THE PHOTOPHYSIOLOGY OF ROCKY INTERTIDAL MICROPHYTOBENTHIC BIOFILMS

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BSc (Hons)

This thesis is presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy of Cardiff University

I declare that this thesis is my own account of my research and contains work which I have undertaken, except where indicated by acknowledgment or reference.

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(Naomi E. Ginnever)

No portion of this work has been previously submitted or is currently submitted for a degree or award in any institution.

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ABSTRACT

Rocky shore microphytobenthic biofilms are areas of high biodiversity, and are protected under UK and European Union legislation. Despite this, little is known about the photophysiology of these biofilms. This study aimed to provide a new contribution to microphytobenthic research with the addition of photophysiological knowledge focussing on the rocky intertidal to add to the extensive photophysiological research which has focussed on mudflats. More specifically the photoregulatory mechanisms of rocky shore biofilms had not been studied prior to this work. This study aimed to determine the effects of ambient environmental conditions, community structure and grazing on the photophysiology of the biofilms and elucidate the complex relationships between the abiotic and biotic factors which influence the biofilm. The community structure of the biofilm changed seasonally, with larger species (> 40 µm valve length and > 25 µm diameter) such as *Parlibellus delognei* being dominant during the winter months (December, January and February) and smaller ones (<40 µm valve length and < 25 µm diameter) such as *Navicula bottnica* during the spring months (March, April and May) indicating an environmental influence on the community structure of the biofilm. The biofilms were found to die-off (biomass below detection levels) in April and May and grow back in the November and December during a 'reproductive phase'. An observed photophysiological 'seasonality' was primarily the result of the timing of the 'reproductive phase' of the biofilm, with higher maximum relative electron transport rates ($rETR_{max}$) being recorded during November and December (on average 85 compared to 60 relative units), when these biofilms were growing after the spring die-off. High temperature and light dose had a negative effect on the $rETR_{max}$, particularly for biofilms on the upper shore sites. It was concluded that the combination of increased temperature and light dose, reducing $rETR_{max}$, and so productivity, and increased grazing contributed significantly to the spring die-off with cells unable to replicate rapidly enough to compensate for increased grazing. By exposing biofilms to different temperatures *ex-situ* it was found that the lower shore biofilms

were more resilient to high (> 25 °C) and low (< 10 °C) temperature with a smaller reduction in $rETR_{max}$, and *ΔF/Fm'* observed in comparison to upper shore. Temperature was found to induce movement in the tube-forming upper shore species *Navicula bottnica*. This was likely to act as a secondary photoregulation strategy as it was found that high temperatures resulted in a reduced ability to induce non-photochemical quenching (NPQ). Biofilms were also treated with Latrunculin A (LAT-A) and DL- Dithiothreitol (DTT) *in situ*, and by comparing the photosynthetic patterns of response over an exposure period it was found that the upper shore biofilms utilised NPQ as the primary means of photoregulation whereas the lower shore biofilms utilised cell movement as the primary photoregulatory mechanisms. The upper and lower shore biofilms also utilised secondary mechanisms, migration in the upper shore samples, and NPQ in the lower shore samples, of downregulation, which allowed the cells to persist on the rocky shore where the rapid changes in environmental conditions result in a high stress environment. The overarching conclusion from this study is that rocky shore biofilms utilise a combination of photoregulatory mechanisms dependent upon life form in order to survive in an environment where many rapidly changing biotic and abiotic factors affect the community structure and photosynthesis of the biofilms.

ACKNOWLEDGEMENTS

It would not have been possible to write this thesis without the help and support of many people, only some of whom are mentioned here.

I would like to express my huge appreciation and thanks to my supervisor, Dr Rupert Perkins (Cardiff University), for the colossal patience he has shown throughout and the help and support he has provided me during this project. I would also like to thank Dr Bryan Spears (Centre for Ecology and Hydrology) for going absolutely above and beyond the call of duty in helping get this thesis ready for submission. I cannot express how much this assistance has helped me and how much I appreciate it.

I would like to thank the Centre for Ecology and Hydrology (CEH) who provided the funding for this project and so allowed it to go ahead and Cardiff University who provided my financial support during this project without which I would not have been able to do this PhD.

Dr Johann Lavaud (Université de La Rochelle) and Dr Paulo Cartaxana (Faculdade de Ciências - Universidade de Lisboa) taught me how to prepare the DL- Dithiothreitol solution and he Latrunculin A solution respectively. This invaluable firsthand experience prevented me from making costly mistakes when preparing the chemicals used in Chapter 4.

My Mum and Dad have been twin pillars of support and have provided every kind of help it is possible to provide; emotional, financial and practical during this project. I am so grateful to them and I cannot express my thanks to them enough. My best friend Barrie has been a huge support during this project and her immense kindness and listening ear has been invaluable to me. My sister Hazel has also been a sounding board and invaluable calming influence to me during this project. Sue and Victor Reeks have welcomed me into their home and I am so

grateful to them for everything they have done for me. My dog Minnie has been absolutely vital in maintaining my sanity and happiness during this project.

To John who has been there for me throughout this project, I cannot offer enough gratitude and thanks. Without your patience, kindness and love I would not have produced this thesis.

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ABBREVIATIONS

- *Alpha*- Coefficient of light utilisation efficiency
- *Dde*- Diadinoxanthin de-epoxidase
- *Dep*- Diatoxanthin epoxidase
- *Dd*- Diadinoxanthin
- *Dt*- Diatoxanthin
- *DTT*- Dithiothreitol
- *Dx cycle*- Diatoxanthin cycle
- *ΔF/Fm'*- Light adapted quantum efficiency
- *Ek*- Light saturation coefficient
- *EPS*- Extra-cellular polymeric substances
- *F*- Operational fluorescence yield
- *F'*- Light adapted operational fluorescence yield
- F_m- Maximum fluorescence yield
- *Fm'* – Light adapted maximum fluorescence yield
- *Fo*- Minimum fluorescence
- *Fo'*- Light adapted minimum fluorescence
- F_v/F_m- Ratio of variable to maximum fluorescence - the quantum efficiency of open photosystem II centres
- *LAT a*- Latrunculin A
- *MDGD*- Galactolipid monogalactosyldiacylglycerol
- *NADP(H)*- Nicotinamide adenine dinucleotide phosphate (reduced form)
- *NPQ*- Non-photochemical quenching
- *PAM*- Pulse amplitude modulated
- *PAR*- Photosynthetically active radiation
- *PFD*- Photon flux density

- *Pmax*- Maximum photosynthetic rate
- *PSI*- Photosystem I
- *PSII*- Photosystem II
- Q_A- Primary quinone acceptor of photosystem II
- *rETR*- Relative electron transport rate
- $rETR_{max}$- Maximum relative electron transport rate
- *Vx cycle*- Violaxanthin cycle

THESIS INTRODUCTION

CHAPTER 1

1.1 GENERAL INTRODUCTION

Our understanding of biofilms in coastal waters is based mainly on mud flat ecosystems with few studies focussing on the rocky shore communities. However rocky shore environments have been extensively studied and the research has focussed primarily on macroalgal diversity and function (Lewis 1964, Cubit 1984, Underwood & Jernakoff 1984, Kaehler 1998) and herbivore behaviour and distribution (Hawkins & Hartnoll 1983, Menge 2000, Forrest et al. 2001, Mieszkowska et al. 2006) . The research which has been undertaken in the area of rocky shore microalgae has not utilised PAM fluorescence and has been performed on tropical shores (Underwood 1984, Murphy et al. 2005, Narváez-Zapata et al. 2005). Rocky shore microphytobenthos makes an important contribution to primary production (Lamontagne et al. 1989), plays a role in carbon and nutrient dynamics of inshore areas (Magalhães et al. 2003) and provides food for grazing species (Hawkins & Hartnoll 1983, Underwood & Jernakoff 1984, Hawkins et al. 1992, Thompson et al. 2000, 2004). A comprehensive study on how different environmental conditions affect the photophysiology, species composition and behaviour of rocky shore microphytobenthos has not been undertaken. The majority of rocky shore microphytobenthos can be defined as epilithic, meaning they are attached to the rocky substratum (Round et al. 1990) and these diatoms produce mucilage which often allows un-attached diatoms and other algal species to become incorporated into the biofilm (Behre 1956). This study aims to provide a new contribution to microphytobenthic research with the addition of photophysiological knowledge which focuses on the rocky intertidal to add to the extensive photophysiological research which has focussed on mudflats (Perkins et al. 2002, 2006, 2010, Serodio 2003, 2004, Consalvey et al. 2005, Jesus et al. 2006 and many others). More specifically the photoregulatory mechanisms of rocky shore biofilms have not been studied and these mechanisms will be studied and this data used to allow interpretation of seasonal data collected from the rocky shore sites. This study aims to allow us to understand the effects of ambient environmental conditions, taxonomy and grazing on the

photophysiology and to elucidate the complex interactions between the abiotic and biotic factors which influence the biofilm.

1.1.2 Dunraven Bay

Dunraven Bay is a rocky shore in Southerndown (51° 44.65'N, 03° 60.73'W), Bridgend County, South Wales. The measurements used in this thesis were taken at 5 sample sites on the upper and 'lower' shore (Figure 1).

Figure 1.1.1*; Aerial photo of the research site,* the sites marked in white are the upper shore sites and those marked in red are the mid/lower shore sites. Source: *(Imagery © 2012 Bluesky, Infoterra Ltd & COWI A/S, DigitalGlobe, GeoEye, Getmapping plc, The GeoInformation Group. Map data © 2012 Google).*

The tidal range at the site is large with the high water mark, during spring tides, reaching the cliff base with no intertidal zone exposed and the low water mark, during spring tides being 153m from the cliff base (pers. measurements). The upper shore sites were between 25-30m from the cliff base and the 'lower' shore sites between 65-75m away. The lowest part of the shore (between 100-155m from the cliff base) is dominated by the colonial worm *Sabellaria alveolata* (Boyden et al. 1977, Holt et al. 1998). This shore level is not covered by algal species and is frequently immersed even during spring low tide periods. This area was therefore not

studied and for ease of reporting the mid/lower shore (60-85m from the cliff face) will be henceforth known as the lower shore.

The studied intertidal zone (25-75m from the cliff face) is made up of a limestone wave cut platform (Figure 2). The rock is heavily pitted with solution holes (Elston 1917) and these contain microalgal biofilm. The lower shore sites investigated in this thesis closely resemble those found in mudflat systems as they form a cohesive biofilm in a thin layer of sediment on the rock surface within the solution holes. The upper shore biofilms are dominated by tube-forming diatoms which live within the solution holes, which are permanently wet during the autumn, winter and spring (for detailed information see Chapter 2). The lower shore wave-cut platform is covered by large flat boulders which are believed to have been formed when the wave cut platform was damaged during a tsunami in 1607 (Bryant & Haslett 2007).

Figure 1.1.2: *Dunraven Bay,* wave cut platform.

Using quadrates it was determined that the depressions on the rock surface cover 50% of the total rocky shore and during the peak biofilm biomass these pools are completely covered in microalgae. Therefore the percentage algal cover of the shore is 50%; however during wet

periods in the winter months the algae can grow on the rock surface and therefore percentage cover can increase to 70% (pers.obs). The biofilms at both shore sites are dominated by diatoms and the site communities do not contain any macro-algal species. This provides a suitable research environment to study the photophysiology of these highly productive areas (Lamontagne et al. 1989). Please refer to Chapter 2 for more detailed information on the research site

1.1.3 Project Introduction

This project assessed the natural seasonal changes in the photophysiology of rocky shore biofilms. The upper and lower shore biofilms were investigated as these sites were very different with the lower shore site more closely resembling a mudflat system and the upper shore site being dominated by tube-forming species. The environmental pressures on the rocky shore are different from those in mudflat systems. By comparing the photophysiological responses of rocky shore biofilms growing on sediment layers, on the lower shore, and biofilms growing on rocky substratum, on the upper shore, this thesis has investigated whether different environmental conditions and biotic factors have different photophysiological effects on the biofilms and what effect, if any, these combined factors have both photosynthetically and taxonomically on the biofilm. The primary investigatory method was pulse-amplitude modulated (PAM) fluorescence which was used in conjunction with taxonomic analysis, weather and light records and herbivory records to understand the photophysiology of the rocky shore communities. Seasonal photophysiological observations were related to controlled field and laboratory experiments. Chemical manipulations using Latrunculin- A (Lat A) (Cartaxana & Serôdio 2008) and DL- Dithiothreitol (DTT), to inhibit diatom movement and prevent the ability of the cells to utilise movement and non-photochemical quenching respectively, were used to assess which photoregulatory mechanisms were being employed by the rocky shore biofilms. As diatoms present on the upper rocky shore cannot move into the

rock surface to regulate their photophysiology as is the norm in mudflat based systems (Kromkamp et al. 1998, Serôdio & Catarino 1999, Paterson et al. 2001, Perkins et al. 2001, 2002, 2010, Jesus et al. 2006) the method of photoregulation that these cells used primarily was unknown. The effect of temperature on the photophysiology of the rocky shore biofilms was also assessed. The effect of temperature on the photophysiology of mudflat based biofilms has been investigated previously (Blanchard et al. 1996, Defew et al. 2004, Salleh & McMinn 2011). However upper shore rocky intertidal biofilms are composed of different diatom species, which have different life-modes, and therefore the effect of temperature on these biofilms may be different. This study provides new information about the effect of temperature on rocky shore biofilms. These laboratory based observations were designed to allow quantification of the specific environmental parameters that were important in regulating the photophysiology of rocky shore biofilms.

These thesis aims were designed to address the lack of information available on the photophysiological activity and behaviour of rocky shore microalgal biofilms. Rocky shores comprise 34% of the coastline of Great Britain (Sutherland 1995), which itself is 19,491 miles in length (British Cartographic Society); thus the length of rocky shore is about 6626 miles in length. Most rocky shore communities consist of a microalgal component and these biofilms can be the predominant autotrophs on the rocky shore (Hill & Hawkins 1991). These cells form the base of near shore food chains which support large numbers of grazing species which in turn support the fry of commercial fish species (Castenholz 1961, Hawkins et al. 1992, Thompson et al. 2000, 2004). Rocky shore habitats may also play a role in the carbon and nutrient dynamics of inshore areas (Magalhães et al. 2003). As such these important environments need to be more fully understood.

Estuarine microalgal biofilms have been intensively studied as a highly productive ecosystem which is of huge importance in supporting coastal food chains (Heip et al. 1995) and providing stabilising properties for sediments in estuaries and mudflats (Underwood & Paterson 2003, Paterson 2004 , Underwood et al. 2004) . The irradiance experienced by the cells influences the behaviour (Kromkamp et al. 1998, Wolfstein & Stal 2002, Paterson et al. 2003, Cohn et al. 2004, Jesus et al. 2006, Mouget et al. 2008, van Leeuwe et al. 2008, Perkins et al. 2010) and photosynthesis of the cells (Falkowski & Owens 1980, Falkowski et al. 1981, Ley & Mauzerall 1982, Sukenik et al. 1987, Olaizola & Yamamoto 1994, Barranguet et al. 1998, Serodio et al. 2004, Perkins, et al. 2010). The photophysiology of these areas has been studied extensively (Underwood et al. 1999, 2005, Perkins et al. 2002, 2006, 2010, Serodio 2003, 2004, Consalvey et al. 2005, Jesus et al. 2006) and fluorescence methodology has been used extensively to study these areas due to the non-invasive properties and portability of this method (Kromkamp et al. 1998, Perkins et al. 2002, Serôdio 2004, 2005, 2006, 2007, Jesus et al. 2005, Perkins et al. 2010). This methodology has not been employed to date to assess the photophysiology of rocky shore biofilms (see section 1.5 for further fluorescence information).

1.2 MICROPHYTOBENTHIC BIOFILMS

1.2.1 Taxonomic analysis

This thesis includes a detailed taxonomic analysis of the rocky shore microalgae at Dunraven Bay. The taxonomy of microalgal biofilms on the rocky shore has been studied in the United Kingdom by (Cox 1977a, b, 1981), this study utilises taxonomic assessment methods and keys produced by Cox, in order to understand the effect of community structure on the photophysiology of the biofilms. Tube-forming rocky shore species require a stable substratum as opposed to epipelic diatoms which favour soft sediments (Cox 1977b, Houpt 1994). These epilithic cells cannot survive in areas with frequent influxes of sediment as these cells are unable to move within the sediment back to the surface after being covered, like epipelic

diatoms can (Cox 1977b). However Cox (1977) noted that in spring the cells can exit the tubes and become epipelic, and in this circumstance the cells may be able to survive within sediment. Cox (1977) noted distinct species assemblages in zones on the rocky shore. As the shore at Dunraven bay is split into distinct areas with an upper shore comprising a wave cut platform, and a lower shore made up of boulders, the species and mode of life of the diatoms in these biofilms was expected to vary. This seasonal and spatial variation is addressed in Chapter 2.

1.2.2 Biofilm structure

Microbial biofilms are a community of microorganisms, which are frequently bound by an extracellular polymeric matrix (Allison et al. 2000). These biofilms can be composed of a combination of organisms including bacteria, fungi, algae and protozoa. Biofilm formation is often an adaptation of the environment, performed by the microbial community, which allows successful colonisation of otherwise extreme or unfavourable environments (Toole et al. 2000). The matrix of extracellular polymeric substances (EPS) of microalgal biofilms acts to protect the microalgal community by providing a microenvironment where adaptation strategies (including chemical, biological and physical strategies) are used to regulate exposure to stresses including nutrient limitation (Wulff et al. 2000), high light (Kromkamp et al. 1998, Serôdio & Catarino 1999, (Perkins et al. 2001, Mouget et al. 2008), predation (Saburova & Polikarpov 2003), and physical disturbance (Hauton & Paterson 2003, Aspden et al. 2004). EPS can be of varying viscosities, from thick gel to a fully dissolved solution (Decho 1990). These polymers are made up of repeating monomers which are attached by glycosidic bonds (Varki et al. 2008). The way in which these molecules interact and the order of the molecules in the chains determines the properties exhibited by the EPS (Verdugo 1993). EPS which contains more cross-linked bonds absorbs and retains more water readily and provides protection against desiccation. This collection of molecules and bonds is stabilised by calcium ion bonds

(Chin et al. 1998). Biofilms can have many structures and EPS plays an important role in this structural formation (Paterson et al. 2001). Diatom biofilms bound by EPS are able to remain in unstable environments such as mudflats and rocky shores and the cells are able photosynthesise within these biofilms and persist in unfavourable environments.

1.3 PHOTOSYNTHETIC FUNCTION

1.3.1 Conversion of excitation energy into chemical potential

The majority of fluorescence recorded from diatom cells is emitted from Photosystem II (PSII). PSII catalyses the oxidation of water molecules in a light driven process. This provides electrons which move through the electron transport chain and delivers the energy required to reduce inorganic carbon (Falkowski & Raven 1997). PSII is an aggregation of proteins and pigment molecules which form the initial electron acceptors of the electron transport chain (Zouni et al. 2001, Barber 2006). Barber (2006) describes PSII as a light harvesting complex and this forms a part of the light harvesting antenna. It is often termed the P680 as it absorbs light at the wavelength 680 nm (Hankamer et al. 1997).

Diatoms are photoautotrophs meaning they must capture photons and convert these to energy. In order for the cells to do this they must remove electrons and protons from water. Irradiance is recorded as energy incident per unit time and area. The photosynthetically active wavelengths are between 400-700 nm of the total spectrum (McCree 1971, 1972). However it is thought that algae are capable of utilising wavelengths as low as 350nm (Geider & Osbourne 1991) and therefore any photon of light between these wavelengths can cause charge separation to occur. The oxidation-reduction reactions which occur in the D1 and D2 proteins are steady state electron transfer reactions which either oxidise the primary quinone acceptor of PSII (Qa) or reduce Qa. Several authors have described the electron transport chain which allows the reduction of NADP (Nicotinamide adenine dinucleotide phosphate) to NADPH

(Nicotinamide adenine dinucleotide phosphate reduced form) including, Taiz & Zeiger (1991) and Falkowski & Raven (1997). The electron transport chain will be described below as reported in Taiz & Zeiger (1991) (see Fig. 1.3.1 for a graphical respresentation).When a photon is absorbed a pigment molecule moves into an excited state. PSII is excited and transfers an electron to the pheaophytin electron carrier, this is termed a charge separation. The electron is then transferred to the first quinone in the chain (Qa). Tyrosine then provides an electron and reduces the PSII. The electron is transferred from Qa to Qb and concurrently manganese provides an electron which reduces tyrosine. Manganese is then also reduced by an electron provided by a water molecule. Therefore as four change separations occur four photons are required to oxidize two water molecules which release oxygen and four hydrogen ions into the lumen of the cell. Qb in a reduced state binds to two hydrogen ions and as this is only loosely bound, it is then released into the thylakoid membrane. In the thylakoid membrane it reaches cytochrome b6-f and attaches to it. The hydrogen ions are released and this forms a pH gradient between the lumen and the stroma of the cell. As this is occurring a plastocyanin protein is transferring the electrons from cytochrome b6-f to the photosystem I (PSI). A reaction centre referred to as P700, which is in the oxidized state, transfers the electron through four other molecules in the electron transport chain and this results in the reduction of ferrodoxin. To reduce one NADP molecule two electrons are required. This is a conversion from the excitation energy from sunlight into chemical potential energy which is used by the cells. This process is oxygenic photosynthesis and is one of the most important steps in the evolution of life on Earth.

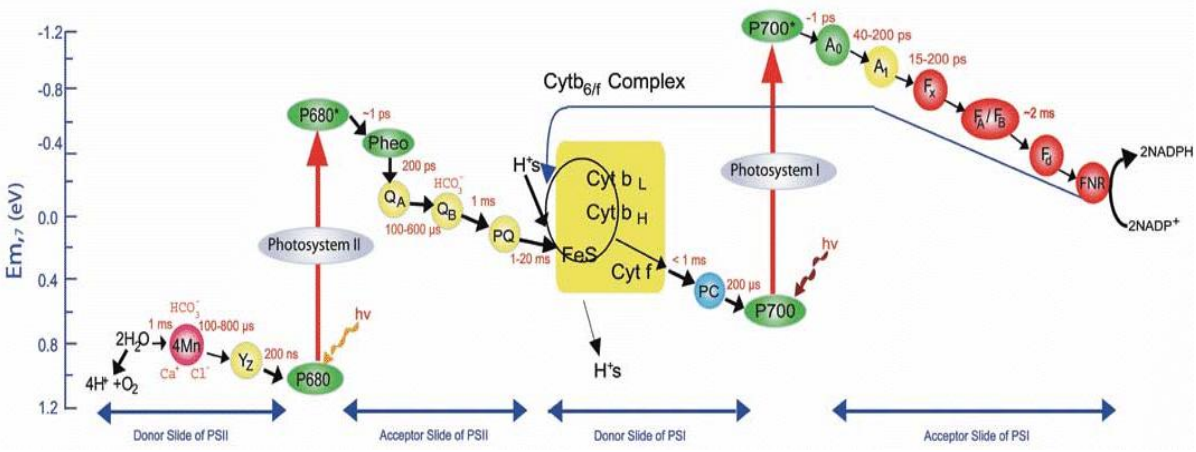


Figure 1.3.1: The Z scheme for electron transport in oxygenic photosynthesis (Reproduced from (Lien & San Pietro 1975)

1.4 PHOTOSYNTHETIC REGUATION (PHOTOREGULATION)

1.4.1 Migration

As mentioned the biofilms found at the lower shore sites more closely resemble mudflat biofilms and it was hypothesised in chapter 4 that the diatom cells in the lower shore biofilm would utilise the same photoregulation strategies as cells found in mudflat biofilms. Microphytobenthic biofilms which are found on mudflats are known to utilise migration as a mechanisms for photoregulation both in a mass movement manner and by micro-cycling (Perkins, et al. 2010, Cohn 2001, Cohn et al. 2004, Consalvey et al. 2004, Apoya-Horton et al. 2006, Jesus et al. 2006). This is a primary method of photoregulation as the cells actively position themselves in the upper layers of the sediment to expose themselves to the most optimum light level. The cells move using extruded EPS and an adhesion-traction mechanism suggested by Edgar and Pickett-Heaps (1984) is commonly accepted. The EPS strands attach to either the substratum in the case of the lower shore biofilms or the inside of the polysaccharide tubes in the case of the upper shore biofilms. These strands are also connected to trans-membrane arrangements which move along the raphe of diatom frustules by interacting with actin filament bundles (Edgar & Zavortink 1983, Underwood & Paterson

1993). The motility of diatom cells is affected by the water content of the environment (Hopkins 1966), the light levels the cells are exposed to (Apoya-Horton et al. 2006, McLachlan et al. 2009, Perkins et al. 2010) and the temperature (Wolfstein & Stal 2002, Cohn et al. 2003). Tube forming cells are known to move within the tubes (Houpt 1987). The factors which effect this movement have not yet been investigated and will be in this thesis.

1.4.2 Non-photochemical quenching

The cells present on the rocky shore are exposed to periods of very high light levels and although prior to this investigation it was not known which photoregulation mechanism was used by these biofilms to regulate their photosynthesis, the cells on the upper shore cannot migrate into the rock surface. A major mechanisms of photoregulation used in circumstances when migration is not available is non-photochemical quenching or NPQ (Cartaxana & Serôdio 2008, Serôdio et al. 2009, Perkins et al. 2010, Cartaxana et al. 2011). This is an enzyme dependent process (Fig. 1.4.1) and therefore can be effected by temperature changes (Olaizola et al. 1994, Olaizola & Yamamoto 1994). Most enzymes are vulnerable to elevated temperatures which can alter the confirmation of the enzyme and therefore reduce or totally destroy its functionality (Sizer et al. 1943, Palmer & Bonner 2007).

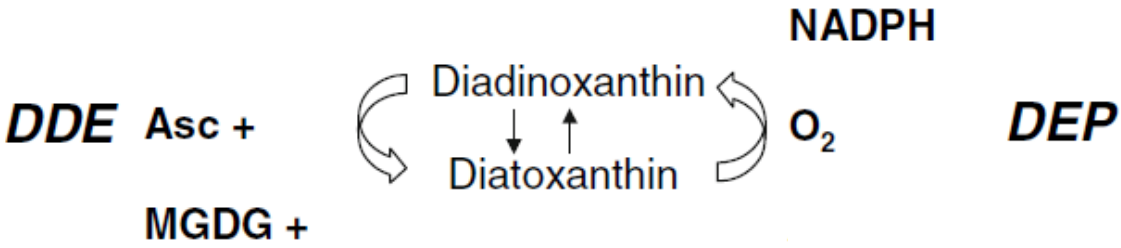


Figure 1.4.1: *The reaction sequence of the diadinoxanthin*, cycle (reproduced from Goss & Jakob 2010). The diadinoxanthin cycle is utilised by the diatoms. The enzymes which catalyse the de-epoxidation reaction require cofactors to function. The + symbols next to the cofactors are to illustrate the low (+) or high (++) concentrations of these substrates which are required for high levels of enzyme activity. DDE- diadinoxanthin de-epoxidase, DEP- diatoxanthin epoxidase, Asc- ascorbate, MGDG- monogalactosyl-diacylglycerole.

Microalgae which use the diadinoxanthin (Dx) cycle are known to synthesis the xanthophylls required for the violaxanthin (Vx) cycle (Lohr & Wilhelm 2001). These pigments are precursors required in the formation of diadinoxanthin (Dd) and diatoxanthin (Dt), which are the enzymes required for NPQ in diatoms, and fucoxanthin which is the main light harvesting accessory pigment. The Dx cycle only has one de-epoxidation step compared to the two required in the Vx cycle (Demers et al. 1991). The Dx cycle reaction sequence requires the conversion of an epoxy-xanthophyll (Dd) to an epoxy-less carotenoid (Dt). This conversion takes place under moderate-high light conditions and the re-epoxidation of diatoxanthin to diadinoxanthin occurs in low light or darkness. However chlororespiration is also known to induce de-epoxidation. The proton gradient built up during respiration induces the conversion of Dd to Dt (Jakob et al. 2001).

Enzymes are required to catalyse stages of the Dx cycle. The ΔpH exerts a high level of control on the induction of the Dx cycle (Goss et al. 2006). Diadinoxanthin de-epoxidase (Dde) is activated at pH 7.2 which is much more sensitive than violaxanthin de-epoxidase (Vde) where activity can be observed at pH 6.5. This means that the slight acidification of the thylakoid

lumen during chlororespiration is sufficient to activate enzyme activity (Jakob et al. 2001). Reduced ascorbate is required by Dde in the reduction and elimination of the epoxy group present in Dd. However secondary co-substrate utilisation means that Dde requires a lower ascorbate concentration within the thylakoid lumen than the Vx cycle (Grouneva et al. 2006). Both the Vx cycle and the Dx cycle require the lipid galactolipid monogalactosyldiacylglycerol (MGDG) (Goss et al. 2007). Once again the diadinoxanthin cycle requires lower concentrations of MGDG for de-epoxidation to occur compared to the violaxanthin cycle due to the better solubility of the lipid (Goss et al. 2005). During light exposure higher levels of diadinoxanthin diffuse from the light-harvesting proteins to the free lipid phase of the membrane for de-epoxidation. This is confirmed by the observed larger pigment pool size in the diadinoxanthin cycle (Lavaud et al. 2003).

The reaction to epoxidase diatoxanthin to diadinoxanthin is catalysed by diatoxanthin epoxidase. This process requires oxygen, flavin adenine dinucleotide (FAD), and NADPH in order to attach the epoxy group to the diatoxanthin molecule (Büch et al. 1995). Diatoxanthin epoxidase (Dep) is activated at a pH of 7.5 and is totally inhibited during high light exposure as the proton gradient is formed (Goss et al. 2006). This inhibition and the activation of Dde allows the high-speed de-epoxidation of diadinoxanthin to diatoxanthin which is necessary in the highly variable light conditions experienced by both mudflat and rocky shore diatom cells.

NPQ is correlated to the concentration of diatoxanthin and the proton gradient does not influence this (Goss et al. 2006). During NPQ PSII and the light harvesting antenna change conformation and this it is then in a heat-dissipating state. PSII is converted rapidly back to the light-harvesting state when the quenching pigment diatoxanthin is removed by epoxidation, a process that requires NADPH. When a cell is moved from high light conditions to darkness the Calvin Cycle uses NADPH and reduces the amount available to diadinoxanthin epoxidase.

When Rubisco is deactivated in the dark this reduces the activity of the Calvin cycle and so more NADPH becomes available to Dep (Macintyre & Geider 1996).

NPQ is composed of several different mechanisms. The quenching of the antenna by the Dx cycle is the most widely known mechanism of NPQ. Results from a study by Grouneva et al. (2008) suggested that in the diatom *C. meneghiniana* there are three clear aspects. The first begins on illumination and relies on the trans-thylakoidal proton gradient and the levels of light the cell is exposed to. This process is regulated by the level of diatoxanthin in the cell prior to illumination. This occurs in the antenna complex of the diatom. The second process occurs during prolonged exposure to high light conditions and is reliant on the Dt produced during light exposure by the Dx cycle. The third is a rapid relaxing process observable when cells are moved directly from high light to darkness. It again is reliant on diatoxanthin synthesised during light exposure and occurs within seconds of transfer into darkness. The pH dependent activation of Dde and the trans-membrane proton gradient are required to initiate Dt non-photochemical quenching (Ruban et al. 2004, Lavaud & Kroth 2006). Lavaud & Kroth (2006) suggest that production of Dt and protonation of antenna complexes changes the shape of the antenna. To prevent relaxation of NPQ when a bulk proton gradient is not present Dt binds to hydrophobic regions of protein in the light harvesting complexes and dislocates proton-binding domains. Goss et al. (2006) support this by observing that if Dt is activated pH no longer affects the efficiency of NPQ. The process of physiologically regulating photosynthesis using NPQ is complex and the effect of environmental variation on this process is investigated in this thesis with regard to rocky shore and mudflat based diatom dominated biofilms.

1.4.3 Photoinhibition and photodamage of PSII

There are several mechanisms by which photoinhibition and photodamage occur including, total reduction of Qa during light exposure, resulting in charge recombinations and singlet oxygen production and direct damage from photons entering an oxygenated environment.

Exposure to high light levels and cold temperatures can inhibit the activity of Photosystem II (PSII) (Baker & Bowyer 1994, Maxwell et al. 1994, 1995). This is termed photoinhibition and it is likely that diatom cells present in the rocky shore biofilms of Dunraven Bay were photoinhibited, as they are exposed to fluctuating temperatures (Lewis 1964) and high light levels during the emersion period. Consistent photoinhibition can lead to permanent photodamage if energy dissipation, by various means (Schreiber et al. 1986, Olaizola et al. 1994, Underwood et al. 1995, Staats et al. 2000, Perkins et al. 2001, Koblízek et al. 2001, Muller et al. 2001, Serodio et al. 2005), is not utilised (Young et al. 1997, Ruban et al. 2004, Tyystjärvi 2008).If energy is not dissipated Qa can be overly reduced by the excess light (Huner et al. 1996). There is a low probability for photodamage to occur when there is photochemical utilisation of light energy and Qa is oxidized (Maxwell et al. 1994, 1995). Excitation energy in the P680 causes a photochemical charge separation. If Qa is reduced when primary charge separation occurs between P680 and pheophytin the electron transfer chain is interrupted and this can lead to photo-oxidative damage (Barber 1995). A charge recombination occurs in the P680 and pheaophytin pair. This reaction can generate a P680 triplet (Vass et al. 1992, Vass & Styring 1993) which is quenched by triplet oxygen (Durrant et al. 1990). This can generate the damaging singlet state oxygen which then damages the P680 reaction centre (Telfer et al. 1994, Telfer 2002).

The exposure of PSII to light results in the production of an oxidant which oxidises water (Prášil et al. 1996). PSII is made up of multiple proteins which act as an oxidoreductase enzyme. The

transmembrane portions of the photosystem act as light harvesting complexes (Barber 1989). The central portion of the PSII contains the D1 and D2 proteins. These are responsible for various processes including electron transfer, water oxidation and light utilisation (Nanba & Satoh 1987). Photons enter the PSII at rates of up to 10,000 per second (Melis 1999) into a highly oxygenated environment which in the presence of oxidants results in photo-oxidative damage (Aro et al. 1993, Tyystjarvi 2008). This can cause direct damage to the P680 reaction centre which inhibits photosynthesis. means The level of photodamage is reliant on the number of photons absorbed rather than the rate of absorption (Park et al. 1995). The photodamaged PSII is disassembled which leads to degradation of the D1 protein. The D1 protein is then synthesised from the degraded proteins. These proteins are then inserted into the damaged PSII protein and the PSII protein is re-assembled (Aro et al. 1993). Diatom cells are able to repair part or all of the photodamage caused to PSII in this way and therefore minimise the effect of photoinhibition (Kim et al. 1993, Neidhardt et al. 1998, Melis 1999, Heraud & Beardall 2000). The degradation and synthesis of the D1 proteins is a limiting factor (Neidhardt et al. 1998) and if the rate of damage to the PSII occurs at a faster rate than the degradation and synthesis of the D1 protein then photoinhibition occurs (Baker & Bowyer 1994, Kim et al. 1993). The re-assembled PSII is then capable of resuming electron transport processes.

1.5 FLUORESCENCE GENERAL METHODOLGY

As mentioned pulse amplitude modulated chlorophyll a fluorescence was utilised in every chapter of this thesis. As such the general methodology and background information about this technique is included.

1.5.1 The Walz Water PAM

Variable chlorophyll fluorescence is a light emission which primarily comes from PSII which contains chlorophyll *a* (Krause & Weis 1991, Govindjee 1995, Papageorgiou et al. 2007). Energy absorbed by the light harvesting complexes in the PSII is utilised for photochemistry and emitted by non-photochemical quenching (heat dissipation) or fluorescence. These different processes are competitive (Krause & Weis, 1991, Kolber & Falkowski, 1993). Therefore an increase in one of these factors results in a decrease in the other factors. The Walz Water PAM (Walz GmbH Germany) (Fig. 1.5.1, A and B), was used in this study to measure relative electron transport rate (*rETR*), light acclimated quantum efficiency (*ΔF/*Fm') and light acclimated maximum fluorescence (*Fm'*) light adapted state is noted with a '. This PAM was used as it is specialised for the study of microalgal biofilms with low biomass.

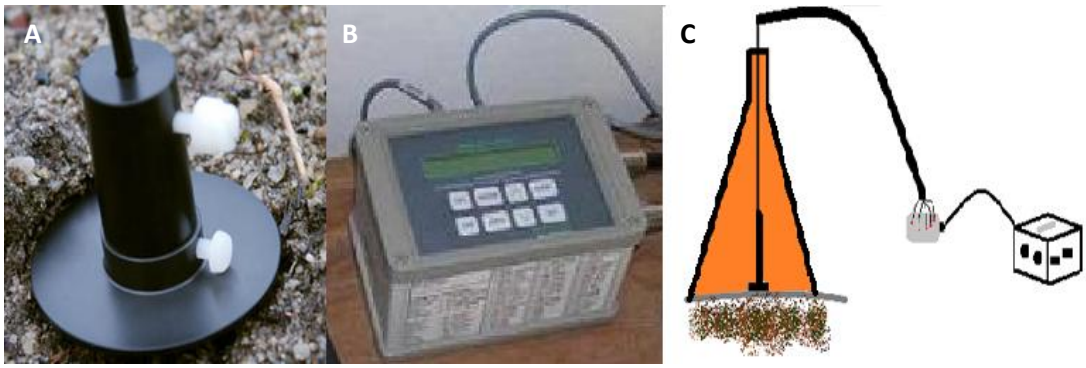


Figure 1.5.1: *Walz water PAM,* A) Measuring head against rock sample, B) PAM control unit, C) Diagram of the rocky shore set-up.

The measuring head (Fig. 1.5.1, A) is water-resistant which allows for investigations of biofilms under a layer of water. This fluorometer is used in conjunction with Wincontrol® software (Walz) which allows detailed adjustments of the functioning of the fluorometer and logs the fluorescence data and automatically calculated fluorescence parameters. The PAM fluorometer uses 3 different lights, which influence the photosynthetic apparatus of the

microalgal cells. A weak measuring light causes an emission of fluorescence which does not stimulate photosynthesis this provides information regarding the fraction of closed PSII reaction centres. The fluorescence emission induced by the weak measuring light is termed the minimum fluorescence. A saturating pulse is then produced which closes all PSII reaction centres which causes a large fluorescence emission. The resulting fluorescence emission is termed the maximum fluorescence. Another light pulse of actinic light is produced and this causes photosynthesis to occur. The spectrum of light emitted as fluorescence is identifiable by the PAM fluorometer as it is different to the spectrum of the absorbed light. The spectrum of the fluorescence emission is a longer wavelength (685nm) than the absorbed light (less than 670nm)(Maxwell & Johnson 2000). The modulated measuring system allows the detection of the fluorescence from the measuring light only and this allowed measurements to be made in the presence of ambient light which is very beneficial for field experiments (Quick & Horton 1984). A Walz Mini IPAM was used in Chapter 4, please see this chapter for information on this PAM fluorometer.

1.5.2 Fluorescence methodology used in this study

Settings on the Walz Water-PAM were as follows: saturating pulse at setting 10 (approximately 8,600 µmol m^{-2} s^{-1} PAR) for 600 ms duration; light curve settings of 30 second light step duration covering 10-1037 µmol m^{-2} s^{-1} in chapters 2 and 4 and 10-2975 µmol m^{-2} s^{-1} in chapter 3. The lower light levels were chosen as the light levels under the water at the field site do not exceed this level. The higher light level was used in Chapter 3 as this was a temperature manipulation experiment *ex -situ* where the likely photosynthetic responses were unknown. The light acclimated quantum efficiency of PSII (*ΔF/Fm'*), the relative electron transport rate *(rETR)*, and maximum fluorescence (*Fm'*) were used in this thesis and these were obtained by creating light response curves. The *Fm'* can be used to ascertain whether downregulation is occurring as a reduction in these values equates to an increase in downregulation.

The coefficients the maximum light use coefficient (α), the light saturation coefficient (*Ek)* and the maximum relative electron transport rate at which light becomes limiting *($rETR_{max)}$* levels are calculated using an iterative curve fitting solution by Eilers & Peeters (1988). This curve-fitting and regression analysis is performed using Sigmaplot V11. *Ek* was calculated from the maximum relative ETR ($rETR_{max}$) and light use coefficient (α);

1. $Ek = rETRmax / \alpha$. Light

Quantum efficiency of PSII is calculated as *ΔF/Fm'*, calculated as;

2. $(Fm' - Fo')/Fm'$)

These fluorescence parameters are calculated from data collected during a rapid light curve.

The initial point on the light curve, representing the quantum efficiency of PSII, taken at 0 µmol m^{-2} s^{-1} was used as a measure of *ΔF/Fm'.* Despite being recorded at the first point of the curve, in the dark, it is termed the light acclimated quantum efficiency as the biofilms were not dark adapter prior to measurement. Dark adaption was not performed as it is known that dark-adapting diatoms can increase non-photochemical quenching and due to practical limitations on the rocky shore, the preferred far-red adaptation (Consalvey *et al*. 2004) was not possible. These measurements also represent the actual efficiency state of PSII rather than the optimum state, which is less relevant to this research.

1.5.3 Rapid Light Curves

Rapid light curves measure effective quantum yield as a function of the irradiance. The fluorescence was recorded at 8 light levels at 30 second increments, consistent with the methodology of Perkins et al (2006). The relative electron transport rate (*rETR)* which is obtained from these curves when plotted against irradiance appear similar to P-E curves achieved from measuring oxygen evolution or CO_2 gas exchange (Flameling & Kromkamp 1995, Hartig et al. 1998, Barranguet & Kromkamp 2000, Perkins et al. 2002) however these curves

should not be analysed in the same way. Rapid light curves provide detailed photosynthetic data over multiple light levels which provide reliable information in situations when light levels fluctuate frequently (Serodio 2003). The major difference between rapid light curves and P-E curves is that *rETR* and quantum efficiency of PSII recorded by a rapid light curve represent the current state of the cell, whereas P-E curves display the optimum photosynthesis (Perkins et al. 2006). Rapid light curves are typically made up of three regions (Figure 1.5.1), the light limited region, the light saturated region and the photoinhibited region of the curve. At initial light levels, the photosynthesis is limited by the light intensity, the slope of this portion of the curve is termed alpha (α) and it is representative of the efficiency of light absorption (this can also be termed the effective quantum yield) (Govindjee & Papageorgiou 2005). The minimum saturating irradiance or *Ek* is the intercept between the α and the maximum photosynthetic rate (Sakshaug et al. 1997). Above the level of Ek the quenching that is occurring is non-photochemical quenching (Henley 1993). At moderate irradiance the photosynthetic limiting factor is capacity of the electron transport chain. This is when the curve plateaus and is said to be saturated and $rETR_{max}$ is reached (Govindjee & Papageorgiou 2005). At high irradiances the *rETR* reduces due to down regulation of PSII.

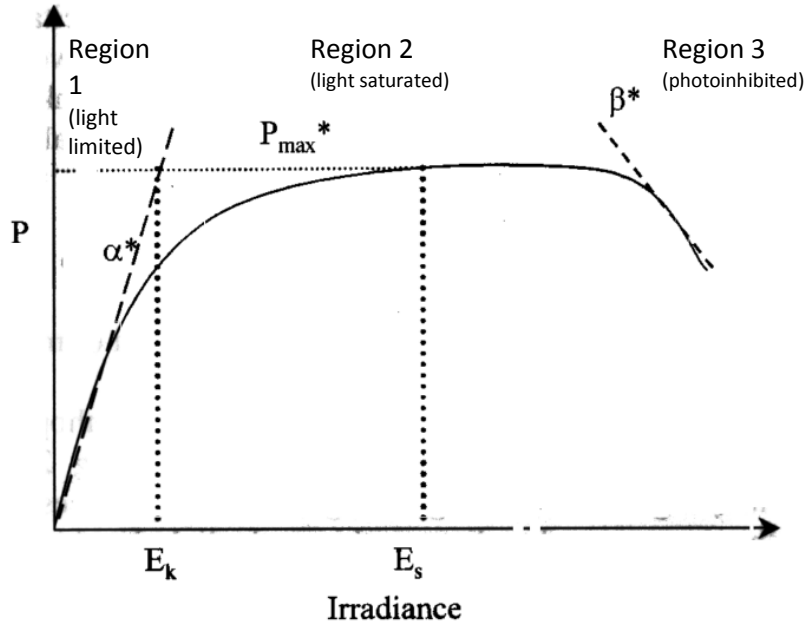


Figure 1.5.1: A representation of a P-E curve taken from Consalvey et al. (2005)

1.6 CONCLUDING REMARKS

This thesis aims to use established (i.e. PAM fluorescence and taxonomic analysis) and novel (i.e. temperature controlled microscopy) techniques to assess the photophysiology, taxonomy and behaviour of rocky shore microalgal biofilms. This is a neglected area of research and these techniques, with some modifications, can be successfully applied to these variable and diverse habitats. This work represents one of the first seasonal investigations of biofilms in the rocky shore. Survey work will be combined with controlled experiments in which temperature and photoregulation mechanisms will be manipulated to examine community behaviour and photophysiological processes.

AN INVESTIGATION INTO THE SEASONAL VARIATION IN THE PHOTOPHYSIOLOGY, BEHAVIOUR AND TAXONOMY OF ROCKY SHORE BIOFILMS AT DUNRAVEN BAY

CHAPTER 2

ABSTRACT

The seasonal variation in the photophysiology of rocky shore biofilms was investigated on an exposed shore on the South Wales coast (Dunraven Bay, Bridgend Borough County). The effect of biotic and abiotic factors, including temperature, light dose and grazing pressure, on the phototphysiology was investigated during the low tide emersion period. This study took place over 3 consecutive years with measurements being made every month, at 5 lower and upper shore sites, whilst the biofilms were intact. The photosynthetic parameters $rETR_{max}$,, *ΔF/Fm' α Ek,* and *Fm'* were measured and calculated in conjunction with analysis of biofilm community structure and grazing pressure. Seasonal variations in photophysiology were linked to the reproductive phase of the biofilm with higher $rETR_{max}$ and *ΔF/Fm'* exhibited in November and December when the biofilm was recovering from an observed spring die-off (on average 85 compared to 60 relative units). Measurements made on months with high temperatures and light dose showed reduced $rETR_{max}$ and *ΔF/Fm'.* These unfavourable environmental conditions in addition to grazing pressure, which was found to increase 2-fold in the spring, resulted in the die-off. Once the environmental conditions became more favourable (cooler temperatures and lower light dose) and the grazing pressure reduced, the biofilms re-grew in the autumn months. The photosynthetic responses of the lower shore biofilm were influenced less by the external environmental conditions. This was likely to be because the biofilm cells were found to live in a thin layer (6mm) of sediment on the rock surface as opposed to the upper shore biofilm cells which were tube-forming and attached to the rocky substratum. There was a pronounced shift in biofilm community structure observed in the upper shore biofilms, with a shift from large cells (*Berkeleya rutilans* and *Parlibellus delognei*) in the winter, to smaller cells (*Navicula bottnica*) in the summer months. This change was less pronounced in the lower shore biofilms where any changes were gradual. These observed changes in community structure did not appear to affect the photophysiology of the biofilm as a whole. This study highlights the complex nature of the biotic and abiotic factors which influence the photosynthesis and photoregulation of rocky shore microalgae and it is clear that several drivers caused reductions and increases in the microphytobenthic biomass on the rocky shore, which in turn influenced the photophysiology of the biofilms.

INTRODUCTION

Intertidal zones are dynamic areas which are exposed to the energetic actions of tides and waves. In addition to this, these areas are also exposed to the wind, rain and fluctuating temperatures of terrestrial systems. These highly changeable environmental conditions pose challenges to rocky shore dwellers, yet despite this these areas are highly diverse (Aleem 1950, Lewis 1964, Underwood & Chapman 1998). This investigation aimed to more fully understand the dynamic relationship between the microalgae present on the rocky shore and the environmental and biological features of this environment.

Rocky shores are typically characterised by zonation (Stephenson 1949, Stephenson & Stephenson 1972) with distinct zones of the shore inhabited by different species of algae and herbivores in response to the spatial variation in environmental conditions. The distribution of organisms in horizontal bands or 'vertical zonation' has been observed for some time and has been linked in a descriptive manner to factors including emergence time and light quality/intensity (Doty 1946, Lewis 1964, Stephenson & Stephenson 1972). In addition, this study site (Dunraven Bay; 51° 44.71'N, 03° 60.87'W) is exposed to large storms and sea swells which move up the Bristol Channel. The cells within rocky shore biofilms at this site are also exposed to extremes of light (240 µmol m^{-2} s^{-}1-1800 µmol m^{-2} s^{-}1) and temperature (-4°C-32°C). Transient changes in environmental conditions are known to affect the photosynthesis (Colijn & Buurt 1975, Grant 1986, Pinckney & Zingmark 1993, Blanchard et al. 1996b, Serôdio & Catarino 1999, Perkins et al. 2001) and behaviour (Admiraal 1977, Perkins et al. 2001, Cohn, Farrell, Munro, Ryan, et al. 2003, Du et al. 2012) of mudflat microphytobenthos. This chapter investigates whether these transient changes and non-transient seasonal changes such as lower light levels and temperature in the winter months also have an effect on the photosynthesis and ecology of the microalgal component of these biofilms. The aim was to determine whether the transient or seasonal changes have a larger impact on these factors.

Biological stress - grazing pressure

Rocky shores are areas of high biodiversity and the microphytobenthic portion of these shores provides an important base of near shore food chains (Castenholz 1961, Hawkins *et al.* 1992, Thompson *et al.* 2000, 2004). These areas also form vital refugia (holes and crevices) for invertebrates (Menge & Lubchenco 1981, Bergey 1999). Rocky shore microbial biofilms are composed of bacteria, fungi, viruses, diatoms and cyanobacteria (Decho 2000). The sites (i.e. upper and lower shore sites) studied during the measurement period, were primarily composed of diatom species present in shallow pools on flat rock surfaces. The biofilms were actively grazed by limpets (Patella spp*)* and other gastropods (e.g. *Melarhaphe neritoides* and *Littorina saxatalis*). The grazing pressure can be assessed using the numbers of grazers present on the rocky shore and the number of grazing marks (Figure 2.1.1) or tooth marks present on upper and lower shore sites (Thompson *et al.* 1997, Forrest *et al.* 2001). Rocky shore habitats have been extensively studied and manipulated (Lewis 1964, Cubit 1984, Underwood & Jernakoff 1984, Kaehler 1998). Lewis (1964) produced accounts of the majority of rocky shore habitats in Britain, describing the grazing species and macroalgal communities present. Herbivory has also been studied in depth (Jones 1948, Hawkins & Hartnoll 1983, Menge 2000, Forrest *et al.* 2001, Mieszkowska *et al.* 2006), particularly by Jones (1948). These studies focused on the grazing behaviour of limpet populations, which Hawkins (1992) found to have influence on the distribution of macroalgal species on the rocky shore. The herbivore communities found on rocky shores are also known to be sensitive to the effects of climate change, and the use of these environments as indicators of a changing climate has been explored (Poloczanska *et al.* 2008). The types of communities found on the rocky shores are determined by both biological (Hawkins & Hartnoll 1983, Menge 2000, Forrest *et al.* 2001, Mieszkowska *et al.* 2006) and physical factors.

The amount of grazing marks made by limpets (Figure 2.1.1) can be indicative of the level of activity of the limpets (Thompson *et al.* 1997, Forrest *et al.* 2001).

Figure 2.1.1: *Limpet grazing marks,* on limestone rock covered with a diatom biofilm.

This grazing data allows deeper understanding of the seasonal pressures on the biofilms, as limpets are known to behave seasonally (Jenkins *et al.* 2001). Microalgae present on lower shores are generally thought to be influenced most by herbivores, and algae present on the upper shore primarily affected by physical stresses (Lawson 1957, Lewis 1964, Chapman 1973, Haven 1973, Underwood 1980, Creese & Underwood 1982) as some herbivores are sensitive to desiccation and predation on the upper shore. This is unlikely to be the case at the Dunraven Bay site since the primary herbivores are gastropods, which use refugia present in the cliff areas of the upper shore, and so protect themselves from these factors.

Physical stress

It is known that physical stresses present in the summer months primarily cause the microalgae present on rocky shores to reduce dramatically in density (Decho 2000, Thompson & Norton 2004). These physical stressors include high light, temperature and desiccation (Van Den Hoek 1982, Souffreau *et al.* 2010). The biofilms present at Dunraven Bay were not subject to desiccation, being largely within small rock pool depressions which retained water throughout the emersion period (pers.obs), so this is not a physical stress at this site. Cubit (1984) found that with exclusion of grazers in the summer months, some macro and

microalgae survived, but in the plots he created without grazer exclusion almost no microalgae survived. This suggests that physical stress reduced the levels of microalgae but in conjunction with grazing it was almost completely removed. This chapter discusses the way in which the combinations of biotic and abiotic pressures influence the biomass and species composition of the microphytobenthic biofilm at Dunraven Bay. These factors may be influencing the photophysiological responses of the microphytobenthos and this is investigated in this chapter.

Taxonomic variability

Patterns in taxonomic variation in microalgal biofilms have been studied but have primarily focussed on the spatial variation related to the vertical zonation on the shore (Aleem 1950, Underwood 1984, Underwood & Chapman 1998), rather than seasonal temporal variation. The temporal variation has been studied by Castenholz (1963, 1967) in the United States and Norway, by Underwood (1984) in Australia, and by Aleem (1950) and Hill & Hawkins (1991) in Great Britain. These studies however were made on very different shores to that at Dunraven Bay and these studies did not investigate the photophysiology of the biofilms in conjunction with these taxonomic observations. Hill and Hawkins (1991) found that the overarching characteristic of rocky shore microphytobenthic biofilms is patchiness. The biofilms do not cover the whole rock, and in those areas covered with biofilm, grazing results in a heterogeneous biofilm. This has implications for sampling, and they suggest using several sample sites to obtain data. They, amongst others (Hawkins & Hartnoll 1982a, Cubit 1984, Hill & Hawkins 1991a), suggest that the patterns of increased biofilm biomass in the autumn could be as a result of the decreased activity of grazing species, due to gonad development hindering movement. The variation in biofilm biomass during the year has implications for total ecosystem productivity, therefore influencing the activity and seasonality of the near-shore food chain (Castenholz 1961, Hawkins *et al*. 1992, Thompson *et al*. 2000, 2004).

In this study the definition of a tube-dwelling diatom will follow that provided by Cox (1975, 1981), who defined tube-dwelling diatoms as 'producing mucilage which is consolidated into a tubular structure around the cells, yet within the mucilage tube individual cells move and divide'. Furthermore, keys within these works will be used to identify the species of tube-dwelling diatom present at Dunraven Bay. Tube-dwelling diatoms, as studied in this investigation, have been the focus of studies related to their distribution along the Severn Estuary (Cox 1977b) and their distribution on the shore. Cox (1981) found that the tube-dwelling species only thrived on hard, submerged substrata. Cox (1981) also noted that the cells were able to move within their tubes and move out of tubes in order to escape events such as burial with sediment. This suggests that the tube-dwelling diatoms at Dunraven Bay may also be able to move in this manner and this behaviour may be related to seasonal environmental pressures. Cox (1981) also found that species appeared to have a wider range of salinity tolerance, and it was speculated that this was a result of the tubes providing a micro-environment.

HYPOTHESES

- In order to assess the seasonal variations in photophysiology a Walz Water-PAM fluorometer was used to take a light curve at each of the five sample sites on the upper and lower shore. The $rETR_{max}$ was measured to provide an understanding of the way in which environmental and biological factors such as light, temperature and grazing affect the overall photosynthetic rate. It was hypothesised that the higher light levels and temperature in the spring months will result in greater $rETR_{max}$ values. However it was also expected that there will be an increased level of energy dissipation, indicated by reduction in the recorded *Fm'*, as the light level will likely be above saturating levels. The seasonal changes in the light adaptation of the cells will be assessed by the recorded *Ek* and *ΔF/Fm'.* It was hypothesised that the changes in *Ek* will reflect the changes in the light adaptation state of the cells, it is likely that *Ek* will increase as the light levels increase, but will not increase indefinitely as physiological limits will

likely restrict this. It was hypothesised that the *ΔF/Fm'* will be affected by the light levels, with higher light levels resulting in a lower *ΔF/Fm',* however it is likely that this will be influenced by overall cell health and function (Kolber *et al.* 1988, 1994, Geider *et al.* 1993), meaning that temperature was also likely to be of influence, with a possible decrease in *ΔF/Fm'* during periods of extremely high and low temperature.

- The number of grazers on the shore, and the activity of the most active grazers (measured by recording radula scrapes) will be recorded to ascertain whether there is a relationship between changes in the photophysiology of the cells and the activity of grazers. Previous work suggests that increased grazer activity will increase the *rETR* (Mccormick & Stevenson 1989, Skov *et al.* 2010), therefore it was hypothesised that increases in grazer activity and numbers occur, then $rETR_{max}$ will increase.

- Photophysiological measurements will be made at the upper and lower shore on a monthly basis. The patterns in photophysiological response will be compared. It was hypothesised that the photophysiological responses will be different with the lower shore sites displaying a greater reduction in $rETR_{max}$ and *ΔF/Fm'* during periods of extreme temperature or high light. This was predicted as the cells are exposed for a shorter period of time on a daily basis and therefore the cells are likely to be less well adapted to extreme changes in environmental conditions.

- It was hypothesised that changes in photophysiology will be as a result of a combination of environmental and biological factors. The effect of these environmental factors and the interactive effects will be examined using principal component analysis. It was predicted that the largest reductions and increases in $rETR_{max}$ and *ΔF/Fm'* will occur on measurement days when extremes of temperature and light combine.

- It was hypothesised that there will be seasonal variation in the microalgal taxonomic community of the biofilm. The photophysiology of the biofilms as a whole may change as a result of changes in the taxonomic community of the biofilms and this will be investigated by identifying the species present in a scrape and counting how many cells of each species are present, whilst estimating their percentage cover of the biofilm (incorporating relative cell size).

METHODS

Sample site and sampling

Seasonal sampling took place at Dunraven Bay (Southerndown, Bridgend Borough County, Wales). 5 sites were chosen from the upper and lower shore (Table 2.2.1) in order to provide a sufficient sample size to take into account the inherent heterogeneity of rocky shore biofilms as mentioned by Hill & Hawkins (1991).

Table 2.2.1: The latitude and longitude of the upper and lower shore sample sites.

Site	Shore level	Geographical location
1	Upper	51° 44.71'N, 03° 60.87'W
2	Upper	51° 44.69'N, 03° 60.83'W
3	Upper	51° 44.68'N, 03° 60.38'W
4	Upper	51° 44.66'N, 03° 60.76'W
5	Upper	51° 44.65'N, 03° 60.73'W
1	Lower	51° 44.69'N, 03° 60.93'W
2	Lower	51° 44.67'N, 03° 60.89'W
3	Lower	51° 44.66'N, 03° 60.85'W
4	Lower	51° 44.65'N, 03° 60.82'W
5	Lower	51° 44.62'N, 03° 60.78'W

The latitude and longitude of the sites was recorded using a hand held GPS (Montana 650, Garmin Ltd). Sites were split into upper shore and mid/lower shore sites ('lower shore'). The actual lower shore areas at the sample site consist of the colonial worm *Sabellaria alveolata* which makes it impossible to sample the true lower shore. The lowest level of rocky shore area not covered by the worm was therefore chosen for the lower shore sites (Figure 2.2.1). Henceforth these areas are termed lower shore for ease of reporting.

Figure 2.2.1: *Aerial photo of the research site,* the sites marked in white are the upper shore sites and those marked in red are the mid/lower shore sites. *(Imagery © 2012 Bluesky, Infoterra Ltd & COWI A/S, DigitalGlobe, GeoEye, Getmapping plc, The GeoInformation Group. Map data © 2012 Google).*

Preliminary field observations indicated that the microphytobenthic biofilms form in permanently wet holes in the limestone surface rather than on the exposed rock surface at Dunraven Bay (Figure 2.2.2). These depressions are believed to have been formed in softer areas in the limestone which erode more quickly than harder areas (Elston 1917), remaining wet through the year and limiting the effects of desiccation experienced in more exposed areas of the rocky shore by biofilms (Lewis 1964; Ji & Tanaka 2002, Souffreau *et al*. 2010).

Figure 2.2.2: *Dunraven bay rocky shore,* with observable permanently wet depressions in the rock surface.

For simplicity and ease of reporting, the measurement years are named 2009, 2010 and 2011 in this thesis. Sampling was conducted monthly from November 2008 through to May in 2009, November 2009- April in 2010 and November 2010- April in 2011. In 2010 and 2011 the month of April was very warm with air temperatures recorded up to 28 °C and there was very little cloud cover which resulted in very high light doses for several weeks. This resulted in an earlier 'bleaching' event, where cells exited the tubes, and biomass became too low in May to viably compare fluorescence results. Therefore the measurements from May in 2010 and 2011 are not included in this chapter. Fluorescence and taxonomic investigation were not performed during the summer months as the biomass on the rocky shore was too low. Observations of grazer activity were made during this period; however these are not included as only a qualitative method could be used.

Fluorescence measurements

By using pulse amplitude modulated (PAM) fluorescence the photosynthetic parameters can be measured in the presence of ambient light (Schreiber *et al*. 1986). The saturating light pulses produced provide momentary saturation of the PSII reaction centre. In diatoms there is a relationship between the rate of photosynthetic oxygen evolution and the electron transport rate (Geel *et al*. 1997) and so these measurements should produce an accurate measure of the level of photosynthesis occurring. The photosynthetic properties of the biofilms were analysed using a Walz Water PAM fluorometer. The measuring head was secured into an opaque orange funnel. This funnel kept the measuring head at a known distance (2mm) from the biofilm surface and prevented the measuring head from moving in the wind or by moving when being held (Figure 2.2.3).

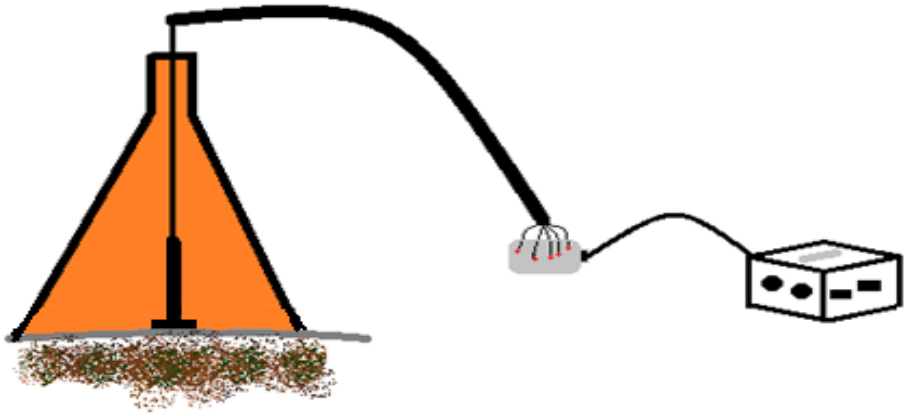


Figure 2.2.3: *Walz Water PAM set-up,* connected to the orange funnel placed onto the rock surface.

The settings of the Water PAM are fully explained in the introduction section 1.5. The fluorescence yields measured by the fluorometer which were used in this analysis were *Fm'*, *ΔF/Fm'* and *rETR*. The percentage change in *Fm'* was calculated as a fraction of the initial maximum yield recorded at the start of a rapid light curve.

Rapid light response curves were made and the measurements were taken at 30 second increments, consistent with the methodology of Perkins *et al.* (2006) - this can be reviewed in more detail in Section 1.5.3 of the general introduction. The light steps covered 10 – 1037 µmol m^{-2} s^{-1} (PAR). 1 light curves were recorded at 5 sites on the upper and lower shores. These measurements were averaged to provide one value for each shore level per sample date. The NPQ levels were calculated from the change in maximum fluorescence yield using Equation 1 in section 1.5.2 of the general introduction.

The measurements were made on days with a low tide period during the middle portion of the day as the sites were totally submerged during high tides. The measurement days also had to be chosen to coincide with satisfactory weather as the fluorometer cannot be used in heavy rain and so a bias may be inherent in the data. However, days with light showers and heavy clouds were used as measurement days, so this bias was minimised. Weather observations were recorded on each measurement day. The Walz Water PAM was used to record the light

dose from the beginning of the emersion period to the measurement time. The length of time from the start of emersion to the fluorescence measurements was fixed at 2 hours. Any difference in light dose was therefore a result of higher light levels on the day of measurement rather than length of exposure time.

Taxonomic observations

Biofilm samples were collected from each sample site (Table 2.2.1). Samples were collected from 1 cm^2 areas from each of the 10 sample sites and on each sample date using a scalpel and tweezers before being placed in containers with a small amount of site water for transport to the laboratory. The samples were collected after the seasonal fluorescence measurements were made in order to minimise the length of time the samples were in the containers. Samples were collected monthly between November and May in 2008 and between November and April in 2010 and 2011. Both taxonomic and photosynthetic observations were not made between May-October, because the biofilms had completely degraded, with initial taxonomic scrapes revealing only dead cells. Care was taken to ensure minimal sediment was removed with the biofilms from the lower shore sites, with samples only stored if a sediment layer of less than 1 mm was collected. The depth of the sediment was recorded by pushing a wooden skewer through the sediment to the rock surface and then measuring the depth of penetration with a ruler. The percentage biofilm cover inside the pool was estimated visually using a 0.25 m^2 quadrat.

Microscopic examination of microalgae

The collected sample was suspended in water, to evenly distribute the tubes, and the width and height of the sample was measured. 10% of the sample was then removed by slicing, using a scalpel, the measured portion as accurately as possible. The sample was placed on a microscope slide with a drop of site water for visual assessment of behaviour. Cox (1981)

suggested that movement behaviour within the tubes allows the diatoms to escape from the tubes when necessary. This activity was monitored throughout the sampling period.

Oil was dropped onto these live samples to facilitate cell identification following behavioural assessments. Observations were made at various magnifications using an Olympus (BX53) bifocal light microscope with an attached camera following methods described by Cox (1996). The number of cells was recorded from a 500 µm area, as defined by a haemocytometer. The species were identified in each of the 5 sites from the upper and lower shore.

Biofilm sub samples were 'cleaned' in a 30% hydrogen peroxide solution at 40 °C on a hot plate to remove organic matter and facilitate taxonomic identification. These samples were centrifuged (MSE Centaur 2) at 3000 rpm for 15 minutes following a hydrogen peroxide contact time of 1 hour. The supernatants were removed and one drop of the pellet was placed onto a slide before addition of 1 drop of Naphrax. A cover slip was placed onto the sample and the Naphrax was then cured by placing the slide under an ultraviolet lamp (Cox 1975b). The algal cells observable in a 500 µm area were counted. The size of the cells affected the relative abundance of the species. For example, a small species in large numbers may constitute a smaller proportion of the biofilm than a larger species with a lower abundance. The relative abundance was estimated as the percentage cover of the optical area, in much the same way as using a standard quadrat in the field.

Light microscope images did not show the external structure of the polysaccharide tubes accurately and therefore an environmental scanning electron microscope (ENVSEM, Veeco FEI (Philips) XL30) was used to investigate the polysaccharide tube structure. The microscope was set at 6.9 Torr, and the temperature was maintained at 15°C by a cooling stage (Linkham, (Guildford) P60 Peletier stage)). Water vapour was used to saturate the sample chamber at 100% relative humidity. This allowed the sample to remain wet and ensured that drying shrinkage of the tubes was minimised (Collins *et al*. 1993). The diatoms and their tubes were

identified using The Atlas of British Diatoms (Hartley *et al*. 1996) and based on observational data provided by Cox (1977). See appendix for the names of the individuals responsible for describing these species.

Biofilm observations

Biofilm 'quality' observations, consisting of the level of biofilm development, from the baseline summer total die-off stage, were made and recorded to aid in the analysis of the fluorescence data. The biofilms were defined as 'excellent' if the algal cover was dense, the colour was dark and there was no grazing damage. The biofilm was defined as 'good' if it was slightly patchy or if the colour had degraded as is seen in the spring months. The biofilm was defined as 'poor' if it was degraded, with more rock surface visible than biofilm.

Assessment of biofilm grazer community and activity

The biofilm herbivores were identified within a 1 m^2 quadrat at each site and on each sample date (Dethier *et al*. 1993). Abundance and community composition was recorded *insitu*, at the species level using the Collins rocky shore guide (Hayward & Nelson-Smith 2001). The herbivores were not removed from the sample sites to minimise the impact of these experiments on the ecosystem and therefore species level identification of limpets was not possible. *Patella vulgata* was identified but other species were termed *Patella* spp. Radula scrapes were counted at each of the sample sites and on each sample date within 1 m^2 quadrats (Forrest *et al*. 2001). The quadrat was placed on the rock surface and the number of radula scrapes in the biofilm was counted and recorded. Radula scrapes are made up of several small scrapes (Figure 2.2.1) these smaller scrapes make up larger individual scrapes. These larger scrapes were counted and this method was used to estimate grazing effort (per m^2).

Light measurements

Light measurements were made during sampling every minute, using a Water PAM light meter (2 πcosine corrected) and these data were used to calculate the light dose from the product of light measurement and the duration of exposure time.

Weather observations

General observations including estimated cloud cover, rainfall and wind strengths were made. These observations were recorded in a field note book. The temperature was recorded using an infrared thermometer (CEM, DT-8818H).

Statistical analysis

Levene's test was used to test for equal variance and the Anderson Darling test was used to test for normality. When the data were normal and variances equal, a nested 3 factor ANOVA was used with temperature/light nested within shore level nested within year. This resulted in 7 temperatures/light levels nested within 2 shore levels nested within 3 years.

A principal component analysis (correlation) was used to observe the relationship between different environmental factors, species and photophysiological responses. The correlation between related eigenfactors was calculated using Minitab 15. The correlation between the percentage light dose, temperature, $rETR_{max}$ and $\Delta F/Fm'$ was calculated using the Spearman correlation function of Sigmaplot 12.0.

Simpson's diversity index and evenness were calculated to understand the changes in community structure over the measurement years.

RESULTS

Observations on biofilm structure of the upper and lower shores

The upper shore of Dunraven Bay was characterised by dense tube-forming diatom biofilms from November to April, with the most common species being *Navicula bottnica* and *Parlibellus delognei* (Fig. 2.3.3). The diatom cells were observed to be living within polysaccharide tubes (Figs. 2.3.1, 2.3.2 and 2.3.3). In contrast, the lower shore biofilms were dominated by free-living species (Figure 2.3.4), the cells living within a thin (6mm) layer of sediment in the depressions on the rock surface (Fig. 2.3.1). The most common species in the lower shore community was *Navicula ramosissima.*

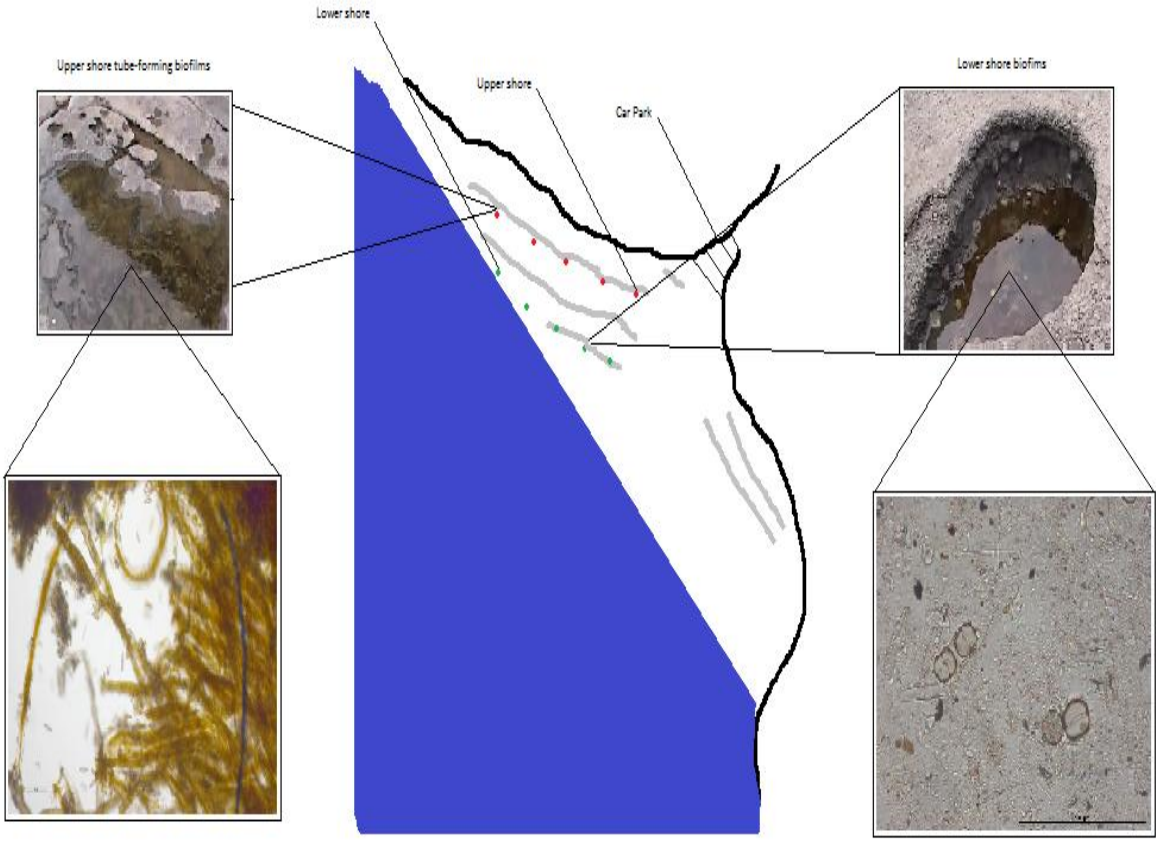


Figure 2.3.1: *Dunraven Bay and its constituent microphytobenthic biofilm.* Representative drawing (Naomi Ginnever, Microsoft Paint) with research sites marked in red (upper shore) and green (lower shore), with photographs of the biofilms and light microscope images illustrating the different biofilm forms.

Figure 2.3.2 (A) shows a thin diatom tube from the upper shore which contained a single file row of diatoms less than 2 µm in width and 10 µm in length. The tubes contained multiple layers of cells, some larger species, and contained polysaccharide interior partitions. Figure 2.3.2 (B) shows a 'cleaned' cyanobacteria tube. The cyanobacteria species *Moorea producens* (formerly *Lyngbya majuscula)* was often observed with epiphytic diatom growth (Fig. 2.3.2, C). *Achnanthes* spp. were common epiphytic species which attached with a polysaccharide stalk to a polysaccharide tube (Fig.2.3.2, D). *Navicula* spp. was recorded in several life modes. These are illustrated in Figure 2.3.2 with free-living cells (Fig. 2.3.2, F) and tube-forming cells being observed at the same location and time on the shore (Fig. 2.3.2, E and G). Evidence of extensive nematode grazing was also observed (Fig. 2.3.2 H). *Parlibellus delognei* was frequently observed in single file within tubes surrounded by a *Nitzschia* spp (Fig. 2.3.3 E and F). *Melosira moniliformis* (Fig. 2.3.3, G) was found at the upper and lower shore sites. This species forms chains which are connected to surfaces and each other by polysaccharide pads. Free-living species were also present, especially in winter months including *Grammatophora marina* (Fig. 2.3.3, H).

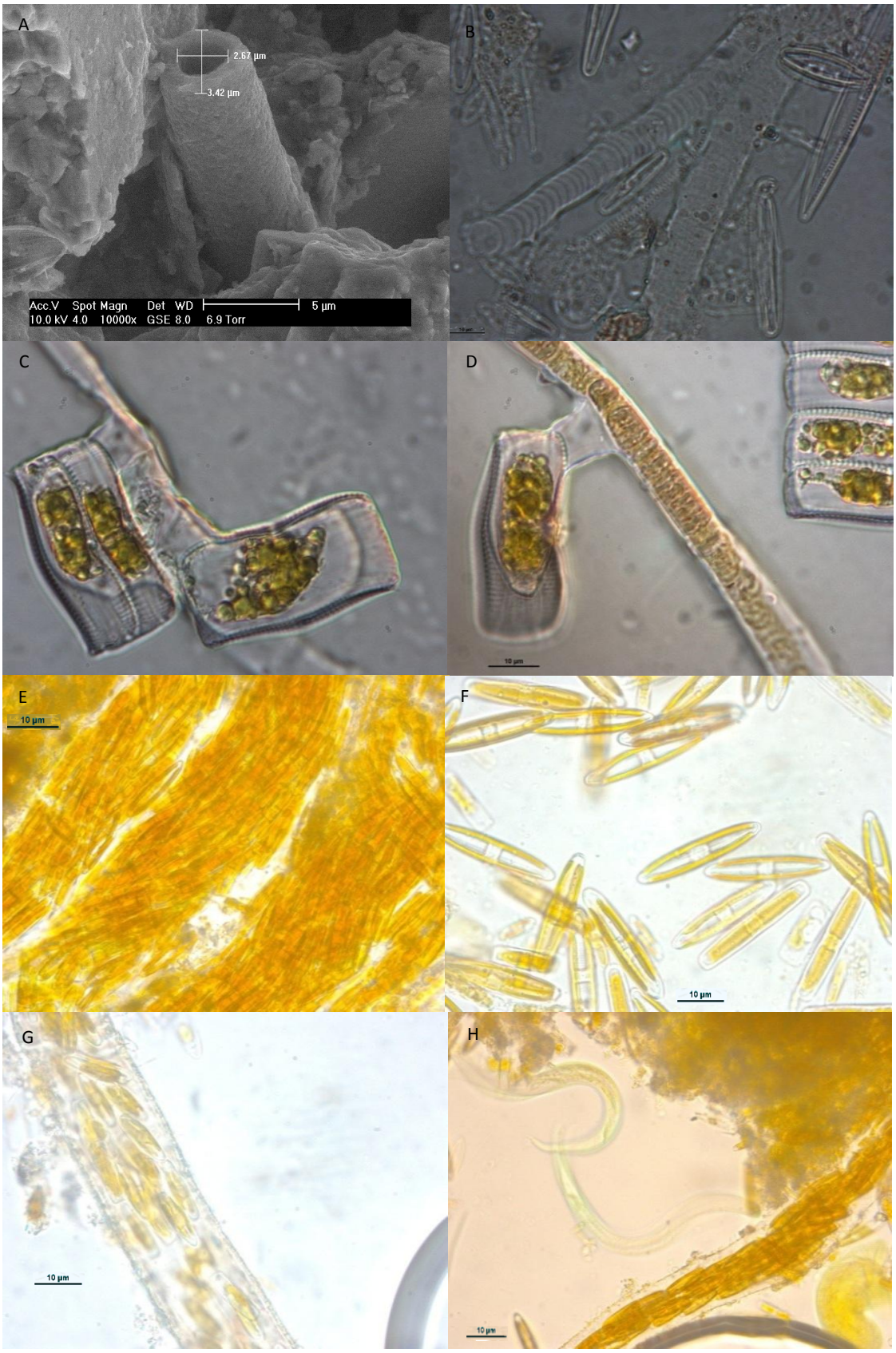


Figure 2.3.2: *Images of summer upper shore microalgal species, with different life-styles.* A) Environmental scanning electron micrograph, of a polysaccharide diatom tube (March). B) Light microscope image, of a cyanobacteria tube (July). C) *Moorea producens* with epiphytic *Achnanthes brevipes* (July), D) *Achnanthes brevipes* with polysaccharide attachment (July). E) *Navicula bottnica* in a tightly packed tube (April). F) *Navicula bottnica* in a free-living form (April). G) *Navicula bottnica* in a loosely packed tube (April). H) *Navicula bottnica* in a tightly packed tube next to a nematode which had grazed on *Navicula bottnica* cells (April).

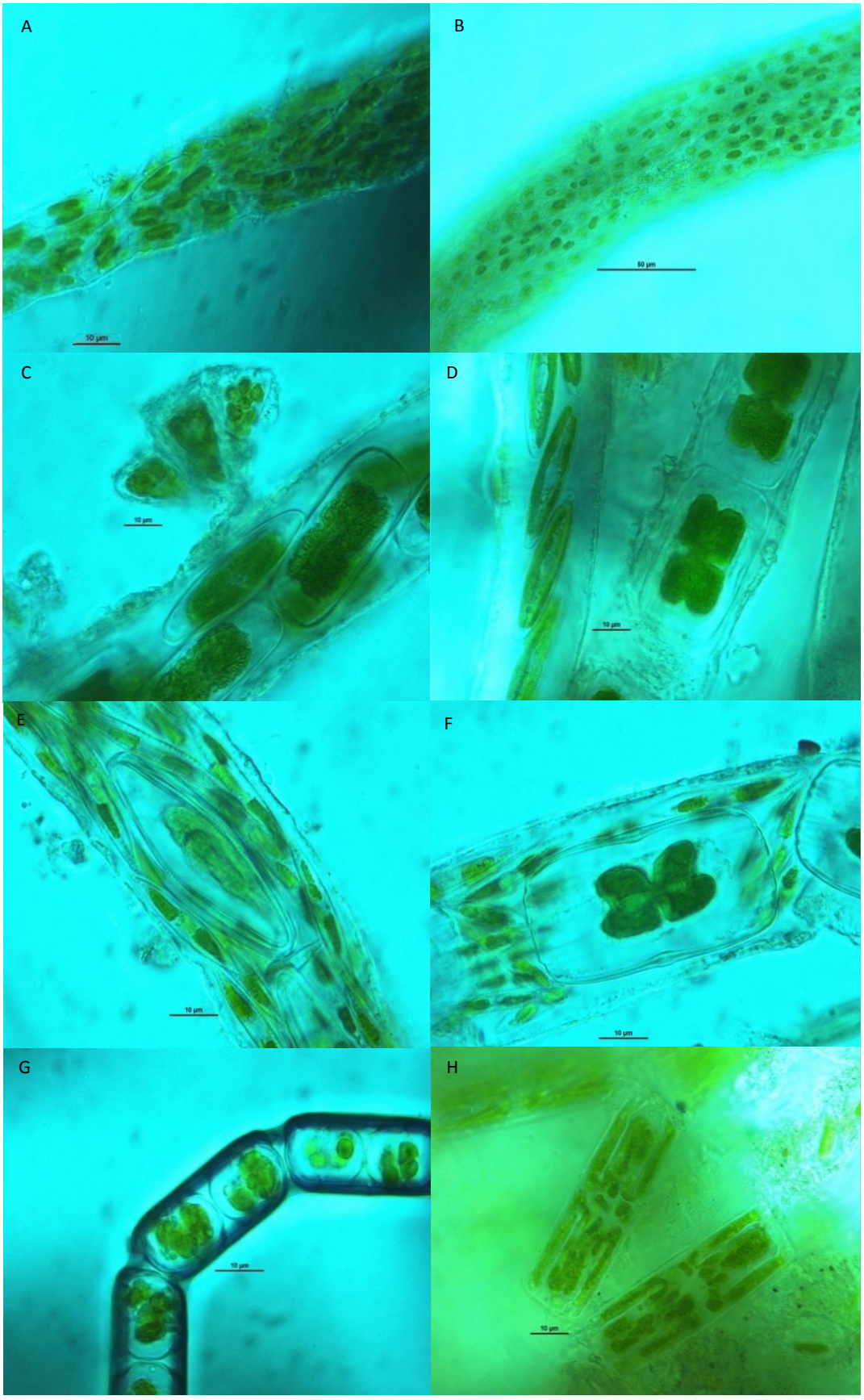


Figure 2.3.3: *Images of winter upper shore microalgal species, with different life-styles.* A) *Navicula ramosissima* and B) *Berkeleya rutilans* in a polysaccharide tube which was tightly packed with cells which lie parallel to one another. C) *Parlibellus delognei* in a slightly overlapping single file from. With attached epiphytic *Licmophora* spp D) Two thin tubes containing *Navicula bottnica* and *Parlibellus delognei* in a single file form. E) *Parlibellus delognei* in association with a Nitzschia species, from the valve view. F) Girdle view. G) *Melosira moniliformis*. H) *Grammatophora marina*.

The lower shore was dominated by free-living diatom species throughout the year (Fig 2.3.4 A and B). The habitat was characterised by a 6 mm layer of sediment within which the free-living cells were present. It was frequently necessary to use cleaned samples to facilitate identification of species which could not be identified using a key (Fig. 2.3.4, C and D). A range of cell dimensions were also common across these species including very large cells (e.g. *Nitzschia linearis,* Fig. 2.3.4 D).

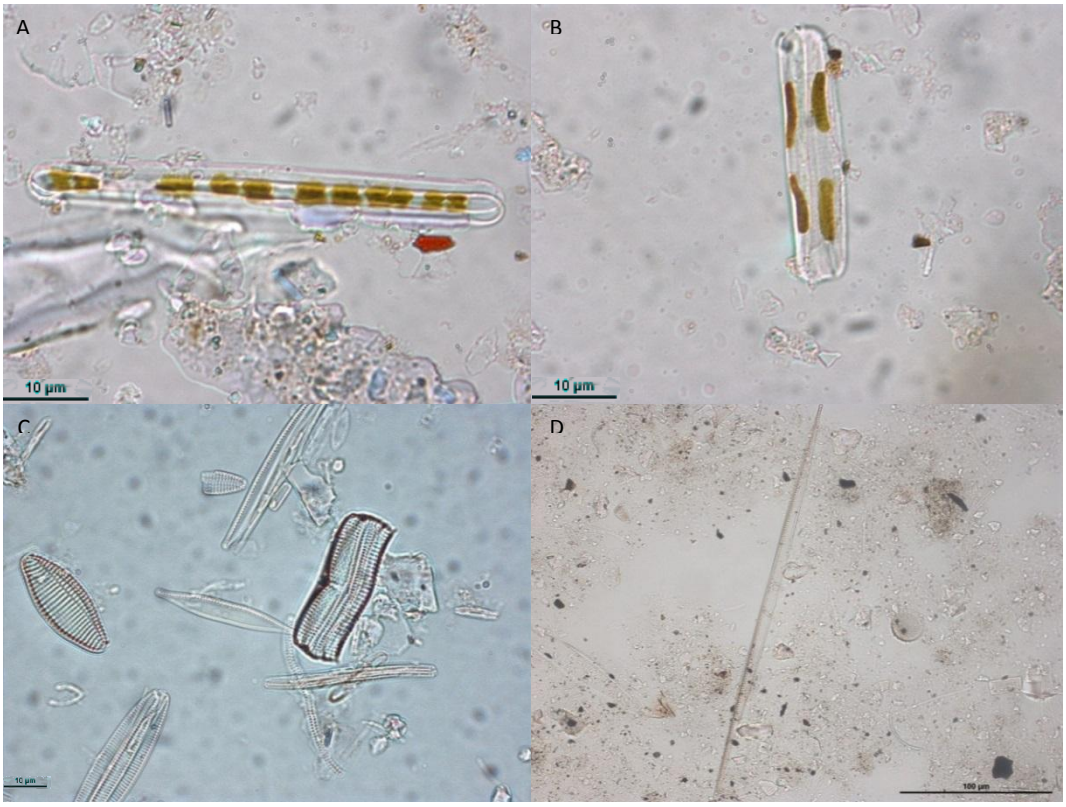


Figure 2.3.4: *Images of lower shore microalgal species*, A and B display live specimens with intact chloroplasts. C and D display samples cleaned with hydrogen peroxide.

Biofilm community analysis

Rocky shores can be termed complex assemblages (Underwood & Chapman 1998). As such the taxonomy of these ecosystems can be difficult to understand and changes that may be occurring can be difficult to see. Monthly cell counts and taxonomic identification indicated when each of the species found at the Dunraven Bay site appeared and disappeared. The species were then separated into two distinct biofilms communities based on these data. The percentage of the total biofilm composed of these communities, displayed below, illustrates the taxonomic changes which occurred during the studied years. The percentage biofilm composition of the two communities changed during the year and there was a distinct change from winter to spring. Figure 2.3.5 A, B and C display the shift in the biofilm composition between biofilm community 1 and biofilm community 2 on the upper (blue) and lower shore (red). The shift was most apparent at the upper shore sites, with total dominance of the winter community observed in 2009.

A)

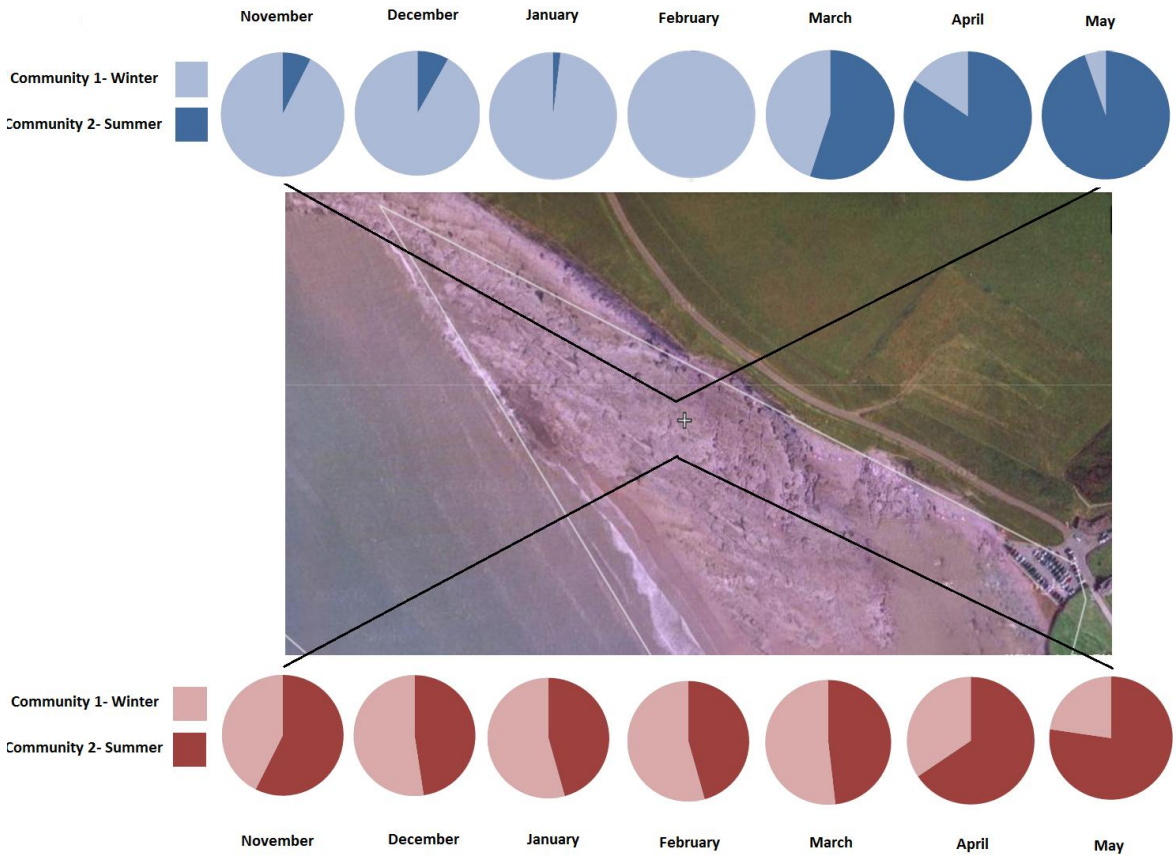


Figure 2.3.5: (A), *The proportion of the biofilm made up of these two communities through the measurement months in 2009. n = 5*

B)

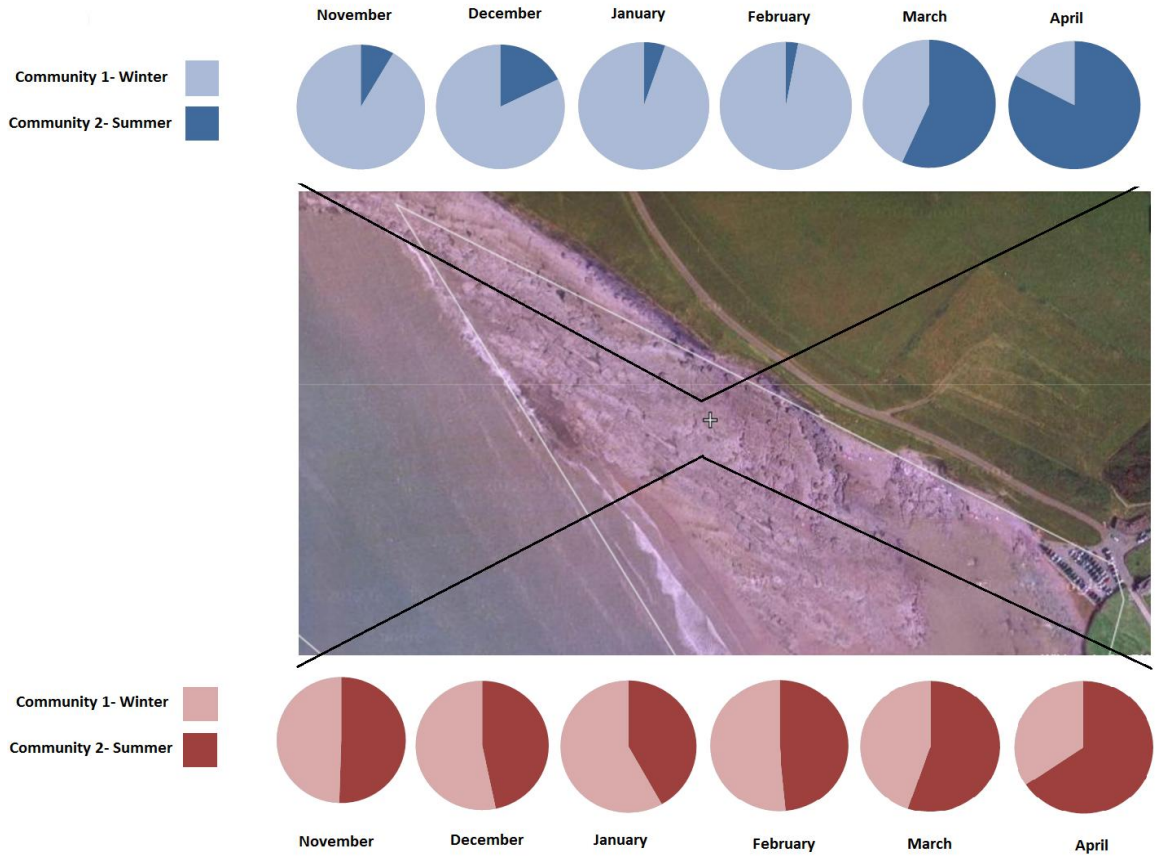


Figure 2.3.5: (B), *The proportion of the biofilm made up of these two communities through the measurement months in 2010. n = 5*

C)

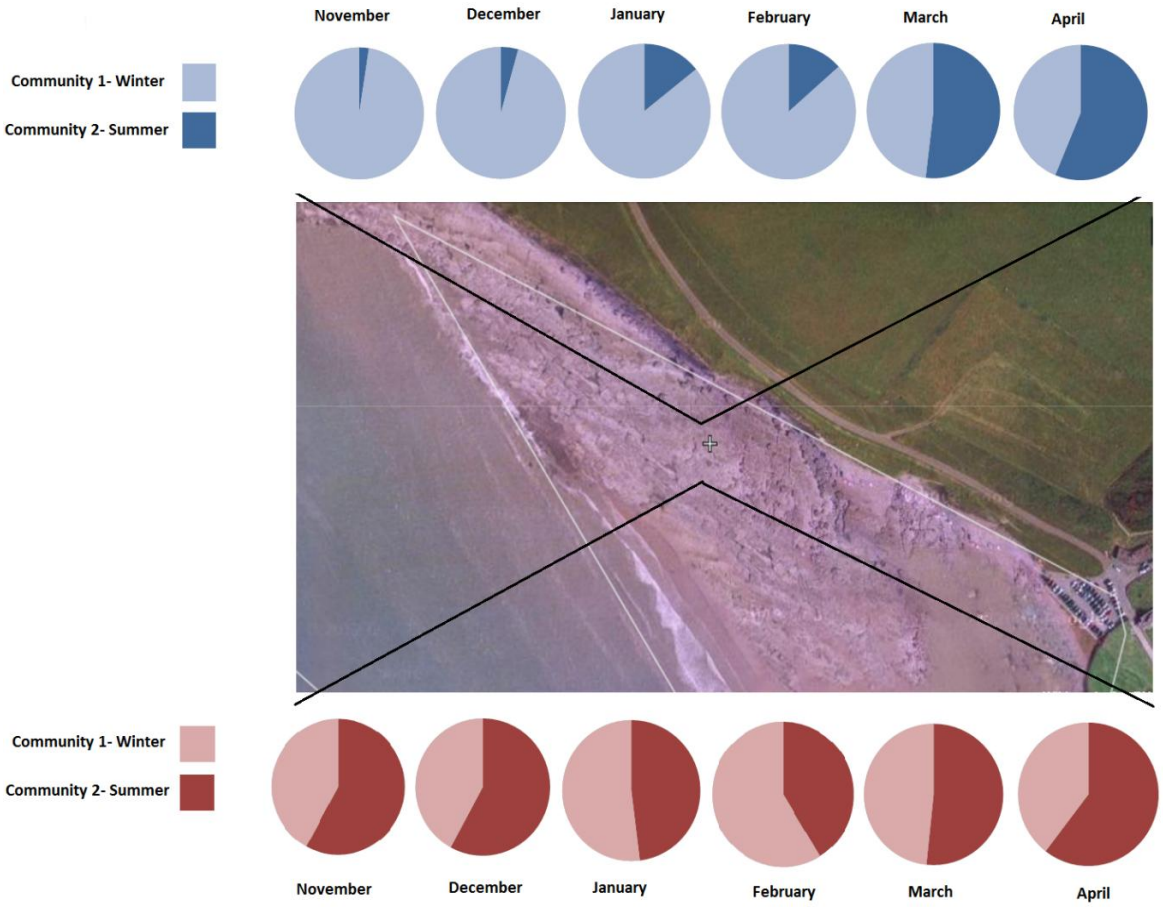


Figure 2.3.5: (C), *The proportion of the biofilm made up of these two communities through the measurement months in 2011. n = 5*

Light dose records

The total light dose was measured during the day up to the measurement time (Fig. 2.3.6). The light levels recorded were only those for the measurement day and did not represent the average light levels experienced by the cells for the preceding days. The light dose was highest in April in the 2009, 2010 and 2011 measurement years. The winter months exhibited the lowest light dose (Fig. 2.3.6). There was a significant moderate positive correlation ($r = 0.54499$, $P < 0.05$, $n = 7$) between the light dose and temperature.

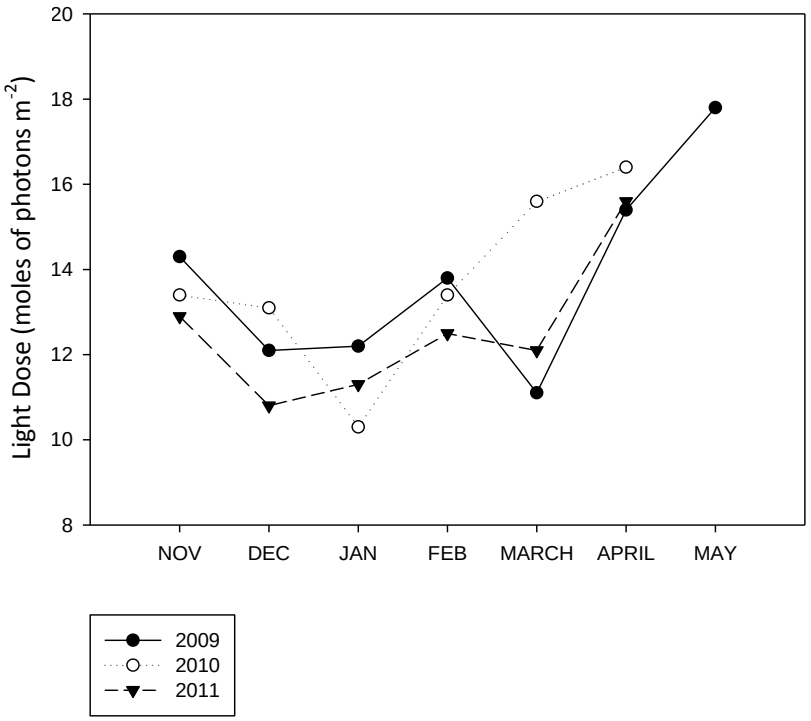


Figure 2.3.6: *Total light dose,* recorded monthly in 2009, 2010 and 2011.

Biofilm condition and weather records

In November the biofilms were still developing and growing (Table 2.3.1). By December the biofilms were fully developed and remained so until grazing began in March and damage was observed. The biofilm was in poor condition by April, with extensive grazing damage and tube bleaching observed.

Table 2.3.1: *Weather and biofilm observations,* on measurement days.

Month and Year	Weather Description	Temp °C	Biofilm condition observations	Additional Information
November 2008	Light Rain, patchy cloud cover	11°C	Good condition- not fully Developed	
December 2008	Light Rain, Total cloud cover	8°C	Excellent condition- fully Developed	
January 2009	High Winds, very patchy cloud cover	4°C	Excellent condition	
February 2009	Sunny day with clear skies and light winds	8°C	Excellent condition	
March 2009	No rain but very clouds, thunder heard	4°C	Good condition-some grazing damage	
April 2009	Sunny and Warm, light wind	14°C	Good condition in parts- heavy grazing damage	BMX bikers practicing on biofilm, damaged upper shore sites
May 2009	Very warm and full sun	18°C	Poor condition- heavy grazing damage and some bleaching	BBC doctor who film crews damaged upper shore biofilms
November 2009	Strong wind, Patchy cloud cover	11°C	Good condition-fully developed	
December 2009	Very cold, light cloud cover	5°C	Excellent condition	
January 2010	Snow, Pools were not frozen, total cloud cover	-1°C	Excellent condition	
February 2010	Snow, Pools were not frozen, clear sky	2°C	Good condition- slight degradation	
March 2010	Cool and full sun	7°C	Good condition slight grazing Damage	
April 2010	Very warm and full sun	18°C	Poor condition- grazing damage and some bleaching	
November 2011	Snow, Pools were not frozen	-4°C	Good condition- not fully Developed	Snow covering the all rocks, which were not covered by water
December 2011	Very cold, total cloud cover	-2°C	Excellent condition- fully Developed	
January 2011	Light rain, patchy cloud cover	6°C	Excellent condition	
February 2011	Windy with patchy cloud cover	9°C	Excellent condition	
March 2011	Warm with patchy cloud cover	17°C	Excellent condition	
April 2011	Very warm with full sun	22°C	Good condition- some grazing damage and some bleaching	Beachgoers sitting around biofilm and walking through the pools

Total cell counts

The shore level had a significant effect on the shore level with the total number of cells on the upper rocky shore being significantly higher than the lower shore ($F = 3.62$, $df = 6, 184$, $P < 0.05$) (Figs. 2.3.7 and 2.3.8). The biofilms responded in the same way to light dose as to temperature, and therefore only the changes in cell counts with relation to temperature have been reported here. There was an observable pattern in the cell numbers between the shore levels, with similar responses to temperature observed. There was a significant effect of temperature on the cell counts ($F = 6.61$, $df = 6, 184$, $P < 0.05$). In 2009 the greatest cell counts were recorded at the coolest temperatures (Figs. 2.3.7 and 2.3.8 A), this pattern was observable but less apparent in 2010 (Figs. 2.3.7 and 2.3.8 B). In 2011 the opposite response was recorded with higher cell counts at higher temperatures (Fig. 2.3.7 C). The measurement year had no significant effect on the cell number recorded.

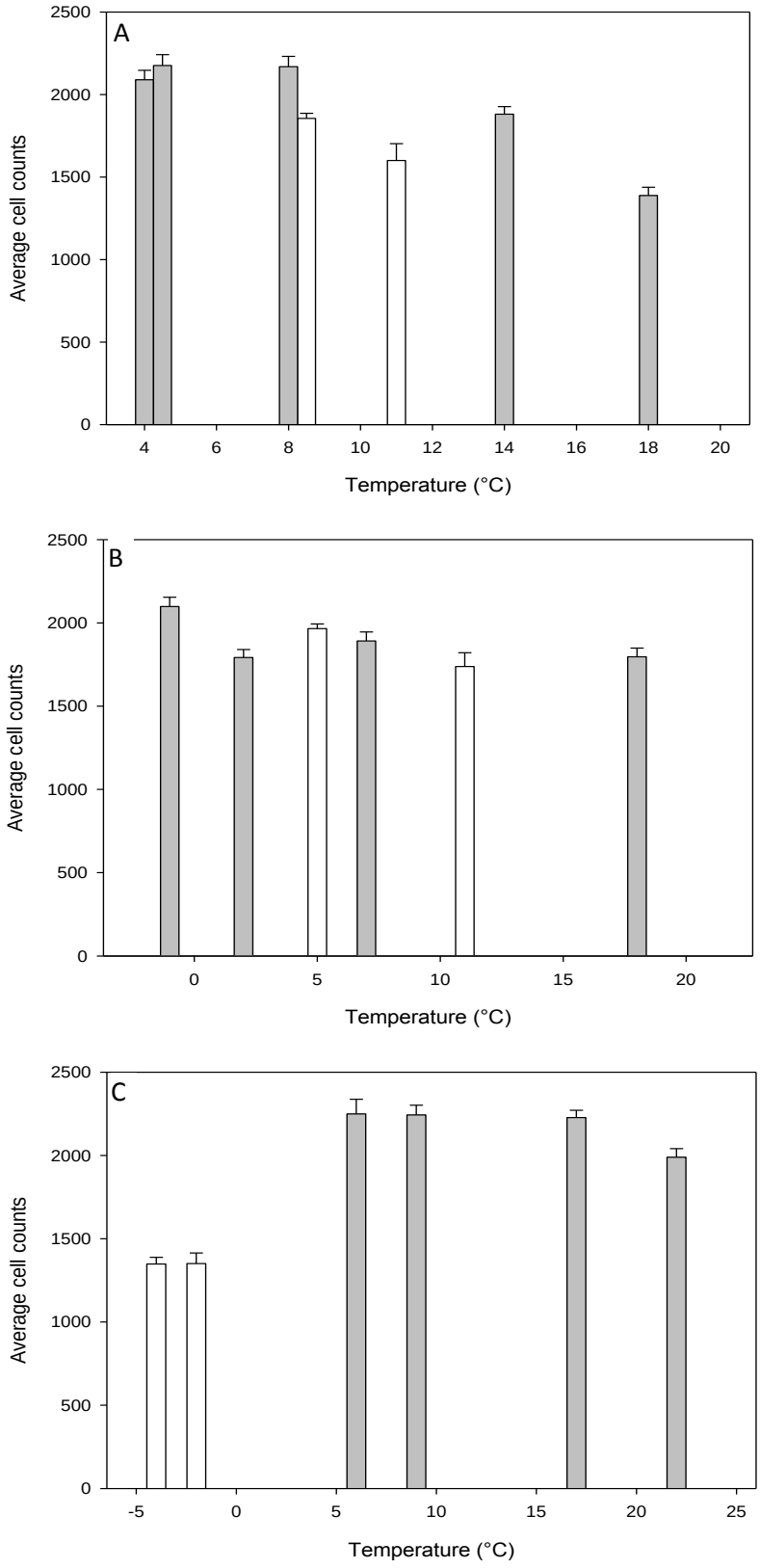


Figure 2.3.7: *Average upper shore cell counts*, of all species from a 500 µm^2 area of a light microscope slide, recorded over the three measurement years (A- 2009, B- 2010, C- 2011) versus measurement day temperature. These include cell counts from inside tubes. Mean + s.e. *n=5*

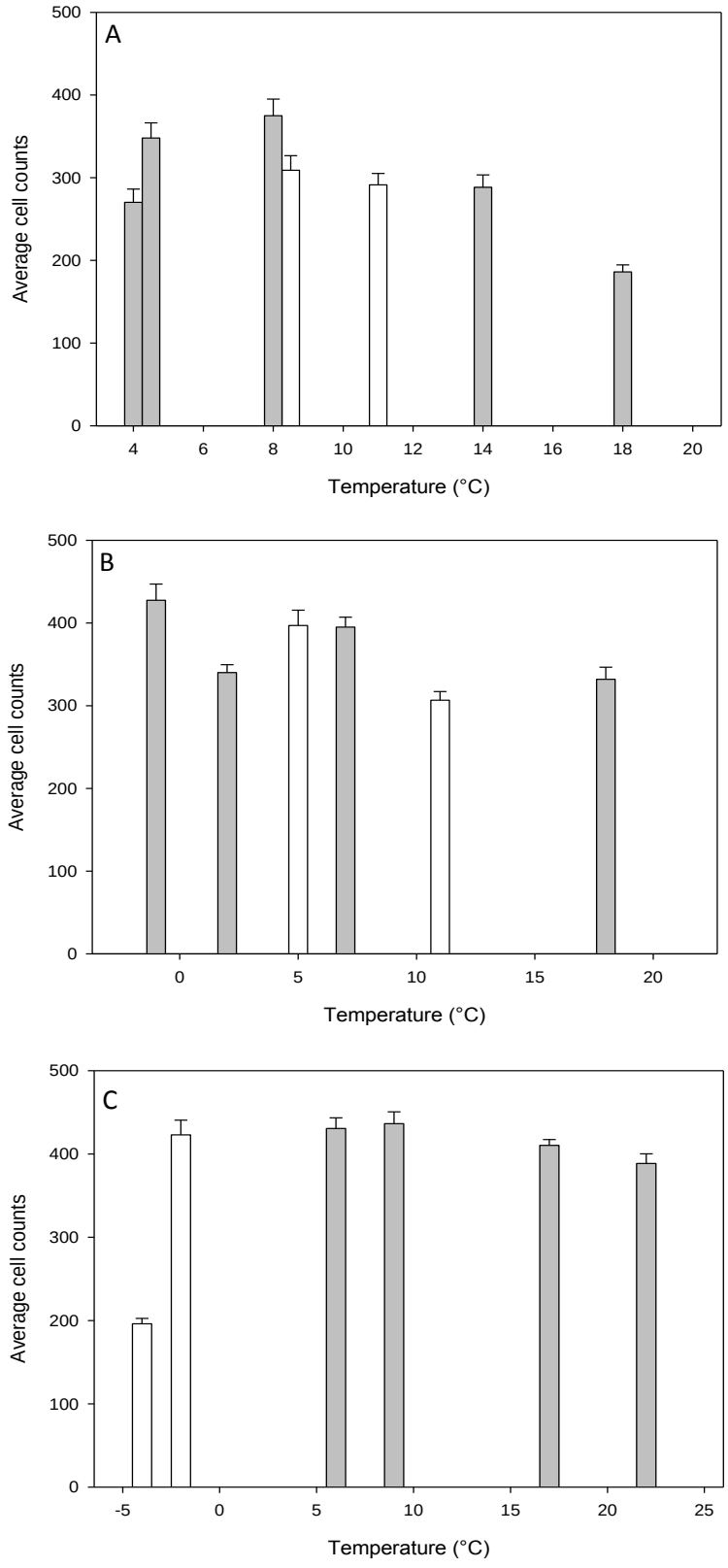


Figure 2.3.8: *Average lower shore cell counts*, of all species from a 500 µm^2 area of a light microscope slide, recorded over the three measurement years (A- 2009, B- 2010, C- 2011) versus measurement day temperature. These include cell counts from inside tubes. Mean + s.e. *n=5*

Variation in diversity and evenness indices

Table 2.3.2 summarises the calculated Simpson's diversity index and evenness of the upper shore sites. The highest species diversity was frequently observed in March and April. The lowest diversity was most frequently recorded in January and February. In contrast species evenness was lowest in March and April and highest in January and February.

Table 2.3.3 contains the calculated Simpson's diversity index and evenness of the lower shore sites. The highest diversity was most frequently recorded in the January. The lowest diversity was most frequently recorded in March. The species evenness of the lower shore sites changed less over the measurement months than the upper shore sites. The evenness was the same throughout the 2010 and 2011 measurement periods. In 2009 the highest evenness was recorded in February and March.

Table 2.3.2: *Simpson's diversity index and evenness,* of algal species, over the measurement months and years at the upper shore

	2009	**2009**	**2010**	**2010**	**2011**	**2011**
	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness
November	0.66	0.07	0.65	0.07	0.65	0.07
December	0.60	0.08	0.60	0.08	0.62	0.07
January	0.57	0.08	0.61	0.07	0.51	0.08
February	0.60	0.08	0.62	0.07	0.58	0.08
March	0.72	0.06	0.70	0.06	0.67	0.07
April	0.69	0.07	0.71	0.06	0.72	0.06
May	0.60	0.08				

Table 2.3.3: *Simpson's diversity index and evenness,* of algal species, over the measurement months and years at the lower shore

	2009	**2009**	**2010**	**2010**	**2011**	**2011**
	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness
November	0.71	0.06	0.77	0.06	0.75	0.06
December	0.81	0.06	0.79	0.06	0.76	0.06
January	0.83	0.06	0.76	0.06	0.81	0.06
February	0.66	0.07	0.76	0.06	0.82	0.06
March	0.67	0.07	0.73	0.06	0.70	0.06
April	0.83	0.05	0.71	0.06	0.78	0.06
May	0.77	0.06				

Variation in relative community composition

Upper shore

The species percentage cover of the microscope slide was not directly comparable to the cell counts as certain species are larger and therefore represent a larger percentage of the slide (Fig. 2.3.9). The cells present on the upper shore were *Achnanthes brevipes*, *Amphora* spp, *Licmophora ehrenbergii* and *Licmophora flabellata* which are epiphytic (cells which grow on the surface of other algae) diatoms. *Berkeleya rutilans, Grammatophora marina, Melosira moniliformis, Navicula bottnica, Navicula ramosissima* and *Parlibellus delognei* are tube forming species and were found at upper shore sites. Free-living cells were observed but they were either the same species as were found in the tubes or planktonic species.

In 2009 the community was dominated by five species *Parlibellus delognei, Navicula ramosissima, Berkeleya rutilans, Melosira moniliformis* and *Navicula bottnica,* the latter being a seasonal diatom species (Fig. 2.3.9 A). *Navicula bottnica* cells appeared in March and the biomass of this species increased in April to be the dominant diatom on the shore. However this dominance was short-lived and the biomass reduced in May. The cells were still present in November and December but did not survive in January and February. During the winter months the most dominant species were *Parlibellus delognei* and *Berkeleya rutilans. Parlibellus delognei* did not remain on the rocky shore after March. The biomass of *Navicula ramosissima* remained constant except in April, where there was a reduction in the percentage cover it represented. There were low levels of *Melosira moniliformis* during all years, though the levels were higher in the winter months. There was an increase in the number of epiphytic species in the spring and early summer months.

In 2010 and 2011 (Figs. 2.3.9 B and C) the species on the upper shore slides were similar to those recorded in 2009 (Fig. 2.3.9 A). In 2010 and 2011 the *Parlibellus delognei* biomass did not completely disappear in the spring. However, the levels did reduce from the winter months

into the spring. In 2010 and 2011 the *Navicula bottnica* did not completely disappear in the winter months. There was a large increase in the *Navicula bottnica* biomass from March. The *Navicula ramosissima* biomass again remains stable during the year with a reduction in biomass observed in 2011 in April. There was a lower level of *Melosira moniliformis* in 2010 and 2011 than in 2009. In 2010 and 2011 there was again an increase in the biomass of epiphytic species recorded in spring.

The community structure of the biofilm changed seasonally, with larger species (> 40 µm valve length and > 25 µm diameter) such as *Parlibellus delognei* being dominant during the winter months (December, January and February) and smaller ones (<40 µm valve length and < 25 µm diameter) such as *Navicula bottnica* during the spring months (March, April and May).

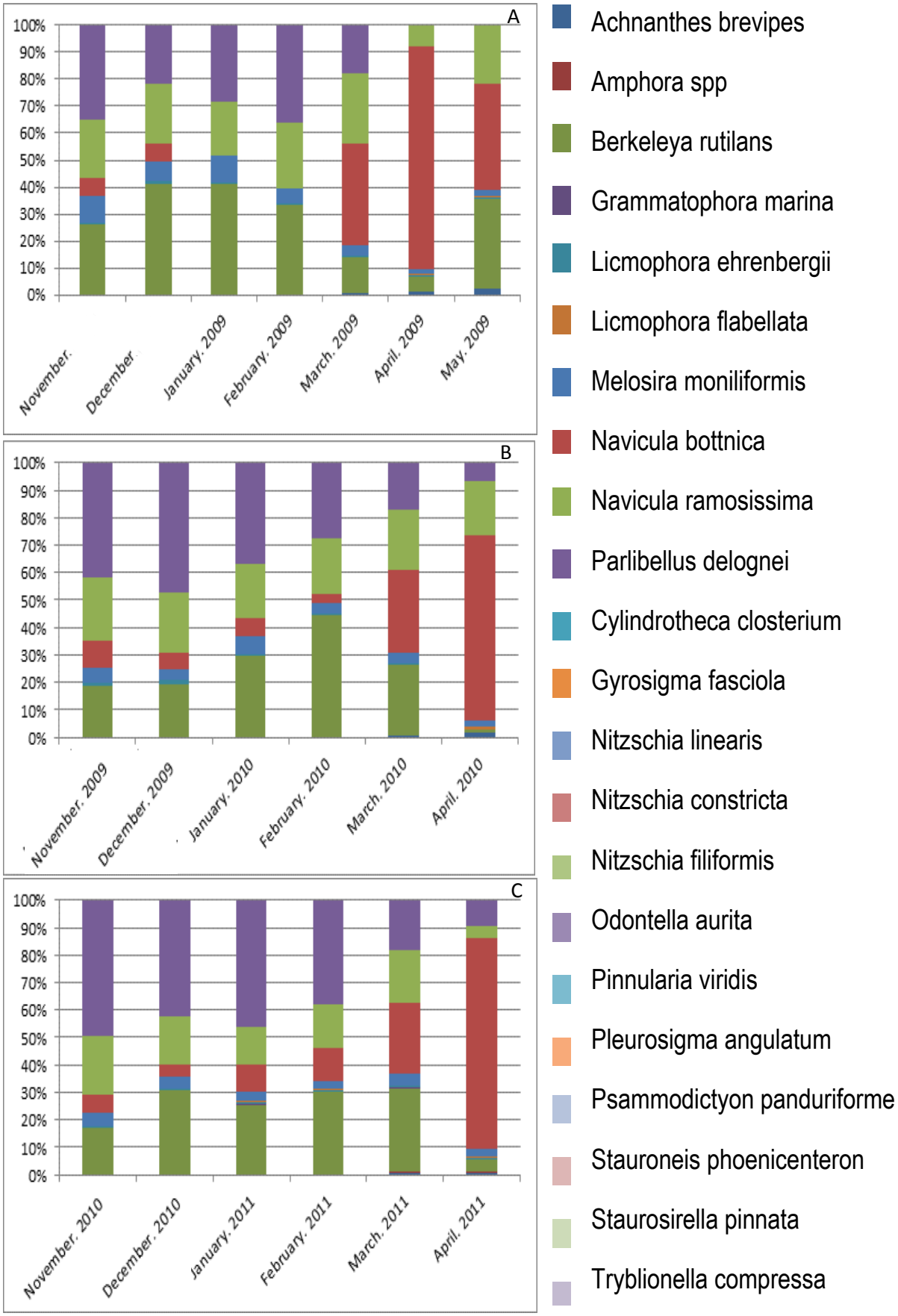


Figure 2.3.9: *Percentage species composition of biofilm,* A) Upper shore at each monthly measurement during the 2009 measurement year. B) Upper shore at each monthly measurement during the 2010 measurement year. C) Upper shore at each monthly measurement during the 2011 measurement year. *N=5*

Variation in relative community composition

Lower shore

In the lower shore sites the relative abundance of *Navicula ramosissima* was dominant and stable throughout the sample period, with the exception of a slight reduction in spring 2011 (Fig. 2.3.10 C) leading to an increase in the relative abundance of rare species including; *Cylindrotheca closterium, Gyrosigma fasciola, Nitzschia linearis, Nitzschia constricta, Nitzschia filiformis, Odontella aurita, Pinnularia viridis, Pleaurosigma angulatum, Psammodictyon panduriforme, Stauroneis phoenicenteron, Staurosirella pinnata* and *Tryblionella compressa. Cylindrotheca closterium* increased from November to February and then decreased from February to May in all years (Figs. 2.3.10 A, B and C) *Odontella aurita* decreased from November to March and increased in April. *Nitzschia linearis* was present in all months but it was present in larger quantities (30 per 500um^2) in November and December. The relative abundance of *Stauroneis phoenicenteron* was stable over the measurement months.

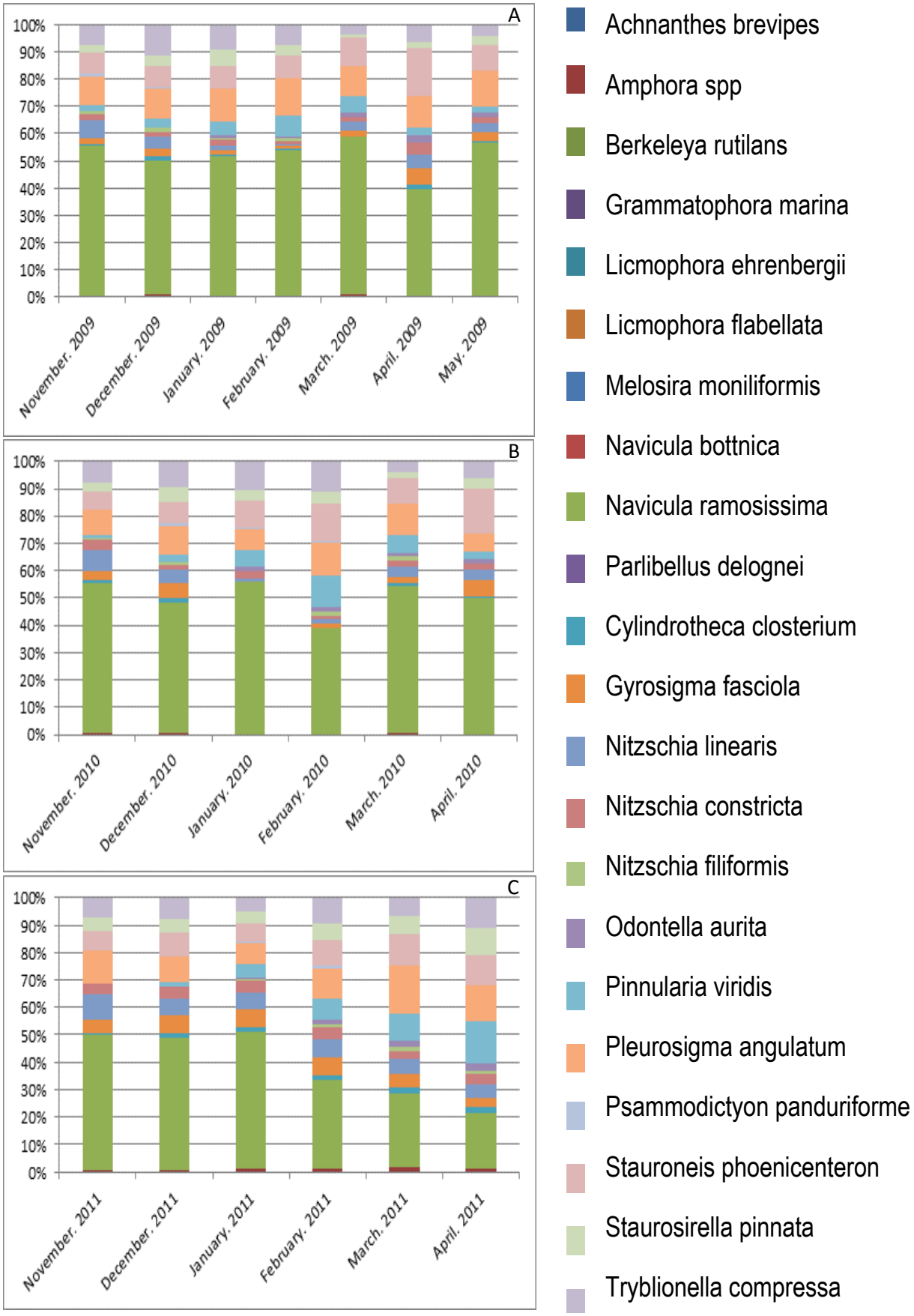


Figure 2.3.10: A) *Percentage species composition of biofilm*, A) Lower shore at each monthly measurement during the 2009 measurement year. B) Lower shore at each monthly measurement during the 2010 measurement year. C) Lower shore at each monthly measurement during the 2011 measurement year. *N=5*

Algal cover with relation to light dose and *Patella vulgata* activity

The lowest percentage cover of biofilm occurred in May of all years (Fig. 2.3.11, A). The highest levels of percentage algal cover occurred between December and February. In all years that were monitored the biofilms present in December, January and February had algal coverage of > 95% for the upper shore and > 85% for the lower shore samples. There was a significant negative correlation between the percentage algal cover and the light dose with the lowest percentage algal cover being recorded when the highest light dose was recorded (2009: $r = -0.72729$ ($P < 0.05$), $n = 14$, 2010: $r = -0.5502$ ($P < 0.05$), $n = 12$, 2011: $r = -0.66783$ ($P < 0.05$), $n = 12$).

The activity of *Patella vulgata* was significantly higher in the spring months from March onwards at the lower shore and April onwards at the upper shore in all years ($F = 7.82$, $df = 29$, $P < 0.05$) There was a negative correlation between the percentage algal cover and the number of radula scrapes (Figs. 2.3.11 A and B) observed on the rocky shore (Upper shore: 2009: $r = -0.93419$ ($P < 0.05$), $n = 7$, 2010: $r = -0.79947$ ($P < 0.05$), $n = 6$, 2011: $r = -0.5164$ ($P < 0.05$), $n = 6$, Lower shore: 2009: $r = -0.83281$ ($P < 0.05$), $n = 7$, 2010: $r = -0.76743$ ($P < 0.05$), $n = 6$, 2011: $r = -0.6224$ ($P < 0.05$), $n = 6$). There was a significant positive correlation between the number of radula scrapes and the light dose (Figs 2.3.11 B, C and D) recorded in 2009 and 2010 (Upper shore: 2009: $r = 0.6756$ ($P < 0.05$), $n = 7$, 2010: $r = 0.8331$ ($P < 0.05$), $n = 6$, Lower shore: 2009: $r = 0.7136$ ($P < 0.05$), $n = 7$, 2010: $r = 0.7328$ ($P < 0.05$), $n = 6$).

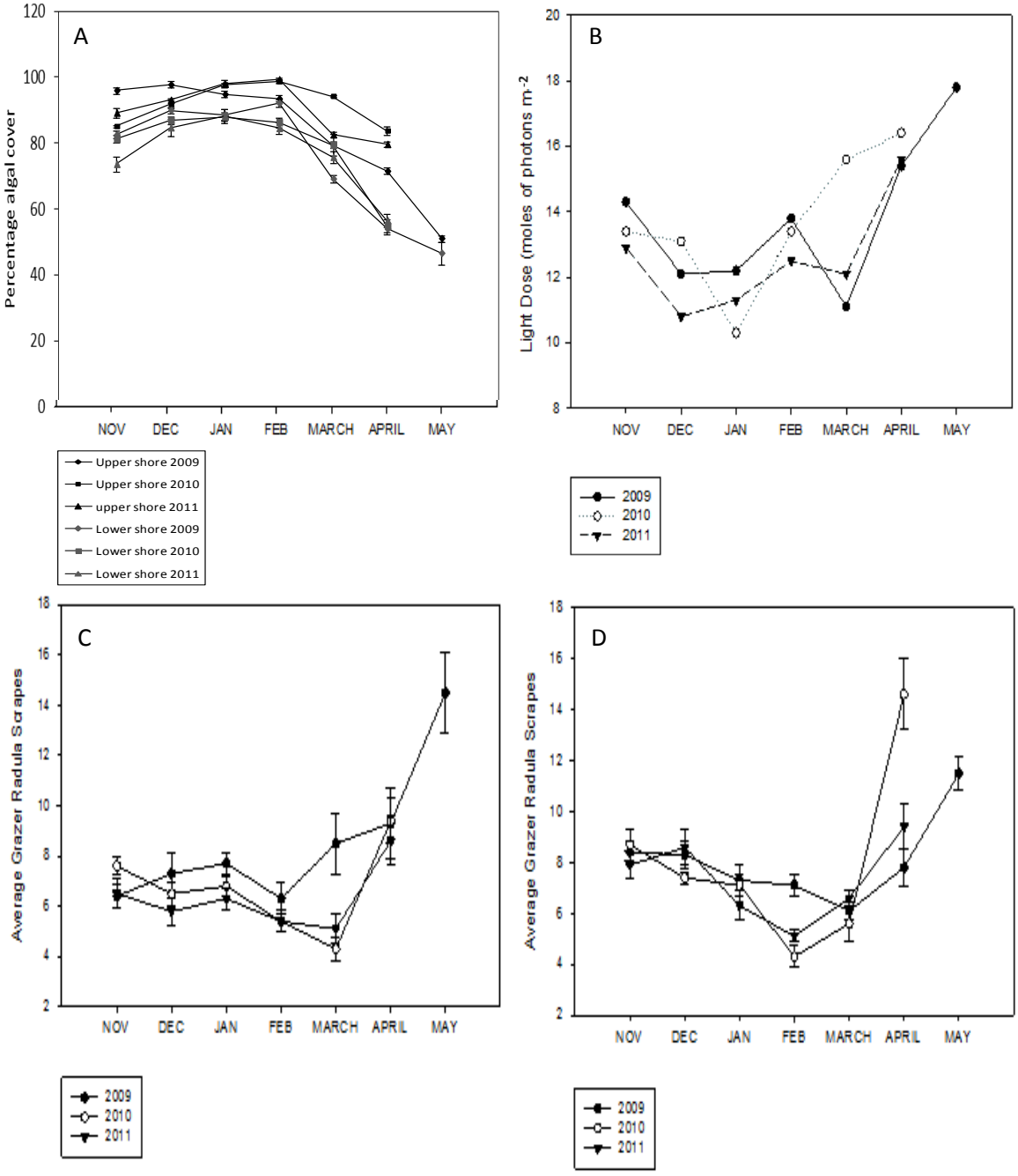


Figure 2.3.11: *Percentage algal cover, the light dose and limpet radula scrapes.* A) The percentage algal cover of the upper shore sites recorded in 2009, 2010 and 2011. B) Light dose (moles of photons m^{-2}) recorded over 2009, 2010 and 2011. C) Average number of radula scrapes recorded at the upper shore sites in 2009, 2010 and 2011, D) and lower shore sites in 2009, 2010 and 2011. mean± SE, *n=5* (C and D).

Herbivore community structure

Tables 2.3.4-2.3.9 contain the herbivore species abundances observed per m^2 on each sample date. *Melarhaphe neritoides* was the most abundant species on the upper shore although *Patella vulgata* was also present in relatively high abundance. *Littorina saxatalis* was the most abundant species on the lower shore although, again, *Patella vulgata* was present in relatively high abundances. There was no consistent pattern in the numbers of *Patella vulgata* on the lower shore.

Nematodes were observed in all sites in all months in both the upper and lower shores and were observed to graze diatoms (Figure 2.3.2, H). However, nematode abundances could not be quantified. *Melarhaphe neritoides* was present in relatively high abundance in the upper shore sites. *Melarhaphe neritoides* were the most numerous herbivores on the rocky shore. *Patella spp* were the only limpet species observed, with the exception of *Patella vulgata*.

Upper shore sites

Table 2.3.4: *Grazing species number records,* from the 2009 measurement season.

Species	Nov 08	Dec 08	Jan 09	Feb 09	Mar 09	Apr 09	May 09
Buccinum undatum	1	0	0	0	0	0	0
Gibbula umbilicalis	1	1	1	0	0	0	0
Littorina littorea	7	4	5	12	4	8	9
Littorina saxatalis	5	4	6	3	0	1	4
Melarhaphe neritoides	75	132	87	77	95	87	46
Nematode spp	P	P	P	P	P	P	P
Nucella lapillus	0	0	0	0	0	1	0
Osilinus lineata	1	1	0	0	0	0	4
Patella vulgata	9	13	7	8	15	7	8
Patella spp	2	1	1	0	3	2	0

Table 2.3.5*: Grazing species number records,* from the 2010 measurement season.

Species	Nov 09	Dec 09	Jan 10	Feb 10	Mar 10	Apr 10
Buccinum undatum	0	0	0	1	0	0
Gibbula umbilicalis	0	2	0	0	1	1
Littorina littorea	3	7	9	6	7	4
Littorina saxatalis	7	2	3	4	4	5
Melarhaphe neritoides	79	34	97	134	74	57
Nematode spp	P	P	P	P	P	P
Nucella lapillus	0	0	0	0	1	0
Osilinus lineata	0	0	2	0	0	1
Patella vulgata	14	5	6	3	5	8
Patella spp	4	2	1	0	4	2

Table 2.3.6: *Grazing species number records,* from the 2011 measurement season.

Species	Nov 10	Dec 10	Jan 11	Feb 11	Mar 11	Apr 11
Buccinum undatum	0	1	0	0	0	0
Gibbula umbilicalis	0	0	1	1	0	0
Littorina littorea	9	15	6	9	7	6
Littorina saxatalis	2	6	8	4	5	2
Melarhaphe neritoides	67	83	59	84	47	56
Nematode spp	P	P	P	P	P	P
Nucella lapillus	0	0	0	0	0	0
Osilinus lineata	2	0	0	0	1	0
Patella vulgata	6	9	3	6	11	6
Patella spp	0	1	2	6	2	1

Lower shore sites

Table 2.3.7*: Grazing species number records,* from the 2009 measurement season.

Species	Nov 08	Dec 08	Jan 09	Feb 09	Mar 09	Apr 09	May 09
Buccinum undatum	2	0	1	0	0	2	0
Gibbula umbilicalis	2	3	3	2	1	3	1
Littorina littorea	4	3	5	2	0	5	3
Littorina saxatalis	13	9	5	16	0	14	23
Melarhaphe neritoides	0	0	0	0	0	0	3
Nematode spp	P	P	P	P	P	P	P
Nucella lapillus	4	3	4	2	3	3	3
Osilinus lineata	4	1	1	5	0	0	0
Patella vulgata	14	3	7	9	11	14	13
Patella spp	4	3	0	0	0	2	3

Table 2.3.8*: Grazing species number records,* from the 2010 measurement season.

Species	Nov 09	Dec 09	Jan 10	Feb 10	Mar 10	Apr 10
Buccinum undatum	0	0	2	1	0	1
Gibbula umbilicalis	2	2	1	0	1	0
Littorina littorea	7	3	4	2	6	7
Littorina saxatalis	5	9	17	14	9	20
Melarhaphe neritoides	0	0	10	0	0	0
Nematode spp	P	P	P	P	P	P
Nucella lapillus	0	3	2	3	2	3
Osilinus lineata	2	1	5	2	0	0
Patella vulgata	11	8	13	9	7	19
Patella spp	3	6	1	0	2	0

Table 2.3.9*: Grazing species number records,* from the 2011 measurement season.

Species	Nov 10	Dec 10	Jan 11	Feb 11	Mar 11	Apr 11
Buccinum undatum	0	1	3	0	0	1
Gibbula umbilicalis	2	2	1	0	1	2
Littorina littorea	4	5	4	2	3	5
Littorina saxatalis	7	0	5	15	27	4
Melarhaphe neritoides	0	0	0	0	0	0
Nematode spp	P	P	P	P	P	P
Nucella lapillus	4	2	0	3	3	2
Osilinus lineata	1	4	0	0	2	3
Patella vulgata	16	15	13	8	9	11
Patella spp	0	2	1	0	4	1

Diversity and evenness of herbivore species

The diversity and evenness of the upper shore herbivore species was investigated using the Simpson's diversity index. Both the diversity and the evenness varied during the year with no discernible seasonal patterns observed over the months and years (Table 2.3.10).

At the lower shore sites there was again no discernible seasonal patterns in the species diversity and evenness. However, the lower shore sites had higher diversity of herbivores and lower evenness of herbivores than the upper shore sites (Table 2.3.11).

Table 2.3.10: *Simpson's diversity index and evenness,* of herbivore species, over the measurement months and years at the upper shore.

	2009	**2009**	**2010**	**2010**	**2011**	**2011**
	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness
November	0.44	0.25	0.44	0.26	0.38	0.29
December	0.28	0.40	0.55	0.20	0.46	0.24
January	0.33	0.33	0.32	0.35	0.43	0.26
February	0.39	0.29	0.18	0.62	0.41	0.27
March	0.33	0.34	0.40	0.28	0.56	0.20
April	0.32	0.35	0.45	0.24	0.37	0.30
May	0.55	0.20				

Table 2.3.11: *Simpsons diversity index and evenness,* of herbivore species, over the measurement months and years at the lower shore.

	2009	**2009**	**2010**	**2010**	**2011**	**2011**
	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness	Simpsons diversity index (1-D)	Evenness
November	0.82	0.14	0.79	0.14	0.73	0.15
December	0.83	0.13	0.83	0.13	0.73	0.15
January	0.85	0.13	0.81	0.14	0.72	0.15
February	0.73	0.15	0.72	0.16	0.64	0.17
March	0.45	0.25	0.79	0.14	0.66	0.17
April	0.78	0.14	0.69	0.16	0.81	0.14
May	0.67	0.17				

Microphytobenthic photophysiology

A 3 factor ANOVA was performed with temperature/light nested within shore level nested within year. The measurement year had a significant effect on all photosynthetic parameters investigated. For brevity this will not be stated again in this results section but will be considered in the discussion.

Relative electron transport rate ($rETR_{max}$)

The upper shore $rETR_{max,}$ plotted against temperature, illustrated that in general the highest $rETR_{max}$ was observed at temperatures in the middle of the recorded temperature range (Fig. 2.3.12). This was particularly apparent in 2009 and 2010 (Fig. 2.3.12 A and B). The open bars represent the November and December measurements when the biofilm was developing, and it was apparent that these months displayed high $rETR_{max}$. In 2011 high $rETR_{max}$ levels were recorded at cold temperatures, but these were again recorded in November and December (Fig. 2.3.12 C). Temperature had a significant positive effect on $rETR_{max}$ ($F= 6.67$, $df = 6, 184$, $P<0.05$).

Unlike the upper shore biofilms, the lower shore biofilms did not display an obvious pattern of response to temperature (Fig. 2.3.13). The shore level had a significant effect on the $rETR_{max}$ ($F= 8.97$, $df = 6, 184$, $P<0.05$). In 2009 there was a trend of increasing $rETR_{max}$ as temperature increases (Fig. 2.3.13 A). This is in contrast to 2010, which showed no trend in relation to temperature (Fig. 2.3.13 B). In 2011, like the upper shore biofilms, the highest $rETR_{max}$ was recorded at the lowest temperatures(Fig. 2.3.13 C).

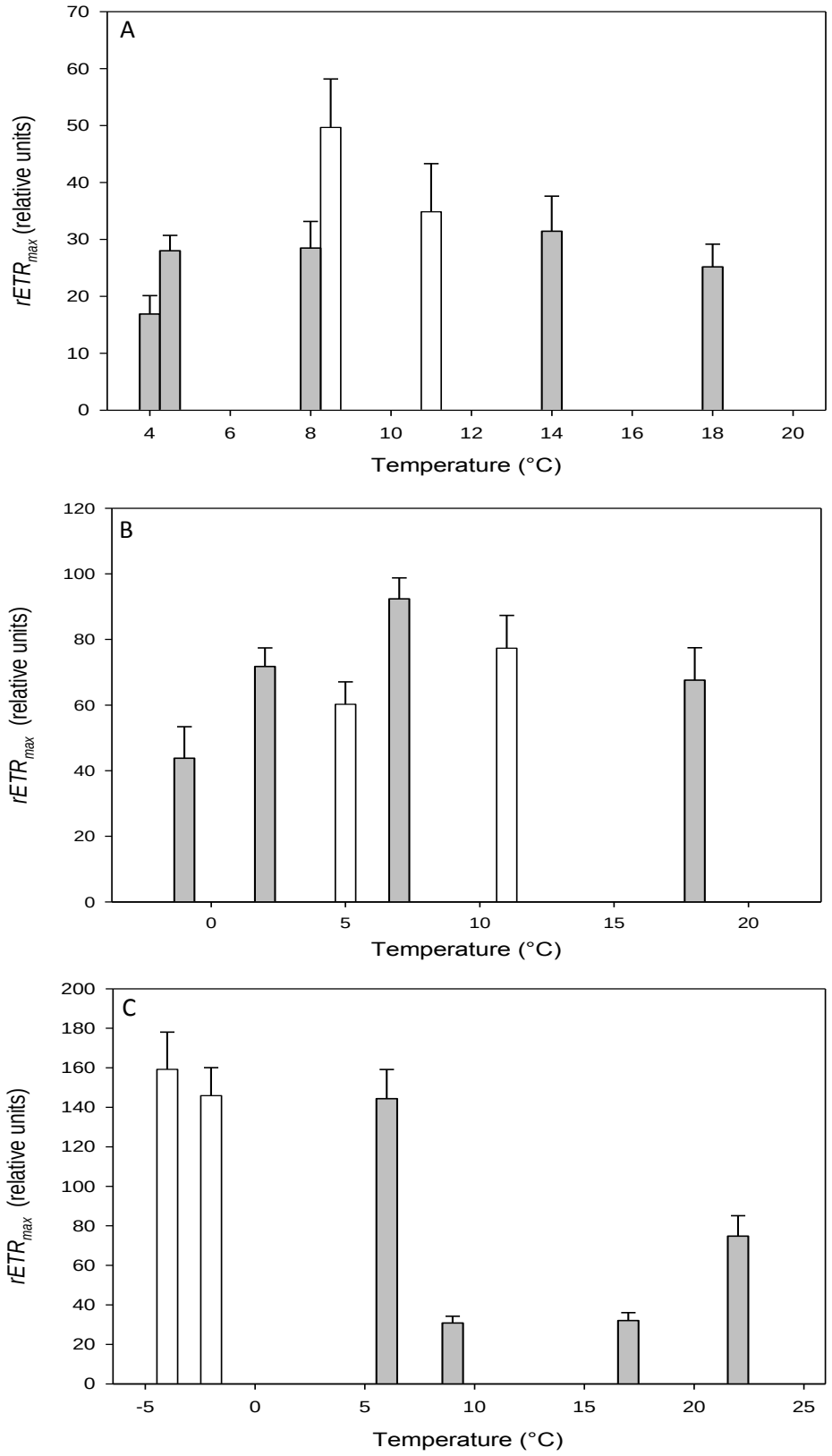


Figure 2.3.12: *Upper shore rETR$_{max}$ levels,* plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

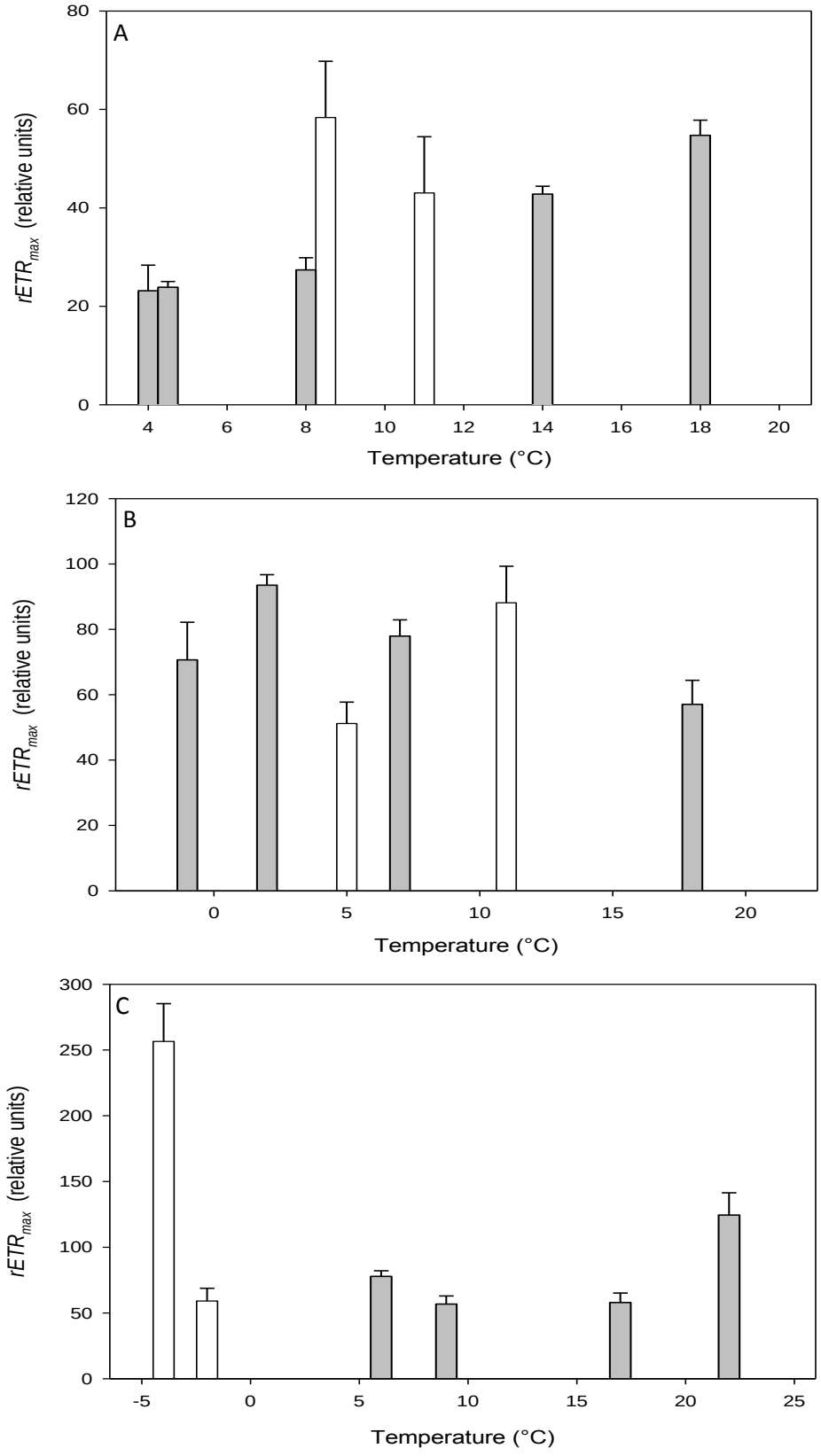


Figure 2.3.13: *Lower shore $rETR_{max}$ levels,* plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

When plotted against light dose it is clear that the $rETR_{max}$ of the upper shore in particular was influenced by this environmental factor (F= 4.90, df= 6, 184, P< 0.05) (Fig.2.3.14). However, the trends in response were not the same over the studied years. There did not appear to be an obvious trend in the $rETR_{max}$ response of the 2009 biofilms (Fig 2.3.14 A). The 2010 and 2011 years exhibited contrasting trends, with the $rETR_{max}$ increasing as light dose increased in 2010 (Fig. 2.3.14 B) and the $rETR_{max}$ decreasing as light dose increased in 2011 (Fig. 2.3.14 C). In 2009 in both the upper and lower shore sites the biofilm development months exhibit higher $rETR_{max}$ levels.

There was again variation in the observed trends over the study years in the lower shore biofilms. In 2009 there did appear to be a trend of increasing $rETR_{max}$ as the light dose increased (Fig. 2.3.15 A). The exception to this was the $rETR_{max}$ recorded in November and December when the biofilms were developing. There was no observable trend in the $rETR_{max}$ recorded, relative to light dose in 2010. However there was a reduction observed as light dose increased to the highest recorded level (Fig 2.3.15 B). In 2011, at the low light doses the recorded $rETR_{max}$ is consistently lower (Fig 2.3.15 C). The highest $rETR_{max}$ recorded was during the growth phase of the biofilm. The shore level did not have a significant effect on the $rETR_{max}$.

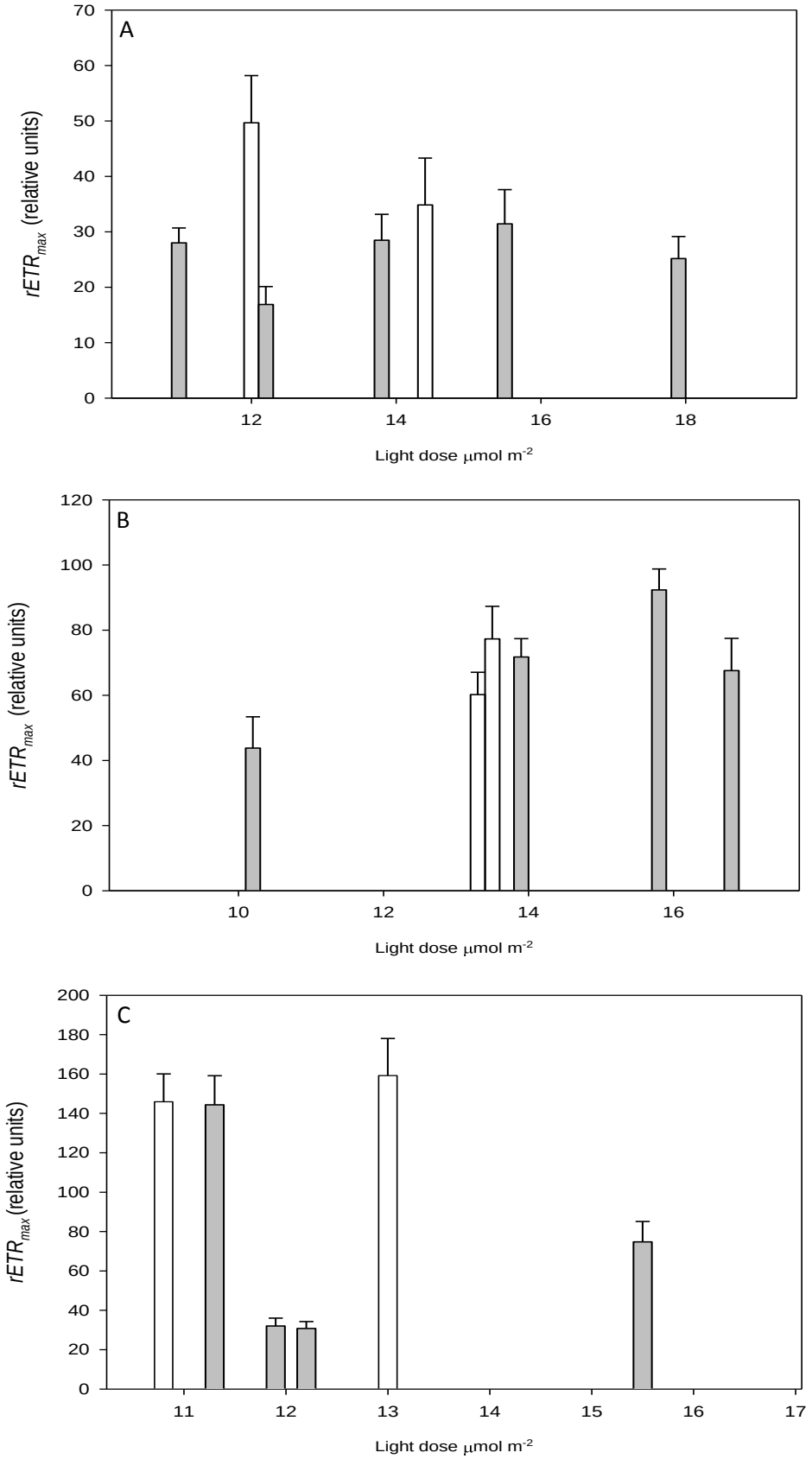


Figure 2.3.14: *Upper shore rETR$_{max}$* levels*,* plotted against light dose µmol m^{-2} recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

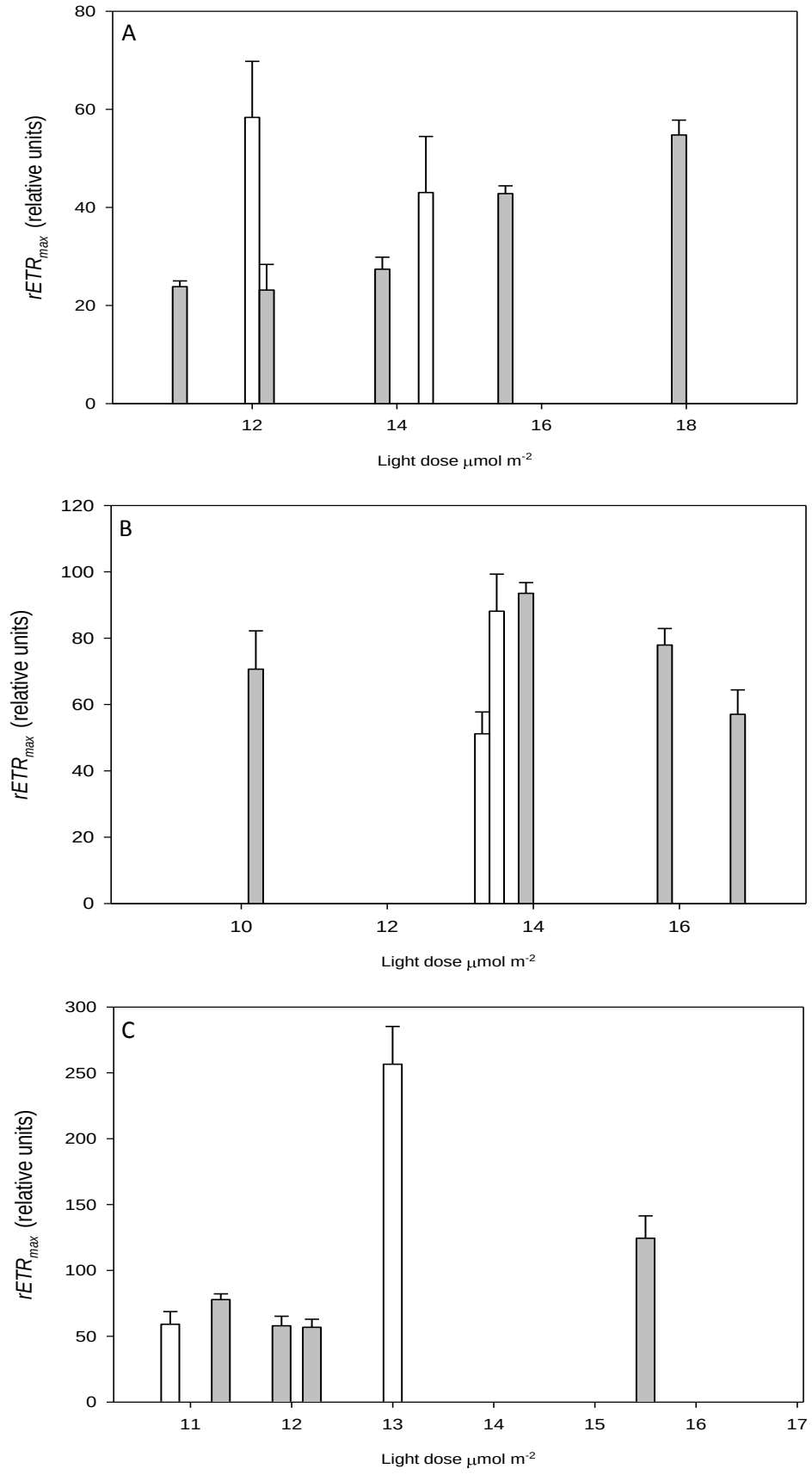


Figure 2.3.15: *Lower shore rETR$_{max}$* levels*,* plotted against light dose µmol m^{-2} recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

Coefficient of light utilisation (α)

Temperature had a significant effect on the α ($F= 12.37$, $df= 6, 184$, $P<0.05$) recorded. The α followed the same patterns as that observed in the $rETR_{max}$ with a trend towards the highest α being recorded at the least extreme temperatures in 2009 (Fig. 2.3.16 A) and 2010 (Fig. 2.3.16 B) in the upper shore samples. This was particularly apparent in 2009. In 2010, the lowest α recorded was during the growing months. In contrast, the highest α recorded in 2011 was during the growing months of 2011 (Fig. 2.3.16 C).

Interestingly, unlike the $rETR_{max}$ data (Figures 2.3.14 and 2.3.15), the pattern of response between the upper and lower shore was virtually identical with no significant difference noted. In 2009 and 2010, data generally displayed the pattern of highest α at mid temperature points (Fig. 2.3.17 A and B). The 2011 measurements again indicate that the highest α was recorded at low temperatures during the growth months of November and December (Fig 2.3.16 C).

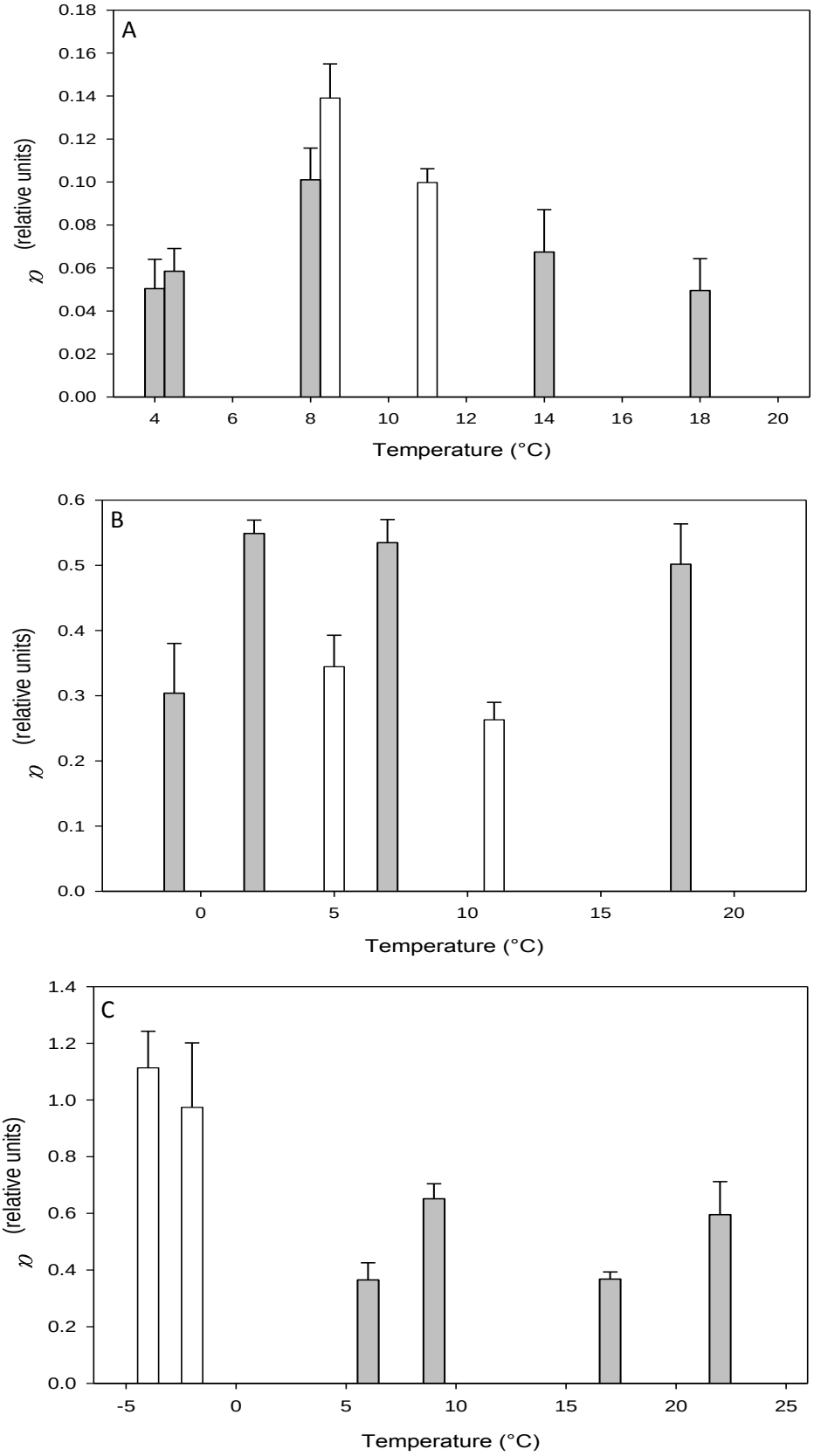


Figure 2.3.16: *Upper shore α* levels, plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

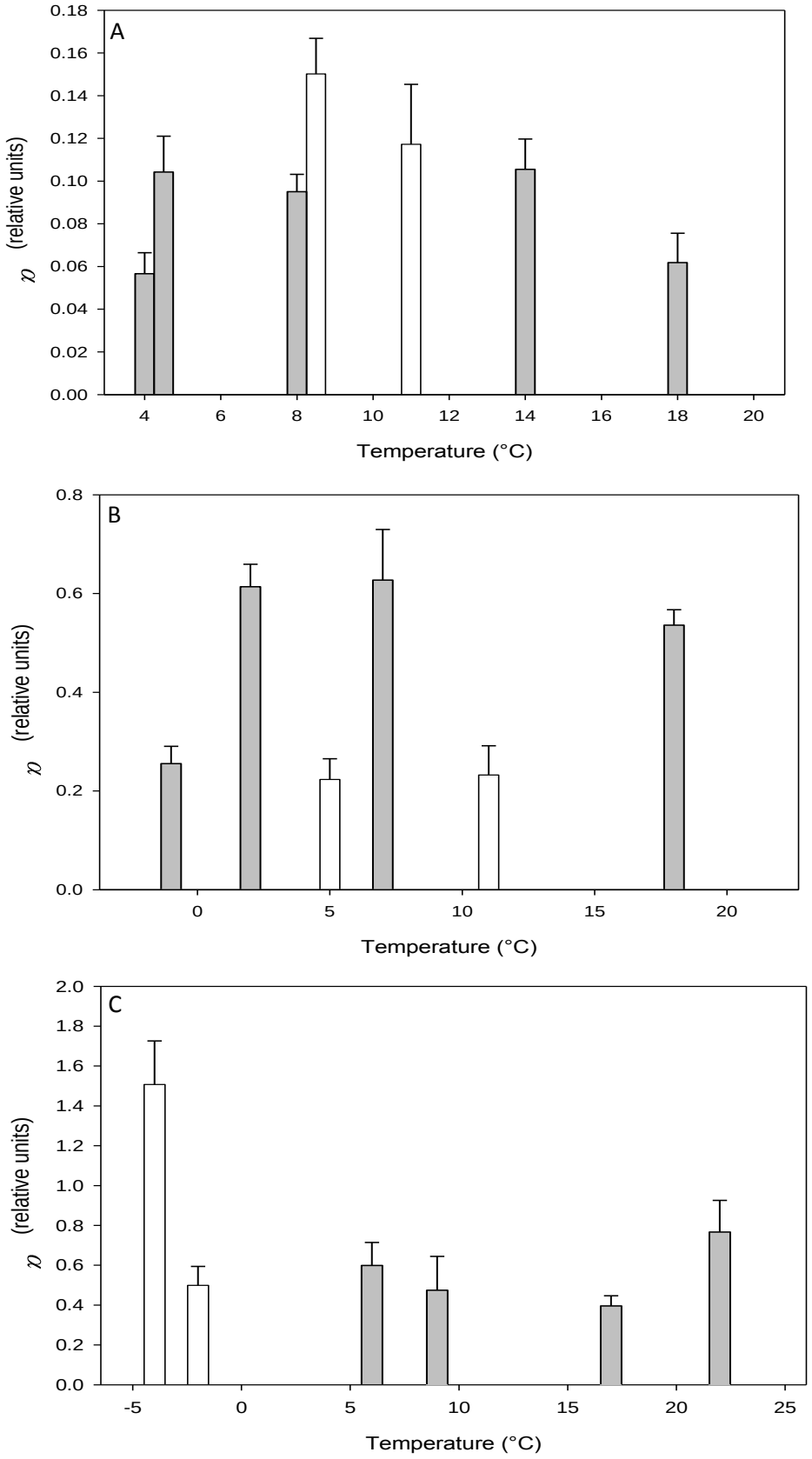


Figure 2.3.17: *Lower shore α levels,* plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

The α, with relation to measurement day light dose, followed the same trends as the $rETR_{max}$ and, as above, there was also very little difference between the upper and lower shore measurements, with no significant effect of shore level noted (Figs. 2.3.18 and 2.3.19). Light dose did have a significant effect on the α ($F=3.22$, $df= 6, 184$, $P<0.05$), with discernible patterns within the 2009 and 2010 upper shore samples (Fig. 2.3.18 A and B). The α reduced as light dose increased in 2009, with the highest α recorded again in the growth months. In 2010 the α appeared to increase as light dose increased with the growth months exhibiting low α levels. There was no discernible pattern in the 2011 records with the highest α clearly being exhibited by the growth months (Fig. 2.3.18 C).

The same patterns were observed in 2009 in the lower and upper shore, however, there was a less obvious reduction in α as light levels increased (Fig. 2.3.19 A). In 2010, the highest α levels were observed at higher light doses (Fig. 2.3.19 B). In 2011, only one of the growth months (November) exhibited the high α levels seen in the upper shore samples (Fig 2.3.19 C).

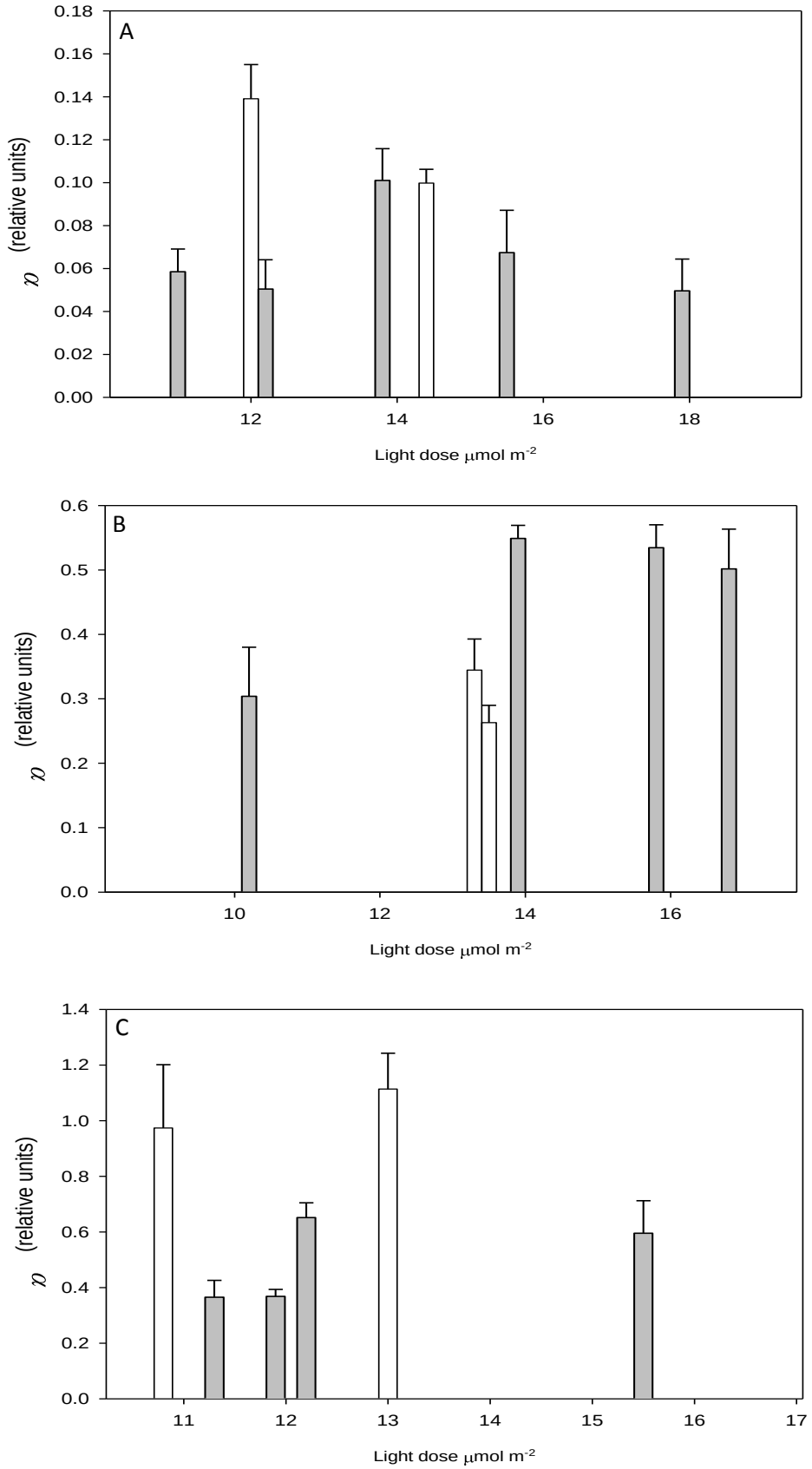


Figure 2.3.18: *Upper shore α levels,* plotted against the light dose recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

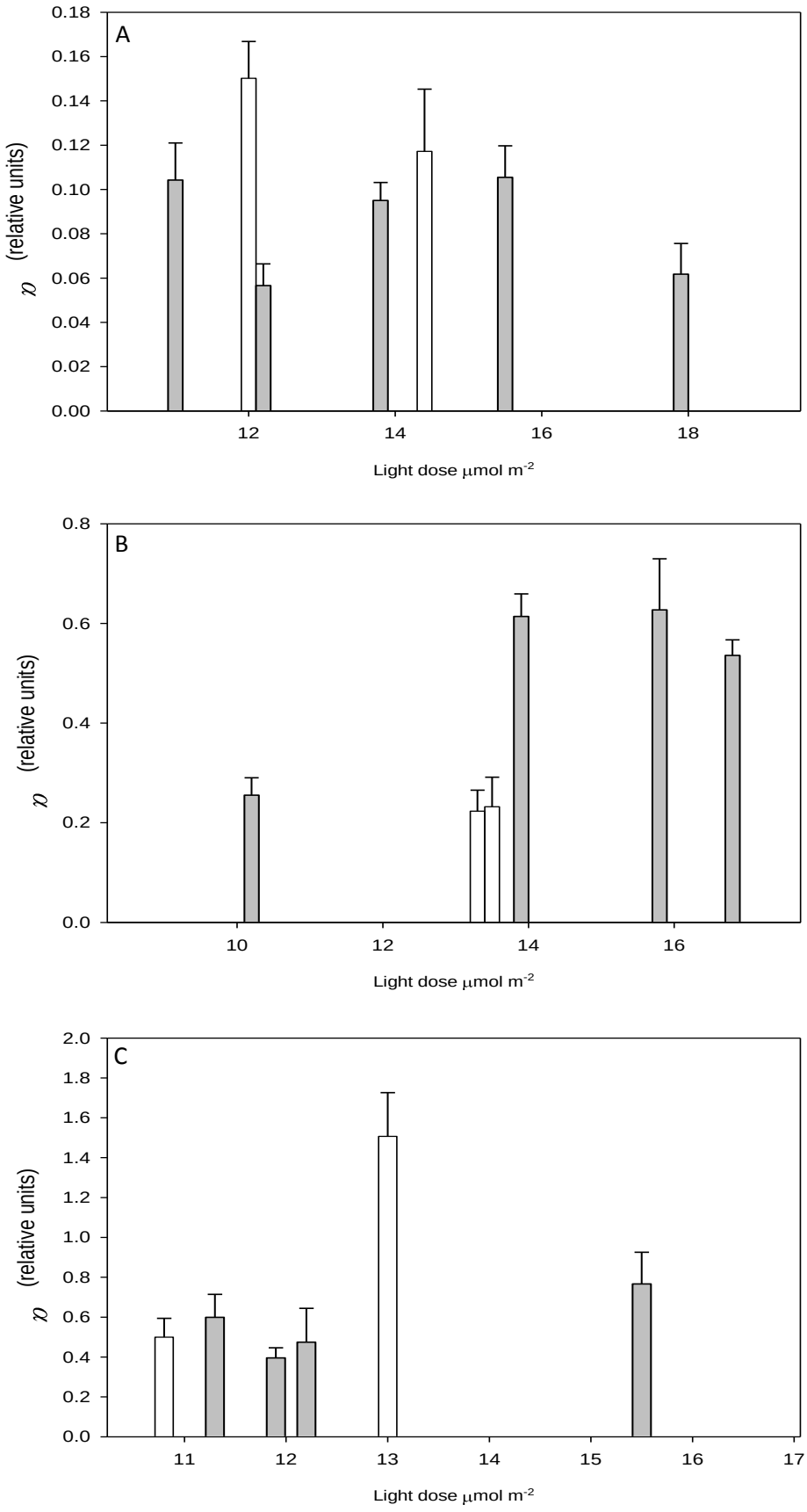


Figure 2.3.19: *Lower shore α levels,* plotted against the light dose recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

Light saturation coefficient (*Ek*)

Temperature had a significant effect on the *Ek* ($F=5.39$, $df= 6, 184$ $p<0.05$). This is most apparent in the upper shore biofilms (Figure 2.3.20). There were, however, no clear patterns in the *Ek* response to temperature over the measurement years. In 2009 the highest *Ek,* at the upper shore sites, was recorded at during months with the highest temperature (Fig. 2.3.20 A). In 2010, there was little difference in the *Ek* recorded, but there was a high *Ek* recorded during the biofilm growing months (Fig. 2.3.20 B). In 2011, the recorded *Ek* was lower during the higher temperatures months (Fig. 2.3.20 C).

Shore level had no significant effect on the *Ek*, with similar patterns observed between the shore levels in 2009 and 2010 (Figs 2.3.20 A and B and 2.3.21 A and B). In 2010 there was a high *Ek* recorded during the highest temperature month, which was May (Fig. 2.3.21 B). In 2010 the *Ek* was highly variable but there was no discernible pattern of response. In 2011, there was again no pattern of response and there was very little variation in *Ek* over the measurement months (Fig. 2.3.21 C).

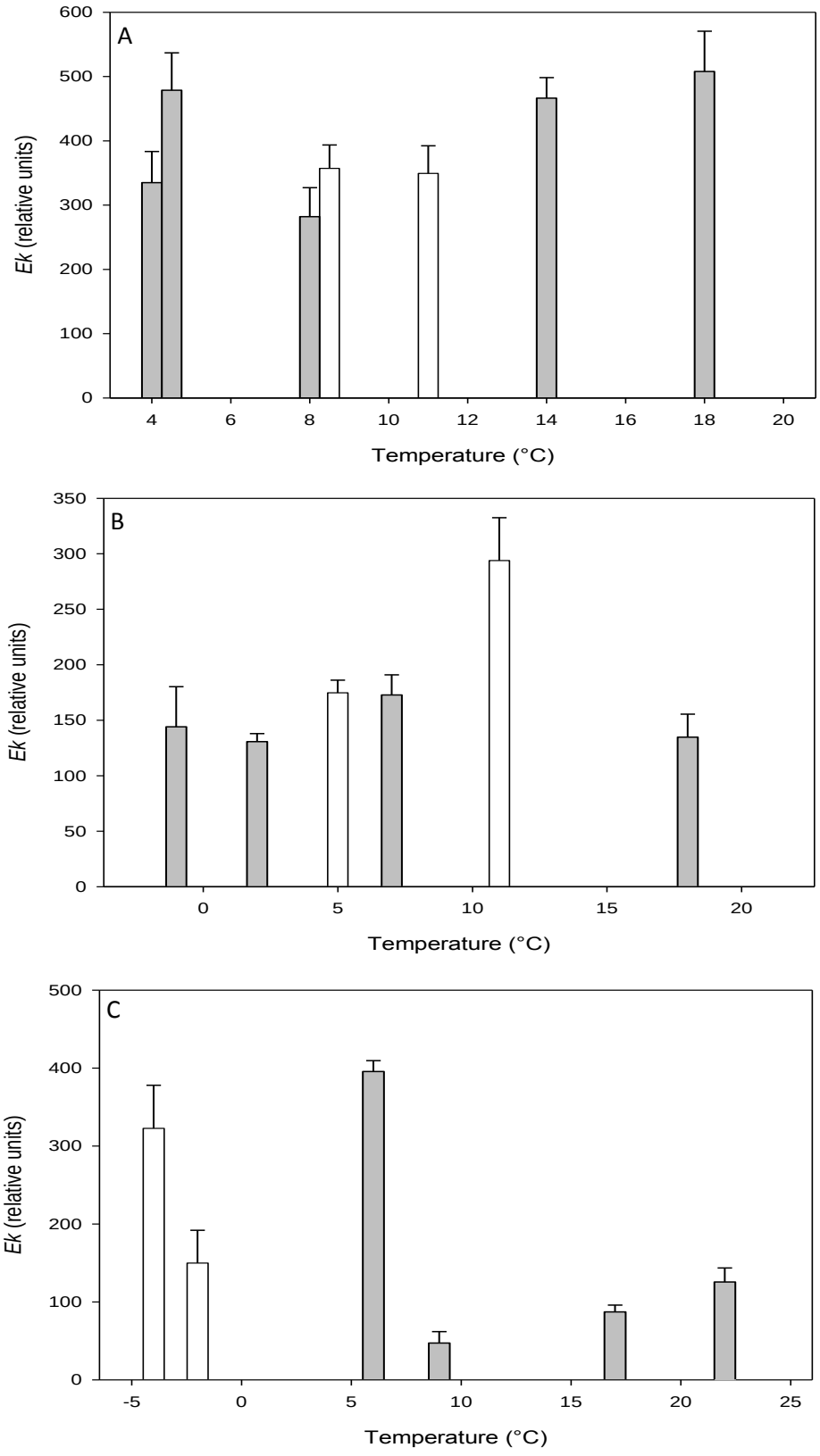


Figure 2.3.20: *Upper shore Ek levels,* plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

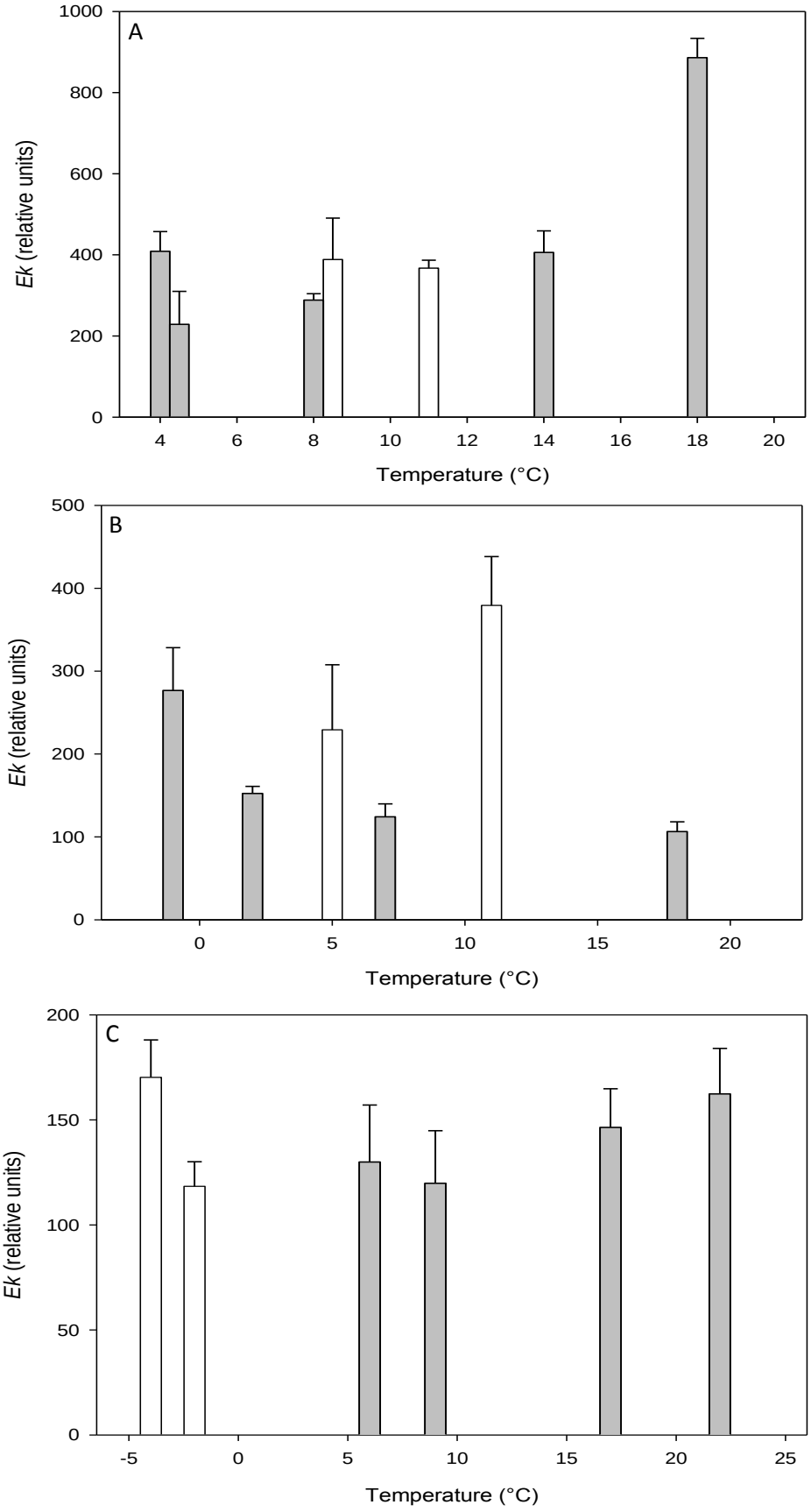


Figure 2.3.21 *Lower shore Ek levels,* plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

Light dose had a significant effect on the *Ek* with patterns of response noticeable ($F=6.52$, $df= 6, 184$, $P<0.05$). In the 2009 upper shore samples, lower *Ek* levels were recorded during the median light levels (Fig. 2.3.22 A). In 2010, the highest *Ek* was recorded during the growth months and again these were measured at median light levels (Fig. 2.3.22 B). In 2011, the highest *Ek* levels were recorded on months where low light levels was recorded (Fig. 2.3.22 C).

The shore level also had a significant effect on the *Ek* ($F=7.12$, $df= 6, 184$, $P<0.05$). In 2009, the highest *Ek* level was recorded at the highest light dose (Fig. 2.3.23 A). There was little difference in the *Ek* levels recorded at the lower light levels. In 2010, the highest *Ek* levels were recorded at low and mid light levels (Fig. 2.3.23 B). The lowest *Ek* levels were recorded at the highest light levels. In 2011, the *Ek* levels slightly increased as light levels increased (Fig. 2.3.23 C).

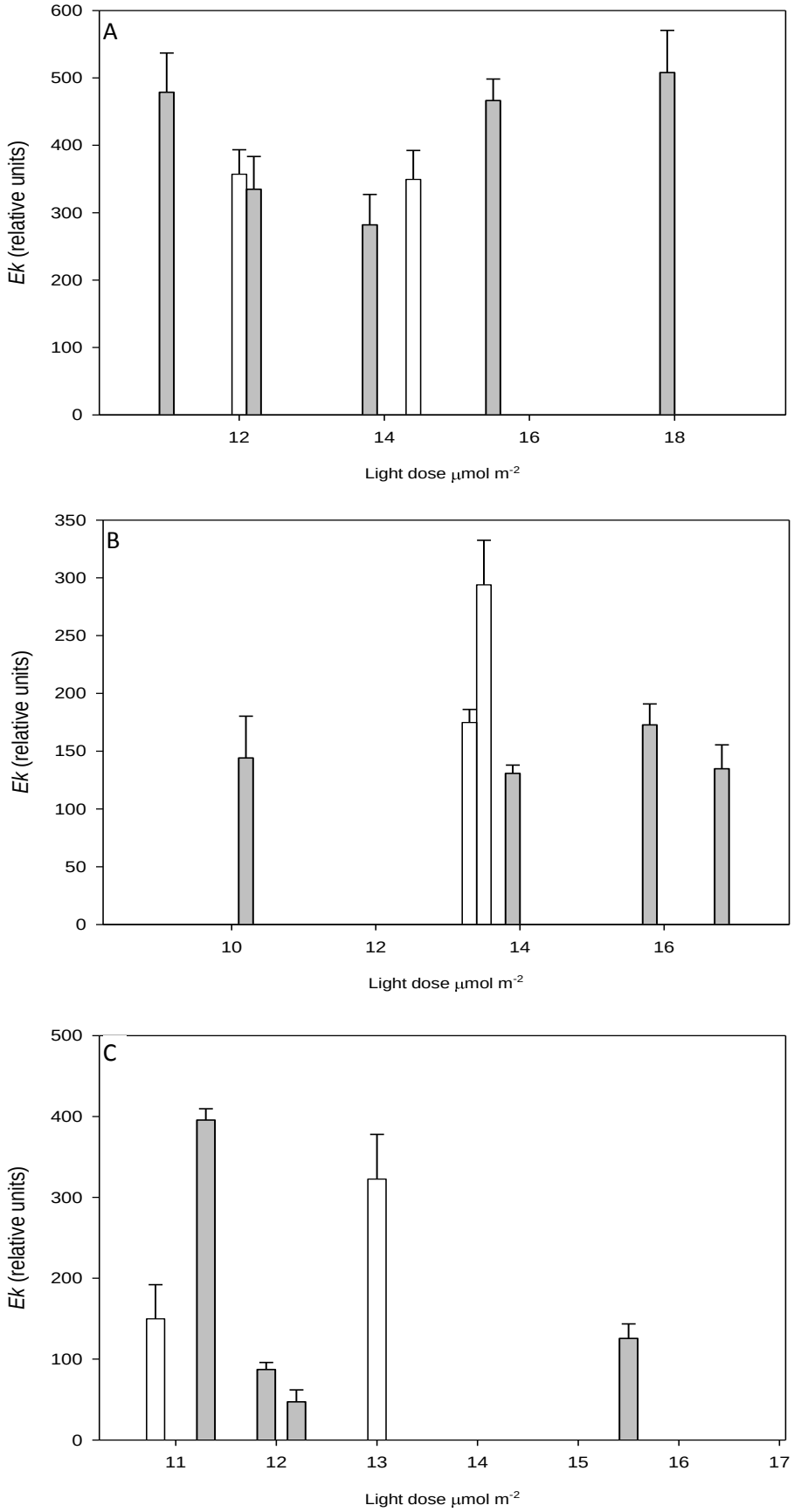


Figure 2.3.22: *Upper shore Ek levels,* plotted against the light dose recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

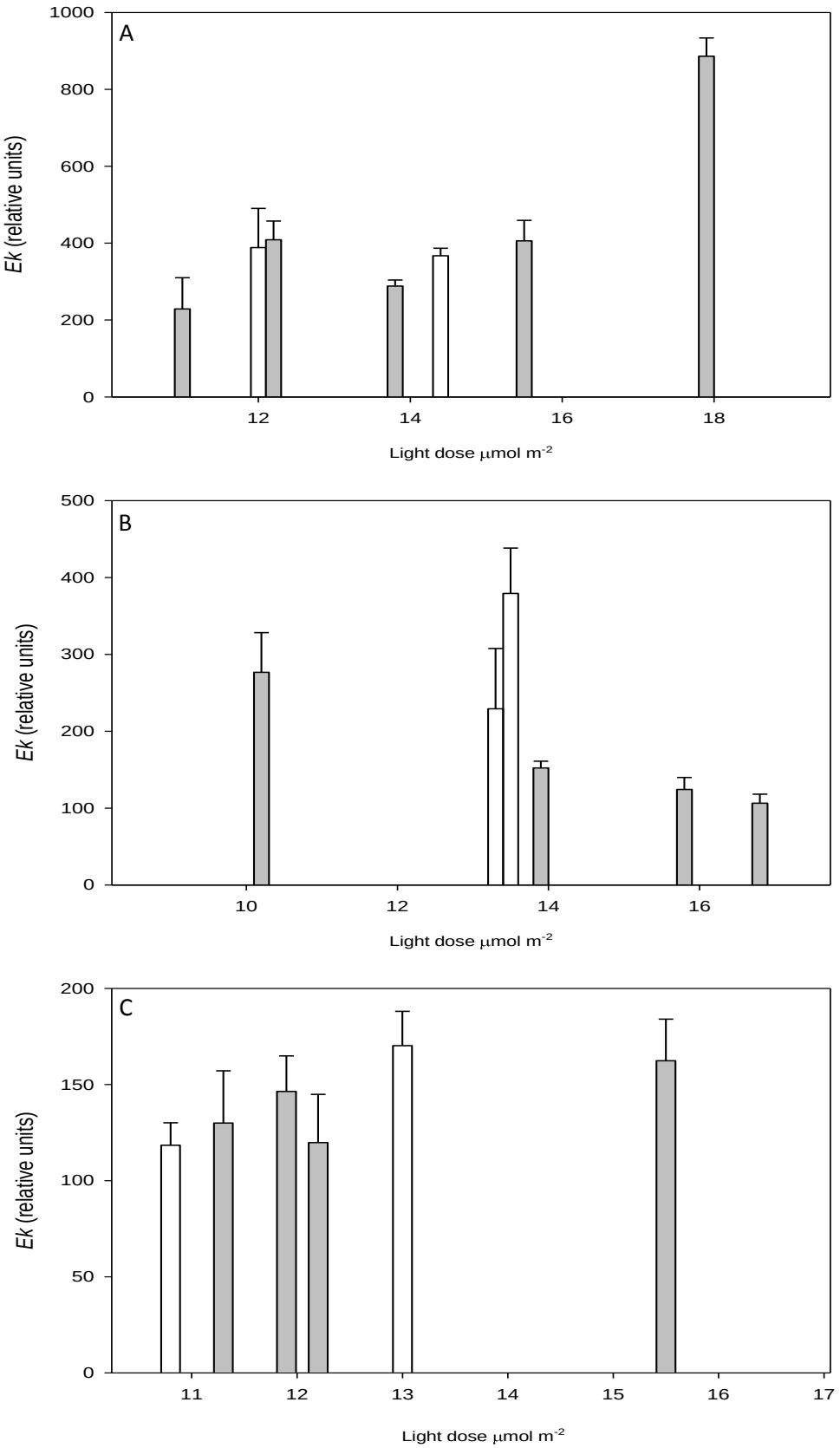


Figure 2.3.23: *Lower shore Ek* levels, plotted against the light dose recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

Light acclimated quantum efficiency *(ΔF/Fm')*

Temperature had a significant effect on *ΔF/Fm'* ($F=8.12$, df= 6, 184, $P<0.05$). There was also a consistent pattern, in the upper shore sites (Fig. 2.3.24), observable over the 3 studied years, which was most apparent in 2009 (Fig 2.3.24 A). There was an increase in *ΔF/Fm'* as temperature increased up to the median temperature where the highest *ΔF/Fm'* was recorded. The *ΔF/Fm'* then decreased as temperature increased. The pattern was less obvious in 2010 but there was still an increase to the median temperatures (Fig. 2.3.24 B). The highest temperature also resulted in a high *ΔF/Fm'* level. In 2011, there was again an observable increase in *ΔF/Fm'* as temperature increased. This was followed by a decrease at the highest temperature (Fig. 2.3.24 C). Light dose had the same effect as the temperature on the *ΔF/Fm'* and so for brevity this data was not displayed.

Unlike the upper shore biofilms there was no observable pattern in the *ΔF/Fm'* response of the lower shore biofilms across the measurement years (Fig. 2.3.25). In 2009 there did appear to be an increase in *ΔF/Fm'* at the median temperatures (Fig. 2.3.25 A). In 2010 there was no observable pattern of response and the *ΔF/Fm'* measured during the growth months of November and December was the lowest recorded (Fig. 2.3.25 B). In 2011 there was again no observable pattern. The lowest *ΔF/Fm'* was recorded at the highest temperature (Fig. 2.3.24). Shore level did not have a significant effect on the *ΔF/Fm'* recorded.

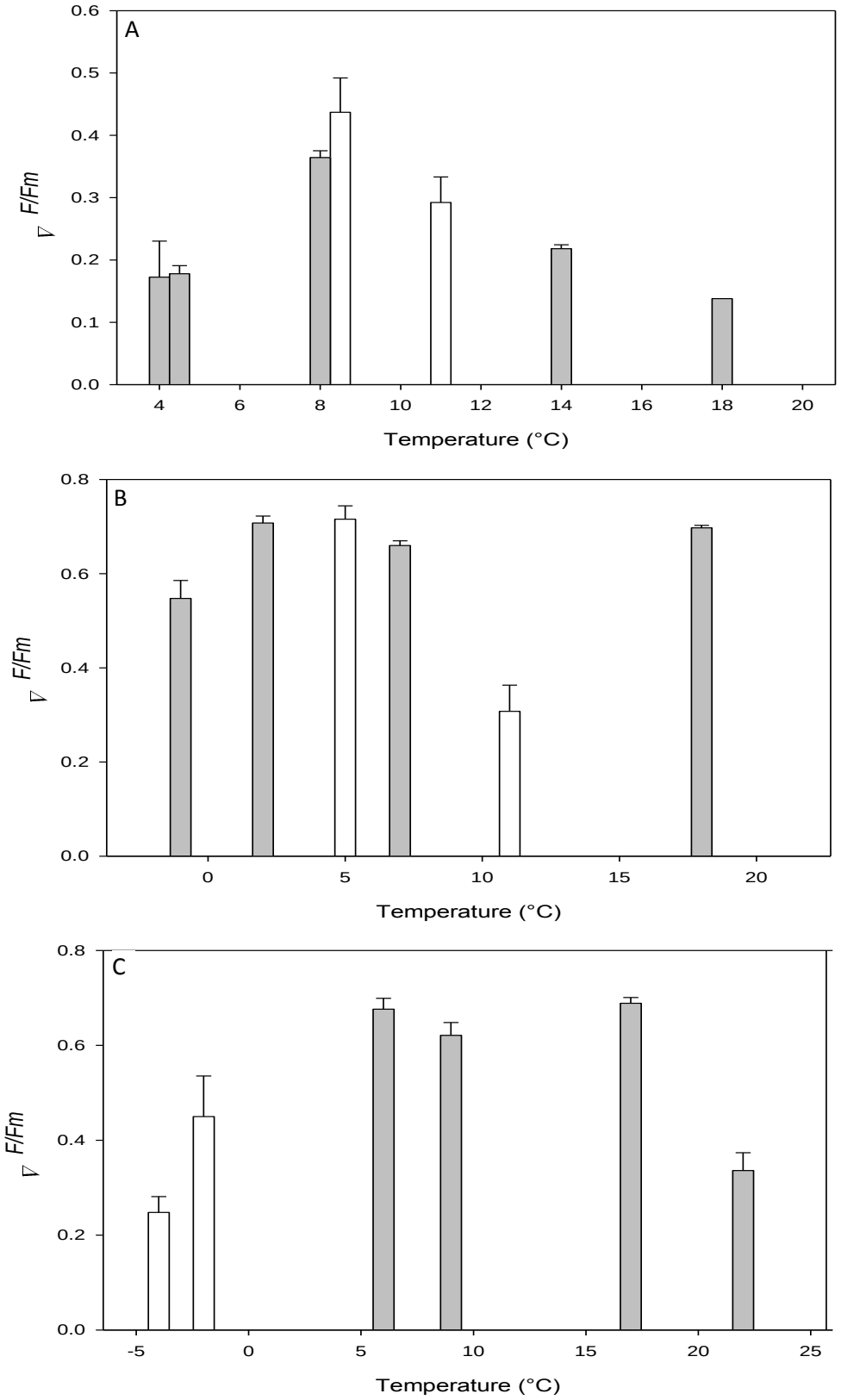


Figure 2.3.24: *Upper shore ΔF/Fm'* levels, plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

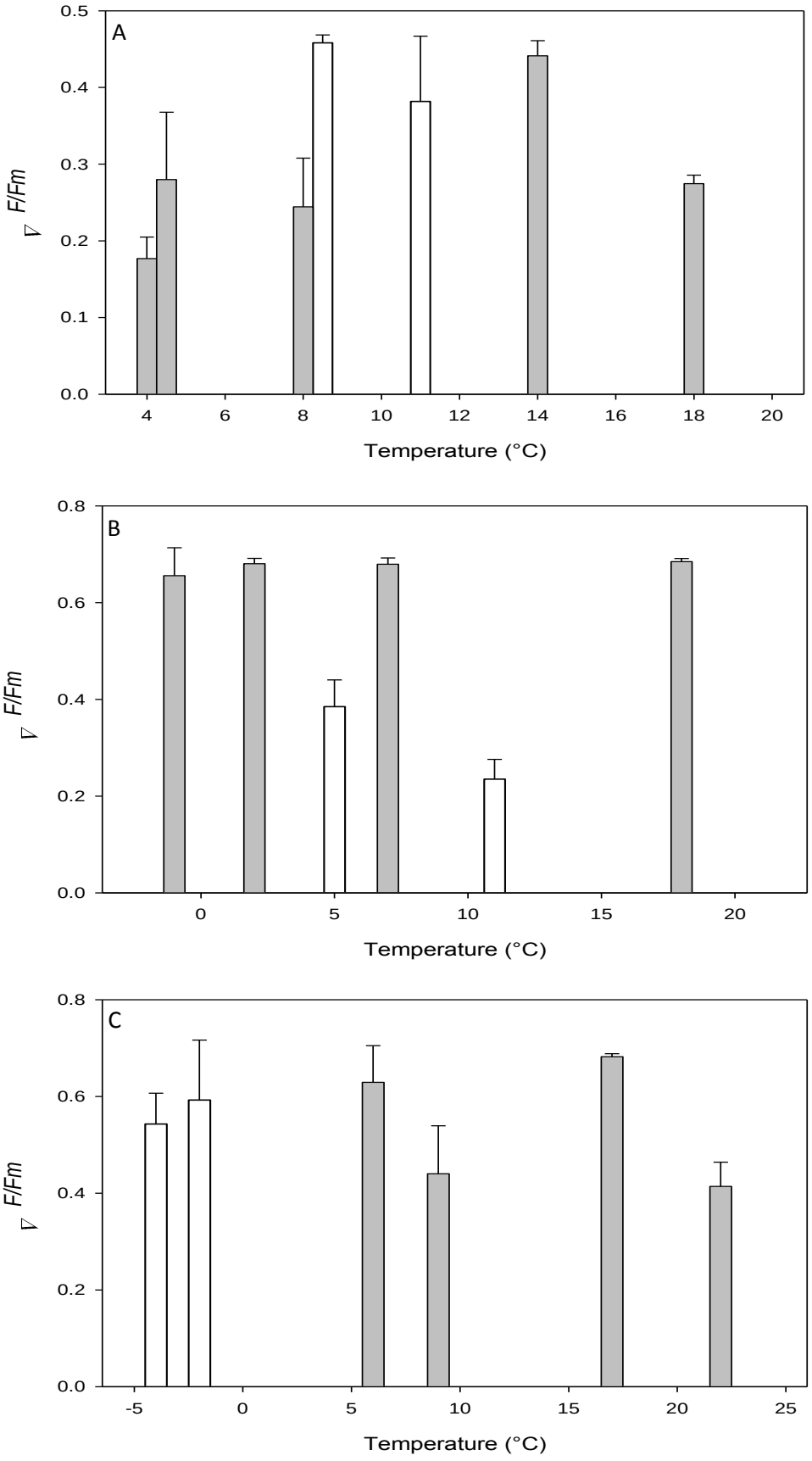


Figure 2.3.25: *Lower shore ΔF/Fm'* levels, plotted against the temperature recorded at each measurement month, over the 3 measurement years (A- 2009, B- 2010, C- 2011). Mean + s.e. *n=5*

Non-photochemical quenching (NPQ)

Maximum fluorescence yield (*Fm')*

During 2009 at the upper shore sites there was a consistent reversal of downregulation from 0 to 603 µmol m^{-2} s^{-1} (Fig. 2.3.26 A). The greatest reversal of downregulation occurred in January and February. There was again little induction of downregulation at the highest light point of 1037 µmol m^{-2} s^{-1}. NPQ reversal was performed by all lower shore biofilms in 2009 from 10 to 603 µmol m^{-2} s^{-1} (Fig. 2.3.26 B). There was then down regulation induced from 603 to 1037 µmol m^{-2} s^{-1}. However the level of downregulation was no higher than that being induced at 10 µmol m^{-2} s^{-1}.

During 2010 the upper shore sites exhibited a very similar pattern of downregulation induction with again a reversal being noted in November from 0 to 603 µmol m^{-2} s^{-1} followed by induction to the 1037 µmol m^{-2} s^{-1} (Fig. 2.3.27 A). At all other months downregulation was induced as light levels increased but this was most apparent in February, March and April. The lower shore biofilms in 2010 only exhibited reversal of downregulation in November, this was from 0 to 603 µmol m^{-2} s^{-1} (Fig. 2.3.27 B). In December and January there was no induction of down regulation from 0 to 603 µmol m^{-2} s^{-1} but there was from 603 to 1037 µmol m^{-2} s^{-1}. In all other months down regulation was induced as light steps increased.

The upper shore biofilms in 2011 more consistently induced downregulation (Fig. 2.3.28 A). In November there was greater variation in the responses of the biofilms indicated by the large error bars. In April there was less induction of downregulation at 603 µmol m^{-2} s^{-1} than at the previous months. In 2011, the lower shore biofilms exhibit a similar pattern to those seen in 2010 with the biofilms exhibiting reversal of downregulation in November (Fig. 2.3.28 B). Again in all other months downregulation occurred as light levels increased.

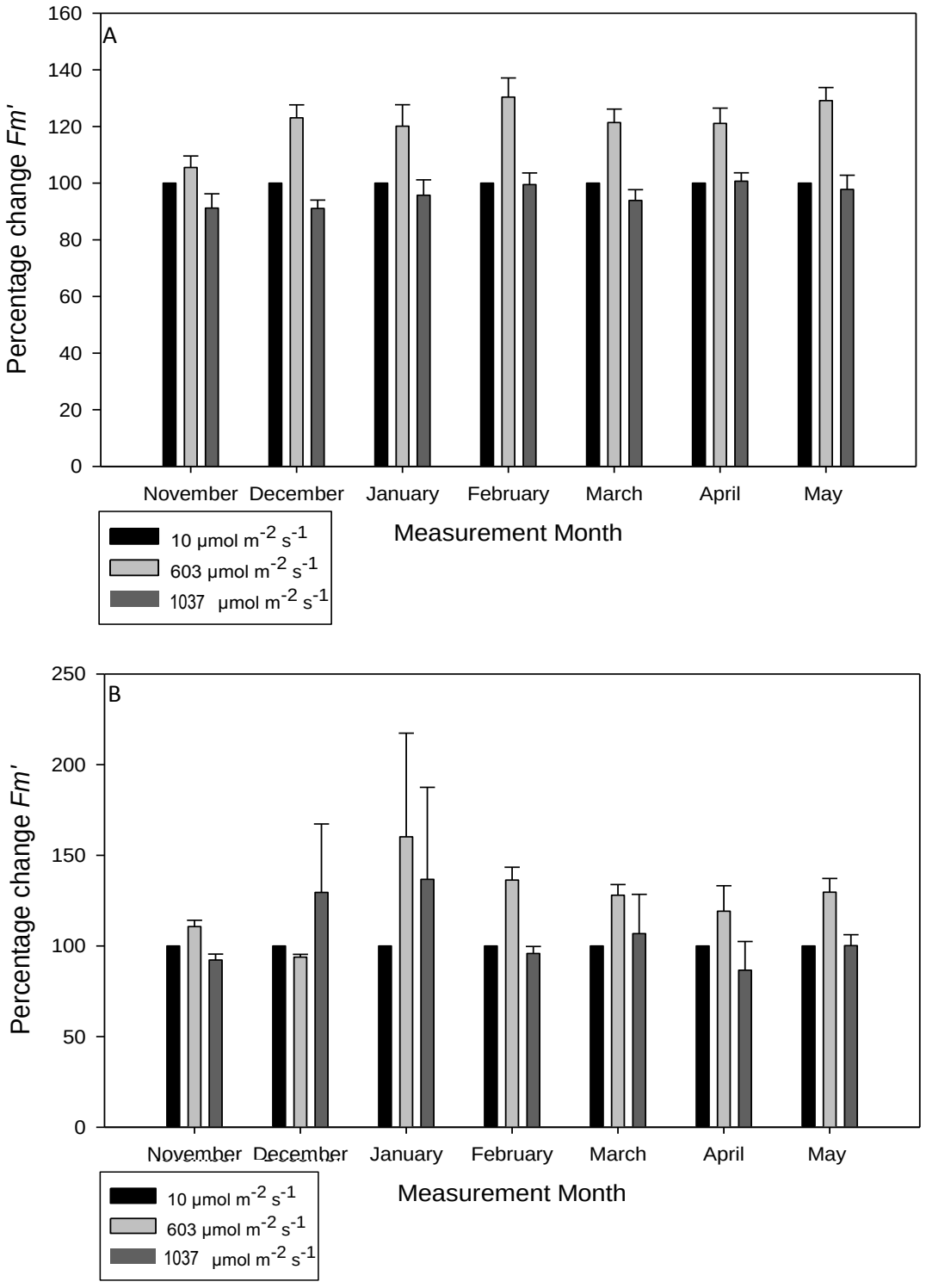


Figure 2.3.26: *The percentage change in Fm' levels from the upper (A) and lower shore (B),* recorded at each measurement month in 2009. Mean + s.e. *n=5*

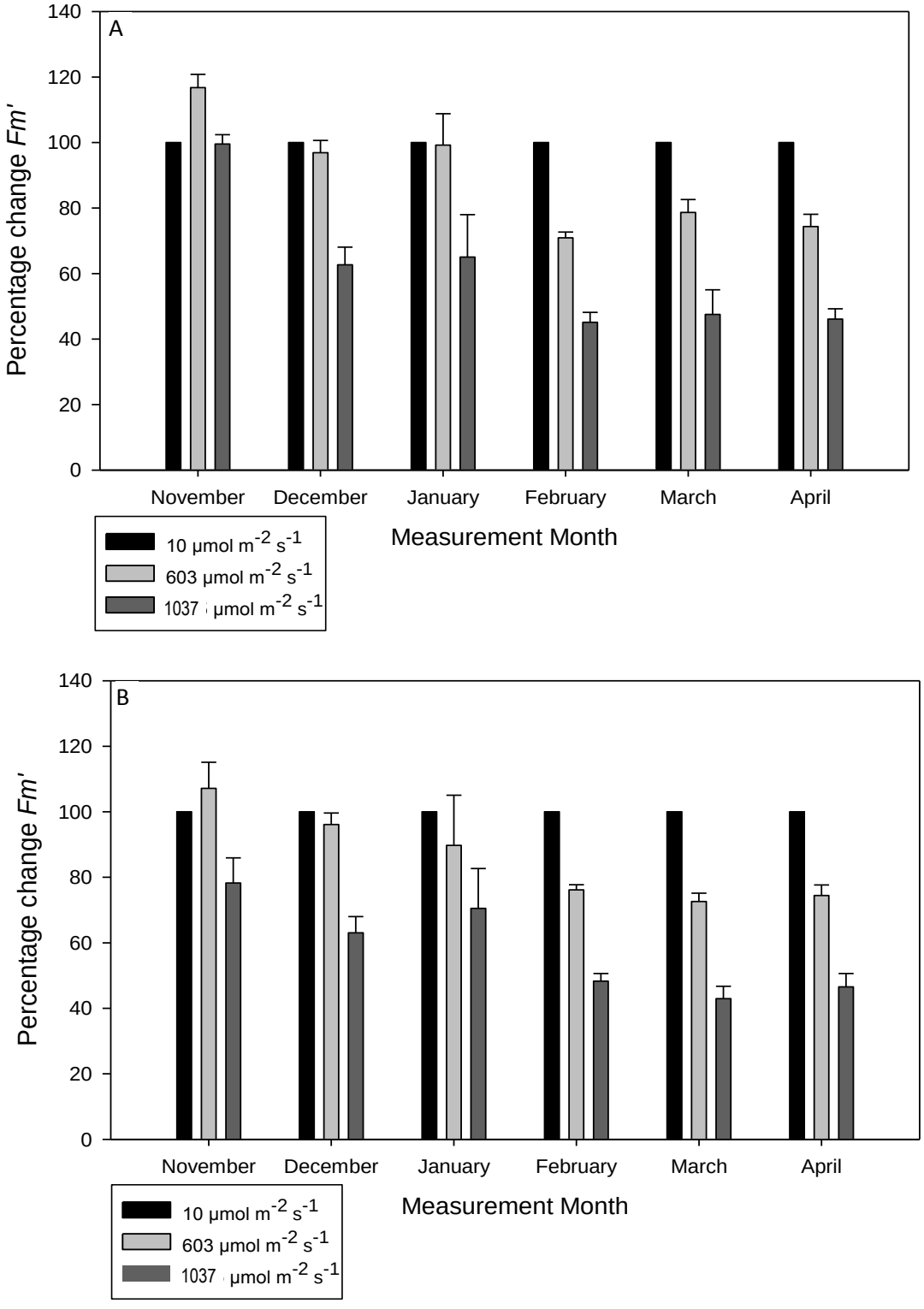


Figure 2.3.27: *The percentage change in Fm' levels from the upper (A) and lower shore (B),* recorded at each measurement month in 2010. Mean + s.e. *n=5*

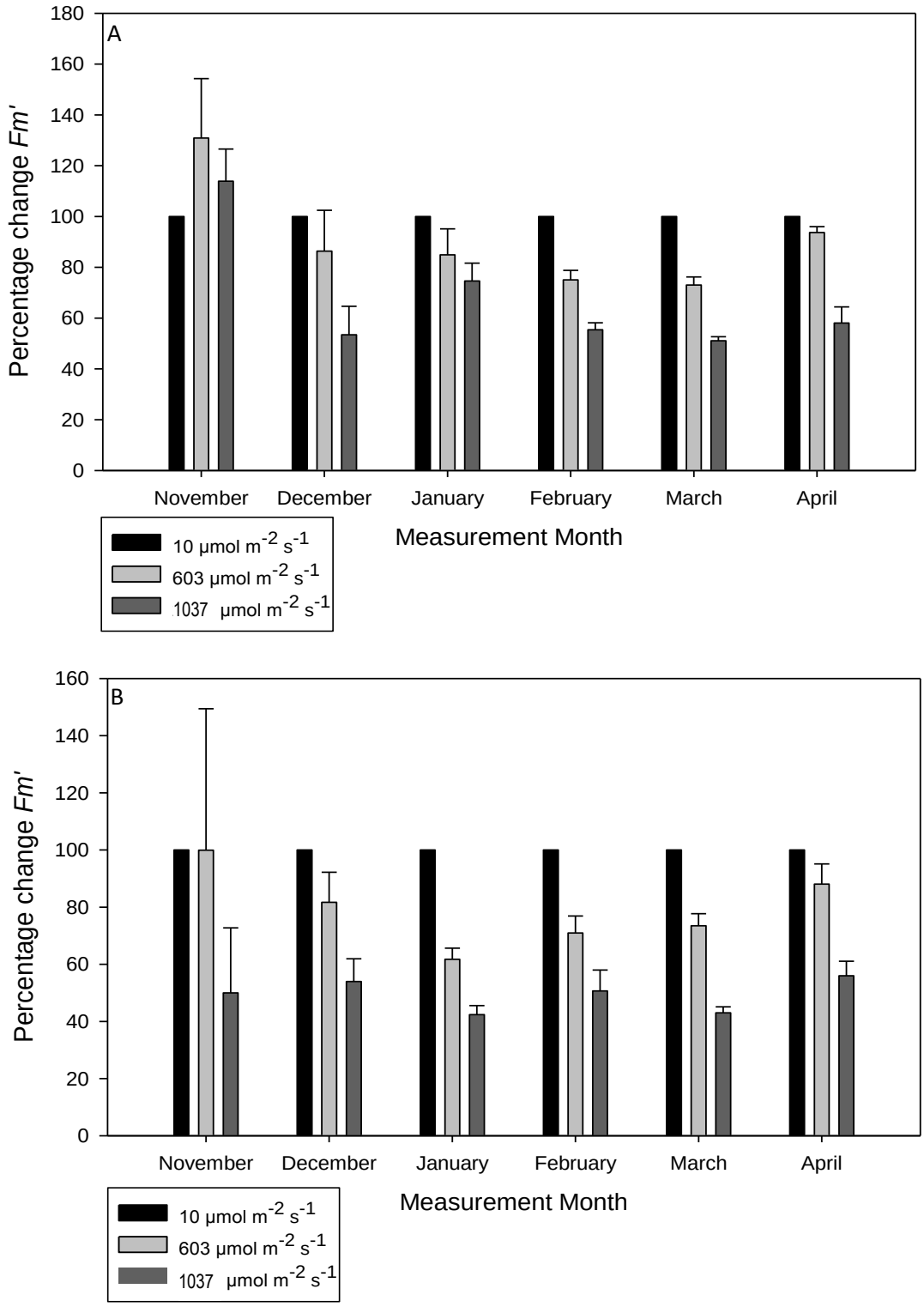


Figure 2.3.28: *The percentage change in Fm' levels from the upper (A) and lower shore (B),* recorded at each measurement month in 2011. Mean + s.e. *n=5*

Multivariate Principal Component Analysis

Upper shore 2009

98% of the data was explained by 5 principal components, and 75% explained by PC1 and PC2 (Fig. 2.3.29). November, December and February were characterised by high $rETR_{max}$, α and *ΔF/Fm'* levels. This was particularly apparent in November, it was also apparent that high algal cover characterised December and February but to a lesser extent November. This indicated that the high $rETR_{max}$, α and *ΔF/Fm'* levels were being recorded during a period of biofilm growth. High cell counts also characterised December, February and January, but the high levels of $rETR_{max}$, α and *ΔF/Fm'* recorded in November and December were not recorded in January. January was characterised by high numbers of *Navicula ramosissima* and the epiphytic *Licmophera ehrenbergii. Licmophera ehrenbergii* could be defined as the winter epiphyte with *Achnanthes brevipes* and *Licmophera flabellata* being the summer epiphytes most commonly associated with the cyanobacteria *Moorea producens* (formerly *Lyngbya majuscula*). As would be expected high temperatures characterised April and May and consequently low temperatures characterised December, February and January. A high *EK* defines May and April. High numbers of radula scrapes were also recorded. There was a linear correlation between the *Ek* and the number of radula scrapes ($r = 0.913$, $n=7$, $P < 0.05$).

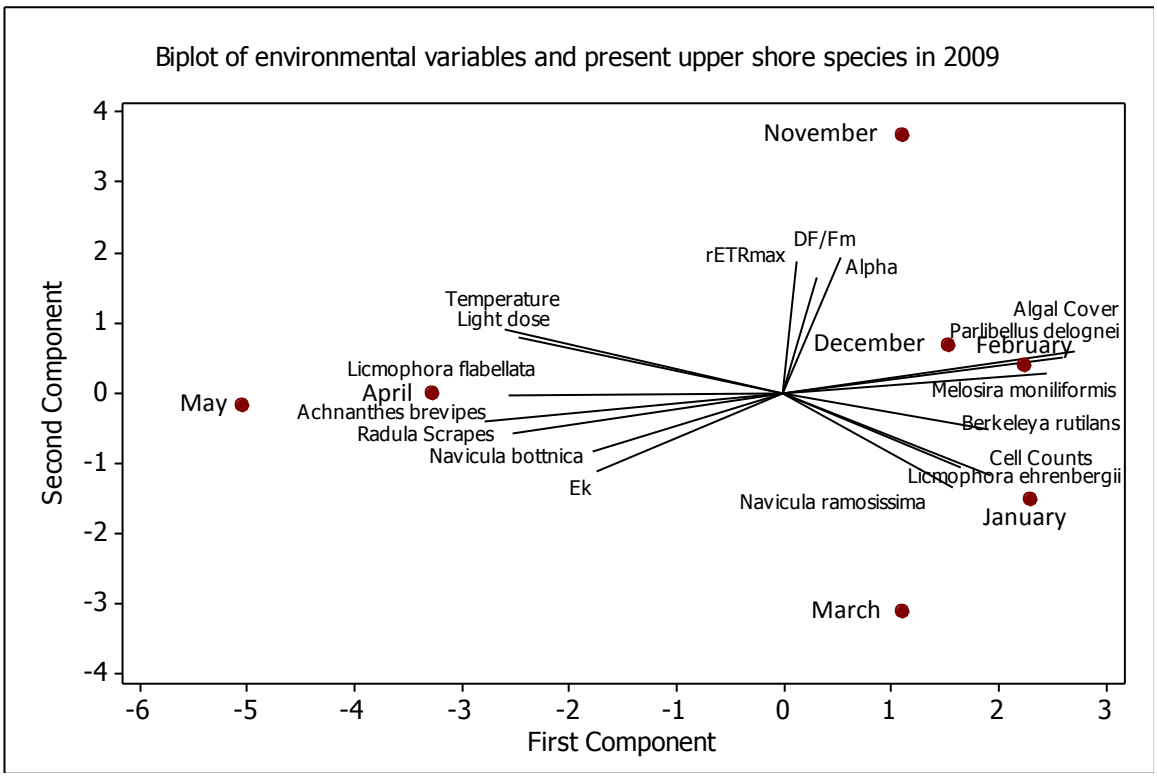


Figure 2.3.29: *Principal component analysis*, of photosynthetic parameters, biofilm taxonomic composition, and environmental factors at upper shore sites in 2009.

Lower shore 2009

95% of the data was explained by 5 principal components, and 60% explained by PC1 and PC2 (Fig. 2.3.30). November and December were separated from the other months and defined by higher $rETR_{max}$ and *ΔF/Fm'* with November in particular being defined by higher *ΔF/Fm.* *Nitzschia linearis* and *Tryblionella compressa* also defined these months with particularly high cell numbers which reduced throughout the remaining measurement months. There was a high density of species defining January and February and the data indicated that these months had the highest level of biodiversity. These also followed the direction of the algal cover, which again indicates that during these months the biodiversity and algal cover in general was highest. In May a large quantity of radula scrapes and high light dose was coinciding with a high *Ek* level. The increase in radula scrapes defined April and May, with the highest levels recorded during this time. There was also a linear correlation between the *Ek* and the number of the radula scrapes ($r = 0.868$, $n=7$, $P < 0.05$), the *Ek* and light dose ($r = 0.911$, $n=7$, $P < 0.05$ and the *Ek* and temperature ($r = 0.817$, $n=7$, $P < 0.05$.

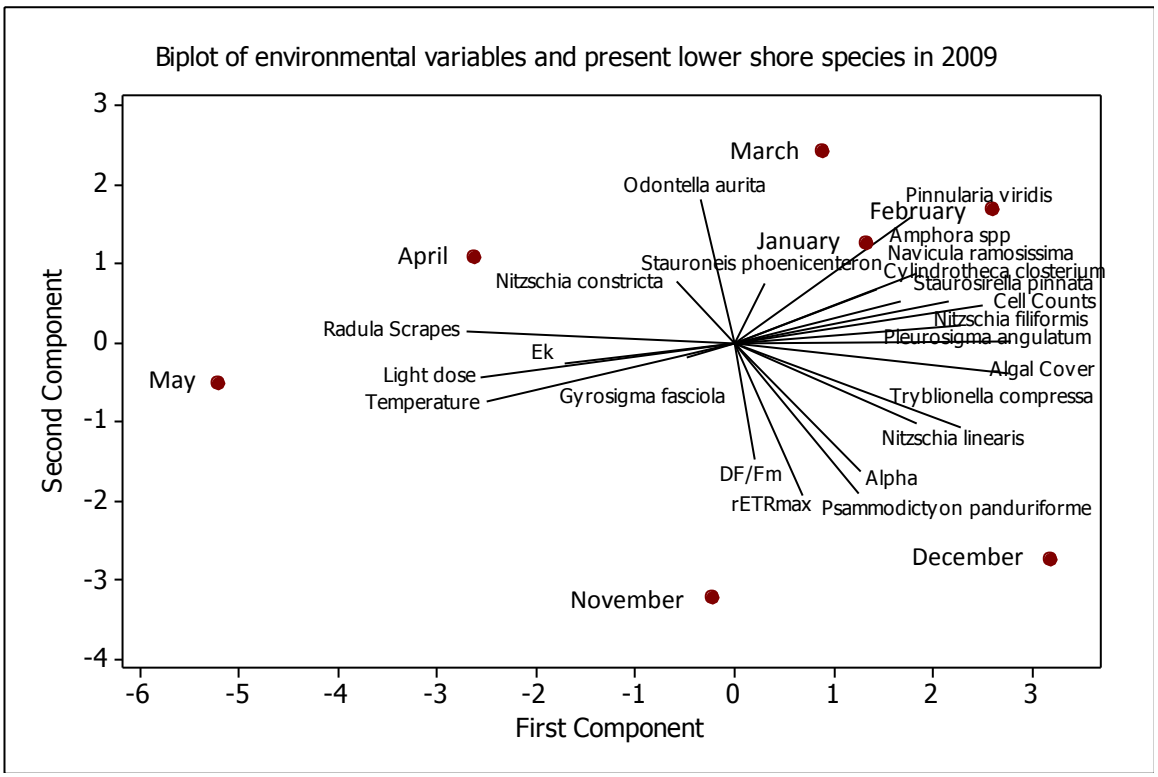


Figure 2.3.30: *Principal component analysis*, of photosynthetic parameters, biofilm taxonomic composition, and environmental factors at lower shore sites in 2009.

Upper shore 2010

100% of the data was explained by 5 principal components, and 68% explained by PC1 and PC2 (Fig. 2.3.31). November was defined by low cell counts and low *Ek* levels. In contrast to the 2009 records, the high levels of $rETR_{max}$, α and *ΔF/Fm'* were recorded in March rather than in November, December and January. Low temperatures characterised December, January and February. Again the high number of radula scrapes characterised April; with the contrasting high levels of Algal cover being recorded in February. There was a linear correlation between the Radula scrapes and the temperature (r = 0.784, n=6, P < 0.05).The same species characterised the seasons with *Licmophera ehrenbergii* defining the winter months with *Achnanthes brevipes* and *Licmophera flabellata* being present in the spring. Again the larger species were present most commonly in the winter months.

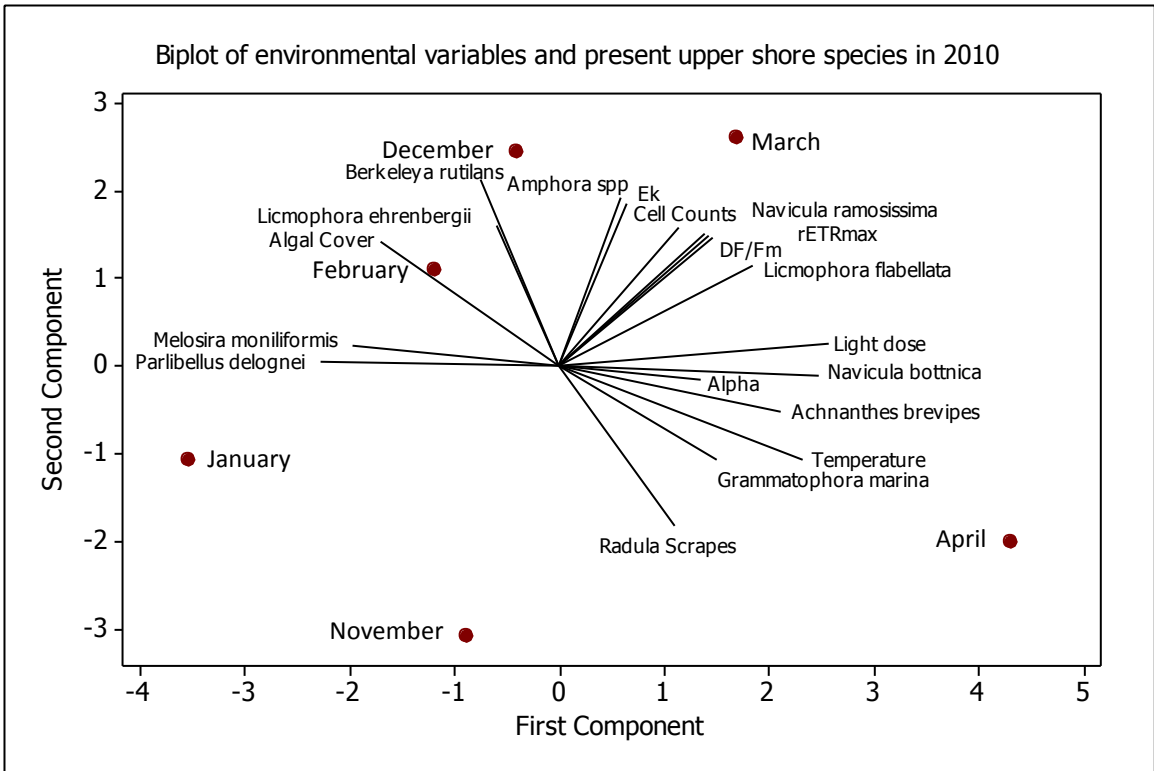


Figure 2.3.31: *Principal component analysis*, of photosynthetic parameters, biofilm taxonomic composition, and environmental factors at upper shore sites in 2010.

Lower shore 2010

100% of the data is explained by 5 principal components, and 66% explained by PC1 and PC2 (Fig. 2.3.32). November and December were characterised by high biodiversity and high cell counts. January and February were defined by high $rETR_{max}$ and by the species *Pinnularia viridis.* There was a linear correlation between the number of *Pinnularia viridis* and $rETR_{max}$, (r = 0.768, n=7, P < 0.05),April was defined by a low *Ek* level, and high light dose, temperatures and radula scrapes. The high numbers of *Navicula ramosissima* also define March and April. There was also a linear correlation between the temperature and the number of the radula scrapes (r = 0.791, n=6, P < 0.05).

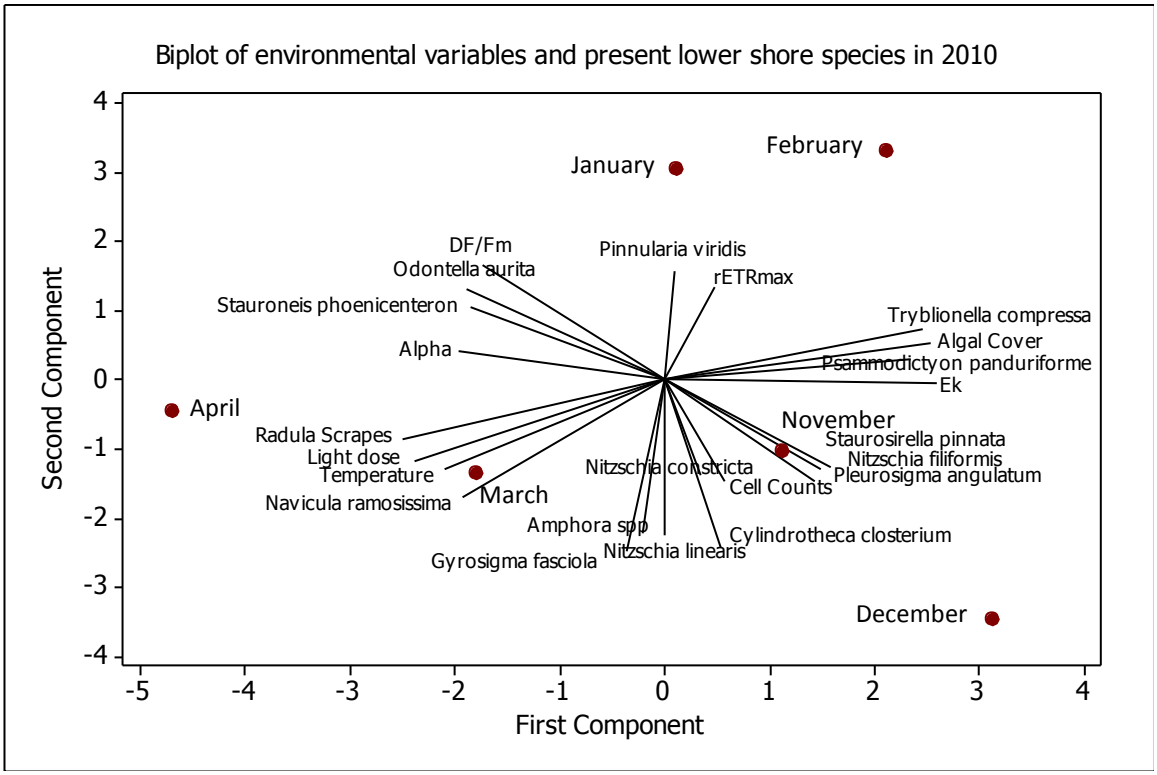


Figure 2.3.32: *Principal component analysis*, of photosynthetic parameters, biofilm taxonomic composition, and environmental factors at lower shore sites in 2010.

Upper shore 2011

100% of the data was explained by 5 principal components, and 75% explained by PC1 and PC2 (Fig. 2.3.33). Again similarly to 2009, November and December were clearly characterised by high $rETR_{max}$ and α, November was also characterised by a high *Ek,* January and February are characterised by high *ΔF/Fm'* and high algal cover and cell counts. In March and April, smaller species such as *Navicula bottnica* were present and a high light dose and temperature characterised these months. There was a linear correlation between the *Ek* and the number of radula scrapes (r = 0.881, n=6, P < 0.05)

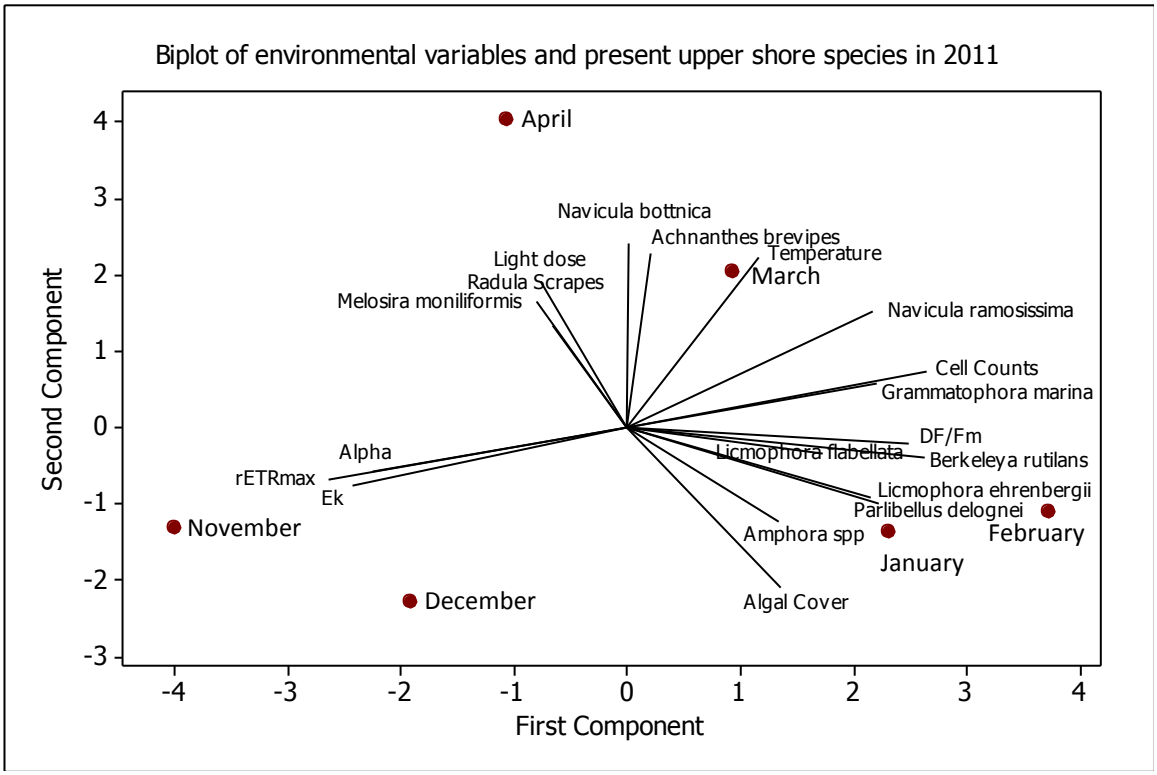


Figure 2.3.33: *Principal component analysis* of photosynthetic parameters, biofilm taxonomic composition, and environmental factors at upper shore sites in 2011.

Lower shore 2011

100% of the data is explained by 5 principal components, and 78% explained by PC1 and PC2 (Fig. 2.3.34). November was characterised by high $rETR_{max}$, α and levels. December was characterised by high *ΔF/Fm'* levels. High algal cover was observed in December, January and February and in contrast low percentage algal cover was observed in April. A cluster of species can be observed around January and February, indicating higher biodiversity during the winter months. March and April were again characterised by high light dose, *Ek* and a large number of radula scrapes. There was a linear correlation between the *Ek* and the number of radula scrapes (r = 0.763, n=6, P < 0.05)

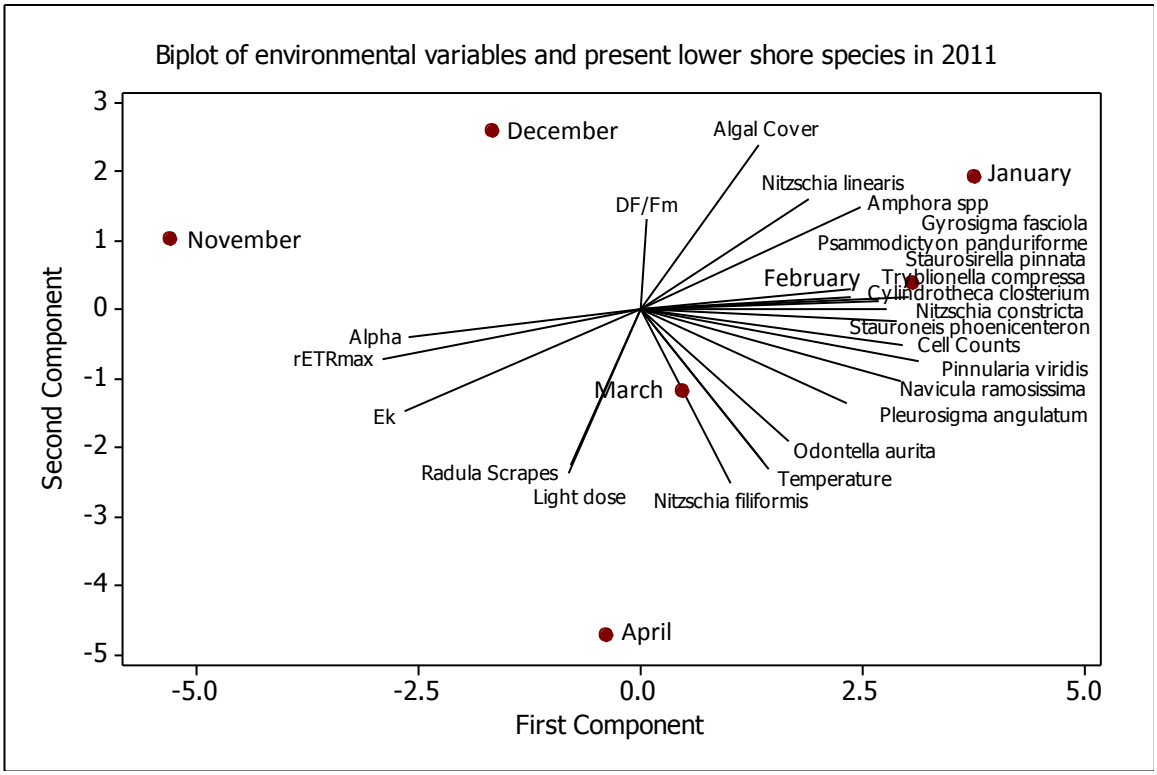


Figure 2.3.34: *Principal component analysis*, of photosynthetic parameters, biofilm taxonomic composition, and environmental factors at lower shore sites in 2011.

DISCUSSION

The effect of seasonal environmental factors on the biofilm photophysiology (Hypothesis 1)

It was hypothesised that seasonal environmental factors such as temperature and light would result in changes in the photophysiology of the biofilm cells. It was predicted that the $rETR_{max}$ would increase as light levels and temperature increased into the spring months. There did appear to be a significant effect of temperature on the $rETR_{max}$ recorded from the upper shore biofilms, with the highest $rETR_{max}$ measured during 2009 and 2010 occurring at median temperatures (Fig. 2.3.12). However, this relationship may not be as clear cut. When viewing the Principal Component analysis from 2009 and 2011 the high $rETR_{max}$ levels were recorded during the growth phases of the biofilm and not during months of median temperature. Both the upper and lower shore biofilms show this relationship. Cell replication and growth is known to increase the *rETR* of diatom cells (Chan 1980) so this may be influencing the electron transport rates of the cells in the biofilm. In 2010, there again appeared to be a relationship between temperature and $rETR_{max,}$ with the highest $rETR_{max}$ levels recorded during the median temperatures. At the upper shore sites the highest level was recorded in March, which was not during the growth phase of the biofilm, and this is supported by the PCA (Fig. 2.3.31) which shows that March was defined by a high $rETR_{max}$ and *ΔF/Fm*. By March, the biofilm was fully developed and had not yet undergone the heavy grazing which begins in April (Fig. 2.3.11). The light dose in March 2010 was higher than that recorded during the other measurement years and this resulted in a high *Ek* and high $rETR_{max}$ in 2010. This has also resulted in a greater percentage reduction in *Fm'* than previous months at the upper shore sites. In 2011, particularly low temperatures were recorded (-4°C, Table 2.3.1) which would be expected to slow the enzymes responsible for photosynthesis down and reduce reaction rates and so cause an imbalance between the absorption and utilisation of light (Anning *et al.* 2001), resulting in photoinhibition. This would be particularly expected in this case as high levels of $rETR_{max}$ were recorded. In the upper shore sites this may be the case as there was a low *ΔF/Fm'* recorded. There did not however appear to be a photoregulatory response with very little

downregulation being initiated (Fig. 2.3.28). In fact, a reversal in downregulation was recorded during the light curve. The cell counts were low during this time as the biofilms were only beginning to recover from the summer die-off period. This is illustrated in Figure 2.3.33 where the cell counts contributed to the spatial spread such that it separated from November. A high *α* was recorded during November and December, meaning that light utilisation was high, so in contrast to the results of Anning *et al* (2001) the low temperatures do not appear to be resulting in an imbalance between absorbed light and utilised light. The high level of utilisation may be because the cells require higher levels of carbon fixation in order to allow for increased growth; therefore, the cells would not downregulate their photosynthesis until sufficient carbon products had been synthesised (Perkins, *et al* 2001). Interestingly, despite the low light dose the month November was characterised by a high *Ek,* which may be as a result of the low algal cover, allowing more light to penetrate into the biofilm and causing the cells to become high light acclimated. At the upper shore sites, temperature had a significant effect on the *ΔF/Fm',* with a similar pattern of response being observed over the 3 measurement years. The lowest *ΔF/Fm'* was recorded during the extremes of temperature and this is clear from Figure 2.3.24. This low level at high temperatures was not expected, as research has shown that PSii has a thermally stable structure up to temperatures of 45 °C (De Las Rivas & Barber 1997, Nishiyama *et al*. 1997, Tang *et al*. 2007). However, as mentioned, temperature directly correlated with light dose and, as the 2010 and 2011 *Fm'* data clearly shows greater downregulation during the months where higher light dose was recorded, this will have caused a drop in *ΔF/Fm'*. In addition, Long *et al*., (1994) state that low temperatures allow the development of persistent photoinhibition, which can be decreased by a canopy effect. It is likely that as the biofilms are still developing a canopy will not mitigate this effect. Similarly, when grazers become active in April and May and begin removing the biofilm any canopy effect acting to self-shade the cells will be removed, resulting in an increased light level and further reduction in *ΔF/Fm'*. Surprisingly, upper shore *Ek* did not increase linearly as light levels increased. On closer inspection of Figure 2.3.22 and the upper shore PCAs (Figs. 2.3.29, 2.3.31,

2.3.33), there is a relationship between the light dose on the measurement day but also on the state of the biofilm (Table 2.3.1). In 2011, despite the low light dose, November was characterised by a high *Ek,* which may be as a result of the low algal cover, allowing more light to penetrate into the biofilm and causing the cells to become high light acclimated.

The measurement year had a significant effect on all of the photosynthetic parameters investigated. This was due to the variation in environmental conditions over the 3 years. The trends in environmental conditions within the year were similar with cold temperature and low light levels recorded in the winter months and high temperatures and light doses recorded in the spring months, causing the seasonality in community structure and biomass. The differences between the years resulted in photosynthetic differences which further supports the conclusion that the photophysiology of the cells was primarily influenced by the environmental conditions on the day of study.

The effect of grazing on the biofilm photophysiology (Hypothesis 2)

It was predicted that the grazing activity would have an effect on the photophysiology with an increase in $rETR_{max}$ due to the removal of detritus (Skov *et al.* 2010) and increased nutrients from excrement (Mccormick & Stevenson 1989). The removal of detritus did have an effect on the photophysiology and at both the upper and lower shore sites. The number of radula scrapes, and so grazing activity, resulted in a higher *Ek* level. This occurred in 2009 and 2011 and is evident from PCA's where there is an correlation of r = 0.913 (n=7, P < 0.05) and r = 0.881 (n=6, P < 0.05) respectively for the upper shore sites and r = 0.841 (n=7, P < 0.05) and r = 0.763 (n=6, P < 0.05) for the lower, between the *Ek* and the number of radula scrapes. However, the predicted increase in $rETR_{max}$ associated with grazing was not observed. Mccormick & Stevenson (1989) observed that increased nutrient availability due to the excrement production of the grazers (Mccormick & Stevenson 1989) resulted in an increase in cell replication in the biofilm, which in turn resulted in higher levels of $rETR_{max.}$ The algal cover

reduced when grazing increased in late March, and data from this field site did not support the findings of Mccormick & Stevenson (1989). Despite an increase in grazing there was no resultant increase in cell replication. In all the PCA plots (Figs. 2.3.29-2.3.34) there was spatial separation between the Radula scrapes and algal cover/cell counts. Grazing was having a negative impact on algal cover, which suggests that the biofilms were not able to replicate enough to replace the lost biofilm. It appeared that the increased grazing was not resulting in an increase in cell replication, possibly due to the increased light and temperature negatively affecting photosynthesis, and thus no increase in $rETR_{max}$ was observed. Cubit (1984) performed grazer isolation experiments on the high rocky shore of the Oregon coast (USA) and he found that herbivory was not the main driver controlling algal abundance on high rocky shore sites, rather, seasonal changes in rate of algal production, likely related to the favourable winter insolation and temperature and unfavourable summer conditions, were responsible for the reduction in algal cover seen in the summer months. He speculated that the high temperatures, desiccation and light levels in the summer months resulted in the cells being unable to replicate quick enough to compensate for the grazing. At these sites, the grazing pressure was greater in the winter months, which was not the case at Dunraven Bay. As such, it seems very likely that the unfavourable conditions reducing algal growth combined with the increased grazing resulted in the almost total loss of algal cover in the late spring and summer months.

Shore level differences in photophysiology (Hypothesis 3)

The lower shore sites are more similar to mudflat systems with the cells living in a mud matrix which was on average 6mm thick. The cells on the upper shore sites grow within tubes attached directly to the rock surface. It is possible to compare the trends of response and it was predicted that the lower shore sites would be more 'vulnerable' to extreme temperatures and light levels. This was predicted because the cells are exposed for shorter periods of time during the tidal cycle, thus it was thought that they would be less well adapted to the variable

and often extreme environmental conditions during emersion. A reduction in $rETR_{max}$ and *ΔF/Fm'* was expected at these extremes and it was expected that this reduction would be more pronounced in the lower shore sites. However, this was not the case, as the lower shore seemed far more resilient to extremes of temperature. In fact, it was apparent that there was very little effect of environmental conditions on the photophysiology of the lower shore sites. This was made clear in the PCA biplots. It is particularly apparent in the 2010 upper shore (Fig. 2.3.31) PCA plots that the spokes associated with light dose face a similar direction to the spokes associated with $rETR_{max}$ and *ΔF/Fm'*. The lower shore PCA biplots do not show this relationship. Unexpectedly, the lower shore biofilms sampled in April were defined by a low *Ek* level, despite the high light dose. It may be that cells on the lower shore can move into the sediment to regulate their photosynthesis, like those found in mudflat environments (Perkins, *et al*. 2010, Cohn 2001, Cohn *et al*. 2004, Consalvey *et al*. 2004, Apoya-Horton *et al*. 2006, Jesus *et al*. 2006). If this was the case then the cells would be able to move into the sediment to optimise their light exposure, and so they would not need to be high light acclimated. The photoregulatory behaviour and mechanisms will be investigated in Chapter 4. If the cells can utilise migration to move into the upper layers of sediment it would explain why there was very little effect of the external environmental conditions on the photosynthesis of the cells. There was a reduction in *Fm'* recorded from the lower shore sites in 2010 and 2011. It is possible that this is an indication of migration as opposed to downregulation as a result of high light exposure (Consalvey *et al*. 2004). Again, this will be examined in detail in Chapter 4. Notwithstanding the apparent negligible effect of environmental factors on the lower shore, the development stage of the biofilms had the same effect on photophysiology as was found at the upper shore sites. The highest $rETR_{max}$ and *ΔF/Fm'* measured during most years was recorded during the months of November and December when the cells were in their growth phases.

The combined effect of Abiotic and Biotic factors on the biofilm photophysiology (Hypothesis 4)

It was predicted in hypothesis 4 that combinations of environmental factors would influence the photophysiology and that in particular periods of combined extreme temperature and light dose would result in the greatest reductions in $rETR_{max}$ and *ΔF/Fm'*. The upper shore sites appear to support this with the spring months, where high light and temperature were recorded (Table 2.3.1 and Fig. 2.3.11), exhibiting low $rETR_{max}$ and *ΔF/Fm'*. In 2009 and 2010 the growth periods (November and December) were not the coldest months (Fig. 2.3.12). During these years the coldest months did result in the lowest upper shore $rETR_{max,}$ however, in 2011, when the growth months were during the coldest periods, the $rETR_{max}$ was very high. This suggests that the negative effects of extreme temperatures can be alleviated. However, at the upper shore sites there did not appear to be any factors which alleviated the negative effect of the high light and temperatures during the summer months. It is likely that this resulted in the biofilm die-off observed in May. It has been suggested by Perkins *et al*. (2001), that once cells have fixed enough carbon, downregulation of photosynthesis occurs to slow the rate of formation of radical oxygen and excess carbon products. Downregulation is being induced as is apparent from the *Fm'* data (Figs. 2.3.27 and 2.3.28), however, if enough carbon products are being produced, this is not resulting in a healthy and growing biofilm, so it is likely that the downregulation is being induced to prevent damage only, not because the cells have produced sufficient carbon products. As mentioned above, the lower shore sites were resilient and were less affected by environmental factors. However, the biofilms still sloughed off and disappeared in the summer months, suggesting that despite being able to maintain higher $rETR_{max}$ and *ΔF/Fm'* levels during periods of extreme temperatures, the biofilms are under stress and are unable to remain all year round on the rocky shore. High temperature and high light dose were consistently associated as is observable in the PCA biplots (Figs. 2.3.29-2.3.34) and these spokes are consistently spatially opposed to those associated with cell counts and

algal cover. It is clear that combined extremes of light and temperature result in less efficient photosynthesis and reduced biomass.

Seasonality of community structure and its' effect on biofilm photophysiology (Hypothesis 5)

This chapter contains a 3 year record of the seasonal variation in species composition of rocky shore biofilms. The upper shore sites were dominated by tube forming species particularly *Navicula bottnica* in the spring and *Berkeleya rutilans* in the winter months. This is in contrast to the lower shore where the majority of cells were free-living forms and were dominated by *Navicula ramosissima.* These cells live in a thin layer of sediment on the rock surface. The hypothesis that seasonal variations would be observed in the species composition of the biofilm has been partially supported. The upper shore biofilms show clear seasonality, whereas the lower shore biofilms are more stable, in terms of the biomass of individual species during the year. *Navicula bottnica* became the dominant diatom in the upper shore biofilm after March (Figure 2.3.9) and despite this dominance the diversity of the biofilm increased in the spring. The relative abundance of rarer species at the upper shore site increased from March (Fig. 2.3.9), which led to this increase in diversity (Table 2.3.3). However the evenness was the lowest during this time (Table 2.3.3). The relative abundance of the more common species reduced in response to the increase in *Navicula bottnica* after March (Fig 2.3.9). It may be that the environmental conditions in the spring favoured the rarer species, or that one of the more common winter species such as *Parlibellus delognei*, whose relative abundance numbers decreased in spring prevent the increase in biomass of the rarer species.

The most dominant species in the lower shore biofilm was *Navicula ramosissima*. The highest diversity was recorded in January and the lowest in March (Table 2.3.4). The increase in *Navicula ramosissima* occurred in March, suggesting that competition for resources and/or light was limiting the biomass of rare species (Hillebrand 2005). The evenness of the biofilms did not change markedly from month to month (Table 2.3.4). This suggests that the lower

shore sites were a more stable environment and that fewer environmental changes result in less taxonomic variation.

The peak biomass of both shore sites was recorded in the winter months, as opposed to early spring in mudflat systems (Sahan *et al*. 2007). As the taxonomic make-up of the lower shore biofilms are similar to mudflat biofilms (Colijn & Dijkema 1981, Underwood & Barnett 2006), this suggests that the rocky substratum was causing the biofilm to be less successful than mudflat biofilms during the spring months. It may be that the cells remain in refugia during the summer months (Bergey 1999). It is possible that the rock is preventing deep migration, which may be preventing successful photoregulation during the spring months and so restricting peak biomass to months with a lower light dose.

The grazing activity of *Patella vulgata*, estimated using radula scrape enumeration, increased in March (Fig. 2.3.11) most likely a result of an increase in water temperature (Jenkins *et al*. 2001). A reduction in the grazing species *Littorina saxatalis* and *Littorina littorea* occurred in late spring and this may be as a result of cyanobacterial growth in the spring. *Moorea producens* is known to cause contact dermatitis in humans because of a toxin produced by the algae (Osborne *et al*. 2001) and is also believed to cause poisoning in grazing species (Capper *et al*. 2005). There was an increase in this species when the diatom biofilm die-off occurs in April. Watermann *et al*. (1999) noted that at higher temperatures cyanobacteria out-competed diatoms on sandy sediment. Therefore, it is likely that the increase in light levels, temperature and grazing activity which reduced the diatom biofilm allowed *Moorea producens* to colonise the shore area.

As mentioned in Table 2.3.1, 'bleaching' events occurred in the late spring. After microscopic observations this phenomenon was not a result of chlorophyll bleaching but a result of cells moving out of the polysaccharide tubes. Houpt, (1990) suggested that as epiphytes are more

prevalent on polysaccharide tubes in the spring months, tube-forming cells may exit the tubes as the light levels become too low for photosynthesis. The epiphytes, however, did not completely conceal the cells inside the tubes at Dunraven Bay and, as the light levels were significantly higher in the spring months, this does not seem to be a plausible reason for the observation of tube-forming cells exiting the tubes in the spring months. Taken collectively, these results indicate that a combination of higher light levels and higher temperatures induce the cells to exit the tubes, rather than the low light levels caused by an increase in the growth of epiphytes in the spring.

The overall changes in the taxonomic makeup of the biofilms did not appear to influence the photophysiology other than the growth phase resulting in high $rETR_{max}$ and *ΔF/Fm'.* However the PCA biplots did appear to show certain species having an effect on the photophysiology. January and February 2010 were defined by high $rETR_{max}$ and by the species *Pinnularia viridis.* The high number of *Pinnularia viridis* correlated ($r = 0.768$, $n=6$, $P < 0.05$) with the high $rETR_{max}$ levels, however, this was not the case in other years and so may be coincidental. The clusters of species around the spokes associated with $rETR_{max}$ did appear to show that during the winter months and correspondingly high $rETR_{max}$ levels larger species were the most abundant. During the summer months, the $rETR_{max}$ levels were lower and smaller species became dominant during this period. *Navicula bottnica*, was the most dominant tube forming species found in the summer months and the cell size of this species is small.

CONCLUSIONS

This study provides new information about the seasonality of microphytobenthic species present on the rocky shore and the effect of herbivores on these important systems:-

- There were seasonal variations in the photophysiology of the cells which was primarily related to the reproductive phases of the biofilm, with higher $rETR_{max}$ and *ΔF/Fm'* exhibited in November and December.
- The lower shore biofilms were less affected by the external environment variables (principally light and temperature) and showed a less pronounced reduction in $rETR_{max}$ when temperatures and light dose increased in the spring. However, this resilience did not prevent the biofilms from degrading and sloughing off in the late spring.
- There is a clear shift from large cells (*Berkeleya rutilans* and *Parlibellus delognei*) to small cells (*Navicula bottnica*) in the summer months. Larger species do not survive when temperature and light dose increases in the spring.
- The overall clear changes in community structure of the upper shore biofilms, with *Navicula bottnica* dominant in the spring and *Berkeleya rutilans* in the winter, did not appear to affect the photophysiology, with the environmental conditions on the measurement day having a more pronounced controlling influence.
- The combination of the higher temperatures, higher light doses and the increased grazing pressure acted to reduce the biomass on the rocky shore and cause the late spring die-off observed at this site. Once the environmental conditions became more favourable (cooler temperatures and lower light dose) and the grazing pressure reduced, the biofilms re-grew in the autumn months.

The hypothesis that the photophysiology would be influenced by a collection of environmental factors, rather than one over-arching factor, such as light, has been supported by these results. It was hypothesised that there would be both short-term effects on the photophysiology, caused by transient and changeable factors such as light and temperature and trends in the photophysiology, $rETR_{max}$ for example, caused by seasonal factors, such as grazing pressure and biofilm development stage. This has also been partially supported by these results, and this medium-term study highlights the complex nature of the biotic and abiotic factors which

influence the photosynthesis and photoregulation of rocky shore microalgae. This study provides new information about the seasonality, in both photophysiology and taxonomy, of rocky shore microalgal biofilms.

THE EFFECTS OF TEMPERATURE ON THE PHOTOPHYSIOLOGY AND BEHAVIOUR OF BENTHIC ROCKY SHORE DIATOM DOMINATED BIOFILMS

CHAPTER 3

ABSTRACT

The tolerance to heat stress of rocky shore microalgal biofilms was investigated *ex-situ* using computer controlled environmental manipulation chambers, which maintained and monitored the temperatures within tidal simulation tanks. Upper and lower shore benthic biofilms were collected from Dunraven Bay in Bridgend County Borough, (South Wales), in both the spring and winter. The lower shore biofilms consist of free-living cells which live in a thin layer of sediment and the upper shore biofilms consist of polysaccharide tube-forming cells which are attached the rocky substratum. These were incubated under ambient light inside acrylic tidal simulation tanks. Due to degradation of the spring biofilms only the upper shore biofilm could be investigated. These samples were incubated at temperatures of 5, 10, 20, 25 and 30 °C for 48 hours. The lower and upper shore winter biofilms were investigated and were exposed to 5 and 10 °C temperatures for 52 hours and 15, 20, 25 and 30 °C temperatures for 60 hours. Biofilm samples which were cooled to temperature lower than was ambient on the day of removal, exhibited a greater reduction in $rETR_{max}$ than those exposed to increased temperature. The spring upper shore biofilms exposed to 25˚C and 30˚C temperatures induced lower levels of non-photochemical quenching (NPQ) than those exposed to 10 °C and 20 °C (average 21% reduction in *Fm'* compared to an average 29% reduction). NPQ is an enzyme dependent process and it is known that enzymes are susceptible to conformation changes under high temperature. The high temperatures during this experiment may have resulted in a loss of enzyme function, which in turn would limit NPQ induction. Cells in these biofilms were found to be capable of moving within their tubes. The movement was induced at temperatures above 14˚C and the speed of movement increased up to 32˚C at which point it then decreased. This movement may allow the cells to self-shade behind other cells and so reduce their exposure to high light, and hence may reduce the need for NPQ induction. The lower shore samples, being more similar to those found in mudflat systems, were known to move into the sediment surface. The lower shore samples were in general more resilient to both increased and decreased temperature, with smaller reductions in $rETR_{max}$ recorded (average of 12%

reduction over the exposure period, compared to an average 22% reduction in the upper shore biofilms). It is likely that the ability of the cells to migrate into the sediment reduced their exposure to the simulated temperatures, reducing the effect of them. This research provides new information about the effects of short–term temperature changes on rocky shore biofilms and complements previous studies which investigated the effect of temperature on the photophysiology of mudflat biofilms.

INTRODUCTION

Despite the ecological importance of rocky shore biofilms, no research has been carried out into the way in which environmental temperature influences the photosynthesis and behaviour of these biofilms. This study used the novel method of observing the behaviour of the biofilm cells at varying temperatures using a temperature controlled microscope stage. This was used in conjunction with PAM fluorescence analysis of the photosynthetic responses of the cells to different temperatures. Rocky shore areas are characterised by extreme changes in temperature, due to their tidal nature (Lewis 1964). During emersion, temperature can fluctuate very quickly despite the constant cover of water experienced by the biofilms at Dunraven Bay. This study increased our understanding of the way in which environmental temperature influences the behaviour and photophysiology of the diatom biofilms present on both the upper and lower shore of Dunraven Bay. For a detailed overview of Dunraven Bay, see the General Introduction (section 1.1.2).

Temperature and microphytobenthos

Temperature is known to influence overall biofilm photosynthesis in two ways; influencing photosynthetic rates (Colijn & Buurt 1975, Blanchard *et al*. 1996a, 1997, Guarini & Blanchard 1997, Serôdio & Catarino 1999, Herlory *et al*. 2007) or inducing changes in the species composition of the biofilm (Admiraal & Peletier 1980, Defew *et al*. 2004). Admiraal & Peletier (1980) showed that temperature has a direct impact on primary production in microphytobenthos cultures collected from the intertidal. Temperature affects the photosynthetic rate of microphytobenthos present on mudflats on a temporal scale of hours in addition to a seasonal impact. Defew *et al*. (2004) found that the biomass of the biofilm measured using F_o^{15} (minimum chlorophyll fluorescence, where cells had been dark adapted for 15 minutes (Honeywill *et al*. 2002)) could be sustained and increased after 21 days of incubation at temperatures of 10 °C and 18 °C, but the samples incubated at 26 °C suffered a

significant loss of biomass after only 14 days. This was attributed to nutrient limitation as the initial development of this heated biofilm was faster and by 14 days the biofilm was thicker than the others. In addition the $rETR_{max}$ was double that of the biofilm incubated at 10 °C prior to 14 days.

A distinct change in the community structure of the biofilms was observed at Dunraven Bay during the year (Chapter 2) and a spring die-off resulted in a period of very low algal cover, where both tube-forming and free-living cells disappeared during the summer months. This spring die-off has been observed by others including Hawkins & Hartnoll (1982), Cubit (1984) and Hill & Hawkins (1991). As the biofilms grow back in the autumn it may be that cells survive in refugia during the summer months (Bergey 1999) and when the environmental conditions become favourable again they may form colonies of tube structures and biofilms. There were several environmental changes which occurred during the spring/summer that may have induced this change in community structure and reduced the community biomass, including increased temperature and increased day length, and hence a significantly higher light dose experienced by the algae in summer. This may have caused some species to die as they were unable to regulate their photosynthesis sufficiently to reduce the damage caused by increased photodose (Barranguet & Kromkamp 1998, Serôdio & Catarino 1999, Perkins *et al*. 2001, Jesus *et al*. 2005, Pinckney & Zingmark 1991). The effect of temperature on European rocky shores has been investigated in the context of climate change. However these studies have focussed on macroalgal taxonomic changes (e.g. Lima *et al*. 2007) and grazer distribution (e.g. Simkanin *et al*. 2005, Mieszkowska *et al*. 2006, Skov *et al*. 2010). The effect of climate change on microalgal rocky shore biofilms is difficult to investigate as rocky shore biofilms cannot be maintained in the laboratory for more than a few weeks (pers. obs). However, short-term changes in temperature can be investigated through manipulation experiments by removing samples from the rocky shore and incubating them under controlled conditions.

In order to persist on the rocky shore, even for a short time, during spring, the cells must be able to dissipate excess energy from the photosystem or oxidative stress will cause damage to the cells (Muller *et al*. 2001, Krieger-Liszkay 2005, Serodio *et al*. 2005, Lavaud 2007). Diatoms use NPQ to help dissipate energy in the form of heat (Olaizola *et al*. 1994, Serodio *et al*. 2005, Serôdio *et al*. 2006, Lavaud 2007, Perkins *et al*. 2010). As NPQ is an enzymatic process, it is therefore possible that it may be influenced by temperature changes *in-situ* (Sizer *et al*. 1943, Palmer & Bonner 2007). Physiological photoregulatory methods are important to diatoms but are likely to be particularly important to those present on the upper rocky shore which are attached to rocky substratum and which cannot therefore move into the upper layers of sediment to shade themselves (Kromkamp *et al*. 1998, Patterson *et al*. 2001, Perkins *et al*. 2002) during periods of high irradiance.

Non-photochemical quenching (NPQ)

The Diadinoxanthin cycle allows energy to be dissipated as heat (Olaizola *et al*. 1994, Young *et al*. 1997, Lohr & Wilhem 1999, Lavaud *et al*. 2002, Goss *et al*. 2006, Goss & Jakob 2010). NPQ is composed of several different mechanisms. The quenching of the antenna by the diadinoxanthin cycle is the most widely known mechanism of NPQ (Young *et al*. 1997, Serodio *et al*. 2005, Dimier *et al*. 2007, van Leeuwe *et al*. 2008, Goss & Jakob 2010). Grouneva *et al*. (2008) suggest that in the diatom *C. meneghiniana* there are three clear aspects: the first begins on illumination and relies on the trans-thylakoidal proton gradient and the levels of light the cell is exposed to. This process is regulated by the level of Diatoxanthin in the cell prior to illumination. The second process occurs during prolonged exposure to high light conditions and is reliant on Diatoxanthin produced during light exposure by the Diadinoxanthin cycle. The third is a rapid relaxing process observable when cells are moved directly from high light to darkness. It again is reliant on Diatoxanthin synthesised during light exposure and occurs within seconds of transfer into darkness. The pH dependent activation of Diatoxanthin de-epoxidase and the trans-membrane proton gradient are required to initiate Diatoxanthin

non-photochemical quenching (Grouneva & Jakob 2006). Lavaud and Kroth (2006) suggest that production of Diatoxanthin and protonation of antenna complexes changes the shape of the antenna. To prevent relaxation of NPQ when a bulk proton gradient is not present, Diatoxanthin binds to hydrophobic regions of protein in the light harvesting complexes and dislocates proton-binding domains. Goss *et al*. (2006) support this by observing that if Diatoxanthin is activated, pH no longer affects the efficiency of NPQ. This complex strategy allows rapid regulation of NPQ and is an important photoregulatory strategy .

HYPOTHESES

This chapter investigates the effect of short-term exposures to different temperatures on the photophysiology of rocky shore microphytobenthos, adding to current knowledge of the effect of temperature on the intertidal zone, by testing the following hypotheses.

1) Lower shore biofilms are emersed for less time and therefore they may be less accustomed to fluctuating temperatures. The upper shore tube-forming diatoms are enclosed by a polysaccharide sheath and it has been speculated that this acts as a form of protection, isolating the cells from the environment and potentially protecting against unfavourable salinity and desiccation (Chastain & Stewart 1985) and potentially also the direct effects of high/low temperature. Therefore it was hypothesised that the upper shore biofilms will be more resilient to higher and lower than usual temperatures. The maximum relative electron transport rate ($rETR_{max}$) and the light acclimated quantum efficiency (*ΔF/Fm')* of the lower shore biofilms was predicted to exhibit a greater reduction on exposure to extreme temperatures than the upper shore biofilms. It was not possible to predict the extent to which this will be the case as no comparison has yet been made between the photophysiology of tube-forming and free-living rocky shore diatoms.

2) It was hypothesised that photosynthetic reaction rates will be controlled by variation in ambient temperature. In response to increased temperature, at both shore levels, it is predicted that there will be an initial increase in $rETR_{max}$ due to the increase in the chemical reaction rates which are known to occur as temperature increases. *ΔF/Fm'* was expected to remain stable as PSII is known to be thermally stable up to temperatures of between 38°C and 45°C (De Las Rivas & Barber 1997, Nishiyama *et al*. 1997, Tang *et al*. 2007). When the biofilms are cooled to temperatures lower than the usual field temperature it was hypothesised that the $rETR_{max,}$ and the *ΔF/Fm'* will reduce and remain suppressed. This is because reaction rates are slowed at cooler temperatures.

3) As the benthic diatom cells on the upper shore cannot move into the rock surface, in comparison to cells in the lower shore biofilms, living in shallow soft sediment substrata (see Chapter 2), they can likely only utilise NPQ as a means of photoregulation It was hypothesised that at the highest light levels of the light curve the upper shore cells will induce a greater level of NPQ than lower shore cells, and hence exhibit a large reduction in fluorescence yield *(Fm')*. It was also hypothesised that the magnitude of these changes will increase as a function of increasing temperature as cells are put under thermal and light stress.

Hypothesis 1 and 2 concerned the different responses to heat exhibited by the biofilms from the different shore levels. As it was not possible to perform the temperature manipulation experiments, during the spring months, on the lower shore sites hypothesis 1 and 2 do not apply to that experiment.

METHODS

Sample site and sampling

The samples used in this study were collected from Dunraven Bay in Southerndown, Bridgend Borough County ((51° 44.65'N, 03° 60.73'W) see Introduction section 1.1.2 for more information). The samples were collected from the sites which were used for monthly data records (exact locations can be found in the Chapter 2). Upper shore and lower shore samples were collected for use in the winter experiments. During the spring months the biofilms present on the lower shore were of insufficient density to register responses using the fluorometer. Samples from the lower shore were therefore not used in the spring experiments. The samples were removed using a hammer and chisel, stored in opaque containers, and moved quickly (within 1 hour) to tidal simulation tanks (Section 2.3.2.). Scrape samples were taken at the sites for taxonomic and behavioural analysis (Chapter 2 for full methodology). Site water was collected using 20L bottles to be used, unfiltered, in the tidal control tanks. The temperature was recorded at the time of sample removal using an infra-red thermometer (CEM, DT-8818H).

Tidal simulation tanks and climate control chambers

Tidal control chambers were built using the material listed below (Fig. 2.3.1 for configuration). The water collected from the sites was used to fill the lower reservoir tank, which was pumped into the sample tank during the immersion period. Even during the summer months, the pools at Dunraven Bay containing the biofilms remained inundated with water and so the tank containing the samples was never completely emptied during the experiment. A timer was applied to the water pump which allowed vigorous water flow, and an increased depth, to occur in the sample tank during what would have been high tides *in-situ*. This simulated the wave action of the tides as closely as possible in a laboratory environment. The depth of water cover during a spring high tide at Dunraven Bay is over 1m and this could not be simulated.

The water level was increased to 20cm during the simulated immersion period and reduced to 3cm during the emersion period, so as to, as closely as possible, simulate the influx of water experienced by the biofilms during immersion. The water was pumped into the sample tank using an aquarium water pump and plastic piping. The water was then drained using smaller tubes which could be sealed or opened to decrease or increase the flow rate of the water.

Figure 2.3.1 illustrates the construction of the tidal tank. Each tidal control tank contained the following equipment: two 30 cm x 25 cm translucent plastic containers, used as the reservoir tank and the sample tank. A 1 m piece of 15 mm diameter plastic tubing was attached to the Eheim compact 600 pump and was used to pump water from the reservoir tank to the sample tank. Four 15cm pieces of 4mm diameter tubing were used to provide variable drainage back into the tanks. The water temperature in the tanks was controlled by an Elite submersible heater (100 watt). The tidal tanks were placed in climate control chambers (Fig. 2.3.1), controlled using Doorway© software which regulates temperature (±2 °C) and humidity. These were then set to the desired temperature which was monitored using internal temperature sensors. The relative humidity was set at the ambient humidity for the time of year, with February being 87% saturation and 74% saturation in May. The chambers were located in a greenhouse and so samples were exposed to near ambient light. The glass attenuated the external light and the highest light level recorded was 650 µmol m^{-2} s^{-1}. No supplementary lighting was used so the spectra experienced by the cells was similar to that of the natural light.

Tidal Tank configuration

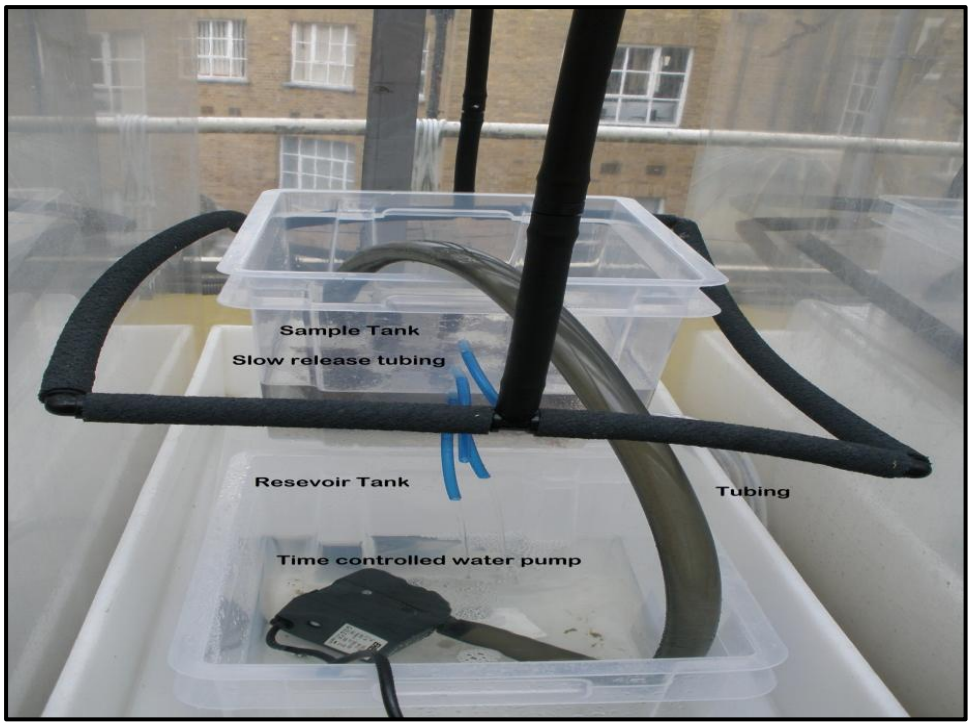


Figure 2.3.1: *Tidal control tanks inside climate control chambers*

Climate control chambers

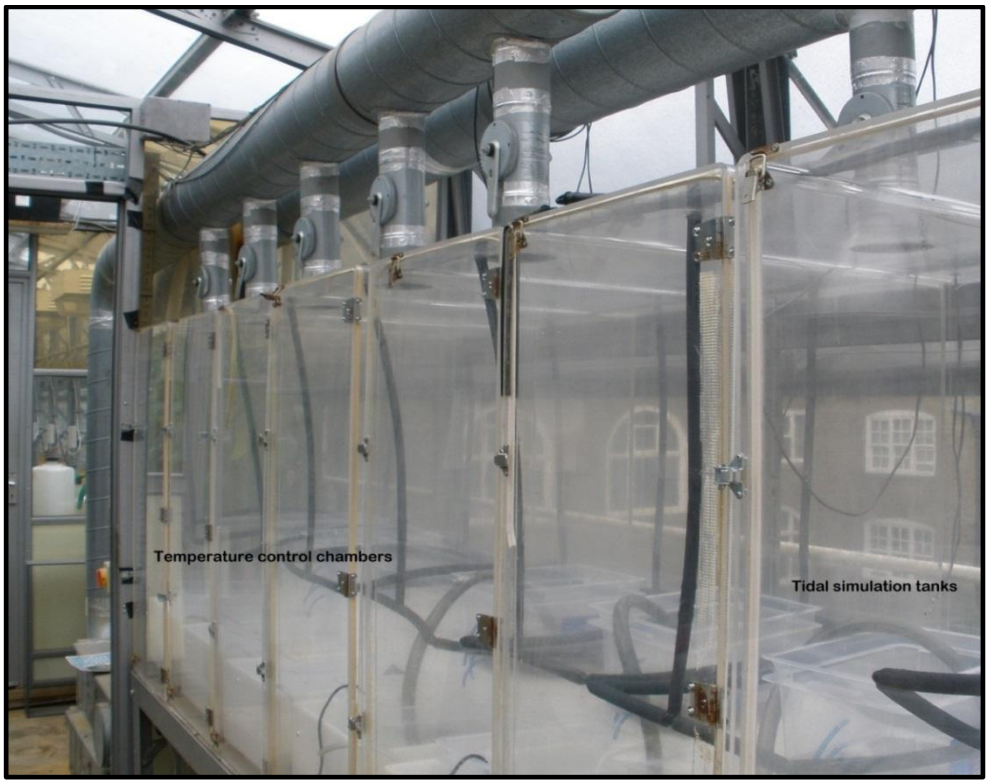


Figure 2.3.2: *The climate control chambers,* containing the tidal simulation tanks

Fluorescence measurements

Rapid light response curves were obtained using a Waltz Water PAM fluorometer. The measurements of each light curve step were taken at 30 second increments, consistent with the methodology of Perkins *et al*. (2006). This can also be reviewed in more detail in Section 1.5.3 of the General Introduction. The light curve light levels were set to 10 – 2975 µmol m^{-2} s^{-1} (PAR). These light levels were decided upon based on the on-going seasonal data collection at these sites, which suggested that these light levels would guarantee saturation and reduction of the relative electron transport rate (*rETR*) in all treatments. Light curve measurements were performed in a random order (determined by random number allocation to samples) amongst the temperature treatments. Three measurements were taken from each temperature treated biofilm. A Walz spacing piece was used to ensure that a fixed distance (2 mm) was maintained from the biofilm. This was used to prevent hand movements altering the fluorescence measurements. The fluorescence parameters used in this investigation were *Fm'*, *ΔF/Fm'*, and *rETR*. The analysis of this data was consistent with that described by Perkins *et al*. (2006). The iterative solution for curve fitting created by Eilers & Peeters (1988) was used to calculate the coefficients relating to *α*, *Ek*, and $rETR_{max}$ (see General Introduction 1.5.2 for more detailed information), using Sigmaplot V11 to calculate the fitted regression.

Statistical analysis

Levene's test was used to test for equal variance and the Anderson Darling test was used to test for normality. When data were not normal with unequal variance and unbalanced a Kruskall-Wallis test was used. When data were normal and variances equal but data too unbalanced, a nested ANOVA was not possible so a one-way ANOVA was used to examine the effect of exposure time and temperature. A student t-test was used to examine the effect of shore level on the $rETR_{max}$. When the data were balanced a nested 3 factor ANOVA was used for the winter experimental analysis, with temperature nested within time nested within shore level. This resulted in either 4 temperature (15, 20, 25 and 30 °C) measurements nested within

13 time points nested within 2 shore levels or 2 temperature measurements (5 and 10 °C) nested within 11 time points, within 2 shore levels. For analysis of the spring experiments a nested 2 factor ANOVA was used with temperature nested within time. This resulted in 5 temperature measurements nested within 11 time points. Data transformations (e.g. Log (n+1) and arcsine (sqrt x) (Zar, 1984) were attempted, but did not affect normality of heteroscadescicity.

Cell behavioural observations at different temperatures

The speed (µm s^{-1}) and pattern of cell movement was recorded by observing the cells using a light microscope (Olympus 1600). A microscope stage with controllable temperature (Biostage 600) was used to maintain the slides at a known temperature for behavioural investigation and to ensure that the slide was not heated by the microscope light source. The light source was an incandescent bulb with a wavelength output range of between 300-1200 nm, with the majority of the output being between 600-1200 nm. The cells were exposed to a high light level of 1430 µmol m^{-2} s^{-1}, whilst on the microscope stage. The cells were exposed to the same light levels for 5 minutes at temperatures ranging from 5°C to 40°C increasing in 1°C increments. Videos were made of the cells at the different temperatures using a Sony video camera (Handycam HDR-XR520), and the speed of movement was estimated using the microscope 16 mm graticule. Care was taken to effectively line up one of the polysaccharide tubes with the graticule. Observations were made using a single tube that was not tightly packed to allow the cells freedom of movement. The movement speeds were measured in selected tubes, as from initial observations it was found that tubes with very dense cell numbers either did not exhibit movement or the movement was sporadic as cells became trapped and had to manoeuvre in order to re-start movement along the tube. The tubes selected were those that on average contained less than 200 cells in a two dimensional plane. A single cell was tracked across the graticule at each temperature. A cell was selected and once it had passed onto a known point on the graticule the stopwatch was started and stopped when the cell reached the other side.

Different cells were used for each temperature observation, as the cells had passed out of the field of view. A single observation was made at each temperature using a single cell chosen because it had reached the start of the graticule point once the temperature had been achieved.

Sampling schedule: Spring temperature manipulation experiment

The spring biofilm samples were removed from upper shore sites at Dunraven Bay. The temperature on removal was 17 ˚C. This highest temperature on the removal day was 21 °C with the average temperature being 16 °C. Scrape samples were taken, per the methods in Chapter 2, for taxonomic analysis. Fifteen rock chips covered in biofilm were then removed from the shore and taken to the lab. Three chips were placed in each tidal tank and prior to any temperature manipulation one light curve was taken from each chip, to assess the 'baseline' photosynthesis. The samples were maintained for 48 h at 5, 10, 20, 25 and 30˚C and one light curve was taken from each of the three rock chips at the exposure periods noted in (Table 3.2.1). These values were then averaged. At T10 (48 hours (h) of exposure) the samples were returned to a pre-manipulation temperature of 17˚C, a process which took 2 h, and final fluorescence measurements were made.

Table 3.2.1: *Sampling schedule,* the table indicates the time periods at which the fluorescence measurements were taken and the time of day that this occurred.

Measurement code	Exposure time (h)	Time of day
T0	0	10am
T1	2	12pm
T2	6	4pm
T3	10	8pm
T4	20	6am
T5	24	10am
T6	28	2pm

T7	32	6pm
T8	36	10pm
T9	46	8am
T10	48	10am

Sampling schedule: Winter temperature manipulation experiment

The winter biofilm experiments were performed on different days, as they had to be separated into cooling and heating experiments due to the large number of chambers required because the manipulations were performed using both upper and lower shore samples. The temperature on removal was 12˚C and upon return to the lab the samples were briefly exposed to a room temperature of 20˚C which must be taken into account when analysing the results. The same sample removal and fluorescence measurement strategy was used as described above in the spring temperature manipulation experiments. The upper and lower shore samples were exposed to 5 and 10˚C temperatures for 52 h (Table 3.2.2). Separate samples were collected using the same method and then exposed to 15, 20, 25 and 30˚C temperature for 60 h (Table 3.2.3). Again the samples were returned to the pre-exposure temperature (12 °C) and final fluorescence measurements taken.

Table 3.2.2: *Sampling schedules,* the time periods at which the fluorescence measurements were taken and the time of day that this occurred.

Measurement code	Exposure time (h)	Time of day
T0	0	11am
T1	4	1pm
T2	8	5pm
T3	12	9pm
T4	22	7am
T5	26	11am
T6	30	3pm
T7	34	7pm

T8	44	11pm
T9	48	9am
T10	52	11am

Table 3.2.3: *Sampling schedule,* the time periods at which the fluorescence measurements were taken and the time of day that this occurred.

Measurement code	Exposure time (h)	Time of day
T0	0	10am
T1	4	2pm
T2	8	6pm
T3	12	10pm
T4	22	8am
T5	26	12pm
T6	30	4pm
T7	34	8pm
T8	44	6am
T9	48	10am
T10	52	2pm
T11	56	6pm
T12	60	10pm

RESULTS

Temperature manipulation cell observations (upper shore spring biofilms)

Cells within the loosely packed tubes were identified to be *Navicula bottnica*. These did not move within their tubes at temperatures lower than 14°C (Fig. 3.3.1). A single cell was observed moving in a single tube. The cell movement speed (μms^{-1}) increased as temperature increased to 32°C. The cell movement speed then decreased to 40°C which was the highest temperature investigated. The highest speed ($\mu m\ s^{-1}$) was recorded at 32 °C. The winter biofilms were observed in the same way but no movement was seen at any of the temperature exposures.

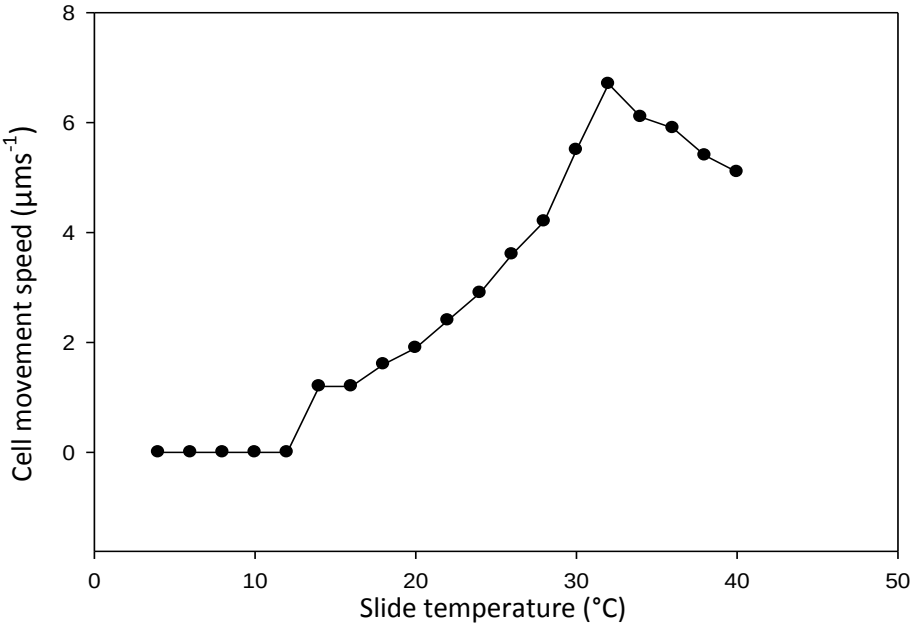


Figure 3.3.1: *Cell movement speed,* for upper shore spring diatoms, with relation to temperature under constant light conditions. *N=1*

Cell movement did not appear to be random during the observations. Figure 3.3.2 is a diagrammatic representation of the movement of the cells during the temperature exposures. The cells on the outside of the tube tended to move upwards towards the tip. Once at the tip they then moved into the centre of the tube and began to move down the tube towards the base. This cycling type of movement was not observed in all tubes but was seen in the majority of those observed.

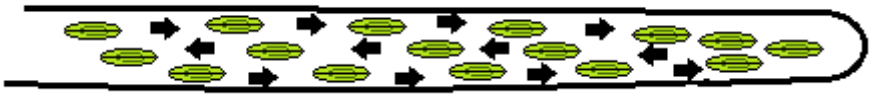


Figure 3.3.2: *Cell movement diagram,* of the cell movement frequently exhibited by the tube forming diatoms on the upper rocky shore. Made using Coral Draw (2.7).

Spring temperature manipulation experiments (upper shore only)

Maximum relative electron transport rate (*rETR$_{max}$*)

Temperature had a significant effect on the *rETR$_{max}$* (F=20.49, df = 4,164, p < 0.001) recorded from the upper shore biofilms in the spring, with the 20 and 25 °C treated biofilms exhibiting consistently higher *rETR$_{max}$* levels (Fig. 3.3.3). The lowest *rETR$_{max}$* levels were recorded from the 5 and 10 °C biofilms. The exposure time did not have significant effect on the *rETR$_{max}$* levels with no discernible patterns observed in the 5, 20 and 25 °C treated biofilms. The *rETR$_{max}$* of the biofilms exposed to 10 and 30°C, reduced consistently as the exposure period increased. The *rETR$_{max}$* of the biofilm exposed to 10 °C temperatures increased at the end of the exposure period.

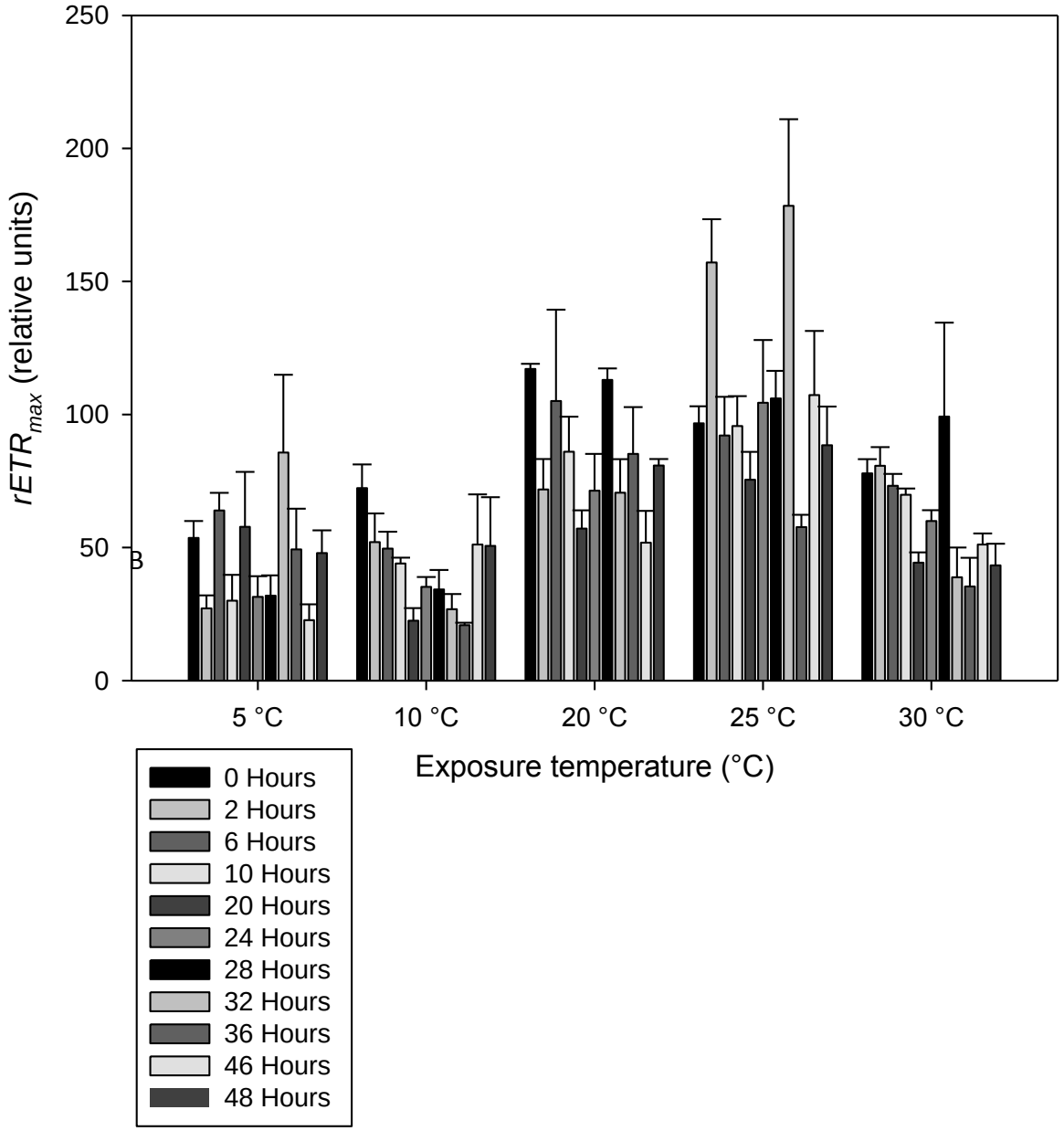


Figure 3.3.3: *Maximum relative electron transport rate* (*rETR$_{max}$*), of each temperature treated biofilm from the upper shore, recorded at preselected time points over the course of 48 h. Mean ± SE, *n=3*

Light utilisation coefficient (*α*)

There was no significant effect of temperature or exposure time on the *α*, however it does appear that the colder temperature (5 and 10 °C) were displaying lower *α* levels (Fig. 3.3.4). Despite exposure time not having a significant effect on the *α* there does appear to be a reduction in the *α* recorded from the 30 °C treated biofilm as exposure time increases.

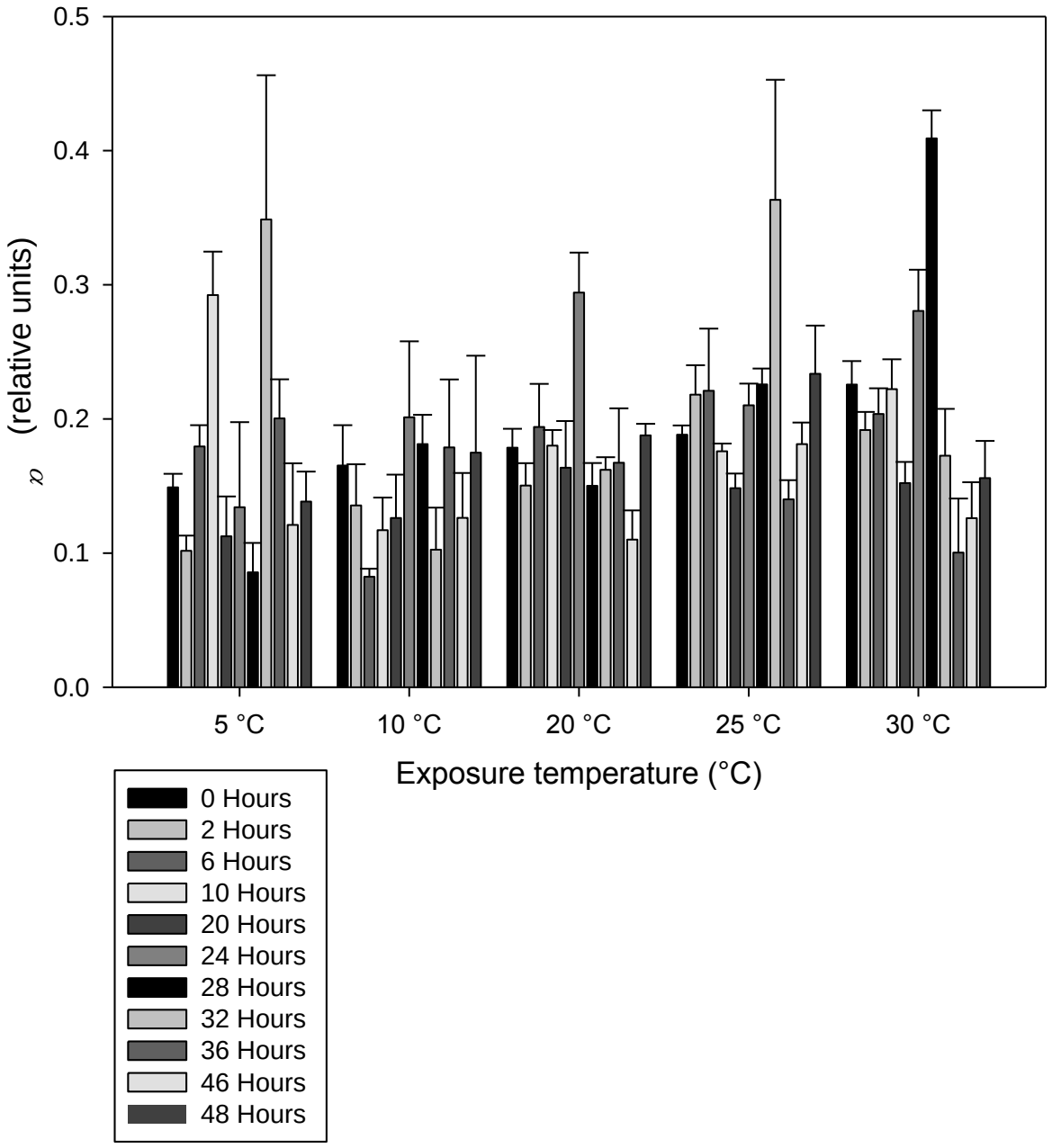


Figure 3.3.4: *Light utilisation coefficient (α),* of each temperature treated biofilm from the upper shore, recorded at preselected time points over the course of 48 h. Mean± SE, *n=3*

Light saturation coefficient (*Ek*)

As expected the *Ek* displayed a similar pattern of response to the $rETR_{max}$ (Fig. 3.3.5), with a significant effect of temperature observed ($F = 10.63$, $df = 4,164$, $p < 0.001$) . Again the 20 and 25 °C treated biofilms exhibited the highest *Ek*. The 5, 10 and 30 °C treated biofilms exhibited lower *Ek* levels. The exposure time had no significant effect on the *Ek,* however the *Ek* did appear to be reducing during the exposure period in the 25 and 30 °C treated biofilms.

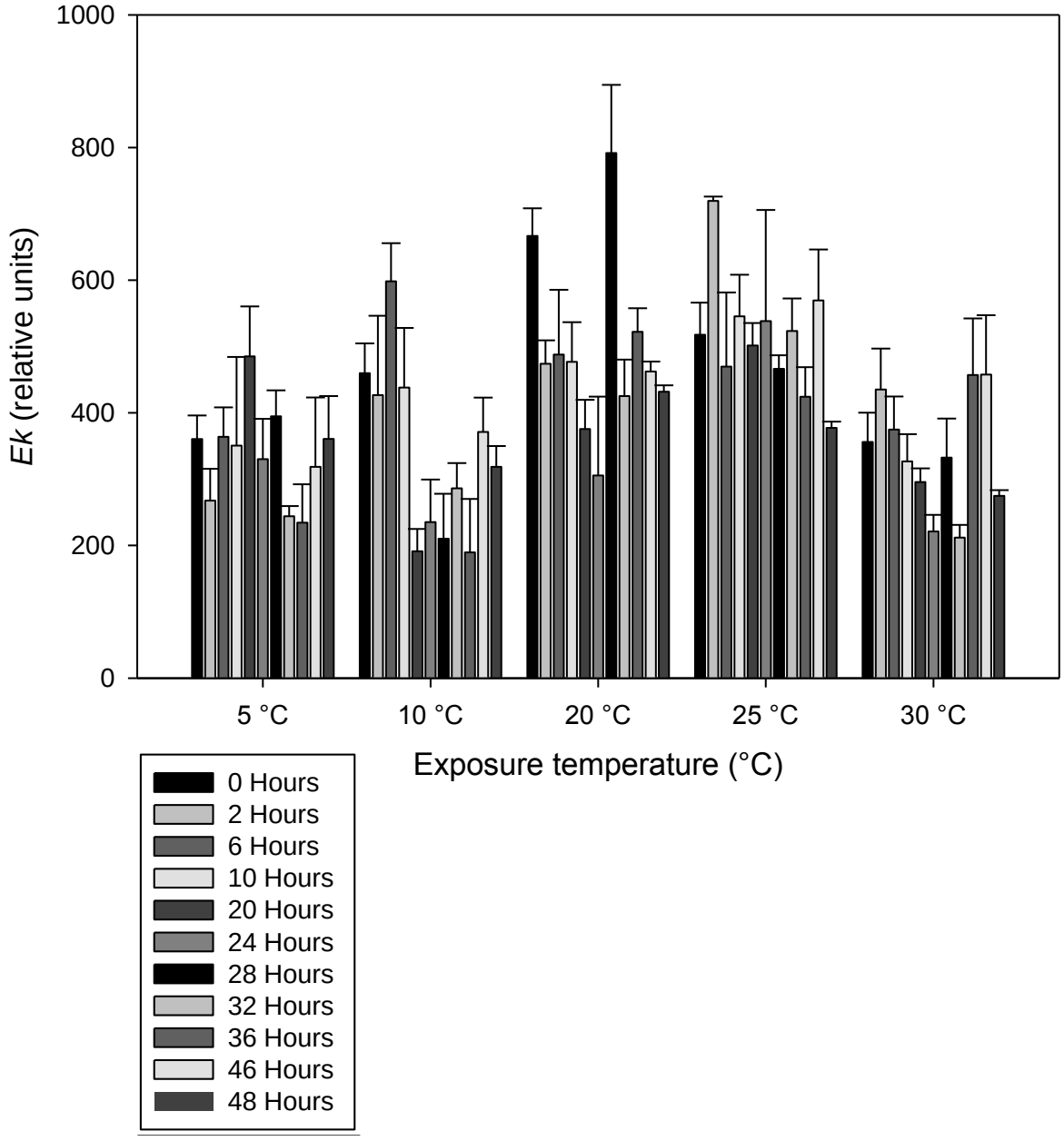


Figure 3.3.5: *Light saturation coefficient (Ek),* of each temperature treated biofilm from the upper shore, recorded at preselected time points over the course of 48 h. Mean± SE, *n=3*

Light adapted quantum efficiency of PSII (*ΔF/Fm')*

Exposure time had a significant effect on the *ΔF/Fm'* ($F = 2.28$, $df = 10,164$, $p < 0.05$) with a general trend of reducing *ΔF/Fm'* as the exposure time increased (Fig. 3.3.6). A recovery of the *ΔF/Fm'* was observed at the final measurement time once the samples had been cooled in the 25 and 30 °C treated biofilms. There was a marginally insignificant effect of temperature on the *ΔF/Fm'* ($F= 2.38$, $df = 4,164$, $p = 0.054$).

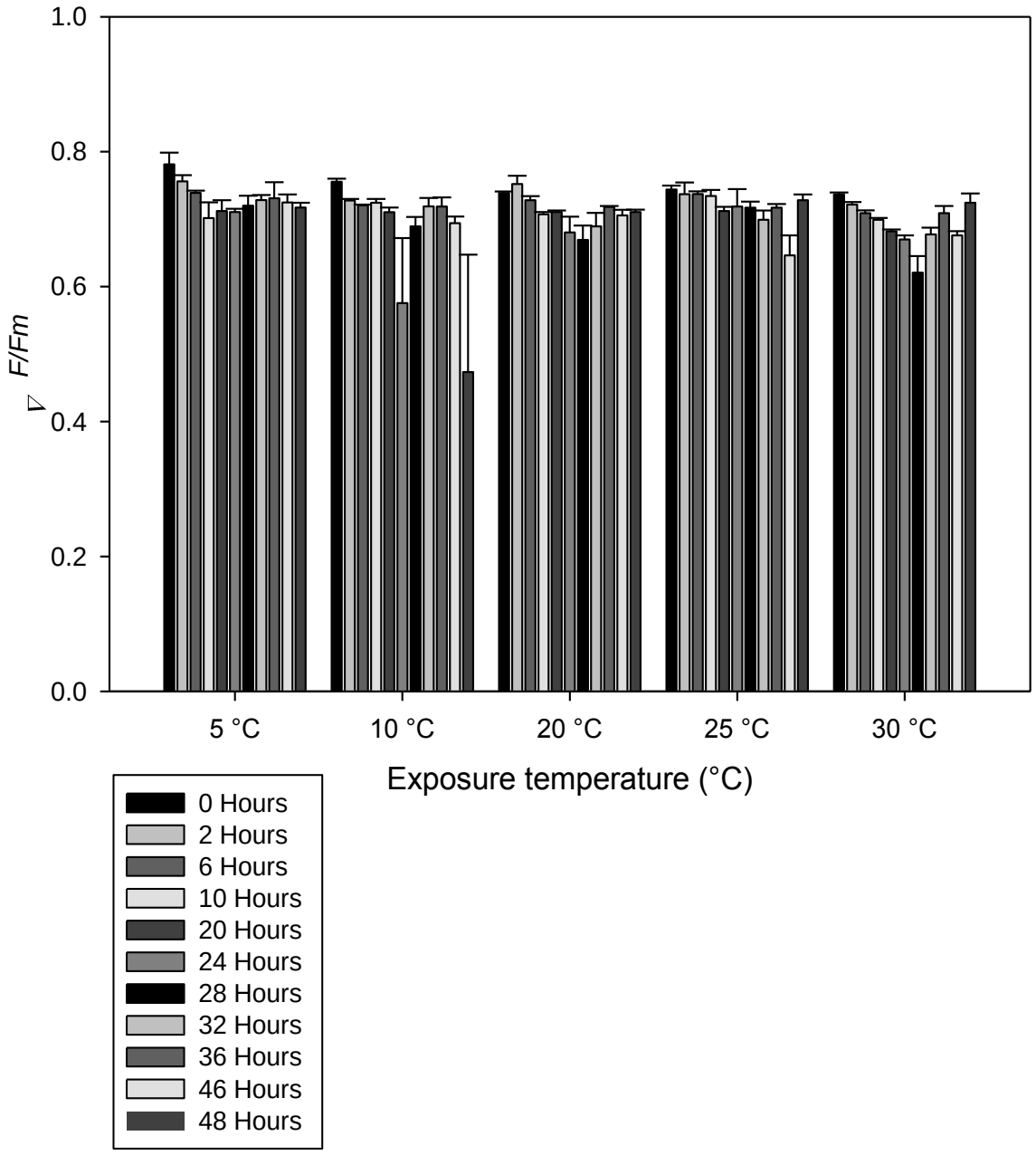


Figure 3.3.6: *Light adapted quantum efficiency of PSII (ΔF/Fm'),* of each temperature treated biofilm from the upper shore, recorded at preselected time points over the course of 48 h. Mean± SE, *n=3*

Downregulation of photosynthesis (Percentage change of Maximum fluorescence (*Fm'*))

At all temperature exposures the greatest reduction in *Fm'* occurred from 0 µmol m^{-2} s^{-1} to 603 µmol m^{-2} s^{-1}. In general, there was then a further smaller reduction from 603 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1} (Figs. 3.3.7 – 3.3.11). The *Fm'* recorded from the 5 ° C was reducing as light levels in the light curve increased at all but hours 0, 10, 32 and 46 (Fig. 3.3.7). The *Fm'* recorded from the 10 °C treated biofilms was reducing as light levels increased at all but the final 48 hour measurement once the sample had been returned to the pre-exposure temperature. At this exposure time there was an increase in *Fm'* from 0 µmol m^{-2} s^{-1} to 603 µmol m^{-2} s^{-1}. This was followed by a reduction in *Fm'* from 603 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1}. There was a greater reduction in *Fm'* observed mid exposure at the 10 and 20 hour exposure points (Fig. 3.3.8). The *Fm'* recorded from the 20 °C treated biofilm reduced as light level increased at all exposure periods (Fig 3.3.9). In contrast to the 10 °C treated biofilm (Fig. 3.3.8) the smallest reduction in *Fm'* recorded from the 20 and 25 and 30°C treated biofilms occurred mid exposure (Figs. 3.3.9, 3.3.10, and 3.3.11). The *Fm'* recorded from the 25 °C treated biofilms reduced as light level increased at all exposure periods (Fig. 3.3.10). The *Fm'* recorded from the 30 °C treated biofilms reduced as light levels increased at all exposure periods (Fig. 3.3.11). There was a similar pattern of response observed in the 25 °C biofilms with the 30 °C treated biofilm exhibiting a smaller reduction in *Fm'* after 24 h (Figs. 3.3.10 and 3.3.11).

The reduction in *Fm'* from 0 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1} was averaged over the 3 measurements. This average reduction was plotted and temperature had a significant effect on the reduction ($F = 6.59$, $df = 4,164$, $P < 0.05$), with the 5 °C, 25 °C and 30 °C treated biofilms exhibiting significantly smaller reductions in *Fm'* (Fig. 3.3.12). This is particularly evident after 24 h and the exposure period had a significant effect on the reduction in *Fm'* ($F=7.92$, $df = 10, 164$, $P < 0.05$).

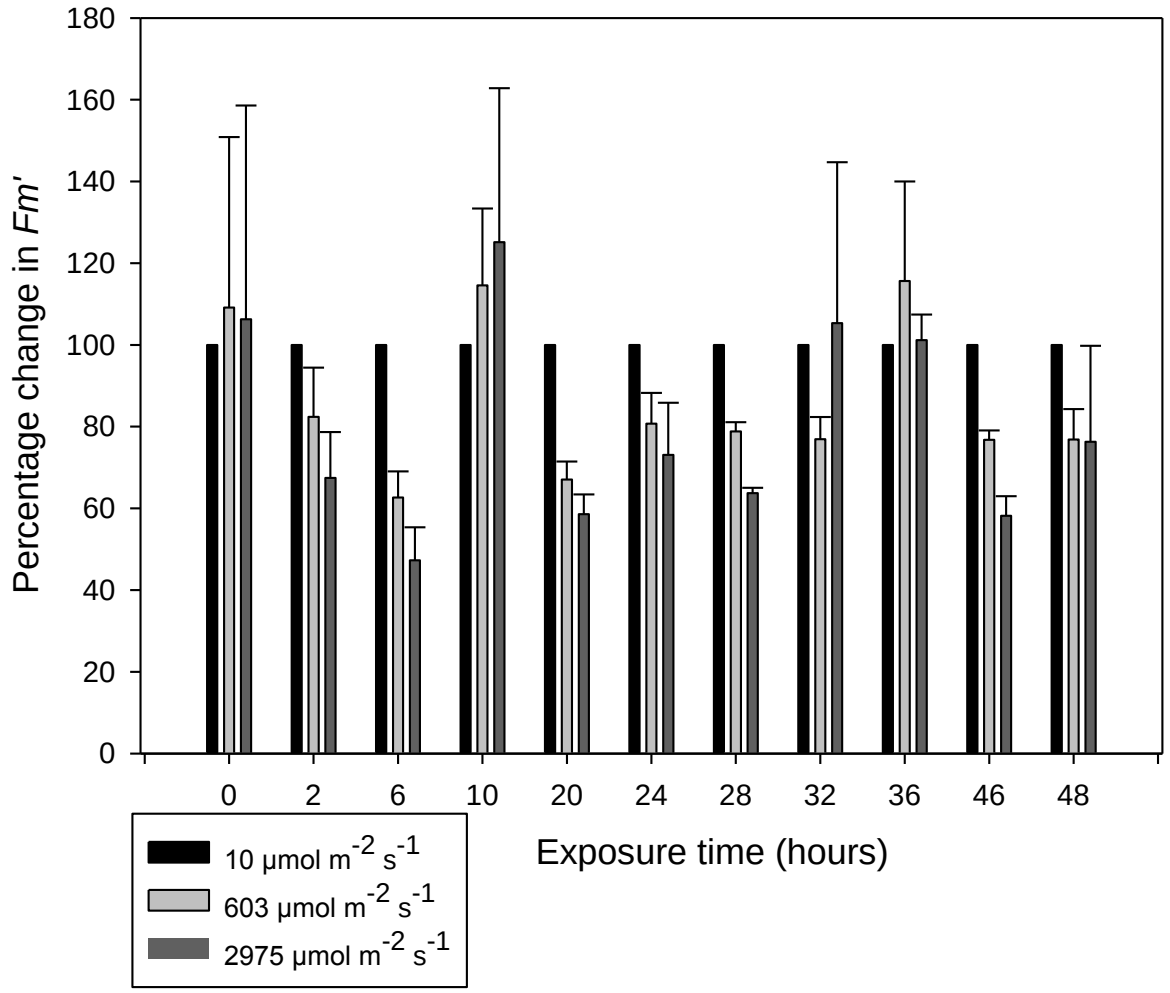


Figure 3.3.7: *The percentage change in Maximum fluorescence,* recorded from the 5 °C treated upper shore biofilm recorded at preselected time points over 48 h of exposure. Mean± SE, *n=3*

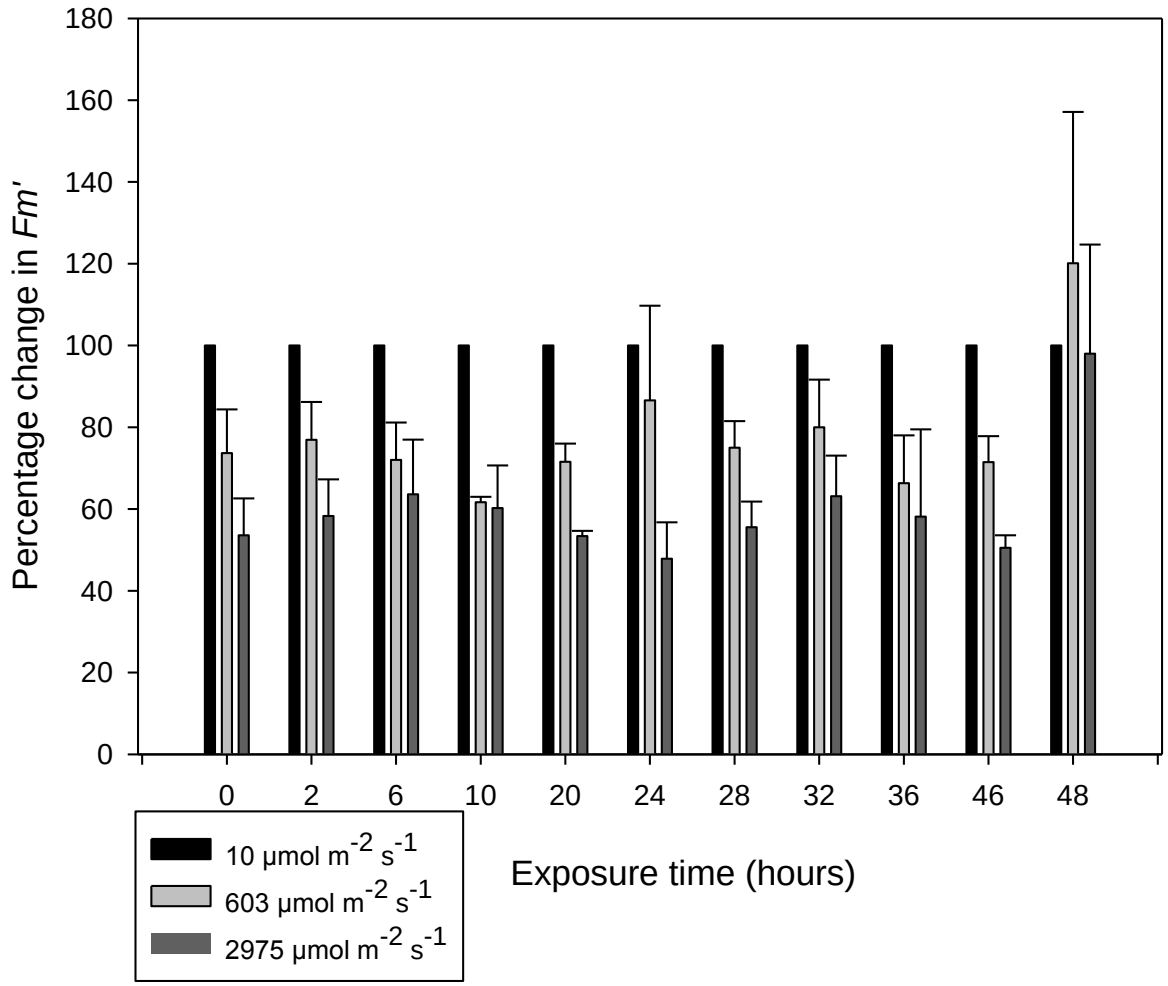


Figure 3.3.8: *The percentage change in Maximum fluorescence,* recorded from the 10 °C treated upper shore biofilm recorded at preselected time points over 48 h of exposure. Mean± SE, *n=3*

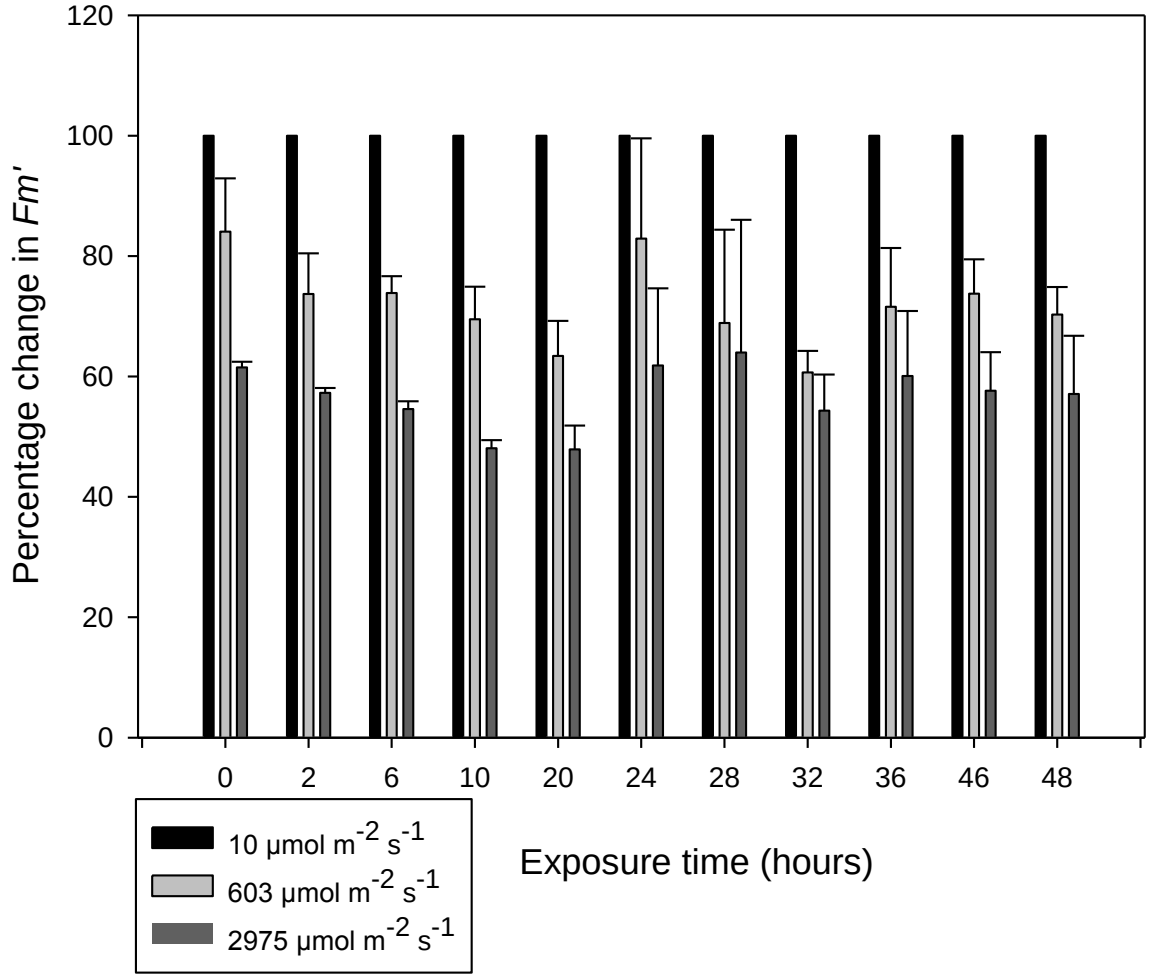


Figure 3.3.9: *The percentage change in Maximum fluorescence,* recorded from the 20 °C treated upper shore biofilm recorded at preselected time points over 48 h of exposure. Mean± SE, *n=3*

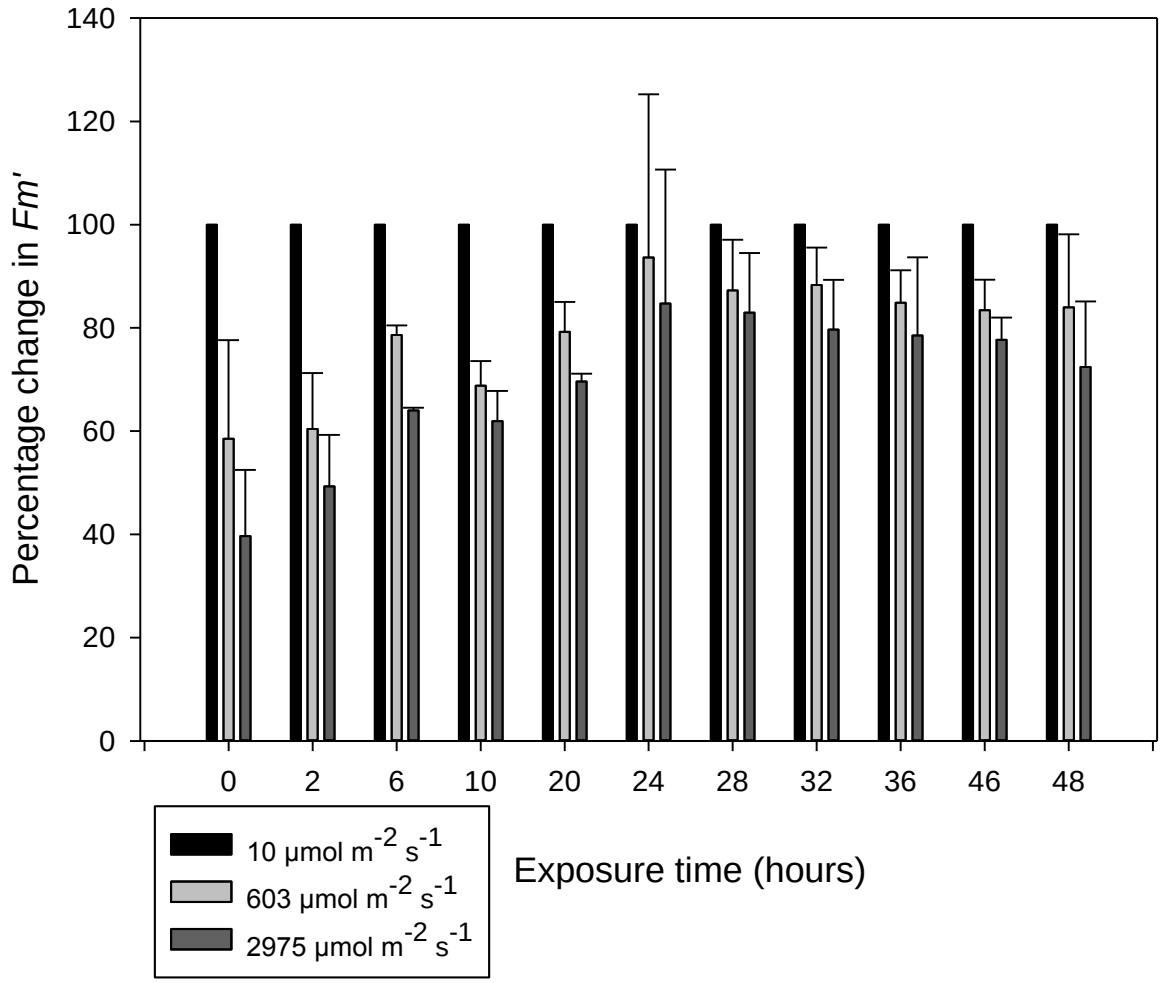


Figure 3.3.10: *The percentage change in Maximum fluorescence,* recorded from the 25 °C treated upper shore biofilm over recorded at preselected time points 48 h of exposure. Mean± SE, *n=3*

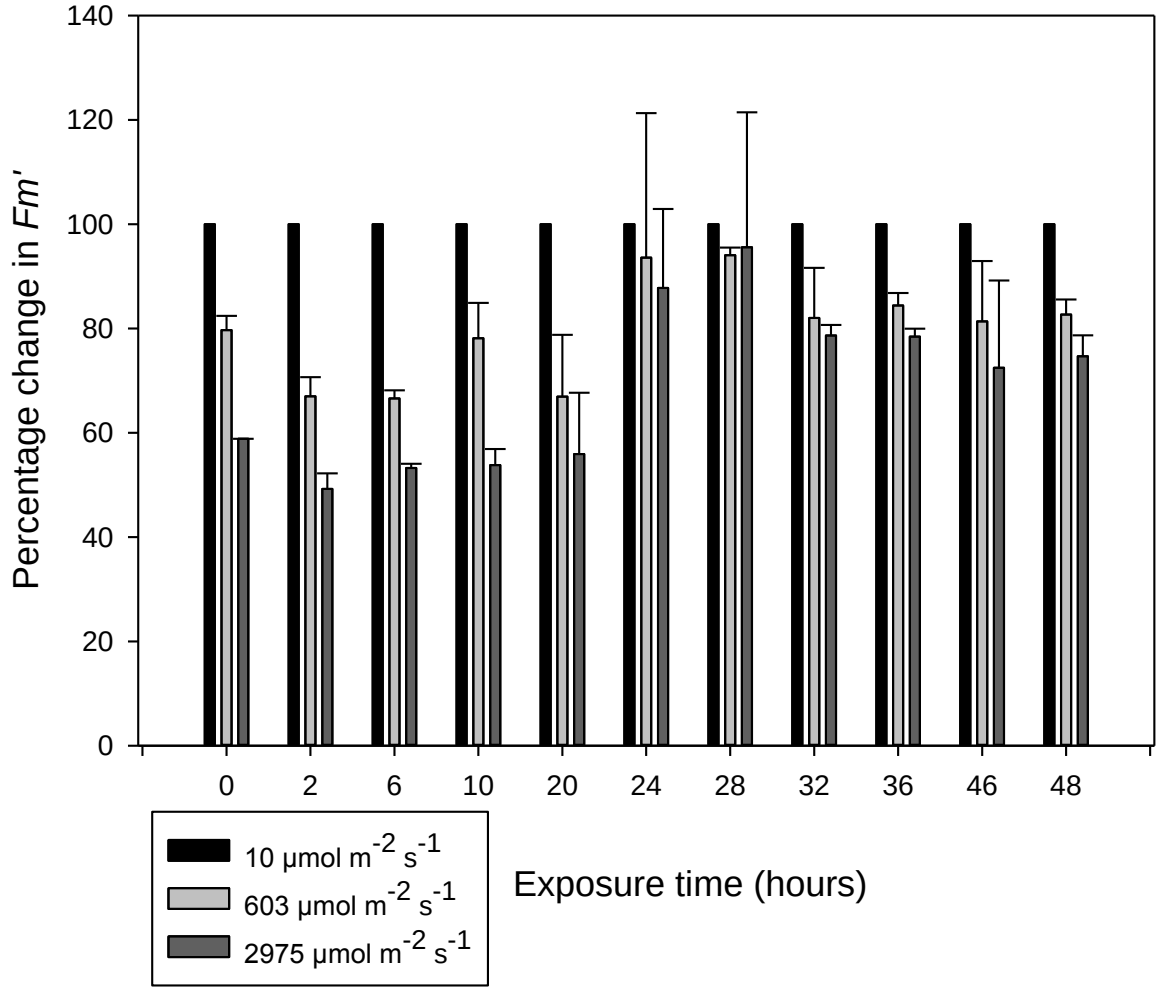


Figure 3.3.11: *The percentage change in Maximum fluorescence,* recorded from the 30 °C treated upper shore biofilm over recorded at preselected time points 48 h of exposure. Mean± SE, *n=3*

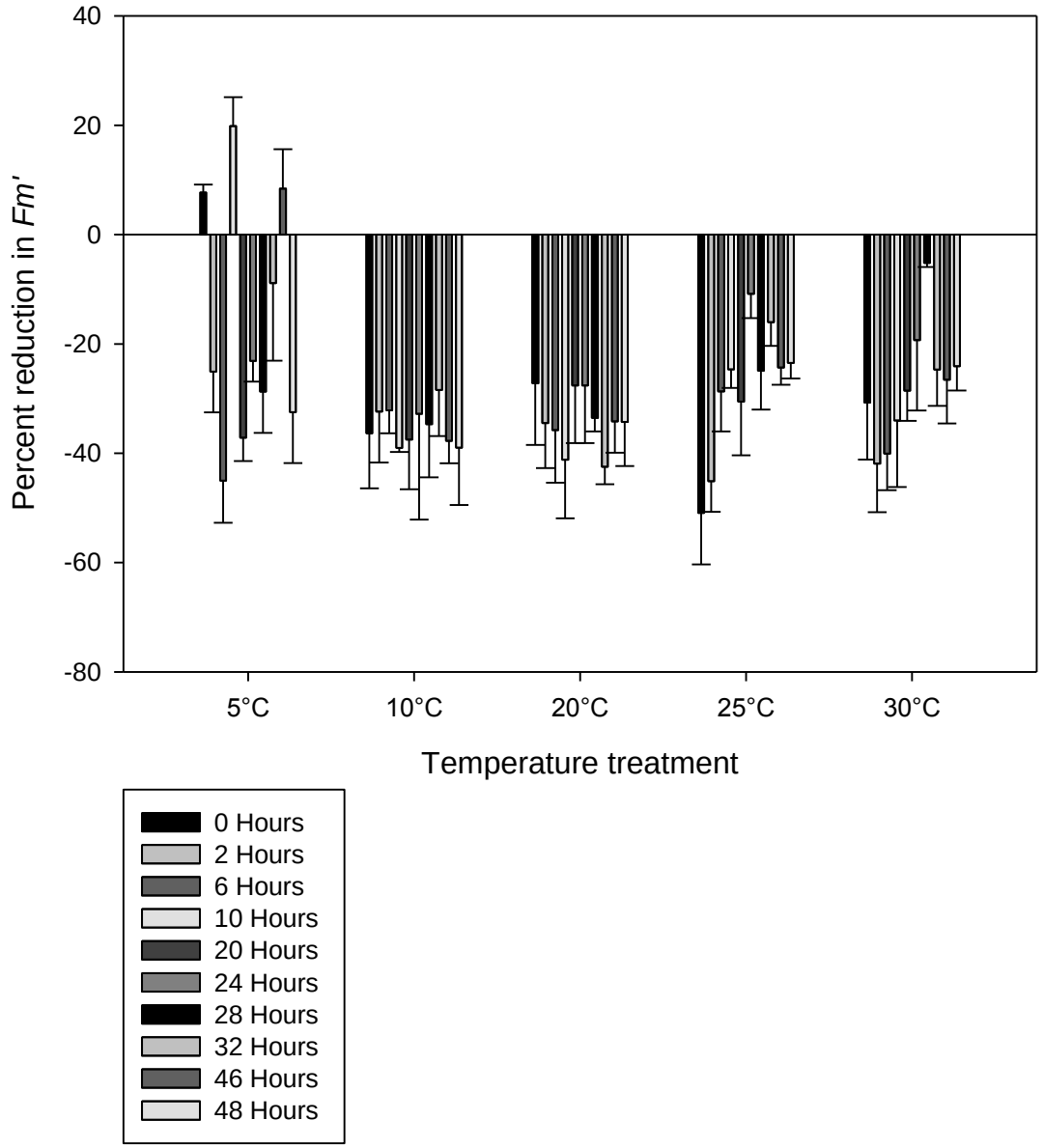
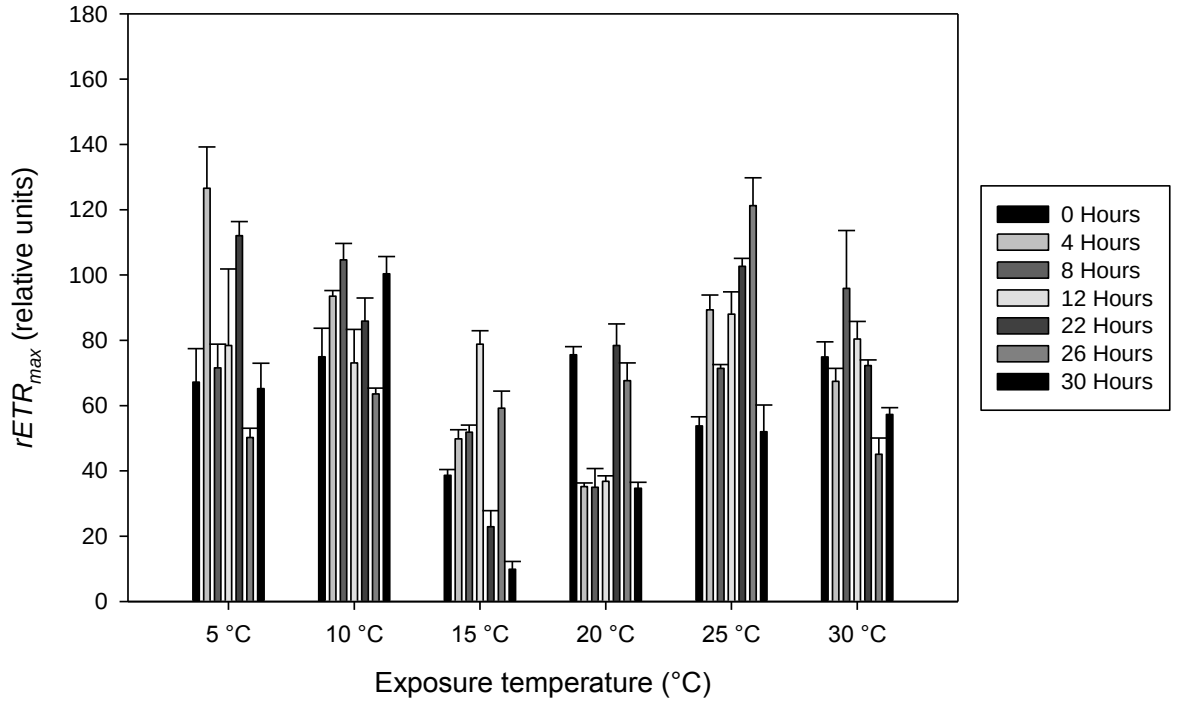


Figure 3.3.12: *The average reduction in Fm' from 0 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1},* recorded from the temperature treated upper shore biofilm recorded at preselected time points over 48 h of exposure. Mean± SE, *n=3*

Photophysiological assessment: Winter temperature manipulations (upper and lower shore)

Maximum relative electron transport rate (*rETR$_{max}$*)

Temperature had a significant effect on the *rETR$_{max}$* (F= 8.02, df=5,443, P<0.001), with lower *rETR$_{max}$* being observed at 15 °C and 20 °C for the upper shore site samples in the first 30 h (Fig. 3.3.13 A). Between 34 and 60 h the 25 °C and 30 °C upper shore biofilms also displayed lower *rETR$_{max}$* levels (Fig. 3.3.13). The 25 °C and 30 °C lower shore biofilms exhibited consistently higher *rETR$_{max}$*, particularly post 34 h of treatment (Fig. 3.3.14). The exposure time also had a significant effect on the *rETR$_{max}$* (F=3.47, df= 12, 443, P<0.001), with the trend being a reduction in *rETR$_{max}$* over the exposure period and this reduction was most pronounced in the 5 °C upper shore biofilm (Fig. 3.3.13) and the 10 °C lower shore biofilm (Fig. 3.3.14). There was a significant difference in the *rETR$_{max}$* recorded at the different shore sites (T= 2.07, df= 441, P<0.05), with different responses to the temperature treatments recorded. The 25 °C and 30 °C lower shore biofilms were unaffected by the temperature over the exposure period, with a consistent *rETR$_{max}$*, which as the *rETR$_{max}$* and as the *rETR$_{max}$* recorded from the other treated biofilms reduced, these became the highest (Fig. 3.3.14). This was in contrast to the upper shore biofilm where the 25 °C and 30 °C biofilms displayed decreasing *rETR$_{max}$* along with the 5 °C and 10 °C treatments (Fig. 3.3.13). Despite initially displaying a lower *rETR$_{max}$*, the 15 °C and 20 °C treatments maintained their *rETR$_{max}$* levels throughout the exposure period.



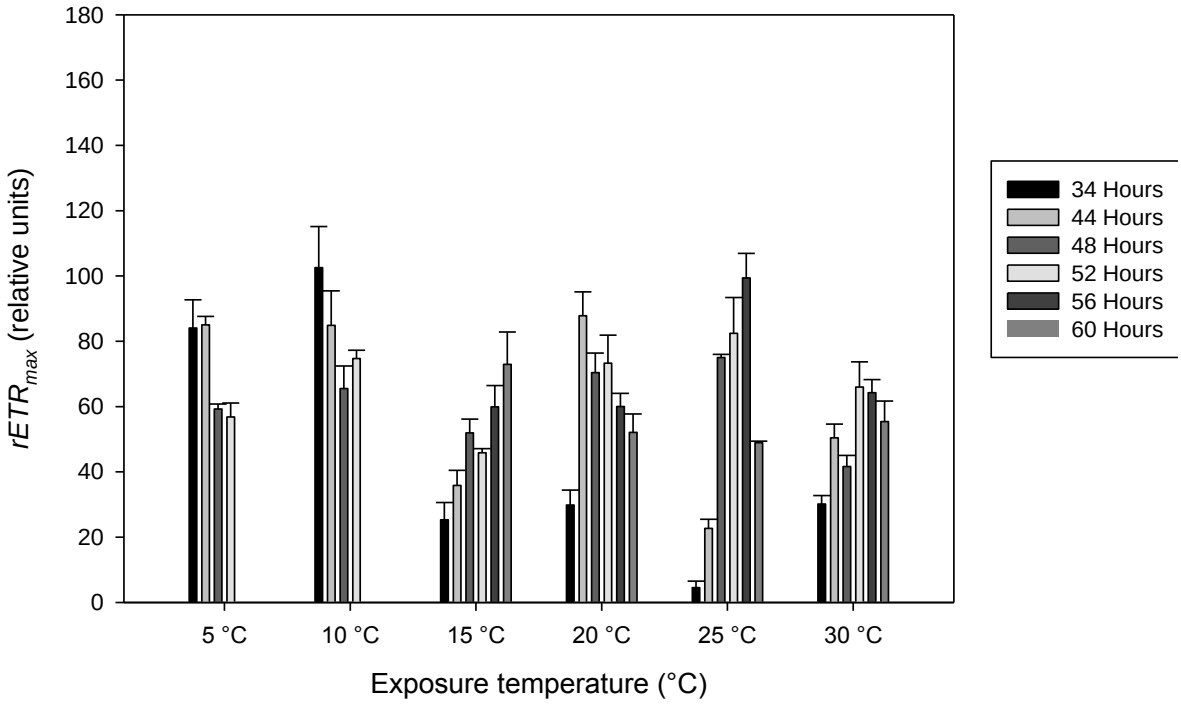


Figure 3.3.13: *Maximum relative electron transport rate* (*rETR$_{max}$*), of each temperature treated biofilm from the upper shore, recorded at preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

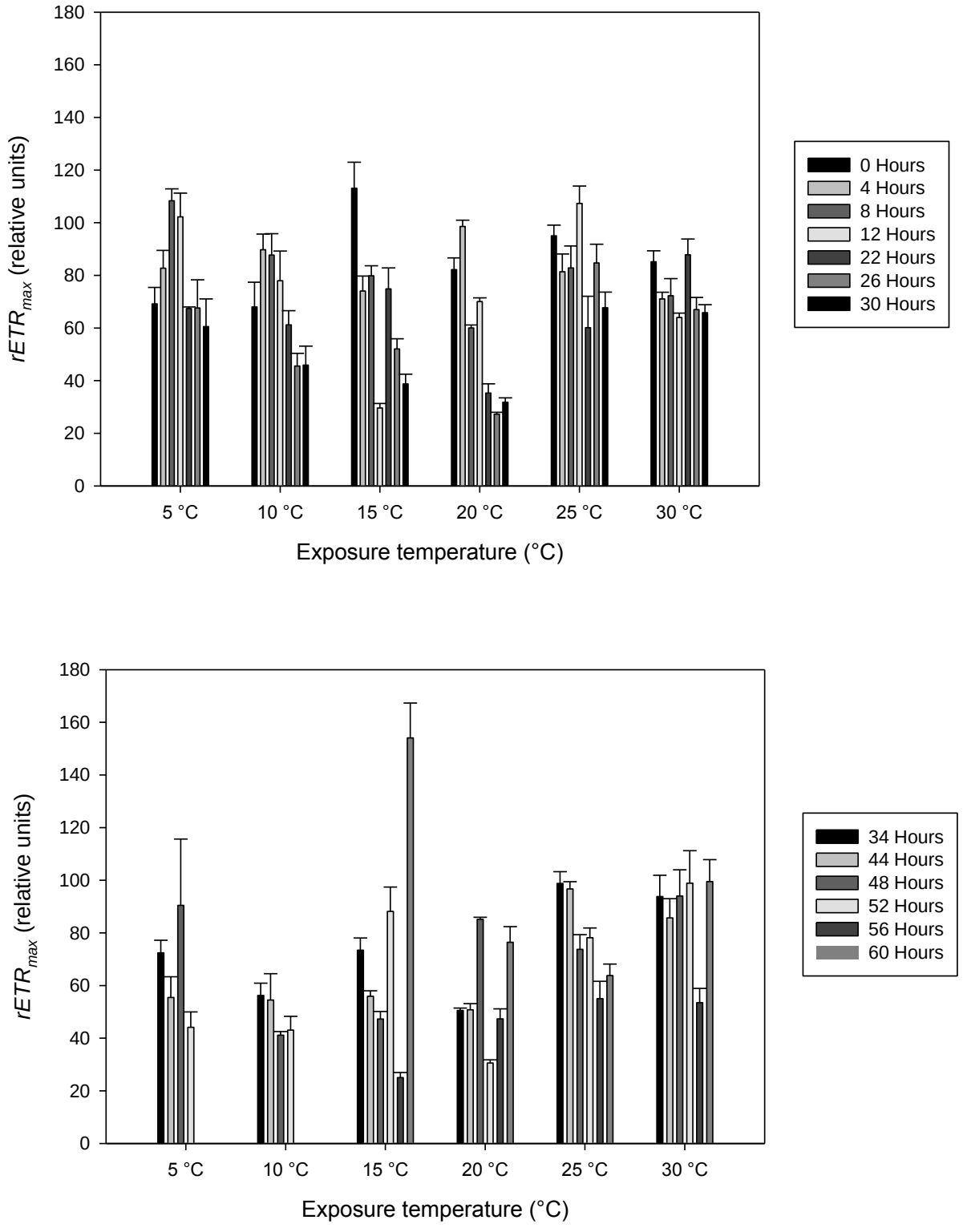


Figure 3.3.14: *Maximum relative electron transport rate* (*rETR$_{max}$*), of each temperature treated biofilm from the lower shore, recorded at preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

Light utilisation efficiency (α)

There was a significant effect of temperature on α recorded from the incubated biofilms (H=25.06, df=5, P<0.001). Similar patterns to that of the $rETR_{max}$ were recorded from the lower shore biofilms with the 25 °C and 30 °C biofilms displaying consistent α, which as a result of a reduction in the α of the other treated biofilms, became the highest post 34 h (Fig. 3.3.16). In addition a similar pattern was recorded in the upper shore sites, with the 15 °C and 20 °C treatments displaying consistent α, which again resulted in these treatments displaying the highest α post 34 h as the other treatments displayed reducing α (Fig. 3.3.15). Exposure time also had a significant effect on α (H=36.71, df=12, P<0.001), with a general trend of reducing α over the exposure period, with a more pronounced effect being observed from the upper shore biofilms (Fig. 3.3.15), although the different shore level had no significant effect on α recorded.

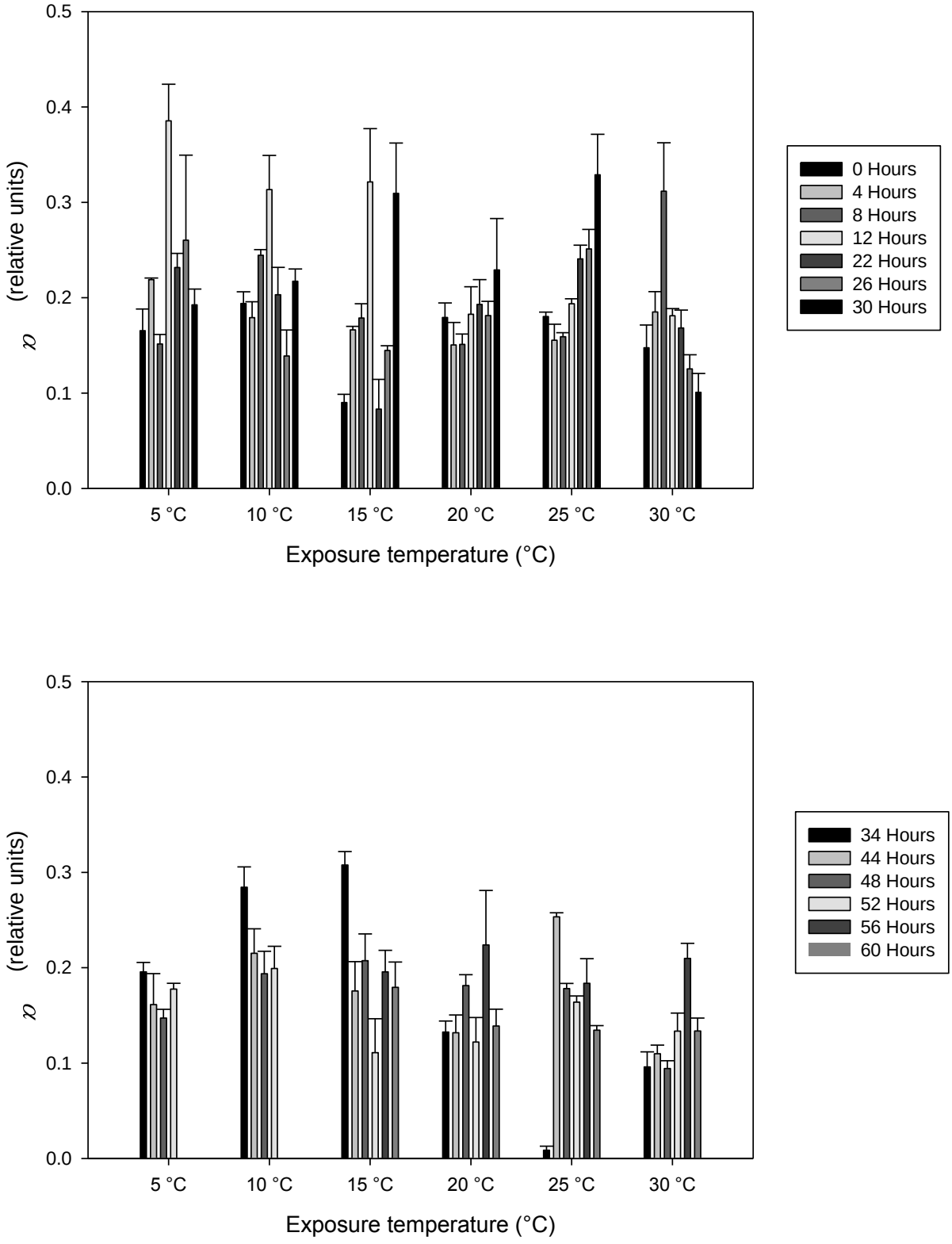


Figure 3.3.15: *Light utilisation coefficient (α),* of each temperature treated biofilm from the upper shore, recorded preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

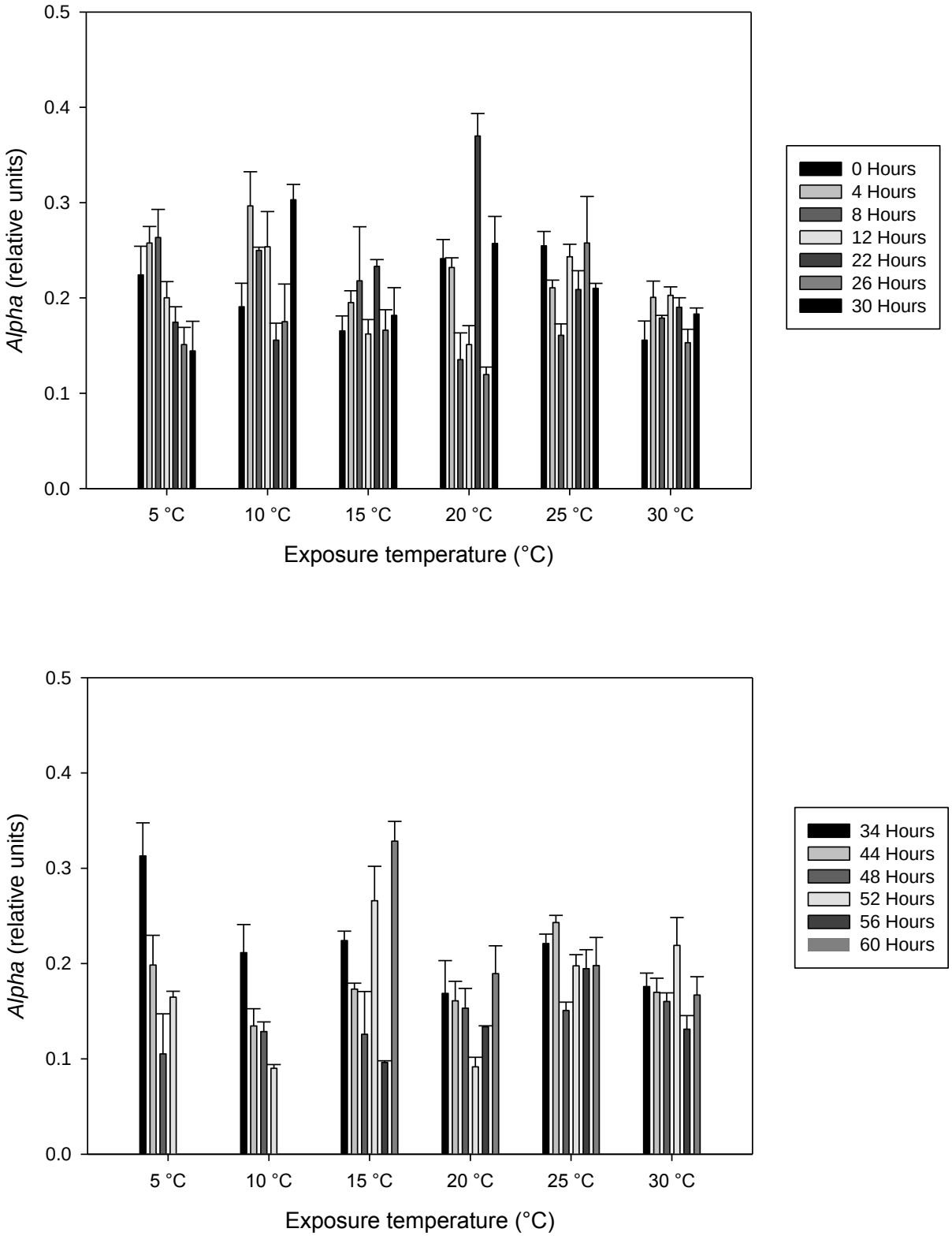


Figure 3.3.16: *Light utilisation coefficient (α),* of each temperature treated biofilm from the lower shore, recorded preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

Light saturation coefficient (*Ek*),

As expected, *Ek* followed a similar pattern to that observed in the rETR_{max}; temperature had a significant effect on the *Ek* (H=33.48, df=5, P<0.001),with lower *Ek* being observed in the upper shore 15 °C and 20 °C biofilms up to 30 h of exposure (Fig. 3.3.17). As the other temperature treated biofilms exhibited a reduction in *Ek* during the exposure period, the 15 °C and 20 °C treated biofilms did not exhibit lower *Ek* after 48 h (Fig. 3.3.17). The 25 °C and 30 °C lower shore biofilms exhibited higher *Ek* levels particularly post 34 h of treatment (Fig. 3.3.18). Exposure time also had a significant effect on *Ek* (H=25.43, df=12, P=0.013), there was a general reduction during the exposure period. In the upper shore treatments the 15 °C and 20 °C treatment was least affected by the increasing exposure time (Fig. 3.3.17). For the lower shore biofilms, the 25 °C and 30 °C treatments were most resilient to increasing exposure time. However, there was no significant effect of the shore level on *Ek* (Fig. 3.3.18).

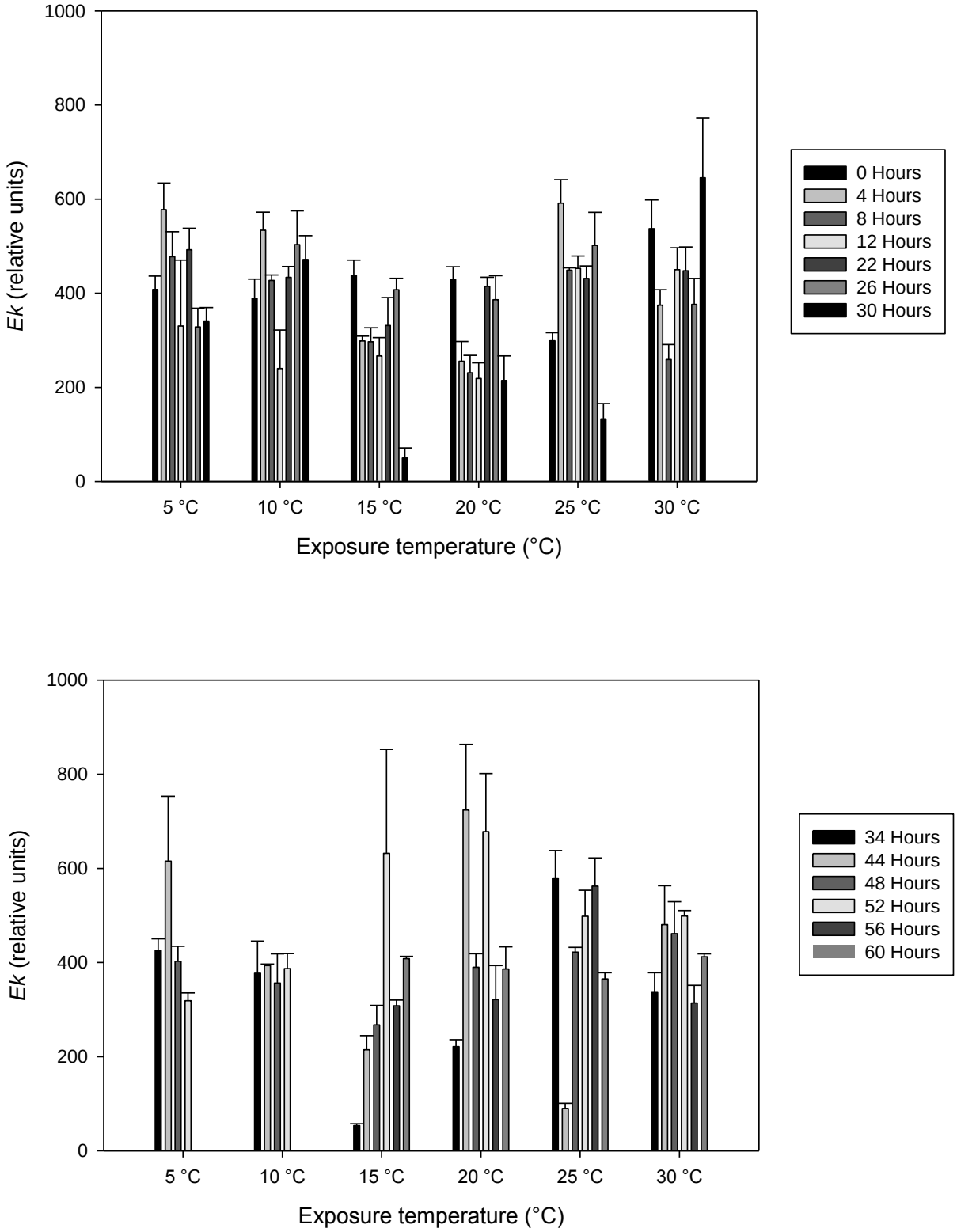


Figure 3.3.17: *Light saturation coefficient (Ek),* of each temperature treated biofilm from the upper shore, recorded preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

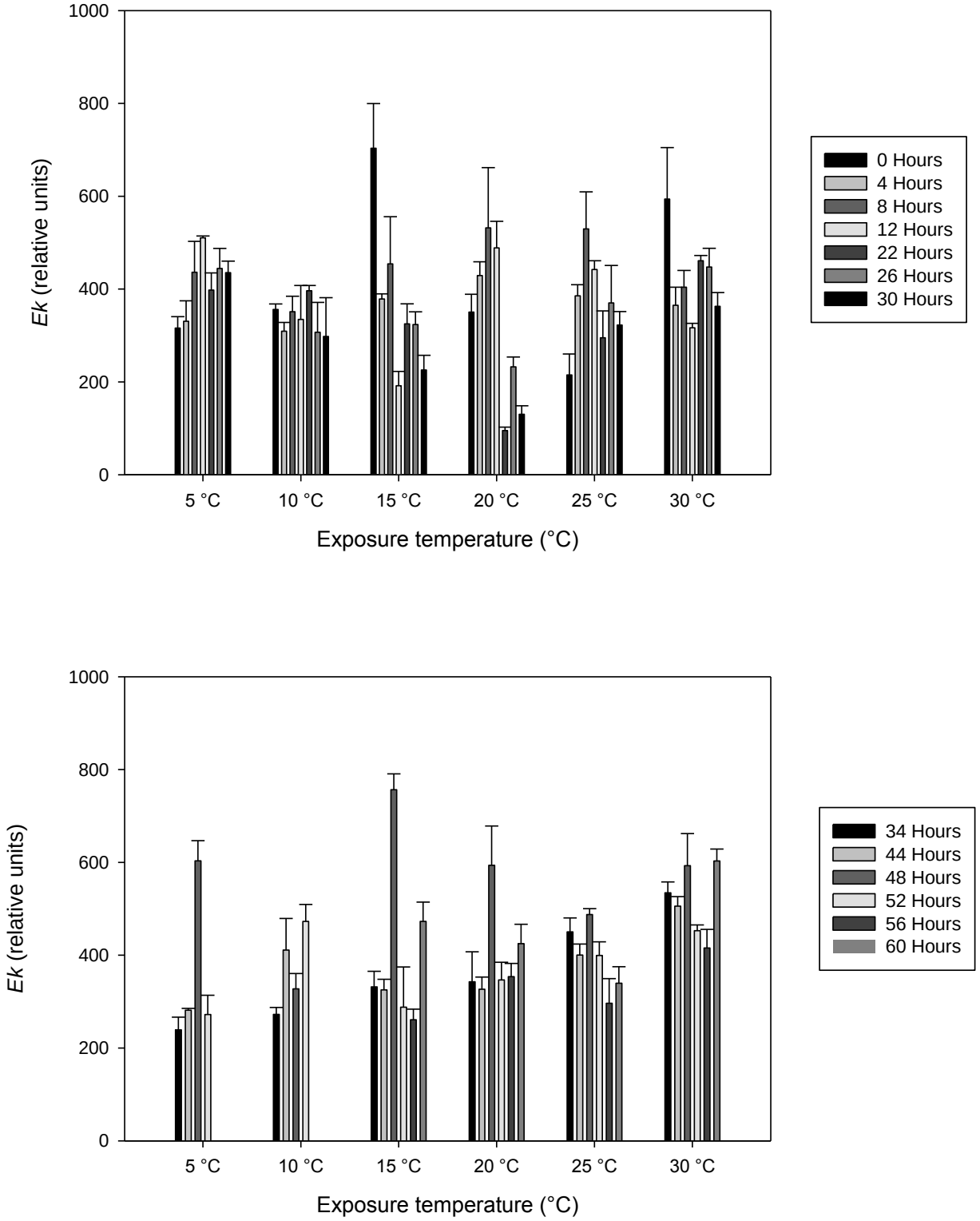


Figure 3.3.18: *Light saturation coefficient (Ek),* of each temperature treated biofilm from the lower shore, recorded at preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

Light adapted quantum efficiency of PSII (*ΔF/Fm'*)

There was a significant effect of temperature on *ΔF/Fm'* (H=225.15, df= 5, p<0.001). The 5 °C and 10 °C treatments from both shore levels exhibited very little difference as a result of changing temperature and very little difference as the exposure period increased (Figs. 3.3.19 and 3.3.20). The 15, 20, 25 and 30 °C from both shore levels displayed much more variable *ΔF/Fm'*. These treatments also exhibited lower *ΔF/Fm'* than the 5 °C and 10 °C treatment. The same trends were observed in the lower shore treated biofilms with higher and more stable *ΔF/Fm'* recorded at 5 °C and 10 °C (Fig. 3.3.20). The exposure time did not have a significant effect on the *ΔF/Fm'* there did not appear to be an observable pattern in the responses over time and there was no reduction after 34 h as observed in $rETR_{max}$. Shore level had a significant effect on *ΔF/Fm',* with higher *ΔF/Fm'* recorded in the 15, 20, 25 and 30 °C lower shore biofilms after 34 h (Fig. 3.3.20).

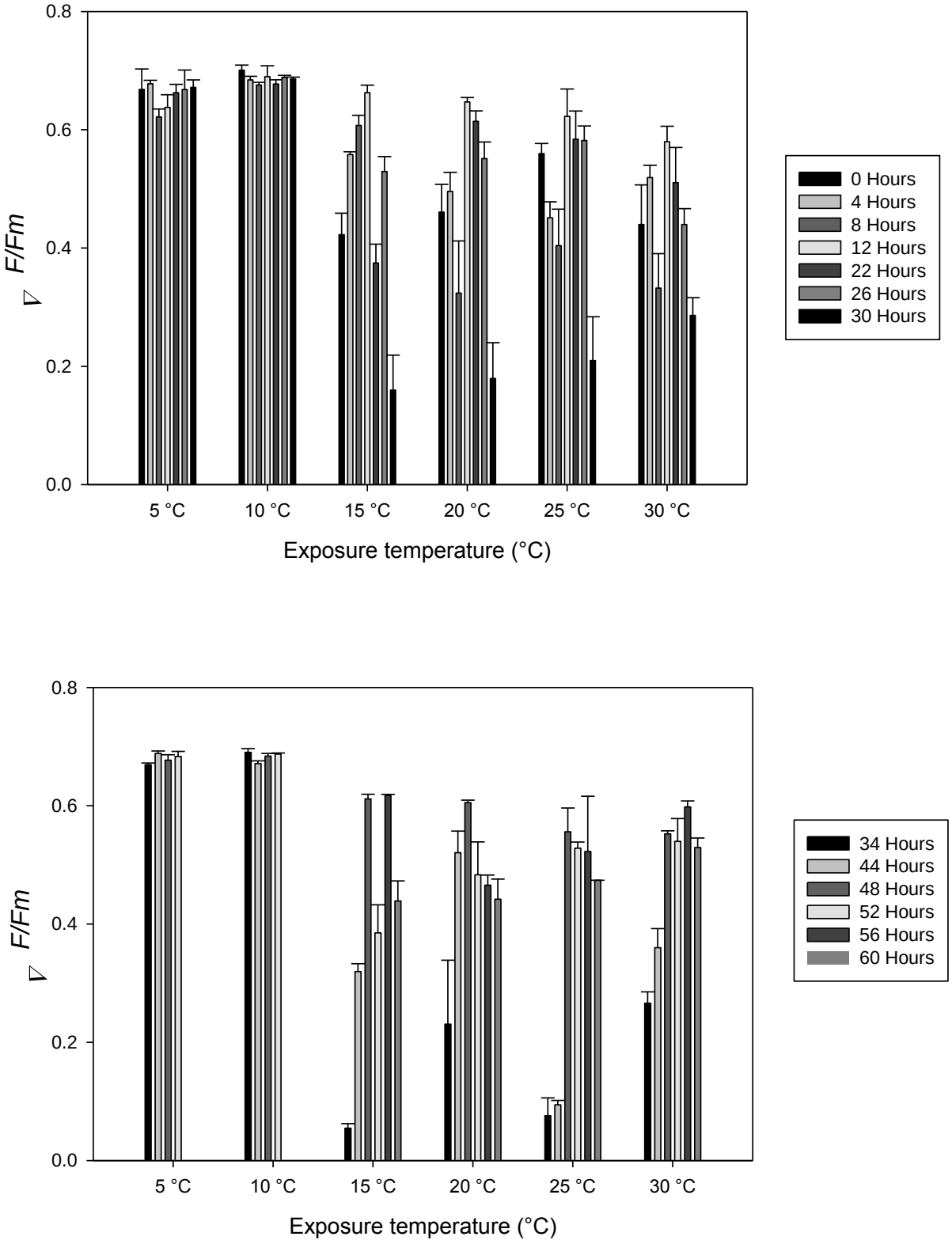


Figure 3.3.19: *Light adapted quantum efficiency of PSII (ΔF/Fm'),* of each temperature treated biofilm from the upper shore, recorded at preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

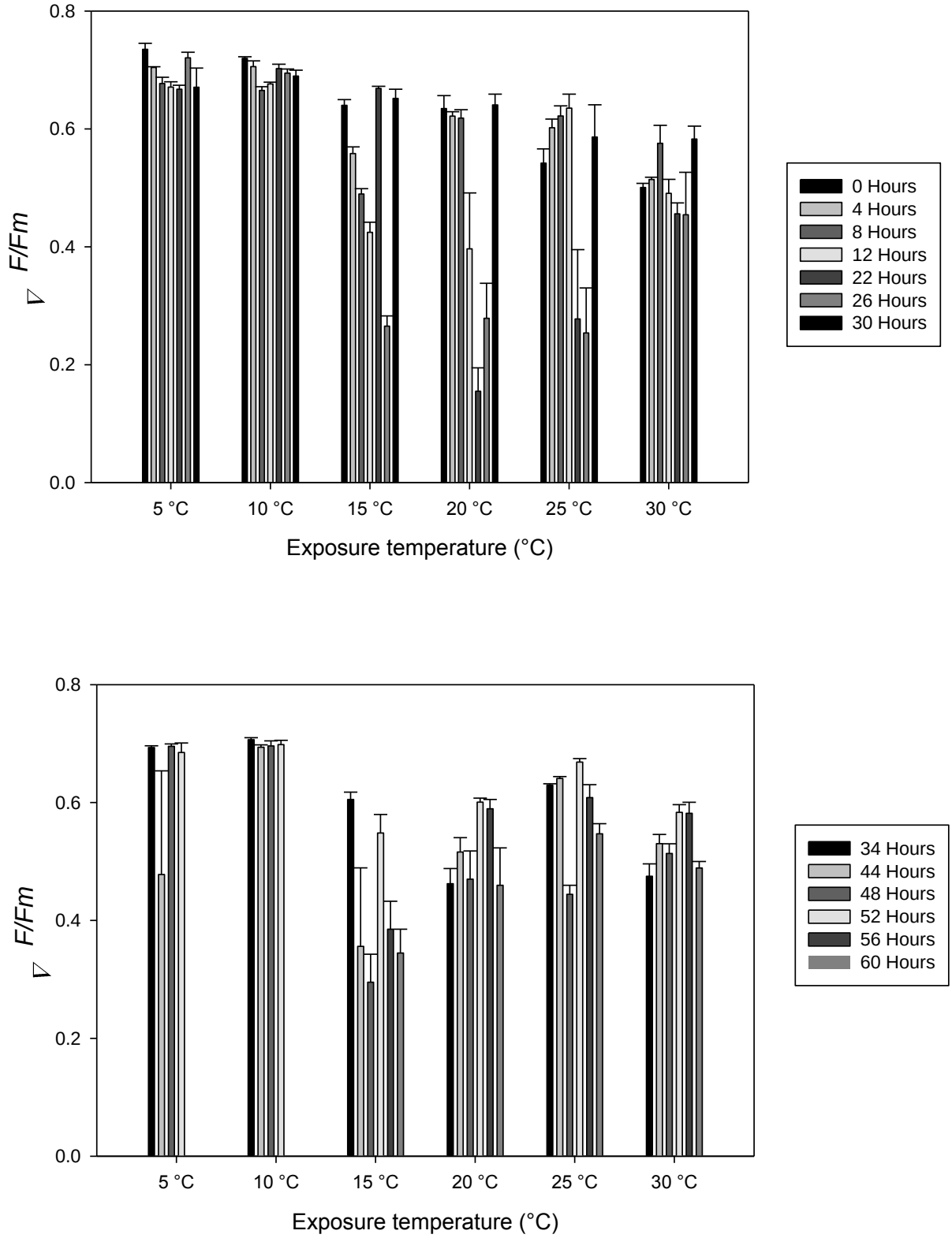


Figure 3.3.20: *Light adapted quantum efficiency of PSII (ΔF/Fm'),* of each temperature treated biofilm from the lower shore, recorded at preselected time points over the course of 52 (5°C and 10°C) and 60 h (15, 20, 25 and 30 °C). Mean ± SE, *n=3*

Downregulation of photosynthesis (Percentage change of Maximum fluorescence (Fm'))

In the upper and lower shore 5 °C biofilms there was an observable reduction in *Fm'* at each time point to 26 h (Fig. 3.3.21 A). After 44, 48 and 52 h there was an increase in *Fm'* from 603 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1}. A similar pattern was observed in the 10 °C upper and lower shore biofilms (Fig. 3.3.22 A and B). The 15 °C upper shore biofilm did not exhibit the same response (Fig. 3.3.23 A). At 8, 12 (h) there was an increase in *Fm'* from 10 µmol m^{-2} s^{-1} to 603 µmol m^{-2} s^{-1}. At 54 h there was an increase in *Fm'* from 603 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1}. At all other times (h) there was a reduction in *Fm'* as light levels increased. In the 15 °C lower shore biofilm at 44 and 60 h there was a slight increase in the *Fm'* from 10 µmol m^{-2} s^{-1} to 603 µmol m^{-2} s^{-1} (Fig. 3.3.23 B). At all other hours there was a reduction in *Fm'* as light level increased. The 20 °C upper shore biofilm exhibited reducing *Fm'* as light levels increased at all exposure times (Fig. 3.3.24 A). The lower shore biofilm again displayed reducing *Fm'* as light levels increase at all exposure times except 26 h where there was a slight increase in *Fm'* from 10 µmol m^{-2} s^{-1} to 603 µmol m^{-2} s^{-1} (Fig. 3.3.24 B). The 25 °C lower and upper shore biofilms exhibited the same *Fm'* response with an increase in *Fm'* from 10 µmol m^{-2} s^{-1} to 603 µmol m^{-2} s^{-1} at 0 h of exposure (Fig. 3.3.25 A and B). At all other exposure periods there was a reduction in *Fm'* as light levels increased. The 30 °C upper and lower shore biofilms exhibited reducing *Fm'* as light levels increased at all exposure times (h) (Fig. 3.3.26 A and B).

At each shore level mid-way through the exposure smaller reductions in *Fm'* occurred as light levels in the rapid light curve increase, however exposure time had no significant effect on the change in *Fm'* (Fig. 3.3.27). In general the lower shore biofilms displayed a smaller reduction in *Fm'*. The change in *Fm'* from 0 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1} was averaged over the 3 measurements. This average change was plotted and temperature had a significant effect on this ($F = 7.29$, $df = 4.164$, $P < 0.05$), with the 25 °C treated biofilms exhibiting a smaller reduction in *Fm'*.

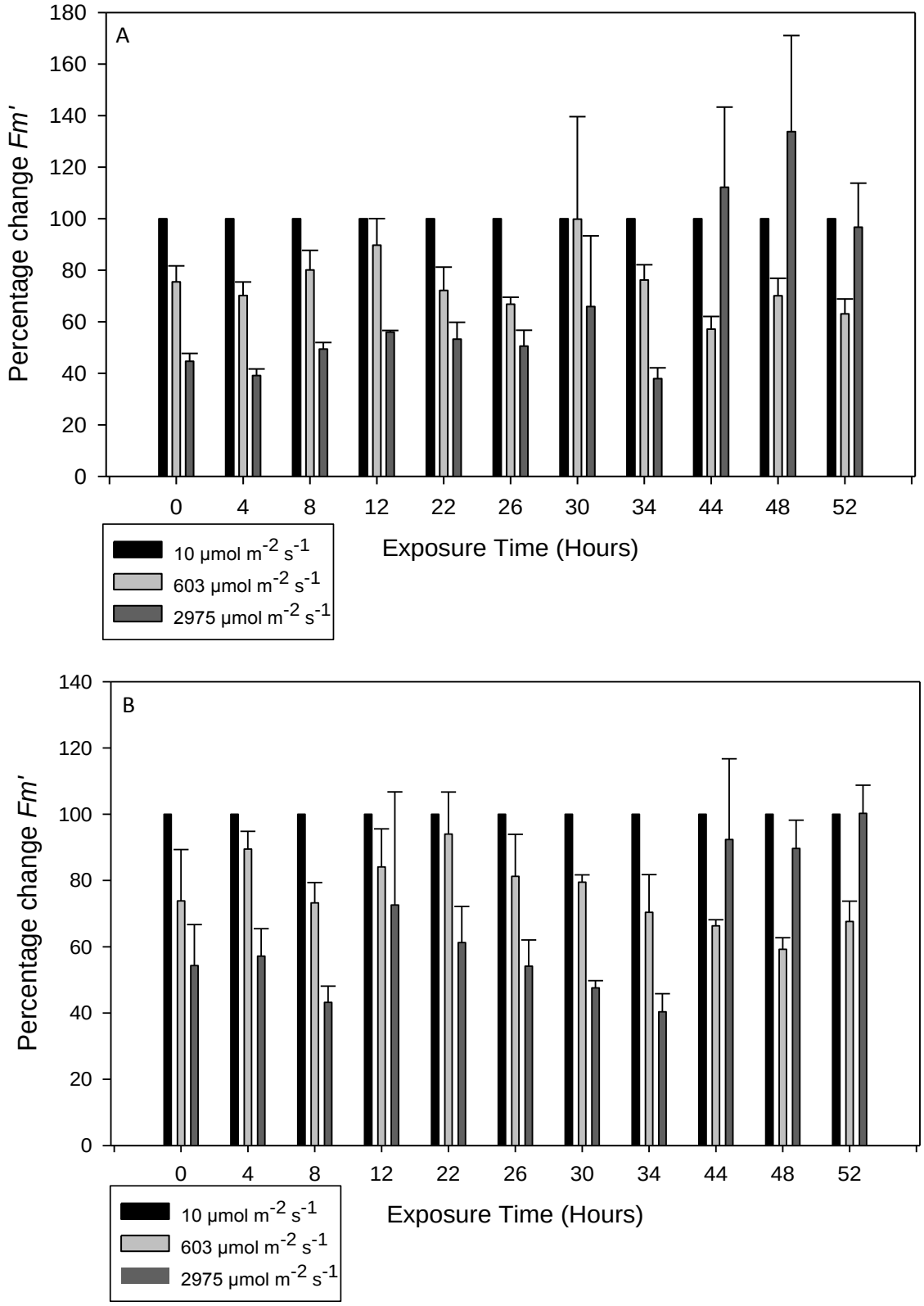


Figure 3.3.21: *The percentage change in Maximum fluorescence,* recorded from the 5 °C treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 52 h of exposure. Mean± SE, *n=3*

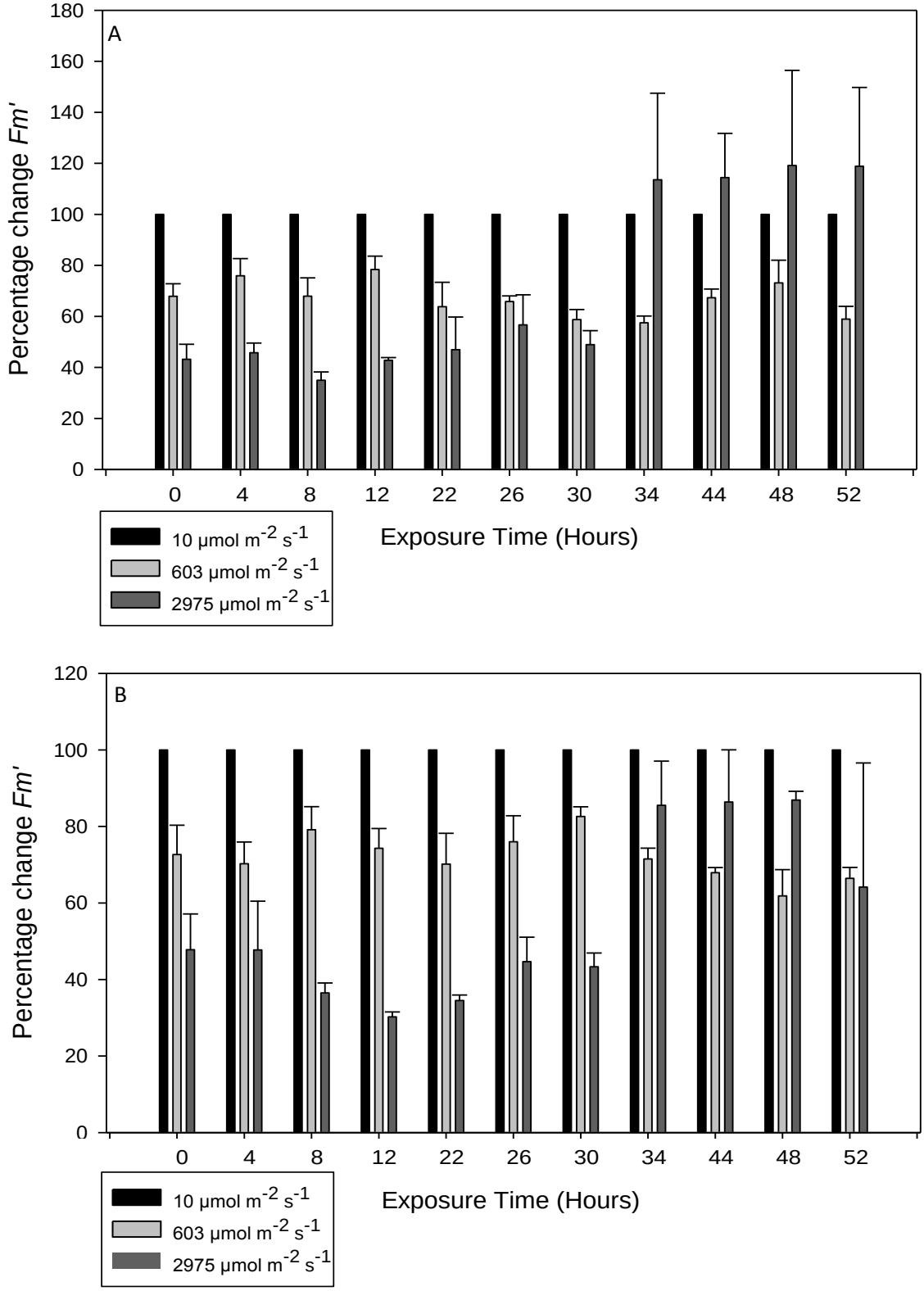


Figure 3.3.22: *The percentage change in Maximum fluorescence,* recorded from the 10 °C treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 52 h of exposure. Mean± SE, *n=3*

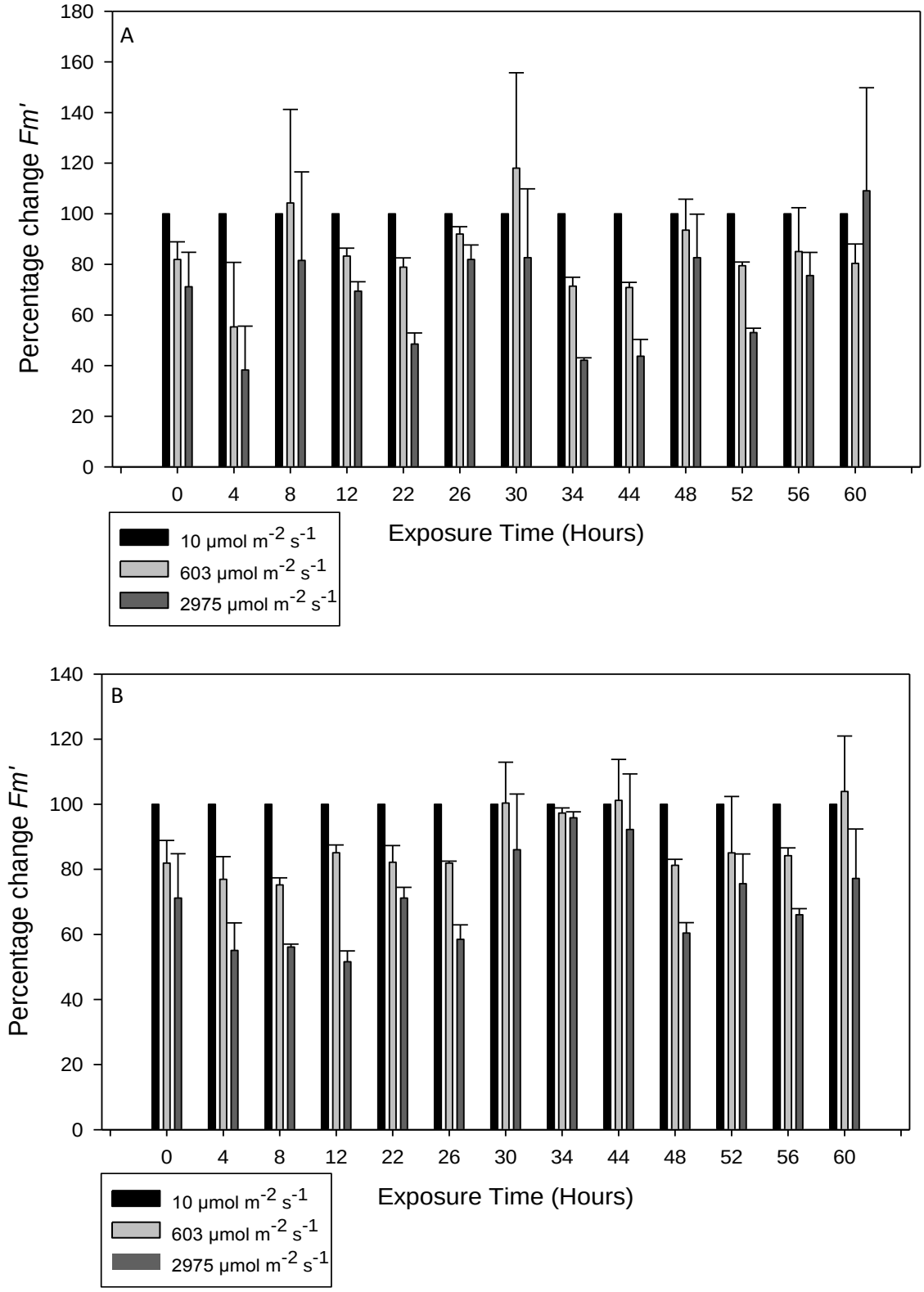


Figure 3.3.23: *The percentage change in Maximum fluorescence,* recorded from the 15 °C treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 60 h of exposure. Mean± SE, *n=3*

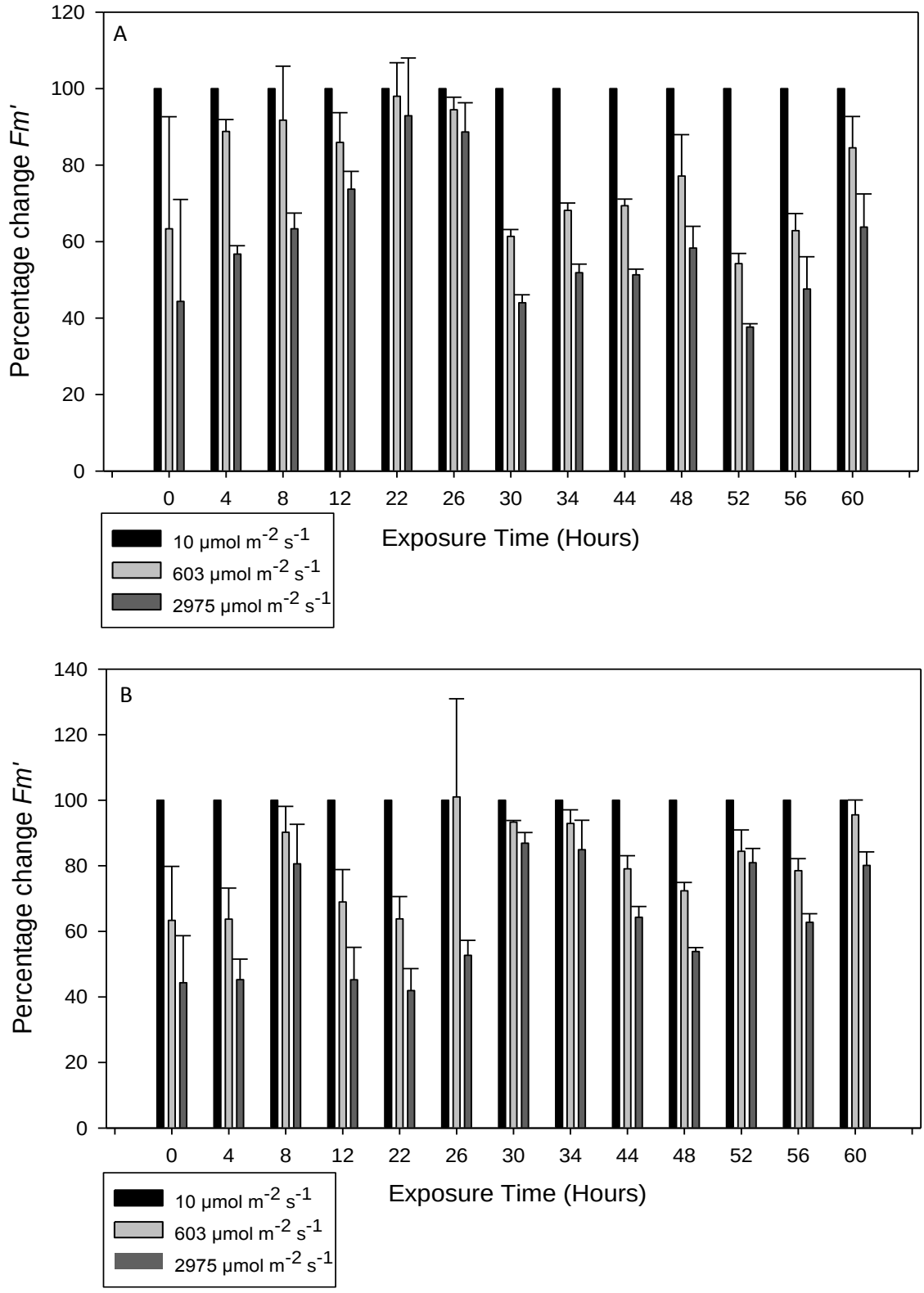


Figure 3.3.24: *The percentage change in Maximum fluorescence,* recorded from the 20 °C treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 60 h of exposure. Mean± SE, *n=3*

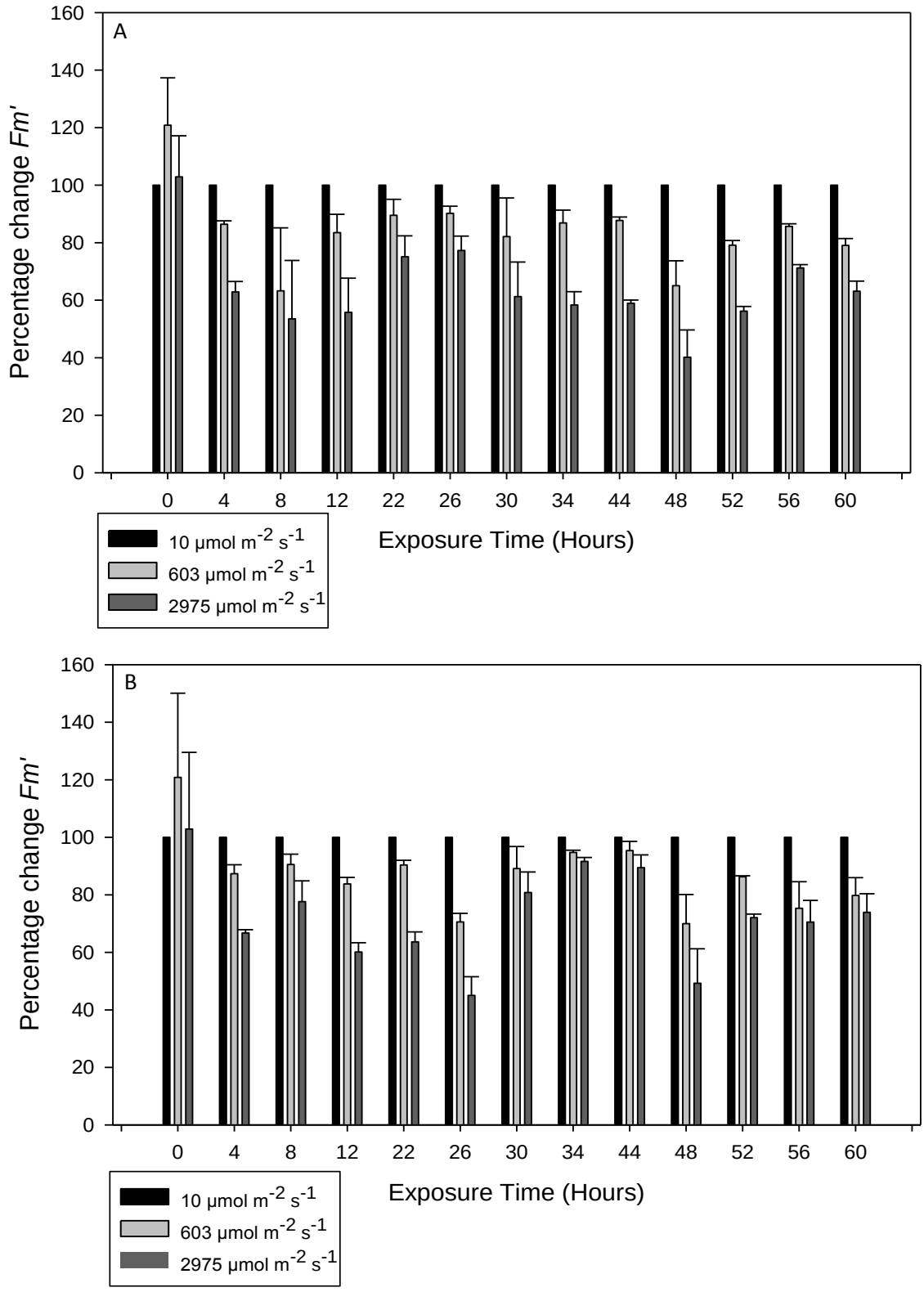


Figure 3.3.25: *The percentage change in Maximum fluorescence,* recorded from the 25 °C treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 60 h of exposure. Mean± SE, *n=3*

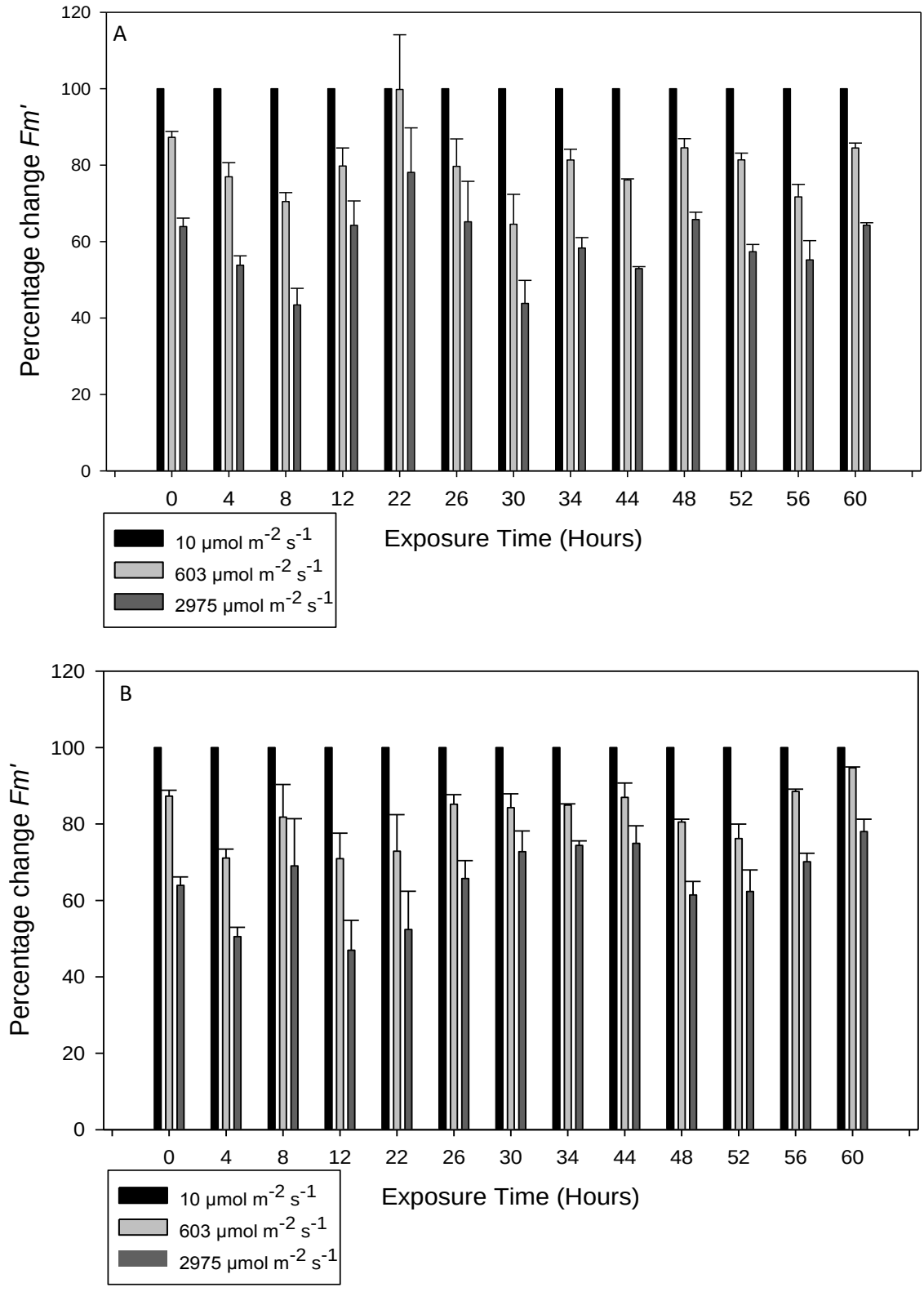


Figure 3.3.26: *The percentage change in Maximum fluorescence,* recorded from the 30 °C treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 60 h of exposure. Mean± SE, *n=3*

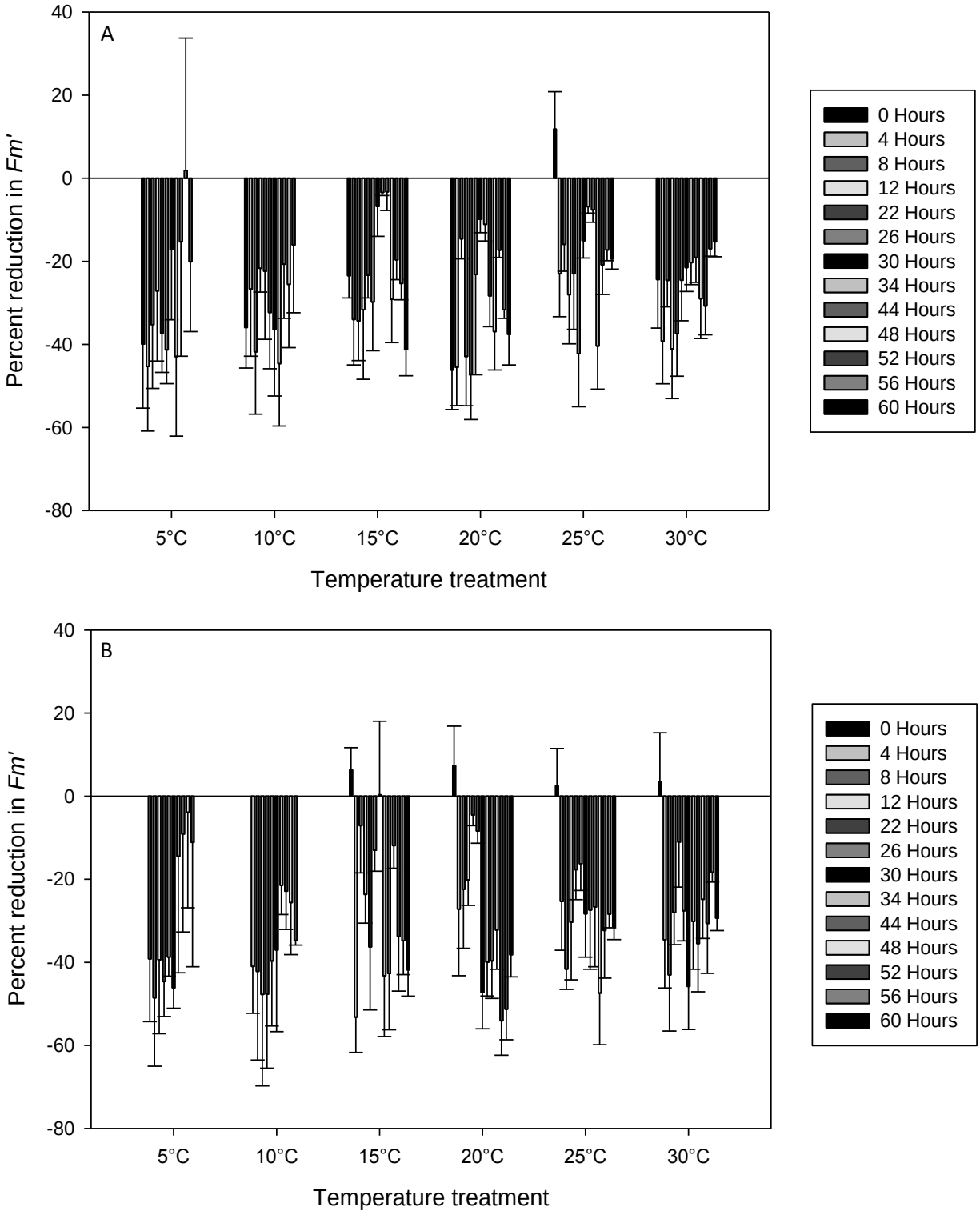


Figure 3.3.27: *The average reduction in Fm' from 0 µmol m^{-2} s^{-1} to 2975 µmol m^{-2} s^{-1},* recorded from the temperature treated upper (A) and lower (B) shore biofilm recorded at preselected time points over 60 h of exposure. Mean± SE, *n=3*

DISCUSSION

Temperature was found to influence the photosynthesis of the rocky shore biofilms. The spring upper shore biofilms exposed to 25˚C and 30˚C temperatures induced lower levels of NPQ, but were capable of limiting their exposure to light in a similar manner to microphytobenthos found in mudflat environments by microcycling within the tubes, spending limited time on the edge of the tubes to reduce exposure to high light. The movement was induced at temperatures above 14˚C and the speed of movement increased up to 32˚C when it then decreased. The winter samples were not observed moving at any of the temperatures investigated. The lower shore samples were in general more resilient to both increased and decreased temperature. It is likely that the ability of the cells to migrate into the sediment reduced their exposure to the simulated temperature, reducing the effect of them. The colder temperature had a more negative effect on $rETR_{max}$ than the warmer ones, recorded from the upper shore biofilms, implying that the increased reaction rates induced by increasing temperature in part mitigated the negative effects of high temperature.

Behavioural observation of tube-forming cells

A reduction in *Fm'* is known to represent either the migratory action of diatom biofilms, as the cells move away from the biofilm surface and the fluorescence yield reduces (Perkins *et al*. 2002, Consalvey *et al*. 2004, 2005), or an induction on NPQ. NPQ as described in the introduction diverts light energy away from the PSII reaction centres (Olaizola & Yamamoto 1994). This results in a reduction in energy in the electron transport chain and therefore a reduction in the energy available for fluorescence induction. When cells are unable to move into the substratum, as is the case in the upper shore biofilms of Dunraven Bay, the cells cannot optimise their light exposure via sediment shading. This is the primary form of photoregulation in mudflat biofilms (Perkins, Lavaud, et al. 2010) and if cells cannot do this then other regulation mechanisms must be employed. NPQ is the most commonly recognised

physiological photoregulatory mechanism, however, the spring upper shore biofilms exposed to 25˚C and 30˚C temperatures only induced low levels of NPQ. Therefore, in order to prevent transient photoinhibition, with the associated reduction in carbon fixation, or permanent photodamage, the cells must be utilising another mechanism. It was noticed during taxonomic observations that the cells were capable of moving within their tubes. An observational experiment was performed to record the patterns and rates of diatom movement. The upper shore cells appeared to be capable of limiting their exposure to light similarly to mudflat microphytobenthos by 'microcycling' (Kromkamp *et al*. 1998, Serôdio & Catarino 1999, Consalvey, *et al*. 2004, Underwood *et al*. 2005, Jesus *et al*. 2006, 2010, Perkins *et al*. 2010) within their tubes. The pattern of cell movement limited their exposure to the high light at the edge of the tube. This movement appeared to be temperature dependent. Movement of cells within their tubes was induced at temperature above 14˚C and the speed of movement increased up to 32˚C when it then decreased.

One of the assumptions of hypothesis 3 was that the upper shore biofilms could not move into the surface of the rock. However it was discovered that the cells can move inside the polysaccharide tubes which make up the biofilm. Therefore, this hypothesis assumption can be partially rejected as the upper shore cells can move, but not into the substratum.

The cells appeared to have 3 modes of life during the spring months (Figs 3.4.1, A, B and C). Despite the significant reduction in biomass in the spring months (see Chapter 2) the free living cells which increased in number in May remained in very low numbers throughout the spring months, reducing in number in October. The cells pictured in Figure 3.4.1 (A) were free-living and retained motility as free-living cells. It is speculated that cells may become free-living as it may be difficult to remain static in polysaccharide tubes during the summer months due to increased grazing pressure (Hillebrand *et al*. 2000) (Chapter 2) and moving into crevices or refugia may prevent herbivory. In addition, the ability to move into crevices may also allow the

cells to avoid the higher light doses and temperatures during the summer months and may also be a driving force to abandon the tubes. Empty tubes, which appeared white, were observed during April and May, suggesting that these cells abandoned the tubes during the spring months. It has also been reported that UV radiation can change the structure of marine polymers (Kieber *et al*. 1990, Mopper *et al*. 1991, Thomas & Lara 1995) and it may be that the increase in light levels and resulting increased UV radiation during the late spring and summer months was causing degradation of the polysaccharides in the tubes. The tubes were observed in the late spring and no visual differences were noted, however, it was not possible to gauge tube thickness and so it may be that a degradation of the tube micro-habitat, and the increased pressures to move into refugia, were the driving forces behind the movement of cells out of the tubes and the subsequent large reduction in diatom numbers. There were two other modes of life, recorded in winter and spring, both of which involved the use of polysaccharide tubes. Figure 3.4.1, (B) illustrates the densely packed tube living mode. Due to the tightly packed nature of these tubes the cells did not move at any temperature. These densely packed tubes contained *Navicula bottnica* and *Navicula ramosissima.* These species were capable of motility as they were observed moving in a free-living form. It is possible that the densely packed cells shaded one another and provided sufficient protection from the higher summer light levels. The cells in the outer areas may move inwards much more slowly and this was not observed during the microscopy observation. *Navicula ramosissima* was not observed moving at any temperature. The densely packed nature of the tubes may reduce the light level sufficiently for those cells not in the outer layers to persist on the rocky shore, despite having a reduced ability to induce NPQ. The third life mode (Fig. 3.4.1 C) was the most common during the spring observations. These were less densely packed tubes which typically contained three or four diatoms next to one another across the tube (in a 2D plane). These cells moved at temperatures above 14°C (Fig. 3.3.1). They moved freely past one another consistently with the same movement pattern (Fig. 3.3.2), where those cells on the outside moved upwards and those on the inside moving downwards. This motion may be a way of

moving within the tubes so that all cells were exposed to lower light doses. Such a pattern, rotating cells from the periphery to the centre, is not dissimilar to the motion penguins utilise so that a particular member of the colony is not exposed to the cold outside temperatures for a long time (Ancel *et al*. 1997).

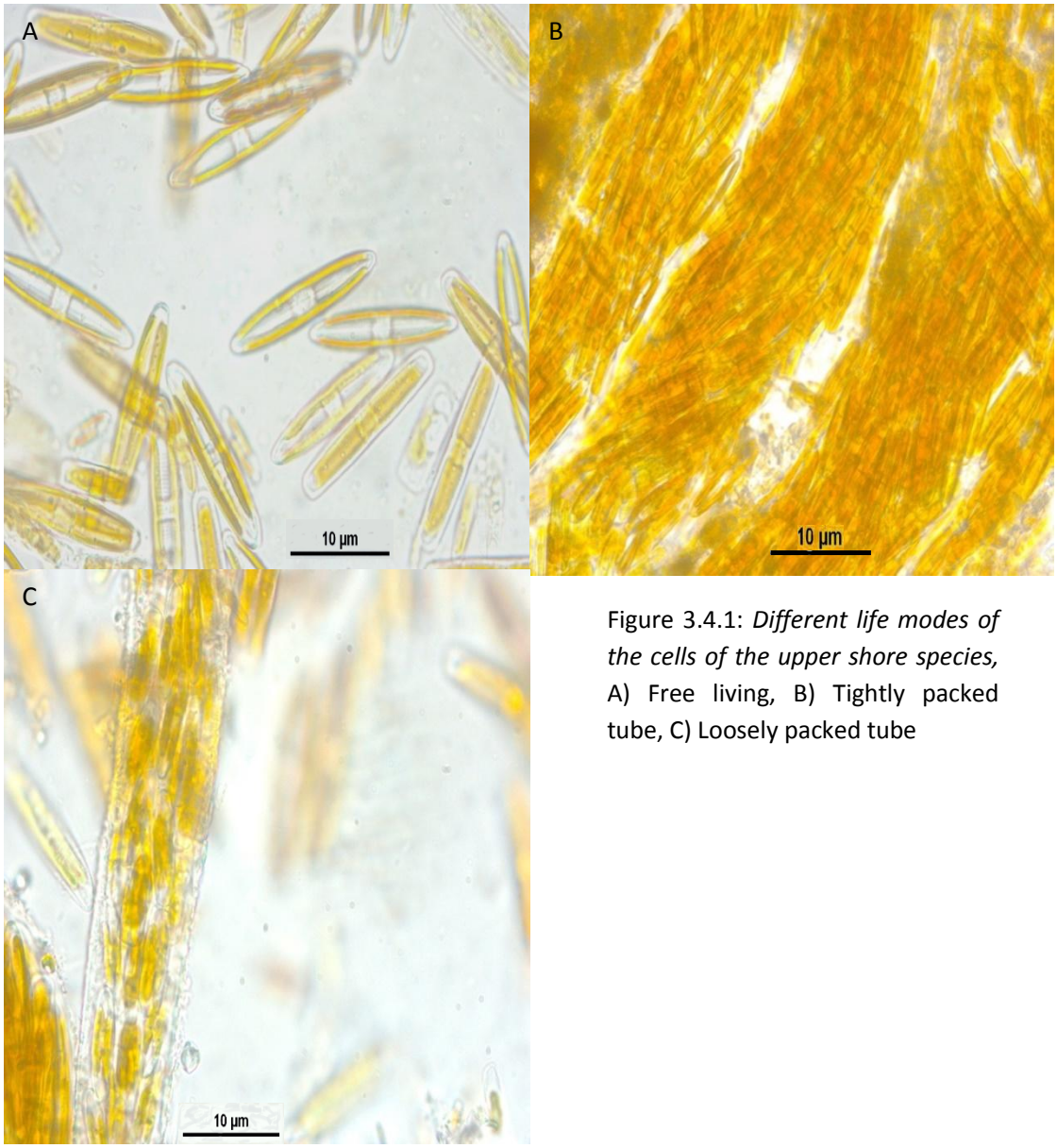


Figure 3.4.1: *Different life modes of the cells of the upper shore species,* A) Free living, B) Tightly packed tube, C) Loosely packed tube

The winter samples were also monitored using a temperature control stage and the light microscope. None of the winter cells were observed moving at any temperatures, despite the fact that *Navicula bottnica,* which could move in the spring months, was present in the winter months (Chapter 2). The winter cells may not have been moving as the trigger for movement may be a combination of high light and temperature, as it is known that multiple stimuli are

believed to be responsible for migration in mudflat based diatom cells (Hopkins 1966, Palmer & Round 1967, Apoya-Horton *et al*. 2006, Du *et al*. 2012). Despite the winter cells being exposed to high light levels on the microscope slide at similar levels to those recorded during the spring months at Dunraven Bay (1430 µmol m^{-2} s^{-1}, measured using the Walz Water PAM light meter) the cells did not begin to move. This could be due to the wavelengths of light produced by the microscope being unsuitable to trigger movement (Cohn *et al*. 2004). It may also be that the spring biofilm cells have been exposed to a higher light dose for a longer period of time and therefore may be 'primed' so there was a quick behavioural response when temperatures were increased. This, unfortunately, is difficult to test as the cells cannot be maintained in the laboratory successfully for longer than 14 days without senescence beginning to occur. The cells therefore could not be exposed to high light for a long period of time and then tested using the temperature manipulation stage. These movements were exhibited by the spring cells possibly as a means of compensating for the fact that *Fm'* reduced less as light levels increased in the 25˚C and 30˚C treated biofilms, and therefore NPQ was only minimally being induced (Fig. 3.3.12). Perkins *et al*. (2010) showed that migration was the most important mechanism for photoregulation amongst mudflat biofilms. The spring biofilms, treated with the higher temperatures, were either utilising this method as a priority over inducing NPQ or were unable to induce NPQ fully, possibly because the elevated temperature altered the enzymes required for NPQ inducement (Sizer *et al*. 1943, Palmer & Bonner 2007). The 5˚C and 10˚C treated biofilms, where no movement was recorded in the thermal microscopy, induced NPQ consistently. It is unlikely that the cells in biofilms exposed to elevated temperature would not utilise NPQ if they were able to induce it. Mudflat biofilm diatoms utilise migration as a primary regulation mechanism, but also induce NPQ in addition to this form of down regulation (Perkins *et al*. 2010).

Above 32˚C there was a decrease in the speed of cell movement (Fig. 3.3.1). It is unlikely that this reduction was a result of motility regulation as the very high temperatures were reducing

the ability to utilise NPQ. therefore, the movement was the only mechanism by which the cells could limit the amount of light that they were exposed to and photoregulate. Diatoms move by secreting extra-cellular polymeric substances (EPS) from the raphe (Edgar 1983, Edgar & Pickett-Heaps 1984). This adheres to the substratum (polysaccharide tube) and the cell moves along this. Wolfstein and Stal (2002) found that EPS production increased to 25°C and then production decreased at temperatures above that. It may be that the speed of movement reduced as the production of EPS became limited. Environmental conditions are also known to effect the composition of the EPS, and the EPS characteristics change as a result (Cohn & Weitzell 1996). It may be that the EPS viscosity changes at higher temperature and the ability of the cell to use it for movement is reduced. Cohn *et al* (2003) suggest that denaturation of myosin proteins were likely to be responsible for the reduction in rates of movement above 32°C, that they observed. They also suggest that damage to enzymes may also be having an effect on the speed of movement, since the increase and decrease follows the activity profile of typical enzymes (Lehninger *et al*. 1975). It was hypothesised (Hypothesis 3) that, as the cells on the upper shore cannot move into the rock surface, NPQ would be utilised and a large reduction in *Fm'* would be observed. This can be partially rejected as the cells can move and therefore may be able to utilise some form of migration, and the cells exposed to high temperatures did not exhibit a large reduction in *Fm'* after a period of exposure. It was predicted that extremes of temperature would result in greater reductions in *Fm'* but, as elaborated upon in the following section, this was not the case.

Spring temperature manipulation experiment (Upper shore only)

The effect of temperature on the $rETR_{max}$ and $\Delta F/Fm'$ (Hypothesis 2)

It was hypothesised (Hypothesis 2) that $rETR_{max}$ (Fig. 3.3.3) and the $\Delta F/Fm'$ (Fig. 3.3.6) exhibited by the biofilms would reduce on exposure to cold temperatures and increase on exposure to high temperatures. The 5 °C, 10 °C and 30 °C treated biofilms collected in the spring exhibited lower $rETR_{max}$ than the 20 °C and 25 °C treated biofilms, therefore this

hypothesis was only partially supported. The extremes of temperature had a negative effect on the $rETR_{max,}$ however the *ΔF/Fm'* was unaffected by the different exposure temperatures (Figure 3.3.6). It was hypothesised that, as PSII is known to be thermally stable up to temperatures higher than the ones examined in this study (De Las Rivas & Barber 1997, Nishiyama *et al*. 1997, Tang *et al*. 2007), *ΔF/Fm'* would not be negatively affected by the increased or decreased temperature, and this hypothesis was partly supported. However, unexpectedly, the exposure time did have a significant effect on the *ΔF/Fm'* with a reduction over the initial exposure period seen at all temperatures. This increased to return to pre-exposure levels once returned to the temperature recorded when the samples were removed from the field. As this was observed in all treatments, it appears that the *ΔF/Fm'* was affected by removing the sample from the shore and incubating in the tidal simulation tanks. It is widely reported that *Fv/Fm* can be used as a measure of the nutrient state of the cell. This has been shown in green phytoplankton (Kolber *et al*. 1994, Beardall *et al*. 2001), sea ice algae (Robinson *et al*. 1998) and diatoms, both in benthic (Underwood *et al*. 1999) and planktoninc forms (Geider *et al*. 1993). It may be that the *ΔF/Fm'* recorded here was reducing as a result of nutrient depletion. Miller & Kamykowski (1986) also noted that diurnal photosynthetic variation and salinity appeared to have an effect on the photosynthesis (Pmax) of diatom cells, and it is possible that between replenishment and changes of the sea water in the tidal tanks, the salinity rose, due to evaporation. This was not measured as it was assumed that the maintained humidity in the chambers would limit evaporation, though it is possible that this was not the case.

As mentioned above, it was hypothesised that higher temperature would result in greater $rETR_{max}$ levels. The higher level of $rETR_{max}$ exhibited by the 25 °C treatment can be explained using Boltzmann's theory which suggests, simply, that increasing temperature increases the speed of movement of all particles and therefore the speed of reaction, since as temperature increases the mass of the particle is reduced and the velocity increases (Trautz 1916). Upon

returning the treated biofilms to the pre-removal temperature, all but the 30°C treatments exhibited increasing $rETR_{max}$ which indicates that the effect of low and moderately high temperature was temporary and no permanent damage was done to PSII or the electron transport chain. At temperatures increase the structure of Rubisco is changed and it binds more readily with oxygen relative to carbon dioxide (Long 1991). This increased affinity for oxygen is also known to maintain electron flow even during periods of limited CO_2, potentially occurring a result of increased chemical reaction rates caused by increasing temperature (Osmond & Grace 1995, Osmond & Badger 1997). This prevents over reduction of Qa, which can result in charge recombinations, which can ultimately lead to the formation of damaging singlet oxygen (Keren *et al*. 1997, Krieger-Liszkay 2005). This activity may prevent any permanent photodamage from occurring in the rocky shore diatom cells.

Like $rETR_{max}$, cells in the 20 °C and 25 °C treated biofilms exhibited the highest light saturation coefficient (*Ek)* (Fig. 3.3.5). This implies that the temperature had increased the capacity of the electron transport chain of the 20 °C and 25 °C treated biofilm cells, and they were able to utilise higher light without the induction of photoregulation. The light utilisation coefficient (α) (Fig. 3.3.4) of the different temperature treated biofilms was not significantly different, suggesting that the higher temperatures were not affecting the functioning of the PSII. The observed fluctuations in α did not follow a consistent pattern during the exposure period, and temperature and exposure time had no significant effect on α. This is likely to be related to the thermal stability of PSII which as can be observed in the *ΔF/Fm'* values (Fig. 3.3.6), which were largely unaffected by exposure temperature. It is known that the effects of high temperatures on PSII are usually permanent and take place at high temperatures (Cajbek *et al*. 1998, Yamane *et al*. 1997). The light adapted quantum efficiency of PSII returned to pre-exposure levels upon cooling in both the winter and spring experiment, suggesting that the effects of temperature on PSII, and/or photochemistry in general, were not permanent at the temperatures examined

in this study. Therefore, photochemistry of rocky shore biofilms appears to be thermally stable.

Winter temperature manipulation experiment (lower and upper shore biofilms

The different shore level responses to temperature (Hypothesis 1)

It was hypothesised that the lower shore biofilms would be less resilient to temperature because the cells are exposed for shorter periods during the emersion period. However these results cause us to reject this hypothesis as the upper shore $rETR_{max}$ exhibited by the biofilms exposed to the extremes of temperature (5, 10, and 30 °C) reduced more during the exposure period (29% compared to 21%). The lower shore cells were found in a matrix which may offer some protection against the external temperature. This again supports the rejection of the hypotheses that the lower shore samples would be more affected by high and low comparative temperature variation.

The samples from the lower and upper shore sites exhibited very different responses to the exposure period in terms of the efficiency of the coefficient of light utilisation at limiting irradiance, α (Figs. 3.3.15 and 3.3.16). The upper shore sites exhibited decreasing α in the 5, 20, 25 and 30 °C treated biofilms. The 10 and 15 °C treated biofilms did not display this reduction. This is likely to be because these temperatures were very similar to those experienced in the field. The samples should be accustomed to these temperatures, but the inherent reduction in reaction rates will slow down electron transport even at unsaturated low light levels which resulted in initially lower α levels. The upper shore biofilms exhibited reducing α during the exposure period, and this further reduced slightly upon warming the samples to pre-removal temperatures. This implies that it may not be temperature which was affecting α, but may have been the experimental conditions.

The light saturation coefficient *Ek* (Figs. 3.3.17 and 3.3.18) recorded from the two shore levels responded in a similar way. A reduction was observed during the exposure period in the *Ek* of the 5 and 10 °C treated biofilms of both shores. This indicates that the cells were becoming more acclimated to low light, possibly as a result of the lower light levels in the greenhouse compared to on the rocky shore. The biofilms from both shore levels were exposed to the same light levels, so a similar *Ek* response pattern was to be expected.

The effect of temperature on the $rETR_{max}$ and *ΔF/Fm'* (Hypothesis 2)

Initially, it appeared that hypothesis (2), that low temperatures would result in reduced $rETR_{max}$, was incorrect, as the 15 °C and 20 °C lower and upper shore treated samples displayed $rETR_{max}$ levels which were initially lower than those recorded from the 5 °C and 10 ° treated biofilm, however the 15 °C and 20 °C treated biofilms were unaffected by the exposure time and the $rETR_{max}$ remained stable, whilst the $rETR_{max}$ recorded from the 5 °C and 10 °C biofilms decreased (Figs 3.3.13 and 3.3.14). The 5 °C and 10 °C treated samples initially displayed high $rETR_{max}$ levels similar to those displayed by the 25 °C and 30 °C samples, however, after 22 h there was a reduction in $rETR_{max}$ which was particularly apparent in the lower shore samples. The cold may well have reduced the speed of photochemical reactions which could have had an impact on *rETR* (Trautz 1916). It has also been shown that colder than usual temperatures, in conjunction with normal light levels, can increase the risk of photoinhibition/photodamage as PSII excitation pressure increases (Tyystjärvi 2008). However, as there was no significant reduction in light adapted *ΔF/Fm'* over the exposure period, this is unlikely. It was also hypothesised that the *ΔF/Fm'* would remain stable in the heated samples but would be suppressed and remain so in the cooled samples. This was not supported as the chilled samples exhibited very stable *ΔF/Fm'* at both shore levels. The lower shore samples (Fig. 3.3.20), exhibited a higher *ΔF/Fm'* prior to temperature exposure but these levels were not regained upon cooling at the end of the exposure. Enzymes and proteins are very sensitive to temperatures above those which they would normally experience (Sizer *et al*. 1943, Lehninger

et al. 1975, Cohn *et al*. 2003, Palmer & Bonner 2007). Damage to bonds between proteins can occur quickly at higher than normal temperatures. It may be that the light harvesting complexes in the PSII were being damaged by this heat (Allakhverdiev *et al*. 2008, Sharkey & Zhang 2010) and this required new protein synthesis to recover, which would have taken longer than the time taken to cool the sample (Behrenfeld *et al*. 1998, Melis 1999). It may also be that due to the higher *ΔF/Fm'* levels earlier in the exposure period, the cells sufficiently fixed carbon and so down regulation occurred in order to reduce the production of reactive oxygen species (Perkins *et al*. 2001). The pattern of *ΔF/Fm'* response was similar with a reduction during the first day of exposure followed by a levelling off, or slight increase, during the remaining exposure period. It is possible that this is a result of the cells utilising the available nutrients during the light period of the day and gradually becoming nutrient deplete. Geider *et al*. (1993) found that under nitrate and iron stress diatoms display a reduction in *Fv/Fm.* As the water was only replaced daily at the end of the light cycle it is possible the nutrients were completely utilised. However, this is unlikely as the samples were small relative to the size of the incubation tanks. The more likely explanation for this apparent pattern is that, as light levels increased during the first day, the cells were becoming light stressed and so were inducing downregulating either in the form of migration or NPQ. The percentage reduction in *Fm'* was observed to increase during the first exposure day (Fig. 3.3.27) indicating that more energy was being dissipated.

Shore level differences in NPQ utilisation (Hypothesis 3)

All treated biofilms exhibited reducing *Fm'* as light levels increased (Figure 3.3.27), meaning that down regulation, likely including NPQ, was being induced by upper and lower shore treated biofilms. NPQ inducement was observed during periods of low-light or darkness. This may be a result of chloro-respiration causing a proton gradient in the thylakoid membranes and activating the xanthophyll cycle (Jakob *et al*. 1999, Grouneva *et al*. 2009). There was a smaller reduction in *Fm'* as exposure time increased, observed in the 30 °C upper shore biofilm

and the 5 and 10 °C lower shore biofilms. This suggests that either the cells were not light stressed at the latter stages of the experiment or that the cells were being prevented from inducing NPQ. It is more likely that the latter is the case as the other treatments did not display this reducing trend. It is possible that the reductions observed in the lower shore samples were as a result of migration of cells into the sediment. Jesus *et al*. (2006) noted that cell migration into sediment resulted in the significant underestimation of NPQ, as the *Fm'* measurement was higher than would be expected. As the lower shore cells are migratory, it is likely that downwards migration during the exposure period resulted in the smaller reduction in *Fm'*. Downward migration by the cells suggests that they were unable to process the light effectively and this is confirmed by reducing α. It was hypothesised (Hypothesis 3) that, as the upper shore cells cannot move into the substratum surface, a greater reduction in *Fm'*, equating to NPQ, would be observed, relative to the lower shore sites. This was not the case and so this part of Hypothesis 3 can be rejected. The second part of Hypothesis 3 predicted that extremes of temperature would result in a greater reduction in *Fm'* and so larger induction of NPQ. This again was not the case and it seems that the extremes of temperature actually reduced the NPQ induced by the cells. The high temperature treated spring biofilms exhibited smaller reductions in *Fm',* indicating less NPQ induction despite the higher light levels. It could be expected then that the winter biofilms exposed to these temperatures would respond in the same way. They appeared however to be more resilient to high temperatures, and so this would suggest that either temperature alone was not responsible for preventing NPQ, or that if the temperature was responsible for preventing NPQ, by damaging the diatoxanthin de-epoxidase and the diatoxanthin epoxidase enzymes, then the winter cells must have mechanisms to protect these proteins. They could be utilising heat shock proteins as produced by cyanobacteria in high light and high temperature situations (Nitta *et al*. 2005) or they may compensate by utilising the violaxanthin cycle as reported by Lohr & Wilhem (1999). It is more probable that a combination of long term high light exposure

and high temperatures were responsible for the reduces ability to induce NPQ in the spring biofilms.

Limiting factors associated with the experiment

During the measurement of the speed of diatom movement a stopwatch was used to time how long the cell took to move across the graticule. The human reaction needed to stop the watch at the correct time was an unavoidable problem, though since one person was responsible for all the measurements, the discrepancy was the same throughout the measurement. The tubes which contained the moving cells also had to be selected which is not ideal. Some tubes contained too many diatoms to get a true measurement of cell speed because the cells would become trapped by other cells. Ideally the tubes would be randomly selected.

The biofilms were stored in tidal simulation tanks in order to keep them alive while the temperature was manipulated. It is assumed that the biofilms were behaving in a natural manner despite being removed from the rocky shore. The tidal simulation tanks were likely to not fully represent the conditions experienced on the rocky shore. The levels of nutrients may be depleted more quickly in the small pools on the rocky shore during the emersion period. The level of wave action was likely to also be higher *in-situ*. This may play an important role in removing detritus and small grazing organisms (Foster 1966). These factors mean that the simple action of removing the samples may impact upon the photosynthetic responses of the cells negatively. However, it could be argued that the circulating water in the tidal tanks, by aerating the water, actually increased oxygenation and CO_2 supply rates compared to static water during an in situ low tide emersion period, and this would likely result in increased photosynthetic rates.

The light levels experienced in the tanks were lower than those experienced *in-situ* due to the tanks being stored in the climate control chambers which were made of acrylic and located in a glass greenhouse. The glass and acrylic attenuated the light levels and the maximum light recorded in the tanks during the spring months was 750 µmol m^{-2} s^{-1} compared to 1975 µmol m^{-2} s^{-1} *in situ*. This was significantly lower and is likely to have influenced the photosynthetic responses of the cells. However, since all the treated biofilms were exposed to this same light level, the differences between samples can be attributed to the temperature manipulations.

CONCLUSIONS

It has been reported that the optimum temperature for photosynthesis of mudflat biofilms was 25 °C (Blanchard *et al*. 1996b, 1997, Guarini *et al*. 2006). This study came to the same conclusion for rocky shore biofilms, with the highest $rETR_{max}$ and *ΔF/Fm'* being exhibited by the 25°C treated biofilms. Despite the fact that the biofilms present on the upper shore were very different to those found on mudflats the response to temperature was similar. Cohn *et al*. (2003) also found that the speed of movement of mudflat diatom cells increased up to 30 °C - 35 °C, after which it reduced. This study again came to a similar conclusion with an increase in the speed of movement of the tube forming cells up to 32 °C. This study provides new information about the response of rocky shore diatoms to temperature which complements information focussing on mudflat biofilms and so provides a more comprehensive overview of the ways in which intertidal biofilms respond to temperature.

- The spring upper shore biofilms exposed to 25˚C and 30˚C temperatures induced lower levels of NPQ. It is likely that the combination of high light exposure and high temperatures prevented the cells from inducing NPQ.
- The upper shore cells were capable of limiting their exposure to light in a similar manner to microphytobenthos found in mudflat environments, by microcycling within the tubes. The cells move within their tubes and limit their exposure to the high light at the edge of the tube.

- The movement was induced at temperatures above 14˚C and the speed of movement increased up to 32˚C at which point it then decreased. As the winter samples, which contain some of the same species as the spring biofilms, did not exhibit movement at any temperature, it is likely that temperature is not the sole trigger for movement.
- The lower shore samples were in general more resilient to both increased and decreased temperature. Therefore the hypothesis that this shore level would be more vulnerable was rejected. It is likely that the ability of the cells to migrate into the sediment, reduced their exposure to the simulated temperatures, reducing the effect of them.
- The decrease in temperature had a more negative effect on $rETR_{max}$ than the increase in temperature, implying that the increased reaction rates induced by increasing temperature in part mitigated the negative effects of high temperature such as damage to protein structure. The chilling process may have slowed reaction rates and thus resulted in a reduction of $rETR_{max}$.

The effect of temperature on the photosynthetic responses of rocky shore biofilms was clearly demonstrated. This must be taken into account by researchers using fluorescence to study rocky shore biofilms in the future as temperature is a variable and this must be acknowledged and controlled if unaffected measurements are to be obtained. The fact that temperature had an impact on the photosynthetic responses and photoregulation of these biofilms has relevance to further photosynthetic studies focussing on microphytobenthos, but there is also a potential larger scale relevance to this study. The IPCC Special Report on Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation (Field *et al*. 2011) predicts that there will be more frequent 'freak' high and low temperature events occurring in Britain as a result of the overall increase in global temperature. If there are high temperature events occurring earlier in the spring, before the cells have the ability to microcycle within their tubes, induced by increasing ambient temperature (to above 14 °C), the tube forming diatoms may not have a mechanism to regulate their photosynthesis and therefore may die-off

much earlier in the year. As these biofilms are an important food source for rocky shore grazers (Chapter 2) this may have trophic implications.

Overall this study has increased our understanding of the way in which rocky shore diatoms respond to different temperatures and light. The photosynthesis, investigated using PAM fluorescence, has not been studied before. The changes in the rates of movement have also not been studied in tube-forming diatoms. Although movement has been observed (Houpt 1987), the factors which affect this movement have not, and this information is new to science.

THE EFFECT OF HIGH LIGHT ON THE PHOTOPHYSIOLOGY AND BEHAVIOUR OF ROCKY SHORE MICROPHYTOBENTHOS

CHAPTER 4

ABSTRACT

Mudflat based diatom dominated biofilms are known to utilise migration as the primary form of behavioural photoregulation. These diatoms then utilise non-photochemical quenching (NPQ) as a 'fine tuning' physiological mechanism, in conjunction with migration. Despite this detailed knowledge of mudflat systems, in contrast very little is known about the photoregulatory mechanisms of rocky shore biofilms. The rocky shore biofilms present at Dunraven Bay occurred in two forms. The lower shore biofilms were similar to mudflat biofilms, as the cells were predominantly free-living within a thin layer of sediment. The upper shore biofilms were dominated by polysaccharide tube-forming species attached to the rocky substratum. In order to elucidate the photoregulatory mechanism of these biofilms Latrunculin-a (LAT-A) was used to inhibit cell movement and DL- Dithiothreitol (DTT) to inhibit NPQ. Photosynthetic parameters were measured ($rETR_{max}$,, *ΔF/Fm' α Ek,* and *Fm'*) at five time points: prior to chemical application, post chemical application, after 2 hours of ambient light exposure, after 4 hours of ambient light exposure and after one week. The upper shore biofilms in all treatments, including the control treatment, showed photoinhibition during the exposure period. This was indicated by a reduction in $rETR_{max}$ and *ΔF/Fm'* and by reduction of *Fm'*. The patterns of response, in the photosynthetic parameters, over the experiment period were most similar between the LAT-A treated and control biofilms at the upper shore sites, indicating that the primary means of photoregulation used by the upper shore biofilms was non-photochemical quenching. In contrast there was more similarity in the photosynthetic parameter patterns between the DTT treated and control lower shore biofilms, indicating that the primary means of photoregulation used by the microalgal cells in the lower shore biofilms was cell movement (vertically within the sediment layer). There was however no significant difference between the chemical treatments, therefore only the patterns of response could be interpreted. Due to the exposed nature of the rocky shore, it is likely that the biofilms cells have multiple methods of photoregulation in order to survive and acclimate to light conditions on the rocky shore. This study found that each shore level was able to utilise a secondary means of photo-regulation (upper shore, NPQ followed by cell movement; lower shore, cell movement

followed by NPQ), which allowed for successful downregulation, preventing permanent photodamage.

INTRODUCTION

Rocky shore biofilms are highly variable, both in terms of community structure and in abundance (Lewis 1964, Williams *et al*. 2000, Hutchinson & Williams 2001). Some shore areas are not covered by biofilms and the shore areas that support the biofilms are influenced by abiotic factors and grazing. These factors drive the patchiness of rocky shore biofilms. Figure 4.1.1 shows the variability in habitat structure at Dunraven Bay. The red, highlighted box displays an area of biofilm on the rocky shore, but the photo as a whole illustrates the large scale heterogeneity on the rocky shore, with dry areas, devoid of biofilm.

Figure 4.1.1*, Dunraven Bay*, Bridgend County: Heterogeneous and patchy biofilms.

Spatial heterogeneity or patchiness has been recognised at all ecological scales including benthic (Morrisey *et al*. 1992, van de Koppel *et al*. 2012) and planktonic (Seuront & Schmitt 1999) microalgal systems. The organisms are rarely distributed evenly, and as heterogeneity is the norm, ecological sampling must take this into account. The patchiness of rocky shore biofilms was apparent at Dunraven Bay (see Chapter 2) at various scales (i.e. centimetres to metres). As such, the small area measured by a Walz Water PAM (Walz GmbH Germany) may not fully represent the photophysiology of the biofilm on a larger scale (Fig. 4.1.2). In fact Spilmont *et al*. (2011) quantitatively inferred that in order to get a true microphytobenthic biomass measure, 225 sediment samples over a 9 m^2 area must be taken. This is a very large sampling effort and in many tidal areas would be impossible to undertake. Therefore it must be assumed that some sampling bias is occurring. Biomass measurements were not being made during this investigation, and as was shown in Chapter 2, taxonomic variation had little effect on the photophysiology of the biofilm. However, the variation in biomass distribution within the biofilms would result in variations in the light adapted maximum fluorescence (*Fm'*) (Jesus, Brotas, & Paterson 2005), and as this photosynthetic parameter was being used to interpret the photoregulatory activity of the biofilm, this is undesirable. A different approach was therefore taken, using an imaging fluorometer; the Walz Mini IPAM imaged a larger area (24 x 32 mm) from which multiple measurement areas could be chosen. In this investigation areas were chosen at random (random block design), but if the area selected contained no biofilm another was selected. An example of the biofilm as visualised using chlorophyll fluorescence from the Walz Mini IPAM is shown in Figure 4.1.2. Please see the methods section for further information regarding the Walz IPAM.

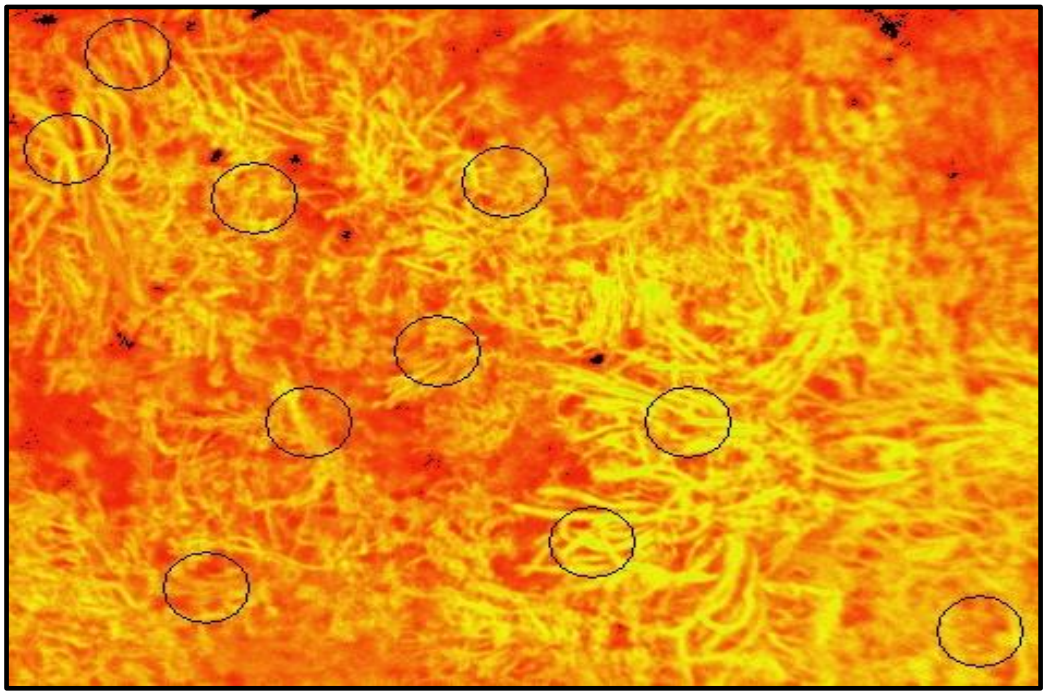


Figure 4.1.2*: IPAM image of tube forming diatoms on the shore.* There were 10 areas selected, from which fluorescence measurements were recorded, and these were internally averaged by the fluorometer.

The photoregulation of mudflat biofilms has been studied extensively, but the present study was the first to investigate photoregulatory mechanisms of rocky shore microphytobenthos. Cells on soft mudflat sediments have been reported to use either behavioural or physiological mechanisms of downregulation. Vertical cell movements of microcycling and bulk migration (Barranguet & Kromkamp 1998, Serôdio & Catarino 1999, Perkins *et al*. 2001, Paterson *et al*. 2001) are behavioural responses of these cells, to a range of stimuli including light and tidal regime. They are known to respond to light by moving into the sediment during periods of high light and moving upwards when more light is required for photosynthesis (Jesus *et al*. 2005, Perkins *et al*. 2010). The cells are known to move within the sediment surface (microcycling) thus regulating their exposure to light by shading themselves within the upper sediment layers (Barranguet & Kromkamp 1998, Serôdio & Catarino. 1999, Perkins *et al*. 2001, 2002, 2010). For more detailed information about diatom movement refer to the general introduction to this thesis (Introduction section 1.4.1). Non-photochemical quenching (NPQ) in the form of the xanthophyll cycle (Young *et al*. 1997, Serôdio *et al*. 2005, Jesus *et al*. 2006, Goss & Jakob 2010,

Perkins *et al*. 2002, 2006, 2010) is used to physiologically regulate photosynthesis. These mechanisms are influenced by the light dose that the cells are exposed to (Barranguet & Kromkamp 1998, Serôdio & Catarino 1999, Perkins *et al*. 2001, Jesus *et al*. 2005, Pinckney & Zingmark 1991). Refer to the introduction (section 1.4.2) for more detailed information about the mechanism responsible for NPQ. Previous work (Lavaud *et al*. 2002, Cartaxana & Serôdio 2008, Cartaxana *et al*. 2008) has shown that both processes of photoregulation, behavioural and physiological, can be inhibited by chemical manipulation. Latrunculin- A (LAT-A) inhibits cell movement by inhibiting actin fibre function (Cartaxana & Serôdio 2008) and DL-Dithiothreitol (DTT) inhibits NPQ by preventing diadinoxanthin to diatoxanthin conversion (Olaizola *et al*. 1994). By using these chemicals photophysiological responses of the biofilms can be measured whilst particular photoregulatory mechanisms are inhibited. In order to ascertain which mechanisms are being used and elucidate which, if any, of these is the primary photoregulatory mechanism.

Lamontagne *et al*. (1989) investigated the photosynthesis of a rocky shore microphytobenthic biofilm over an emersion period using C^{14}. These biofilms were very different to those at Dunraven Bay as they were completely exposed during emersion. The cells did not appear to have any photoregulation strategy with the cells being completely photoinhibited during emersion. However, this photoinhibition did not appear to affect the overall productivity of the system which was equal to that seen in mudflat systems (data compared to review by Colijn & de Jonge (1984). This being the case a detailed investigation into photoregulation using fluorescence would provide important new insight into these highly productive areas and allow us to more fully understand the processes of rocky shore communities and the intertidal in general.

HYPOTHESES

1. It was hypothesised that the lower shore biofilms, being similar to mudflat biofilms, would utilise 'microcycling' vertical cell movement as the primary means of photoregulation. This was investigated be recording the fluorescence patterns of response over a period of exposure to ambient light under the influence of two chemical treatments whilst monitoring a control, untreated biofilm. In contrast, it was hypothesised that the upper shore, tube-dwelling biofilm community would utilise NPQ as the primary means of photoregulation, being unable to move vertically away from high light into sediment. As such, the photosynthetic parameters recorded at lower shore sites would be more similar between the DTT treated biofilm and the untreated control biofilm, whereas the upper shore biofilm would show more similarity between the LAT-A treated biofilm and the control biofilm.

2. It was hypothesised that the control biofilms would exhibit the highest $rETR_{max}$ and *ΔF/Fm'* as these biofilms were able to utilise normal photoregulation methods.

3. It was hypothesised that the biofilms treated with the chemical (LAT- A or DTT) which was to inhibit the predicted primary photoregulatory mechanism, would show the greatest reduction in photosynthetic parameters of $rETR_{max}$ and *ΔF/Fm'* as the cells would become photoinhibited.

4. It was hypothesised that the cells treated with LAT-A, which cannot move, would exhibit greater *Ek* levels, as the cells would be forced to photoacclimate to a higher light level. The *Ek* levels were expected to increase quickly and after this photoacclimation had taken place, an increase in the $rETR_{max}$ and *ΔF/Fm'* was expected at the last time point.

5. It was hypothesised that chemically treated biofilms would also exhibit lower $rETR_{max}$ and *ΔF/Fm'* one week after the initial treatment as it was expected that this period of stress would have had a permanent photodamage effect.

METHODS

Experimental overview

To determine what forms of downregulation were being utilised (behavioural, through cell vertical movement, or physiological, through non photochemical quenching), chemical inhibitors described above (LAT-A and DTT) were added to small rock pools on Dunraven Bay (51° 44.65'N, 03° 60.73'W), containing dense biofilm growth (determined visually), at the upper and lower shore sampling sites used throughout this thesis. Chemical treatments were LAT-A, to inhibit cell motility (Cartaxana & Serôdio 2008), and DTT, which inhibits NPQ (Lavaud, *et al*. 2002).

Experimental Design

The experiment was completed in the field at Dunraven Bay on the 13 April 2011. The temperature on the measurement day was 17 ℃. Nine sites were chosen on the lower and upper shore, 3 control pools, 3 LAT-A treated pools and 3 DTT treated pools (*n=3*). The overlaying water from all pools was carefully removed prior to the experimental period. Chemical solutions (LAT-A or DTT, see below for details) were poured onto the 6 sites at each shore level after the removal of the overlying water. Water used for making the solutions required for each chemical treatment was collected from the sample site the previous day and stored at 4 °C overnight prior to treatment. Control sample sites consisted of similar pools, where the overlaying water was also removed and this was replaced at the same time as the addition of the chemical solutions. Initial, 'pre chemical' application, fluorescence measurements were made before the chemical application, when the biofilm was unaltered and covered in site water. This measurement was made one hour after emersion and so after

one hour of exposure to ambient light (photodose = 6.5 mol/photons m^{-2} (Fig. 4.3.1)). The chemical solutions or site water for the control biofilm were then applied and the samples were covered in black polythene for one hour to prevent light exposure. The 'post chemical' application measurement was made after this dark period (photodose = 6.5 mol/photons m^{-2}). The 'T1' measurements were taken after 2 hours of ambient light exposure (photodose = 13 mol/photons m^{-2}). The 'T2' measurements were taken after a further 4 hours (photodose = 21 mol/photons m^{-2}). 'One week post experiment' measurements were taken at the sites to investigate whether the effects of the chemicals persisted. The measurement points were termed; pre chemical, post chemical, T1, T2 and one week post experiment.

Light measurements

The ambient light levels were recorded using the cosine corrected light meter of the Walz Water PAM. The light dose was calculated from the product of light measurement and exposure time from the beginning of the emersion period. Units of light dose were mole of photons m^{-2} as a simple integration of light dose over time.

Chemical preparation and application

DL- Dithiothreitol (DTT)

The DTT treatment was prepared on the morning of the experiment. A 160 mM initial stock solution was prepared by diluting the dry DTT in ethanol. This was then diluted using the site water to a 1600 µM concentration. A different volume of either LAT-A or DTT solution in site water was added to each pool, but resulting in the same water depth overlaying the biofilms. As the level of water cover affects the light levels that the cells were exposed to, variation in this would result in the biofilms being exposed to different light levels between replicates and treatments, in turn affecting the photosynthetic response. Thus the total magnitude of chemical in each pool varied but the concentration was the same for each replicate.

The concentration of DTT required to inhibit NPQ was determined by performing a preliminary experiment with the concentration used by Perkins *et al*. (2010) which was 800 µM and double this concentration of 1600 µM. This was done as the diatoms were tube-forming and it was unknown whether these tubes would reduce the level of chemical that the cells were exposed to. Measurements were made using the Walz Mini IPAM and it was determined that in order to successfully inhibit NPQ (as observed from the inhibition of quenching in maximum fluorescence yield under exposure to high light (*Fm'*)) the higher concentration should be used on the rocky shore to ensure complete suppression of NPQ, i.e. no reduction in *Fm'*.

Latrunculin-a (LAT-A)

A 1 mM solution of Latrunculin-A was prepared on the morning of the experiment. The Latrunculin-A waxy solid was diluted using dimethylsulfoxide. This liquid was then diluted using site water to a concentration of 12.5 µM. Due to the expense of Latrunculin-A only a small amount of solution could be produced. Smaller pools were chosen, as the LAT-A sites, so that the liquid level in the pools could be maintained with the smaller amount of solution available. The concentration was determined by Perkins *et al*. (2010) to be sufficient to inhibit the movement of the cells in mud-flat based biofilms, but this could not be increased, as in the case of the DTT treatment, due to the limited availability.

Fluorescence measurements

Rapid light curve were obtained using a Walz IPAM fluorometer at light levels increasing in steps (applied by the internal actinic light source) from 0 µmol m^{-2} s^{-1} to 1037 µmol m^{-2} s^{-1}. Measurements were obtained after 30 s (Perkins *et al*. 2006) incubation at each light level. The highest light level output produced light saturated data from the biofilms in this area as this is above the light saturation coefficient (*Ek*) determined in previous work (Chapter 2). ImagingWin® software (Walz, Effeltrich, Germany) was used to control the light steps produced by the fluorometer. Light curve measurements were taken in a random (random

block design using assigned random numbering of sites) order between chemical treatments. At each time point, however, lower shore measurements were made prior to upper shore measurements due to tidal restrictions. The fluorescence parameters were calculated using the equations outlined in the General Introduction, section 1.5.2. The percentage change in *Fm'* was used to illustrate the cellular downregulation, rather than the absolute *Fm',* as the change is what is important and this is best illustrated as a percentage change.

Like the Walz Water PAM, the Walz Mini IPAM measures chlorophyll fluorescence yield. LED lights generate the pulse-modulated measuring light, actinic light and saturating pulse. The light was applied to the whole biofilm area and measurements were recorded from the selected areas of the biofilm (see Walz (2009) Imaging PAM manual for more information). This method provided a non-destructive way of assessing the photophysiology of the biofilms. The results were averaged internally to provide a mean biofilm response, which is less subjective than the Water PAM. This analysis of several sample areas at the same time provided parallel results which had been exposed to the same light regime. This minimises the variables that can cause differences in the responses of the samples, such as the distance of the measuring head from the sample and slight movements of the measuring head. The usual field set-up of the Water PAM involves using a clamp stand to minimise these variables, but wind can still move the measuring head and undulations in the rock can mean that the head is not maintained at a constant distance from the test surface.

The IPAM (Figure 4.2.2) provided a solid base which sat on the rock surface so the distance from the measuring head and the sample did not change. As the equipment is heavy, the wind did not affect the measuring head. The Walz Mini IPAM uses a 1/3" CCD camera with a F1.2/f=12mm objective lens. The image area is 24 x 32 mm and it was illuminated using a Luxean LED array which uses four groups of three LEDs. This allowed the IPAM to maintain variable light levels over a large area without the addition of heat to the investigation area,

providing an excellent non-invasive method to observe spatio-temporal variations in the photophysiology of the biofilm.

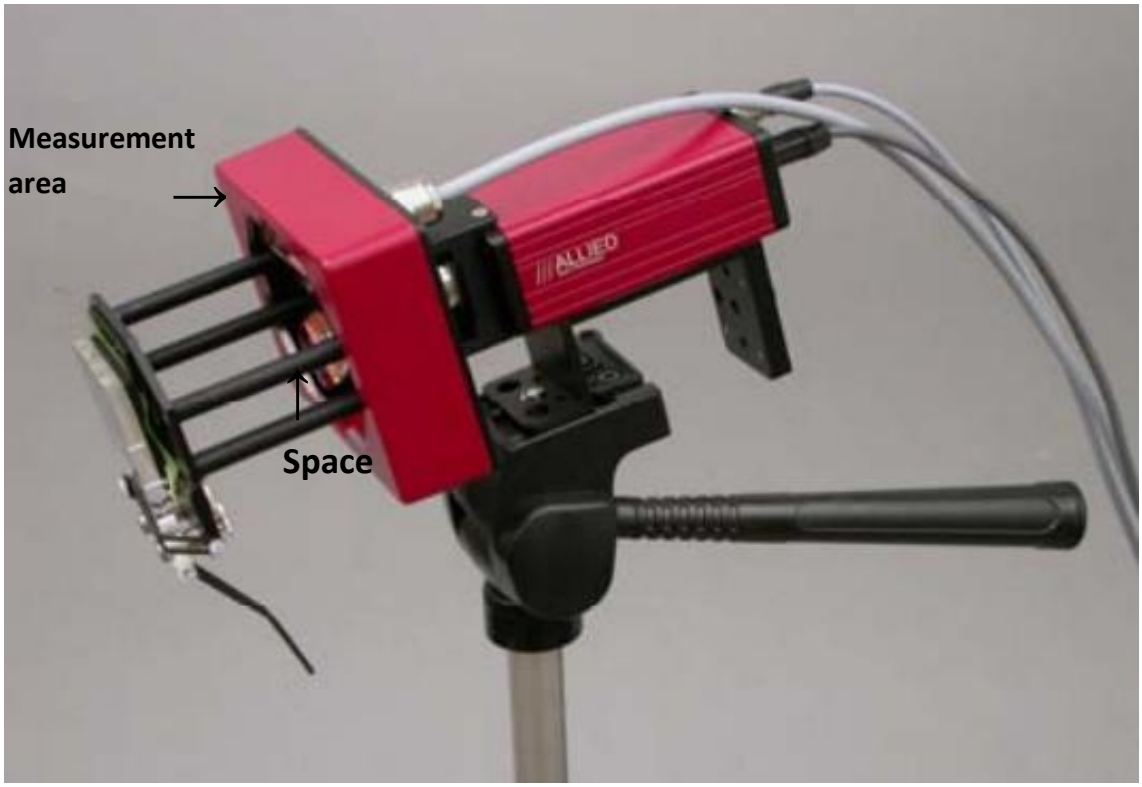


Figure 4.2.2*:* The Mini Walz IPAM provides a stable measuring head which permanently maintained the distance of the measuring head from the biofilm.

Statistical analysis

Normality and homogeneity of variance of data were tested using the Kolmogorov-Smirnov test, followed by Bartlett's test respectively. The data were balanced so significant difference was determined using a 3 factor nested ANOVA with chemical treatment (LAT-A, DTT or controls) nested within time (Pre chemical, post chemical, T1, T2 and one week post), nested within shore level (upper and lower). This resulted in triplicates for each of the 3 chemical treatments nested within 5 time points within 2 shore levels.

RESULTS

Light measurements

Figure 4.3.1 illustrates the ambient PAR µmol m^{-2} s^{-1} over the day before and during the experiment. Once the chemical treatments were applied the samples were covered to allow the LAT-A and DTT to take effect without the ambient light affecting the cells. Two further shorter periods of darkness then refer to the periods of fluorescence measurements (indicated in red).

The measurements were taken at 1200, 1300, 1500 and 1700 hours. The PAR levels were recorded from the beginning of the emersion period at 11.00, and were measured every 30 seconds. The PAR levels reduced from the beginning of the measurement period. The PAR levels were 0 for one hour after chemical addition. The cumulative light dose increased during the experiment period. The light levels did not fluctuate greatly during the day as there was no cloud cover.

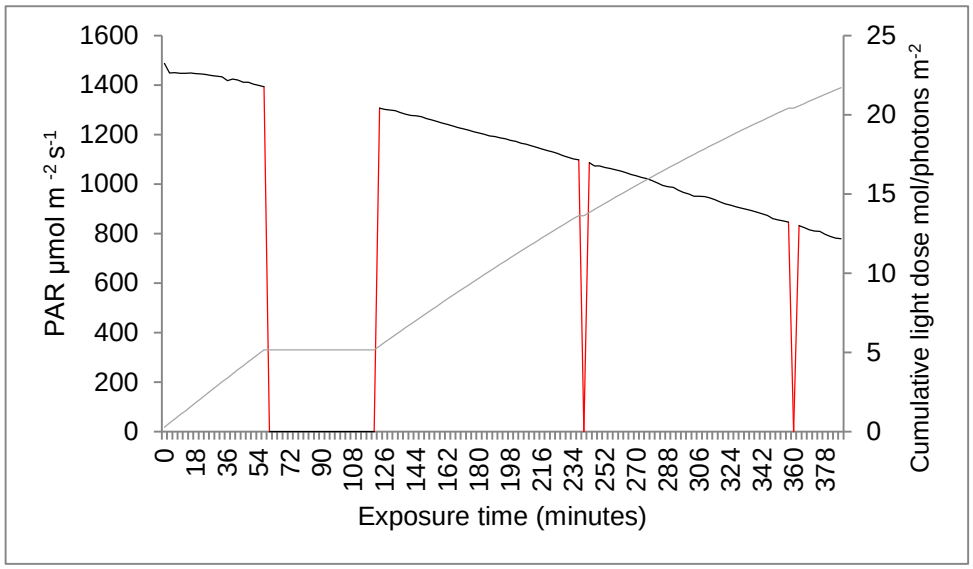


Figure 4.3.1*: Light dose (mols.photons m^{-2}) and photosynthetically active radiation (PAR) (µmol m^{-2} s^{-1})* levels taken at three minute intervals throughout the emersion period. Red lines represent measurement times.

Photophysiological measurements

Maximum relative electron transport rate (*rETR$_{max}$*)

The shore level, and hence taxonomic community, from which the biofilm was removed, had no significant effect on the *rETR$_{max}$* over the experimental period (Figs. 4.3.2 and 4.3.3), due to the fluctuations in the *rETR$_{max}$* levels. The measurement time point did have a significant effect on the *rETR$_{max}$* ($F = 2.787$, $df = 8, 89$, $p < 0.05$). The control biofilms from both shores exhibited the same general pattern with an increase after the 1 hour dark period (for chemical addition). This was followed by a decrease in *rETR$_{max}$* to T2. There was then a slight increase after one week of 'recovery time'.

There was a difference in the pattern of response in the lower (Fig. 4.3.3) and upper shore LAT-A treated samples (Fig. 4.3.2). The upper shore LAT-A treated biofilms showed a similar pattern to the control biofilms with an increase after the dark period followed by a decrease to T2. There was a pronounced increase after one week of recovery. The *rETR$_{max}$* response recorded from the lower shore LAT-A treated biofilms was not similar to the control biofilm. There was a decrease after the dark period followed by an increase to T1 and again a decrease to T2. There was also a small decrease after the one week 'recovery period'.

The DTT treated biofilms of the upper shore exhibited a different pattern of response to the control biofilms. There was an increase in *rETR$_{max}$* to T1 followed by a decrease. In contrast the lower shore DTT treated biofilms exhibited the same pattern in $rETR_{max}$ response as the control biofilms. Despite these trends there was no significant effect of the chemical treatment on the *rETR$_{max}$*.

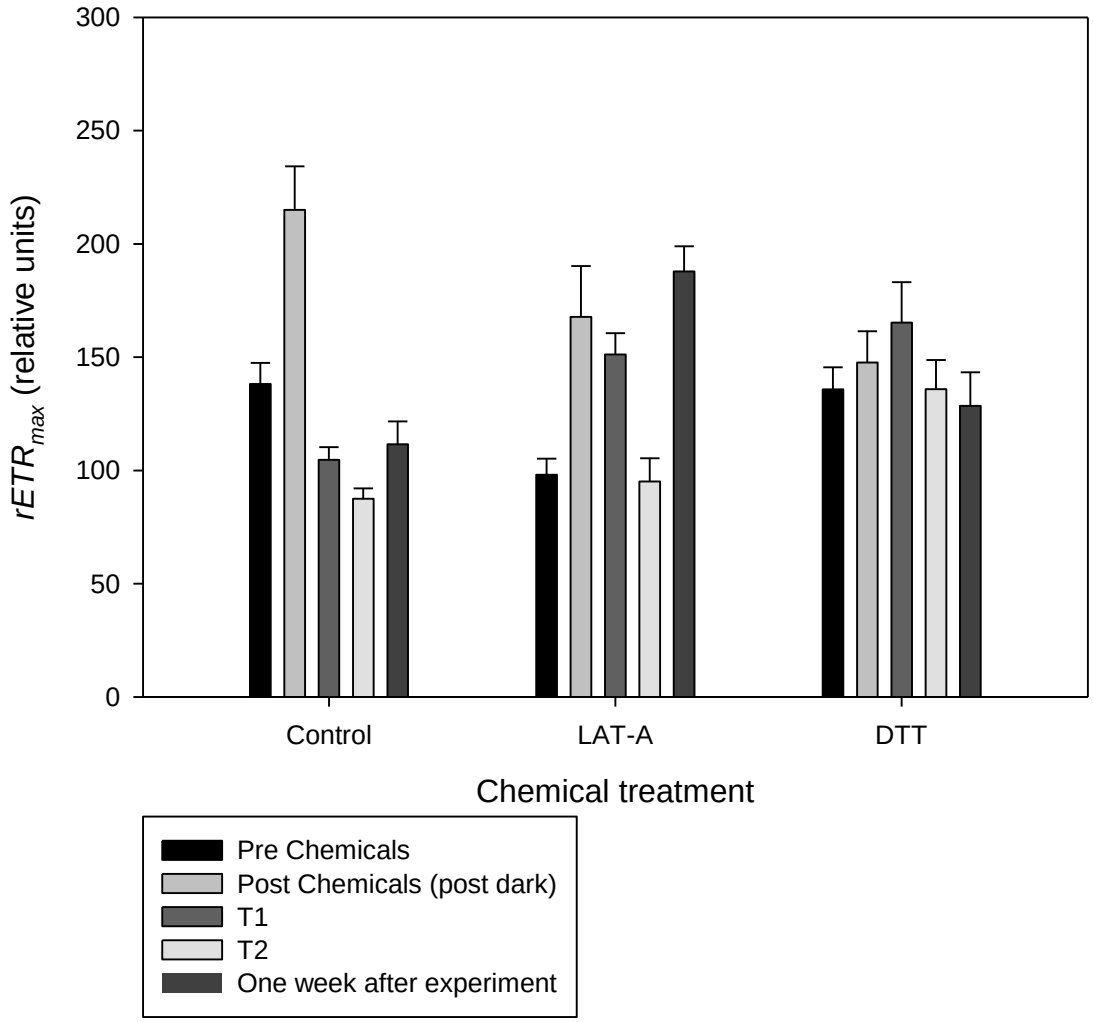


Figure 4.3.2: *Maximum relative electron transport rate* (*rETR$_{max}$*), of each chemical treated and control biofilm from the upper shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

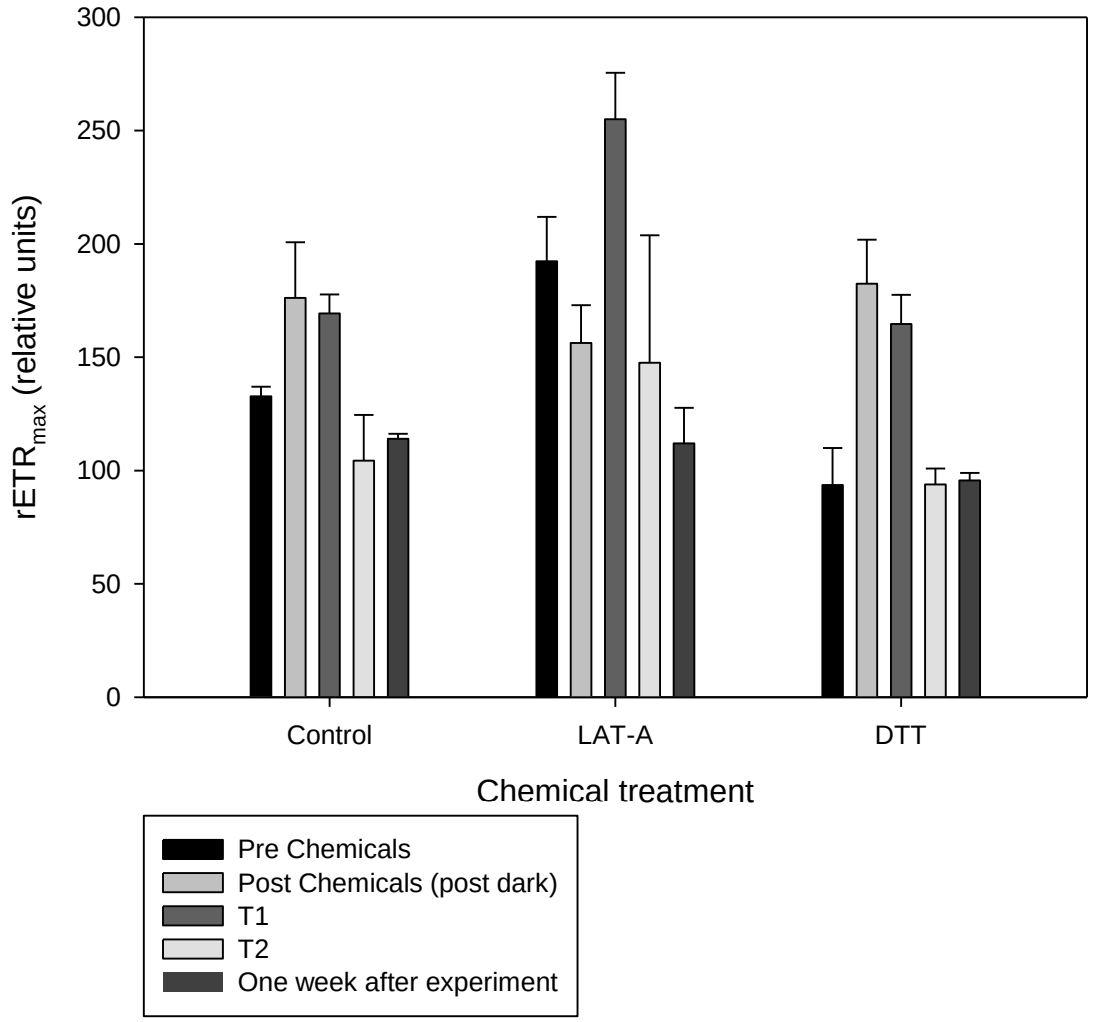


Figure 4.3.3: *Maximum relative electron transport rate* (*rETR$_{max}$*), of each chemical treated and control biofilm from the lower shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

Efficiency of light utilisation (α)

Shore level had no significant effect on α over the experimental period due to a large range in measured values (Figs. 4.3.4 and 4.3.5). However, there was a significant effect of measurement time point on α ($F = 2.787$, $df = 8, 89$, $p < 0.05$). Unlike $rETR_{max}$, the control biofilms of the upper and lower shore did not exhibit the same general pattern of response, although both exhibited an increase in α after the dark period. On light exposure, the upper shore biofilms then exhibited a decrease at T1 and T2, followed by an increase after the one-week recovery period. The lower shore biofilm exhibited a decrease from the post dark measurement to T1, but there was then an increase from T1 to T2, again followed by an increase after the one-week recovery period (Fig. 4.3.5).

As in the $rETR_{max}$ measurements the pattern of response in the upper shore biofilms was most similar between the LAT-A treated biofilms and the control biofilm (Fig. 4.3.4). The lower shore DTT treated biofilms exhibited a similar response to the control biofilm.

The upper shore DTT and lower shore LAT-A treated biofilm, i.e. the samples treated with chemicals which inhibit the expected primary photoregulatory response, exhibited the same pattern of response, with a decrease after the dark period followed to T1 followed by an increase to T2 and to the one-week recovery period. The chemical treatment did not have a significant effect on α although this was marginal ($F = 1.290$, $df = 20, 89$, $P = 0.086$).

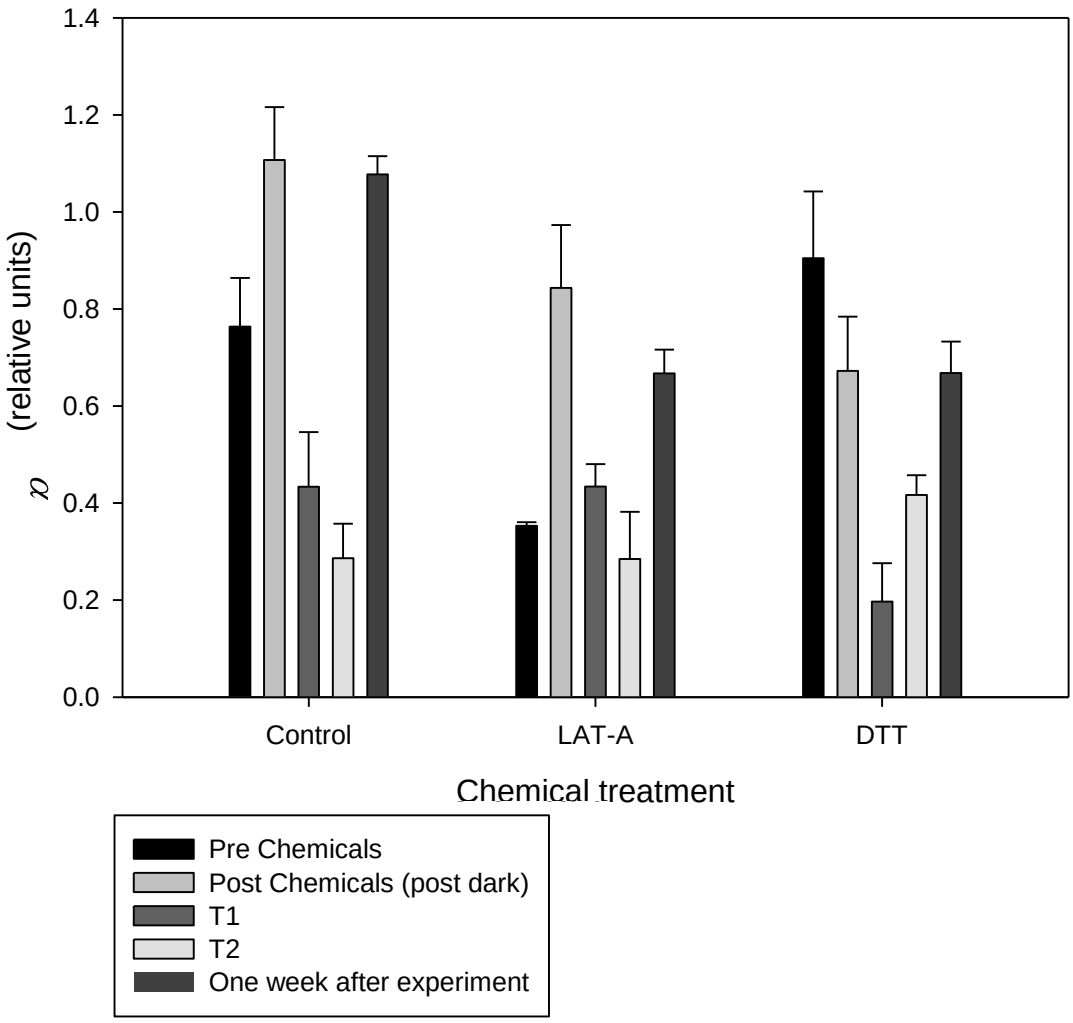


Figure 4.3.4: *Efficiency of light utilisation* (α), of each chemical treated and control biofilm from the upper shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

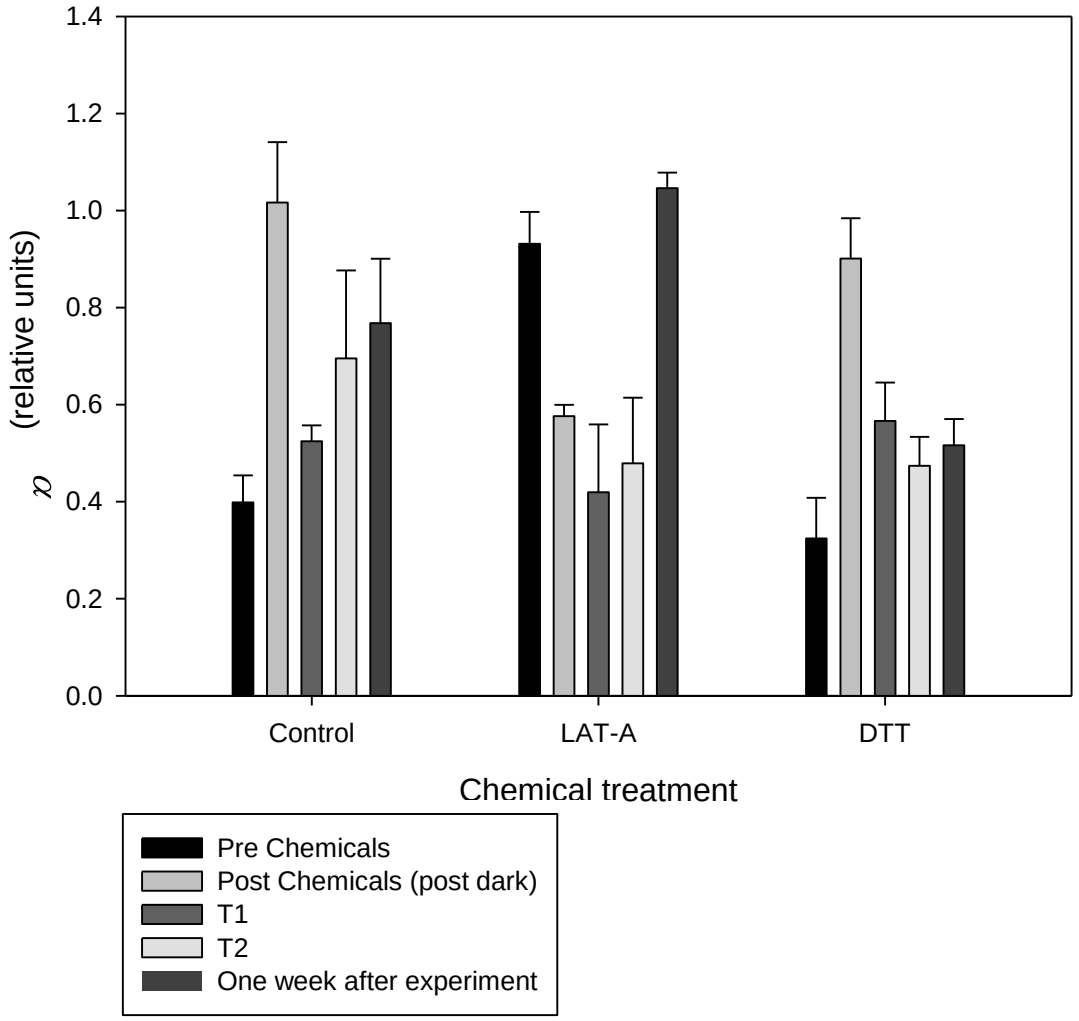


Figure 4.3.5: *Efficiency of light utilisation* (α), of each chemical treated and control biofilm from the lower shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

Light utilisation coefficient (*Ek*)

The shore level had no significant effect on *Ek* (Figs. 4.3.6 and 4.3.7). There was also no significant effect of the measurement time point on the *Ek*. Unlike $rETR_{max}$ and α, the 3 treatments within the upper shore sites exhibited a similar pattern of response to each other, with the DTT treated biofilm again being most similar to the control biofilm (Fig. 4.3.6).

The lower shore biofilm in contrast was more similar to the patterns observed for $rETR_{max}$ and α, with the DTT treated biofilm exhibiting the same pattern of response as the control biofilms, with an initial decrease after the dark period, followed by an increase to T1 and a subsequent decrease to T2 (Fig. 4.3.7). Both of these treatments exhibited a slight decrease in *Ek* after the one-week recovery period.

In contrast, the LAT-A treated biofilm exhibited an increase in *Ek* from the pre-chemical measurement point to T1, followed by a slight decrease to T2. There was a large decrease in *Ek* after the one-week recovery period. Despite these differences noted in the lower shore biofilms the chemical treatment had no significant effect on the *Ek.*

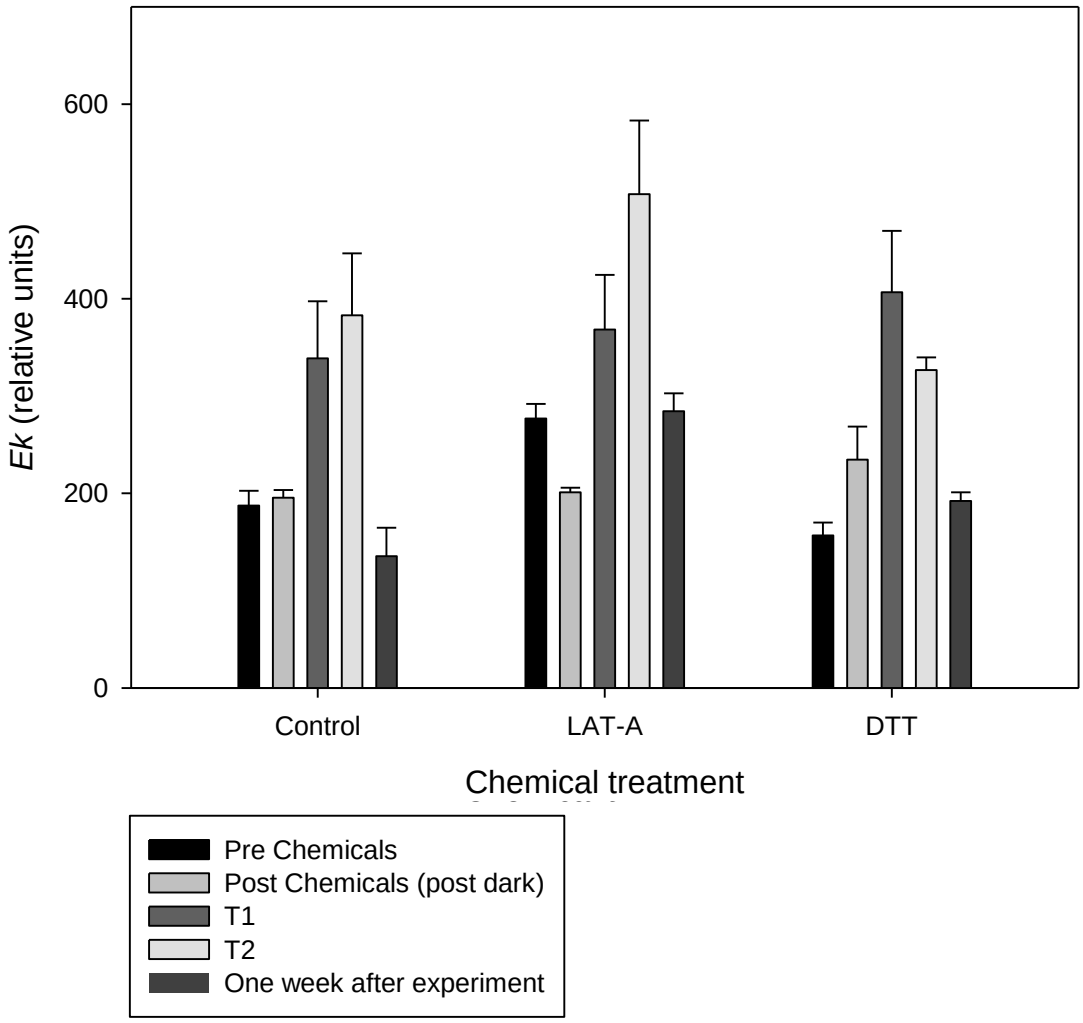


Figure 4.3.6: *Light utilisation coefficient* (*Ek),* of each chemical treated and control biofilm from the upper shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

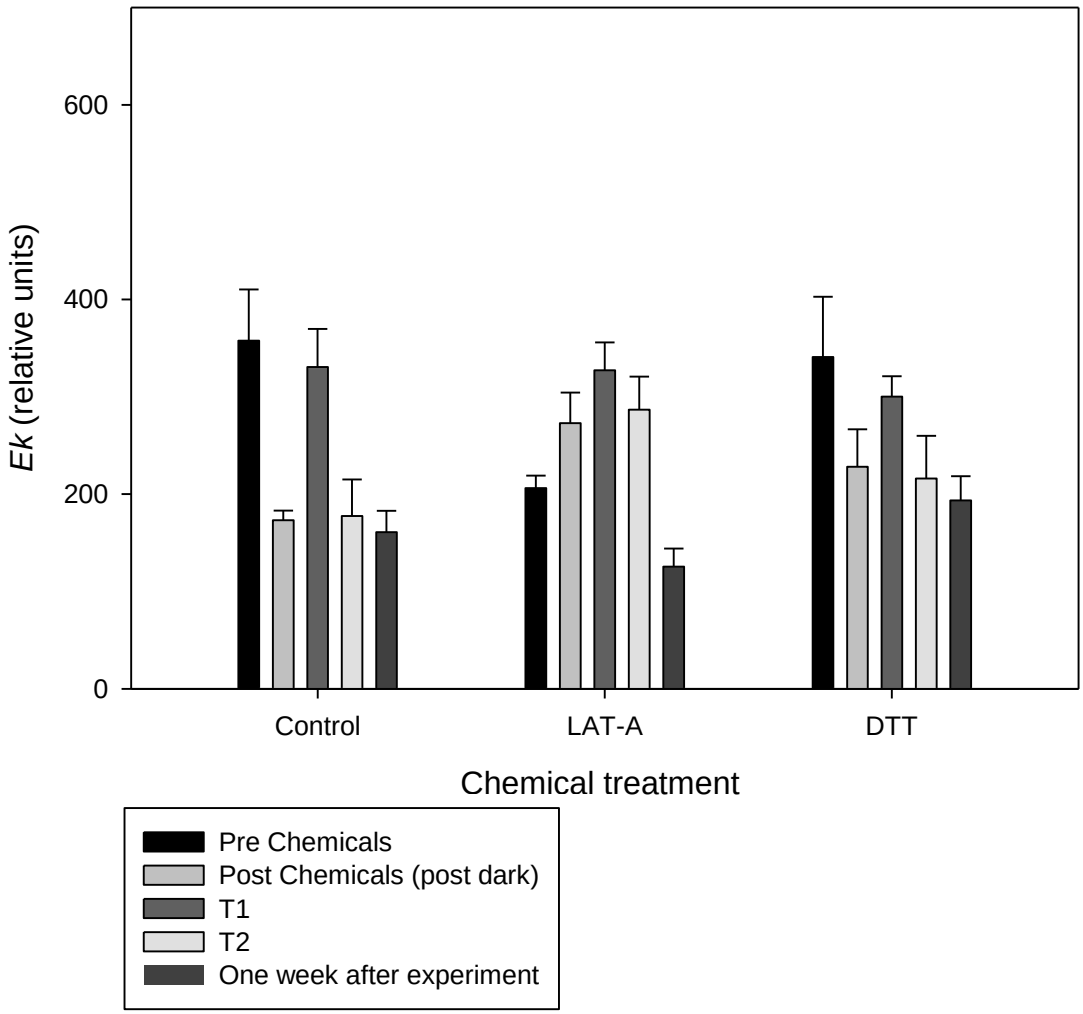


Figure 4.3.7: *Light utilisation coefficient* (*Ek),* of each chemical treated and control biofilm from the lower shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

Light adapted quantum efficiency (*ΔF/Fm'*)

The *ΔF/Fm'* responded very differently to the other photosynthetic parameters. The control treatments did not display the same pattern of response as either of the chemical treatments at the lower or upper shore sites (Figs. 4.3.8 and 4.3.9), and chemical treatment had no significant effect on *ΔF/Fm'* of the biofilms. There was also no significant effect of both the shore level or the measurement time on the *ΔF/Fm'*.

The control treatments at each shore level had opposing patterns in *ΔF/Fm'*. The upper shore control biofilm exhibited decreasing *ΔF/Fm'* as the experiment progressed, which only increased after the one-week recovery period (Fig. 4.3.8). The lower shore biofilm exhibited a general increase in *ΔF/Fm'* with a decrease only occurring at T1 after the dark period (Fig 4.3.9).

The lower shore DTT treated biofilm appeared to exhibit lower *ΔF/Fm'* than the control and LAT-A treated biofilm, however as mentioned this was insignificant. Despite the high photodose experienced by the cells by T2, the efficiency of PSII remained high in the lower shore biofilms.

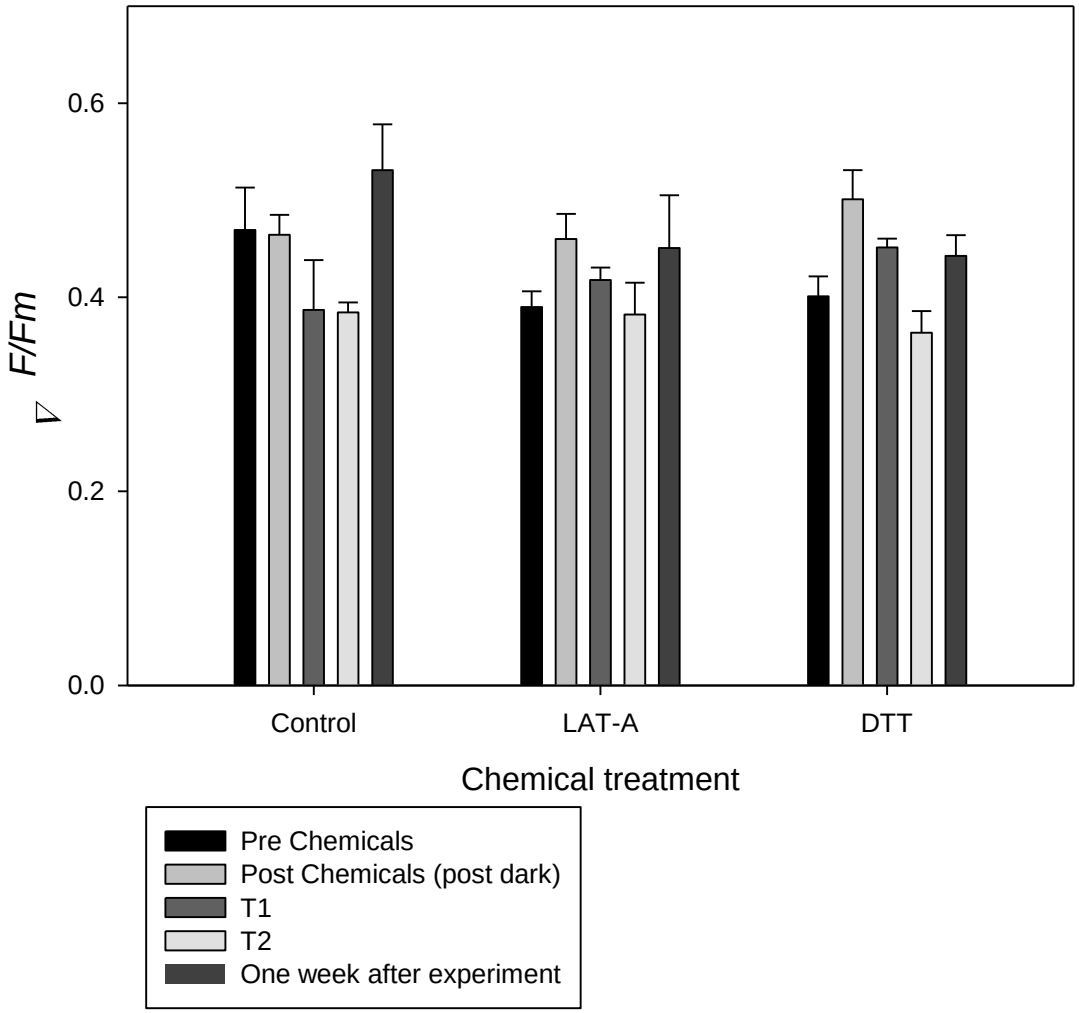


Figure 4.3.8: *Light adapted quantum efficiency of PSii (ΔF/Fm'),* of each chemical treated and control biofilm from the upper shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

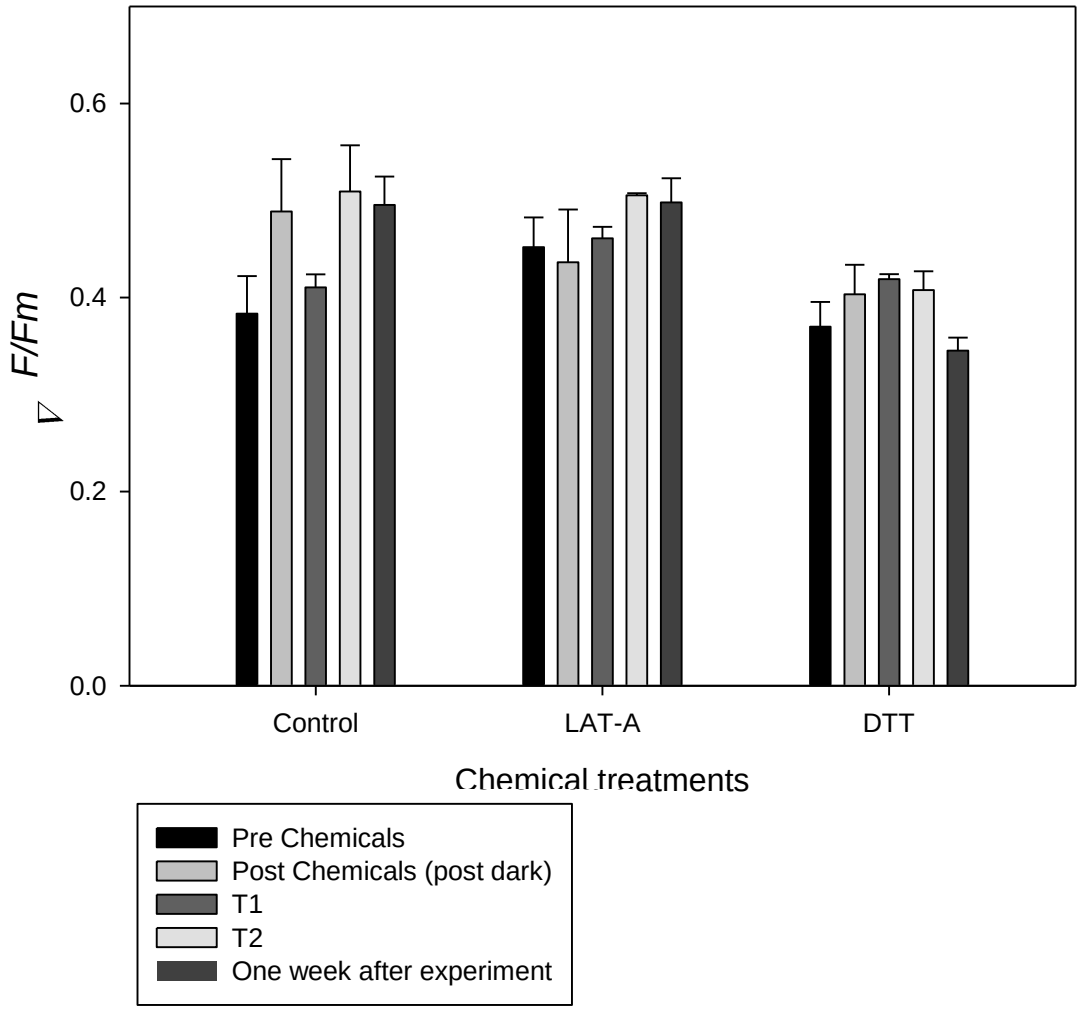


Figure 4.3.9: *Light adapted quantum efficiency of PSii (ΔF/Fm'),* of each chemical treated and control biofilm from the lower shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

Photophysiological Downregulation - percentage change in light adapted maximum fluorescence (*Fm'*)

At the upper shore sites the greatest percentage reduction in *Fm'* occurred during T1 and T2 when the cells had been exposed to 2 hours (photodose= 13 mol/photons m^{-2}) and 4 hours (photodose= 21 mol/photons m^{-2}) of light, respectively. This was apparent in the upper shore biofilms (Fig. 4.3.10). The upper shore biofilms at T1 and T2 displayed an initial ~ 60% decrease in *Fm'* over the pseudo light curve steps from 10 µmol m^{-2} s^{-1} to 603 m^{-2} s^{-1}. There was then very little further decrease from 603 to 1037 m^{-2} s^{-1} suggesting saturation of NPQ.

The lower shore biofilms exhibited a large reduction in *Fm'* at T1, but there was a smaller reduction in *Fm'* at T2 (Fig. 4.3.11). It was clear at both shore levels that downregulation was occurring in all treated biofilms including those treated with DTT. A reduction in *Fm'* was observed in the lower shore LAT-A treated biofilms. The ~ 60% reduction in *Fm'* seen in the LAT-A treated samples was the same as that seen in the control and DTT samples. It was also the same as that observed in the upper shore samples.

After the one-week recovery period all of the upper shore biofilms exhibited a smaller percentage reduction in *Fm'* during the incremental steps of the pseudo light curves, whereas the LAT-A treated lower shore biofilm exhibits a similar level of downregulation to that observed at T1 during the experiment. The control and DTT treated biofilm, one-week after, also exhibited a percentage reduction in *Fm'* similar to that observed at T2 on the experimental day.

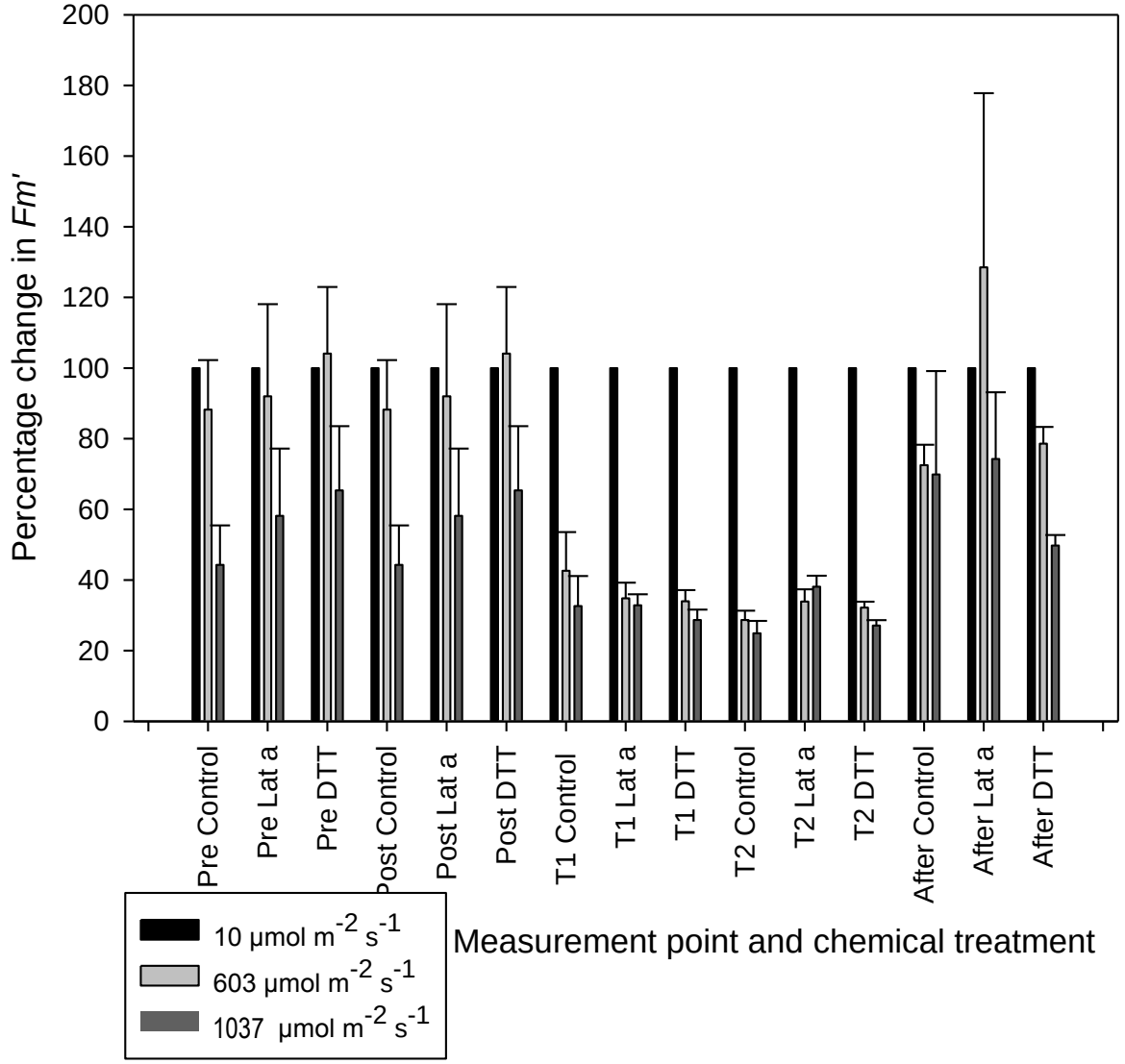


Figure 4.3.10: *The percentage change in maximum fluorescence (Fm'),* taken at 3 points from a light curve, of each chemical treated and control biofilm from the upper shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

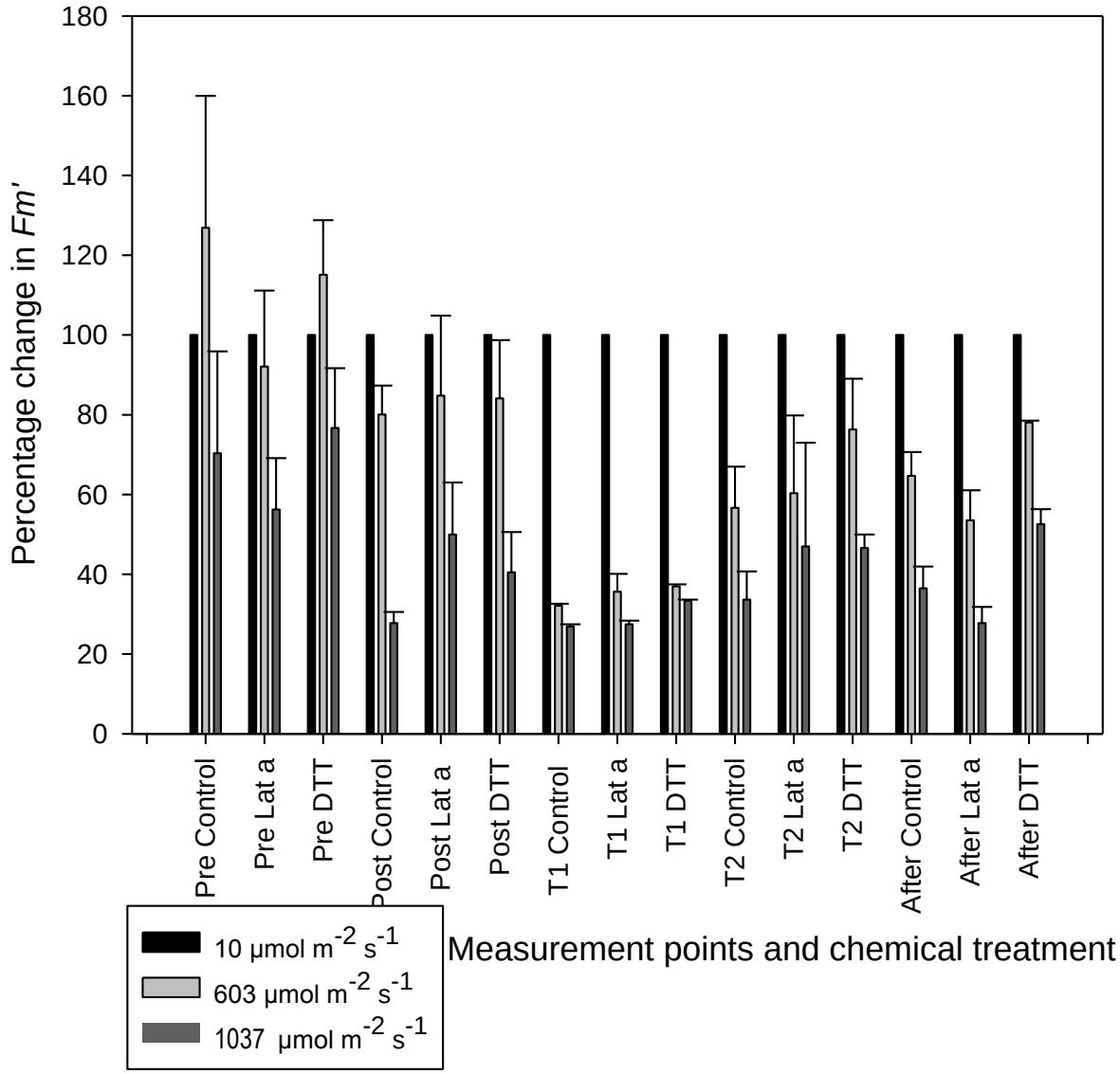


Figure 4.3.11: *The percentage change in maximum fluorescence (Fm'),* taken at 3 points from a light curve, of each chemical treated and control biofilm from the lower shore, recorded at 5 preselected time points. Mean ± SE, *n=3*

DISCUSSION

Despite there being observable patterns in the photophysiological responses of the different chemical treated biofilms, there were few significant results. These patterns were compared and conclusions drawn based on the recorded responses. Overall, diatoms on rocky shores utilise a combination of cell movement and NPQ downregulation, possibly in addition to other mechanisms not measured, but discussed, in this study. The observed patterns of response indicated that tube-forming diatoms appeared to utilise NPQ as a primary means of photoregulation and the lower shore biofilms appeared to use migration as a primary means of photoregulation.

Photoregulation strategies of the lower and upper shore (Hypothesis 1)

It was hypothesised that the lower shore biofilms, being similar to mudflat biofilms, would utilise 'microcycling' vertical cell movement as the primary means of photoregulation and that the upper shore, tube-dwelling biofilm community would utilise NPQ as the primary means of photoregulation, being unable to move vertically away from high light into sediment. The lower shore control and DTT treated samples responded to the high ambient light levels in a similar manner, with the same pattern of response for $rETR_{max}$, α, and *Ek* (Figs. 4.3.3, 4.3.5 and 4.3.7 respectively) as light dose increased over the experiment period, indicating that, as hypothesised, the lower shore biofilm cells' primary form of photoregulation was vertical cell movement. The upper shore control samples responded most similarly to the LAT-A treated samples, again with the same pattern of response for α, *Ek* and $rETR_{max}$ as light dose increased, suggesting, again as hypothesised, that the upper shore biofilms were primarily using a physiological means of photoregulation. It was hypothesised that the upper shore cells would likely not utilise cell vertical movement (microcycling or bulk migration) as a secondary means of photoregulation due to the nature of the substratum. However, these upper shore cells live in polysaccharide tubes and they were observed to be able to move within their tubes at higher temperatures (Chapter 3). The temperature on the experimental day was above the

14°C level at which the cells began to move (Chapter 3), and therefore it is likely that cell movement within the polymer tubes was occurring. There was no significant effect of the chemical treatments on the $rETR_{max,}$ *ΔF/Fm'*, *Ek* and *α*, indicating that either the chemical treatments were ineffective or that the cells were able to compensate for losing one photoregulatory mechanisms by using another. As movement has been observed in previous experiments, this was likely to have been the case here. As the cells were unable to move into a sediment layer or into the rock's surface, using a physiological means combined with limited cell movement was likely to be the photoregulatory strategy of tube-dwelling diatoms.

Photoregulation strategies of the control biofilm (Hypothesis 2)

The cells in all upper shore biofilms, including the control biofilm, showed photoinhibition during the exposure period, presumably due to the high photodose. It was predicted that the control biofilms would exhibit greater $rETR_{max}$ and *ΔF/Fm,* however this was not the case at either shore level. The control biofilms exhibited reducing *Fm'*, indicating that downregulation was occurring and this resulted in lower $rETR_{max}$ and *ΔF/Fm,* protecting the cell from permanent photodamage.

Inhibition of primary photoregulatory response (Hypothesis 3)

It was predicted in hypothesis 3 that there would be a greater reduction in the $rETR_{max}$ and *ΔF/Fm'* in the upper and lower shore biofilms treated with chemicals, which would inhibit the predicted primary photoregulatory response. This can be roundly rejected, as the chemical treatments had no significant effect on these parameters. The patterns of response were different between the treatments, but there was very little difference in the overall level of response. However, a reduction, particularly in the upper shore $rETR_{max}$, was observable in the biofilms, including the control. The exposure period did have a significant effect on the $rETR_{max}$, with an observable reduction during light exposure, indicating that photoinhibition

occurred. There was very little reduction and change in *ΔF/Fm',* indicating that PSII did not undergo significant inhibition at either the lower or the upper shore sites. This was unexpected as photoinhibition of PSII at high light doses is commonly reported to result in a decrease in *ΔF/Fm'* (Tyystjärvi & Aro 1996, Behrenfeld *et al*. 1998, Kromkamp *et al*. 1998, Murata *et al*. 2007). At T2 there was little induction of downregulation by the lower shore biofilms (Fig. 4.3.11) which is likely to mean that the cells had moved downwards to reduce their light exposure, or in the case of the LAT-A treated biofilm, using alternative methods of photoregulation, discussed below. These strategies may have prevented a reduction in the efficiency of PSII. The upper shore cells cannot move into a layer of sediment to reduce their light exposure and so are more likely to experience permanent photodamage and possible cell death, unless alternative strategies are employed, such as the movement within the polysaccharide tubes, which acts to limit the exposure to high light levels at the tube edge. These 'secondary' mechanisms appeared to be able to completely compensate for the loss of the 'primary' photoregulation mechanism, this is discussed in more detail below. The upper shore biofilms exhibited an increase in *ΔF/Fm'* after the one week recover period, which suggests that this biofilm was able to recover from any photodamage caused by the high light dose exposure. Any damage to PSII appears to have been repaired/reversed during the week by, for example, the degradation and synthesis of the D1 protein (Dwivedi 1995, Rintamaki *et al*. 1996). To test the repair function of the cells a further experiment could be performed using the chemical Lincomycin which inhibits the formation of the D1 protein (Tyystjärvi & Aro 1996).

The effect of LAT-A on the cellular light acclimation state (Hypothesis 4)

It was predicted, in hypothesis 4, that the cells treated with LAT-A would exhibit greater *Ek*, as cells forced to remain on the surface would be exposed to higher light and so become high light acclimated. As there was no significant effect of the chemical treatments on the *Ek,* this hypothesis can be rejected. However, *Ek* tells us a great deal about the photoregulatory

strategies of the different biofilms. It was clear that despite the limited ability of the upper shore cells to move within their tubes, they were not able to shade themselves totally from high light as at T1 and T2 after 2 hours and 4 hours of light exposure respectively, *Ek* was increasing in all treatments. This indicates that the cells were exposed to high light levels and were having to acclimate accordingly. This increase in *Ek* did not result in higher $rETR_{max}$, due to the induction of NPQ in the LAT-A treated biofilm and control biofilm. The lower shore biofilms exhibited very different *Ek* responses, with the control and DTT biofilm generally exhibiting little change, or a decrease, in *Ek* during the exposure period. This indicates that the cells were not exposed to high light levels as they had moved into the sediment surface. There was a decrease in *Ek* observed in both biofilms after the dark phase, which would be expected as the cells either became low light acclimated or migrated downwards. In contrast the LAT-A treated biofilms exhibited an increase in *Ek* at T1 which resulted in an increase in $rETR_{max,}$ which is supportive of previous work of Perkins *et al*. (2010), performed on mudflat based biofilms, which showed *Ek* increasing in LAT-A treated biofilms exposed to increasing light dose. The lower shore cells were primarily free living and live in a thin layer of sediment in depressions on the rock surface, and it would appear they behave more like mudflat diatom biofilms, rather than the upper shore, rocky tube-dwelling diatom biofilms. It is possible that the cells in the lower shore biofilms were 'microcycling' (Kromkamp *et al*. 1998, Patterson *et al*. 2001, Perkins *et al*. 2002) which may account for the stable *ΔF/Fm'*. This stable *ΔF/Fm'* indicates that it is likely permanent photodamage was not occurring at the lower shore sites (Aro *et al*. 1993, Long *et al*. 1994, Gray *et al*. 1996, Behrenfeld *et al*. 1998, Blanchard *et al*. 2004, Murata *et al*. 2007, Serôdio *et al*. 2008). A reduction in *Fm'* was observable and this acted to reduce the $rETR_{max.}$ Higher electron transport rates result in a greater production of reactive oxygen species (Telfer *et al*. 1994, Telfer 2002). The plastoquinone a (Qa) can become overly reduced by the excess light and charge recombinations of the acceptor side and donor side of PSII can create reactive oxygen species (Vass *et al*. 1992, Vass & Styring 1993). The PSII

reaction centre is then damaged by the reactive oxygen species (see Introduction section 1.4.3 for further information).

There was an observable reduction in *Fm'* in the DTT treated biofilms, indicating that some form of downregulation was occurring which appeared to mimic NPQ, such as migration in the lower shore samples (Jesus et al. 2006). In the upper shore biofilm changes in the activity of Rubisco may act to prevent permanent photodamage and may be required when photoregulatory strategies are unavailable. Rubisco binds more readily with oxygen relative to carbon dioxide as temperature increases (Long 1991). This increased affinity for oxygen is also known to maintain electron flow even during periods of limited CO_2, potentially occurring a result of increased chemical reaction rates caused by increased temperature (Osmond & Grace 1995, Osmond & Badger 1997). This prevents over reduction of Qa, which can result in charge recombinations, which can ultimately lead to the formation of damaging singlet oxygen (Keren *et al*. 1997, Krieger-Liszkay 2005). As CO_2 levels are known to reduce dramatically in rock pools over the emersion period (Pers. comm Christopher Williamson), this may be a means to prevent photodamage under CO_2 limiting conditions during periods of high temperature. Alternatively, it is also possible that the cells may have been utilising extracellular polymeric substances (EPS) to 'dump' excess carbon products (Staats *et al*. 2000, De Brouwer & Stal 2001, De Brouwer *et al*. 2002, Stal 2003).

Chemical persistence in the environment (Hypothesis 5)

It was predicted in hypothesis 5 that the chemically treated biofilms, particularly those where the expected primary photoregulation mechanisms was inhibited, would exhibit reduced $rETR_{max}$ and *ΔF/Fm'* after the one week recovery period. One week after the experiment the $rETR_{max}$, α and *Fv/Fm* of the upper and lower shore cells had returned to the levels prior to the experiment. This indicates that there was no long-term impact of the chemical on the biofilm. This either indicates that the chemical was not persistent in the environment and/or that the

cells had replicated and 'turned over' so the biofilm constituted of non-treated cells. One week after the experiment the *Ek* was lower. As this is a measure of photoacclimation, the light history prior to the measurement was likely to be responsible for this.

Experimental limitations

Both the tube-forming species and the free-living species may not have been exposed to the light level which was emitted by the fluorometer or the light dose recorded in the field. The tube-forming species lived inside a polysaccharide tube which is likely to attenuate some of the light. The lower shore free-living forms lived in a thin layer of sediment which, like mudflat based diatoms, they can move into, thereby shading themselves from high light levels (Levinos & Garrity 1983, Paterson & Consalvey 2004, Jesus *et al*. 2006, Serôdio, Coelho, *et al*. 2006, Mouget *et al*. 2008b, Perkins *et al*. 2006, 2010, Du *et al*. 2012). The samples were also permanently covered with a layer of water unlike those present on the mudflat. This layer of water attenuated light levels (Kirk 2003). The light levels were reduced by approximately 20% per cm of water cover (pers.obs from preliminary experiment). Therefore, the results must be interpreted with the understanding that the light levels which were emitted by the fluorometer may not be those that the cells were exposed to, since the light source from the Mini IPAM is not in direct contact with the biofilm surface. However, saturation of the light curve did occur at the highest light level.

Despite the overall patterns described above, in the lower and upper shore biofilms there was no significant effect of the chemical treatments on the photosynthetic parameters $rETR_{max,}$ *ΔF/Fm'*, *EK* and *α*. This was unexpected, as in a preliminary experiment performed on mudflat biofilms, which was consistent with the results of Perkins *et al*. (2010), higher levels of $rETR_{max}$ and *Ek* were recorded in the LAT-A treated biofilm, as the cells were forced to remain on the surface and so were forced to acclimate to the higher light levels. It may be that the concentration of the LAT-A chemical was insufficient in this experiment. The chemical

concentrations were determined from those which successfully inhibited the movement and non-photochemical quenching of mudflat based diatoms (Olaizola *et al*. 1994, Lavaud, Rousseau, *et al*. 2002, Cartaxana & Serôdio 2008, Perkins, Lavaud, *et al*. 2010). Due to the expense of LAT- A, the concentration of the chemical could not be increased. The effectiveness of the DTT concentration was investigated in the lab using samples collected from Dunraven Bay. The concentration used by Perkins *et al*. (2010) was investigated and double that concentration of DTT was also trialled. The lower concentration was found to not be fully effective at inhibiting the NPQ of tube-forming species. The higher concentration was found to be effective, though this concentration did not appear to be fully effective in the field as the level of NPQ induction (reduction in *Fm'*) reduced but did not appear to be fully inhibited. The levels of NPQ observed may not be the levels which were actually occurring due to the impact of vertical cell movement, which acts to reduce *Fm'* (Perkins *et al*. 2006, Jesus *et al*. 2006, Perkins, Kromkamp, *et al*. 2010), or by the presence of existing xanthophylls produced by the rocky shore biofilms in high light prior to the experiment, which could have induced NPQ during the experiment.

CONCLUSIONS

- The primary means of photoregulation used by the microalgal cells in the lower shore biofilms was cell movement (vertical within the sediment layer).
- The primary means of photoregulation used by the upper shore samples was non-photochemical quenching, although some cell motility within the polymer tubes was also likely to have assisted in photoprotection.
- The upper shore biofilms in all treatments, including the control treatment, showed photoinhibition during the exposure period. This is illustrated by a reduction in $rETR_{max}$ and by induction of *Fm'* indicating downregulation was required.
- Due to the exposed nature of the rocky shore the biofilms must have multiple methods of photoregulation in order to survive and acclimate to light conditions on the rocky

shore. This study found that each shore level was able to utilise a secondary means of photo-regulation, which allowed for successful downregulation, preventing any permanent photodamage.

Overall, diatoms on rocky shores utilise a combination of cell movement and NPQ down regulation, possibly in addition to other mechanisms not measured, but discussed, in this study. The observed patterns of response indicated that tube-forming diatoms appeared to utilise NPQ to a greater extent than motility and vice versa for free living cells in soft sediment, however data was not significant. Indicating that, as is observed in mudflat biofilms (Mouget *et al*. 2008, Perkins, Lavaud, *et al*. 2010) there is a preferable photoregulation strategy, however the so called 'secondary' photoregulation strategy was an effective means of photoregulation. This likely reflects the need for cells on the rocky shore to be highly adaptable to the rapidly changing environmental conditions at these sites.

GENERAL DISCUSSION

This study aimed to provide a new contribution to microphytobenthic research with the addition of photophysiological knowledge which focussed on the rocky intertidal to add to the extensive photophysiological research which has focussed on mudflats (Perkins et al. 2002, 2006, 2010, Serodio 2003, 2004, Underwood et al. 2005, Consalvey et al. 2005, Jesus et al. 2006 and many others). More specifically the photoregulatory mechanisms of rocky shore biofilms had not been studied prior to this work. This study aimed to determine the effects of ambient environmental conditions, taxonomy and grazing on the photophysiology of the biofilms and to elucidate the complex interactions between the abiotic and biotic factors which influence the biofilm, including daily light dose, temperature and grazing pressure.

The effect of community structure

This detailed study is far longer than any other rocky shore study focussing on the microalgae, as opposed to studies on herbivore populations and behaviour which are generally more long-term (Cubit 1984, Garrity 1984, Mieszkowska et al. 2006). One of the key findings of the seasonal study was the lack of effect of community structure on the photophysiology. There were marked changes in taxonomy during the year observed at the upper shore sites, with a shift from a 'winter type' to a 'summer type' biofilm in March. This change occurred every year despite the different weather conditions recorded. Despite this, there were no corresponding changes in the photosynthetic parameters, suggesting that the different species in the biofilms responded to daily environmental conditions in a similar manner, and the photophysiology of the integrated biofilm did not change. This was unexpected as it has been found that light history has an effect on the photophysiological responses of mudflat biofilms (Perkins et al. 2001, Jesus et al. 2008) and that different diatom species display different photophysiological traits (Underwood et al. 2005). However this work focussed on mudflat biofilms and it is believed that different species adapt photosynthetically to fill 'light-related niches' within the biofilm and sediment and move to optimise their position, and hence light environment, and

productivity (Kromkamp et al. 1998, Serôdio & Catarino 1999, Perkins et al. 2002, Jesus et al. 2006). This should not be the case for the upper shore biofilms on a rocky shore as these microalgal cells cannot move into a sediment layer and behavioural observations made in this study also suggest that the movement exhibited by the upper shore cells is limited to particular species, cell sizes and environmental temperatures. As such, it is clear that species present at upper rocky shore sites are most likely forced to adapt to one 'light-related niche' and so respond photophysiologically in the same way. This is a new contribution to our knowledge of microphytobenthos and yet compliments the work by others (Cubit 1984, Underwood 1994, Anderson 1995, Hutchinson & Williams 2001 and others). The lower shore sites were more similar to mudflat biofilms and contain species more commonly found at mudflat sites (e.g. *Cylindrotheca closterium*, *Gyrosigma fasciola, Odontella aurita*) (Colijn & Dijkema 1981, Underwood & Paterson 1993b, Underwood & Barnett 2006, Méléder et al. 2007) and the upper shore sites were dominated by tube-forming diatom species attached to the rocky substratum, for example the most dominant species were, *Navicula ramosissima, Navicula bottnica,* and *Berkeleya rutilans*. Seasonal changes in community structure were observed at both shore levels. Therefore it would be expected that there would be an effect of community structure on the photophysiology of the biofilms. However, again there was no observable seasonality in the photophysiological responses. There was far less variation in the community structure of the lower shore biofilm, and this may explain the lack of a seasonal pattern in photophysiology there. The PAM fluorescence measurements were made at the same emersion point after tidal exposure and so any diel and/or tidal induced patterns of 'microcycling' (Kromkamp et al. 1998, Paterson et al. 2003) occurring over the exposure period, which may have resulted in species specific photophysiological changes (Underwood et al. 2005), would not have been observed.

The dominant cells in the biofilm, reduced in size in the summer months. This has not been observed before at rocky shore sites, however observations of cell size differences and biofilm composition, resulting in photophysiological changes have been observed in mudflat biofilms

(Underwood et al. 2005). It was found that smaller naviculoid species had lower $rETR_{max}$ and lower E_{sat} or *Ek* levels. There was no indication that the size change, observed in the present study, resulted in lower $rETR_{max}$ levels but it does appear that the larger cells were either unable to survive during the spring months or were outcompeted by the smaller cells. It was found in Chapter 3 that smaller cells on the upper shore were able to move within polymer tubes, and the subsequent investigation into the photoregulation capabilities (Chapter 4) found that this ability compensated for the loss of NPQ as a result of the DTT chemical treatment. It may be that, as the larger cells cannot move within their tubes, they are unable to successfully photoregulate when the ability to induce non-photochemical quenching saturates in high light conditions in spring.

It was observed, during the seasonal study, that the biofilms at different shore levels were very different both in the community structure and the immediate substratum. Therefore it was hypothesised that the different biofilms would utilise different photoregulatory mechanisms. It was found that the different shore levels used different primary photoregulatory mechanisms with the lower shore biofilms using migration and the upper shore biofilms using non-photochemical quenching.

The effects of light and temperature

Data from the seasonal study showed that the lower shore biofilms were less affected by the external environmental variables, principally light and temperature, and maintained a more stable $rETR_{max}$ and *ΔF/Fm',* this result led to a rejection of the hypothesis that the cells on the lower shore would be less resilient as they are exposed for less time during the tidal cycle. This study has shown that, if the microalgal cells were less resilient, they would be unable to persist at this shore level. The ability of the cells to move into the sediment, as was confirmed in Chapter 4, to protect themselves from the environmental conditions results in a more stable biofilm both photosynthetically and taxonomically.

Although daily environmental conditions did have an effect on the photophysiology, the most apparent photophysiological differences were observed in the autumn growth phase, with higher $rETR_{max}$ levels recorded during these periods despite the fact that this period often coincided with extremely cold temperatures. It is clear that the primary driver of photophysiological changes in the rocky shore was the growth phase of the biofilm and this could be viewed as a seasonal photophysiological response, with short-term drivers being daily light dose and temperature.

Chapter 3 investigated the effect of temperature on the photophysiology of the Dunraven Bay biofilm. The lower shore biofilms showed more resilience when exposed to extreme temperatures (<10°C and >20°) again reflecting the results of Chapter 2 where the lower shore biofilms exhibited more stable photophysiology, which was less affected by environmental condition. It was noticed, however, that during the first year of the seasonal study, temperature appeared to affect the photophysiology of the biofilms, with the highest $rETR_{max}$ levels generally recorded during 'moderate temperatures' (between 10°C and 20°C). It was found that photophysiological down regulation in the form of NPQ was inhibited above 25°C. The summer upper shore biofilms exposed to 25˚C and 30˚C temperatures induced lower levels of NPQ than those exposed to temperature below 25˚C. This is an important conclusion which has not been reported elsewhere in photosynthetic research. Both summer and winter biofilms were exposed to the different temperatures in a controlled laboratory setting. Biofilms removed from the rocky shore in winter did not exhibit this response and so it is likely that the combination of high temperatures, in the laboratory tidal tanks, and a history of high light in the environment, caused this response. Temperatures above an enzyme specific optimum level are known to permanently damage the conformation of the enzyme structure (Palmer & Bonner 2007) and therefore it may be that diadinoxanthin de-epoxidase and diatoxanthin epoxidase, which are required to induce NPQ, were damaged and unable to function

effectively. In Chapter 4 the photoregulatory mechanisms employed by the cells were examined. It was found that upper shore biofilms treated with DTT, to inhibit NPQ, were able to compensate for the loss of this photoregulatory mechanism, as was apparent by the amount of downregulation occurring. It is likely this secondary means of photoregulation was the cell movement observed in Chapter 3. The tube-forming cells inhabited tubes in different ways, with some tubes being densely packed with cells and other being loosely packed. The spring biofilm cells in the less densely packed tubes exhibited movement at temperatures above 14°C, whereas the cells in the densely packed tubes were not moving or were moving too slowly to be observed in this experiment. The cells in the loosely packed tubes moved freely, mostly in what appeared to be a pattern, moving up the outside of the tubes, then when they reach the end, twisting and moving down the centre. The cells appeared to be coordinating this behaviour and it may be that they were shading each other or moving to orientate themselves into a more favourable light niche, as is seen in mudflat biofilms (Underwood et al. 2005). The speed of movement increased up to 32°C, which was higher than the hottest water temperature recorded at this site. Cohn et al. (2003) found very similar results with an increase in diatom motility from 2 °C to between 25 and 35°C depending on the species. They found that after 35°C movement rates decreased and ceased completely at temperatures above 40 °C. They speculated that the adhesion of the mucilage responsible for movement may have been reduced, but this was shown to be incorrect and they concluded that the production of the mucilage must be reduced or inhibited at temperature above 35°C. This is likely to be why a reduction in speed after 32°C was observed in this study; it also becomes clear that at temperatures above 32°C the cells lose their secondary photoregulation mechanism and in these situations they must rely on other methods or face permanent photodamage and ultimately cell death. It has been suggested that microphytobenthos can also down-regulate their photosynthesis via another physiological mechanism with the transfer of photosynthetically fixed carbon to extracellular polymers (e.g. low molecular weight colloidal EPS) as an energy overflow mechanism used during periods of high light (Staats et al. 2000, De

Brouwer & Stal 2001, De Brouwer et al. 2002, Stal 2003). This unfortunately could not be tested due to the high polysaccharide signal produced by the tubes, which could only be examined using the basic phenol-sulphuric method (DuBois et al. 1956). During a preliminary study (data not shown) mudflat diatom biofilms exposed to high light levels produced greater amounts of low molecular weight carbohydrates during a measurement day with a high photodose compared to one with low photodose. There are other more speculative potential downregulation mechanisms that could be utilised by the cells. Photorespiration is known to increase as temperature increases (Long 1991) as Rubisco binds more readily with oxygen relative to carbon dioxide (Long 1991). This increased affinity for oxygen is also known to maintain electron flow even during periods of limited CO_2 potentially caused by the increase electron flow and chemical reactions caused by the increased temperature (Osmond & Grace 1995, Osmond & Badger 1997). This prevents over reduction of Qa which can result in charge recombinations which can ultimately lead to the formation of damaging singlet oxygen (Keren et al. 1997, Krieger-Liszkay 2005). In addition photorespiration leads to the production of glutathione, which acts as an anti-oxidant and so helps to prevent oxidative damage to cells during periods of high light (Wingler et al. 2000).

Colder temperatures were found to limit $rETR_{max}$, particularly after a period of exposure longer than 30 hours. It was found that $rETR_{max}$ was more negatively affected by the colder (5°C and 10°C) temperatures than warmer temperatures (25°C and 30°C). An increased temperature results in an increase in all reaction rates (Trautz 1916) which is likely to compensate for any reduction in the electron transport rate as a result of a reduction in the efficiency of PSII. It is known that exposing photosynthesising organisms to colder temperatures results in a reduction in the irradiance threshold for photoinhibition, by slowing down the rate of repair of the photosynthetic apparatus (Baker & Bowyer 1994). In contrast to high temperatures cold temperatures result in slower rates of reaction, which directly results in lower $rETR_{max}$ levels. In addition the reduced rates result in a shift in the steady-state redox level of Qa (Huner et al.

1996) which increases the risk of charge recombination in the preceding electron transport chain (Vass et al. 1992, Keren et al. 1997). During the seasonal study the growth phase of the biofilm appeared to mitigate any reduction in $rETR_{max}$ that occurred as a result of cold temperature in the autumn months. However when cold temperatures occurred in January and February, when the biofilm was fully established, lower $rETR_{max}$ levels were recorded. Anning et al. (2001) illustrated how low temperatures can act to increase the xanthophyll pool in diatoms, which in turn acts to increase the energy dissipation via NPQ. This could be investigated by using an HPLC to quantify the amount and type of the xanthophylls pigments present and could be used to confirm what is effectively a thermal acclimation mechanism employed by the rocky shore biofilms. Chapter 3 provides valuable information about the effects of temperature on rocky shore biofilms. This has relevance for future photosynthetic research on rocky shore biofilms as temperature must be controlled as a variable in order to gain accurate measurements. It also has direct relevance to conservation and climate change. The IPCC Special Report on Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation (Field et al. 2011) predicts that there will be more frequent 'freak' high and low temperature events occurring in Britain as a result of the overall increase in global temperature. As extremes of temperature were found to negatively affect the photosynthesis of the rocky shore biofilm cells, this may have important implications for the productivity of rocky shore biofilms, resulting in lower biomass, which in turn has trophic impacts for grazing species and the birds and fish which predate them. These biofilms are highly biodiverse (Lewis 1964, Connell 1972, Narváez-Zapata et al. 2005) and provide food for many grazing species (Cubit 1984, Kaehler & Williams 1998, Menge 2000, Forrest et al. 2001). If these communities are to be preserved they must be monitored for the effects of these 'freak' weather events on them.

The combined effect of biotic and abiotic factors

A spring die-off was observed each year in either late April or May which occurred regardless of the weather conditions during the year. As mentioned above high light and temperature had a negative effect on the $rETR_{max}$ and photoregulation, particularly at the upper shore sites. The behaviour of the cells was also altered by these conditions with movement being induced during periods of high temperature. The cells at both shore levels were able to downregulate by utilising multiple methods, however it is clear that this downregulation to prevent permanent photodamage (Lavaud et al. 2004, Lavaud 2007, Tyystjärvi 2008 and others) has resulted in decreased productivity. This reduction in productivity is likely to have resulted in the cells being unable to replicate quickly enough to compensate for the increased grazing (Cubit 1984) observed in the spring. This resulted in the spring die-off of diatoms, with the result being an increase, albeit not in quantities great enough to form dense biofilm, in the toxic cyanobacteria *Moorea producens*.

Further work

In order to elucidate the photoregulatory mechanisms of these biofilms, the patterns of response in measured photosynthetic parameters were observed whilst the biofilms were exposed to them chemicals LAT A and DTT The photosynthetic responses of the chemically treated biofilms were compared with the control biofilms. This experiment was performed in full sunlight and a high light dose was recorded during the day. As a result, all of the biofilms were undergoing dynamic photoinhibition during the day and downregulation was recorded in the control and chemically treated biofilms. Further fluorescence measurements were made one week after the experiment, to ascertain whether the chemical treatment had a prolonged effect on the cells. This was not the case and the treated biofilms returned to responding in the same way as the control biofilms. The chemical was either not persistent in the environment or a cell-turnover had occurred during the week so the biofilm was made up of cells no longer

exposed to the chemicals. This is important to note as it means that these chemicals can be used without permanently damaging the biofilms, however the effect on grazers is unknown. PSII had recovered and had not been permanently damaged, however further research would be valuable. The diatom cells died back in late spring. The functionality of the D1 protein and the ability of the cells to repair the PSII after photoinhibition may be a key aspect to this die-off and an investigation into this would be valuable. The degradation and synthesis of the D1 protein (Dwivedi 1995, Rintamaki et al. 1996) is known to be responsible for the repair of PSII. To test the repair function of the cells a further experiment could be performed using the chemical Lincomycin which inhibits the formation of the D1 protein (Tyystjärvi & Aro 1996). This would provide valuable further information as to how the cells on the rocky shore repair photosynthetic function.

General conclusions

To return to the overarching aims mentioned at the beginning of this discussion, the overarching aim of this investigation was to gain knowledge about the photophysiology of rocky shore microphytobenthos. More specifically to investigate whether rocky shore microalgal biofilms have a seasonal photosynthetic patterns and which environmental factors influence this, with relation to shore level.

- The observed photophysiological 'seasonality' was primarily the result of the timing of the reproductive phase of the biofilm, with higher $rETR_{max}$ levels being recorded during these periods in November and December. Therefore the rocky shore photosynthetic 'seasonality' is the spring die-off and subsequent recovery.
- Extreme temperature and light had a negative effect on the $rETR_{max}$, particularly that observed at the upper shore sites. It can be concluded then that the combination of increased temperature and light dose reducing $rETR_{max}$ and the increased grazing

caused the spring die-off with cells unable to replicate rapidly enough to compensate for the increased grazing.

- Temperature induced previously unobserved, movement in the upper shore tube-forming cells. This was likely to act as a secondary photoregulation strategy as it was found that high temperatures resulted in a reduced ability to induce non-photochemical quenching.
- The upper and lower shore biofilms utilised secondary mechanisms of downregulation This allowed the cells to persist on the rocky shore which is an extreme and quickly changing environment.

This study has provided novel and valuable information about the photophysiology of rocky shore microalgal biofilms at different shore levels, exposed to different environmental conditions and different grazing regimes. These key aims were addressed and the results from this work have added to our knowledge of intertidal biofilms and complemented work already completed. These conclusions have also raised questions which could be further investigated and which would further add to our understanding of these important systems.

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APPENDIX

Table 1.1: *Taxonomists*, The names of the original taxonomic describer and the year in which the species was originally described.

Species	Species describer	Year of description
Achnanthes brevipes	Cleve	1895
Amphora spp	Engene	2012
Berkeleya rutilans	Grunow	1880
Grammatophora marina	Kützing	1844
Licmophora ehrenbergii	Grunow	1867
Licmophora flabellata	C. Agardh	1831
Moorea Producens	Harvey	1833
Melosira moniliformis	C. Agardh	1824
Navicula bottnica	Grunow	1879
Navicula ramosissima	Cleve	1895
Parlibellus delognei	Cox	1988
Cylindrotheca closterium	Ehrenberg	1859
Gyrosigma fasciola	Griffith & Henfrey	1856
Nitzschia linearis	W. Smith	1853
Nitzschia constricta	Grunow	1880
Nitzschia filiformis	Hustedt	1937
Odontella aurita	C. Agardh	1832
Pinnularia viridis	Ehrenberg	1843
Pleurosigma angulatum	W. Smith	1852
Psammodictyon panduriforme	Mann & Round	1990
Stauroneis phoenicenteron	Ehrenberg	1843
Staurosirella pinnata	Ehrenberg	1843
Tryblionella compressa	Poulin	1990