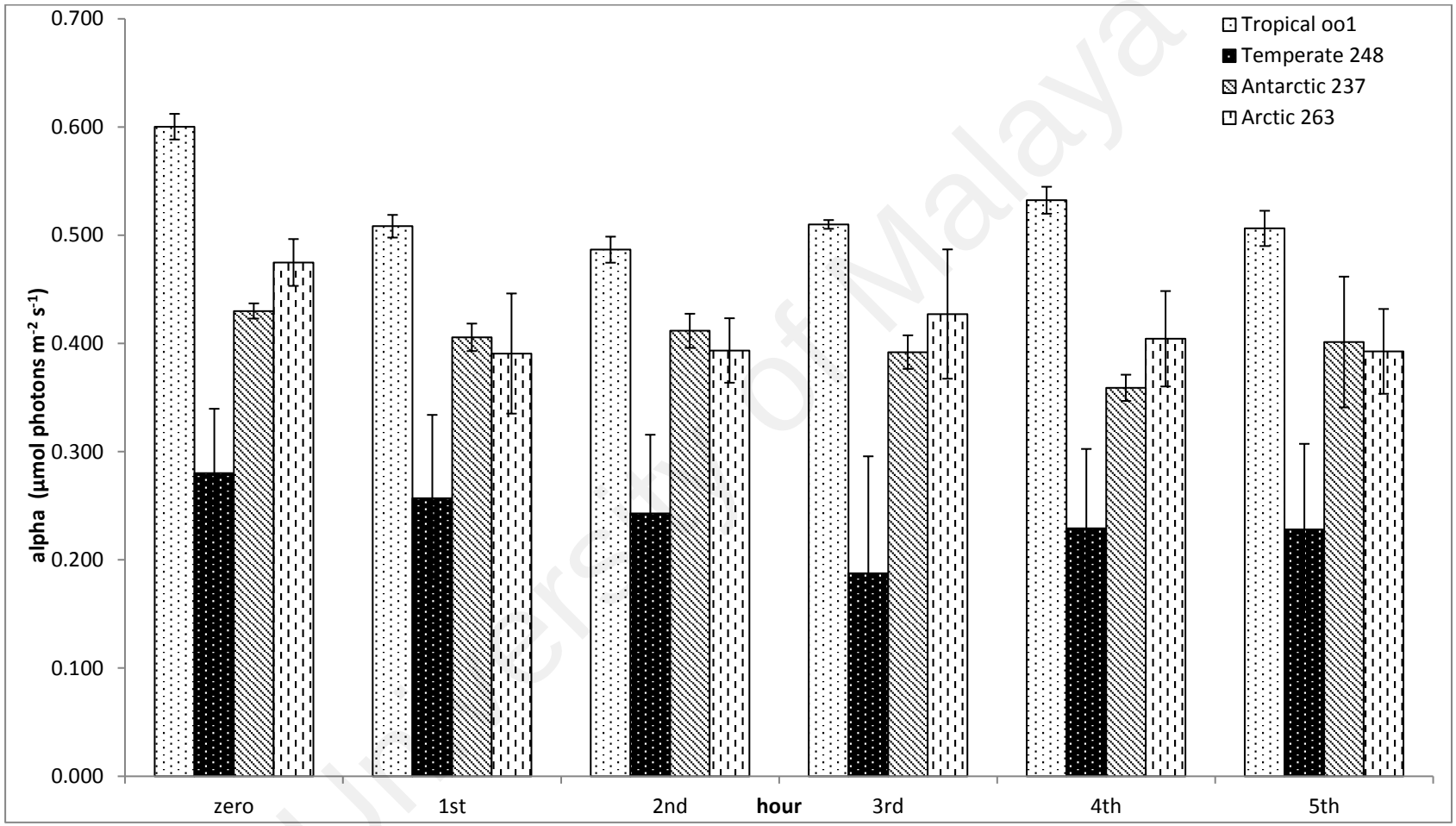


Figure 4.7: Photosynthetic efficiency (α) of all *Chlorella* subjected to a five hours PAR light treatment (control). Data as means ± S.D. (n=3).

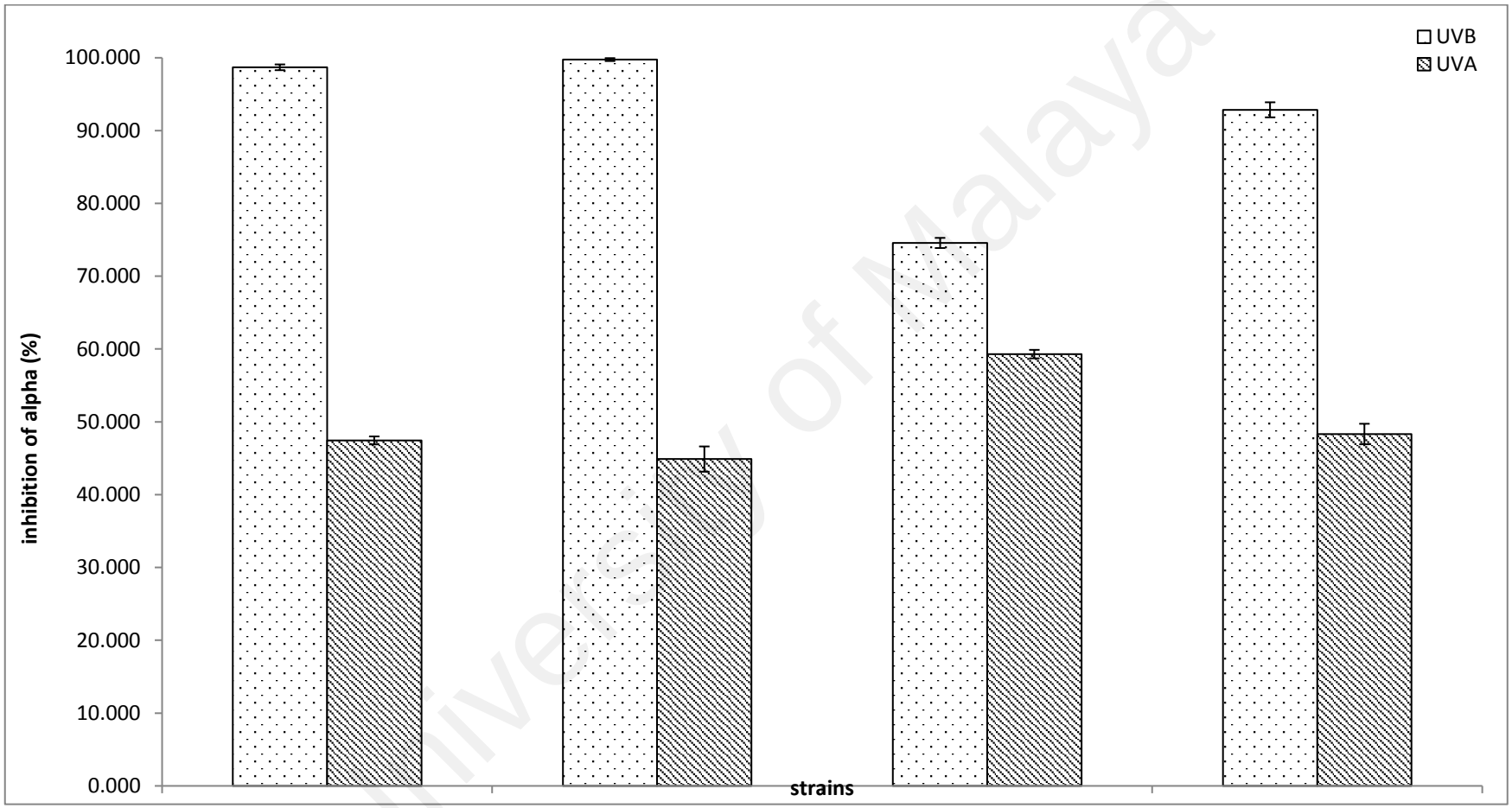


Figure 4.8: Inhibition of photosynthetic efficiency (α) of all *Chlorella* strains subjected to a five hours light treatment. Data as means ± S.D. (n=3).

4.1.3 Maximum electron transport rate (rETRm)

A steadily declining trend in rETRm was observed in the *Chlorella* strains upon exposure to UVB stress (Figure 4.9). The rETRm values in the temperate, Antarctic and Arctic *Chlorella* were observed at less than 100 µmol electrons m^{-2} s^{-1} after an hour of UVB exposure whereas the tropical strain recorded rETRm at less than 50 µmol electrons m^{-2} s^{-1} after being subjected to two hours of UVB treatment. The rETRm values from all *Chlorella* strains were less than 2.00 µmol electrons m^{-2} s^{-1} ($P < 0.05$) after a continuous five hours UVB treatment, in which rETRm terminated at 0.415, 0.009, 1.676 and 1.230 µmol electrons m^{-2} s^{-1} in the tropical, temperate, Antarctic and Arctic strains, respectively. On the other hand, in the UVA treatment, a significant reduction in rETRm was observed in all *Chlorella* strains ($P < 0.05$) but not in the tropical strain ($P > 0.05$) (Figure 4.10).

No significant changes were observed in all *Chlorella* strains which were subjected to PAR treatment ($P > 0.05$) (Figure 4.11). Antarctic *Chlorella* UMACC 237 exhibited a higher initial rETRm value (rETRm = 283.164 µmol electrons m^{-2} s^{-1}) compared to the tropical (158.166 µmol electrons m^{-2} s^{-1}), temperate (89.816 µmol electrons m^{-2} s^{-1}) and Arctic (193.262 µmol electrons m^{-2} s^{-1}) *Chlorella* strains.

Again, UVB resulted in higher % inhibition in rETRm compared to that caused by UVA, with an overall reduction of 99% in all *Chlorella* strains ($P < 0.05$) after a continuous five hours UVB treatment. In UVA treatment, the inhibition was lowest in the tropical strain ($P > 0.05$). A significant reduction in rETRm by UVA ($P < 0.05$) was observed in all temperate, Antarctic and Arctic *Chlorella* at 54%, 60% and 70%, respectively (Figure 4.12).

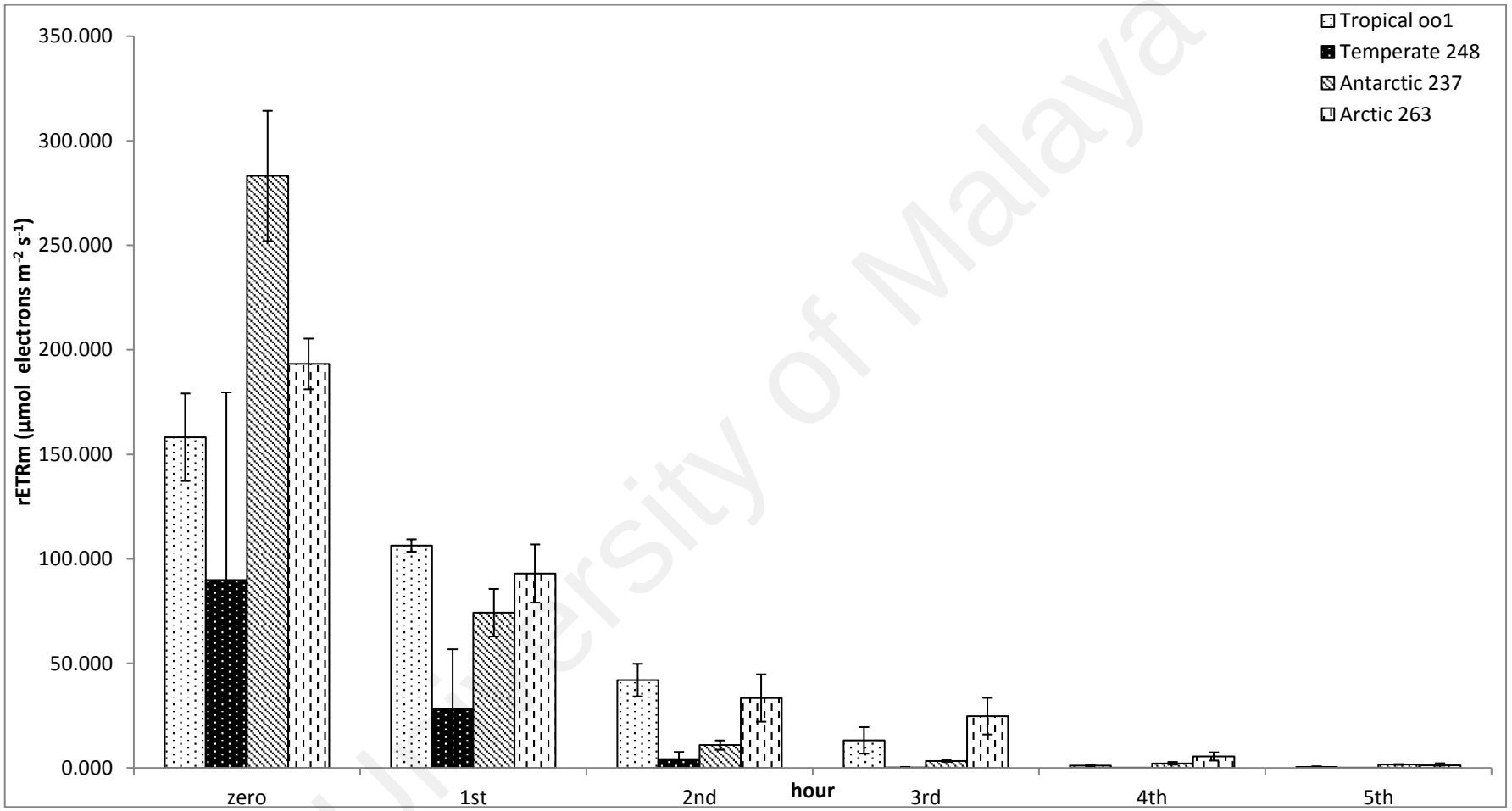


Figure 4.9: Maximum electron transport rate (rETRm) of all *Chlorella* subjected to a five hours UVB treatment. Data as means ± S.D. (n=3).

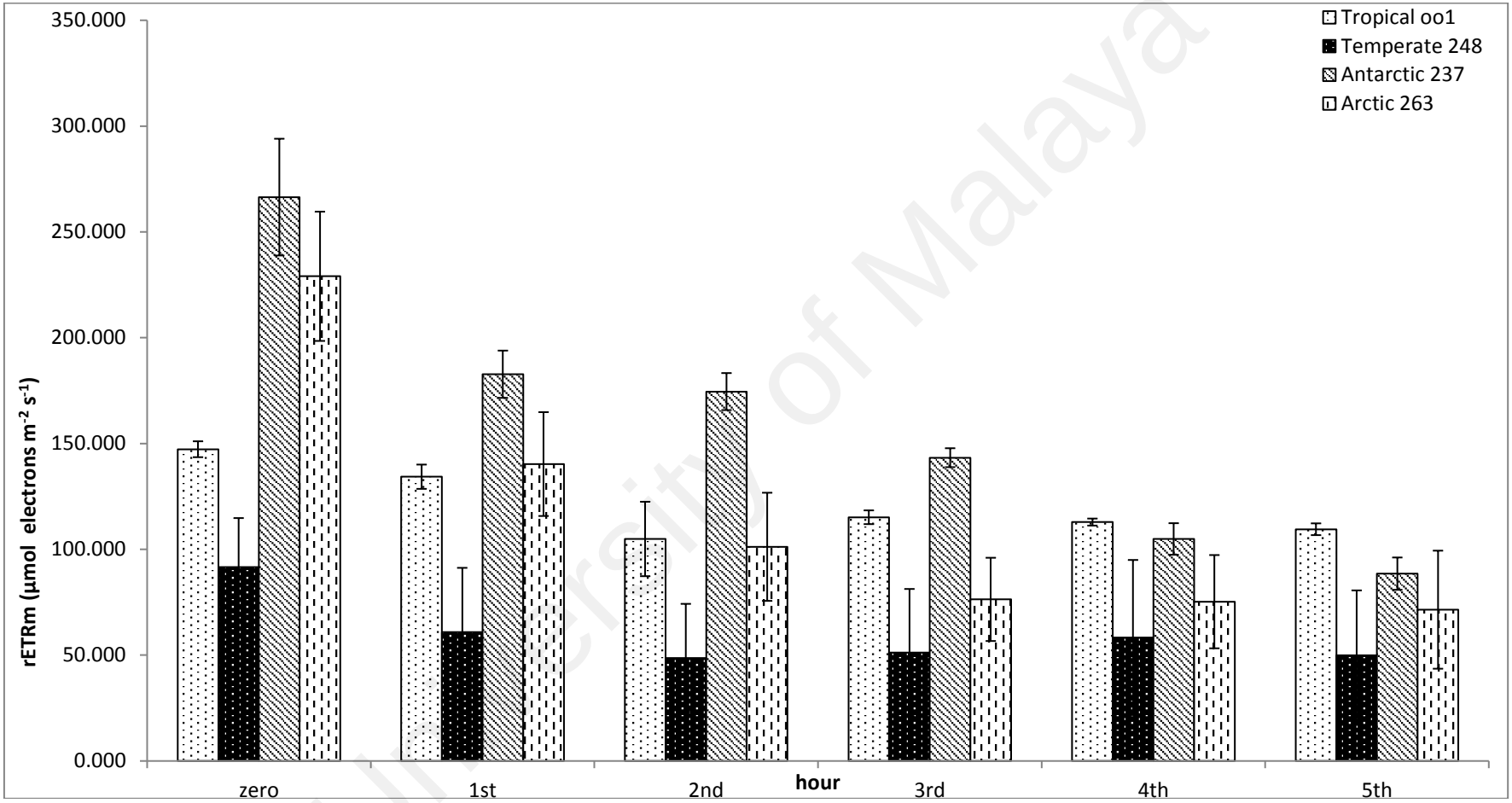


Figure 4.10: Maximum electron transport rate (rETRm) of all *Chlorella* subjected to a five hours UVA treatment. Data as means ± S.D. (n=3).

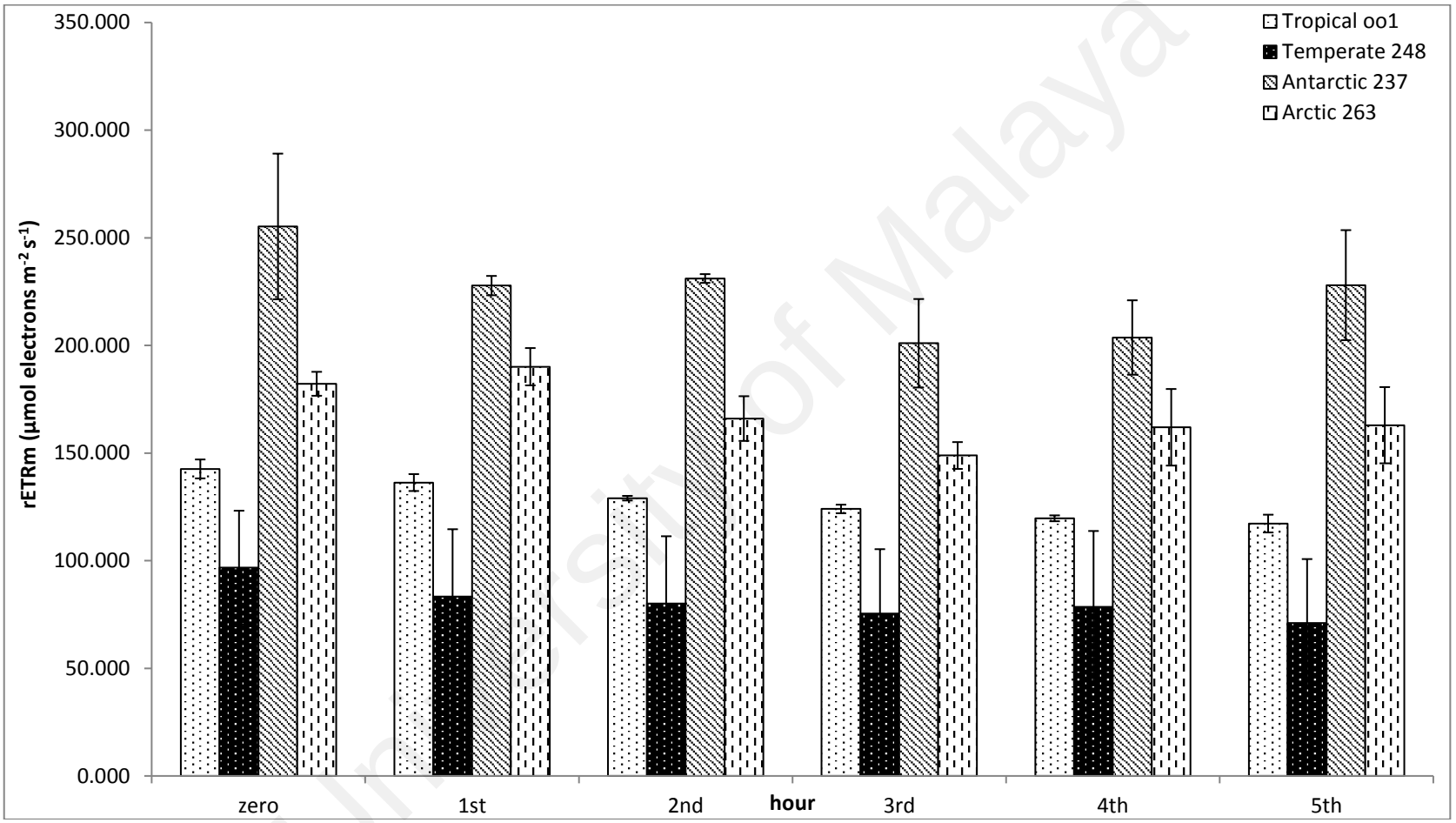


Figure 4.11: Maximum electron transport rate (rETRm) of all *Chlorella* subjected to a five hours PAR light treatment (control). Data as means ± S.D. (n=3).

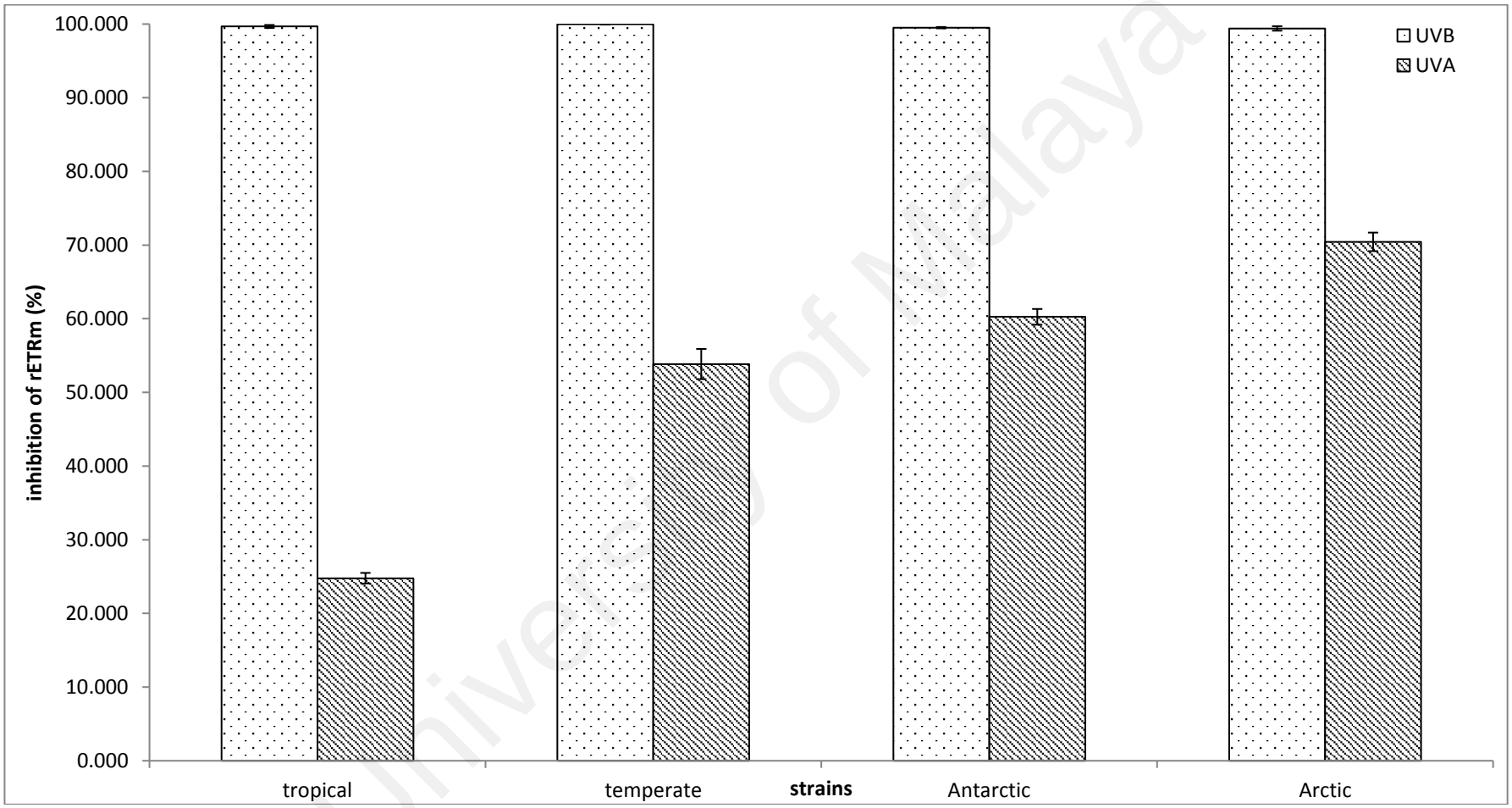


Figure 4.12: Inhibition of maximum electron transport rate (rETRm) of all *Chlorella* strains subjected to a five hours light treatment. Data as means ± S.D. (n=3).

4.1.4 Photoadaptive index (Ek)

Figure 4.13 shows the Ek value in the tropical, temperate, Antarctic and Arctic *Chlorella* strains subjected to five hours of UVB exposure. An overall inhibition in Ek of 96.70, 99.08, 98.60 and 95.55 % were observed in the tropical, temperate, Antarctic and Arctic *Chlorella* strains respectively, as shown in Figure 4.16 ($P < 0.05$). In the tropical *Chlorella*, a slight increased in Ek value was observed after the first hour of UVB exposure but the value decreased after two hours of exposure. However, increase in Ek was observed again at the fifth hour of UVB treatment. For the temperate and polar strains, UVB caused significant impacts in photoadaptive ability as shown by the declining Ek trend.

A significant decrease in Ek values after five hours of UVA exposure was only observed in the Arctic *Chlorella*, with a reduction of 47.13 % ($P < 0.05$) (Figure 4.14 and 4.16).

All strains of *Chlorella* displayed generally similar Ek values as that from the beginning of treatment throughout the entire five hours PAR treatment (Figure 4.15). Antarctic *Chlorella* UMACC 237 recorded a higher initial Ek value (694.34 µmol photons m^{-2} s^{-1}) compared to the tropical, temperate and Arctic *Chlorella* strains with Ek values of 251.63, 319.905 and 400.17 µmol photons m^{-2} s^{-1}, respectively.

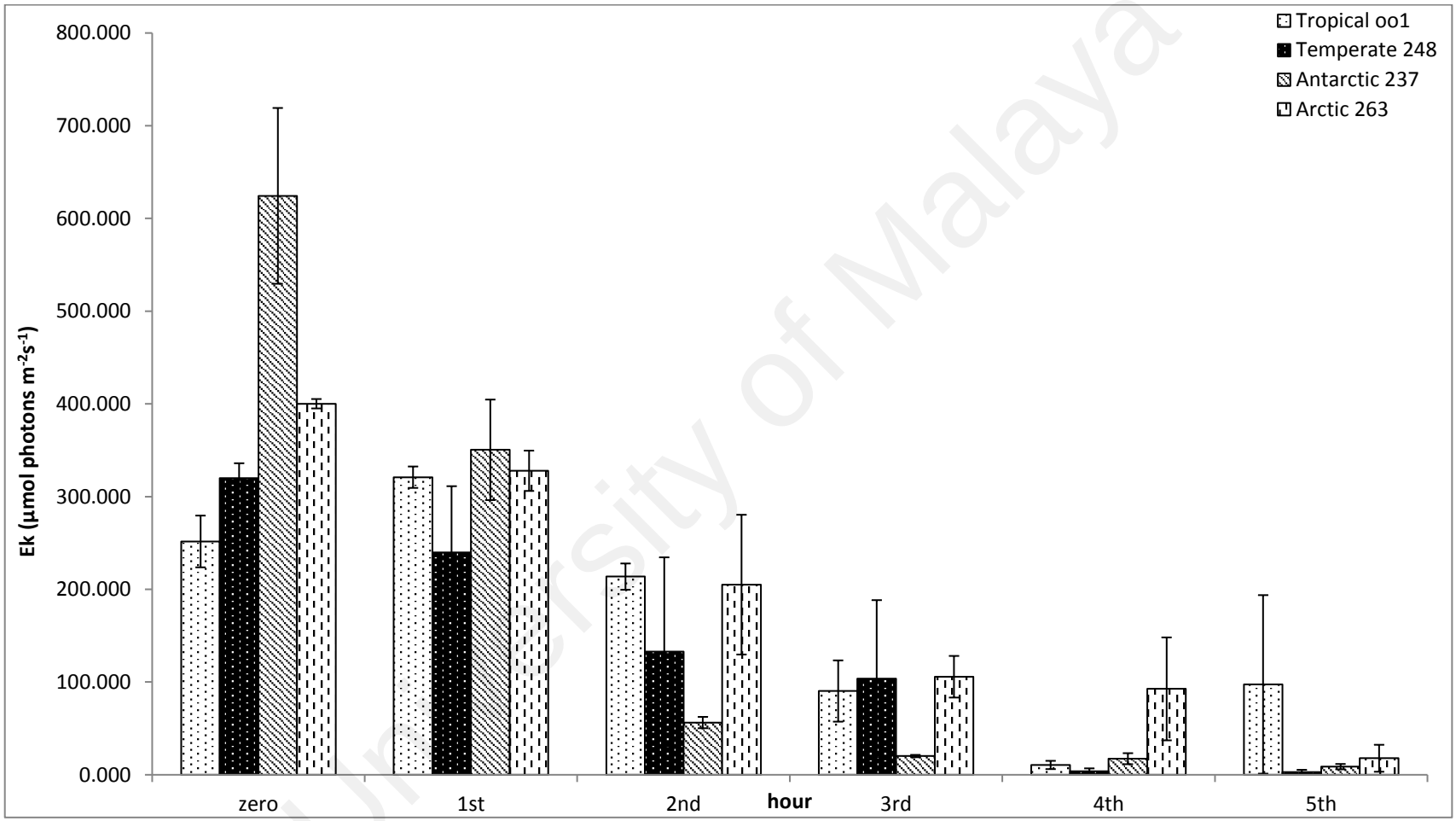


Figure 4.13: Photoadaptive index (Ek) of all *Chlorella* subjected to a five hours UVB treatment. Data as means ± S.D. (n=3).

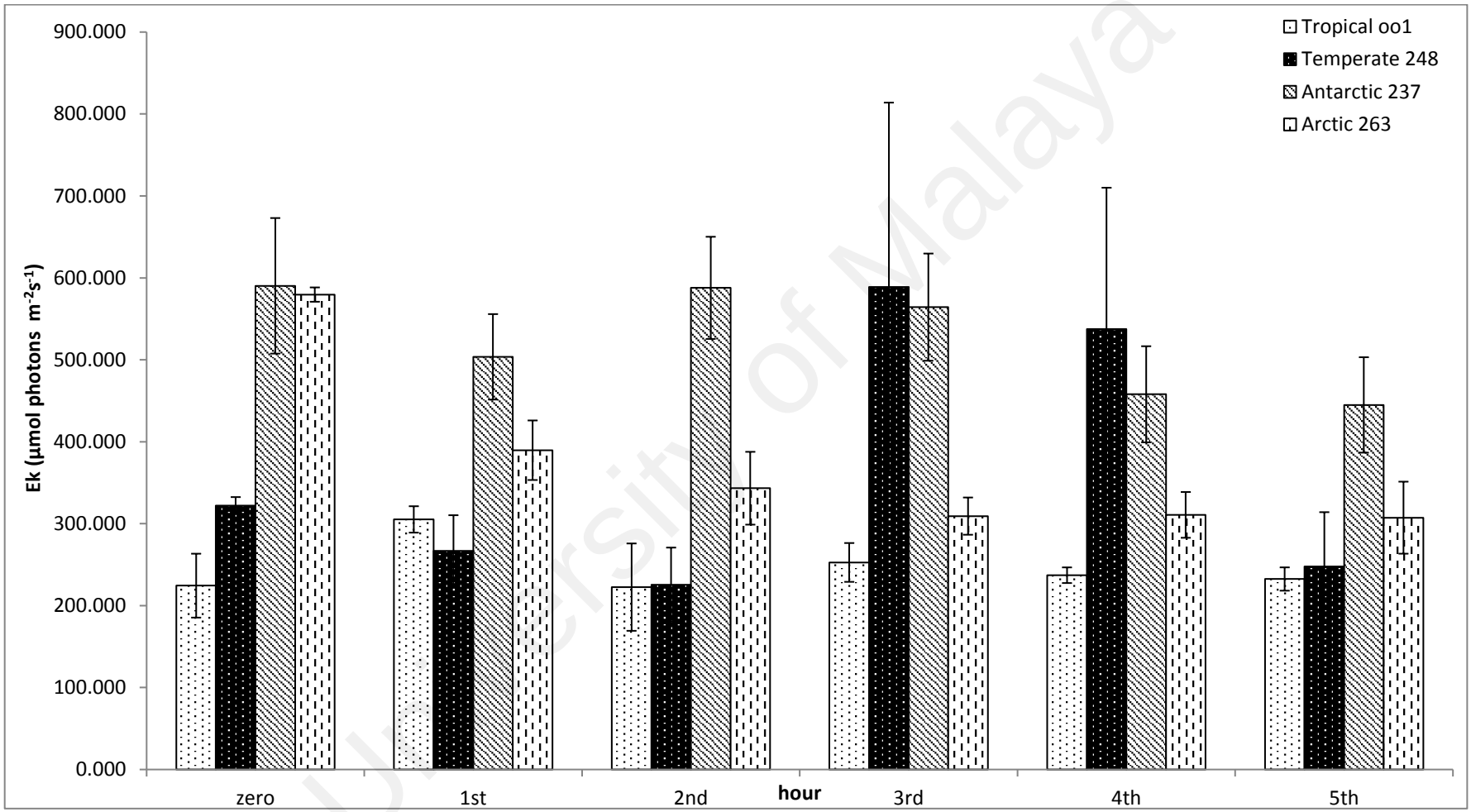


Figure 4.14: Photoadaptive index (Ek) of all *Chlorella* subjected to a five hours UVA treatment. Data as means ± S.D. (n=3).

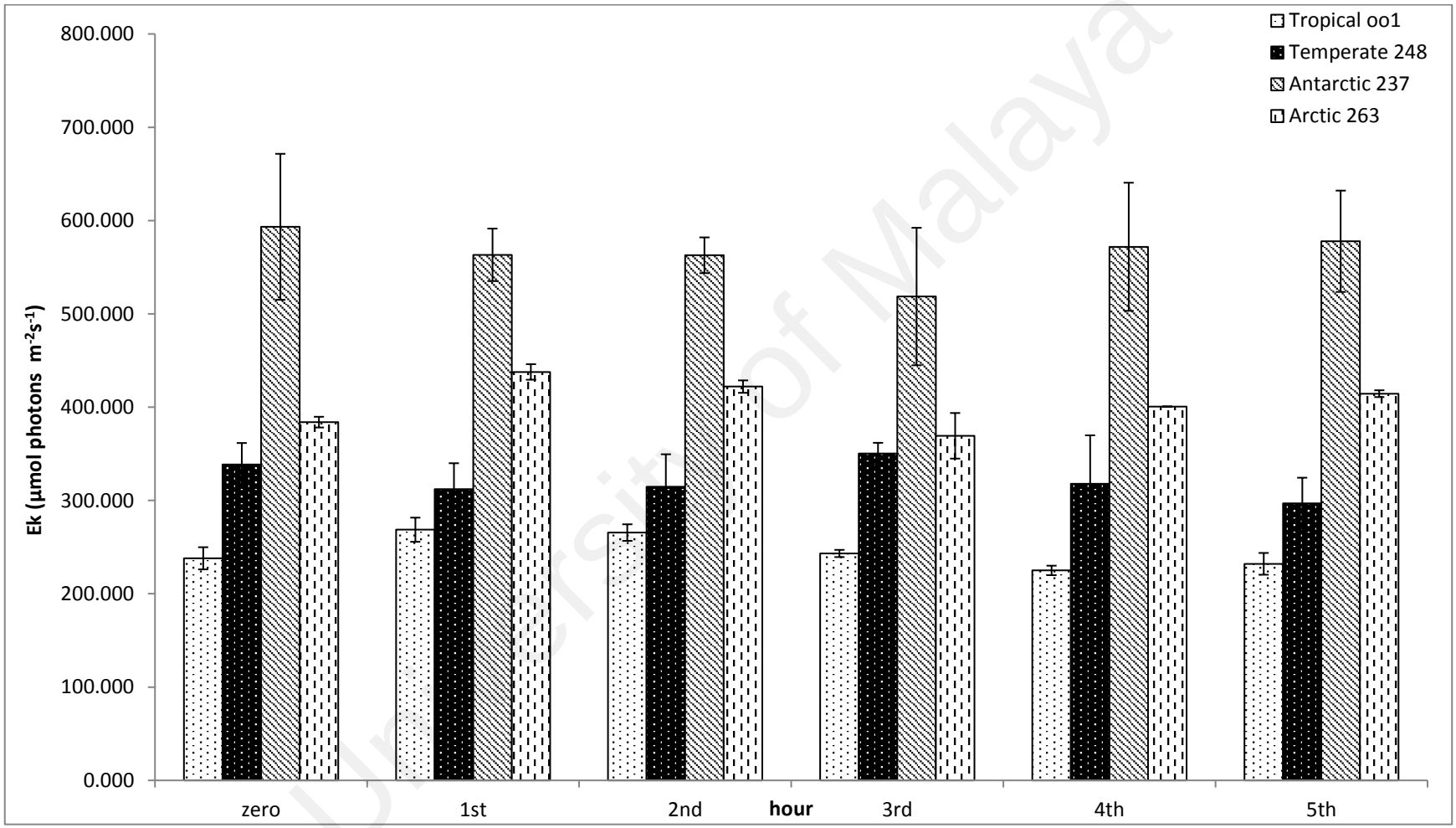


Figure 4.15: Photoadaptive index (Ek) of all *Chlorella* subjected to a five hours PAR light treatment (control). Data as means ± S.D. (n=3).

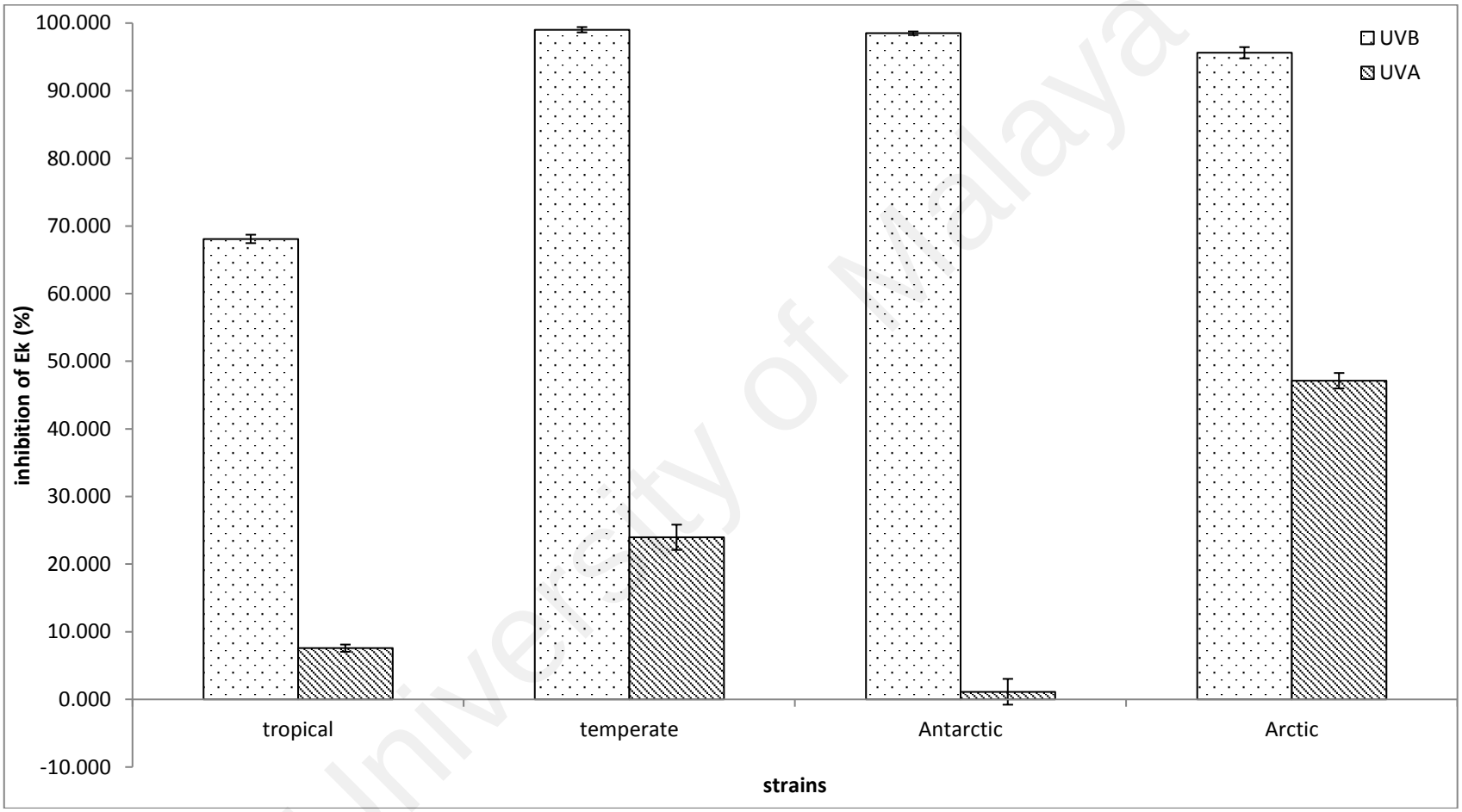


Figure 4.16: Inhibition of photoadaptive index (Ek) of all *Chlorella* strains subjected to a five hours light treatment. Data as means ± S.D. (n=3).

4.2 Photosynthetic pigments

No significant differences in chl *a* per cell, carotenoids per cell and chl *a* : carotenoids ratio were observed in all *Chlorella* strains under UVB, UVA and control treatments as shown in Figure 4.17, 4.18 and 4.19 ($P > 0.05$).

The natural Chl *a* and carotenoid content per cell were higher in both polar *Chlorella* followed by the temperate and tropical strains. Chl *a* : carotenoids ratio was highest in Arctic strain as compared to the other *Chlorella* strains.

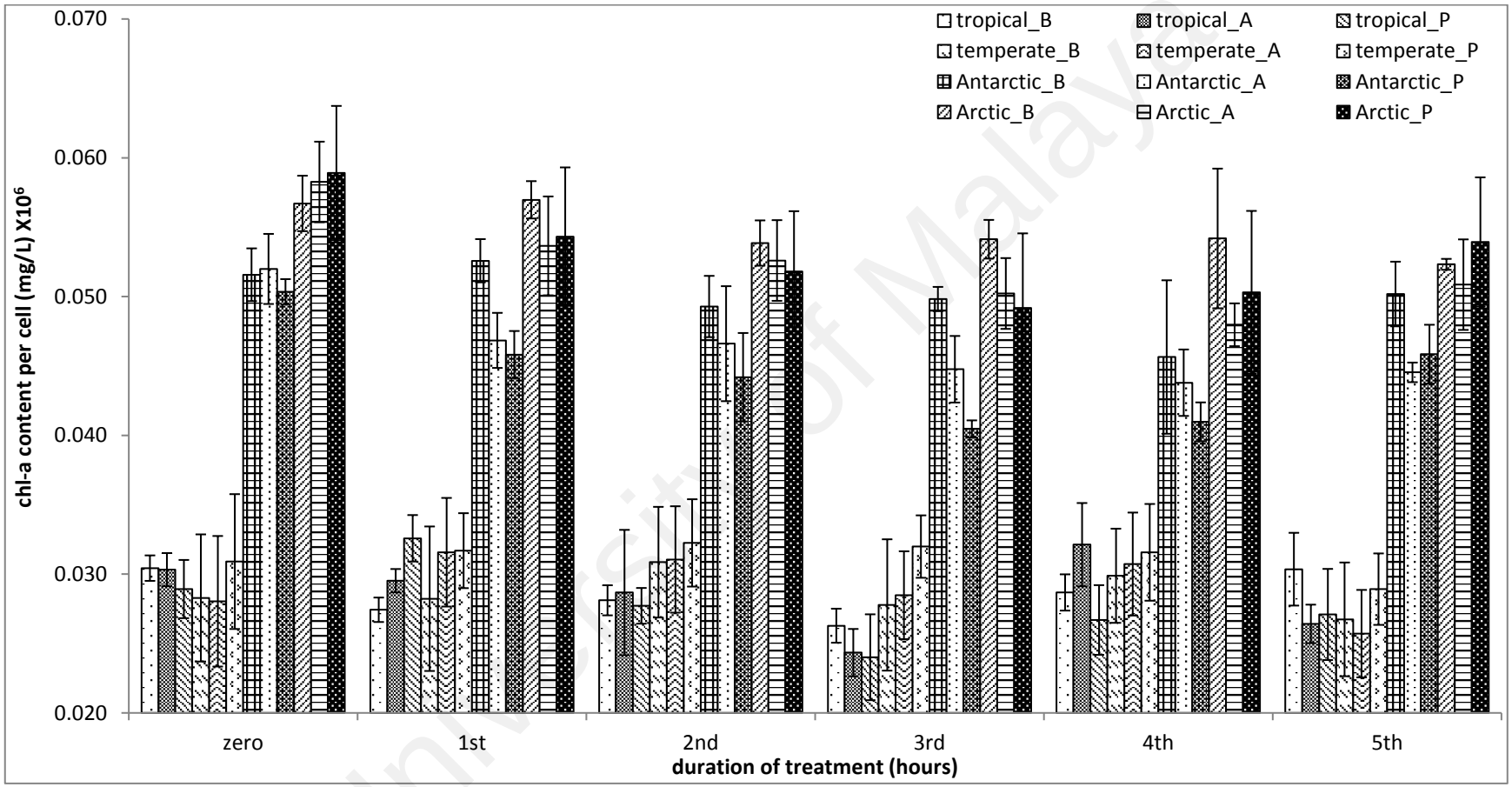


Figure 4.17: Chl *a* content per cell for all *Chlorella* strains subjected to UVB+UVA+PAR (B), UVA+PAR (A) and PAR alone (P) (n = 3). Vertical bars denote standard deviations from triplicate samples. Data as means ± S.D. (n=3).

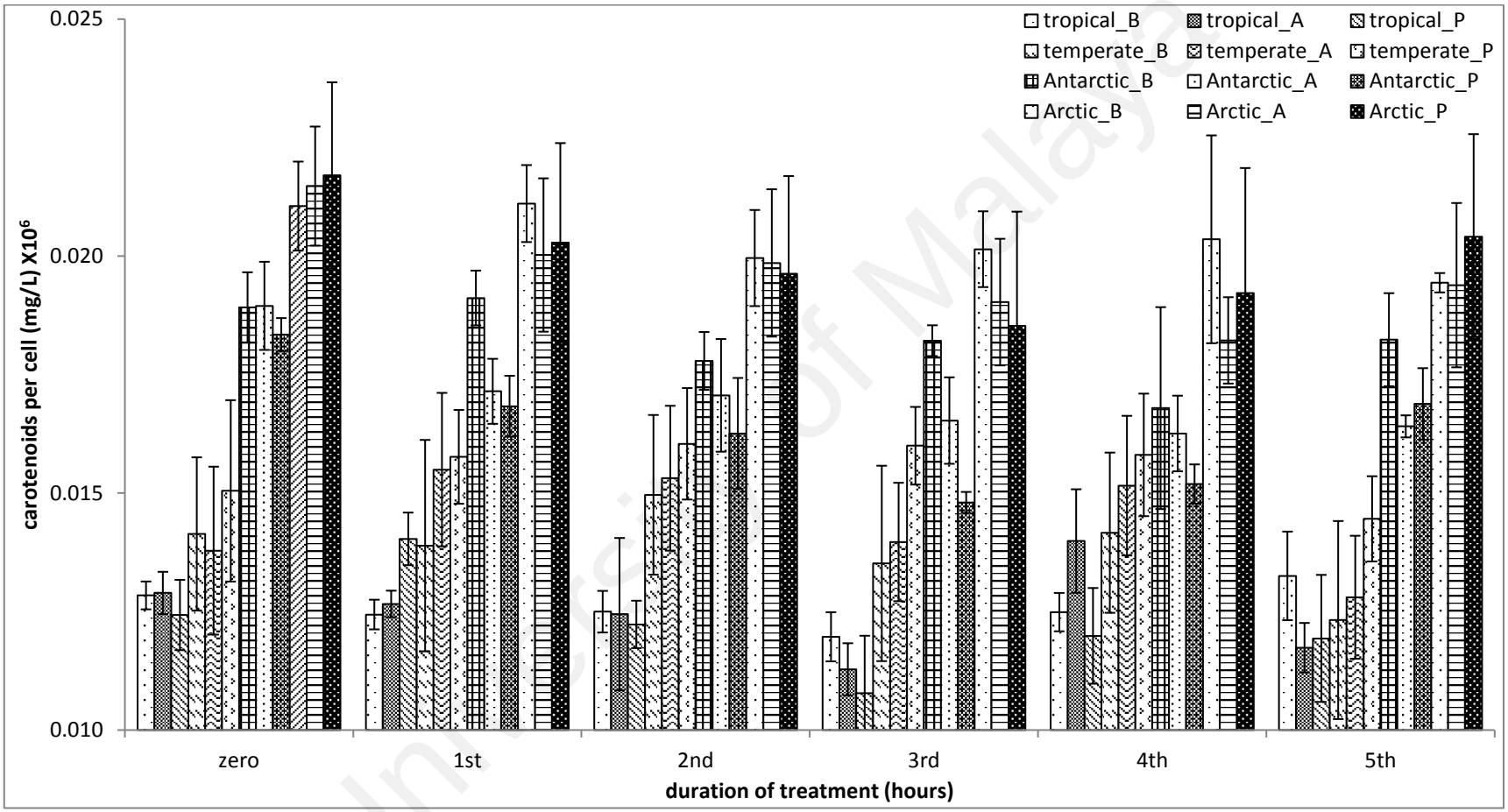


Figure 4.18: Carotenoids content per cell for all *Chlorella* strains subjected to UVB+UVA+PAR (B), UVA+PAR (A) and PAR alone (P) (n = 3). Vertical bars denote standard deviations from triplicate samples. Data as means ± S.D. (n=3).

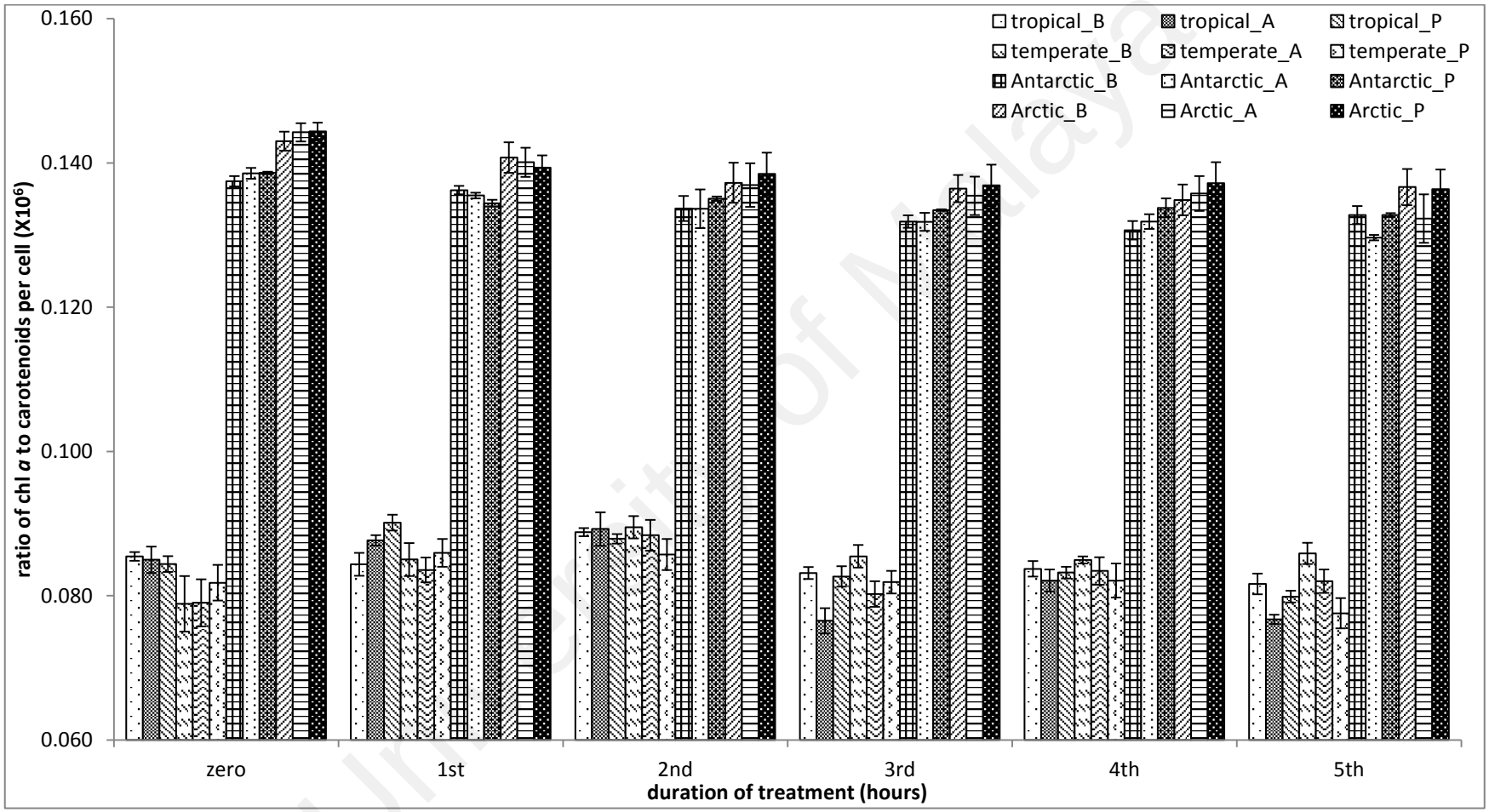


Figure 4.19: chl *a* : carotenoid content ratio per cell for all *Chlorella* strains subjected to UVB+UVA+PAR (B), UVA+PAR (A) and PAR alone (P) (n = 3). Vertical bars denote standard deviations from triplicate samples. Data as means ± S.D. (n=3).

4.3 Phylogenetic analyses of *Chlorella* strains based on 18S rDNA

The phylogeny of the *Chlorella* strains was based on the nuclear 18S rDNA, in which similar tree topologies were observed using the maximum likelihood (ML) and Bayesian Inference (BI) approaches (Figure 4.20). A total alignment length of 505 bp was used to construct the phylogeny. Only the phylogenetic tree inferred from ML was presented here as BI analysis also gave similar tree topology.

Chlorella and its closely related species spanned the two main classes, Trebouxiophyceae and Chlorophyceae, which were not strongly supported based on the 18S rDNA dataset. Two major sister clades were observed within Chlorellaceae, namely the *Chlorella* and *Parachlorella*. The strains UMACC 237, 248, 263 and 001 used in the present study were inferred to be monophyletic with other *Chlorella* samples from GenBank, thus confirming their identity as *Chlorella*.

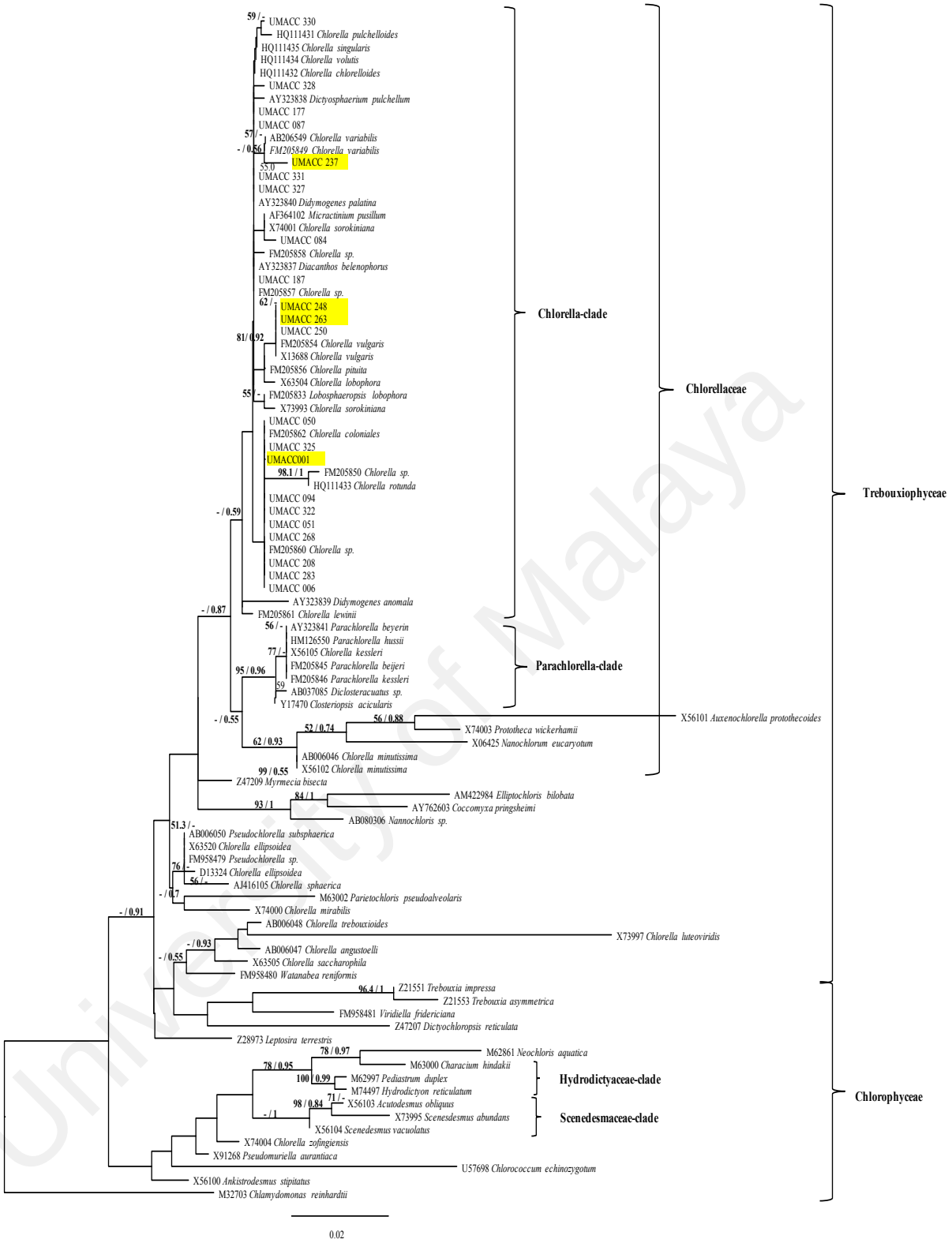


Figure 4.20: Likelihood (ML) phylogenetic tree of Trebouxiophyceae and Chlorophyceae. ML bootstrap values and Bayesian posterior probabilities are shown above branches.
The scale indicates number of substations.
Samples used in the present study are highlighted yellow.

4.4 Random Amplified Polymorphic DNA (RAPD) analysis

Based on the study on photosynthetic efficiency in response to short term UVR exposure (refer to Chapter 4.1), the *Chlorella* which were photosynthetically inactive (which Fv/Fm value was less than 0.1) were used for RAPD analysis. RAPD was used to assess the DNA damage in *Chlorella* sp. after five hours of exposure to light treatment, i.e. PAR treatment (control), UVA and UVB treatment. The effect of UVR on DNA was analyzed by detecting changes of DNA bands profile between control culture and treated cultures.

4.4.1 Degree of polymorphism

In a total of sixty 10-mer priming oligonucleotides, 12 of them showed complete amplification in four of the *Chlorella* strains used (Table 4.1). Further analyses were performed on the 12 selected primers in detecting changes of DNA band profiles and polymorphisms between the control culture, UVA and UVB-treated cultures.

Table 4.1 shows the details and degree of polymorphisms of the 12 selected RAPD primers. The first six primers displaying highest degree of polymorphism are S33, S90, S93, S29, OPA10 and S7, with polymorphism values of 44.83, 38.71, 29.63, 22.95, 21.74 and 19.61 %, respectively (Figure 4.21).

Table 4.1 : Degree of polymorphism of selected RAPD primers.

RAPD Primers	Sequence (5' - 3')	G+C content	Total number of bands	Total number of polymorphic bands	Percentage polymorphism (%)
S 33	CAGCACCCAC	70	58	26	44.83
S 90	AGGGCCGTCT	70	31	12	38.71
S 93	CTCTCCGCCA	70	27	8	29.63
S 29	GGGTAACGCC	70	61	14	22.95
OPA 10	GTGATCGCAG	60	23	5	21.74
S 7	GGTGACGCAG	70	51	10	19.61
OPK 7	AGCGAGCAAG	60	49	8	16.33
S 23	AGTCAGCCAC	60	68	11	16.18
S 42	GGACCCAACC	70	56	8	14.29
S 62	GTGAGGCGTC	70	43	6	13.95
S 48	GTGTGCCCCA	70	49	6	12.24
S 24	AATCGGGCTG	60	72	8	11.11

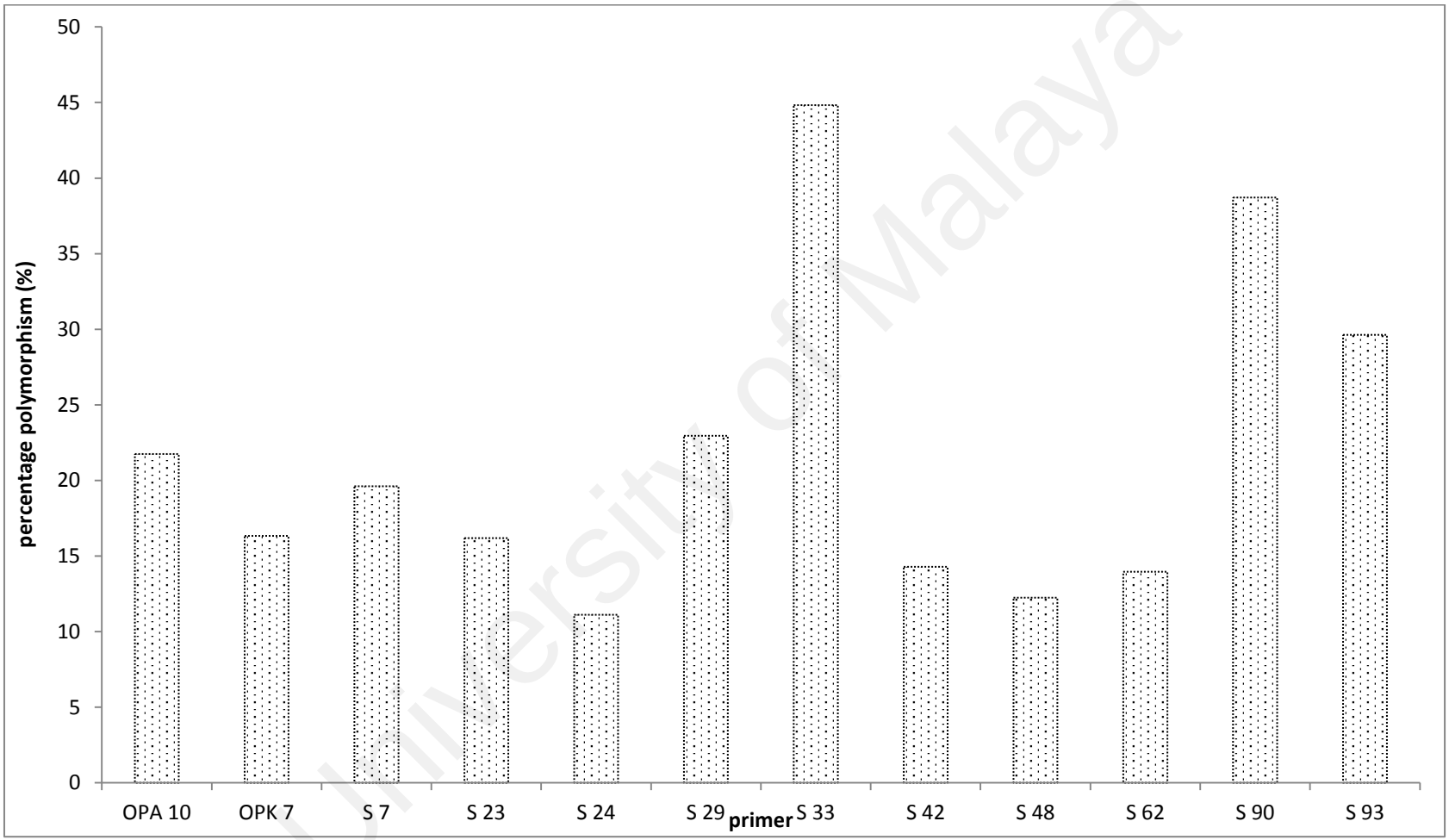


Figure 4.21: The total polymorphism (%) of 12 selected RAPD primers.

4.4.2 Variation of bands

Figures 4.22 to 4.25 show the band variations, defined as the disappearance of existing bands and emergence of new bands in each specimen of the four *Chlorella* strains after five hours of UVA and UVB treatments.

Figure 4.22 shows the individual percentage of bands variation amplified by selected RAPD primers in tropical *Chlorella* sp. (UMACC 001) after five hours of UVA and UVB treatment. The number of RAPD band variations for samples subjected to UVB treatment were greater than that subjected to UVA stress. Disappearance of RAPD bands for samples subjected to UVA was detected only by primer S7 whereas primers S33, S42 and S90 showed band emergence upon treatment with UVA. Primers OPK7, S23, S48, S62 and S90 were capable of detecting both disappearance and emergence of RAPD bands for samples subjected to UVB treatment.

For temperate *Chlorella* UMACC 248, DNA mutation due to UVA stress was less as compared to that of UVB treatment. Disappearance and emergence of RAPD bands due to UVA treatment were detected by OPK7, S29, S33, S90, respectively. Primers OPK7, S42 and S90 were able to identify both disappearance and emergence of RAPD bands by UVB (Figure 4.23).

Polar (Antarctic and Arctic) strains exhibited higher number of band variations compared to the tropical and temperate strains. In Antarctic *Chlorella* UMACC237 (Figure 4.24), primer S93 was the only primer capable of detecting both disappearance and emergence of RAPD bands caused by UVA and UVB treatment. In Arctic *Chlorella* UMACC 263 (Figure 4.25), the number of new bands in all strains was very low. The highest number of band disappearance was detected by primer S29 and S23,

for UVA and UVB treatment, respectively. The primer S33 detected disappearance of bands in both UVA and UVB treatments in the Arctic *Chlorella*.

The primer displaying the highest levels of polymorphism were primer S33 (polymorphism = 44.83%), primer S93 (polymorphism = 29.63%) and primer S29 (polymorphism = 22.95%) as shown in Table 4.1, which yielded more band variations in both UV treatments. Primer S90 with a polymorphism value of 38.71% and primer OPA10 with polymorphism value of 21.74% generated band variations in the tropical and temperate strains only.

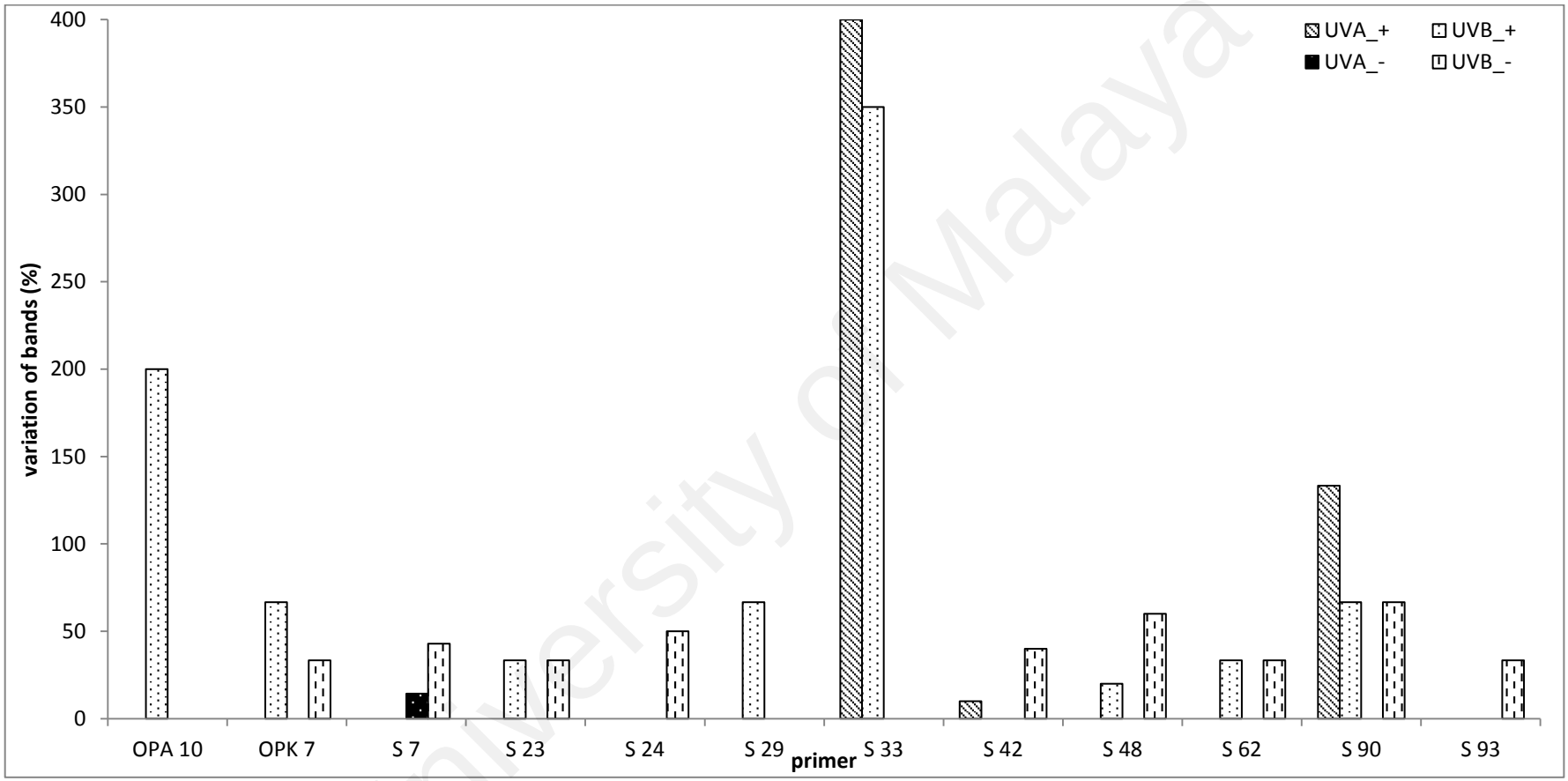


Figure 4.22: Variation of bands (%) in tropical *Chlorella* UMACC 001 from selected RAPD primers. "UVA_+" and "UVB_+" refer to emergence of bands after UVA, and UVB treatments respectively. "UVA_-" and "UVB_-" refer to disappearance of bands after UVA and UVB treatments respectively.

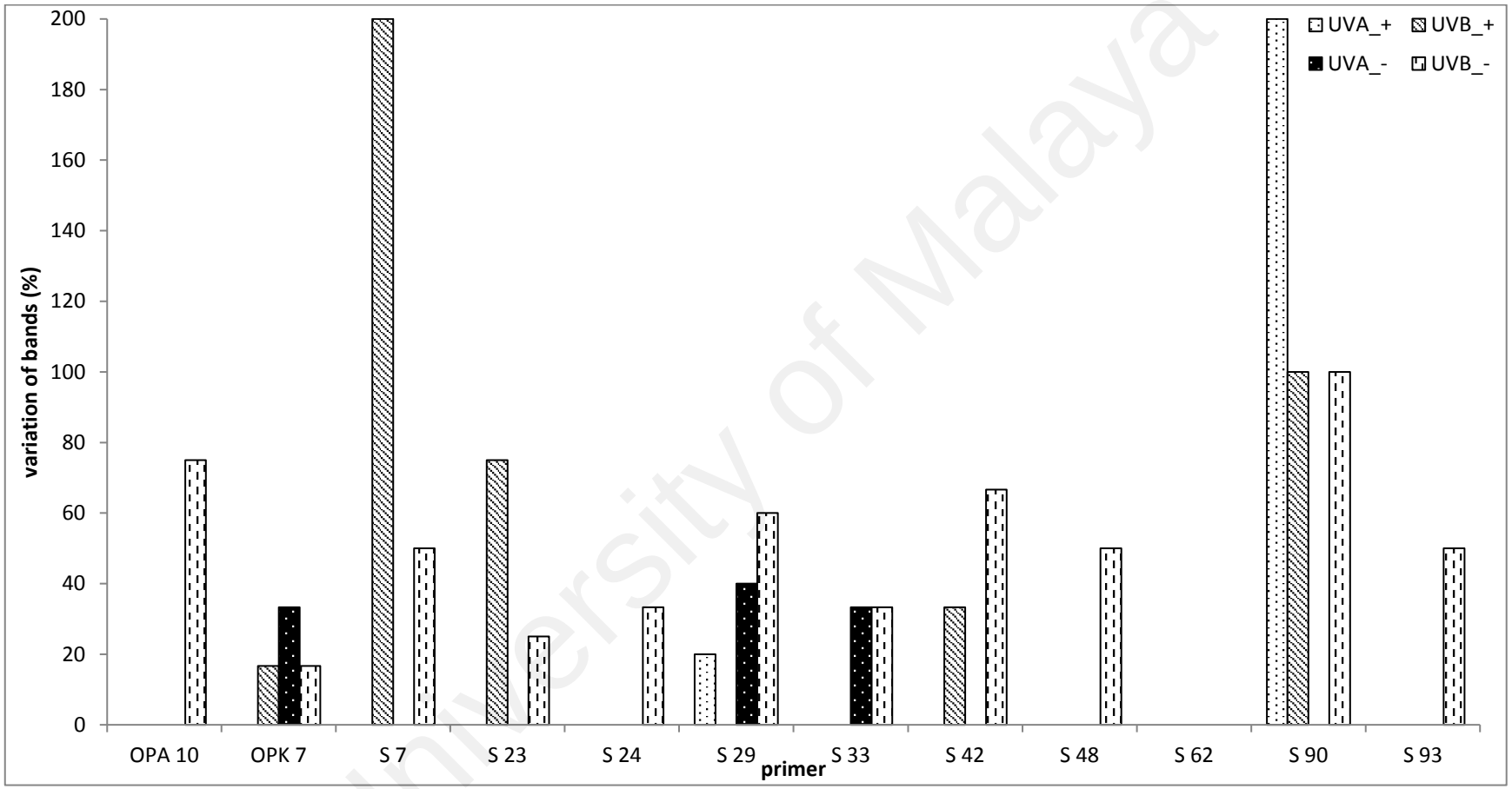


Figure 4.23: Variation of bands (%) for temperate *Chlorella* UMACC 248 from selected RAPD primers. "UVA_+" and "UVB_+" refer to emergence of bands after UVA, and UVB treatments respectively. "UVA_-" and "UVB_-" refer to disappearance of bands after UVA and UVB treatments respectively.

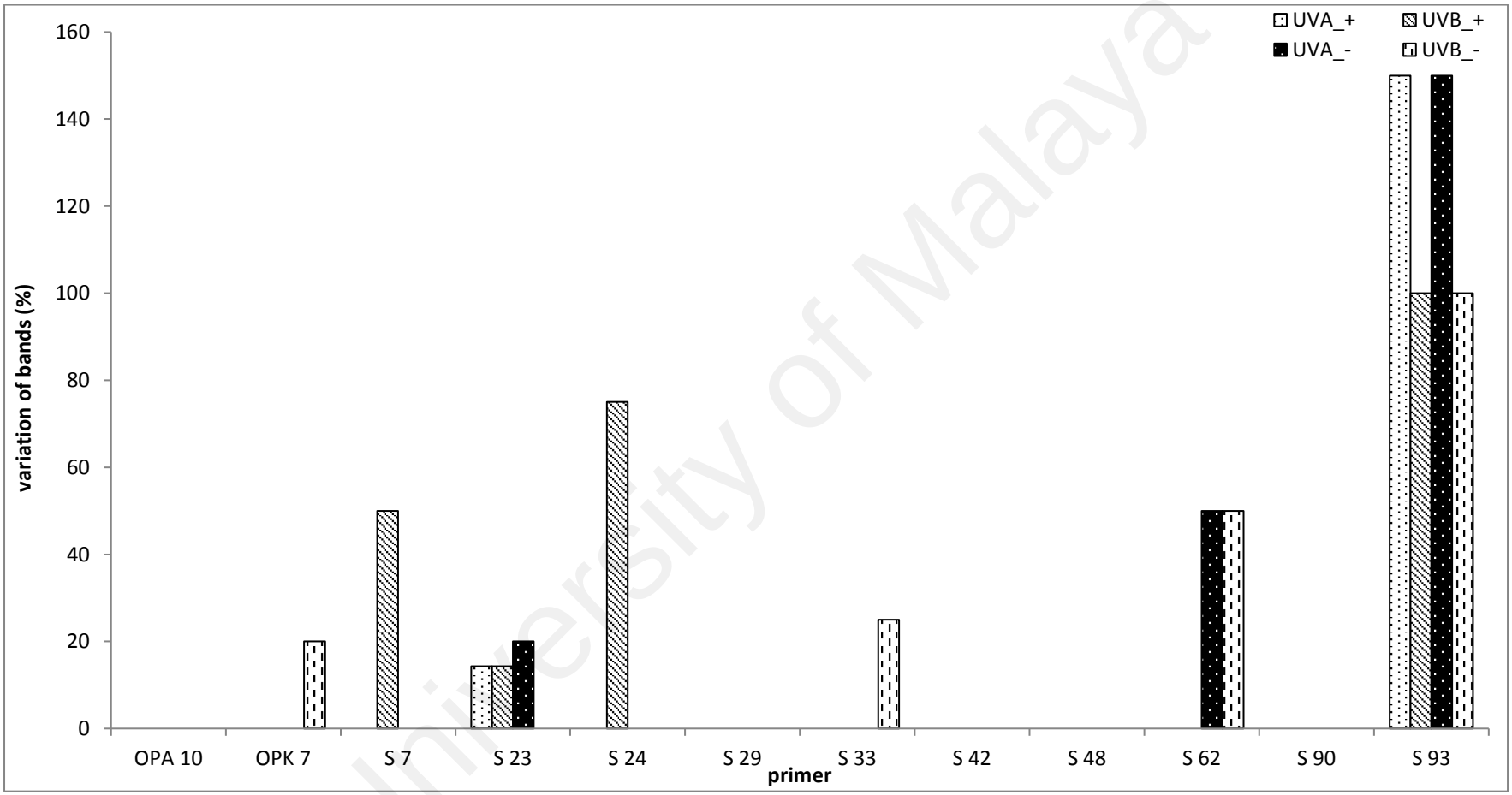


Figure 4.24: Variation of bands (%) for Antarctic *Chlorella* UMACC 237 from selected RAPD primers. "UVA_+" and "UVB_+" refer to emergence of bands after UVA, and UVB treatments respectively. "UVA_-" and "UVB_-" refer to disappearance of bands after UVA and UVB treatments respectively.

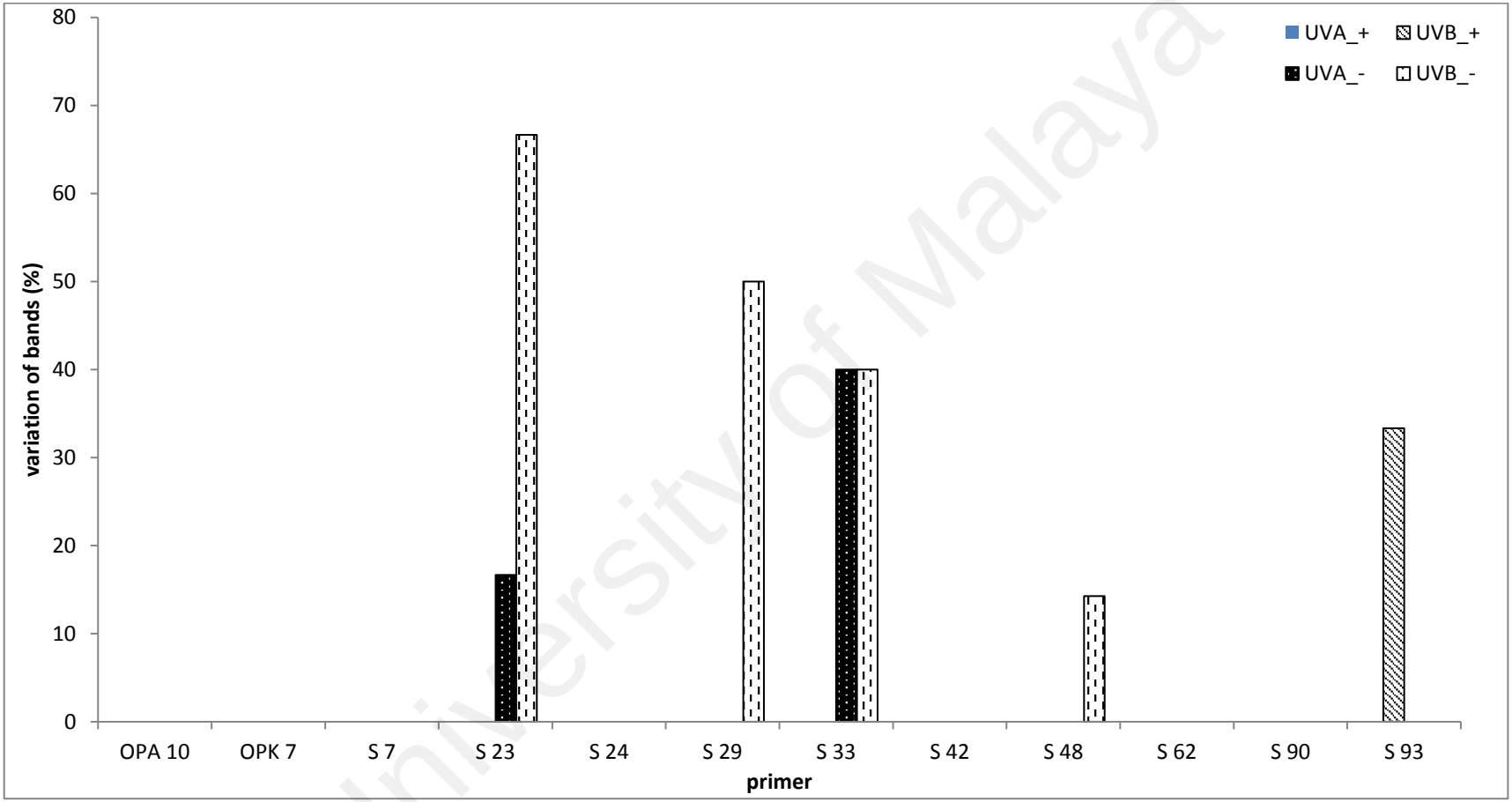


Figure 4.25: Variation of bands (%) for Arctic *Chlorella* UMACC 263 from selected RAPD primers. "UVA_+" and "UVB_+" refer to emergence of bands after UVA, and UVB treatments respectively. "UVA_-" and "UVB_-" refer to disappearance of bands after UVA and UVB treatments respectively.

Table 4.2 Variation of bands (%) after 5 hours light treatment in all *Chlorella* strains, by selected primers with high polymorphism degree. Symbol "+" denotated emergence of new bands while "-" denotated disappearance of bands.

Primers	polymorphism (%)	Strains	Tropical		Temperate		Antarctic		Arctic	
		Band variation (%) / Treatment	+	-	+	-	+	-	+	-
OPA10	21.74	UVB	200	_	_	75	_	_	_	_
		UVA	_	_	_	_	_	_	_	_
S29	22.95	UVB	66.67	_	_	60	_	11.11	_	_
		UVA	_	_	20	40	_	22.22	_	50
S33	44.83	UVB	350	_	_	33.33	_	25	_	40
		UVA	400	_	_	33.33	_	_	_	40
S90	38.71	UVB	66.67	66.67	100	100	_	_	_	_
		UVA	133.33	_	200	_	_	_	_	_
S93	29.63	UVB	_	33.33	_	50	100	-	33.33	_
		UVA	_	_	_	_	150	-	_	_

4.4.3 Similarity of bands

Figures 4.26 to 4.29 show the band similarity of four *Chlorella* strains after exposure to light treatments. In tropical *Chlorella* (Figure 4.26), the lowest band similarity caused by UVA and UVB was 85.71% and 20%, amplified by primers S7 and S48 respectively, and 60% and 25% by primers S29 and OPA10, respectively, in UVA and UVB treatments, for the temperate strain (Figure 4.27). The Antarctic strain recorded a lowest band similarity of 50% and 50% caused by UVA and UVB based on primer S62. The lowest band similarity in Arctic *Chlorella* was 60% as a result of UVA stress (which was detected by primer S33), and 33.33% due to UVB treatment (which was detected by primer S23.

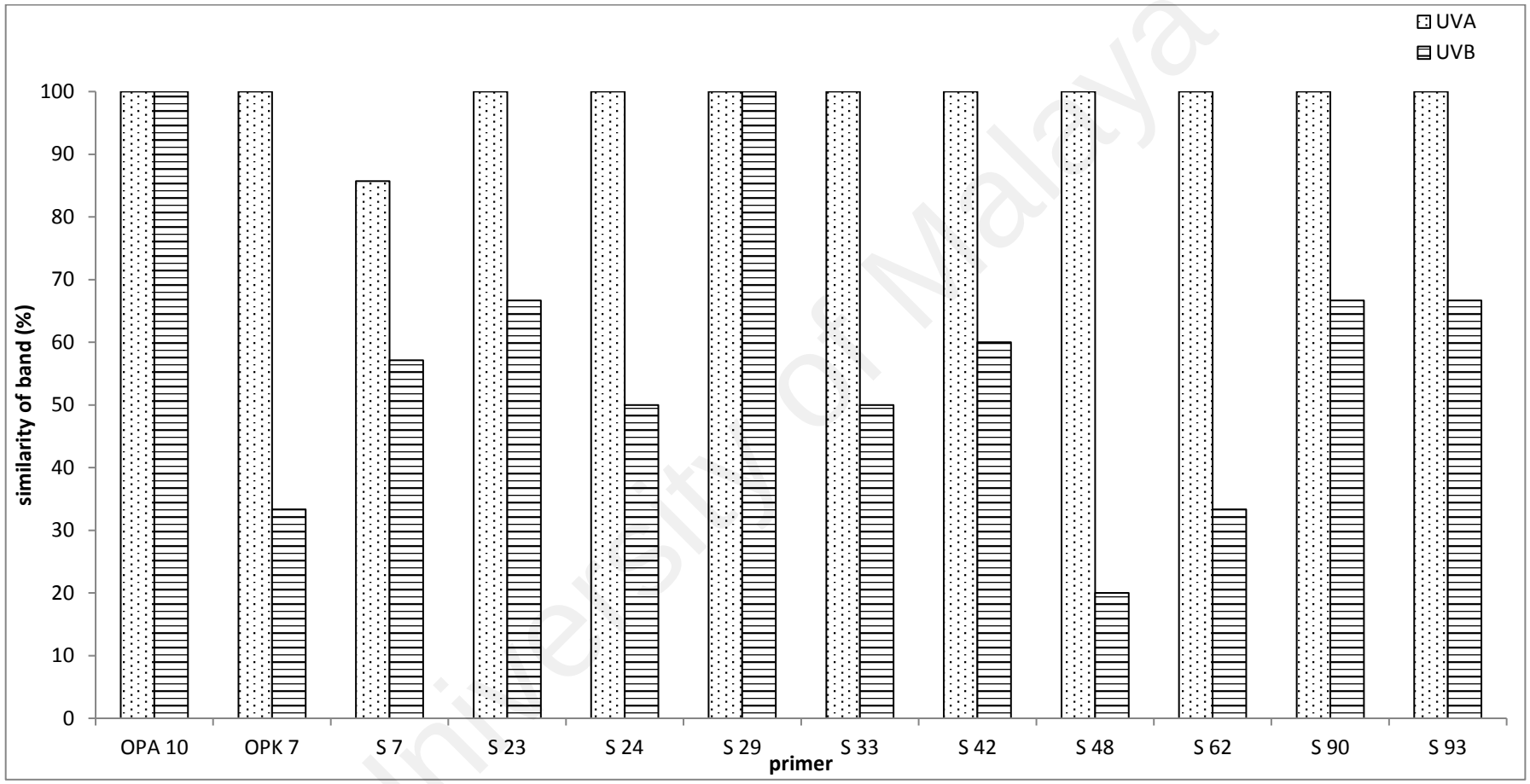


Figure 4.26: Similarity of bands (%) for tropical *Chlorella* UMACC 001.
"UVA" refers to samples treated by UVA; "UVB" refers to samples treated by UVB.

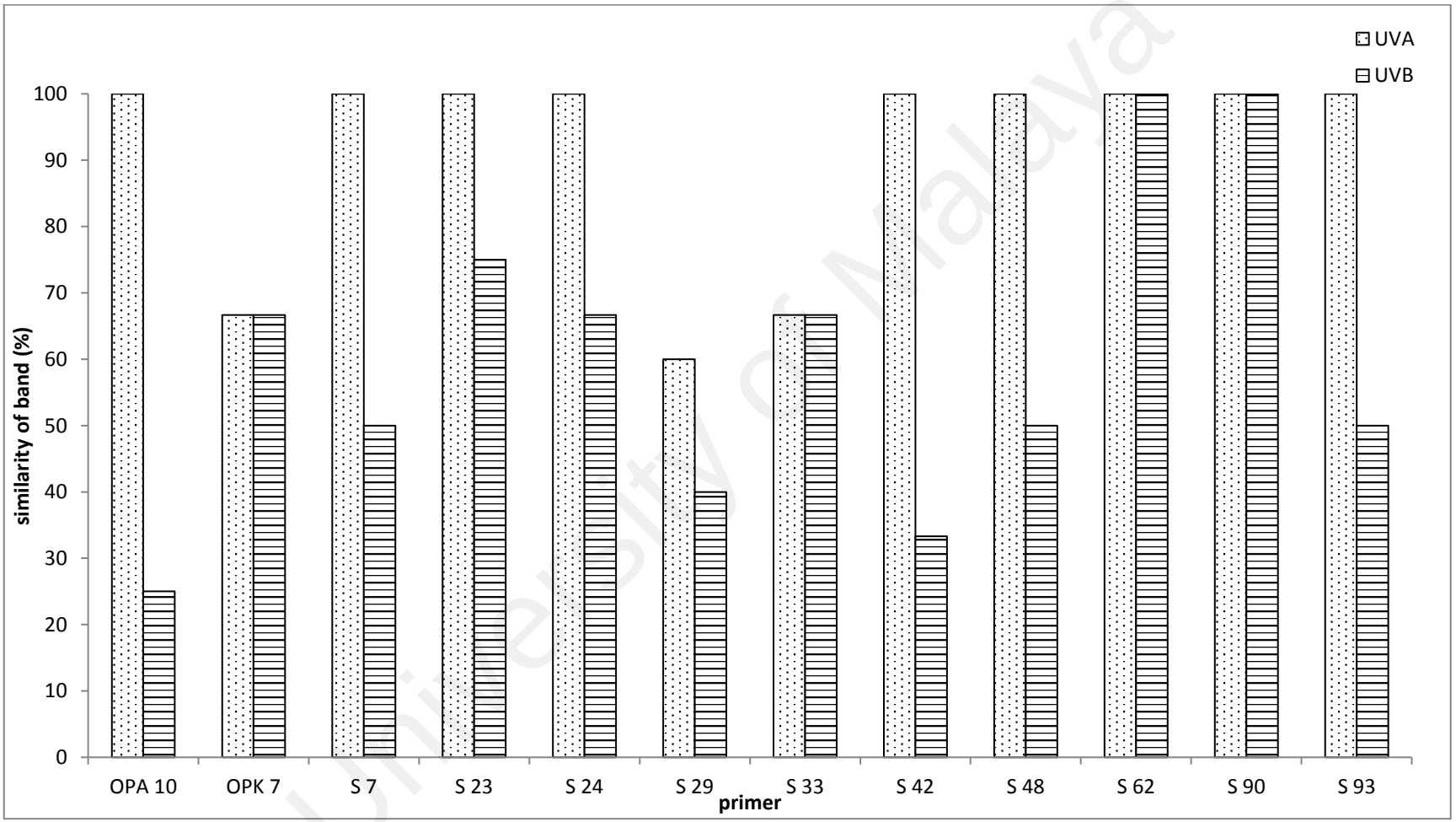


Figure 4.27: Similarity of bands (%) for temperate *Chlorella* UMACC 248.
"UVA" refers to samples treated by UVA; "UVB" refers to samples treated by UVB.

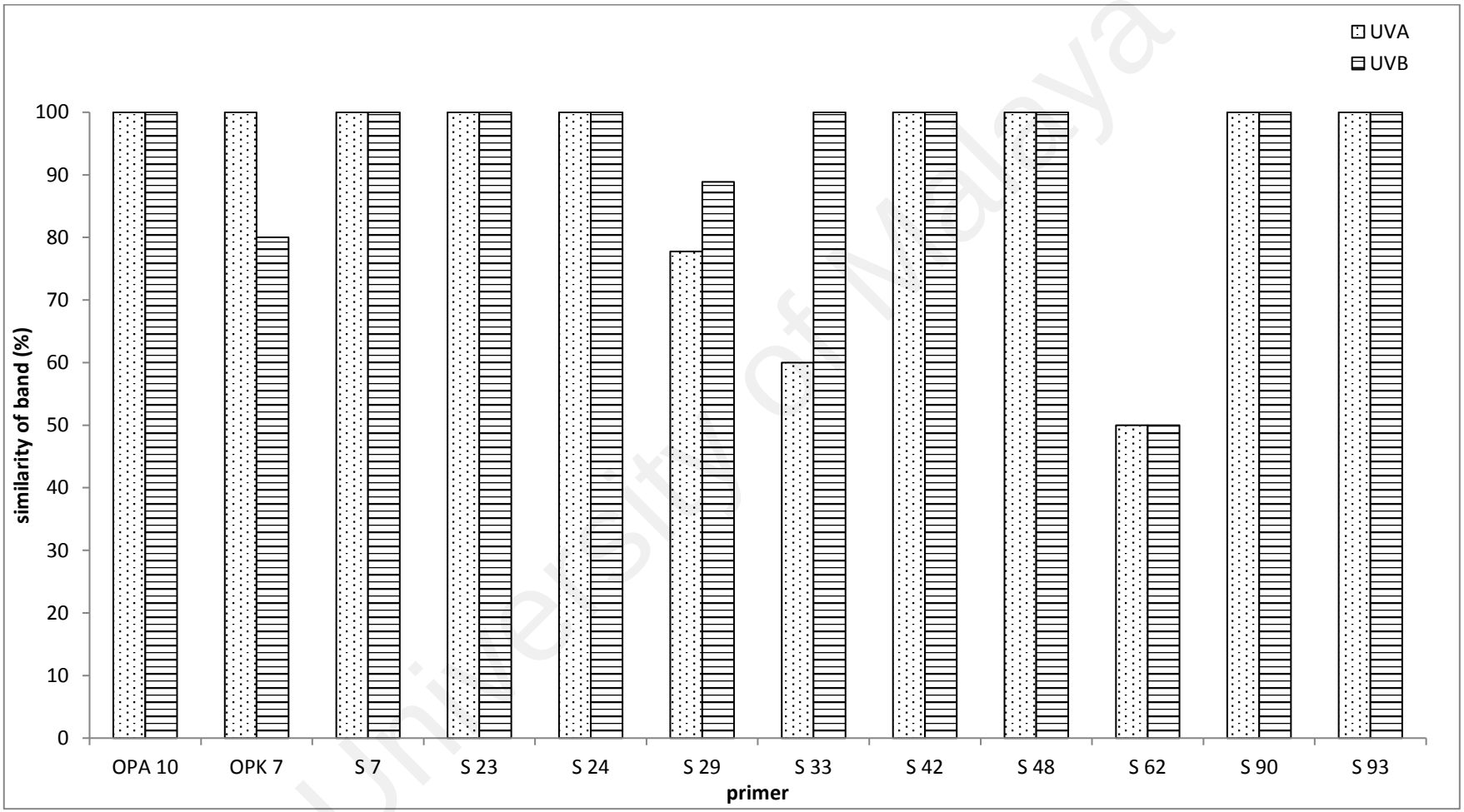


Figure 4.28: Similarity of bands (%) for Antarctic *Chlorella* UMACC 237.
"UVA" refers to samples treated by UVA; "UVB" refers to samples treated by UVB.

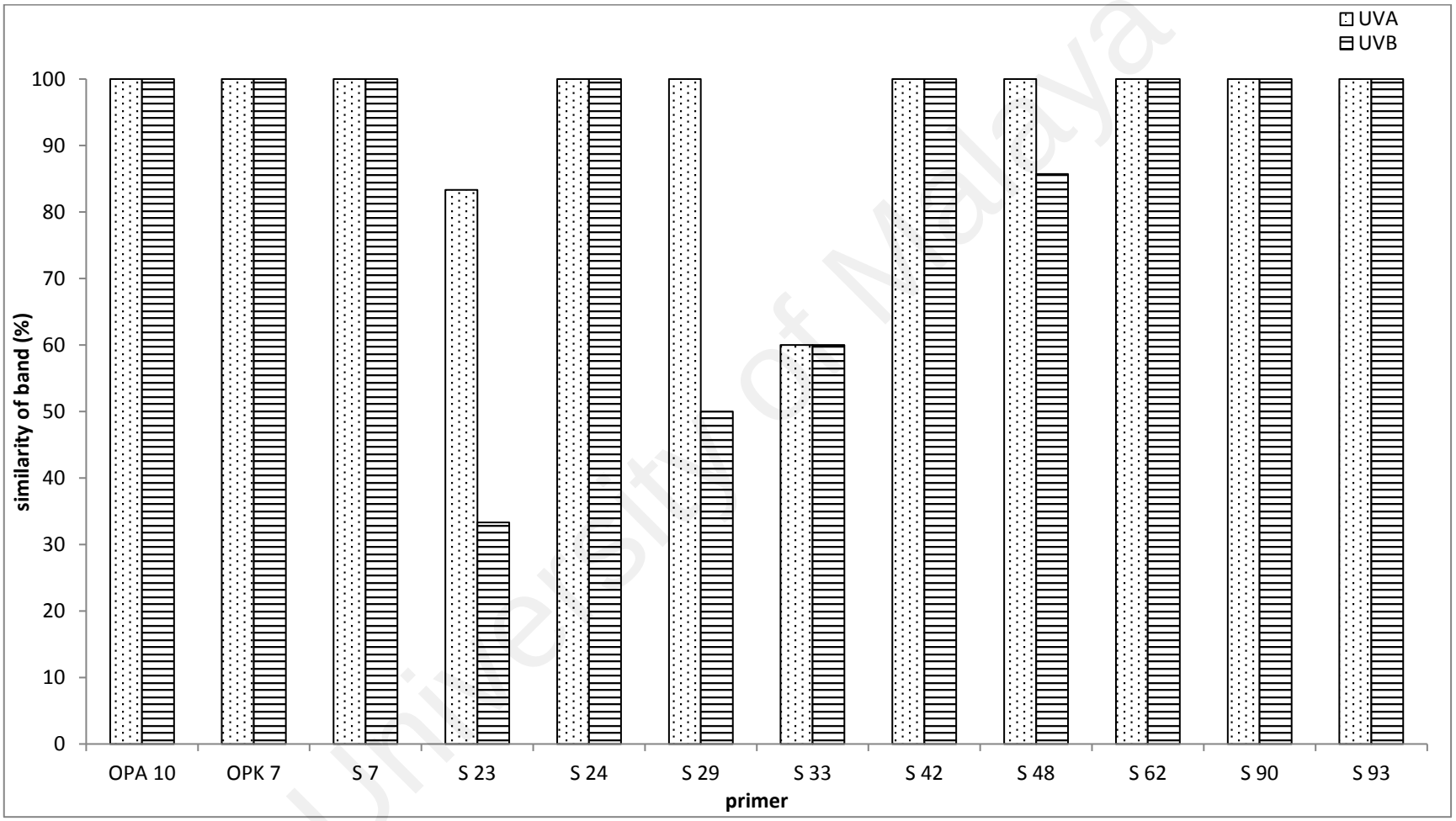


Figure 4.29: Similarity of bands (%) for Arctic *Chlorella* UMACC 263.
"UVA" refers to samples treated by UVA; "UVB" refers to samples treated by UVB.

4.4.4 Genomic template stability (GTS)

Figures 4.30 to 4.32 show the genomic template stability (GTS) after being subjected to UVA and also UVB treatments. GTS in control samples was set at a full value of 100%.

With regards to the UVA treatment, the lowest GTS values obtained were 95.12%, 97.56%, 98.10% and 98.60% in tropical, temperate, Antarctic and Arctic strains, respectively, as detected by primers S33, S29, S93 and S93 and S33 (Figure 4.26).

Figure 4.27 shows that the changes of GTS due to the UVB treatment were lower compared to that of UVA treatment. For samples subjected to UVB stress, the lowest GTS values were at 95.12% (tropical), 95.94% (temperate), 98.10% (Antarctic) and 97.20% (Arctic), as detected by primers S33, S7, S93 and S23, respectively.

Based on Figure 4.28, the total lowest GTS value caused by both UVA and UVB was detected by primer S33 (90.24%) in the tropical strain, followed by primer S29 (95.12%) in temperate strain, S93 (96.84%) in the Antarctic strain and S23 (96.50 %) in the Arctic strain.

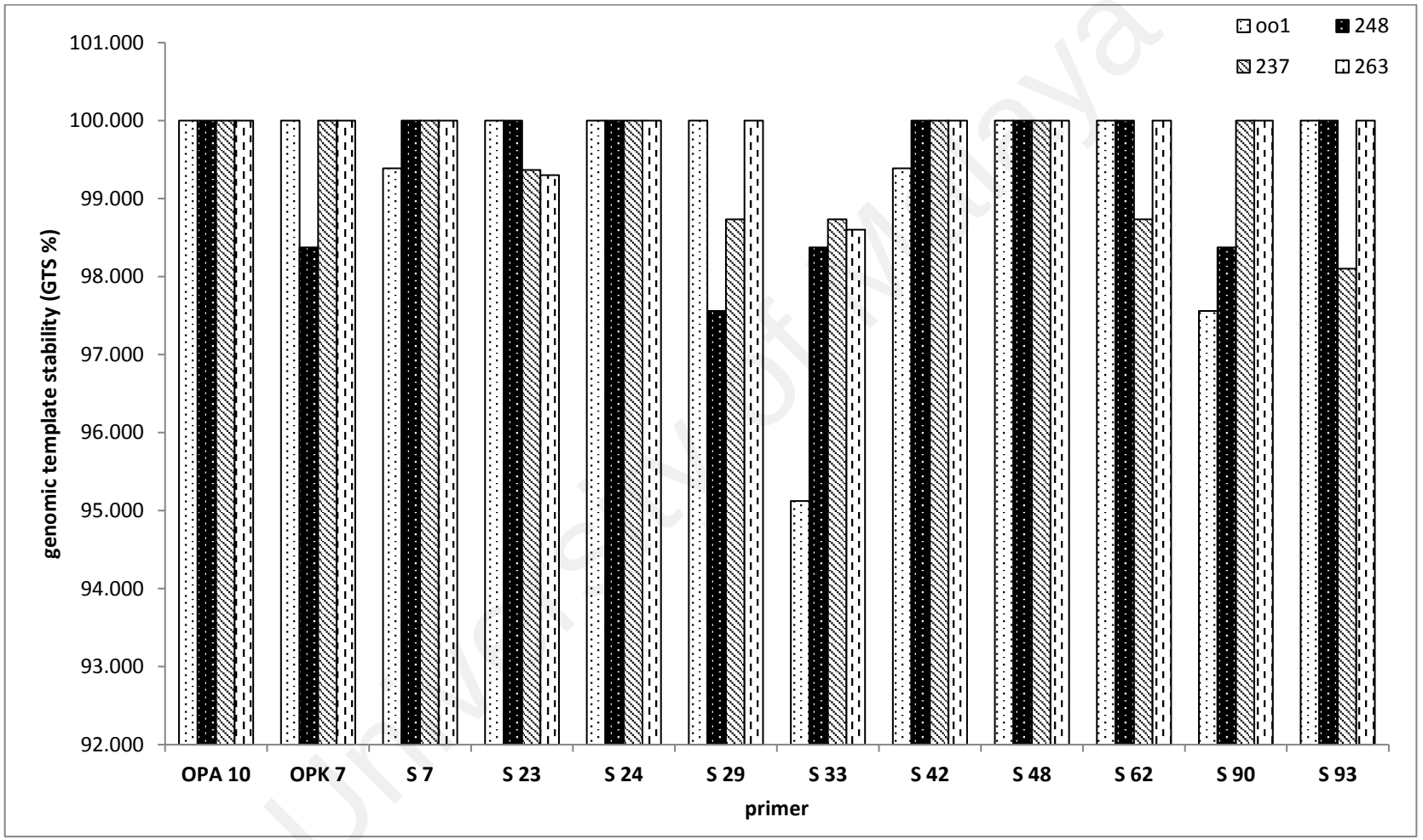


Figure 4.30: Changes of genomic template stability (%) (UVA) for selected RAPD primers in this study.

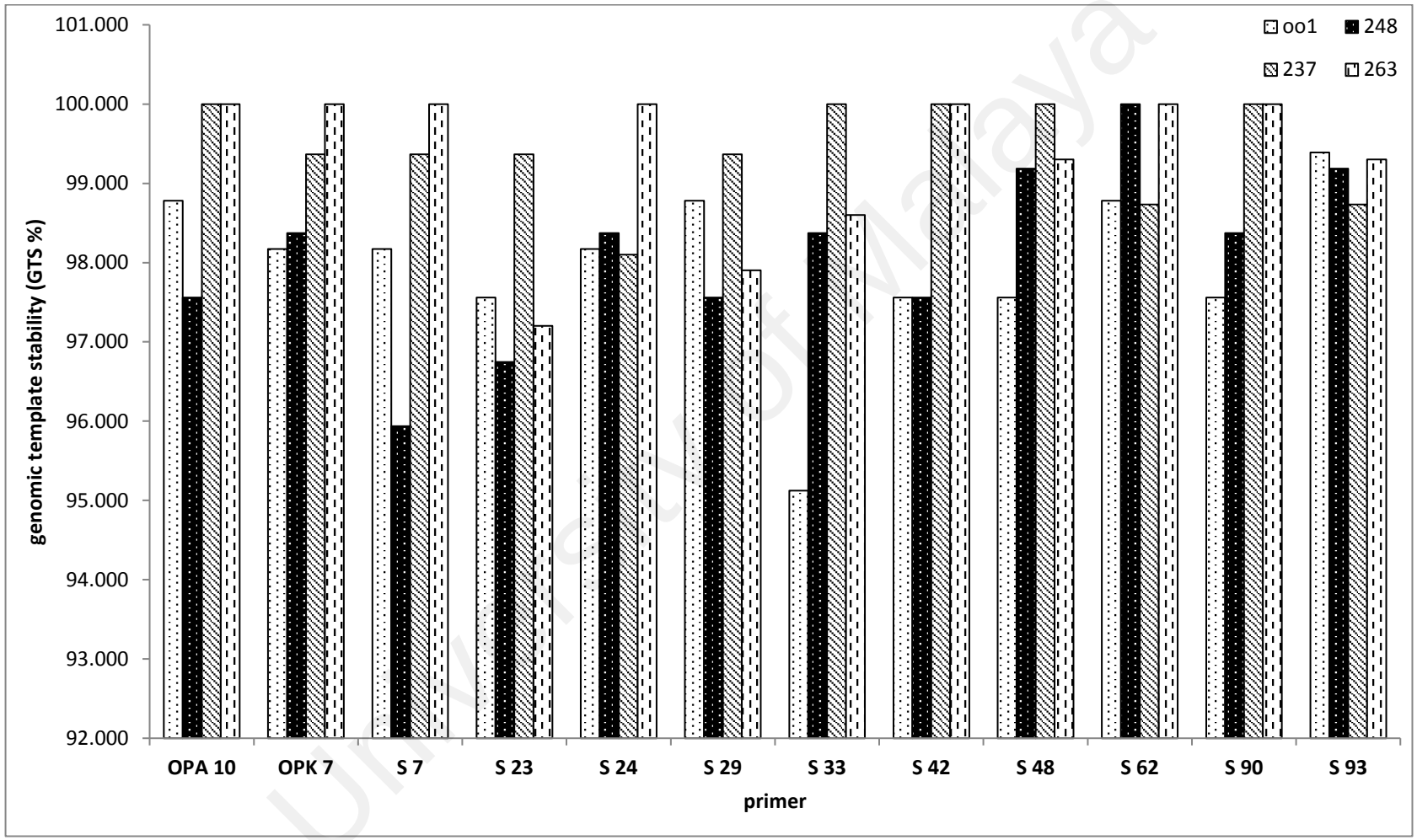


Figure 4.31: Changes of genomic template stability (%) (UVB) for selected RAPD primers in this study.

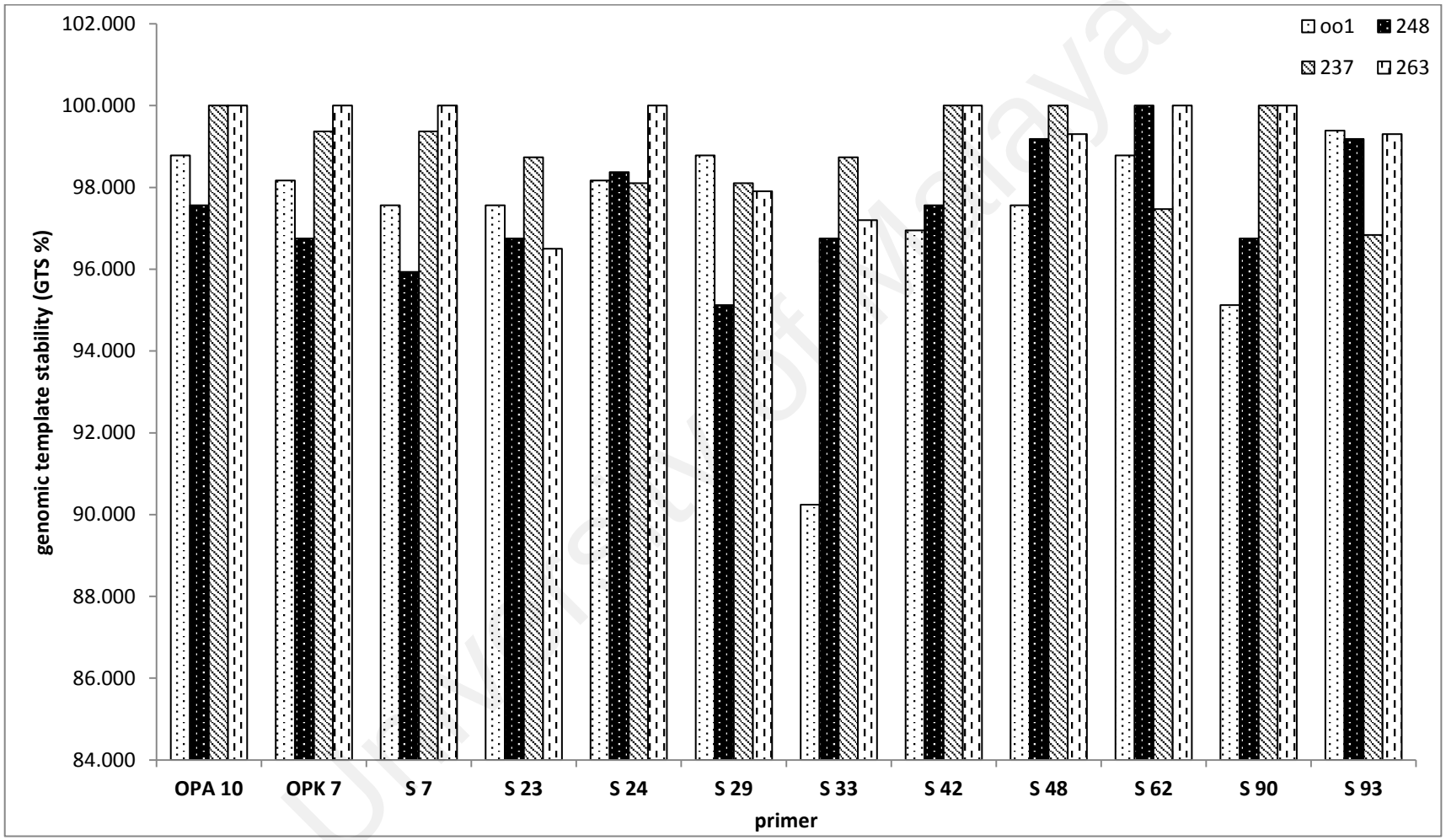


Figure 4.32: Genomic template stability (%) (UVA and UVB) for selected RAPD primer.

CHAPTER 5: DISCUSSION

5.1 Photosynthetic performance assessed by PAM fluorometry

Measurement of chlorophyll fluorescence by PAM makes the screening of photosynthetic performance of algae towards stress relatively easy and fast as it provides an estimation to photosynthetic electron flow (Schreiber et al., 1995; Maxwell and Johnson, 2000). In this study, a clear and significant UVB inhibitory effect on photosynthetic health was observed since the first hour of UVB treatment for all *Chlorella* strains. Strid (1993) and Mackerness et al. (1998) suggested that microalgae sacrifice photosynthetic capacity of the chloroplast under UVB stress in order to protect the rest of the cells. UVA effect in photosynthesis performance was less significant as there is much published evidence to demonstrate that UVA is an additional source of light energy to drive photosynthesis (Pakker et al., 2000; Helbling et al., 2003; Mengelt and Prezelin, 2005).

Herlory et al. (2013) suggested that most of the microalgae populations in good photosynthesis condition should possess a Fv/Fm value around 0.70, which indicates that about 70% of light absorbed was used for photosynthesis. Changes in Fv/Fm is widely used as the reliable diagnostic indicator of photoinhibition (He et al., 1996). In the present study, the initial Fv/Fm of *Chlorella* sp. were considered in healthy stage, which was between 0.50 to 0.70, with tropical and polar strains having a higher initial Fv/Fm (around 0.70) than that of the temperate strain (around 0.50). There was no negative effect on the Fv/Fm of *Chlorella* subjected to PAR treatment, showing the ability of the culture to cope the light energy well under control conditions, by switching on energy dissipating mechanisms (Eggert et al., 2006).

Fv/Fm is proportional to the PSII quantum efficiency which can be used for estimating PSII efficiency (Bjorkman and Demming, 1987). Fv/Fm dropped

dramatically to less than 0.10 after two or three hours of UVB exposure, indicating that UVB resulted in a strong degree of photoinhibition in the photosynthetic mechanisms in the treated *Chlorella*. It was supported by Barber and Andersson (1992) and Eggert et al. (2006) who reported that low Fv/Fm was caused by irreversible photoinhibitory damage to the photosynthetic apparatus. Reeves et al. (2011) discovered that a culture with Fv/Fm value of less than 0.10 was generally unable to recover due to the inactivity and lack of cell viability. Fv/Fm value of nearly zero in *Chlorella* strains subjected to UVB radiation in this study indicated that UVB was destructive and eventually hindered PSII activity. This was supported by the almost 99 % inhibition of Fv/Fm due to UVB stress in all four *Chlorella* strains, as shown in Figure 4.4. Franklin et al. (2009) reported that Fv/Fm value of 0.1 equates to a 10 to 15 % PSII efficiency, which meant that more than 90 % of the cells, would be photosynthetically non-functional. As there is a close linkage between the reactive oxygen species (ROS), UVB exposure and stress (Hideg et al., 2013), oxidative stress might cause a chronic damage to the photosynthetic apparatus (such as the PSII) which might be indicated by a reduction in Fv/Fm (Krause, 1994). Isolates with higher Fv/Fm value are less susceptible to photoinhibition and able to cope with stress by down-regulating PSII instead of getting damaged (Eggert et al., 2006). The present study supported this postulation, as the tropical strain exhibited initial highest Fv/Fm value (~ 0.70), as well as lowest inhibition in Fv/Fm value under UVA treatment, showing that the isolate from the tropics might be more tolerant in withstanding UVR stress as compared to isolates from the temperate and polar regions, due to the different adaptation to the light regime with respect to the natural habitats of the isolates from different origins (Schreiber, 2004).

Photosynthetic efficiency (α) refers to the photosyntehtic effiency and indicates the amount of ETR per photon. Changes of α value might be a result of the variations in

the pigment composition of light harvesting centers, or variations of the efficiency of energy transfer from light-harvesting antennae to the PSII reaction centers (Ralph and Gademann, 2005; Serodio et al., 2006). A significant negative effect from UVB in all *Chlorella* strains was reflected from the declining α (Figure 4.5), with high inhibition at about 99% in the tropical and temperate isolates while 75 and 93 % in Antarctic and Arctic strains (Figure 4.8). In the present study, the negative effect of UVA on α was not significant in all isolates, recorded with about 50% inhibition in all strains. Decline in α value which indicates a decline in the efficiency of light capture (Ralph and Gademann, 2005), is always associated with PSII damage (Powles, 1984). Meindl and Lutz (1996) and Lütz et al. (1997) suggested that the alteration in the protein structures that are involved in photosynthesis, such as the photosystems, might lead to photoinhibition. Based on the present findings, the short wavelength of UV is strong enough to damage the PSII, which would indirectly inhibit the electron transportation in the electron transport chain and reduce the photosynthetic efficiency.

The overall photosynthetic performance and the rate of electron flow via PSII into the photosynthetic electron chain can be assumed from the rETRm values (Juneau et al., 2005). rETRm reflects the maximum relative electron transport rate under light-saturating conditions, and the acclimation of photosynthetic rates can be discussed by comparing short-term with long-term effects on rETRm (Eggert et al., 2006). In this study, a distinct response to UVB stress reflected by declining rETRm values at a nearly 99% inhibition was observed, indicating the *Chlorella* was sensitive to UVB and lacked the ability to adapt the photosynthesis under intense UVB exposure. Inhibition of rETRm by UVA stress was high and significant in isolates from temperate and polar regions whereas it was not significant in the tropical strain. High reduction of rETRm values under UVR treatment indicated the lack of photoacclimation ability in response

to UVR, as supported by Eggert et al. (2006), in which a drop in rETRm was probably due to decrease in enzyme activity of the Calvin Cycle, or a strong stimulation of non-irradiative dissipation of excitation energy. Tropical isolates was shown to be more tolerant towards UVA in this present study.

Ek refers to the light adaptation index used to examine the photoacclimation status (Woelfel et al., 2014). Sakshaug et al. (1997) reported that algae will adjust their Ek values upwards under increasing irradiance. In this present study, a trend of UV acclimatization was only observed in the tropical *Chlorella* at the beginning of UVB treatment, and after 5 hr of UVB exposure. However, increasing photoadaptive index was not found in other *Chlorella* strains in the present study, as only significant decreases in Ek values were observed even after an hour of UVB exposure (Figure 4.13). This may be because the acute UVB stress had suppressed the photosynthetic activity before the temperate and the two polar strains could respond and acclimate to the UVB stress. Adaptive and tolerance level of microalgae towards UVR depend on their original habitat (Wong et al., 2007). The tropical strain showed better adaptation towards UVB stress, with the lowest inhibition among all at 68.09% whereas the other strains had inhibition of 95% and above. Microalgae originating from localities with high irradiance, i.e from the tropics, is believed to adjust their photosynthetic apparatus relatively faster to a new light regime (Kuhl et al., 2001; Karsten et al., 2006). Serodio et al. (2006) suggested that organisms that naturally photoacclimated to high light posses higher Ek as a preparation to withstand direct exposure to solar radiation. Máté et al. (1998) reported that the intensive recovery capacities in some tropical algae is due to the UVB-induced transcription of *PsbA* genes which encode for D1 protein. Thus, more research such as molecular analysis like gene expression study have to be

conducted in order to study the photoacclimation and photorecovery processes of microalgae towards UVB.

This present study suggested that short term and intense UVB stress undoubtedly led to photoinhibition whereas acute UVA stress partly affected the photosynthetic health in the *Chlorella* studied

, except for that from the tropics. All available data on photosynthetic performances clearly indicate a high photophysiological plasticity of the tropical *Chlorella* when subjected to UV stress. It is supported by Davison et al. (1991) and Georgieva (1999) that species from warm and high light habitats develop an increase in light-harvesting efficiency as they are acclimatized to high temperature. Tropical ecosystems have a long evolutionary history of greater flux of UVR exposure than that at higher latitudes (Frederick et al, 1989), thus possessing greater UV tolerance due to the natural ability to adapt to the high irradiance fluxes at low latitude environments (Helbling et al., 2001). Numerous algae growing in tropic areas possess high resistance to UV as well as strong DNA and PSII-repair mechanisms (Hader et al., 2011). Phototrophic organisms that are exposed to long-term high irradiance conditions develop adaptive mechanisms such as the alteration of the size and composition of the photosynthetic units with consequences for increased electron transport capacity (Richarson et al., 1983). In contrast, polar species exhibited low reliance on chloroplast-encoded protein synthesis under UV (Öquist et al., 1992; Hanelt et al., 2003; Marija & Dieter, 2014). Thus, it was observed that isolates from polar regions are more susceptible to UV-induced photoinhibition.

5.2 Photosynthetic pigments

Photosynthetic pigments act as light harvester at low light level, or as a photoprotector which prevents the phototroph from damage under high solar radiation

(Goncalves et al., 2001). Strauss-Debenedetti and Bazzaz (1991) suggested that photosynthetic pigments i.e. chlorophyll and carotenoids are plant responsiveness indicator to light intensity. Phytoplankton with extreme shade adaptation exhibited higher amount of chlorophyll pigments resulting in higher efficiency at converting light to photosynthetic energy (Lizotte and Priscu 1992). Goncalves et al (2001) suggested that both synthesis and carotenoid accumulation are important mechanisms for attenuating stresses caused by high irradiance. Siefermann-Harms (1987) suggested that carotenoids function as light-harvesting pigments with the additional role of photoprotection in microalgae. The increase in carotenoid would be associated with an increased need for energy dissipation (Ralph, 2000).

In the present data as shown in Figure 4.13 to 4.15, both polar *Chlorella* possessed naturally higher content of photosynthetic pigments than the tropical and temperate *Chlorella* ($P < 0.05$). As reported by Mock and Hoch (2005), high content of photoprotective pigments is one of the adaptation strategies in polar microalgae. Boardman (1977) reported that shade-adapted leaves tend to have a higher chlorophyll concentration per unit leave weight compared to leaves exposed to brightly lit conditions. It is because shade leaves exhibited higher and broader grana thylakoid stacks while sun leaves have less light-harvesting chl *a/b* proteins and more reaction centers on a total Chl basis (Liehtenthalar et al 1981; 1982). Similar theory can be applied on other phototrophs like green microalgae from the polar region i.e. Antarctic and Arctic *Chlorella* which live in lower irradiance situation whereas tropical and temperate strains have longer exposure to high irradiance.

Most studies reported decrease in chlorophyll content in response to UVR stress (Wong et al. 2007; Surabhi et al. 2009; Wong et al. 2011). Sakaki et al. (1983) postulated the decrease in total chlorophyll concentration induced by UVB might be due

to rapid chlorophyll degradation, inhibition of new chlorophyll synthesis and structural destruction of chloroplasts. Lüder et al. (2002) suggested that reduction in chlorophyll *a* content may be related to degradation of the photosystem. However, there was no significant UVR-induced effect ($P < 0.05$) in pigment concentration (chlorophyll *a*, carotenoids and chl *a* : carotenoids content) in all *Chlorella* strains throughout the five hours light treatments, including the UVB radiation, although there were obvious changes in photosynthetic parameters as detected by PAM fluorometry. It indicated that short-term UVR stress does not affect photosynthetic pigments. Degradation rate of pigment concentration might not overtake the rate of pigment synthesis, therefore reduction in photosynthetic pigments was insignificant. Similar results were obtained in photosynthetic performance studies i.e. Antarctic red algae exposed to extended darkness (Weykam et al., 1997); Antarctic macroalgae living in ice-covered and ice-free environments (Runcie and Riddle, 2006), as well as short-term UVR and PAR treatments in seagrass *Halophila* (Kunzelman et al., 2005). Besides, photostability of chlorophylls and carotenoids under UVB environment was reported by Tevini and Teramura (1989).

It was reported by Dohler and Lohmann (1995) that damaging effects of UVR on the pigments are dependent on UV wavebands and the exposure time. Pigments take several days to weeks for acclimation toward changing environment or stress (Ralph et al. 2002). Therefore, we postulate that the response of photosynthetic pigments towards short-term UVR stress is not as sensitive as the photosynthetic performance measured by the PAM fluorometry.

5.3 Random Amplified Polymorphic DNA (RAPD) analysis

Screening of suitable RAPD primers was done before applying the RAPD technique, due to the lack of information regarding the suitable primers to detect DNA mutation by UVR stress. Primer selection is the most influential parameter in a successful RAPD (Belkum et al., 1993) assay, with the considerations of suitable selection of primer sequence, primer length and primer concentration (Power, 1996). In the present study, a total of 60 RAPD primers were screened in order to select primers which are able to amplify all *Chlorella* strains used under three light treatments. Table 4.1 shows 12 primers which have been selected as the most suitable RAPD primers to study the DNA mutation caused by UVR, as they are able to produce successful amplification in four *Chlorella* strains under three light treatments. By selecting and using the suitable primers, detection of the differences in RAPD profiles between the control and treated samples can be assessed easily. Based on Table 4.1, the selected primers were high in GC content (60-70%). Primers rich in GC content is key for successful PCR amplification, as the presence of G or C bases within the last five bases from the 3' end of primers (which also known as GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases (Sheffield et al., 1989; Rettedal et al., 2010).

Power (1996) reported that use of different PCR reagents for every reaction, i.e. *Taq* DNA polymerase, will cause differences in the quality of amplified products, as some enzymes or reaction buffer may allow polymerization to proceed and eventually increase the number of priming sites on the template. In the present study, the RAPD reactions were repeated to confirm the changes in band variations were not due to a variation in template DNA concentration, or in PCR reagent concentration, as the same starting materials (DNA) were used and a master mix was performed to minimize

uneven volume of each reaction buffer. The same thermocycler was used for every reaction to prevent model-to-model variability in the rate of heat transfer to the reaction mixture which might affect the reproducibility and quality of RAPD products, as suggested by Power (1996).

Mutation, that is mutation caused by UVR stress, can induce changes in the genome sequence which would then affect the primer annealing sites and therefore change the DNA banding profiles in a RAPD assay (Sianipar et al., 2015), as one base difference in genome sequence may inhibit the annealing of primers (William et al 1990). Thus, differential RAPD profiles generated by selected RAPD primers were used to determine DNA mutation in *Chlorella* in response to UVR stress. Besides, RAPD assay has the potential to detect mutations outside the priming site (Bowditch et al., 1993). Variation in the number of amplified DNA fragments generated by RAPD can be categorized into monomorphic and polymorphic bands, which the polymorphic bands give important information in detecting genetic changes because it showed mutation in DNA sites that served as primer annealing sites (Sianipar et al., 2015). According to the calculation of band variations of treated samples in comparison with the control (refer to Section 3.5.5.1), the banding pattern in this present study is considered as polymorphic. Of the primers used, primer S33 exhibited the highest degree of polymorphism at 44.83%, followed by S90 at 38.71% and S93 at 29.63% (Figure 4.21). Based on Figure 4.22 to 4.25, the banding patterns of the control and treated *Chlorella* exhibited clear differences in the RAPD assay, with disappearance and emergence of RAPD bands. DNA profiles from samples subjected to UV treatment were clearly different from those generated in the control group whereas higher band variation was observed in samples subjected to UVB stress than that treated to UVA treatment. Based on Table 4.2, band variation was easily detected in the tropical and

temperate *Chlorella* compared to the other two polar strains. Among the tropical and temperate strains, emergence of bands was highly detected in the tropical isolate whereas disappearance of bands was largely detected in the temperate *Chlorella*. Other than that, band similarity level of *Chlorella* strains after exposure to UVB treatment was generally lower than that of UVA stress (Figure 4.26 to 4.29), showing that UVB caused greater change in DNA bases compared to UVA.

UVR could result in changes in DNA sequences or DNA structure on parts of the oligonucleotide priming sites. Changes that occurred cause the oligonucleotide priming sites to be accessible or inaccessible to the oligonucleotide primers, thus resulting in disappearance and emergence of RAPD bands (Unal et al., 2009). In detail, UVA wavelengths cause an indirect damage to DNA through the photodynamic production of hydroxyl radicals which leads to strand breaks and DNA cross-links (Peak and Peak, 1990). Meador et al. (2009) reported a strong correlation between UVB and DNA damage in marine microorganisms, where the DNA photoproducts increase with increasing UVB irradiance (Atienzar et al., 2000). Presence of DNA photoproducts, i.e. pyrimidine dimers and 6-4 photoproducts, can act as a bypass event to block or reduce the polymerization of DNA in a PCR reaction (Donahue et al. 1994; Nelson et al., 1996). This bypass event is complicated as it depends on the enzymatic properties of the DNA polymerase, the structure of the lesion and the sequence context of its location (Ide et al., 1991). DNA photoproducts induced structural alterations (Wang and Taylor, 1993) by: (i) influencing the availability of the *Taq* DNA polymerase and primers (resulted in blocked priming sites which caused the loss of bands); (ii) blocking the PCR enzyme at certain sites and allowing a more efficient amplification on non-damaged genomic DNA; and (iii) a better availability of 10-mer primers ; which both (ii) and (iii) could result in increase banding patterns.

Genomic template stability (GTS) is a quantitative measurement that measures the changes in RAPD profiles by relating three aspects: (i) level of DNA damage, (ii) the efficiency of DNA repair, and (iii) the efficiency of DNA replication (Cansaran-Duman et al., 2015). Alterations in GTS may be used to detect genetic damage (Ozturk et al., 2010; Rocco et al., 2014; Doganlar and Doganlar, 2015). Based on Section 4.4.3, GTS after UVR exposure was detected, whereby *Chlorella* subjected to UVB has a lower GTS value than that by UVA stress, revealing stronger degree of genomic instability by UVB. In this present study, the lowest GTS detected in tropical, temperate, Antarctic and Arctic *Chlorella* after 5h UVB treatment was 90.24 %, 95.12 %, 96.84 % and 97.20 % respectively, as detected by primers S33, S29, S93 and S33, individually (Figure 4.31). As for UVA stress (Figure 4.30)., the lowest GTS was 95.12 % in the tropical strain (primer S33), 97.56 % in the temperate strain (primer S29), 98.10 % in Antarctic strain (primer S93) and 98.60 % in the Arctic strain (primer S33). Atienzer et al. (1999) suggested that the alterations to RAPD profiles due to genotoxic exposure can be regarded as alterations to the GTS. DNA replication and error-prone DNA repair are involved in generating mutations (Livneh et al, 1993). A slight decline or even a one-band change in GTS is important in the RAPD assay because high level DNA damage does not necessarily decrease the genomic template stability in comparison to a low level DNA alteration, due to the inhibition of DNA repair and replication as a result of a high frequency of DNA damage (Atienzar et al., 2000; Cansaran-Duman et al., 2015). Doganlar and Doganlar (2015) reported that genomic template instability might result in appearance of new bands in RAPD profiles.

Danylchenko and Sorochinsky (2005) suggested that genetic differences resulted from DNA damage due to irradiation caused changes in DNA structure. Irradiation can induce genome, chromosome, gene and organelle mutation (Qosim 2006). Atienzar et al.

(2000) suggested that change in DNA sequences was due to: (i) mutations (resulted in new annealing event); (ii) deletions (bringing two pre-existing annealing sites closer), or (iii) homologous recombination (posing two sequences that match the sequence of the primer). This postulation was then supported by Liu et al. (2005) who suggested that the loss of bands was due to changes in oligonucleotide priming sites caused by genomic rearrangement, DNA damage to the primer binding site, and also interactions between DNA polymerase and damaged DNA in the test organism. In this present study, time of UV exposure might be too short to cause permanent DNA damage and mutations, but UV is capable to induce some structural modifications and possibly resulting in new annealing events (Atienzar et al., 2000). Besides, suitable primers with known short oligosequences that appeared promising in generating differential RAPD profiles of UV-induced DNA mutation was detected, which are primers S33 and S93. With this study, future work including determination and design of specific molecular markers for real-time PCR and transcriptome study, should be conducted to detect the effects of UV towards microalgae at the molecular level.

5.4 Limitations of this study

Due to technical limitations of the incubator, the PAR irradiance that was used as the "background" irradiance (42 µmol photons m^{-2} s^{-1}) was about fourfold lower than that of natural PAR (Table 3.2). It was not possible to adjust the irradiance or level of UV similar to the natural condition which has much higher irradiance as shown in Table 3.2. However, the application of 42 µmol photons m^{-2} s^{-1} of PAR was considered healthy to the *Chlorella* strains studied as it caused no damaging effect as judged from the Fv/Fm value during its growing phase (data not shown). The experimentally applied

UVB irradiance in the present study (1.17 Wm^{-2}) was within the range of natural UVB level as it imitated the experimental UVB condition by Wong (2011) who studied the growth, fatty acid profile and SOD activities of *Chlorella* sp. under both low and high level of UVB at 1.17 Wm^{-2} and 5.15 Wm^{-2}, respectively. The PAR : UVA : UVB ratio in this study (7.8 : 7.3 : 1) showed that the PAR and UVA was less than that in the natural environment. The effect of UVA might be underestimated. The low sensitivity of *Chlorella* to UVA in this study could be also due to the low UVA radiation applied.

Temperate species that live in habitats with annual seasonal fluctuations, as well as polar strains that experience diurnal temperature and light fluctuations, should possess seasonal acclimatization of photosynthetic characteristics. Plants originated from regions with large annual temperature variations display a stronger photosynthetically acclimatization ability than species from habitats with more stable thermal regimes (Santameria and Hootsmans, 1998; Xiong et al. 2000). However, in the present study, polar and temperate strains did not show significant acclimation response to the short-term UV treatments. This is most probably due to the long-term cultivation of isolates under laboratory conditions (Table 3.1), as the culture collections are subjected to regular 12h day-light:12h dark cycle at their optimum growth temperature. Their natural acclimation mechanisms towards introduction of stresses, such as irradiance or temperature stress, may have been 'lost" during the long-term laboratory cultivation. It is known that long-term cultivation may cause accumulation of mutations and in-vitro selection (Lakeman et al., 2009). Photosynthesis studies on long-term cultivated isolates should be conducted to study the differences of photosynthetic activities between laboratory-acclimatized culture with freshly-isolated samples towards induced light or UV stress. It will probably provide a new insight on the development of photosynthetic mechanisms between on-site and indoor cultures. However, a study done

by Marija & Dieter (2014) revealed that photosynthesis sensitivity of *Cosmarium* strains is dependence on its geographical distribution patterns despite its long-term cultivation under constant laboratory conditions. Stamenkovic and Hanelt (2014) also postulated that some microalgae strains may show high genetic stability despite long-term cultivation.

Despite the limitations in simulating the field parameters in the laboratory, the PAM data reported in this study is still relevant in providing a general idea on the differential sensitivity in photosynthesis performance to UVR on microalgae from different habitats, as well as allow a comparative evaluation of photosynthetic responses in *Chlorella* sp. towards UVR stress. The type and level of UVR that induced stress in *Chlorella* based on the photosynthesis performance was identified (Wong et al. 2015). As for RAPD assay, it is difficult to estimate and differentiate the real contribution of DNA damage caused by UVA and UVB. However, it can provide an insight that UVR, especially UVB stress, can result in DNA mutation even in a short term UVR exposure. With that, we can conduct detailed molecular investigation i.e. Real-time PCR, or transciptomic analysis in order to study the specific gene, or even pathway of damaging effect in microalgae due to UVR stress.

CHAPTER 6: CONCLUSION

6.1 Summary of the research findings

1. Acute UVB induced intense photosynthesis impairment in all *Chlorella* strains in comparing to UVA stress. Exposure of UVB exerted an additional stress on the photosynthetic apparatus of the *Chlorella* strains studied, with the decline in all photosynthetic parameters obtained from PAM.

2. UVA resulted in less severe photoinhibition. The tropical strain was less affected compared to isolates from the temperate and polar regions.

3. Response of UV exposure in *Chlorella* was determined by physiological acclimation. Phototrophic *Chlorella* living in polar and temperate regions may be prone to be more responsive towards the damaging effects of UVB while tropical *Chlorella* tends to have a higher tolerance level towards short-term UVB stress.

4. Response of photosynthetic pigments towards acute UVR stress is not as sensitive as the photosynthetic performance measured by the PAM fluorometry. There was no significant changes in the photosynthetic pigments studied.

5. RAPD molecular markers had successfully detected genetic changes (UV-induced DNA mutation) between UVB-treated samples in comparison with the control and samples subjected to UVA stress, observed from the presence of new DNA bands, the loss of DNA bands and also the variation of DNA bands intensity. It shows its potential as a reliable and reproducible assay for UVR-stress study.

6.2 Future research directions

1. To study the differences of photosynthetic activities between laboratory-acclimatized cultures with freshly-isolated samples towards induced light or UV stress.

2. More biochemical studies in detecting oxidative stress and protein contents can be done to further evaluate the deleterious effects of *Chlorella* in photosynthesis performances. Studies like decrease in protein contents can be done, i.e. examination of the reduced amount of RuBiSCO by two dimensional separation of total proteins can be conducted in order to correlated with reduced photosynthetic ability (Sarkar et al. 2015). It takes several days for acclimation of protein turnover as reported by Quigg and Beardall (2003), thus extended exposure of UV experiment can be considered in order to study the long-term acclimation of microalgae towards UV.

3. Combination of ambient stresses, such as temperature and light, can be conducted simultaneously to study the general effects of global warming (that led to increase global temperature and level of irradiance) on photosynthetic activities of microalgae. That will provide more understanding on the responses of photosynthetic mechanisms towards stress.

4. RAPD seems fundamental but it is able to determine the sensitivity and selectivity of microalgae towards UVR stress. RAPD assay can be applied in conjunction with transcriptome and gene expression study in order to provide details and powerful tool for UV stress study.

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LIST OF PUBLICATIONS AND POSTER PRESENTED

Posters presented

1. "Comparison of photosynthetic response of Antarctic and tropical *Chlorella* subjected to acute UVR stress" at World Conference on Marine Biodiversity held at Huanghai Hotel, Qingdao, China from 12 to 16 October 2014.

2. "Photosynthetic performance and genomic response of *Chlorella* species from different regions to ultraviolet radiation (UVR)" at XXXIV SCAR Biennial Meetings (2016 Open Science Conference) held at Kuala Lumpur Convention Centre (KLCC) from 20 to 30th August 2016.

Publication

1. Poong S.W., Lim P. E., Lai Jeannette W.S. and Phang S. (2017). Optimization of high quality total RNA isolation from the microalga, *Chlorella* sp. (Treabouxiophyceae, Chlorophyta) for next-generation sequencing. *Phycological Research, 65* (2): 146 - 150.