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# ASSESSMENT OF THE EFFECTS OF UV-B IN MARINE MACROALGAE: POTENTIAL BIOMARKERS OF EXPOSURE AND EFFECT

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# ASSESSMENT OF THE EFFECTS OF UV-B IN MARINE MACROALGAE: POTENTIAL BIOMARKERS OF EXPOSURE AND EFFECT

by

## BRITT CORDI

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

## DOCTOR OF PHILOSOPHY

Department of Biological Sciences
Faculty of Science

May 1999

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*I dedicate this work to Nature*

*for her beauty*

*her wonders*

*her mysteries*

# ASSESSMENT OF THE EFFECTS OF UV-B IN MARINE MACROALGAE: POTENTIAL BIOMARKERS OF EXPOSURE AND EFFECT

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Studies were undertaken to investigate the suitability of several molecular and physiological responses as biomarkers of UV-B exposure in several marine macroalgal species. Investigations into the sensitivity of mature plants and the reproductive unicells were also carried out. Furthermore, experiments were conducted to determine the interaction between UV-B radiation and the antifouling compound Irgarol 1051 in both a fouling alga and two non-target algal species.

Chlorophyll fluorescence, *in vivo* thallus absorptance and ion leakage were investigated for their suitability as physiological biomarkers of UV-B exposure in the intertidal alga *Enteromorpha intestinalis* and the subtidal alga *Palmaria palmata*. DNA damage (measured by Random Amplification Polymorphic DNA fingerprinting, RAPD) and the cellular stress response (measured by induction of the heat shock 70 protein, HSP 70) were evaluated as molecular biomarkers of UV-B exposure. Measurements of thallus growth were used as a measure of adverse biological effects. $F_v$ / $F_m$ ratio showed potential as a sensitive, non-specific general biomarker of UV-B exposure in both *E. intestinalis* and *P. palmata*. *In vivo* absorptance at wavelengths corresponding to chlorophyll *a*, phycoerythrin and/or carotenoids, as well as phycoerythrobilin and phycocyanin decreased in a dose-response dependent manner with UV-B exposure. These changes were associated with decreases in growth rate in *P. palmata*. The RAPD technique used for measuring DNA damage, showed potential as a tool for assessing UV-induced toxicity. These results illustrated that utilising several responses from different levels of biological organisation offer greater possibilities for detecting UV-B induced effects than do single responses.

Experiments with 12 h old reproductive unicells of *E. intestinalis* demonstrated that asexual zoospores were up to 6 times more sensitive to UV-B exposure than mature thalli (measured as variable fluorescence). After 1 hour exposure to elevated UV-B (equivalent to 27 % ozone depletion) reproductive unicells experienced decreases in variable fluorescence, accompanied by a 50 % inhibition of germination success and 16.4 % reduction in growth rates. Moreover, consistent patterns of greater sensitivity in the sexual reproductive part of the life cycle compared to the asexual part of the life cycle emerged throughout the experiments.

The interactive relationship between UV-B radiation and the s-triazine Irgarol 1051 was investigated in multi-factorial experiments. Inhibitions in optimal quantum yield of approximately 20 % were found after exposure to UV-B or Irgarol 1051 (applied singly). When these two stressors were applied simultaneously, however, an additive effect resulting in further reductions of up to 19.6 % compared to a single treatment occurred.

These decreases in $F_v$ / $F_m$ were accompanied by up to a 38.5 % reduction in growth rates. Simultaneous exposure of the same stressors to two non-target macroalgae, *P. palmata* and *P. umbilicalis*, revealed that these algae were less sensitive to Irgarol 1051 compared to *E. intestinalis*. However, similar additive effects measured as reductions in both $F_v$ / $F_m$ ratio and growth rates occurred after simultaneous exposure. These results underline the importance of investigating combination effects between UV-B radiation and xenobiotic compounds, if an under-estimation of the ecological implications of elevated UV-B exposure in the marine environment is to be avoided.

# CONTENTS

**5.2 EVALUATION OF THE HEAT SHOCK PROTEIN 70 (HSP 70) RESPONSE AS A BIOMARKER OF UV-B EXPOSURE IN TWO MACROALGAL SPECIES.**

**CHAPTER 6**                                   114
**THE INFLUENCE OF UV-B RADIATION ON THE REPRODUCTIVE CELLS OF THE INTERTIDAL MACROALGA, *ENTEROMORPHA INTESTINALIS.***

**CHAPTER 7**                                   133
**INTERACTIVE EFFECTS OF UV-B RADIATION AND THE ANTIFOULING COMPOUND IRGAROL 1051 IN THE MARINE MACROALGA *ENTEROMORPHA INTESTINALIS.***

**CHAPTER 8**                                   154
**EFFECTS OF SIMULTANEOUS EXPOSURE TO UV-B AND IRGAROL 1051 IN TWO NON-TARGET MACROALGAE.**

**CHAPTER 9** <span style="float:right">177</span>
**DISCUSSION AND CONCLUSIONS**

# LIST OF FIGURES AND TABLES

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Effects of UV-B radiation on the reproductive unicells of the marine macroalgal species *Enteromorpha intestinalis.* B. Cordi, J. Peloquin, M. E. Donkin, D. Price and M. H. Depledge. Bordeaux, France 1998.

Damaging effects of UV-B radiation on macroalgae: implications for marine conservation. B. Cordi, M. E. Donkin, D. N. Price and M. H. Depledge. Sydney, Australia 1998.

Development of a genotoxicity assay in aquatic organisms using arbitrarily-primed polymerase chain reaction. F. Atienzar, B. Cordi, A. Evenden, A. Jha, and M. Depledge. Toulouse, France 1997.

DNA fingerprints obtained by AP-PCR to detect DNA damage. F. Atienzar, M. Conradi, B. Cordi, A. Evenden, A. Jha, and M. Depledge. York, UK 1998.

Application of the arbitrarily-primed polymerase chain reaction (AP-PCR) method to detect DNA damage. F. Atienzar, M. Conradi, B. Cordi, A. Evenden, A. Jha, and M. Depledge. Bordeaux, France 1998.

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Signed...*Blatt Crok*......

Date.*3.6.99*.................

# CHAPTER 1

# INTRODUCTION

## 1.1 Ozone depletion and interactions with global warming

The ozone hole over Antarctica was first discovered in the late 1970s, but has now become a predictable event in the springtime atmosphere (Farman *et al.* 1985, Solomon and Schoeberl 1988). Ozone depletion develops because of several physical properties of the springtime Antarctic atmosphere, i. e. the polar vortex, unique characteristics of polar stratospheric cloud formations, decreased temperatures and the presence of air pollutants (Hofmann 1989). Depletion of ozone in the stratosphere (the atmospheric layer located between 10 - 30 miles above the earth's surface), which creates the ozone hole over Antarctica, takes place within the polar vortex during September and October, and minimum levels can be sustained for several weeks. The ozone depleted area disappears when the polar vortex dissipates and ozone concentrations equilibrate with surrounding air masses. The extent of ozone depletion and the area of the depletion varies greatly from year to year. The consequence of the decreased ozone concentration has resulted in the entire Southern Antarctic Ocean now being subjected to reductions in ozone reaching 90 %. Thus, while there is inter-annual variations in the depth of the ozone hole, it is clear that the effect is close to saturation (Pyle 1997).

Recent data indicate that the loss of ozone is now also affecting temperate regions (Kerr and McElroy 1993). Numerous modelling studies confirm that quite significant losses can be expected (Pyle 1997). Current estimates predict average ozone reductions, over northern mid-latitudes, of 13 % during winter and 7 % during summer and autumn months compared to the

late 1960s (WMO 1994). Contrary to the Antarctic polar vortex, which is centred close to the pole, the Arctic polar vortex is more variable and more mobile (Pyle 1997). Consequently, the vortex is often located over Northern Europe, where a maximum depletion of 50 % was measured for a number of days in March 1996 (Pearce 1996).

It has recently been emphasised that ozone depletion and global climate change are linked through the dual roles of trace gases such as carbon dioxide ($CO_2$), nitrous oxide ($N_2O$), chlorofluorocarbons (CFCs), methane ($CH_4$), halons (used in fire-fighting), and tropospheric ozone ($O_3$) (Worrest *et al.* 1989). These compounds function as greenhouse gases in the troposphere, the atmospheric layer located up to 10 miles above the earth's surface. Many of these atmospheric trace gases are relatively transparent in the visible region of the solar spectrum. They do, however, absorb long wavelength solar radiation that is radiated back from the surface of the earth, resulting in the green house effect (Worrest *et al.* 1989). Furthermore, many of these gases also destroy the concentration of ozone in the stratosphere. Gases such as CFCs and halons gradually migrate upward into the stratosphere where they contribute through complex chemical processes to the destruction of ozone (US EPA. 1987). Thus, because of these atmospheric interactions between global warming and ozone depletion, greater environmental damage may ensue. Moreover, other changes such as acid deposition / air pollution may also be closely linked to climate change (Worrest *et al.* 1989), thereby adding to the complexity of atmospheric interactions. The longest atmospheric lifetimes for some of the trace gases are 150 years for $N_2O$, 110 years for halons and up to 110 years for CFCs (Worrest *et al.* 1989). Thus, stratospheric ozone is expected to continue to decrease into the middle of the next century even if emissions are reduced world-wide (Crawford 1987).

## 1.1.2 General aspects of UV-B photobiology

UV wavelengths are subdivided into four categories (Jagger 1985): vacuum UV (< 200 nm), UV-C (200 - 280 nm), UV-B (280 - 315 nm) and UV-A (315 - 400 nm). Vacuum and UV-C are completely absorbed in the atmosphere. In contrast, both UV-A and UV-B penetrate to the earth's surface. UV-A is both biologically damaging and beneficial, and the transmission of these wavelengths through the atmosphere is not significantly affected by ozone concentrations. The reduction of the ozone layer, however, results in a very specific increase in solar UV-B, primarily between 290 and 315 nm. Within this waveband the solar irradiance decreases by over 4 orders of magnitude (Figure 1.1).
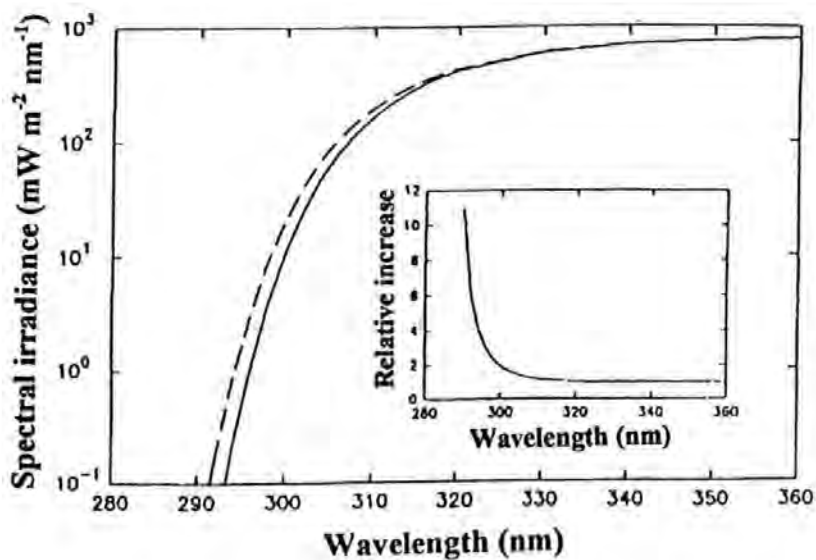


Figure 1.1. Solar global spectral irradiance computed for normal ozone concentrations, for midday in the summer at temperate latitudes (40°) with no ozone reduction (Continuous line) and with 20 % ozone reduction (dashed line). The inset shows the factor for relative increase of spectral irradiance at each wavelength due to ozone depletion (adapted from Caldwell and Flint 1997).

The relative increase in UV-B is highly wavelength dependent, with the greatest increases occurring with decreasing wavelength (Figure 1.1, inset).

Thus, the enhanced solar radiation from ozone depletion becomes important if biological responses are particularly sensitive to shorter wavelength radiation (Caldwell and Flint 1997).

UV-B radiation has repeatedly been reported to be biologically harmful to a diversity of terrestrial and aquatic life (Dahlbeck *et al.* 1989, Häder *et al.* 1995, Lowe *et al.* 1998, submitted). Solar UV-B radiation has been reported to cause damage to DNA, photosynthesis, chlorophyll fluorescence, enzyme activity and nitrogen incorporation, and to bleach cellular pigments for several algal species (Wood 1987, 1989, Döhler *et al.* 1991, Larkum and Wood 1993, Mitchell and Karentz 1993, Häder *et al.* 1996a, b).

The biological effects of UV-B are strongly wavelength dependent, even within a range of a few nanometers. Therefore, any increases in radiation due to ozone depletion are expected to result in disproportionately large biological effects. For example, due to the higher energy content of quanta at 295 nm compared to 320 nm, 295 nm radiation can cause 1000 times more erythemal damage on human skin (Moseley 1988). Caldwell (1977) estimated that a 1 % decrease in stratospheric ozone concentration would result in an increase of approximately 2 % in biologically effective UV-B radiation at temperate latitudes (calculated according to the generalised plant action spectrum, Caldwell 1971). Thus, the result of the predicted 7 to 13 % stratospheric ozone reduction (WMO 1994) would result in 26 % increase in biologically effective UV-B. However, the relationship between ozone depletion and magnitude of the associated increase in biologically weighted UV-B irradiance is highly dependent on wavelength and the biological response of the particular organism in question (Caldwell and Flint 1997). Thus, much emphasis is currently placed on establishing biological spectral weighting functions, which incorporate the biological response with the relative

effectiveness of different wavelengths of UV radiation. Reports that the Arctic ozone hole in the winter of 1995-1996 was the "deepest ever seen in the Northern Hemisphere" (Pearce 1996) have underlined the need for comparable studies in northern marine environment.

## 1.1.3 Algae and the marine environment

UV-B radiation must be considered as a potentially damaging factor in aquatic systems even for submerged organisms. UV-B can penetrate to 60 m in Antarctic waters during spring (Smith *et al.* 1992); and biological effects have been reported down to 20 m (Karentz and Lutze 1990, Smith *et al.* 1992). In moderately productive waters, containing average amounts of dissolved organic matter, attenuation is between 2.5 and 6 m (Smith and Baker 1979). In coastal waters, with their higher concentrations of suspended particulates, 90 % UV-B attenuation at only 0.5 m was reported for the German Bight and 3.5 - 6 m in the Fladen Ground of the northern North Sea (Højerslev 1988).

UV-B can adversely affect many structural components and metabolic processes in aquatic organisms (Worrest and Häder 1989, Holm-Hansen *et al.* 1993, Vincent and Roy 1993). Even without ozone depletion, UV-B is a continuous environmental stressor, and ambient levels at any latitude can potentially cause adverse effects in marine organisms. Solar UV-B radiation has been found to cause damage to early developmental stages of fish, shrimp, crab, amphibians and other animals (Häder *et al.* 1995). The most severe effects are decreased reproductive capacity and impaired larval development (Häder *et al.* 1995). Thus, even small increases in UV-B caused by ozone depletion events are of great concern (Bidigare 1989, Karentz 1991, Karentz 1992, Häder *et al.* 1995). A major consideration (which generally goes unmentioned) in evaluating the ecological impact of spring time ozone depletion is that the ozone hole has now been in existence for two decades. Consequently, any biological and

subsequent wider ecological effects may already have been initiated, especially for organisms with short generation times such as phytoplankton and some macroalgal species.

All primary producers occupy the upper layers in the water column, where they are simultaneously exposed to high levels of ultraviolet radiation. Investigations into the effects of enhanced UV-B radiation on phytoplankton and macroalgae are of great importance, since algae are vital primary producers, the foundation on which the very survival of aquatic food webs depends (Houghton and Woodwell 1989). A major loss in primary biomass productivity would necessarily cause decreases in biomass at higher trophic levels, thus affecting food productivity. It has been estimated that a 16 % ozone depletion could result in a 5 % loss in phytoplankton, which equals a loss of approximately 7 million tons of fish per year or a 7% reduction in fishery and aquaculture yields (Nixon 1988, Häder *et al.* 1995).

In addition, the oceans play a key role with respect to global warming. Marine algae are a major sink for atmospheric carbon dioxide, and they play a decisive role in the development of future trends of carbon dioxide concentrations in the atmosphere (Häder *et al.* 1995). A long-term global warming of surface air temperature by 1.5-4.5 °C is predicted for a doubling of the $CO_2$ concentration accompanied with a 1 m rise in the sea level by 2080 (IPCC 1992, Weaver 1993). The increase in fossil fuel burning and deforestation accounts for an annual increase of 7 Gt of carbon into the atmosphere, where only 3 Gt is retained in the atmosphere. The remaining 4 Gt are taken out of the global atmosphere by the biological pump in the oceans and possibly also by the terrestrial biosphere (Lampitt *et al.* 1993, Toggweiler 1993).

In contrast to the wealth of research concerning detrimental UV-B effects on microalgae, the macroalgae have largely been overlooked. Most macroalgae, as opposed to phytoplankton, are attached to their growing site, and thus have no opportunity to avoid high levels of UV

radiation by vertical migration as do phytoplankton. This suggests that UV-B together with photosynthetically active radiation (PAR) are limiting factors which determine the zonation of macroalgae. Moreover, springtime increases in UV-B coincide with the production of the potentially most vulnerable life stages of many macroalgae. Macroalgal systems are highly productive. Beds of kelp species such as *Laminaria* and *Macrocyctis* produce 1000-2000 g carbon $m^{-2}$ $yr^{-1}$ (Mann and Chapman 1975). These rates are similar to those in the most productive terrestrial ecosystems. Although they occupy a smaller total global area, benthic macrophytes in bays and estuaries have three times the productivity of phytoplankton on a per-area basis. Thus they are vital primary producers in near shore ecosystems and provide a source of food for a wide variety of invertebrates and fish (Mann 1972a).

## 1.1.4 UV protective mechanisms

It must be emphasised that the springtime increase in UV-B is a relative temporal occurrence. Therefore, the biological consequences of increased UV-B do not rest entirely upon the ability of algae to cope with UV exposure, but on their ability to cope with summer levels of UV-B in early spring (50 % ozone depletion over Plymouth, Devon, 50°22' N latitude and 4°17' W longitude, in March is equivalent to above May levels, calculated using Caldwells generalised plant action spectra and the computer programme of Björn and Murphy 1985). With the occurrence of the ozone hole, the seasonal gradient of increasing UV levels no longer exists, hence elevated UV-B is experienced during the early spring, immediately after minimum levels during winter. Thus, if an increase in the UV-B / PAR ratio occurs, the algae will be exposed to enhanced short wavelength radiation to which they may not be adapted. This is an important consideration because, although there is an increasing body of literature on the effects of UV-B on algae, little is known about the adaptive responses of marine algae in the field (Britt 1997).

A primary hazard of UV exposure is the absorption by DNA (Zölzer and Kiefer 1989). Nuclear DNA is present in a very low copy number, and acts as the template for its own synthesis, it is therefore particularly vulnerable to UV induced damage (Britt 1997). Even a single persisting UV-induced lesion can potentially be a lethal event. This is particularly true for haploid tissues such as pollen grains (Britt 1997) and gametes and zoospores of macroalgae. UV induced lesions consist mainly of dimeric photoproducts, the cyclobutane pyrimidine dimer (CPD) and the pyrimidine (6-4) pyrimidinone dimer (the 6-4 photoproduct) (Mitchell and Nairn 1989). These lesions alter the structure of DNA and consequently interfere with critical aspects of DNA metabolism such as transcription, replication and recombination (Taylor *et al.* 1997). Thus, the degree of UV sensitivity exhibited by algae is related to the number and efficiency of its protective mechanisms and repair systems. Furthermore, dose rates and duration of exposure must also be considered in assessing biological damage and capabilities for protection and repair.

Since algae are constantly exposed to sunlight, they have acquired protection mechanisms during their development and evolution. Thus, the actual biological UV-B doses received will partly depend on the differential UV screening properties of external layers. Structural protection from UV radiation may be provided by secondary cell walls (which the reproductive uni-cells are lacking) and membranes, however, very limited data is available on the optical properties of such cellular structures. Biochemical protection from UV exposure appears to be another common strategy. For example, UV-absorbing substances such as mycosporine-like amino acids (MAAs) have been observed in marine phytoplankton and macroalgae, from Antarctic, temperate as well as tropical species (Dunlap *et al.* 1986, Mitchell *et al.* 1989, Karentz *et al.* 1991a). The protective role of these compounds has been inferred from their UV-absorbing properties (they have a range of absorption maxima from

8

310-360 nm), decreasing concentrations with depth, and increased concentrations in response to UV exposure (Franklin and Forster 1997).

The cellular repair mechanisms responsible for the removal of damage induced by UV-B radiation include: photoreactivation, excision (dark) repair, and recombination repair (Sancar and Sancar 1988). These repair pathways are not specific for UV-induced damage, but are activated to repair DNA damage in response to for example heat, mechanical stress and free radicals. Photoreactivation (photo-enhanced repair) involves a light-dependent enzyme, DNA photolyase, which utilises photons of light in the UV-A and blue light region (Sancar 1994). However, although UV-A plays a very important role in the repair mechanism of DNA lesions, it is also biologically damaging (Forster and Lüning 1996). Very little is understood about the interaction of detrimental and beneficial wavelengths of solar radiation.

Although repair mechanisms for UV damage exist for most living tissues, this does not imply that there are no detrimental net-effects. For example, even if no net reduction in photosynthesis is detected over longer periods of time, the energy reserves needed to resynthesise damaged proteins and pigments may still result in a net metabolic loss which could potentially affect the long-term survival fitness of the organism (Calkins and Thordardottir 1980). For example, it has been shown in a cyanobacterium, that 10 % of the total net protein synthesis was required for repair processes such as resynthesis of the D1 protein in PS II complex (Raven and Samuelsson 1986).

In addition to genetic damage, other effects of UV exposure are related to absorption by RNA, proteins (enzymes, histones, hormones etc.), pigments and other biological molecules (Caldwell 1981). Furthermore, UV absorption can initiate changes in membrane structure and

the chemical environment of cells, interfering with normal metabolic processes (respiration, photosynthesis etc.) and resulting in decreased growth, impairment of reproduction, or death.

## 1.2 Organic pollutants and the marine environment

A vast number of organic compounds synthesised by man arrive in the aquatic environment via atmospheric transport, spray drift, groundwater leaching, soil run-off and sewage input (Zhou *et al.* 1996). Some by virtue of their nature are highly toxic and can adversely affect the health of the marine biota (Pridmore *et al.* 1992). These compounds are transported by currents and wave motion and are therefore distributed widely. Thus algae, perhaps more so than plants in the terrestrial environment, are subjected to numerous xenobiotic compounds. It is therefore important to consider elevated UV-B radiation as an additional environmental stressor. Investigations into combination effects of UV-B radiation and these compounds are of great importance if we are to accurately estimate the ecological implications of ozone depletion. The s-triazine compound Irgarol 1051 was chosen for investigations in Chapter 7 and 8 for a number of reasons explained below.

## 1.2.1 The antifouling compound Irgarol 1051

Antifouling agents, to which Irgarol 1051 belongs, are used in paints to prevent biofouling of submerged surfaces in the sea. They were introduced as a substitution for tri-*n*-butyltin (TBT), which in the 1980s was reported to cause severe damage to non-target populations of bivalves and gastropods at very low concentrations (Bryan and Gibbs 1991). The non-selective biotoxicity of TBT led to its regulation in antifouling paints by the OECD countries in 1988 (Readman *et al.* 1993).

Irgarol 1051 (2-methylthio-4-tert-butylamino-6-cyclopropylamino-s-triazine), with the chemical structure $C_{11}H_{19}N_5S$ (Fig. 1.2), is a newly developed herbicidal additive for use in copper-based antifouling paints. This compound inhibits colonisation and the growth of fouling algae. However, increasing concern has arisen following reports of high concentrations in coastal waters in the south of France (Readman *et al.* 1993) and in southern England (Gough *et al.* 1994).
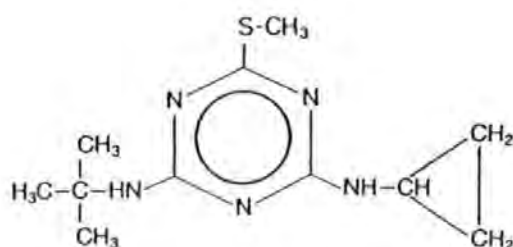
Figure 1.2. Irgarol 1051.

It is feared that Irgarol 1051, because of its mode of action, can inhibit growth of non-target algae along shorelines, thereby changing biological communities and the ecology of coastal areas (Tolosa *et al.* 1996).

Due to its solubility in water of 7 mg l$^{-1}$, Irgarol 1051 is leaking dynamically and may, therefore, circulate in the water column (Ciba-Geigy 1995a). Evaporation is negligible. While some biodegradation and photolytic reactions do occur (photolysis half-life of 273 days), it is assumed that the biologically less active triazine core is persistent (Ciba-Geigy 1995a). Moreover, aerobic degradation is relatively slow with half-lifes of 200 days in marine sediments and 800 days in sea water (EPA 1994, Ciba-Geigy 1995a). Anaerobic degradation

is considerably slower (half-life between 2052-8503 days, EPA 1994). Thus, the long-term concentration of Irgarol 1051 in the marine ecosystem depends on complex processes, among them the rate and place of release, the low rate of degradation, the binding to sediment and several dilution factors in the sea (Ciba-Geigy 1995a). $LD_{50}$ for Bobwhite quail is 2250 mg $kg^{-1}$, for Rainbow trout 0.74 ppm (mg $l^{-1}$) and for *Daphnia magna* 8.2 ppm. Moreover, early life cycle experiments revealed very high toxicity for the reproduction of Rainbow trout of 4.02 ppb ($\mu g\ l^{-1}$) and 110 ppb for Mysid shrimp (EPA 1994). This compound was concluded by the EPA (1994) to be practically non-toxic to quail, moderately to highly toxic to fish, and highly toxic to invertebrates.

Irgarol 1051 belongs to the s-triazine group known as photosystem II inhibitors. They inhibit the photosynthetic electron transport in chloroplasts (Samson and Popovic 1988) and reduce electron transfer between $Q_A$ and $Q_B$, the primary and secondary plastoquinone electron acceptors (Vermaas *et al.* 1984). These inhibitors bind to the D1 polypeptide of the PS II core complex at the $Q_B$ site (Vermaas *et al.* 1984). Moreover, a second site of triazine action, located on the donor site of PS II has also been established (Purcell *et al.* 1990). Thus, due to the mode of action this compound is extremely toxic to macroalgae, a minimum inhibition concentration (MIC) of 10 ppb was quoted by the manufacturers (Ciba-Geigy 1995b).

## 1.2.2 Analysis of combination effects

Simultaneous or sequential exposure of organisms to two or more chemicals may alter biological responses in qualitative and quantitative ways relative to that for single compounds (Altenburger *et al.* 1993). Combination effects of compounds have therefore been assessed in numerous studies in pharmacology, toxicology and biometrics. However, studies in interactions between xenobiotic compounds (i.e. heavy metals or organic compounds) and

UV-B irradiance have, with the exception of Polycyclic Aromatic hydrocarbons (PAHs) (Gala and Giesy 1992), rarely been attempted (Dubé and Bornman 1992).

Combination effects of compounds are investigated in relation to the expected effects of mixtures. Synergism is a term defined as the effect of a mixture which is larger than would be expected on the basis of the potency of its constituents. If the effect is smaller than would be expected it is termed antagonistic. If, however, the combined effect of the compounds is as expected from the effects of the individual substances, the combination effect is additive (Altenburger *et al.* 1993). Although the above mentioned definitions are based on work performed on inorganic or organic agents, combination effects involving radiation can also be identified using the above criteria.

The empirical foundation for defining expected combination effects lies in dose-response analysis (Berenbaum *et al.* 1989, Altenburger *et al.* 1993). Expected effects are calculated using biometrical models based on either the concept of effect summation, effect multiplication or concentration addition. These terms as defined by Altenburger and co-workers (1993) are:

• Concentration addition is a concept based on the assumption that any constituent of a mixture can be replaced totally or partly by the equieffective amount of another while the effect of the mixture remains constant.

• Effect multiplication is based on the assumption that the effect of a mixture equals the product of the effects of the components applied singly.

• Effect summation, which is the concept underlying the rational of Chapters 7 and 8 is based on the assumption that the effect of a mixture equals the sum of the effects provoked by the individual constituents.

Several experimental designs have been used to investigate interactive effects between two or more compounds. The combination effects of the s-triazine Irgarol 1051 and UV-B irradiance were tested in Chapter 8, using an experimental design which has often been applied to organic and inorganic agents. UV-B and Irgarol 1051 were tested alone at an irradiance $i_1$ and a concentration $c_2$ and subsequently in the combination $i_1 + c_2$ ("2 x 2 design"). Moreover, dose-response curves for each stressor, tested singly, were established prior to the combination experiments. In Chapter 7, the experimental approach in which one substance is tested at various levels while the other is kept constant was applied i.e. a "2 x n design" (Altenburger *et al.* 1993). This approach is an extension of the previously outlined method. Here several UV-B irradiances were used in combination with only one concentration of Irgarol 1051. Irrespective of the approach chosen to investigate combination effects of several stressors, both advantages and disadvantages are attached. The experimental design chosen in a particular experiment will depend on the purpose of the intended study, the assessment model to be used for data analysis and resource constraints underlying the experiments (Altenburger *et al.* 1993).

## 1.3 The biomarker approach

The coastal marine environment is one of the major recipients of anthropogenic pollution, as outlined in detail in the previous sections. Animals and algae in such areas are therefore threatened by the potential toxicity of various chemical compounds in addition to natural stressors and UV-B irradiation (Viarengo and Nott 1993, Lobban and Harrison 1994, Franklin and Forster 1997). Current ecotoxicological test procedures focus on ranking the relative toxicities of chemicals by establishing no observable effect levels in controlled laboratory conditions (Depledge 1994). Predictions of whether adverse effects are likely to occur are then made by determining the fate and concentrations of chemicals in ecosystems based on extrapolations from toxicity tests. A more

direct approach is to investigate the well-being of individual organisms or plants, populations and communities *in situ*. The biomarker approach aims to provide measures for evaluating exposure of organisms to xenobiotics and / or radiations and also information regarding the adverse effects on the organisms.

With regards to photobiology, the emphasis has in the past been to investigate atmospheric processes responsible for ozone depletion (Voytek 1990). Moreover, most research addressing UV effects on the biosphere has focused on establishing action spectra and weighing functions for different responses, or has assessed the spectral penetration of UV in water. Often only limited UV-B regimes were applied (Wood 1987, Caldwell 1971, Cullen *et al.* 1992, Hanelt *et al.* 1997, Häder *et al.* 1996b, Häder 1997a, Nilawati *et al.* 1997) However, finding biochemical, cellular or physiological responses which can be related in a dose dependent manner to UV-B exposure, and therefore act as early warning signals to changes in UV-B levels, has less frequently been attempted (Campos *et al.* 1991, Cullen and Lesser 1991, Uhrmacher *et al.* 1995). If these early warning signals can be identified they might be used to signal a change in biological fitness that may have consequences for entire populations.

Marine plants exposed to contaminants and / or UV radiation may elicit responses at all levels of biological organisation, from the molecular level, where pollutants or radiation may cause damage to critical cellular macromolecules initiating detoxification and repair mechanisms, to the individual level (Figure 1.3). If the impact of the toxicant and / or UV radiation at, for example, the biochemical, cytological or the physiological level is large enough to exceed compensatory responses, then its effect passes to successively higher levels of organisation (Stebbing 1985). Effects at the individual level may result in reduced growth or reproduction, which eventually may become evident at the population or community levels where changes in species diversity, density and genotypic frequencies may arise (Moriarty 1983; McCarthy and Shugart 1990). The temporal

sequence shown in Figure 1.3 is important because responses to pollution and / or UV radiation at lower levels of organisation enable the ecotoxicologist to anticipate and predict effects that may occur later at higher levels of organisation. In fact, biochemical responses such as DNA damage, or physiological responses such as reduction in chlorophyll fluorescence, as two examples, may provide an early warning signal of exposure to a particular toxicant or UV radiation. Utilising such responses at lower levels of organisation to predict possible effects that may occur at higher levels of organisation is a key feature underlying the rationale of the biomarker approach.
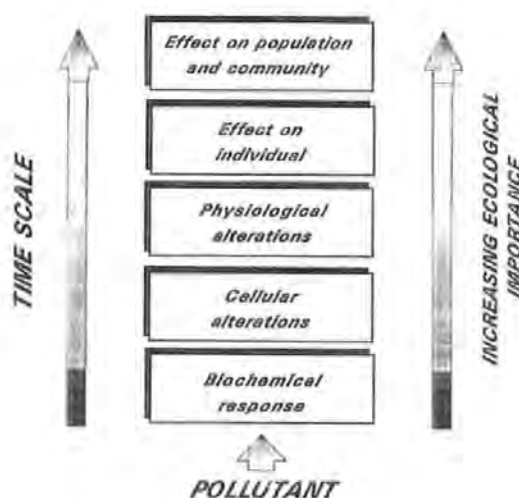


Figure 1.3. Relationship between responses to pollutants at different levels of biological organisation and their modifications along gradients of response time, toxicological and ecological relevance (adapted from Fossi and Loenzio 1994).

Although the relationship between responses at different levels of biological organisation originated from research on anthropogenic contaminants described by Peakall (1992), McCarthy and Shugart (1990) and Fossi *et al.* (1994), based on the earlier work of Adams *et al.* (1989) this approach can equally well be used regarding UV irradiation.

Depledge (1994) extended the definition of biomarkers proposed by the National Academy of Sciences (NRC 1989) to: "An ecotoxicological biomarker is a biochemical, cellular, physiological, or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms (either individuals or populations) that provides evidence of exposure to and / or effects of one or more chemical pollutants (and / or radiations)". Biomarkers have been used extensively in the evaluation of occupational health risks (Grandjean 1991). For example acetylcholinesterase (AChE) inhibition was used as a biomarker of pesticide exposure in humans (Brock and Brock 1990) to detect potentially toxic exposures long before adverse effects would arise.

Depledge (1994) proposed a division of ecotoxicological biomarkers into four classes.

• The first class contains biomarkers of exposure, which signal exposure of an organism, a population, or a community to chemical pollutants. These biomarkers may range from generalised indicators of UV-B radiation and / or pollution to specific indicators of exposure. An example is reduction in chlorophyll fluorescence and changes in pigment absorptance spectra.

• The second class contains biomarkers of effect, which signal that an organism, a population, or a community has been affected by one or more pollutants. They do not necessarily provide information concerning the nature of the pollutant stress. Altered gill Na, K-ATPase activity as a measure of osmoregulatory disfunction in fish was mentioned by Depledge (1994) as an example. Additionally, ion leakage also belongs to this group, since it is a measure of membrane damage.

17

• The third class are the so called exposure / effect biomarkers, in which the acethylcholinesterase inhibition belongs. These biomarkers not only indicate exposure to one or several xenobiotics, but specifically link the exposure to an effect.

• The fourth class, the latent effect biomarkers, deals with possible changes in the capacity of organisms to adapt to future environmental changes because of a previous pollutant exposure. These biomarkers indicate the exposure of apparently normally functioning organisms, by pointing towards limitations in the ability to adapt or survive. Depledge (1994) mentions altered Scope-for-Growth as an example. Reduced algal growth rate can also be regarded as a latent effect biomarker since the algae may survive perfectly well with a slightly reduced growth rate, but the possibility that these individuals are more vulnerable to environmental changes remains.

In addition, Depledge (1994) introduced the term functional biomarker to identify biomarkers that are integral components of the compensatory response or detoxification mechanism initiated by pollutant or UV exposure. The induction of heat shock protein 70 (HSP 70) or Mycosporine-like amino acids (MAAs) are examples of functional biomarkers. Independent of which kind of biomarker is being evaluated, attempts should be made, wherever possible, to establish the dose-response relationship between the biomarker response and the stressor. Furthermore, it is important to underline that biomarkers do not necessarily provide information regarding the significance of the exposure to UV-B or pollutant for the well-being of the exposed organisms or algae (Depledge 1994). However, the third class of biomarkers mentioned above, the exposure / effect biomarkers, identify responses which are linked to declining health.

The "multiple response concept" illustrated in Figure 1.4a has created a frame work for evaluation of the health status of individuals and populations by using biomarkers to identify progressive changes in the physiological condition of organisms (Moore 1992, Depledge 1989, Depledge et al. 1993, Depledge 1994). A healthy individual exposed to a pollutant may experience a deterioration

in health, as it moves along the "physiological condition" scale exceeding homeostasis at the point (h), from where it moves into a compensation zone. As the organism or plant is exposed to increasing doses of a toxicant or UV-B radiation, compensatory biochemical and physiological responses are seen, with no obvious signs of disease. If the pollutant exposure exceeds the organism's tolerance level, i. e. that can be compensated for (by excretion, repair and / or compensatory mechanisms) (point c), a movement into the non-compensation zone with signs of overt disease will result. If point (r) on the curve is crossed, recovery through repair mechanisms is impossible and the organism or the alga will finally suffer from pathological processes leading to death (Depledge 1994).

Figure 1.4b show a number of hypothetical biomarker responses compared to the health status curve. Particular biomarkers (B1 - B5) may be present with more or less specific responses in each of the individual "zones". Thus, there may be potential for determining, where individuals and whole populations samples *in situ* or in the laboratory lie on the health status curve (Depledge 1993, 1994). Early warning of potentially detrimental effects may be given by biomarker responses occurring in the compensation zone, which make these of special interest. Moreover, this figure also illustrates that the simultaneous use of more than one biomarker can offer greater possibilities for the detection of effects than do single biomarkers.
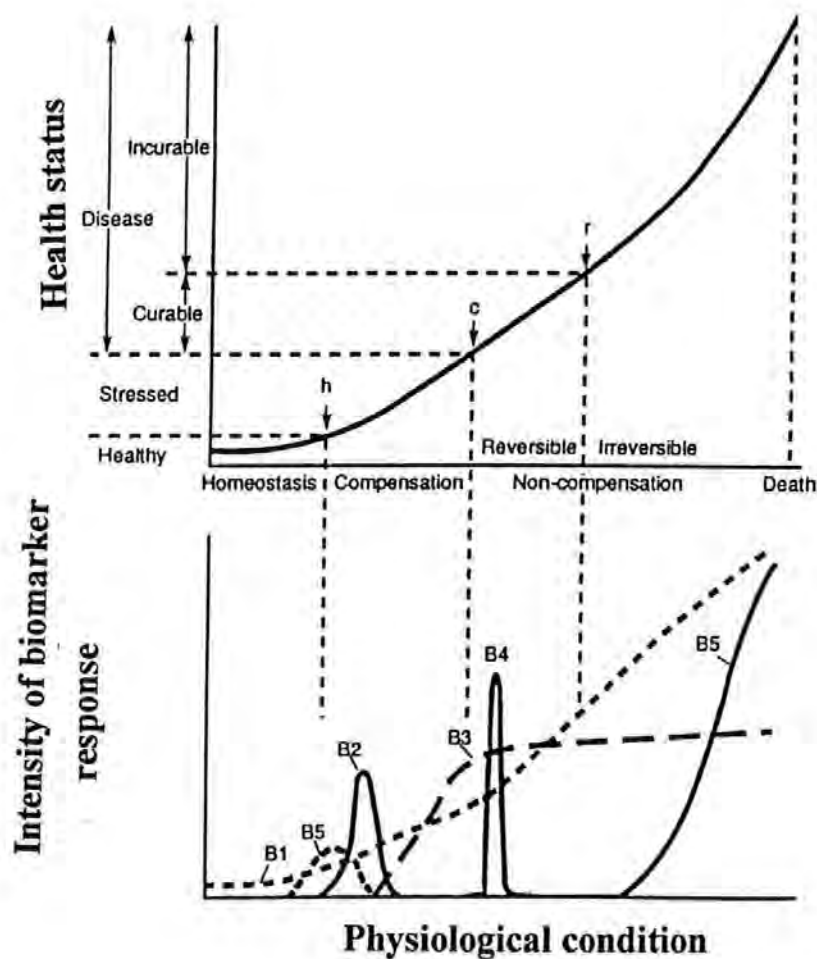
Figure 1.4. The relationship between health status and physiological condition in a plant or an organism. In the upper panel, changes in the physiological condition are shown during progressive deterioration in health status. In the lower panel several biomarker responses are shown (adapted from Depledge 1994).

## 1.3.1. Non-destructive biomarkers

The use of non-destructive biomarkers has proved particularly useful in research involving animals (Leonzio and Fossi 1994). However, many of the advantages for animals are also valid for research with plants and algae. For example, sequential sampling on the same individual allows the individual to act as its own control and long-term studies of responses in individuals are possible (Peakall 1992; Depledge 1994). Non-destructive sampling does not involve loss of individuals from the population, which is of special importance when rare or threatened species are investigated. In addition, the role of endogenous (sexual cycle, age, nutritional status, etc.) and exogenous (temperature, daylight, etc.) factors in variations in biochemical biomarkers can be investigated in the same individual, and thus exclude intraspecific variation (Fossi *et al.* 1994). *In vivo* chlorophyll fluorescence, *in vivo* pigment absorptance and monitoring of growth are examples of non-destructive measurements applied throughout this thesis.

## 1.3.2 Choice of potential biomarkers

One of the primary aims of this study was to investigate potential biomarkers, both molecular and physiological, which can be related in a dose-response dependent manner to UV-B radiation and therefore can act as early warning to elevated UV-B. *In vivo* chlorophyll fluorescence was investigated as it is a rapid and non-destructive method, and thus has considerable potential for use *in situ*. It provides information on inhibition or damage to transfer of electrons from Photosystem II, photochemical quantum yield and is a sensitive indicator of photoinhibition.

In addition, *In vivo* pigment absorptance was investigated as a non-destructive, general biomarker of exposure. It has the potential to reveal *in vivo* changes in the absorptance spectra of pigments following UV exposure, to characterise sites of damage to pigments and

to establish the time course of damage and repair (Smith and Alberte 1994). High performance liquid chromatography (HPLC) was applied to identify and quantify chlorophyll and carotenoid pigments which were only tentatively identified by *in vivo* spectrophotometry.

Ion leakage was used to assess the extent of cell damage and to evaluate its suitability as an early indicator of UV-B stress. To investigate possible responses to UV-B irradiance at the molecular level, DNA damage (measured as Random Amplification Polymorphic DNA fingerprinting) and the cellular stress response were examined. The induction of heat shock 70 (HSP 70) which belongs to the group of functional biomarkers was measured as the cellular stress response. These parameters were linked to effects on growth rate and reproduction, two Darwinian fitness parameters. Furthermore, wherever possible, several biomarkers were used simultaneously to aid the detection of exposure and/or effects.

### 1.3.2.1 Chlorophyll fluorescence measurements

Chlorophyll *a* and *b* and several carotenoids are the light-absorbing molecules in the antenna complexes of Photosystem II (PS II) of higher plants and algae. The normal electron transfer in Photosystem II, reflected by a fast chlorophyll fluorescence decay (Holzwarth 1991) is as follows: Absorbed energy is transferred to the primary electron donor of the reacting centre, P680 (van Grondelle *et al.* 1994), where an electron is moved to the primary acceptor pheophytin (Figure 1.5).
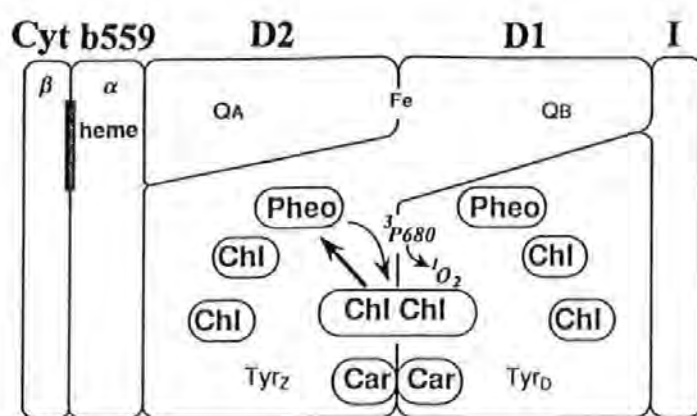
Figure 1.5. Schematic model indicating the structure of the isolated PS II reaction centre (adapted from Telfer and Barber 1994).

An electron transfer to the primary quinone $Q_A$ ensures charge stabilisation (Schatz *et al.* 1988). The electron is then transferred to the secondary quinone $Q_B$, which is bound at a specific site of the D1 protein. The doubly reduced $Q_B$ quinone is then protonated and released as a quinol to the plastoquinone-pool (Conrad *et al.* 1993). An oxidised plastoquinone molecule binds in exchange at the D1-protein (Kyle and Ohad 1987) and the oxidised donor, P680$^+$, is then reduced by an electron of the water splitting complex.

The D1-protein which plays an important role in electron transfer out of PS II, as described above, can be damaged by various stressors such as chemical pollutants or UV radiation. For example, herbicides such as phenylureas (DCMU) and triazines (i. e. atrazine and Irgarol 1051) possess a high specific affinity at the $Q_B$-binding site of PS II. This results in a block of photosynthetic electron flow causing an increase in PS II dependent chlorophyll *a* fluorescence (Regner 1986, Van Reensen 1982, Draber *et al.* 1991). Moreover, it has been shown that UV-B exposure inactivates the PS II →-centres, but not PS II ↑-centres, causing a decrease in the electron transfer (Tevini and Pfister 1985, Renger *et al.* 1989, Tevini *et al.* 1989, reviews by Franklin and Forster 1997 and Häder and Figueroa 1997). Furthermore, the

water-splitting site of PS II and the reaction centre are damaged following UV-B irradiance (Bhattacharjee and David 1987). Thus, *in vivo* PS II fluorescence emission show a promising potential as a biomarker of exposure to photosynthesis-inhibiting herbicides and UV-B radiation in the aquatic environment.

Chlorophyll fluorescence induction kinetics were originally applied for fundamental studies of photosynthesis. A leaf illuminated with a constant light irradiance will fluorescence steadily, however if a leaf is kept in darkness and then brightly illuminated, a characteristic pattern of fluorescence appears, the fluorescence induction curve -the Kautsky curve (Figure 1.6).
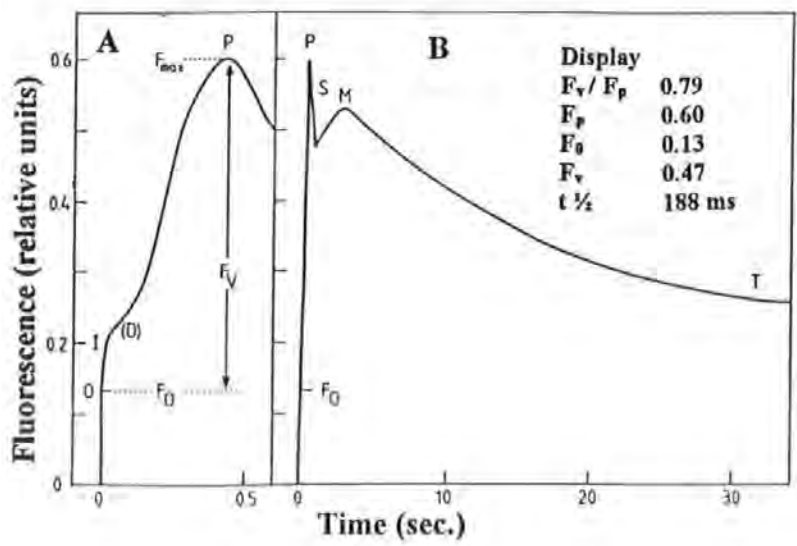


Figure 1.6. Kautsky Curve showing fast A) and slow B) kinetics. The different phases of the Kautsky Curve are denoted O I D P S M T (adapted from Bolhàr-Nordenkampf *et al.* 1989).

The period following the first second of illumination, when the fluorescence rises from O to P, is often denoted the fast phase. The following phase from P to T of several minutes duration is denoted the slow phase. However, it is the fast phase which is related to the primary processes in photosynthesis in PS II. During the first seconds, the fluorescence time course reflects the events of water-splitting and subsequent electron transport (Papageorgiou 1975). A portion of the energy trapped by the PS II antenna which is not able to transfer beyond $Q_A$ via electron transport is reemitted as variable fluorescence, $F_v$. Consequently, variable fluorescence increases as the primary acceptor $Q_A$ is reduced or is quenched as $Q_A$ is oxidised. $F_o$ is the level of fluorescence emission when all reaction centres of $Q_A$ are open and fully oxidised, before electrons are trapped. $F_m$ is the maximum level of fluorescence attained in the induction curve, usually at P, when all reaction centres are closed and $Q_A$ is fully reduced. $F_v$ / $F_m$ ratio is proportional to the quantum yield of photochemistry (Butler and Kitajima 1975) and is highly correlated with the quantum yield of net photosynthesis of intact leaves (Demming and Björnman 1987). The area above the curve between $F_o$ and $F_m$, the complementary area, is proportional to the pool size of electron acceptors (plastoquinone) on the reducing side of PS II and is therefore also proportional to the photochemical capacity of PS II (Lavorel *et al.* 1986). Its value is decreased when PS II photochemistry is lowered by effects of copper or trizine-herbicides (Samson and Popovic 1988).

Algal photosynthesis varies greatly from one taxonomic group to another. Only green algae have pigment and chloroplast configurations similar to those which have been well studied in higher plants (Büchel and Wilhelm 1993). Consequently, care is required when interpreting fluorescence measurements from algae. For example, in green algae and higher plants a $F_v$ / $F_m$ of approximately 0.8 was measured, contrary to values as low as 0.4 to 0.7 found in other algae (Büchel and Wilhelm 1993). This may be due to variations in the supramolecular organisation of the thylakoid membrane system (Cunningham *et al.* 1996). However, despite

the need for careful signal interpretation, Cunningham and co-workers (1996) concluded that the pulse-amplitude modulated (PAM) instrumentation appears satisfactory regarding measurements on macroalgae. Dring *et al.* 1996a added that $F_v$ / $F_m$ appeared to provide a sensitive indicator of the response of the photosynthetic apparatus to UV radiation in red algae.

### 1.3.2.2 *In vivo* pigment absorptance and HPLC analyses

*In vivo* pigment absorptance was selected to obtain spectral diagnostics for different algal species and to provide a means to determine whether spectral features could be used to identify UV-B stress. *In vivo* spectral analyses can sensitively and rapidly characterise pigment diversity, non-invasively, especially when pigment identities are known (Smith and Alberte 1994). A diversity of accessory pigments have been documented through analyses of *in vivo* absorption spectra for a range of unicellular algae (Jeffrey 1980, Larkum and Barrett 1983), and some chlorophyte macroalgae (Haxo and Blinks 1950, Kageyama *et al.* 1977, Jeffrey 1980, Smith and Alberte 1994), phaeophytes (Dring 1982) and rhodophytes (Haxo and Blinks 1950, Jeffrey 1980). The advantages of this method come from the fact that these spectra are independent of the need for corrections due to hypsochromatic shifts (decrease in wavelength) which occur when the tissue is disrupted by extractions into organic solvent (Smith and Alberte 1994). This method has been used for several macroalgae species throughout this thesis to investigate *in vivo* changes of absorptance spectra of pigments following experimental manipulations, to characterise sites of damage to pigments and to establish time-courses of recovery after UV-B exposure. Additionally, there is a potential of using this method as a non-invasive means to evaluate subtle responses of the photosynthetic apparatus of algal populations in a variety of aquatic environments where stressors such as chemical pollution, eutrophication, UV or other impacts might be realised (Smith and Alberte 1994).

Some of the limitations associated with spectrophotometry are inaccuracies associated with spectral interferences between several pigments, and identification of specific pigments can therefore be difficult. Moreover, decreases in pigment absorptance are not directly related to changes in pigment concentrations, since changes in *in vivo* absorptance spectra can also arise from changes in the package effect, caused by chloroplast movements, thylakoid organisation and changes in mean cell size (Forster, personal communication). Therefore, high performance liquid chromatography (HPLC) was performed to identify and quantify the chlorophyll and carotenoid pigments.

The use of HPLC also allows a rapid separation and quantification of up to 50 chloropigments and carotenoids (Gibb *et al.* 1998). This method is used to obtain information on the taxonomic composition of phytoplankton communities and their biomass abundance (Gibb *et al.* 1998). Moreover, HPLC has been applied to separate Mycosporine-like compounds, MAAs, from marine organisms and to investigate if the induction of MAAs can be related in a dose-dependent manner to UV-B exposure (Nakamura and Kobayashi 1982, Wood 1989, Garcia-Pichel *et al.* 1993). In addition, this method has also been utilised to investigate the linear relationship between zeaxanthin content and the degree of photoinhibition following varying doses of PAR in brown macroalgal species (Uhrmacher *at al.* 1995). In this study HPLC analyses were also used to further investigate if chlorophyll and carotenoid pigment concentrations can be related in a dose-dependent manner to UV-B exposure in macroalgae.

### 1.3.2.3 Membrane damage

Cellular disruption causing membrane damage is partly the mode of action of several aquatic herbicides, ozone, heavy metals and possibly UV. Determining the leakage of ions in plant or

algal cells is a common method of assessing the extent of cell damage resulting from both natural and anthropogenic stressors (Koch *et al.* 1995). Three groups of pollutants which have membrane disruptive properties have been distinguished in the aquatic environment (Watts and Moore 1988, Gulyas *et al.* 1993). Group a) includes several aquatic herbicides and peroxide-based bleaches which cause membrane disintegration by the generation of oxygen radicals or loss of adenosine triphosphate (ATP) needed to maintain cellular integrity (Macdonald *et al.* 1993). Group b) includes organic solvents that interact with membrane lipids because of their nonpolar nature. Group c) includes detergents that damage membranes by disintegrating the lipid bilayer and by solubilizing membrane constituents (Marcomini *et al.* 1988). However, findings suggest that almost all severe damages on vital processes lead inevitably to death of damaged cells and an increase in the plasma membrane permeability (Axelsson and Axelsson 1987).

The method used in this work to investigate mambrane damage is based on the ability of seaweeds to leak ions when placed in distilled water. Ion leakage is then measured as electrical conductivity and a "health index" is calculated based on the ion loss. This method has been modified by Axelsson and Axelsson (1987) from the original work of Gessner and Hammer (1968). This method was found suitable to detect harmful substances in industrial waste water (Axelsson and Axelsson 1987). Moreover, quantitative structure-activity relationships, using ion leakage, have also been established for the effect of non-polar narcotic alcohols in the green alga *E. intestinalis* (Schild *et al.* 1995). Although research into membrane damage caused by UV-B has been limited, it is known that the integrity of the membranes is affected by UV-B (Murphy 1983, Koch *et al.* 1995), and is due to a decrease in lipid content and damage to membrane transport systems.

## 1.3.2.4 Molecular biomarkers

The major advantage for utilising molecular biomarkers such as DNA damage and stress proteins is that these responses are detected before adverse effects can be observed on the individual or population level (Depledge 1994). The following sections describe the use of DNA fingerprinting and Heat Shock 70 protein induction.

**Random Amplification Polymorphic DNA fingerprinting**

In the field of genotoxicity, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis. These assays include for example DNA finger printing, RFLP (Restriction Fragments Length Polymorphisms) analysis and a number of techniques, which rely on the amplification of DNA sequences using the polymerase chain reaction (PCR). All these methods have the potential to detect DNA damage by virtue of their ability to recognise changes in the structure of the DNA molecule (Atienzar *et al.* 1998).

Random Amplification Polymorphic DNA (RAPD) is a semiquantitative method which has previously been used for genetic mapping, taxonomy, phylogeny (Welsh *et al.* 1991) and detection of various kinds of DNA damage (Kubota *et al.* 1992;

Peinado *et al.* 1992; Ionov *et al.* 1993; Savva 1996; Shimada and Shima 1998). This technique does, however, also offer a useful procedure for the detection of genotoxic effects of environmental stressors (Savva 1996). In short, the assay is based on an aliquot of isolated DNA which is subjected to PCR and then detected by agarose gel electrophoresis. Next, staining with ethidium bromide is performed to allow visualisation of the DNA under UV irradiation. Following extraction of the genetic material, DNA template is amplified by using

one oligonucleotide primer of random sequence (Welsh *et al.* 1995). This method relies on the comparison of amplification products from control (unexposed) and exposed tissue DNA template produced by oligonucleotide primers of random sequence (Welsh *et al.* 1995). Modification following UV radiation is represented by RAPD as either appearance or disappearance of bands, which is a representative of UV induced DNA damage. The RAPD assay has shown potential for detection of environmental chemicals (Savva 1996) and UV-induced genetic damage (Atienzar *et al.* 1998).

The extreme sensitivity of PCR ensures that only very small quantities of tissue are needed and neither radioactivity nor chemical or enzymatic degradation of the DNA is necessary (Grossweiner and Smith 1989). However, the sensitivity of the PCR method can also be a potential drawback, in that minute quantities of contaminating DNAs may be amplified and thus invalidating the fingerprint. The assay used in this study underwent careful optimisation of reaction conditions to facilitate reproducible amplification profiles (Atienzar *et al.* 1998).

**Heat shock 70 protein induction**

The cellular stress response is involved in protecting organisms from both natural and anthropogenic stressors. It occurs when cells undergo changes in gene expression resulting in the synthesis and accumulation of a suite of proteins referred to as stress proteins (Lindquist 1986). The cellular stress response is highly conserved, and has been found in all species tested from bacteria to humans (Schlesinger *et al.* 1982). It is characterised by the rapid synthesis of a small number of proteins, stress proteins, while a simultaneous inhibition of normal protein synthesis occurs. The stress proteins are heat inducible, and were for this reason initially referred to as heat shock proteins. However, it has now been established that stress proteins are involved in protecting organisms from damage related to exposure to a

variety of both natural and xenobiotic stressors such as oxidative stress, heavy metals and UV-B (Sanders 1990, Bauman *et al.* 1993, Bradley 1993, Dyer *et al.* 1993, Döhler *et al.* 1995, Sanders and Martin 1994).

Four main heat shock families are evident according to molecular weight: 90, 70, 60 and 16-24 kDa, which are termed heat shock protein (HSP) 90, HSP 70, chaperonin and low molecular weight (LMW) stress proteins (Sanders 1993). The heat shock protein response is a highly conserved genetic system. The gene for HSP 70 in humans is 73 % homologous to the HSP 70 gene in *Drosophila.* (Bauman *et al.* 1993). The stress 70 family which is the largest of the stress protein families has been extensively studied (Schlesinger *et al.* 1990) and a minimum of 21 proteins belonging to this multigene family have been characterised (Nover 1991). Members of the stress-70 family are located in the mitochondrion, chloroplasts, nucleus and endoplasmatic reticulum (Vierling 1991). The biochemical function of the constitutive forms which are expressed under normal non-stress conditions is primarily to bind with target proteins to modulate protein folding, transport and repair (Sanders 1993). These constitutively synthesised stress proteins play an important role in regulating protein homeostasis and are often referred to as molecular chaperones (Beckmann *et al.* 1990, Hightower 1993).

Under adverse environmental conditions the synthesis of HSP 70, 90 and chaperonin increase and they take on additional roles such as repair of denatured proteins and protection of cellular proteins from damage. It has been suggested that HSP 70 may be used as a biomarker of sublethal toxicity (Sanders 1993). Moreover, several studies have investigated whether heat shock proteins are mediators of heat-shock-induced UV-B resistance in human epidermal keratinocytes (Maytin *et al.* 1993, Trautinger *et al.* 1995). However, less information is available regarding the role of these proteins in algae (Müller *et al.* 1992, Vayda and Yuan

1994, Reith and Munholland 1991). Whether these proteins are induced in algae following UV-B exposure without an initial heat shock treatment is largely unknown.

## 1.4 The use of macroalgae in aquatic ecotoxicology

Growing interest in the world-wide problem of marine pollution has recently focused attention on the search for suitable biological indicators. With the exception of pesticide registration, algal tests are rarely performed. For example, phytotoxicity tests are conducted less frequently than acute toxicity tests with animal species (Fletcher 1991). Only 10 % of the toxicity test results submitted for the Toxic Substance Control Act (TSCA) premanufacturing notification process, contained phytotoxicity data (Benenati 1990). Although the use of marine macroalgae as bioindicators has been neglected on the basis of some misgivings about their insensitivity to pollutants (Jensen 1984), increasing attention has been paid within the last 20 years to the potential use of macroalgae as pollution indicators and test organisms (Thursby *et al.* 1985). Macroalgae are a primary energy source for coastal ecosystems, they play an important role in nutrient cycling, controlling water quality, and they provide habitat and shelter for aquatic life. Thus, their ecological importance suggests that they should be used in environmental assessment. Despite the recent use of some algal species in toxicity testing, there are no standard procedures with regard to the use of macroalgae to date. Most of the phytotoxicity information available to the regulatory community in the United States, for example, is based on results for a few green freshwater algal species (Lewis 1995). Recently, though, there has been a move in the US towards evaluating the toxicity of contaminants on both micro- and macroalgae (Thursby *et al.* 1993). The use of macroalgae in such studies has recently been reviewed by Fletcher (1991) and prior to this by Levine (1984).

Assessment of pollution levels, particularly heavy metal content of surface waters have been determined by analysis of algal tissue (Hellenbrand 1978, Kelly 1989). Furthermore, several workers have reported that certain species of macroalgae such as *Ulva lactuca* and *Enteromorpha* spp. are good bioindicators of heavy metal pollution (Ho 1990, Say *et al.* 1990). Moreover, toxic effects of oils, Polycyclic Aromatic Hydrocarbons (PAHs) and organic pollutants on marine macroalgae have been reported (Schramm 1972, Shiels *et al.* 1973, Hsiao *et al.* 1978, Cross *et al.* 1987, Maroli *et al.* 1993). Although herbicides have not been directly applied to the marine environment, they enter estuarine areas through river discharge and runoff. The presence of these compounds in the marine environment has recently received attention, as they affect non-target plants and algae through their mode of action (Tietjen *et al.* 1991, Bester and Hühnerfuss 1993). Likewise, the assessment of antifouling toxicity on non-target algae has been performed by several workers (Boney 1963, Hopkin and Kain 1978).

Using macroalgae in pollution assessment has several intrinsic advantages. They are, for the most part, sessile and can therefore be used to characterise or assess one location repeatedly over time. Furthermore, the occurrence and abundance of macroalgal taxa has previously been used successfully as an indicator of the general health of a particular marine habitat (Levine 1984). Many seaweeds such as *Enteromorpha intestinalis* and *Porphyra umbilicalis* are physiologically simple, and pieces cut from these plants will remain alive and regenerate easily. Repeated sampling of algal tissues can be undertaken because of the relative ease (compared to animals) with which they can be maintained and cultured in the laboratory (Fletcher 1991). Algae are also eminently suitable for use in the most contemporary biomarker approaches in which markers are employed not only to signal the extent of exposure to anthropogenic disturbances but also to provide some measure of ecologically relevant adverse effects. For example, the rapid growth and the different life history stages of

macroalgae can be used as sensitive "end-points" of pollution stress (Levine 1984, Thursby *et al.* 1985). Indeed, young spore-settling stages of algae have been used for antifouling studies, and it has been considered that the reproductive cells, in particular, are the most suitable to assess the toxicity of a biocide (Goodman and Russel 1977, Scanlan and Wilkinson 1987, Fletcher 1991, Scarlett *et al.* 1997). Macroalgae are the primary producers in the near shore environment, and effects on this, the lowest level of the food chain, will have consequences in other trophic levels (Joubert 1980). It has been concluded, that macroalgae are very suitable organisms for the determination of the impact of toxic substances on the aquatic environment (van Coillie *et al.* 1995). Reports that the reproductive stages of some rhodophyte macroalgae are more sensitive to pollutants than some of the most sensitive test animals will no doubt promote their future use as experimental test organisms (Thursby *et al.* 1985).

## 1.4.1 Choice of macroalgal species

Over 5000 macroalgal species, distributed in the green, brown and red algal groups exist world-wide (Grant 1963). Despite this large number of macroalgae available, surprisingly few species have been used as experimental test organisms. In many cases the choice of experimental alga has been largely determined by its ecological dominance in habitats of particular interest. Alternatively, the choice of alga has reflected its ease of laboratory culture and handling (Levine 1984). For this reason a popular choice has been to use the *Enteromorpha* spp. (Furtado and Fletcher 1987, Fletcher 1989, Schild *et al.* 1995, Lewis 1997, Scarlett *et al.* 1997). Some algae are used because of their apparent sensitivity to pollutants (North and James 1987, Thursby *et al.* 1985). Red macroalgae (Rhodophyta) have also been utilised in pollution assessment studies, because they, in terms of species composition, make up the largest component of seaweed communities (Levine 1984). Red algae can also contribute the majority of biomass in the subtidal habitat. *Enteromorpha*

*intestinalis*, *Palmaria palmata* and *Porphyra umbilicalis* were chosen as test organisms in the different investigations throughout this thesis for a number of reasons which are explained below.


## 1.4.2 Selected aspects of the ecology and reproduction of *Enteromorpha intestinalis*


*Enteromorpha intestinalis* has previously been referred to as an ideal marine bioassay test organism, in that it is cosmopolitan in distribution and is found in differing coastal habitats, both open and sheltered, marine and estuarine, polluted and unpolluted (Fletcher 1989). In temperate regions, maximum abundance of *Enteromorpha intestinalis* occurs in the spring and early summer periods. *Enteromorpha* spp are opportunistic, intertidal colonisers, which can be found exposed on rock or in tidal pools. *Enteromorpha* spp. are also common on sheltered, tidal mud and sand flats, and in harbours and estuaries polluted with sewage effluent (Fletcher 1991). *E. intestinalis* was chosen in several experiments throughout this work as an example of an intertidal, opportunistic green seaweed species. Furthermore, it provided a rapid, reproducible test method and it was possible to successfully maintain cultures in the laboratory. *E. intestinalis* was also the chosen species in the reproduction experiments described in Chapter 6, because of the relative ease with which spore release and the subsequent germination could be controlled in the laboratory. Since *E. intestinalis* is a common fouling alga and therefore "provides a realistic test for antifouling compounds" (Furtado and Fletcher 1987, Fletcher 1991), it was chosen in Chapter 7 to investigate the toxicity of the antifouling compound, Irgarol 1051, during simultaneous exposure to UV-B irradiance.

*Enteromorpha intestinalis* (L.) belongs to the family Ulvaceae within the class Chlorophyceae of the division Chlorophyta (Silva and Moe 1994). It forms a hollow tube bounded by a single layer of cells, which vary greatly in shape and size (10-30 cm) (Fig. 1.7). The tubes are parenchymatous in construction (soft tissue composed of relatively undifferentiated cells), and can be both branched or unbranched. As *E. intestinalis* was chosen for the reproductive experiments, selected aspects of the reproduction are mentioned below (from Fletcher 1989).



Figure 1.7. *Enteromorpha intestinalis.*

The reproductive sporangia are formed by direct conversion of the vegetative cells, in the terminal regions of the tube and are clearly recognised by the light-coloured senescent tips. Two types of sporangia are produced which are formed on separate plants, these are gametangia which contain the sexual gametes and zoosporangia which contain the asexual

zoospores (Figure 1.8) (Fletcher 1989). The sexual gametes are genetically identical to the gametangia bearing plants (the gametophytes). Spore release in *Enteromorpha* species is driven by tidal / lunar rhythms (Smith 1947). Zoospores, released from the sporophytes, are pear-shaped and larger than gametes (with an approximate length of 8-10 µm) with four terminally inserted flagella. The zoospores are weakly negatively phototactic. Their movements are relatively slow and they settle rapidly. In contrast, the haploid gametes (approximate length of 6-8 µm) are highly motile with only two flagella and they exhibit strong positive phototactic behaviour.
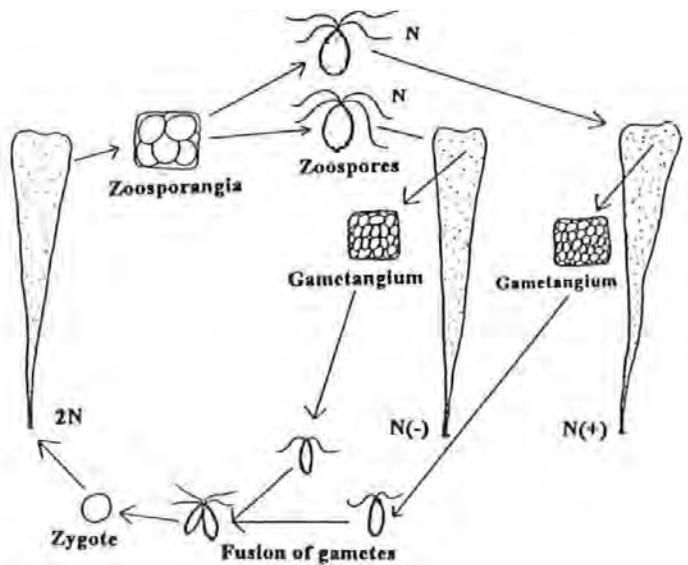


Figure 1.8 Life history of *Enteromorpha* sp. (adapted from Fletcher 1989).

When pairing between two gamete types occurs, a quadriflagellate gametic pair is formed. Following settlement cell and nuclear fusion occurs to form a diploid zygote. Unfused gametes can remain motile for several days under suitable conditions (Jones and Babb 1968).

Germination of the zygote produces a diploid thallus, morphologically and anatomically identical to that of the haploid gamete-producing plants. However, on these diploid plants zoosporangia are formed.

In contrast to the gametes, the haploid zoospores are produced after an initial meiotic division in the zoosporangia. After release the zoospores settle directly on to the substrata and germinate into haploid male and female gametophytic plants. Thus, the life history can be summarised as an isomorphic alternation of generations (alternation of asexual and sexual reproduction from one generation to the next) with the gametophyte producing the biflagellate gametes and the sporophyte producing the quadriflagellate zoospores (Fig. 1.8). It should be noted, however, that there are deviations from the life cycle described above; often the cycle is not completed in full.

### 1.4.3 Selected aspects of the ecology of *Palmaria palmata*.

*Palmaria palmata* (L.) belongs to the family Palmariaceae within the order of Palmariales of the class Rhodophyceae in the division Rhodophyta (Silva and Moe 1994). *P. palmata* which is largely subtidal can be found in rock pools and up to a depth of 20 m in both sheltered and moderately exposed areas. It can generally be found throughout the British Isles, and it is distributed from Arctic Russia to Portugal, the Arctic Canada to California in the USA and in Japan and Korea (Irvine 1995).

*P. palmata* forms a holdfast and is simple below with the blade gradually expanding above, dichotomously or palmately (i.e. shaped like a hand) divided into broad segments (Figure 1.9). The total length is between 50-100 cm and the width ranges between 3-8 cm. The colour is purplish red. *P. palmata* was chosen in several experiments throughout this work as

an example of a subtidal red seaweed species. Furthermore, it was found to be very sensitive to exposure of UV-B radiation and is regarded a non-target species to contamination of antifouling compounds.



Figure 1.9. *Palmaria palmata.*

## 1.4.4 Selected aspects of the ecology of *Porphyra umbilicalis*

*Porphyra umbilicalis* (L.) belongs to the family Bangiaceae within the order of Bangiales of the class Rhodophyceae in the division Rhodophyta (Silva and Moe 1994). *Porphyra umbilicalis* is primarily intertidal, but can occasionally be subtidal. It is generally found

throughout the British Isles and is by far the most common member of the genus. It is widely distributed from North West Europe to the Mediterranean. *Porphyra* ssp. is found in the coastal regions throughout the world and is one of the most extensively used species in mariculture in Japan and China. *P. umbilicalis* forms a broad, tough membranous frond up to 20 cm in diameter (Figure 1.10). It is olive to purple-brown in colour and has an irregular shape which is divided into lobes. The holdfast is central. It is attached to rocks and grows on exposed beaches, where it tolerates wave exposure and desiccation extremely well.



Figure 1.10. *Porphyra umbilicalis.*

*P. umbilicalis* was chosen in several experiments throughout this work as an example of an intertidal red seaweed species. It proved to be eminently suitable to investigate adaptive mechanisms in response to UV-B radiation. Furthermore, it could be readily cut into pieces and was successfully maintained in the laboratory for long periods of time. This species was also used as an example of a non-target rhodophyte species in investigations of the toxic effects of Irgarol 1051.

## 1.5 Aims & approaches

In the previous sections general aspects of ozone depletion and the contamination of the marine environment with organic compounds have been outlined. As coastal regions often act as sinks for industrial and agricultural compounds, it has been emphasised in the previous sections that elevated UV-B must be regarded as an additional stressor. It has also been pointed out that macroalgae generally are permanently attached and as such may be very vulnerable to elevated UV-B and to anthropogenic contamination (Fletcher 1991, Häder *et al.* 1995). Macroalgae are vital primary producers in the near shore ecosystems, play an important role in nutrient cycling and provide habitat and shelter for aquatic life (Houghton and Woodwell 1989, Häder *et al.* 1995). However, despite their significance, relatively limited data, compared to phytoplankton, is available on UV-B effects on macroalgae, and very little attention has been focused on their use in toxicity testing (Lewis 1995, Franklin and Forster 1997). Moreover, almost nothing has been published concerning the effects of elevated UV-B on the early life stages of algae, despite the evidence that the reproductive cells are particularly sensitive to marine pollutants, and that springtime increases in UV-B coincide with increased production of the reproductive cells (Thursby *et al.* 1985, Scanlan and Wilkinson 1987, Fletcher 1991). The biomarker approach, of finding suitable responses from lower levels of biological organisation which can anticipate and predict effects of

xenobiotic compounds, is now a widely used concept in ecotoxicology. However, less information is available in photobiology regarding responses which can be utilized as early warning signals of exposure to elevated UV-B irradiance.

To evaluate the ecological implications of continued springtime UV-B stress on macroalgae, research efforts need to focus on i) finding indicators which can act as early warning signals to effects of elevated UV-B radiation, ii) investigating the effects of UV-B on the early life stages of algae and iii) assessing the interactive effects of UV-B radiation and xenobiotic pollutants. To this end, the overall aims of the studies in this thesis were to:

**a )** investigate the suitability of several molecular and physiological parameters as biomarkers of UV-B exposure and/or effect in marine macroalgae, and to relate these physiological parameters to consequent biological effects measured as growth rate.

**b)** assess differences in susceptibility to UV-B radiation of several macroalgal species from habitats which differ with regard to solar UV and PAR irradiation levels.

**c)** estimate the relative sensitivity of the reproductive unicells of *E. intestinalis* to UV-B exposure.

**d )** examine interactive effects of UV-B radiation and the antifouling compound, Irgarol 1051, using selected biomarkers on both target and non-target macroalgal species.

The following approaches were applied:

• A validation of UV-B measurement procedures ise included in Chapter 2 with special emphasis placed on the procedures used throughout this thesis.

• Laboratory experiments were conducted to investigate the suitability of *in vivo* chlorophyll fluorescence and *in vivo* absorptance spectra as non-destructive physiological biomarkers of UV-B exposure. Dose-response relationships between these parameters and varying UV-B irradiances were established for several macroalgae species in Chapters 3. Furthermore, UV-B induced membrane damage was also assessed by measuring the extent of ion leakage (Chapter 3).

• An experiment was performed in Chapter 4 using HPLC to identify and quantify chlorophyll and carotenoid pigments in several macroalgal species.

• Responses to UV-B exposure on the molecular level were investigated by measuring DNA damage and the induction of HSP 70 (Chapter 5).

• The potential effects of UV-B radiation on the reproductive cells of *E. intestinalis* were investigated in Chapter 6.

• Finally, the effects of simultaneous exposure to UV-B radiation and the antifouling compound Irgarol 1051 were examined using selected biomarkers on both a fouling alga (Chapter 7) and two non-target algal species (Chapter 8).

# CHAPTER 2

# VALIDATION OF UV MEASUREMENT PROCEDURES

Broad-band UV-sensors in laboratory and field based experiments are used world-wide (Karentz *et al.* 1991b, Middleton and Teramura 1993, Quesada 1995, Dring *et al.* 1996a,b), as they are relatively inexpensive and easy to operate compared to spectroradiometers. However, a major disadvantage of these sensors is their lack of spectral information. To facilitate comparison of results between different experiments and laboratories, it is necessary to calibrate these broad-band sensors against a pre-calibrated spectroradiometer. To achieve and maintain a reliable absolute calibration of the spectroradiometer, it must regularly be calibrated against a standard source of spectral irradiance, such as deuterium and tungsten halogen lamps (Tüg and Baumann 1994). These, in turn, should be calibrated by proficient national laboratories such as the National Physics Laboratory (Bais 1997). Special care was taken throughout this study to calibrate the broad-band sensors against a pre-calibrated spectroradiometer, as described below. Moreover, estimates of uncertainties and variations in UV irradiance are listed. Filter material chosen for UV experiments is described and details on how to calculate the correlation factor between unweighted and weighted UV-B values are given.

## 2.1 UV measurements

Throughout this thesis UV-B was supplied by preburned UV-B tubes (Philips TL' 20W/12 RS™) and UV-A by UV-A tubes (Philips 1609™ 15 W). UV-A was always kept constant and independent of the UV-B irradiance, which was changed by adjusting the height of the

UV-B tubes. Figure 2.1a depicts the emission spectra of UV-B and UV-A from UV tubes fitted with 35 μm cellulose diacetate foil, and UV-B tubes fitted with Mylar 125 D (please refer to paragraph below for more details about filters). Photosynthetically Active Radiation (PAR) was supplied throughout by fluorescent PAR tubes, Phillips TLD 32W/83 HF™ and all indoor exposures were performed in growth cabinets. Figure 2.1b shows the PAR emission spectrum.

UV irradiance was measured routinely with UV-A (MP-236) and UV-B (MP-229) cosine sensors (MicroPulse Technology Ltd). The calibration of these sensors was performed against a double monochromator spectroradiometer (model SR 9910™, Macam Photometrics Ltd.) inside the growth cabinet, identical to the experimental set-up. Errors related to calibration of the spectroradiometer such as wavelength calibration, signal linearity, temperature dependence, long-term stability, repeatability, sensitivity and dynamic range (Bais 1997) were minimised by using a double monochromator which was calibrated by an experienced physicist from Macam Photometrics. The calibration of the spectroradiometer was performed with a deuterium and tungsten halogen lamp, which in turn was calibrated by the National Physics Laboratory. The calibration of the broad-band sensors was performed before and after experiments on several occasions by the operator from Macam and by the author.

The ideal broad-band sensor of the kind used here, will measure one spectral range with a rectangular spectral sensitivity (adapted from Blumthaler 1997). Such broad-band sensors use combinations of filters to achieve a shape of the spectral sensitivity as close as possible to a rectangle. This means that all wavelengths within the range of sensitivity are measured with their true weight, and that there is no contribution to the total signal from wavelengths outside this range. This was not quite the case with the broad-band sensors used in these studies since this would result in 1:1 correlation between the calibrated spectroradiometer and

the cosine sensors. Correlations between measurements made by the spectroradiometer and the cosine sensors are presented in fig. 2.2. The fitted models used to calculate the actual UV-B and UV-A output, as measured by the spectroradiometer, are included in the figures. PAR was measured with a PAR meter connected to a quantum sensor (model SKP 200, Skye Instruments Ltd) and calibrated on several occasions by the manufacturers.

Prior to the various experiments, measurements of UV-B, UV-A and PAR irradiances were made at 9 different points, approximately 12 cm apart, covering the entire area where the algal material was placed during the UV exposure. The irradiance was then calculated as an average. Standard deviations, calculated as a percentage of the average irradiance, measured in a particular experiment, are listed in Table 2.1. Moreover, throughout the experimental period, measurements were made to investigate changes in lamp output (Table 2.1).

## 2.2 Filters

UV light was filtered with 35 μm cellulose diacetate foil (AC Converters Ltd.) which showed 0 % transmission below 286 nm (UV-C), (fig. 2.1a). For the control thalli UV tubes were covered with 125 μm Mylar 125 D (DuPont Ltd) which showed 0 % transmission below 320 nm (i.e. filtering out UV-B). Thus, control treatments in all experiments throughout this thesis consisted of no UV-B, but identical UV-A and PAR irradiance levels to the UV-B treatments. However, since UV-B radiation is naturally present in the environment, the ambient level of UV-B, corresponding to the month of the experiments, was always included for comparison as a separate treatment. Transmittance of the filters was measured before and after the experiments with an ATI Unicam UV/Vis spectrophotometer, which can measure transmittance above 230 nm. The filter material was placed in a Labsphere (RSA-UC 40™), a diffuse reflectance and transmittance accessory, inside the spectrophotometer. Filters were

replaced at least every 10 hours and within this time period the filters caused no change in emission spectra of the tubes, as measured by the double monochromator spectroradiometer. A decrease in total irradiance, between 5 - 10 % (maximum 30 % as measured on one occasion), was compensated for by adjusting the height of the UV tubes. Filter ageing has also been reported by others (Middelton and Teramura 1993).

## 2.3 Calculation between unweighted and weighted UV-B irradiance and dose

Throughout this thesis the generalised plant response action spectrum, PAS (Caldwell 1971) has been used to calculate weighted UV-B irradiances, doses and equivalent estimated percentage ozone depletion. This action spectrum has been chosen to permit comparison with other work in the UV field. Using the computer model of Björn and Murphy (1985) the ratio between PAS and the unweighted solar UV-B irradiance and dose range was between 0.07 and 0.18 for Plymouth (50°22' N latitude and 4°17' W longitude). This ratio changes seasonally, and is highest during the summer months, when there is relatively more shorter wavelength UV-B. The comparable ratio under TL 40 UV-B tubes filtered with cellulose diacetate for both UV-B irradiance and dose is 0.282 ± 0.00466 (mean ± SE). This ratio was calculated from PAS weighted and unweighted measurements made by the spectroradiometer. The unweighted UV-B irradiances and doses throughout the experiments were multiplied with this ratio to obtain comparable PAS 300 weighted values.
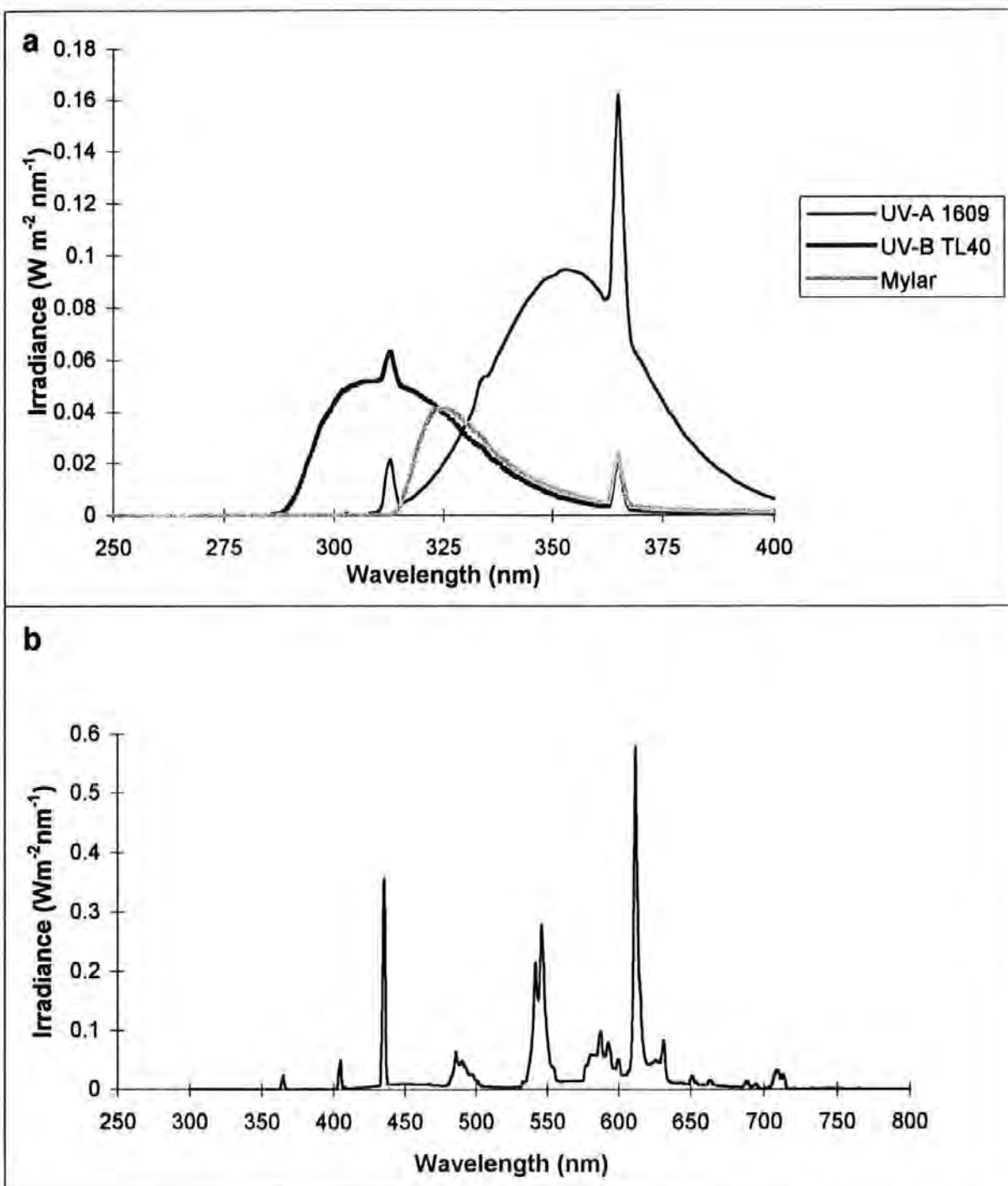
Figure 2.1 a) Emission spectra of UV-B and UV-A tubes used in the different experiments throughout this thesis. UV-B tubes (Philips TL' 20W/12 RS) and UV-A tubes (Philips 1609 15 W) were covered with 35 μm cellulose diacetate foil to filter out UV-C, or UV-B tubes were fitted with Mylar 125 D. b) Emission of fluorescence tubes, Phillips TLD 32W/83 HF, used as background Photosynthetically Active Radiation. Since varying UV and PAR irradiances were used throughout this work, the total irradiance shown on the figure should be regarded as an example.
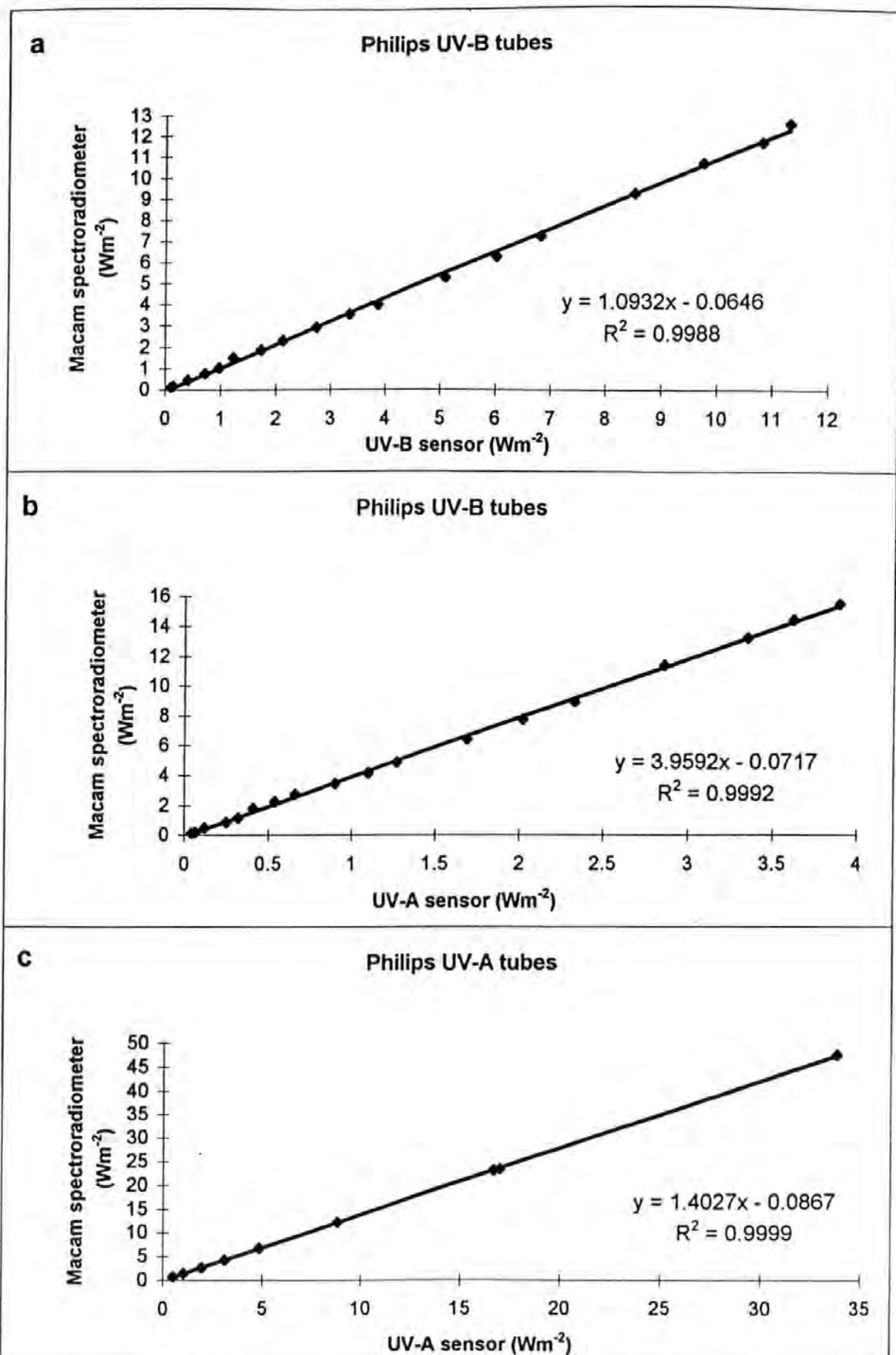
Figure 2.2. Correlation between a) UV-B (280 - 315 nm, MP-229) broad-band cosine sensor and b, c) UV-A (316 - 400 nm, MP-236) broad-band cosing sensor against a double monochromator spectroradiometer using a, b) UV-B Philips TL' 20W/12 RS and c) UV-A tubes Philips 1609 15 W.

Table 2.1. Variation in UV or PAR irradiance as percentage standard deviation, SD. The variation was calculated for different irradiances and then averaged. n represents number of experiments from which SD was determined.

| UV-B | UV-A | PAR |
| --- | --- | --- |
| Variation in irradiance measured at different points under UV or PAR tubes | | |
| SD = 12.04 %, n = 15 | SD = 21.65 %, n = 21 | SD = 15.02 %, n = 15 |
| Variation in irradiance from irregular output of UV tubes | | |
| SD = 4.62 %, n = 9 | SD = 7.4 %, n = 12 | |

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# CHAPTER 3

# EVALUATION OF CHLOROPHYLL FLUORESCENCE, *IN VIVO* SPECTROPHOTOMETRIC PIGMENT ABSORPTANCE AND ION LEAKAGE AS BIOMARKERS OF UV-B EXPOSURE IN MARINE MACROALGAE.

## 3.1 ABSTRACT

A comparison of seven common species from the intertidal and subtidal zones at Wembury beach revealed that the subtidal *Palmaria palmata* was the most sensitive alga to UV-B radiation. The ratio $F_v$ / $F_m$, *in vivo* absorptance spectra and ion leakage were evaluated as biomarkers of ambient and elevated UV-B exposure in *Enteromorpha intestinalis* and *P. palmata*. Measurements of thallus growth were also used to assess adverse biological effects.

Ambient and elevated UV-B significantly inhibited photosynthesis in both species. It was shown that the $F_v$ / $F_m$ ratio is a sensitive, non-specific general biomarker of UV-B exposure in both species. Moreover, *in vivo* absorptance of what was tentatively identified as chlorophyll *a*, phycoerythrin and/or carotenoids, as well as phycoerythrobilin and phycocyanin decreased in a dose-response dependent manner and was associated with a decrease in growth rate in *P. palmata*. The intertidal alga *E. intestinalis* showed a greater degree of tolerance to UV-B exposure.

These results indicate that reductions in - and recovery of optimal quantum yield ($F_v$ / $F_m$ ratio), together with changes in *in vivo* thallus absorptance, and inhibitions in growth rates, could provide an early quantitative warning of the detrimental effects of UV-B in marine macroalgae.

## 3.2 INTRODUCTION

Stratospheric ozone depletion, resulting from the release of anthropogenic chlorofluorocarbons (CFCs), is associated with increased UV-B irradiation (280-315 nm) of the earth's surface (Frederick 1993, Kerr and McElroy 1993). Even with strict international controls on the use of CFCs, ozone depletion is predicted to continue well into the next century (NASA Reference Publication 1990), affecting both Antarctica (Farman et al. 1985) and the northern hemisphere (Stone 1993). In this context investigations into the effects of enhanced UV-B radiation on phytoplankton and macroalgae is of great importance, since these vital primary producers form the basis of aquatic food webs (Houghton and Woodwell 1989). Macroalgae in particular are major primary producers in near-shore and intertidal ecosystems. Rates of productivity are comparable with those measured in the most productive terrestrial ecosystems (Mann 1972a,b, Duggins et al. 1989).

The biomarker approach has been used extensively by ecotoxicologists for detecting exposure to and effects of environmental contamination during the past decade (Depledge 1989, McCarthy and Shugart 1990, Fossi and Leonzio 1994). Biomarkers raise the possibility of determining where an organism lies on the health status curve (Depledge 1989). Thus, they potentially provide an early warning of pollutant or UV-B induced reversible departures from homeostasis, or signal the triggering of compensatory biochemical and physiological responses (please see Section 1.3 for more details). The simultaneous use of more than one biomarker enhances the detection of effects since different biomarker responses are induced at different stages of the plant or organism's health status curve (Depledge 1994). This clinical approach is comparable to that used in human medicine (Peakall 1992). Biomarkers can be identified that indicate exposure to stress (general non-specific biomarkers), specific

categories of stress (natural or related to pollutant exposure), and specific types of stressors (such as acetylcholinesterase inhibition following exposure to organophosphate pesticides). The biomarker approach has proven especially useful when these responses can be linked to changes in Darwinian fitness parameters (Depledge 1989).

The emphasis to date in photobiology has been to establish a correlation between ozone depletion and elevated UV-B. Action spectra and weighting functions have also been derived (Caldwell 1971, Cullen *et al.* 1992). However, finding biochemical, cellular or physiological responses which can be related in a dose dependent manner to UV-B exposure, and therefore act as early warning signals to changes in UV-B levels, has less frequently been attempted (Campos *at al.* 1991, Cullen and Lesser 1991, please see Section 1.3 for more details). If these early warning signals can be identified they might be used to signal a change in biological fitness that may have consequences for entire populations.

The aims of this study were to,

a) compare the sensitivity of selected species of macroalgae from different tidal heights.

b) investigate the suitability of chlorophyll fluorescence, changes in *in vivo* pigment absorptance and ion leakage as biomarkers of UV-B exposure in marine macroalgae.

c) to relate these physiological parameters to consequent biological effects measured as growth rate.

## 3.3 MATERIALS & METHODS

### Algal collections

Several intertidal and subtidal macroalgae species from the divisions Chlorophyta and Rhodophyta were collected at low tide from Wembury on the South West coast of Devon,

UK (50°22' N latitude, 4°17' W longitude) in February (sea water temperature: 5 °C) and in June (sea water temperature: 10 °C). The algal material was collected by cutting at the base of thalli which were attached to the substratum. The thalli were harvested 24 hours before the experiments and kept in filtered sea water at constant temperature (15 °C) and in low light conditions (25 µmol $m^{-2}$ $s^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF™) in growth cabinets. All algae were kept in 12:12h light:dark regime except *Ulva lactuca* and *Enteromorpha intestinalis* which were kept under constant light and no nutrients were added to avoid sporulation.

## UV treatments and experimental design

Thalli of all species, cut to an approximate length of 3 cm and covered to a depth of 1.5 cm with filtered sea water, were exposed in open Petri dishes. PAR and temperature conditions were identical to those in the acclimatisation period. UV-B was supplied by two UV-B tubes (Philips TL 20W/12 RS™) and UV-A by two UV-A tubes (Philips 1609™ 15 W). UV-A was kept constant at 1.3 W $m^{-2}$, which is approximately 4 times less than that calculated at 50° latitude (Cornwall) in December (personal comm., Driscoll *et al.* 1992). This was held independent of the UV-B irradiance. UV-A radiation was included in the exposures in view of the finding of Middleton and Teramura (1993) that it modifies the effectiveness of UV-B radiation for several plant characteristics. For instance, UV-A has been found to be part of photorepair and photoprotection (Beggs *et al.* 1985).

UV irradiance was measured routinely with UV-A (MP-236) and UV-B (MP-229) cosine sensors (MicroPulse Technology Ltd), which were calibrated in the experimental light field against a double monochromator spectroradiometer (model SR 9910™, Macam Photometrics Ltd) (please see Chapter 2 for more details). PAR was kept low and constant during the entire experimental period at an similar irradiance to the acclimatisation period. PAR was

measured with a PAR meter connected to a quantum sensor (model SKP 200™, Skye Instruments Ltd) and calibrated by the manufacturers. The UV and PAR measurements and the choice of lamps described here were used throughout this thesis.

For UV-B treatments, the following unweighted irradiances were measured with varying exposure times: 0.2, 1.4, 2.6, 3.6, 8.6, 9.0, and 12.7 W m$^{-2}$ (equivalent to weighted irradiances of 0.06, 0.4, 0.7, 1.0, 2.4, 2.5 and 3.6 W m$^{-2}$, calculated according to the generalised plant response action spectrum, PAS 300, Caldwell 1971). The weighted irradiances correspond to: ambient irradiance in March, 20 %, 32 %, 34 % ozone reduction respectively and between 35 and 36 % ozone reduction for the last three irradiances. Both weighted irradiance and ozone depletion levels were calculated at Plymouth (at an air pressure of 1000 mbar, relative humidity of 0.5 and aerosol level of 0), at noon 12.00 of the 15.07.97 during clear sky conditions using the computer model Björn and Murphy (1985). The relatively high values of UV-B irradiance were chosen to investigate the entire range of the dose-response curves. The different UV-B irradiances were obtained by adjusting the height of the UV-B tubes above the thalli. UV light was filtered with 35 micron cellulose diacetate foil (AC Converters Ltd.) which showed 0 % transmission below 286 nm (UV-C). For the control thalli UV tubes were covered with Mylar 125 D (DuPont Ltd) which showed 0 % transmission below 320 nm (i.e. filtering out UV-B). Transmission of the filters was measured before and after the experiments with a ATI Unicam UV/Vis spectrometer. The filter material described above was used throughout the thesis. Where reference to UV-B irradiance or dose is made in this and subsequent chapters, unweighted values have been used, unless specifically stated otherwise.

## Measurements of chlorophyll fluorescence induction

*In vivo* chlorophyll fluorescence was measured with an non-modulated Plant Efficiency Analyser™ (Hansatech Instruments Ltd). The ratio $F_v$ / $F_m$ was determined before, immediately after, 24 and 48 hours after UV exposure. $F_m$ is the maximal fluorescence in the dark adapted state i. e. when all reaction centres are closed (reduced). $F_0$ is the initial fluorescence in the dark adapted state i. e. when all reaction centres are open (oxidised) and $F_v$ is the variable fluorescence calculated as $F_m$ - $F_0$. Before the measurements the thalli were dark-adapted for 10 min to ensure an oxidised electron transport chain. Prolonged dark adaptation (25 min) showed no change in $F_0$ and $F_m$. The fluorescence was initiated by 1 s red light pulses with a peak wavelength of 650 nm and an irradiance of 3000 $\mu$mol m$^{-2}$ s$^{-1}$. Illumination was provided by an array of 6 high irradiance light emitting diodes (LEDs, Hansatech Instruments Ltd) which were focused onto the thallus surface to provide even illumination.

## *In vivo* spectrophotometric measurements

Algal pigment absorptance spectra were measured *in vivo* at room temperature to avoid hypsochromic shifts. The samples were placed in a Labsphere (RSA-UC 40™), a diffuse reflectance and transmittance accessory, inside the UV/Vis spectrophotometer (Unicam). Thallus transmittance and reflectance spectra were recorded for both species before exposure (0 hour), immediately after, 24 and 48 hours after an initial 3 hours of exposure. The tissue samples were placed in the sphere and a baseline measurement was performed for each thallus. A light trap was placed behind the sample to avoid scattering. All spectra were gathered with 4 nm bandpass from 215 to 830 nm. To obtain the percentage absorptance spectra the following equation was used

% absorptance = 100% - ( % transmittance + % reflectance)

## Ion leakage measurements

Ion leakage was measured using a modified version of the method of Axelsson and Axelsson (1987). One g tissue of *E. intestinalis* and 0.4 g of *P. palmata* were used for each replicate, which was split into duplicates to investigate method variation. An average variation of ΰ 10 % was found. Ion leakage was measured as electrical conductivity with a Corning 220 conductivity meter at room temperature. The tissue was rinsed very quickly in 50 ml ultra pure water and transferred to 25 ml ultra pure water (sample 1). After 2 minutes the tissue was drained quickly and transferred to a second beaker containing 25 ml ultra pure water and boiled for 5 minutes (sample 2). The conductivity of both samples was measured at room temperature and the "Health Index" was calculated on the basis of the ion loss as:

Sample 2 / (Sample 1 + Sample 2) expressed as percentage.

The minimum value obtained from dead thalli by boiling the tissue in instant ocean for 5 min and then leaving it to equilibrate in instant ocean for 30 min was 20 % (Schild *et al.* 1995). Health Index values for healthy algae were: 60 % ± 1.3 % for *E. intestinalis* and 80 % ± 1.2 % for *P. palmata*.

## Growth measurements

The physiological variables were related to differences in thallus growth by daily measurements of the projected thallus areas on a Quantimet 570 Automatic Image Analyser during a two week period. This image analysis system consisted of a MOS camera (Hitachi, VK-C150ED™), fitted with a 55 mm lens. The process of image capture, image processing and measurements was programmed using QBASIC the command language of the Quantimet.

Distance and light intensity were calibrated prior to measurements. This procedure was used in the different experiments throughout this thesis.

## Statistical analysis

Statistical analyses were conducted using StatGraphics 6.1. Species differences in $F_v / F_m$ were tested in one-way ANOVAs. For $F_v / F_m$ data in all other experiments, the spectrophotometric measurements and growth data, sequential sampling of each thallus at all time periods was performed so each thallus could act as its own control. Inter-individual variability was thus taken into account in the statistical analysis. A multivariate analysis of variance for repeated measurements was applied. A repeated measures design differs from normal ANOVAs in that the different times are not independent. The special Huynh-Feldt (H-F) condition must, therefore, be fulfilled before the test can be correctly applied (Milliken and Johnson 1984). If the above test showed both significant differences between groups and an overall significant time-treatment interaction, Bonferoni tests were conducted (calculated according to Milliken and Johnson 1984) to find where significant differences occurred. A random design was chosen to analyse ion leakage data and statistically tested by performing one-way (single factor) analysis of variance (ANOVA). Tukey's HSD test was used to reveal statistical differences. The underlying assumptions of any parametric test of normal distribution, independence within and between groups, and equal variances were checked each time, for all experiments throughout this thesis before conducting the analyses.

Growth data were analysed using multiple regression analysis (performed according to Mendenhall and Sincich 1989, Ricketts 1995). The Relative Growth Rate $= (\log_e A_1 - \log_e A_0) / T_1 - T_0$, where $A_1$, $A_0$ = area at two times (T), is equal to the slope of the regression lines, determined on log transformed data (Hunt 1978).

# 3.4 RESULTS

## Measurements of chlorophyll fluorescence

Seven common species from the intertidal and subtidal zones at Wembury beach were exposed to unweighted UV-B irradiance of 1.7 W m$^{-2}$ , and an unweighted UV-A irradiance of 1.4 W m$^{-2}$ for a 3 hour period (fig. 3.1). $F_v$ / $F_m$ ratio of Chondrus crispus, Porphyra umbilicalis (Rhodophyta) and Enteromorpha intestinalis (Chlorophyta) were almost unaffected by UV radiation compared to unexposed controls. The most affected species was the subtidal alga Palmaria palmata with a reduction of 65 % in $F_v$ / $F_m$ ratio. For more detailed investigation, described below, E. intestinalis was selected as an example of a tolerant green alga and P. palmata was selected as a very sensitive red alga.

In fig. 3.2a and b significant decreases in $F_v$ / $F_m$ ratio were found for all UV-B irradiances at almost all exposure times for both E. intestinalis and P. palmata. After 3 hours exposure to unweighted UV-B irradiance of 2.6 W m$^{-2}$, a 50 % decrease compared to pre-exposure values was found for E. intestinalis and 75 % for P. palmata. Following 1.5 hours exposure to unweighted UV-B irradiance of 12.7 W m$^{-2}$ a decrease of 75 % in $F_v$ / $F_m$ was found for E. intestinalis and 80 % for P. palmata. These results presented in Figure 3.2c and d as dose-response curves, show an exponentially decreasing relationship between UV-B dose and the $F_v$ / $F_m$ ratio for both species. One curve was fitted for each species and one equation was calculated as a mean of all the points.

A similar experiment performed in June showed recovery to control $F_v$ / $F_m$ ratio for E. intestinalis after an initial 3 hours of exposure to all UV-B irradiances tested (fig. 3.3a).
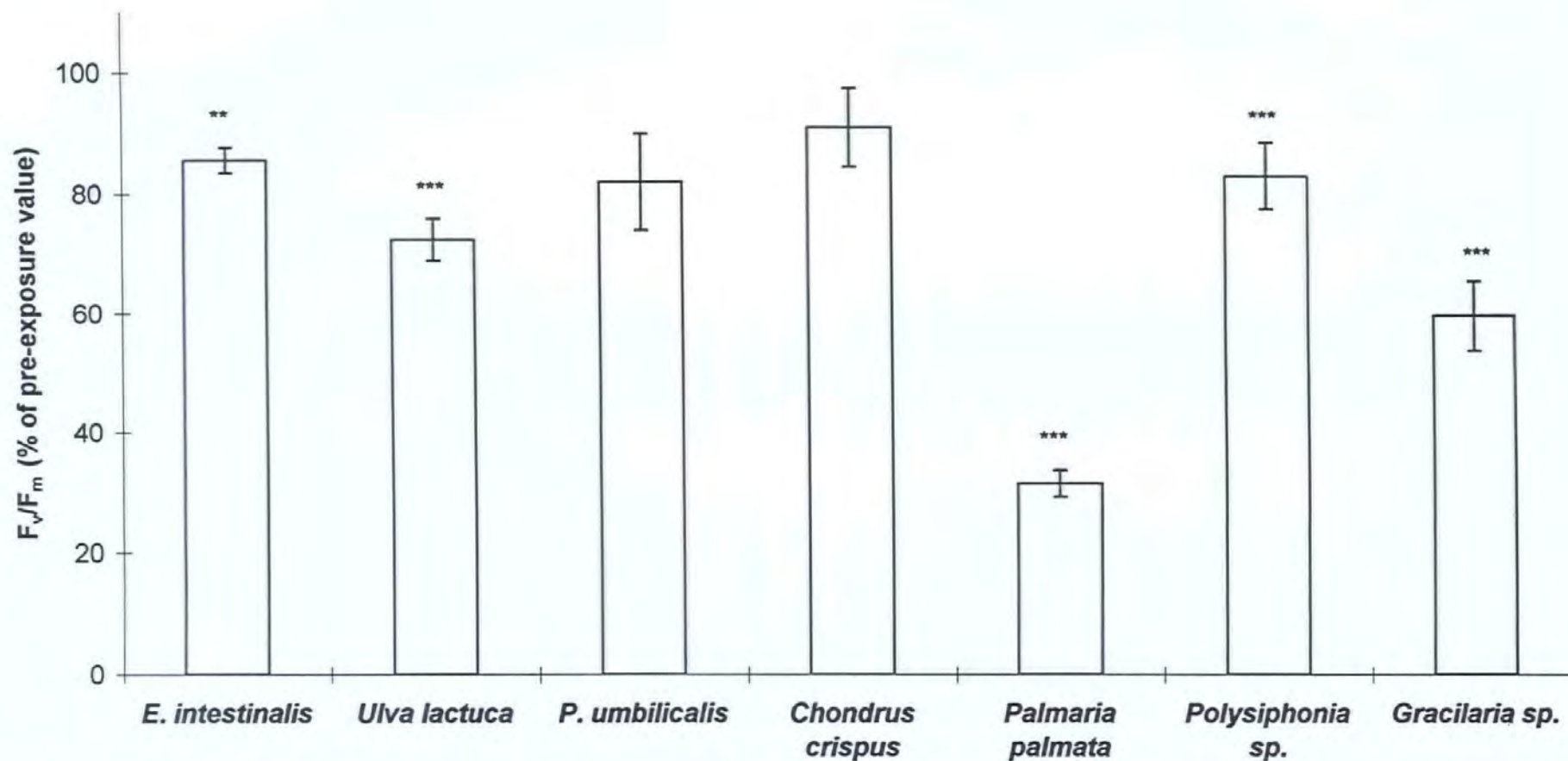
Figure 3.1. Chlorophyll fluorescence $F_v / F_m$ ratio in several species of macroalgae from different tidal heights (Chlorophyta and Rhodophyta) following a 3 hr exposure to unweighted UV-B irradiance of 1.7 W m$^{-2}$. Means +/- SE, n = 5. **, ***; p<0.01, p<0.0001
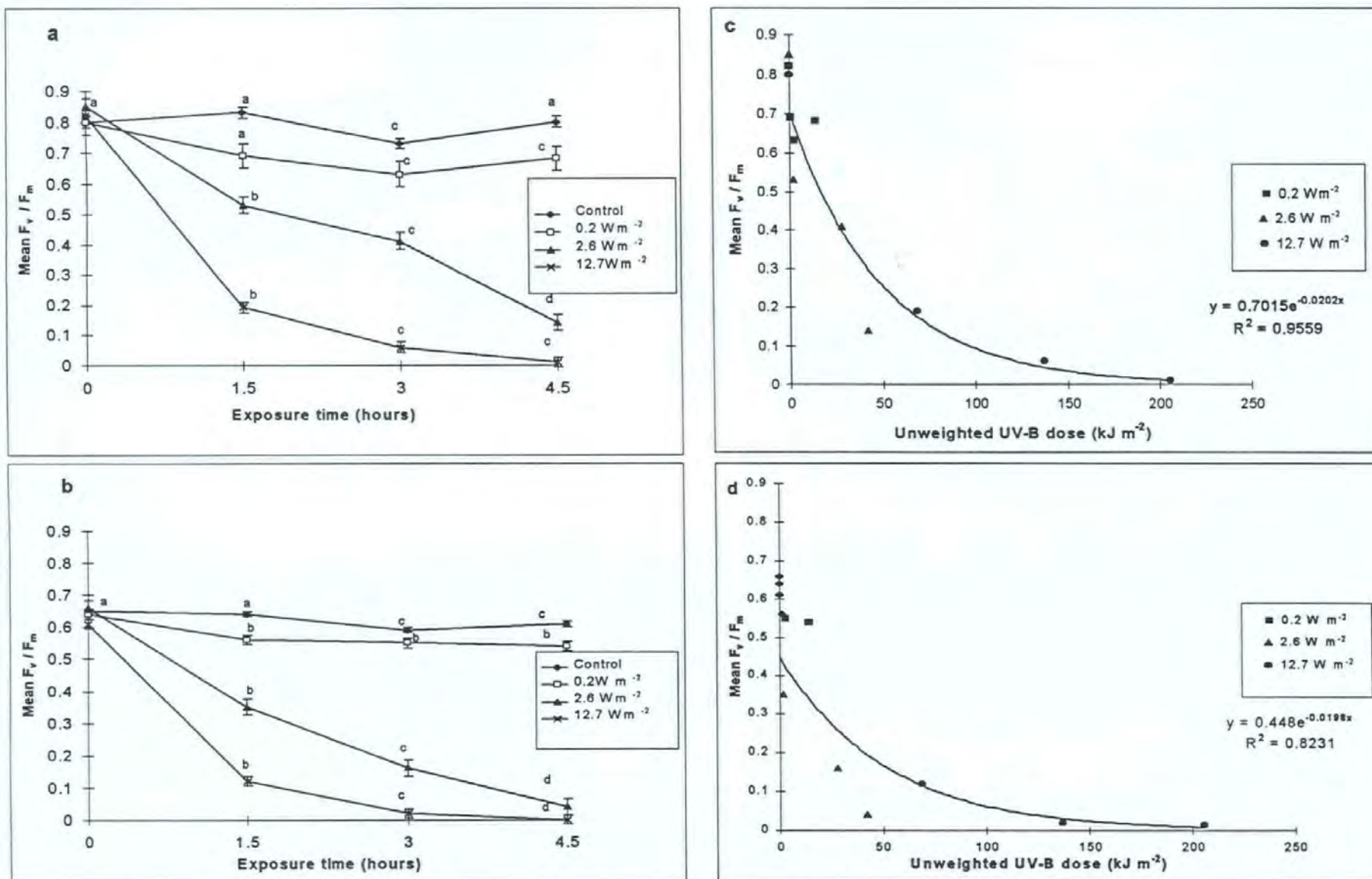
Figure 3.2. *Enteromorpha intestinalis* (a, c) and *Palmaria palmata* (b, d). a, b Chlorophyll fluorescence $F_v / F_m$ ratio as a function of duration of exposure to different UV-B irradiances; data are means +/- SE (n = 5). Means with different letters within the same treatment differ significantly from each other, (ANOVA; $p < 0.05$). c, d unweighted UV-B dose-response curves.

However, for *P. palmata* (fig. 3.3b) an 18 % recovery to 25 % of the pre-exposure value was only found for the lowest irradiance after 48 hours.

To investigate the effect of using different action spectra on doses from an artificial UV-B source the data for *P. palmata*, presented in fig. 3.2b, were expressed both in absolute doses of several weighting functions (fig. 3.4a) and relative to the maximum July dose calculated for Plymouth (fig. 3.4b). The following action spectra were chosen in fig. 3.4a: PAS 300 which is Caldwell's generalised plant action spectrum, normalised to 300 nm; Quaite, Sutherland and Sutherland's (1992) action spectrum for DNA damage in intact alfalfa seedlings; the whole plant action spectrum of Steinmüller (in Caldwell *et al.* 1995); Thimijan which is a modified mathematical fit to Caldwell's generalised action spectrum where a UV-A tail is incorporated; Erythemal which is the standard CIE erythemal (sunburn) action spectrum (Mckinlay and Diffey 1987).

Figure 3.3 *Enteromorpha intestinalis* a) and *Palmaria palmata* b). $F_v / F_m$ as a function of time after exposure to different UV-B irradiances; exposure time for all UV-B irradiances was 3 h; data are means +/- SE (n = 5). *** significant differences between UV-B-treated thalli and control thalli at p <0.001; overall significant time-treatment interaction (p <0.001) was found for both species.

Figure 3.4. Response of $F_v / F_m$ ratio in *Palmaria palmata* at increasing UV-B dose expressed as a) absolute doses of several weighting functions and b) doses relative to the maximum July dose in Plymouth for Caldwells generalised plant response spectrum (PAS300), Quaite or the Erythemal action spectrum. Data are from Figure 3.2b.

## Spectrophotometric measurements

The *in vivo* absorptance spectrum for *E. intestinalis* (fig. 3.5a) demonstrates the presence of chlorophyll *a* and chlorophyll *b* as evidenced in absorptance at approximately 440 nm and 680 nm, and 470 nm and 657 nm, respectively. The broad shoulder at approximately 490 nm is assigned to ß-ß carotene, lutein and xanthophylls in Chlorophyta (Britton 1984, please see also Chapter 4.2 for further identification and quantification of the different chlorophylls and carotenoids). However, no decrease in absorptance at any irradiance or time interval occurred. In contrast, a highly significant decrease in absorptance was found for *P. palmata* 48 and 72 hours after 3 hours exposure to 2.6 W m$^{-2}$ (fig. 3.5b), (p<0.001, Multiple ANOVA). Moreover, a profound decrease in absorptance was observed immediately after 3 hours exposure to 9.0 W m$^{-2}$ (fig. 3.5c), (p<0.001, Multiple ANOVA).

Figure 3.6 shows for *P. palmata*, 48 hours after UV-B exposure, that the greatest decreases in absorptance occurred at 498 nm (characteristic of the phycourobilin chromophore of rhodophycean phycoerythrin and/or carotenoids) and at 568 nm (characteristic of the phycoerythrobilin chromophore of phycoerythrin (Smith and Alberte 1994)). The absorptance maxima at 436 nm and 678 nm are attributable to the presence of chlorophyll *a* (Smith and Alberte 1994) and the band at 626 nm probably arises from phycocyanin (Kursar and Alberte 1983) (please see Chapter 4.2 for further identification and quantification of the different chlorophylls and carotenoids). The peak at 336 nm may arise from mycosporine-like amino acids such as porphyra-334 (peak at 334 nm), shinorine (peak at 334 nm), and / or glycine:valine (peak at 335 nm) (Karentz *et al.* 1991a).
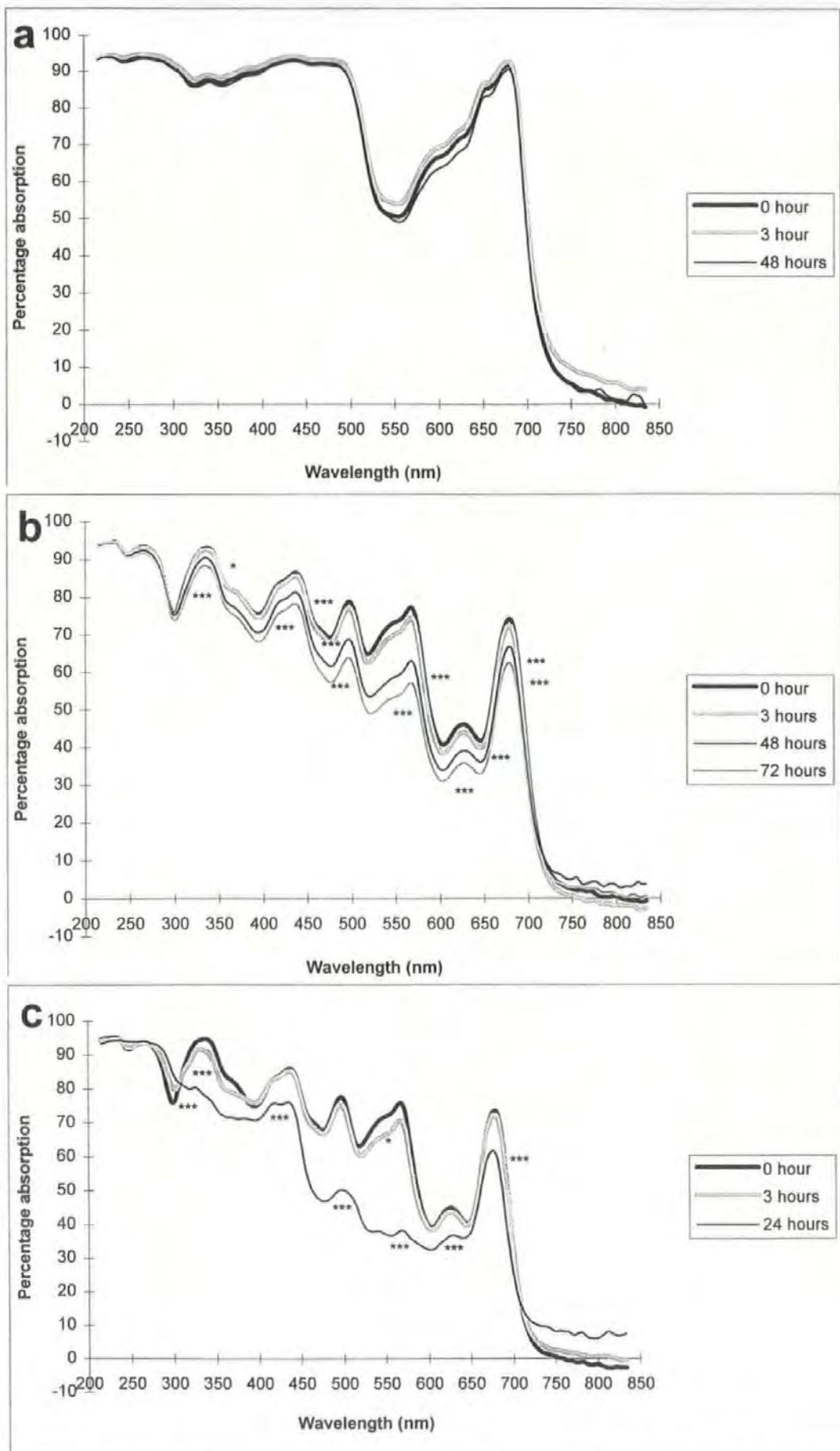
Fig. 3.5 *Enteromorpha intestinalis* a) and *Palmaria palmata* b, c). Average *in vivo* absorptance spectra, before, and after initial 3 h exposure to 2.6 W m$^{-2}$ (unweighted UV-B irradiance) (a, b); and before and after initial 3 h exposure to 9.0 W m$^{-2}$ (c). (*, **, *** significant differences between absorptance maxima before and after exposure at $p < 0.05$, $p < 0.01$; $p < 0.001$, respectively; n = 5).

Figure 3.6. Percentage absorptance in thalli of *Palmaria palmata* as a function of different absorptance maxima, 48 hours after an initial 3 h exposure to different UV-B irradiances; data are means +/- SE; n = 5. (*, **, *** significant differences between UV-B treated thalli and control thalli at p <0.05, p <0.01, p <0.001, respectively).
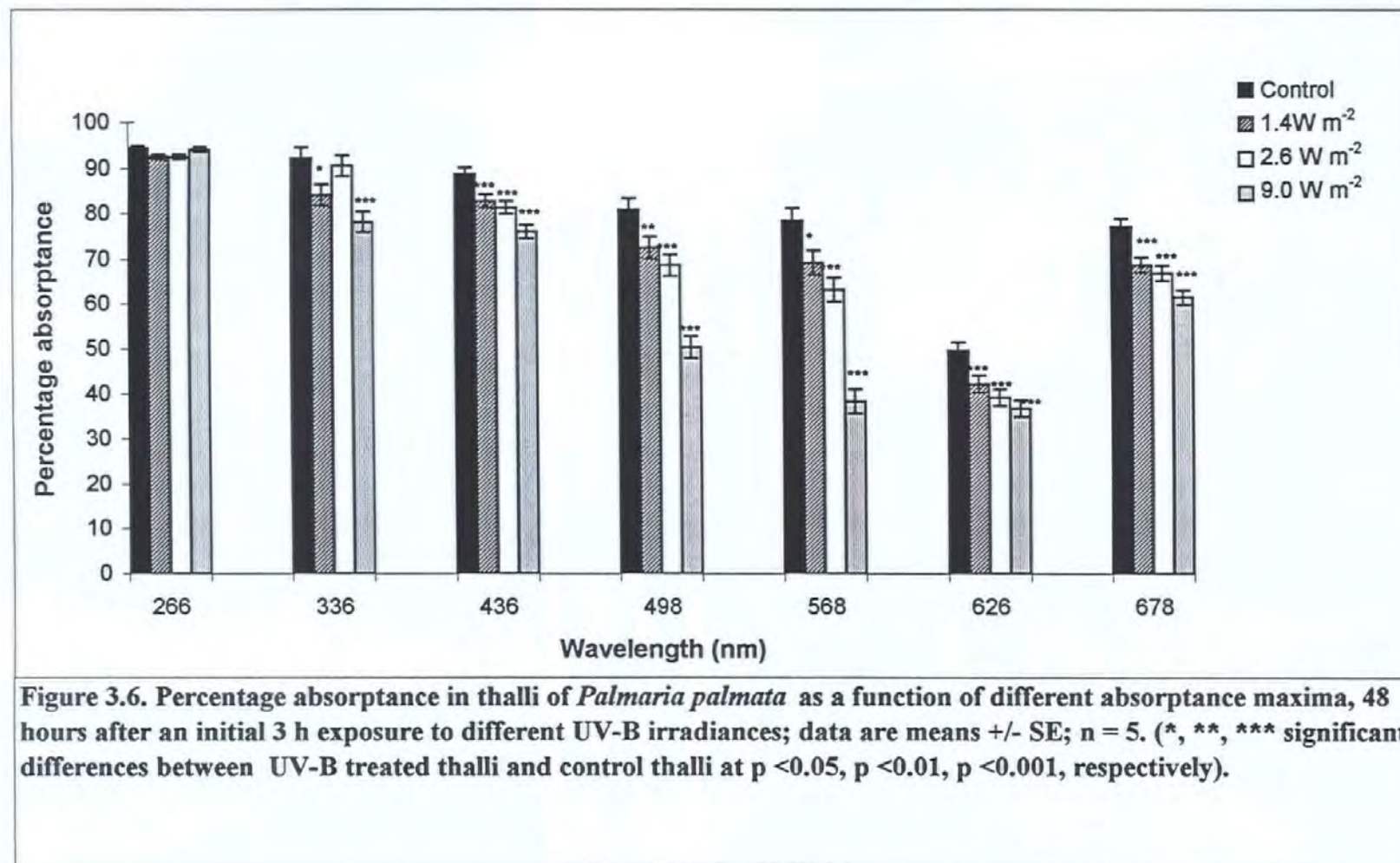
## Ion leakage measurements

A general decrease in Health Index compared to the control value was found for *P. palmata* 24 hours after exposure to relatively high UV-B irradiances (unweighted UV-B of 8.6 W m$^{-2}$), and 48 hours after exposure to unweighted UV-B irradiance of 3.6 W m$^{-2}$ and 8.6 W m$^{-2}$ (Table 3.1, p<0.001, ANOVA). No significant decrease in Health Index were found for *E. intestinalis*.

## Growth measurements

Highly significant reductions in growth rate, measured as % area increase of thallus, were found for *P. palmata* at 1.4 W m$^{-2}$ (unweighted dose of 15.1 kJ m$^{-2}$) and for *E. intestinalis* at 9.0 W m$^{-2}$ (unweighted dose of 97.2 kJ m$^{-2}$) compared to controls (fig. 3.7), (p<0.001, multiple regression). Growth data for *P. palmata* were analysed statistically until day 11, as the growth rate approached zero after this day. This plateau was thought to be due to the artificial growth conditions in the laboratory. Growth rate for the exponential equation for *P. palmata* control was $\alpha = 0.009$ as opposed to $\alpha = 0.005$ for the treated group (p<0.001). For *E. intestinalis*, the growth rate for the exponential equation control group was $\alpha = 0.018$ as opposed to $\alpha = 0.008$ for the treated group (p<0.001).

Table 3.1. *Enteromorpha intestinalis* and *Palmaria Palmata*. Ion leakage calculated as Health index for thalli 24 and 48 h after an initial 3 h exposure to different UV-B irradiances. ** indicate significant differences between UV-B treatments and control group (p < 0.001, n = 5).

| UV-B irradiance | Health index (%) | | |
|---|---|---|---|
| Unweighted | | | |
| (W m$^{-2}$) | Prior to exposure | 24 h after exposure | 48 h after exposure |
| *Enteromorpha intestinalis* | | | |
| Control | 51.9 | 50.4 | 47.2 |
| 2.6 | - | 55.9 | 53.6 |
| 3.6 | - | 57.0 | 54.6 |
| 8.6 | - | 56.5 | 52.3 |
| Dead thalli | 20.0 | | |
| | | | |
| *Palmaria palmata* | | | |
| Control | 86.9 | 84.8 | 86.1 |
| 2.6 | - | 80.7 | 83.0 |
| 3.6 | - | 80.8 | 73.5 (**) |
| 8.6 | - | 46.2 (**) | 53.2 (**) |
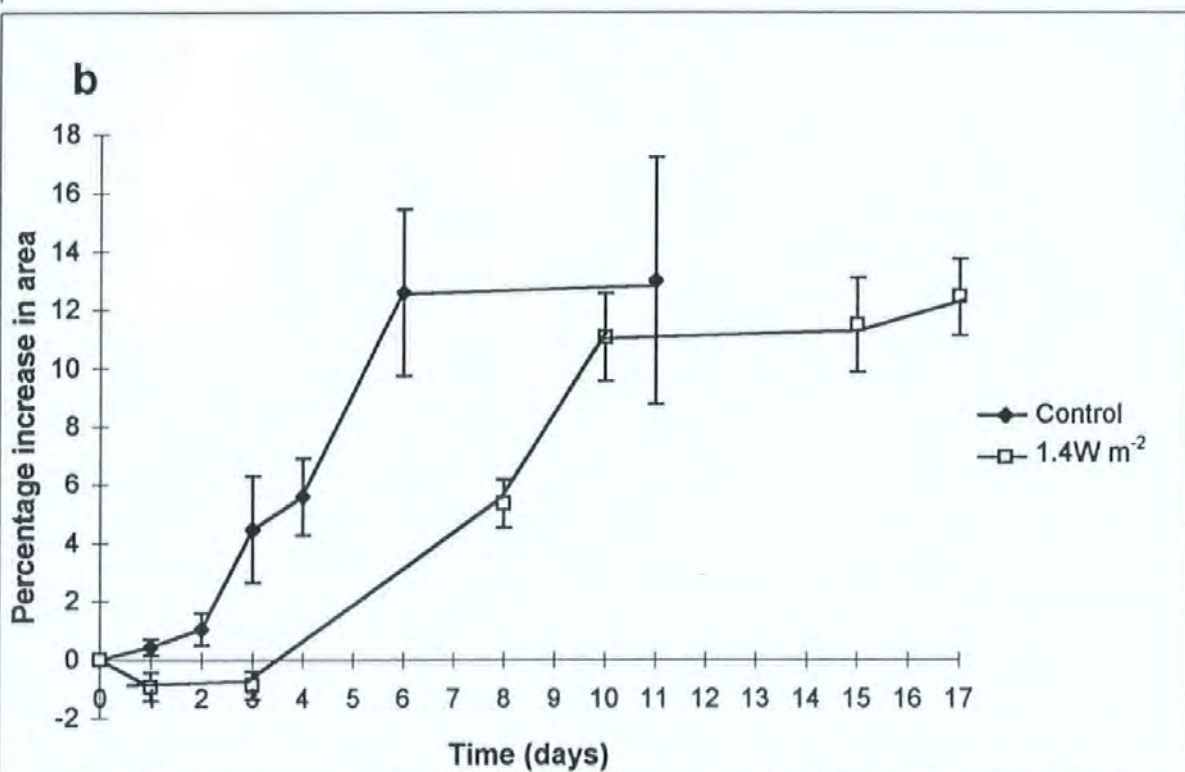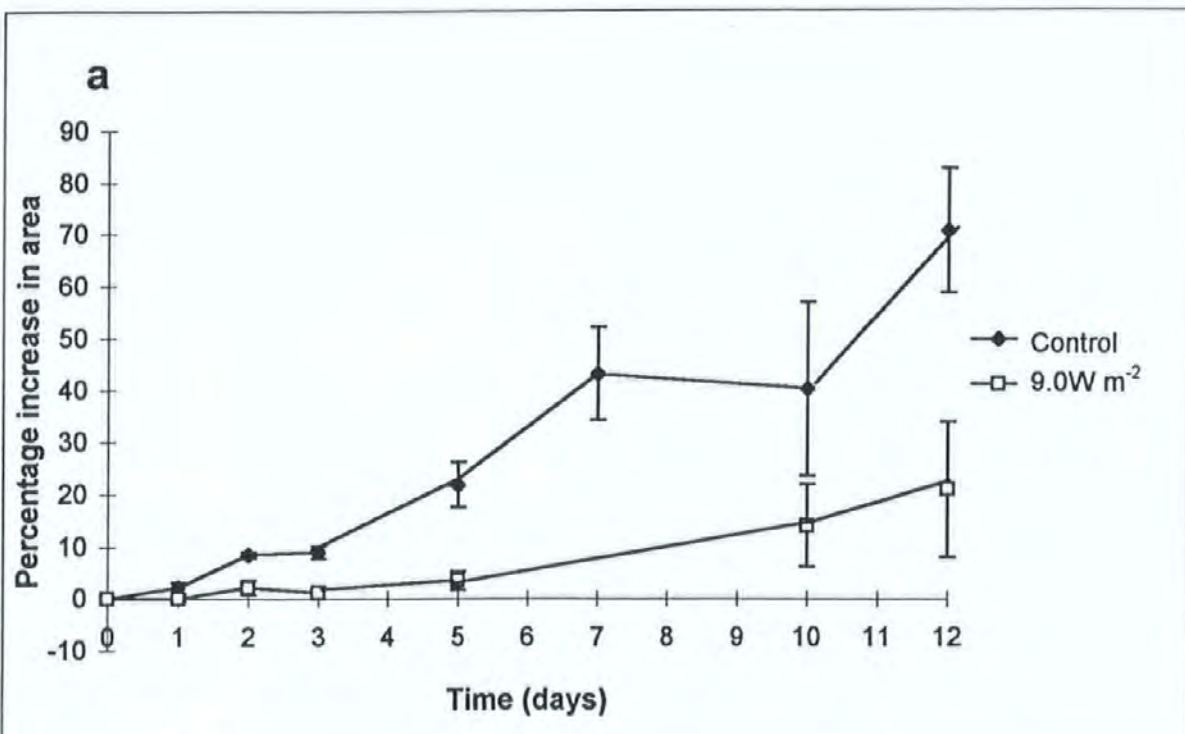
Figure 3.7. *Enteromorpha intestinalis* a) and *Palmaria palmata* b). Growth of mature thalli calculated as percentage area increase from day 0 measured over 12 and 17 d periods after an initial 3 h exposure to 9.0 or 1.4 W m$^{-2}$ (unweighted UV-B irradiance) respectively. Data are means +/- SE; n = 5.

# 3.5 DISCUSSION

It has been shown in this study that $F_v / F_m$ ratio is a sensitive general biomarker which responds in a dose-response dependent manner to exposure to UV-B (fig. 3.2). Furthermore, species-specific changes in $F_v / F_m$ ratio were found, with *P. palmata* exhibiting the greatest reductions (fig. 3.1). Such species-specific patterns have also been observed by others in response to UV (Dring *et al.* 1996a) and during photoinhibition by high irradiances of white light in green and red algae (Hanelt *et al.* 1992, Herrmann *et al.* 1995). In addition to visible light, solar UV radiation is a strong environmental stress factor which modifies the photosynthetic activity of both plants and algae. In several species of marine benthic algae and phytoplankton, UV-B exposure affects several targets in photosynthesis. UV-B impairs the D1 protein associated with photosystem II, which results in a decrease of the noncyclic photosynthetic electron transport (Renger *et al.* 1989, Tevini *et al.* 1989, Franklin and Forster 1997 and references therein). Furthermore, the water splitting site of Photosystem II, and the reaction centre of photosystem II can be damaged (Bhattacharjee and David 1987, Bhattacharjee *et al.* 1987). Herrmann *et al.* (1995) found decreased $F_v / F_m$ levels both in macroalgae exposed to solar PAR alone and in algae exposed to solar UV-B and PAR, and were therefore unable to isolate the cause for the decreased chlorophyll fluorescence. Moreover, Helbling *et al.* (1992) concluded that UV-A radiation accounted for over 50 % of the photoinhibition caused by total solar UV. However, in this study the linear relationship between time of exposure and $F_v / F_m$ values in the control group (low PAR and low UV-A) indicates that the decreases in the treated groups were due to UV-B exposure alone.

Björkman (1987) and Demming and Björkman (1987) reported that a decrease in $F_v / F_m$ ratio was linearly related to a decrease in the optimal quantum yield of photosynthesis. In this study, decreased $F_v / F_m$ and also $F_v$ (data not shown) with no recovery, and reduced growth rates clearly

showed irreversible damage to PSII in *P. palmata*. Similar results of decreased $F_v$ values in chloroplasts and leaves of higher plants have been reported (Björn *et al.* 1986, Renger *et al.* 1986). Trocine *et al.* (1981) found that gross photosynthesis is sensitive to UV-B, and Larkum and Wood (1993) concluded that $F_v$ may be used as an indicator of UV-B inhibition. This study confirms these results. In addition, the finding of decreased $F_v$ / $F_m$ after 1.5 hours exposure to an UV-B unweighted irradiance of 0.2 W m$^{-2}$ (equivalent to ambient irradiance at 12.00 in March) in *P. palmata* (fig. 3.2) clearly indicates the sensitivity of this parameter to UV-B.

Predictive dose dependent relationships between $F_v$ / $F_m$ and UV-B exposure were measured in both *P. palmata* and *E. intestinalis*. Photoinhibition which is a reversible process that only inactivates PS II for limited time periods, is also associated with decreases in optimal quantum yield (Franklin and Forster 1997). Photoinhibition can therefore be regarded as a compensatory response, a protective mechanism belonging to the compensatory zone (please see Section 1.3 for further details). Since chlorophyll fluorescence is known to respond to a wide variety of stressors, both natural (Henley *et al.* 1991, Herrmann *et al.* 1995) and xenobiotic (Conrad *et al.* 1993, Sgardelis *et al.* 1994), it can be characterised as a very sensitive general biomarker. Thus, chlorophyll fluorescence, together with recovery and changes in growth rates may offer a valuable early warning of solar UV induced, reversible departures from homeostasis or from the compensatory zone. The greater the response of $F_v$ / $F_m$, the further the macroalgae measured here had progressed along the health status curve. The inhibition in growth found for *P. palmata* following ambient UV-B levels confirm the detrimental effects of UV-B radiation. Similar results of inhibited growth were found by Grobe and Murphy (1994) in the intertidal alga *Ulva expansa* exposed to ambient and elevated UV-B radiation. Moreover, inverse linear relationships between growth rate and duration of UV-B irradiance were also demonstrated (Grobe and Murphy 1994). Since $F_v$ / $F_m$ for *E. intestinalis* showed full recovery 24 hours after exposure, although a depressed growth rate of 53.9 % was observed 12 days after exposure to 9.0

W m$^{-2}$ (fig. 3.7a), "endpoint effects" such as impairment of growth should be measured with chlorophyll fluorescence when assessing effects of UV exposure.

The weighted dose-response relationship between $F_v$ / $F_m$ and doses expressed relative to the maximum July dose calculated for Plymouth, clearly shows that increases in UV-B has a greater effect in terms of Quaite's weighting function compared to both the Erythemal and PAS 300 action spectra in *P. palmata* (fig. 3.4b). Thus, according to these calculations, it is this spectrum which predicts the most severe UV-B effects in $F_v$/ $F_m$ ratio. PAS 300 suggested the least severe effects in $F_v$/ $F_m$ of the three action spectra presented in fig. 3.4b. The large reductions in $F_v$/ $F_m$ to a value of 0.27, even after ambient UV-B radiation, using PAS 300, either indicate that a "flatter" action spectrum would be more appropriate (such low values have not been measured *in situ*, personal observations, Häder 1997b (fig. 9, p. 184)) or that reductions in $F_v$ / $F_m$ of this magnitude reflect natural variations, and increases in UV-B would therefore have little additional effect. Recently, establishing action spectra for aquatic phytoplankton have also been attempted (Cullen and Lesser 1991, Cullen *et al.* 1992). However, much more research is needed to identify the weighting functions existing between optimal quantum yield and UV-B in different macroalgal species. The results presented here confirm the conclusion made by Paul (1997) that the choice of action spectrum greatly affects both the percentage changes in biologically effective radiation for a given ozone depletion and the relationship between experimental doses and those occurring in the field.

Since establishing or identifying suitable weighing functions were not included in the overall objectives of the work presented here (please see Section 1.5), irradiances and doses will be weighted according to Caldwell's generalised plant response action spectrum throughout this work to permit general comparison with other work in the UV field.

## Ion leakage

The decreased Health Index for *P. palmata* after UV-B exposure (Table 3.1), indicates damage to the plasmalemma. However, since significant reductions were only detected after relatively high UV-B irradiances, membrane damage was not found to be a particularly sensitive parameter of UV-B exposure. Moreover, no decrease in Health Index was found for *E. intestinalis*. Although research into membrane damage caused by UV-B has been limited, it is known that the integrity of the membranes is affected by UV-B (Murphy 1983, Koch *et al.* 1995), and is due to a decrease in lipid content and damage to membrane transport systems. Although the cellular and physiological mechanisms responsible for membrane damage are uncertain, the results in this study are important for the identification of the mechanisms of UV-B exposure, especially in view of the lack of bibliographic information on the effects of UV-B on membranes.

## Pigment measurements

*P. palmata* exhibited a steady decline in what has been identified as chlorophyll *a* absorptance maxima, as well as phycoerythrin and/or carotenoid, phycoerythrobilin and phycocyanin absorptance with increasing UV-B irradiance (fig. 3.5 and 3.6) (please see also next chapter for further identification of chlorophylls and carotenoids). The loss of chlorophyll absorptance was detected to be slower than that of phycoerythrin and/or carotenoids and phycoerythrobilin indicating that the accessory pigments were impaired first. Döhler (1987) and Zündorf and Häder (1991) reported similar results with pigment changes in marine diatoms and cyanobacteria after UV exposures. This study shows dose-response dependent decreases in chlorophyll, phycoerythrin and/or carotenoid and phycoerythrobilin

absorptance with UV-B radiation, indicating that these pigments may be suitable as general biomarkers of exposure.

The presence of UV-B screening compounds in algae has been well documented since the early work of Sivalingam *et al.* (1974) and mycosporine-like amino acids have been identified as major screening compounds in algae, corals and a number of other animals (Dunlap and Chalker 1986, Karentz *et al.* 1991a). These shielding compounds are effective from 310 nm to 335 nm. Karentz and co-workers (1991a) demonstrated the existence of several of these compounds such as porphyra-334, shinorine, and glycine:valine, in *Palmaria decipiens*. The peak at 336 nm found in this study may indicate the existence of such compounds in *P. palmata*.

Several studies have recorded evidence of induced levels of mycosporine-like amino acids with decreasing depth in the water column (Sivalingam *et al.* 1974, Dunlap *et al.* 1986, Post and Larkum 1993). It is interesting to note the window found in pigment absorptance between 290 nm and 315 nm in *P. palmata* (approximately 75 % absorptance) as opposed to *E. intestinalis*, with 95 % absorptance in this region. This may be interpreted in terms of increased screening compounds in intertidal algae compared with subtidal algae. *E. intestinalis* showed no UV-B related pigment changes. The observed lack of UV-B-induced effects on the photosynthetic efficiency further confirms that algae from high illumination habitats develop protective mechanisms against the detrimental effects of UV-B. These results, together with the findings of other workers, of a site dependent induction in these compounds suggest a dose dependent induction to UV radiation. However, more research is necessary to establish such a relationship and to investigate the suitability of these compounds as specific biomarkers of UV exposure.

## 3.6 CONCLUSIONS

$F_v / F_m$ ratio exhibited species-specific changes and is a sensitive, non-specific general biomarker of UV-B exposure in *P. palmata*. Additionally, *in vivo* absorptances identified as being due to chlorophyll *a*, phycoerythrin and/or carotenoids, as well as phycoerythrobilin and phycocyanin decreased in a dose-response dependent manner to UV-B exposure. Reduction in these physiological parameters was associated with detrimental manifestations such as membrane damage and reduced growth rate in the subtidal *P. palmata*. To establish biomarkers of exposure, which can be used as early warning signals and which are related to biological effects, further research, outlined in the next chapter, sought to determine whether the physiological parameters used in this study follow the same predictive pattern after long term exposure to solar UV-B.

# CHAPTER 4

# THE EFFECTS OF UV-B EXPOSURE ON PIGMENT DISTRIBUTION IN MARINE MACROALGAE.

## 4.1 ABSTRACT

Investigations were carried out, by reverse phase high performance liquid chromatography (HPLC), into the effects of UV-B on the pigmentation of three species of macroalgae indigenous to the Southwest coast of England, *Enteromorpha intestinalis* (Chlorophyta), *Palmaria palmata* (Rhodophyta) and *Porphyra umbilicalis* (Rhodophyta). Identification and quantification of the principle signature pigments in the three macroalgal species were undertaken. The subtidal species *P. palmata* exhibited greatest sensitivity to UV-B in that concentrations of chlorophyll *a*, lutein and ß-carotene were highly responsive to increased UV-B exposure. Although not achieving statistical significance, changes in pigment concentrations were found in *P. umbilicalis* with a general trend of higher concentration with increasing UV-B irradiance. In contrast, *E. intestinalis* exhibited no significant response to increasing UV-B exposure. These observations were similar to results found in *in vivo* absorptance spectra in the previous chapter. It can, thus, be concluded that *in vivo* spectrophotometric analyses appear to provide reliable, non-invasive means to characterise subtle responses of macroalgae to UV-B exposure.

## 4.2 INTRODUCTION

To identify and quantify the chlorophyll and carotenoid pigments which in Chapter 3 were tentatively identified by *in vivo* absorptance spectra, UV-B experiments were performed with

three species of macroalgae using HPLC. *In vivo* spectral analyses can sensitively and rapidly characterise pigment diversity non-invasively, especially when pigment identities are known (Smith and Alberte 1994). The advantages of this method come from the fact that these spectra are independent of the need for corrections for hypsochromatic shifts which occur when the tissue is disrupted by extractions into organic solvent (Smith and Alberte 1994).

HPLC analyses have previously been used to characterise plant pigments from different classes of algae, as markers to determine sources of carbon input into aquatic ecosystems (Mantoura and Llewellyn 1983, Millie *et al.* 1993, Bianchi *et al.* 1997). Moreover, several studies have used HPLC to determine whether Mycosporine-like compounds MAAs can be related in a dose-dependent manner to UV-B exposure, and therefore potentially act as early warning signals of changes in UV-B levels (Wood 1989, Garcia-Pichel *et al.* 1993). HPLC analyses were also used in this study to further investigate whether chlorophyll and carotenoid pigment concentrations can be related in a dose-dependent manner to UV-B exposure in macroalgae.

The aims of this study were to:

a) identify and quantify different chlorophylls and carotenoids in *Palmaria palmata*, *Porphyra umbilicalis* and *Enteromorpha intestinalis* using high performance liquid chromatography (HPLC)

b) investigate the effects of short-term UV-B exposure on chlorophyll and carotenoid concentrations in these three species.

## 4.3 MATERIALS & METHODS

### Collection of algae

The intertidal algae *Enteromorpha intestinalis* (Chlorophyta) and *Porphyra umbilicalis* (Rhodophyta), in addition to the subtidal alga *Palmaria palmata* (Rhodophyta) were collected at low tide from Wembury beach in November (sea water temperature: 8 °C). Algal material was collected by cutting attached thalli at the base. The thalli were harvested 12 hours before the experiments. They were kept in filtered sea water at constant temperature (15 °C) and in low light conditions (40 μmol m$^{-2}$ s$^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF™) in growth cabinets. A 12:12h light:dark regime was maintained throughout the experiment. Nitrate (NO$_3^-$) and Phosphate (PO$_4^{3-}$) (5:1) were added to the sea water.

## UV treatments and experimental design

Thalli of *E. intestinalis* and *P. palmata* were cut to an approximate length of 30 mm and *P. umbilicalis* were cut into discs of 18 mm diameter. Three replicates were used for all species. The thalli were covered to a depth of 15 mm with filtered sea water and exposed to UV for a 3 h period in open Petri dishes. PAR and temperature conditions were identical to those in the acclimatisation period. The UV tubes used were identical to those in the previous chapters and measurements of UV and PAR were performed as in Chapter 3. UV-A was kept constant at 1.3 W m$^{-2}$, which is approximately 4 times less than that calculated at 50° latitude (Cornwall) in December (personal comm., Driscoll *et al.* 1992). This was held independent of the UV-B irradiance.

The following unweighted UV-B irradiances were used in the experiment: 0.5 and 1.8 W m$^{-2}$. These levels correspond to 0.14 and 0.49 W m$^{-2}$ weighted UV-B irradiance levels, and to 1.5, 5.34 kJ m$^{-2}$ weighted doses respectively. The weighted values were calculated according to the generalised plant response action spectrum (Caldwell 1971) normalised at 300 nm. The

weighted doses correspond to ambient doses in October / September and July respectively (Björn and Murphy 1985). However, since the experiments were performed in November these doses were relatively high. The weighted doses were calculated for Plymouth (at an air pressure of 1000 mbar, relative humidity of 0.5 and aerosol level of 0) during clear sky conditions (Björn and Murphy 1985).

## Algal conditions following UV exposure

Following the 3 h UV exposure the algae were transferred to growth cabinets (PAR and temperature conditions identical to those in the acclimatisation period) for a 24 h period. The samples were then frozen in liquid nitrogen and stored -80 °C until pigment analysis.

## HPLC analysis

Algal tissues were ground to a powder using a pestle and mortar with liquid nitrogen. A known weight of powdered sample was transferred to a centrifuge tube and chlorophyll and carotenoid pigments were extracted from the sample using 90 % acetone with the aid of ultrasonication. Extracts were centrifuged to remove debris and then analysed for a range of chlorophylls, carotenoids and pheopigments by reverse phase HPLC (Gibb et al. 1998) as follows:

Extracts were held at 2 °C in an autosampler unit, and vortex mixed with ammonium acetate buffer (1:1 v/v) before injection. Pigments were separated on a C-8 column using a binary mobile phase system with linear gradient (methanol / 1.0 M ammonium acetate; 100 % methanol; Barlow et al. 1997). Pigments and pheopigments were detected by absorbance at 440 nm and 667 nm respectively using diode array detection. Pigment identity was secured by co-elution with authentic pigments (VKI, Denmark) and confirmed through spectral

correlation with standard UV-visible spectra (300-750 nm). Pigments were quantified with respect to a *Canthaxanthin* internal standard *via* relative response factors, whilst pheopigments were quantified using response ratios.

## 4.4 RESULTS

The rhodophyte *P. palmata* was characterised by a pigment signature of chlorophyll *a* (CHL*a*), lutein (LUT) and ß-carotene (CAR) and concentrations of these pigments were highly responsive to UV-B exposure (Table 4.1). Significant decreases in the concentration of chlorophyll *a* were observed after the 3 h exposure to both UV-B irradiances (Table 4.1). CHL*a*, LUT and CAR decreased to 56, 53 and 38 % of their respective concentrations in the control samples after exposure to the highest UV-B irradiance ($1.8 \ W \ m^{-2}$).

Like *P. palmata* CHL*a*, lutein and ß-carotene were also the principle signature pigments in *Porphyra umbilicalis* (Rhodophyta). Although no significant changes in pigment concentrations were found, CHL*a*, lutein and ß-carotene all showed a general trend of higher concentration with increasing UV-B irradiance in this species (ANOVA, $p > 0.2$). Concentrations of CHL*a*, LUT and CAR showed increases to 147, 168 and 150 % their respective concentrations in the control samples (Table 4.1).

The chlorophyte *Enteromorpha intestinalis* exhibited chlorophyll *a* (CHL*a*), chlorophyll *b* (CHL*b*), violoxanthin (VIO), lutein and ß-carotene as principle pigments. Concentrations of these pigments showed no significant response to increasing UV-B exposure (ANOVA, $p > 0.2$), (Table 4.1).

Table 4.1. Chlorophyll and Carotenoid concentrations in three species of marine macroalgae (Concentrations in ng mg$^{-1}$)

| | UV-B exposure (W m$^{-2}$) | Chlorophyll $a$ (CHL$a$) | Chlorophyll $b$ (CHL$b$) | Violaxan-thin (VIO) | Zeaxanthin (ZEA) | Lutein (LUT) | ß-Carotene (CAR) | Phaeophytin a |
|---|---|---|---|---|---|---|---|---|
| *P. palmata* | Control | 1585.0 a | - | 0.3 | 0.1 | 216.5 a | 120.4 a | 9.0 a |
| | 0.5 | 1187.2 b | - | 0.1 | 0.0 | 159.1 a | 94.2 a | 4.2 b |
| | 1.8 | 889.4 c | - | 0.0 | 0.0 | 113.7 b | 45.9 b | 2.3 b |
| *P. umbilicalis* | Control | 1644.9 | - | 0.2 | 26.3 | 196.3 | 98.5 | 7.8 |
| | 0.5 | 1910.0 | - | 0.5 | 37.2 | 256.4 | 117.9 | 7.4 |
| | 1.8 | 2428.2 | - | 0.3 | 44.8 | 330.4 | 147.5 | 7.7 |
| *E. intestinalis* | Control | 1263.6 | 531.8 | 63.6 | 2.3 | 120.6 | 44.2 | 4.8 |
| | 0.5 | 922.6 | 392.8 | 55.0 | 2.2 | 95.2 | 35.9 | 3.9 |
| | 1.8 | 1014.2 | 453.2 | 64.4 | 2.8 | 111.7 | 33.9 | 4.3 |

UV-B irradiances are equivalent to weighted doses of 1.5 and 5.3 kJ m$^{-2}$ respectively, calculated according to the generalised plant action spectrum. These weighted doses correspond to ambient doses in October/September and July respectively (Björn and Murphy 1985). Different letters indicate a significant difference in pigment concentration; ANOVA, $p < 0.05$.

Table 4.2. Concentrations of photosynthetic carotenoids (PSC = peridinin, 19'-butanoyloxyfucoxanthin, fucoxanthin, 19'-hexanoyloxyfucoxanthin and prasinoxanthin) and photoprotectant carotenoids (PPC = diadinoxanthin, alloxanthin, zeaxanthin and CAR) in UV-B exposure studies of three macroalgae species.

| | UV-B exposure (W m$^{-2}$) | Total photosynthetic carotenoids (PSC; ng mg$^{-1}$) | Total photoprotective Carotenoids (PPC; ng mg$^{-1}$) | PPC / (PPC + PSC) Photo. car / total car |
|---|---|---|---|---|
| *P. palmata* | Control | 1.7 | 122.4  a | 0.01 |
| | 0.5 | 0.2 | 95.7  a | 0.00 |
| | 1.8 | 0.5 | 47.2  b | 0.01 |
| *P. umbilicalis* | Control | 3.0 | 128.2 | 0.02 |
| | 0.5 | 4.6 | 161.6 | 0.03 |
| | 1.8 | 5.1 | 200.1 | 0.02 |
| *E. intestinalis* | Control | 18.8 | 48.7 | 0.28 |
| | 0.5 | 14.9 | 39.3 | 0.28 |
| | 1.8 | 14.1 | 38.3 | 0.27 |

Different letters indicate a significant difference in pigment concentration; ANOVA, $p < 0.05$.

In Table 4.2 significant decreases in Bidegare total photoprotecting carotenoids and and photosynthetic carotenoids were found for *P. palmata*. The greatest decreases occurred after exposure to the highest UV-B irradiance (ANOVA, $p<0.05$).

## 4.5 DISCUSSION

The general decrease in pigment concentration with increasing UV-B exposure found for *P. palmata* was consistent with the findings in Chapter 3 of an exposure dependent decrease in *in vivo* thallus absorptance (Cordi *et al.* 1997). Furthermore, these decreases were also associated with reductions in chlorophyll fluorescence and growth rate (Chapter 3). Döhler (1987) and Zündorf and Häder (1991) reported similar changes in pigment concentration in marine diatoms and cyanobacteria after UV exposure. The general trend observed for *P. umbilicalis* of increasing concentrations of both chlorophylls and carotenoids following UV-B exposure indicates that this intertidal red macroalga is well adapted to cope with short-term exposure to UV-B irradiance.

The carotenoids determined using HPLC may either be classified as photosynthetically active or photoprotecting species. Photosynthetic carotenoids (PSCs) absorb light energy and transfer it to chlorophyll (possibly via the Dexter exchange mechanism, S. Gibb, pers. comm.). Photoprotecting carotenoids (PPC), on the other hand screen the organism against stressful high light conditions. These carotenoids are capable of quenching excited radicals, and converting their excess energy to heat and dissipating it harmlessly. Thus, the ratio of PSC and PPC may be used to provide some additional information of the light stress placed on the algae.

Although a number of different operational definitions exist for groupings of photosynthetic and photoprotectant pigments, those proposed by Bidegare *et al.* (1990) were adopted here, i.e. the PSCs include peridinin, 19'-butanoyloxyfucoxanthin, fucoxanthin, 19'-hexanoyloxyfucoxanthin and prasinoxanthin, whilst the PPCs include diadinoxanthin, alloxanthin, zeaxanthin and CAR.

Concentrations of both PSC and PPC increased in *P. umbilicalis* in response to UV-B exposure, decreased in *P. palmata* and remained stable in *E. intestinalis* (Table 4.2). In each case, no significant changes were found in the ratio of PSC or PPC to total carotenoid concentration (PSC + PPC). However, the algal carotenoids referred to above absorb in the 350 - 500 nm region of the spectrum. It is thus apparent that PPCs, which absorb excess PAR, offer no protection against UV-B (280-315 nm) and are not synthesised in response to UV-B. Hence, due to the constant exposure of the macroalgae in this study to light in the spectral band 350 - 500 nm the stability of the ratio of PSC and PPC may be expected (Table 4.2). The inference of these two observations is that macroalgae are capable of selective synthesis of photoprotecting pigments i.e. stressful light conditions do not trigger activation of all photoprotection mechanisms.

Since the HPLC method used here can not resolve water soluble phycobilin pigments, such as phycoerythrin in rhodophytes, this method should be used in conjunction with other methods such as *in vivo* spectrophotometry. A suitable HPLC methodology for separation and quantification of phycobilins from natural assemblages does not exist at present (Swanson and Glazer 1990, Bianchi *et al.* 1997).

A group of UV absorbing compounds, the mycosporine-like amino acids (MAAs), have been detected in various organisms including macroalgae. These compounds have a strong

absorption maxima in the range 310 - 360 nm, which corresponds to the wavelength of UV-B, and they appear to offer protection against harmful UV-B dosages received by cells (Garcia-Pichel *et al.* 1993), thus reducing physiological damage. Although, not studied in this work, it may be postulated that concentrations of UV absorbing compounds would have increased significantly in response to UV-B.

## 4.6 CONCLUSIONS

Identification and quantification of the principle signature pigments in three species of macroalgae were achieved. The results from this study provide useful preliminary insights into the differing effects of UV-B on pigment concentrations in macroalgae. The subtidal species *P. palmata* exhibited greatest sensitivity to UV-B, and although no significant changes in pigment concentrations were found in *P. umbilicalis* a general trend of higher concentration with increasing UV-B irradiance was observed. In contrast, *E. intestinalis* exhibited no significant response to increasing UV-B exposure. These observations were similar to results found in *in vivo* absorptance spectra in the previous chapter. With confirmation provided by these HPLC studies, it can be concluded that *in vivo* spectrophotometric analyses from the previous chapter provided reliable, non-invasive means to characterise subtle responses of macroalgae to UV-B exposure. By investigating both photosynthetic pigments and MAAs simultaneously using *in vivo* spectrophotometry and HPLC, it is possible to further our understanding of the response of algae to UV radiation.

# CHAPTER 5

To investigate UV-induced responses at the molecular level, DNA damage (measured as Random Amplification Polymorphic DNA fingerprinting) and the cellular stress response were examined. These parameters were linked to inhibitions in growth rate to assess subsequent adverse effects. The induction of heat shock 70 (HSP 70) which belongs to the group of functional biomarkers was measured as a cellular stress response.

## 5.1 COMPARISON OF UV-INDUCED GENOTOXICITY DETECTED BY RANDOM AMPLIFICATION POLYMORPHIC DNA FINGERPRINTING WITH PHYSIOLOGICAL AND FITNESS PARAMETERS IN *PALMARIA PALMATA*.

### 5.1.1 ABSTRACT

The Random Amplification Polymorphic DNA (RAPD) technique was used to detect DNA damage in the subtidal macroalga *Palmaria palmata* (Rhodophyta) exposed to both ambient and elevated irradiances of UV-B. To investigate the potential of this method in ecotoxicological assessments, the qualitative and quantitative modifications in RAPD profiles were compared with changes in a number of physiological and fitness parameters. RAPD detectable modifications in DNA profiles were observed in all UV exposed individuals compared to controls. Changes in RAPD profiles, chlorophyll fluorescence ($F_v$ / $F_m$ ratio), *in vivo* pigment absorptance and thallus growth, examined simultaneously, provided a sensitive measure of UV-induced toxicity. The application of the RAPD DNA profiling technique, in

conjunction with other suitable physiological and fitness measurements, may prove a valuable tool for investigating the effects of genotoxicity upon marine algal populations. Ultimately, this method may allow the ecotoxicological examination of the link between molecular alterations and measurable adverse effects at higher levels of biological organisation.

## 5.1.2 INTRODUCTION

The primary goal of ecogenotoxicology (Wurgler and Kramers 1992), is to understand the consequences of genotoxicity in individuals for population and community structures (Anderson and Wild 1994, Depledge 1998). Altered fertility, growth, and embryonic survival are ecologically significant, because they reduce reproductive success and thus alter population size or structure (Anderson et al. 1994 Sadinski et al. 1995). Surprisingly, little has been done to explore the reproductive and developmental effects which may result from genotoxic responses. In addition, the simultaneous use of several molecular and physiological responses to detect UV-B induced biological effects, has rarely been attempted.

In the field of genotoxicity, advances in molecular biology have led to the development of a number of selective and sensitive assays for DNA analysis (Ionov et al. 1993). A promising technique is the Random Amplification Polymorphic DNA (RAPD), a semiquantitative method, which has previously been used for genetic mapping, taxonomy, phylogeny (Welsh et al. 1991) and detection of various kinds of DNA damage (Kubota et al. 1992, Peinado et al. 1992, Ionov et al. 1993, Savva 1996, Shimada and Shima 1998), please see Section 1.3.2.4 for further details.

One of the potential applications of the RAPD method is its use in the detection of UV-induced genetic damage. This is a topic of particular concern because ozone depletion, and

the associated higher levels of UV-B radiation, have the potential to adversely affect marine biota (Häder *et al.* 1995). Although the effects of UV irradiance on genetic material (Stapleton *et al.* 1997), physiological parameters (e.g. photosynthesis, chlorophyll fluorescence, reviewed by Franklin and Forster 1997) and decreased growth rates (Grobe and Murphy 1994) have been studied separately, there have been only few attempts to explore the potential consequences of UV-induced DNA damage on the physiology and Darwinian fitness of exposed algal populations.

The objectives of this study, therefore, were to:

a) further evaluate the suitability of the RAPD method to detect UV-induced DNA damage in the marine macroalga *Palmaria palmata*.

b) investigate the relationship between molecular, physiological and fitness parameters following UV exposure.

## 5.1.3 MATERIALS & METHODS

The DNA analysis in this experiment was conducted simultaneously with the experiment described in Chapter 3 and the experimental conditions and UV exposures were identical. Thus, to investigate the relationship between biomarkers from different levels of biological organisation, an overview is presented in this chapter of the physiological and fitness data described in detail in Chapter 3.

### Sampling of macroalgae

*Palmaria palmata* plants were collected at low tide from Wembury beach, Devon, between May and June 1997 (sea water temperature: 13°C). The thalli were harvested 24 hours before the experiments and kept in filtered sea water at constant temperature (15°C) and in low light conditions (25 $\mu$mol m$^{-2}$ s$^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF™) in growth cabinets.

## UV treatments

Thalli were cut to a length of 30 mm and covered with filtered sea water to a depth of 1.5 cm per Petri dish. Each dish was exposed to varying UV irradiance for 3 hours (n=4 per dish). PAR and temperature conditions were identical to those in the acclimatisation period. PAR was measured with a PAR meter (Sky Instruments). UV-B was supplied by two UV-B tubes (Philips TL' 20W/12 RS™) and UV-A by two UV-A tubes (Philips 1609™ 15 W). A constant UV-A irradiance of 1.3 W m$^{-2}$ was included in all UV-B treatments. The total irradiance of both UV-A and UV-B tubes was measured as described in Chapter 3. Irradiances and doses used in the experiments are listed in Table 5.1. The weighted UV-B doses were calculated according to the generalised plant response action spectrum normalised at 300 nm (Caldwell 1971). The corresponding percentage ozone depletion was calculated for 15.07.97 at Plymouth during clear sky conditions (Björn and Murphy 1985). The relatively high UV-B irradiances were chosen to investigate the entire range of the exposure-response curves.

Table 5.1. UV irradiances and doses used in the experiment. The doses were calculated for a 3 h exposure and weighted UV-B doses were calculated according to the generalized plant response action spectrum normalized at 300 nm (Caldwell 1971). The corresponding percentage ozone depletion was calculated using the computer model of Björn and Murphy (1985).

| UV-B irradiance ($W\ m^{-2}$) | 0 | 1.4 | 2.6 | 9.0 | 12.7 |
|---|---|---|---|---|---|
| Unweighted UV-B dose ($kJ\ m^{-2}$) | 0 | 15.1 | 28.1 | 97.2 | 137.2 |
| Weighted UV-B dose ($kJ\ m^{-2}$) | 0 | 4.3 | 7.9 | 27.4 | 38.7 |
| Calculated ozone depletion (%) | 0 | Ambient August | 17 | 41 | 100 |

## DNA isolation and RAPD reactions

Approximately 0.1 g of thalli from individual samples was frozen in liquid nitrogen, ground with a mortar and pestle, and homogenised in 400 µl of sperm lysis buffer (100 mM Tris-HCl pH 8, 500 mM NaCl, 10 mM EDTA, 1 % SDS, 2 % mercaptoethanol). The DNA was treated with RNase (40 µg, 1.5 h at 37°C) and extracted twice with phenol (pH 8), followed by an extraction with chloroform:isoamyol alcohol (1:1). DNA was precipitated from the aqueous phase with 0.1 volume sodium acetate (pH 4.8) and 2 volumes of cold ethanol at -80°C for 6 hours. Precipitated DNA was harvested by centrifugation, air dried, and the final pellet was then dissolved in sterile analytical grade water. The amount and quality of DNA was estimated using agarose gel electrophoresis. All chemicals used throughout the described procedure were obtained from Sigma™.

DNA profiles were generated in RAPD reactions performed in a reaction volume of 25 µl as described previously (Atienzar *et al.* 1998). The decamer oligonucleotides OPA9 (GGGTAACGCC) or OPB1 (GTTTCGCTCC), or OPB14 (TCCGCTCTGG), or OPB 17 (AGGGAACGAG) (sequences given from 5' to 3') were obtained from Operon Technologies Inc. (Oswell), Southampton, UK. Approximately 10 ng of genomic DNA was subjected to RAPD amplification with a primer concentration of 2 µM, a deoxy-trinucleotide phosphate (dNTP) concentration of 0.33 mM, and a $MgCl_2$ concentration of 5.11 mM. This was performed in the presence of 2.8 units of <u>Taq</u> DNA polymerase and 1X reaction buffer (10 mM Tris-Cl, pH 8.3; 50 mM KCl, 0.1% Triton-X 100, 0.01% gelatin, 2.5 µg bovine serum albumin (IGi, Sunderland, UK)). Thermal cycling parameters consisted of 5 min denaturation (95 °C) followed by 40 cycles of 1 min denaturation (95 °C), 1 min annealing at 50 °C, and 1 min extension (74 °C). Reaction mixtures were stored at 4 °C prior to use.

**Agarose gel electrophoresis and analysis of DNA profiles**

Electrophoresis of RAPD reaction products was performed in 1.2% (w/v) agarose, using a Tris-Borate-EDTA buffer system (1X TBE= 90 mM tris-base, 90 mM boric acid and 2 mM EDTA). Amplified DNA was mixed with 1/5th volume of gel loading buffer (analytical grade water containing 25 % ficoll, 0.25 % bromophenol blue, and 0.25 % xylene cyanol). 15µl of this solution was then loaded onto the agarose gel. A DNA molecular size marker (M = 1Kb marker, Gibco BRL (Paisley, UK)) was run for each agarose gel. Bands visualised, from top to bottom, were: 3054, 2036, 1636, 1018, 522, 517, 506, 396, 344, and 298 bp. DNA samples were subjected to electrophoresis at 80 volts for 7 hours, after which the gels were stained in a 1X TBE solution containing ethidium bromide (0.015% v/v) for a period of minimum 40 min. Gels were photographed under UV illumination using a Polaroid camera (CU-5). Images of each gel were also captured using a Kodak DC40 digital camera with the DNA profiles. Changes in band intensity were subsequently analysed using Kodak Digital Science™ 1 D Image Analysis Software.

**Estimation of genomic template stability**

Each change observed in RAPD profiles (i.e. appearance of new bands, disappearance of bands and variation in band intensity compared to control RAPD profiles) was given the arbitrary score of +1. The average value was then calculated for each experimental group exposed to UV. The template genomic stability (%) was calculated as 100-(100a/n) where a is the average number of changes in DNA profiles, and n is the number of bands detected in control DNA profiles.

**Statistical analysis and transformation of data**

To compare the sensitivity of each parameter (genomic template stability, chlorophyll fluorescence ($F_v$ / $F_m$), and growth rate), a change in these values was calculated as a percentage of the control value (set at 100 %). Changes in RAPD profiles were statistically tested by performing one way analysis of variance, ANOVA. The LSD (least significant differences) test was performed to reveal statistical differences. The statistical analyses applied to physiological and fitness parameters have previously been described in Chapter 3.

## 5.1.4 RESULTS

## Measurements of physiological and fitness parameters

The effects of UV exposure on $F_v$ / $F_m$ ratio, *in vivo* absorptance spectra, and growth rates of *P. palmata* are recorded in Table 5.2 (please see Chapter 3 for a more detailed presentation of the results). Reduced levels of *in vivo* absorptance and chlorophyll fluorescence were measured with increasing UV-B irradiance. After a recovery period of 48 hours, only thalli exposed to UV-A or the lowest UV-B irradiance (1.4 W $m^{-2}$) exhibited increases in chlorophyll fluorescence, suggesting complete and partial recovery respectively. No reductions in growth rate, measured over an 11 day period, were found after exposure to UV-A. Thalli exposed to 1.4 W $m^{-2}$ unweighted UV-B, however, suffered a 40 % reduction in growth rate compared to controls. Thalli exposed to higher UV-B levels were unable to grow. Thus, these physiological data suggested irreversible damage following exposure to 2.6 W $m^{-2}$ (equivalent to 17 % reduction in atmospheric ozone levels in July). However, these results may be influenced by the sampling time, due to seasonal variations in the sensitivity of algae to UV exposure. Please see Chapter 3 for a more detailed account of these results.

Table 5.2. Physiological and fitness parameters measured after exposure of *Palmaria palmata* to varying UV irradiances. ↓: little decrease, ↓↓: medium decrease, ↓↓↓: high decrease. For details, please see Chapter 3).

| Time after UV exposure (hours) | Physiological and fitness parameters | UV irradiance (W m$^{-2}$) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Control | UV A | | | UV B | |
| | | | 1.3 | 1.4 | 2.6 | 9.0 | 12.7 |
| 0 | Chlorophyll fluorescence, $F_v / F_m$ ratio (%) | 100 | 80 | 42 | 45 | 2 | 3 |
| 24 | Recovery experiment Chlorophyll fluorescence, $F_v / F_m$ ratio (%) | 100 | 90 | 55 | 35 | 0 | 0 |
| 48 | Recovery experiment Chlorophyll fluorescence (%) $F_v / F_m$ ratio | 100 | 100 | 60 | 5 | 0 | 0 |
| 48 | *In vivo* thallus absorptance | 100 | 100 | ↓ | ↓↓ | ↓↓↓ | |
| 264 | Growth rate (%) | 100 | 100 | 60 | 0 | 0 | 0 |

**A)**

amb UV-A 1.4  2.6  9.0

M - a b c d e f g h i j k l m n o p q r M

**B)**

M - a b c d e f g h i j k l m n o p q r M

**C)**

M - a b c d e f g h i j k l m n o p q r M

**D)**

M - a b c d e f g h i j k l m n o p q r M
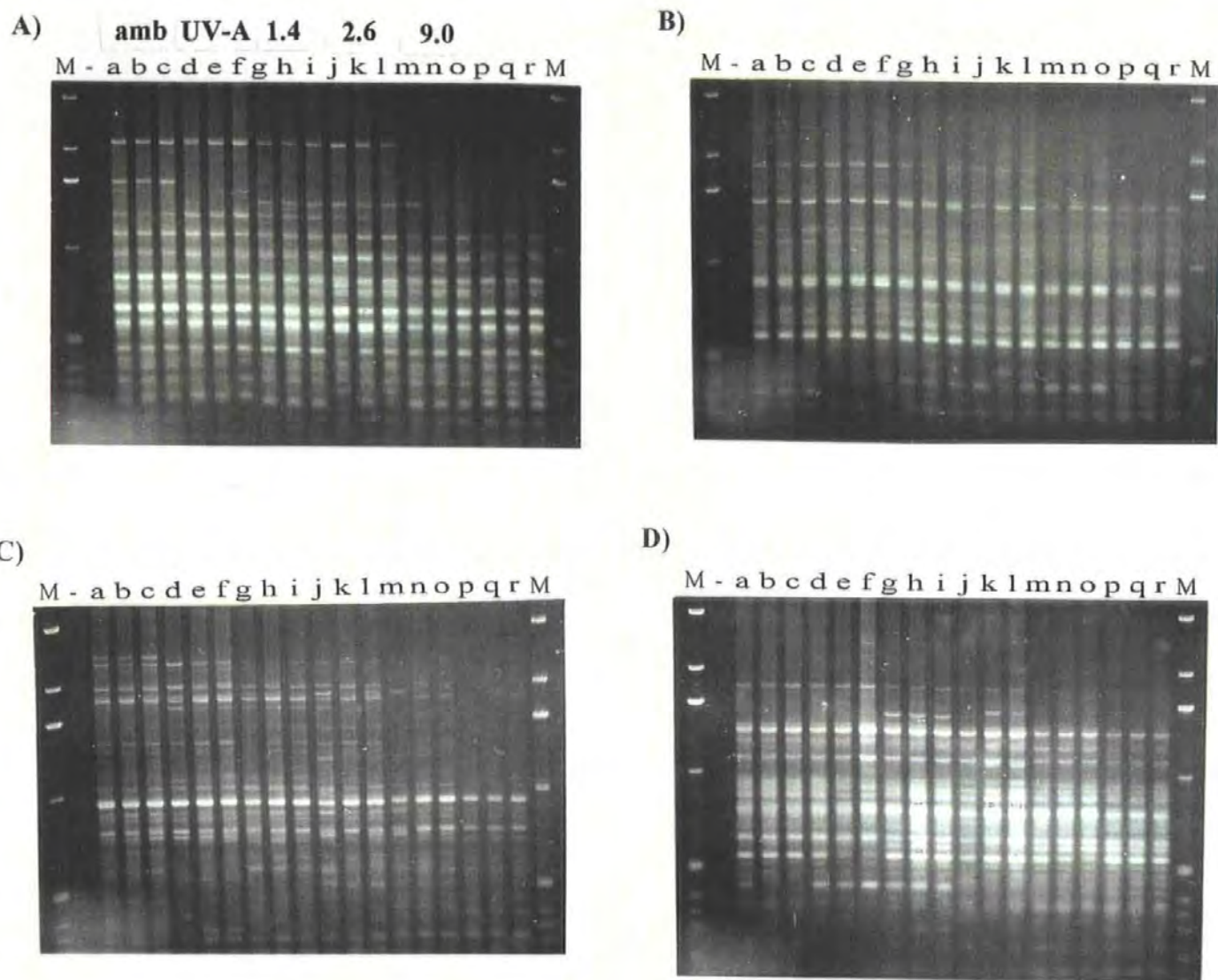
Figure 5.1. RAPD profiles of genomic DNA from *Palmaria palmata* exposed to varying UV irradiances. M = DNA molecular size marker (1 Kb ladder, BRL; see Material & Methods), - = no DNA control. RAPD reactions were performed using oligonucleotide primers OPA9 (A), OPB1 (B), OPB14 (C), OPB17 (D). Each small letter represents an individual alga. Lanes a-r : algae exposed to varying UV irradiances; a-c: ambient UV levels (1) (control alga collected directly from the field site), d-f: 1 + 1.3 W m$^{-2}$ UV-A (2), g-i:  2 + UV-B (1.4 W m$^{-2}$), j-l: 2 + UV B (2.6 W m$^{-2}$), m-o: 2 + UV B (9.0 W m$^{-2}$), p-r: 2 + UV B (12.7 W m$^{-2}$ ). The RAPD and thermocycling conditions are described in Material & Methods.

## RAPD DNA profiling

For all oligonucleotide primers tested, RAPD patterns generated by UV-exposed algae were different from those obtained using control DNA (fig. 5.1). Moreover, DNA patterns generated by each treatment group were reproducible, although each RAPD profile was obtained from individual algae. The principal events observed following UV exposure were variations in band intensity, as well as disappearance and appearance of new bands (fig. 5.1). Table 5.3 and fig. 5.2 present a summary of these RAPD profile modifications. The decrease in band intensity was particularly obvious for algae exposed to the two highest UV-B irradiances (9.0 and 12.7 W m$^{-2}$). In contrast, an increase in band intensity occurred for the three lowest UV irradiances (UV-A and UV-B irradiances of 1.4 and 2.6 W m$^{-2}$) (fig. 5.2). Further experiments showed that variations in band intensity were not caused by variations in the concentration of template DNA (data not shown). As a master mix was performed, no variations in the PCR reagent concentration (e.g. Taq DNA polymerase) were possible. In Table 5.3 it is shown that the number of disappearing bands, which occurred for all UV irradiances, was greater at higher UV-B irradiance. Extra bands appeared for the three lowest UV irradiances (UV-A, UV-B irradiances of 1.4 and 2.6 W m$^{-2}$), but rarely for 9.0 W m$^{-2}$, and never for 12.4 W m$^{-2}$. Additionally, only bands of molecular size greater than 1Kb were shown to disappear.

## Comparison of chlorophyll fluorescence, growth parameters and RAPD profiles.

In fig. 5.3 the genomic template stability, a qualitative measure reflecting changes in RAPD patterns, was used to compare the modifications in RAPD profiles with reductions in
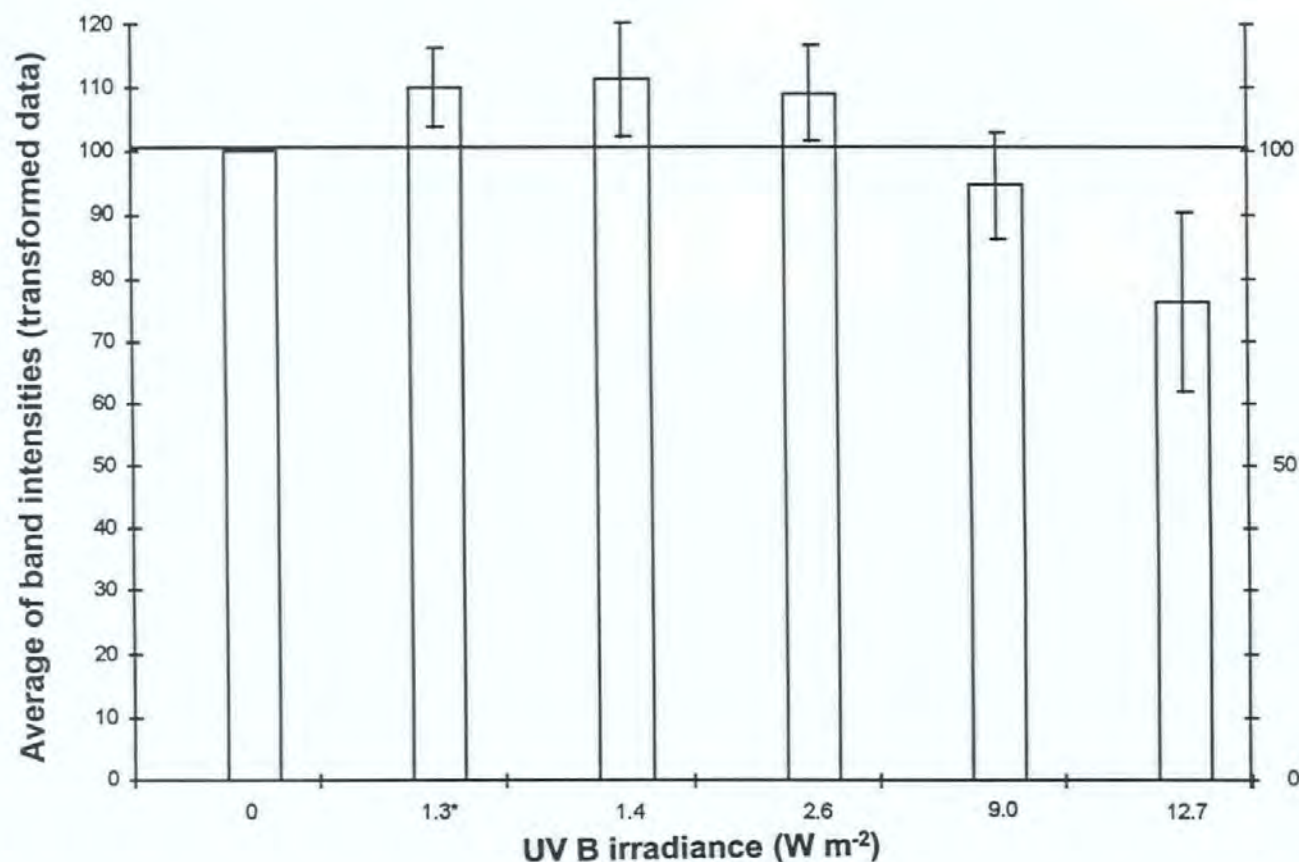
Figure 5.2. Variation of band intensities selected from RAPD profiles of genomic DNA from *Palmaria palmata* exposed to varying UV irradiances. A total of 10 bands appearing across the width of the gels were chosen from the RAPD profiles presented in Fig. 5.1 a, b, c, and d. The band intensity of controls was given the arbitrary score of 100% and band intensities obtained from UV-exposed algae were expressed in % of the control value. Average and standard deviation, SD, were calculated from the 10 selected bands. * indicate that the algae was irradiated with UV A only (in W m⁻²).

Table 5.3. RAPD profile changes after UV exposure of *Palmaria palmata*. ++++: major events, +++: high frequency event, ++: medium frequency event, +(+): between medium and low frequency event, +: low frequency event, +/-: very rare event, and -: absence of the event.

| Changes in RAPD profiles compared with controls | UV irradiance (W m$^{-2}$) | | | | |
| --- | --- | --- | --- | --- | --- |
| | UV-A | UV-B | | | |
| | 1.3 | 1.4 | 2.6 | 9.0 | 12.7 |
| Appearance of bands | + | +(+) | +(+) | +/- | - |
| Disappearance of bands | + | + | + | ++ | +++ |
| Decrease in band intensities | +/- | + | + | ++++ | ++++ |
| Increase in band intensities | ++++ | ++++ | ++++ | + | - |
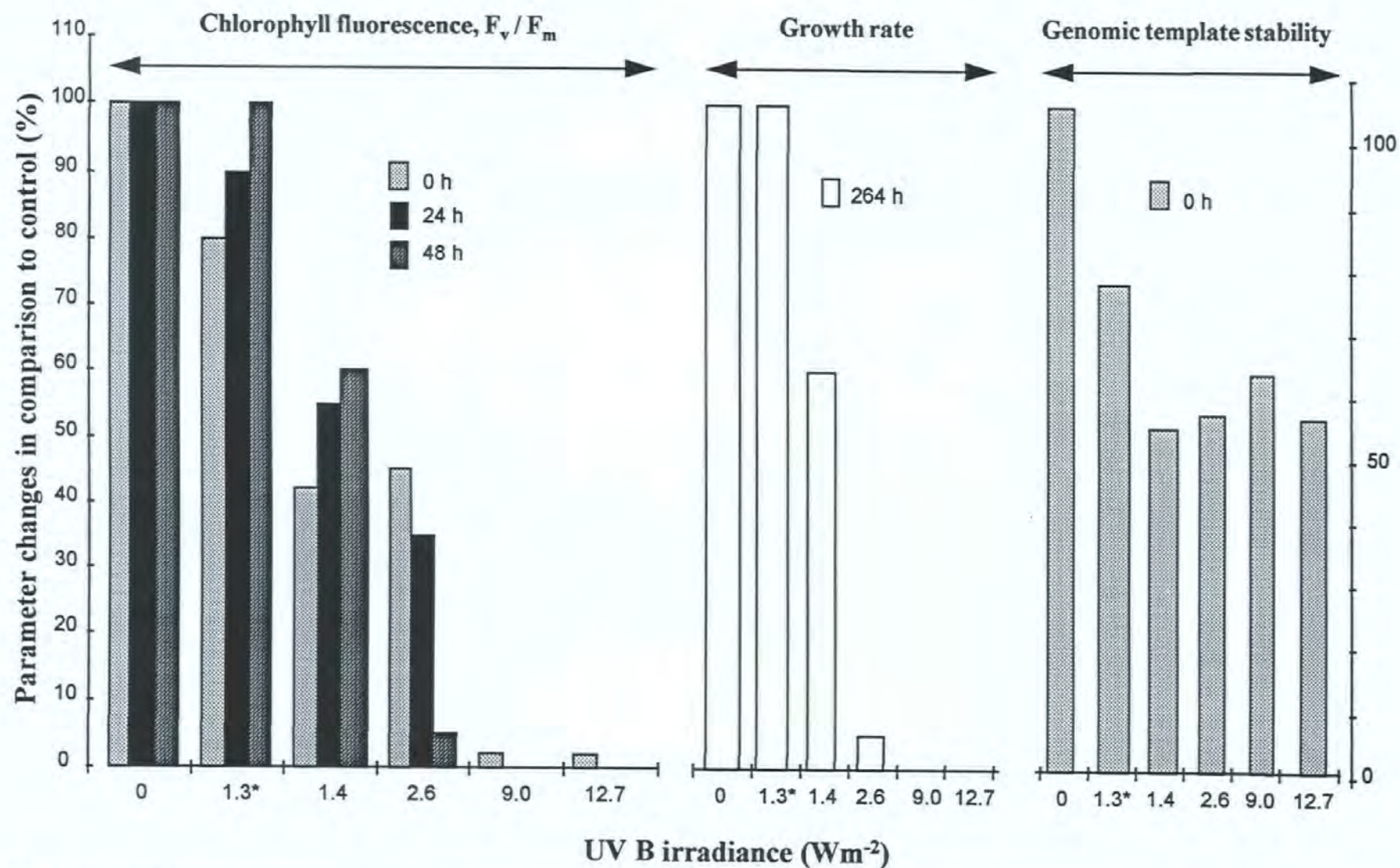
Figure 5.3. Comparison between chlorophyll fluorescence, $F_v / F_m$ ratio, growth rate, and genomic template stability in populations of *Palmaria palmata* exposed to UV radiation. * indicate that the algae were irradiated with UV-A only.

chlorophyll fluorescence ($F_v$ / $F_m$ ratio) and growth rates. Following exposure to increasing UV-B irradiance, chlorophyll fluorescence and growth rates decreased gradually to zero. In contrast, the genomic template stability remained similar (between 52 - 60 %) after all UV-B irradiances.

## 5.1.5 DISCUSSION

Although the generation of RAPD profiles has previously been criticised as unreliable (Ellsworth *et al.* 1993), suitable optimisation of the PCR conditions (Atienzar *et al.* 1998), and the judicious choice of oligonucleotide primers for each species-specific DNA template, suggest that the assay performs well. Indeed, reproducible DNA profiles have been generated from a range of aquatic invertebrates, plants and bacterial species (unpublished data, Atienzar *et al.*). Once optimised, the use of RAPD for the detection of DNA damage presents a number of advantages. The RAPD assay, which is suitable for any extracted DNA (of sufficient quality), allows rapid analysis of a large number of samples. As arbitrary primers are used, specific details of the DNA damage or the sequence of the genome are not needed. Further, no radioactivity nor chemical or enzymatic degradation of the DNA is required before analysis, in contrast to chromatographic techniques (Grossweiner and Smith 1989). Finally, the RAPD method does not depend on the molecular weight of the DNA sample, unlike for example the endonucleic assay used by Quaite *et al.* (1994).

Previous studies using DNA fingerprint analyses, have shown that changes in band patterns, reflect DNA alterations from single base changes to complex chromosomal rearrangements (White *et al.* 1990, Welsh *et al.* 1991). Similarly, in the present study DNA damage, induced by UV radiation reflected changes in RAPD profiles. Variation in band intensities and the disappearance of bands may be attributed to the presence of DNA photoproducts, which act to

block the polymerisation of DNA in the PCR reaction (Donahue *et al.* 1994, Nelson *et al.* 1996). Furthermore, in case of a bypass event, the polymerisation of DNA may be greatly reduced. The bypass process depends on a) the enzymatic properties of the DNA polymerase, b) the structure of the lesion, and c) the sequence context of its location (Ide *et al.* 1991). Therefore, the data suggest that the frequency of DNA photoproducts increased with increasing UV-B irradiance.

As well as disappearance of some bands, extra bands were also detected in the RAPD profiles. Appearance of new bands occurred principally for the three lowest UV irradiances (Table 5.3). This suggests that relatively high UV-B irradiances may inhibit repair and DNA replication in plant tissue (Howland 1975). New PCR amplification products may reveal a change in the DNA sequence due to mutations, which result in (a) new annealing event(s), and/or (b) large deletions (reducing the distance to pre-existing annealing sites), and/or (c) homologous recombination (connecting 2 sequences that match the sequence of the primer). For instance, following exposure to mutagens, DNA replication (Christner *et al.* 1994) and error-prone DNA repair (Sancar and Sancar 1988) are generally involved in generating mutations (Livneh *et al.* 1993). Unlike in mammals, plant cells enter meiosis only after significant vegetative growth. Thus, the induction of mutations, which accumulate over time in the somatic tissue, may be passed to the gametophytes (Walbot and Cullis 1985). This, in turn, could have implications for the long-term survival of the species (Wurgler and Kramers 1992).

A high level of DNA damage does not necessarily decrease the genomic template stability because DNA repair and replication may be inhibited by the high frequency of DNA damage. Hence, the plateau effect observed in fig. 5.3 can be ascribed to each change in RAPD profiles counter-balancing each other. In other words, the genomic template stability is related to the level of DNA damage, the efficiency of DNA repair and replication. Thus, the genomic

template stability can be misleading. In contrast, the actual pattern and frequency of appearing and disappearing bands may be a more suitable parameter of UV-induced stress, as indicated in Table 5.3. In earlier studies, using the AP-PCR technique, a non-mammalian test system for germ-cell mutagenesis, was developed for detecting DNA alterations in $F_1$ progeny descended from the gamma-irradiated male medaka fish. In these studies, DNA alterations were indeed detected as changes in the pattern of appearing and disappearing bands (Kubota et al. 1995). Moreover, the frequency of disappearing bands increased with increasing radiation dose (Kubota et al. 1992). In another study on RAPD profiles, similar changes in the pattern of RAPD bands compared to controls were found in rats exposed to Benzo(a)Pyrene (Savva et al. 1994).

The simultaneous use of more than one biomarker enhances the detection of toxic effects since different biomarker responses are induced at different stages of the plant's health status curve (Depledge 1994). In this study, changes in RAPD patterns, reductions in optimal quantum yield and growth rates, indicated that UV-B radiation (equivalent to an ozone reduction of 17 %) can cause irreversible damage in P. palmata.

Whilst the RAPD technique clearly shows promise in the detection of pollutant-induced DNA effects, a great deal of further experimentation and validation is required. Future work will focus on determining the sensitivity and selectivity of the RAPD technique. A comparison will be made with pre-existing DNA analysis methodologies. These will include ^{32}P-post-labelling assay (Gupta 1984), large scale chromosome aberrations (Jha et al. 1996), and DNA strand breaks. Future work will also determine whether the RAPD technique is a sufficiently powerful tool to detect mutations. This will be performed using material obtained from hypermutating cultured mammalian cells and from bacterial isolates. Applying this approach,

the general applicability of the RAPD technique for detection of DNA damage, in both *in vitro* and *in vivo* systems, can be further evaluated.

## 5.1.6 CONCLUSIONS

The RAPD technique showed potential as a reliable and reproducible assay for genotoxicity, when used with caution. The pattern of appearing and disappearing bands, in particular, showed potential as a sensitive tool for detecting UV-induced genetic damage in *P. palmata*. Coupled with other suitable physiological and fitness measurements, this technique may promote a better understanding of the link between molecular alterations and measurable adverse effects at higher levels of biological organisation.

# 5.2 EVALUATION OF THE HEAT SHOCK PROTEIN 70 (HSP 70) RESPONSE AS A BIOMARKER OF UV-B EXPOSURE IN TWO MACROALGAL SPECIES.

## 5.2.1 ABSTRACT

The induction of heat shock proteins (HSP 70) following UV-B irradiance was studied in *Palmaria palmata* and *Enteromorpha intestinalis* to investigate the potential use of HSP 70 as a molecular marker of UV-B exposure. However, a 3 hour exposure to relatively high irradiances of UV-B (unweighted UV-B: 2.6 and 8.6 W m$^{-2}$) showed no significant induction of HSP 70 for the two species. As increases in band intensity with increasing concentration of purified standard HSP 70, clearly showed that optimisation of the slot-blotting technique was successful, it was concluded that HSP 70 was not induced in these two species after exposure to UV-B radiation.

## 5.2.2 INTRODUCTION

Heat shock proteins (HSP), defined as proteins whose rate of synthesis is increased by exposure to a 5-10 °C rise in optimum temperature, have been found in all species tested from bacteria to humans. Despite the widespread nature of these proteins, their role in the cellular response to stress is not yet understood (Bauman *et al.* 1993). Numerous studies have investigated the suitability of stress protein induction as a potential biomarker of trace metal exposure in marine organisms (Sanders 1990, Bradley 1993, Sanders and Martin 1994, Lewis *et al.* 1998). Several studies have also investigated the possible role of heat shock proteins as mediators of heat-shock-induced UV-B resistance in human epidermal keratinocytes (Maytin

*et al.* 1993, Trautinger *et al.* 1995). However, less information is available regarding the role of these proteins in algae (Müller *et al.* 1992, Vayda and Yuan 1994, Reith and Munholland 1991). The major heat shock proteins, the 70 kDa family, was induced as a response to UV-B exposure after an initial heat shock treatment in marine diatoms (Döhler *et al.* 1995). Likewise, Nicholson and Howe (1986) reported induction of 71 kDa and 65 kDa in *Chlamydomonas reinhardtii* following UV-B irradiation.

The aim of this study was to investigate if induced levels of HSP 70 could be related in a dose-response dependent manner to UV-B exposure, without an initial heat shock treatment. Thus, the potential of HSP 70 induction as a biomarker of short-term UV-B exposure in marine macroalgae was assessed.

## 5.2.3 MATERIALS & METHODS

### Collection of algae

*E. intestinalis* and *P. palmata* were collected at low tide from Wembury in May and June (seawater temperature: 13 °C). The thalli were harvested 24 hours before the experiments and kept in filtered seawater at constant temperature (15 °C) and in low light conditions (25 µmol $m^{-2} s^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF™) in growth cabinets.

### UV treatments

Thalli cut to a length of 3 cm and covered to a depth of 1.5 cm with filtered sea water, were exposed for 3 hours in open Petri dishes (n = 3). The UV exposures were performed as

described in Chapter 3. The following unweighted UV-B irradiances were chosen: 2.6 and 8.6 W m$^{-2}$ (equivalent to weighted irradiances of 0.7 and 2.4 W m$^{-2}$ and weighted doses of 7.9, 26.2 kJ m$^{-2}$, calculated according to the generalised plant response action spectrum, Caldwell 1971). The weighted doses correspond to 17 % and 40 % ozone depletion, calculated for 15.07.97 at Plymouth. UV-A and PAR irradiances were identical to levels used in Chapter 3. Immediately after UV exposure the thalli were frozen in liquid nitrogen and then transferred to - 80 °C until analysis.

## Western blotting and slot blotting

### Preparation of algal tissue

One g of pooled tissue of *E. intestinalis* and 0.4 g of pooled tissue of *P. palmata* were used for each replicate, which was split into duplicates to investigate method variation. The frozen plant tissue was grounded to a powder using liquid nitrogen and a pestle and mortar. The powdered sample was weighed and 2 volumes of cold homogenisation buffer was added (25 mM Tris-HCL, pH = 7.8, 10 % glycerol 0.05 % Triton-X 100 and 5 mM DTT) with several protease inhibitors such as PMSF (100 µM), antipain and pepstatin-A (1 µg ml$^{-1}$ for *E. intestinalis* and 10 µg ml$^{-1}$ for *P. palmata*). After further homogenisation using a Polytron, the smooth homogenate was sonicated with extra PMSF. The samples were then centrifuged at 100,000 g for 60 min at 4 °C. The supernatant was transferred to Centricon 30, with a 30 kDa cut off, and centrifuged for 75 min at 5000g. An aliquot of the concentrated extract was then assayed for total protein and the rest was frozen.

### Western blotting

The cross-reactivity of monoclonal antibody with *P. palmata* samples, using western blotting, was measured using the method of Lundebye *et al.* (1995), as later adapted by Lewis (1997). Proteins were extracted from the tissue of both species and the total protein content of samples was determined by the Bradford method (Bradford 1976). For western blotting, samples were diluted in Laemmli buffer (Laemmli 1970) and boiled. Proteins were separated by one dimensional SDS-PAGE (with 5 % stacking gel on 10 % resolving mini-gels) run at 35 mA per gel in a Hoeffer Se 250 Mighty Small Electrophoresis System™. Molecular weight markers as well as the samples were loaded. Proteins were then transferred to nitrocellulose membranes, using a Pharmacia Nova Blot semi-dry blotter. The nitrocellulose membranes were blocked overnight with 5 % dried-milk powder in tris-buffered saline (TBS). The membranes were then rinsed in TBS with 0.05 % Tween and incubated with 1: 1000 mouse monoclonal anti-HSP 70 antibody for 1.5 hours. After this incubation, more rinses with TBS were followed by 1.5 hours incubation with alkaline phosphatase linked goat anti-mouse IgG. Finally, the membranes were developed for 30 min with p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate ptoludine (NBT and BCIT), to produce a colour signal.

**Slot/dot-blotting**

The slot blotting method was applied to determine relative quantity of HSP 70 in the different algal samples. Nitrocellulose membrane and blotting papers were placed in a Bio-Dot SF microfiltration apparatus (BioRad Ltd). After a dilution in TBS with 1 % SDS (sodium dodecyl sulphate) and a 3 min boiling, algal samples and a commercial purified standard of human HSP 70 were applied to the membrane under gentle vacuum. Samples contained 25 μg total protein content for *E. intestinalis* and 60 μg for *P. palmata*. Each blot was loaded with samples of equal total protein and duplicate calibration curves of the purified standard. The membranes were then blocked in 5 % TBS and rinsed with TBS and Tween before it was

incubated with the two antibodies as above. The membranes were developed with a Amersham ECL™ kit as per manufacturers instructions and the blots were exposed to Hyperfilm-ECL for 10 minutes. The films were scanned using a densitometer (Pharmacia LKB Ultrascan). Calibration curves for each blot were plotted (Au*mm) versus total protein content of standard and a square root x regression model was fitted (Lewis 1997). The resulting equation was used to determine relative quantity of HSP 70 (AU) in the different samples.

## 5.2.4 RESULTS

Figure 5.4 shows the western blot with molecular markers and one band indicating a strong cross-reactivity of monoclonal antibody with *P. palmata* samples. Clear bands appeared for samples of *P. palmata* (fig. 5a) and *E. intestinalis* (fig. 5b) and the two calibration curves (made with the commercial standard of human HSP 70) on both blots. The duplicate calibration curves did not differ significantly from each other (ANOVA, p>0.2). The purified HSP 70 standard was applied at concentrations of 5.63 - 180 µg total protein for both species. No significant difference in HSP 70 expression was found for *P. palmata* or *E. intestinalis* exposed to UV-B compared to un-exposed controls (ANOVA p>0.2).
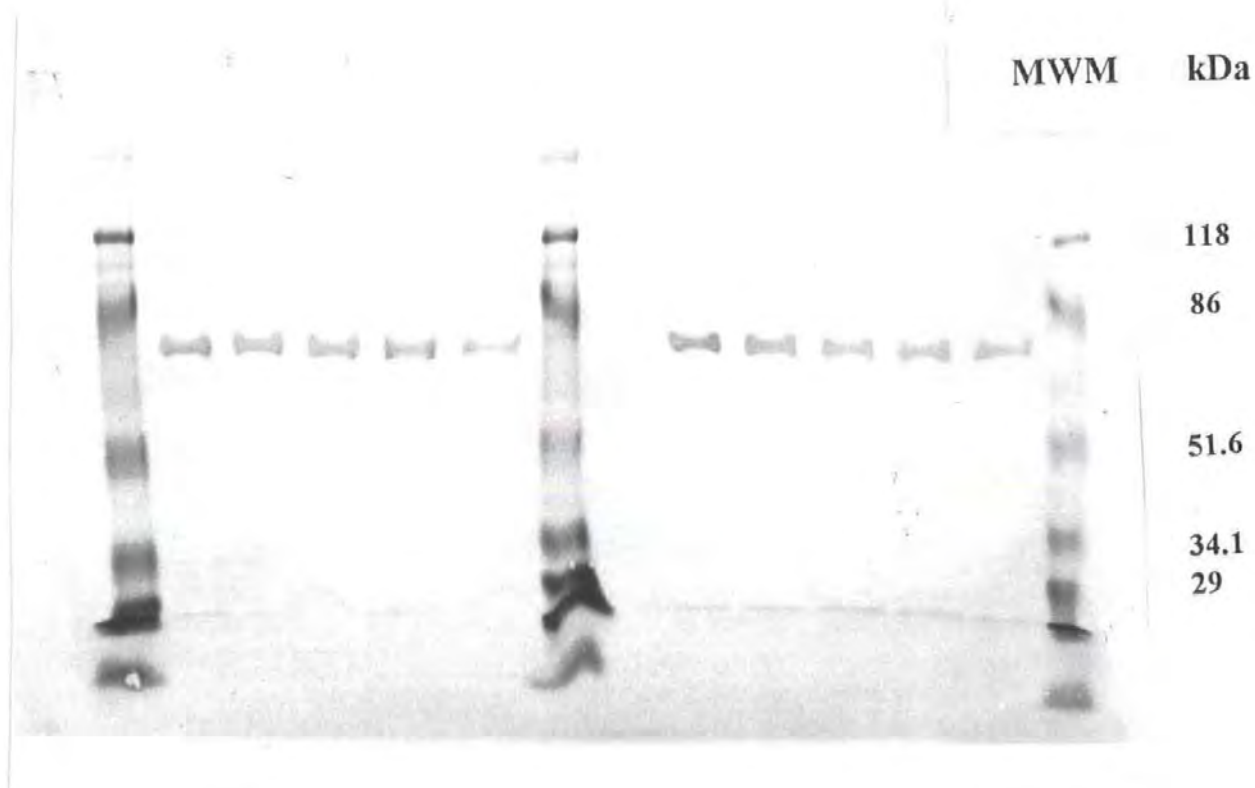
Figure 5.4. Western blot of *Palmata palmaria*. The molecular weight markers (MWM) are highlighted
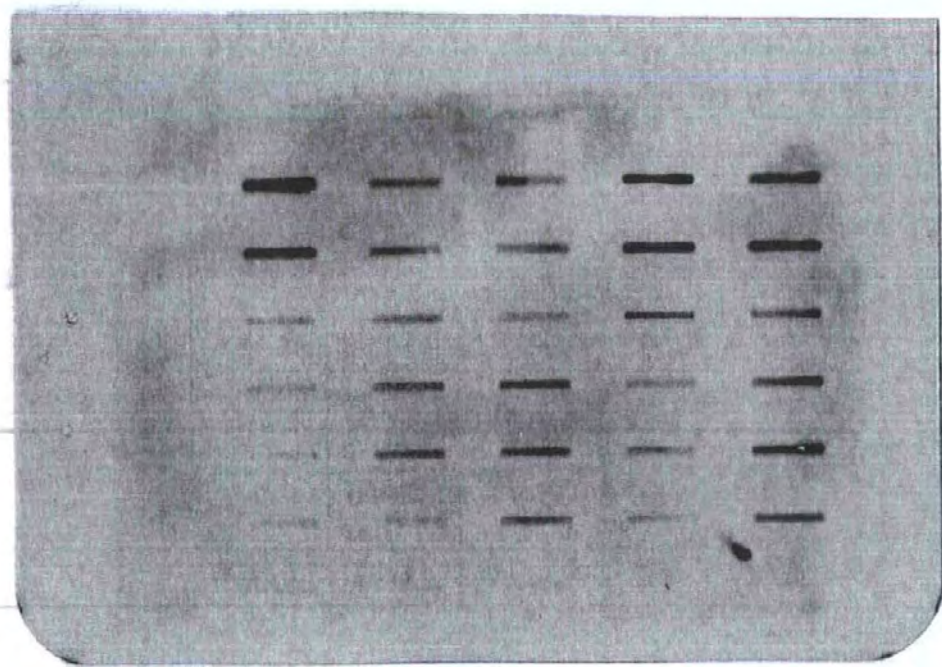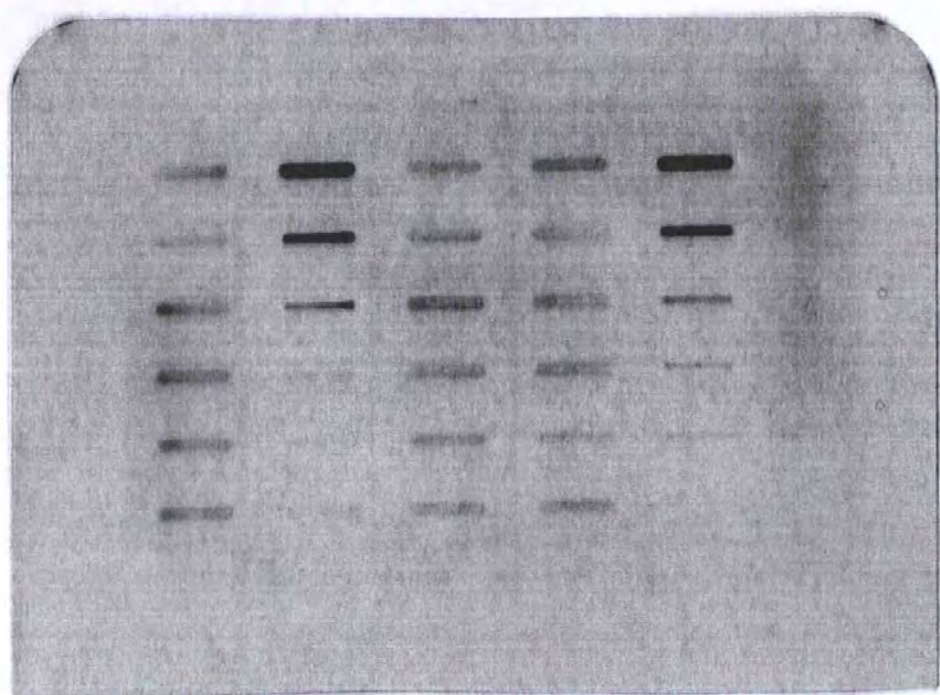
a

| | | | | |
|---|---|---|---|---|
| C 1[a] | Cal. 6 | C 2[a] | C 3[a] | Cal. 6 |
| C 1[b] | Cal. 5 | C 2[b] | C 3[b] | Cal. 5 |
| UV-B 1 1[a] | Cal. 4 | UV-B 1 2[a] | UV-B 1 3[a] | Cal. 4 |
| UV-B 1 1[b] | Cal. 3 | UV-B 1 2[b] | UV-B 1 3[b] | Cal. 3 |
| UV-B 2 1[a] | Cal. 2 | UV-B 2 2[a] | UV-B 2 3[a] | Cal. 2 |
| UV-B 2 1[b] | Cal. 1 | UV-B 2 2[b] | UV-B 2 3[b] | Cal. 1 |

b

| | | | | |
|---|---|---|---|---|
| Cal. 6 | C 1[a] | C 2[a] | Cal. 6 | C 3[a] |
| Cal. 5 | C 1[b] | C 2[b] | Cal. 5 | C 3[b] |
| Cal. 4 | UV-B 1 1[a] | UV-B 1 2[a] | Cal. 4 | UV-B 1 3[a] |
| Cal. 3 | UV-B 2 1[a] | UV-B 1 2[b] | Cal. 3 | UV-B 1 3[b] |
| Cal. 2 | UV-B 2 1[b] | UV-B 2 2[a] | Cal. 2 | UV-B 2 3[a] |
| Cal. 1 | Blank | UV-B 2 2[b] | Cal. 1 | UV-B 2 3[b] |

Figure 5.5. Slot blot of protein extracts from a) *Palmaria palmata* and b) *Enteromorpha intestinalis*, exposed for 3 hours to different UV-B irradiances. Unweighted UV-B irradiances of 2.6 (UV-B 1) and 8.6 W $m^{-2}$ (UV-B 2) were used. Cal. 1 - 6: two Calibration curves were performed with purified commercial standard of human HSP 70 at concentrations 5.6, 11.3, 22.5, 45, 90, and 180 µg total protein (1 - 6). Three replicates were used (1 - 3) which was split into duplicates ([a], [b]) to investigate method variability (although for *E. intestinalis*, UV-B 1 replicate no. 1, no duplicate was made).

# 5.2.5 DISCUSSION

As the antibody used here was raised against human HSP 70, the strong cross-reactivity of monoclonal antibody with *P. palmata* samples illustrated that HSP 70 is highly conserved (fig. 5.4). Very similar results were found by Lewis (1997) for *E. intestinalis*. The lack of variation between the calibration curves for both species indicate that variability between samples was minimal. Furthermore, the gradual increases in band intensity with increasing concentration of purified standard HSP 70 clearly showed a successful optimisation of the slot-blotting technique. Hence, the lack of response observed indicates that HSP 70 was not induced in these two species following UV-B exposure.

Induction of HSP 70 and HSP 60 (60 kDa) following heavy metal exposure has been reported for several marine organisms (Cochrane *et al.* 1991, Sanders *et al.* 1991, 1992, 1994, Sanders and Martin 1993, Ryan and Hightower 1994, Sanders and Dyer 1994). In addition, induced HSP 70 has also been measured in marine macroalgae following heat treatment. Lewis (1997) found induced HSP 70 in *E. intestinalis* after 8 h exposure to 30 °C and Reith and Munholland (1991) reported similar induced levels in *Porphyra umbilicalis* after 75 min exposure to 30 °C. An initial heat shock treatment, followed by 2 hours exposure to UV-B (Caldwell weighted dose of 1.5 kJ m$^{-2}$), produced induced levels of both HSP 70 and 57 kDa in marine diatoms (Döhler *et al.* 1995). Likewise, Nicholson and Howe (1986) reported induction of 71 kDa and 65 kDa in *Chlamydomonas reinhardtii* following UV-B irradiation. Moreover, heat shock proteins may be mediators of heat-shock-induced UV-B resistance in human epidermal keratinocytes (Maytin *et al.* 1993, Trautinger *et al.* 1995).

The lack of induction found here could not be attributed to a reduced protein synthesis due to nutrient limitation (Coleman *et al.* 1995, Lewis 1997), since the algae were placed in nutrient

enriched seawater. Referring to the above-mentioned studies involving UV-B exposure, it therefore seems likely that a response in HSP 70 levels to UV-B exposure is linked to an initial heat shock treatment in algae.

## 5.2.6 CONCLUSIONS

Since relatively high UV-B doses (weighted UV-B of 7.9 and 26.2 kJ m$^{-2}$) were used, it was concluded that induction in HSP 70 is an unsuitable indicator of UV-B exposure in *P. palmata* and *E. intestinalis*. Stress protein analyses are both time consuming and relatively expensive, thus, no further studies were conducted using HSP 70 in assessing UV-induced effects.

# CHAPTER 6

# THE INFLUENCE OF UV-B RADIATION ON THE REPRODUCTIVE CELLS OF THE INTERTIDAL MACROALGA *ENTEROMORPHA INTESTINALIS.*

## 6.1 ABSTRACT

In laboratory experiments the relative sensitivity of the reproductive cells of the seaweed *Enteromorpha intestinalis* to UV-B was investigated by measuring chlorophyll fluorescence ($F_v$ variable fluorescence), germination success and growth rates. It was demonstrated that zoospores (the asexual reproductive cells) exhibit up to approximately 6 fold higher sensitivity to UV-B exposure than the mature thalli (measured as chlorophyll fluorescence, $F_v$), and differences in growth rates were also found. Consistent patterns emerged throughout these experiments in variable fluorescence, germination success and growth rates, of a greater sensitivity in the sexual reproductive part of the life cycle compared with the asexual part of the cycle. Furthermore, inhibitions in germination success (up to 50 %) and in growth rates (up to 16.4 %) of settled gametes and zoospores after 1 h exposure to elevated levels of UV-B (equivalent to 27 and 31 % ozone depletion) showed irreversible damage of reproductive cells. Clearly, the ecological significance of elevated UV-B exposure in the marine environment may be seriously under-estimated if effects on the early lifestages of algae are not considered.

## 6.2 INTRODUCTION

Recent estimates suggest that primary productivity of phytoplankton in areas influenced by the ozone hole in the Southern Antarctic Sea is reduced by 6-23 % (Smith *et al.* 1992, Weiler and Penhale 1994). No such assessment on the coastal primary productivity in the northern hemisphere has been undertaken. UV-B effects in macroalgae have previously been detected, although primarily in mature thalli (Larkum and Wood 1993, Grobe and Murphy 1994, reviews by Häder *et al.* 1995 and Franklin and Forster 1997). However, effects on reproductive unicells and early life stages, which are usually particularly sensitive to stressors (Hanelt *et al.* 1997, Thursby and Steele 1986) have yet to be assessed.

There are several aspects of ozone depletion that are important when considering damaging effects on primary producers in the marine environment:

(i) springtime ozone depletion is a recent phenomenon relative to evolutionary time scales (Karentz 1991). Consequently, marine algae may be poorly adapted to deal with increased UV-B exposure.

(ii) lowered tolerance in algae, due to long-term adaptation to low irradiation levels during the previous winter months may coincide with increases in UV-B during spring.

(iii) spring time increases in UV-B coincide with the production of reproductive cells (unicells) and early life stages (sexually immature gametophytes and sporophytes) in many macroalgae.

*E. intestinalis* is an opportunistic alga which exhibits an alternation of generations which consists of isomorphic sporophytes (2n) and gametophytes (n) and unicellular reproductive cells. The zoospores are released from the mature sporophyte and the gametes are released from the mature gametophyte. The success of this alga is primarily determined by its ability to

distribute itself widely and to rapidly colonise exposed substrata by means of its motile zoospores and gametes (please see Section 1.4.2).

The aims of this study were to:

a) investigate the relative sensitivity of free-swimming zoospores and gametes of the macroalga *E. intestinalis* to ambient and elevated UV-B irradiances.

b) relate changes in chlorophyll fluorescence, $F_v$, with consequent effects on germination success and growth rate.

c) compare effects of UV-B exposure in 12 h old free-swimming zoospores with mature thalli of the same species.

## 6.3 MATERIALS & METHODS

## Algal collection and preparation

Thalli of *Enteromorpha intestinalis* were collected at spring tide (1.4 m from the mean high water mark, with a water temperature of 11-15 °C and salinity of 25 ‰) from Wembury beach between April and June 1997. The proportion of gametophytes to sporophytes at Wembury was approximately 1:1. Following transfer to the laboratory, reproductive cells were released synchronously from fertile thalli by transfer from 13 ° C to 20 °C artificial seawater (Instant Ocean, 33 ‰). Gametes (released from gametophytes) and zoospores (released from sporophytes) were identified using a microscope. The cell density of both gametes and zoospores was $2.9 \times 10^8$ cells per ml, pooled from 15 plants each. Both gametes and zoospores were kept in suspension for 12 h prior to UV exposure at constant temperature

and PAR conditions (15 °C and 25 $\mu$mol m$^{-2}$ s$^{-1}$). The mature thalli were cut to a length of 2 cm and exposed in open Petri dishes covered with 1 cm artificial seawater.

## UV exposure conditions

The following UV-B (280 - 315 nm) exposure regimes were used: unweighted irradiance of 0.5, 1.8 and 2.2 W m$^{-2}$ (equivalent to weighted irradiances of 0.14, 0.51 and 0.62 W m$^{-2}$ respectively (Björn and Murphy 1985), calculated according to the generalised plant response action spectrum (Caldwell 1971) normalised at 300 nm). This weighted function was chosen to permit general comparison with other work in the UV field. The weighted irradiances correspond to ambient irradiance in September, 27 % and 31 % ozone reduction (calculated for 15.07.97), respectively. Both weighted irradiance and ozone depletion levels were calculated for Plymouth (at an air pressure of 1000 mbar, relative humidity of 0.5 and aerosol level of 0), at noon 12.00 of the 15.07.97 during clear sky conditions (Björn and Murphy 1985). Calculating with a correction factor of 0.5 due to cloud cover, the corresponding reduction in ozone of the two elevated UV-B irradiances are 34 and 35 % (calculated for 15.07.97).

UV radiation was supplied by UV-B and UV-A tubes, filters and measurements of UV were performed as described in detail in Chapter 3. UV-A was kept low and constant (5.1 W m$^{-2}$ approximately equivalent to December levels in Cornwall (Driscoll *et al.* 1992)) and independent of the UV-B irradiance. PAR was kept low and constant during the entire experimental period (25 $\mu$mol m$^{-2}$ s$^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF™). PAR irradiation was measured with a PAR meter connected to a quantum sensor (model SKP 200, Skye Instruments Ltd) and calibrated by the manufacturers. UV exposures ranging from 10 to

60 min were performed in a constant temperature cabinet (15° C). The surrounding water was either agitated by placing the Petri dishes on a shaking table or kept still.

## Measurements of chlorophyll fluorescence induction

*In vivo* chlorophyll fluorescence was recorded with an non-modulated Plant Efficiency Analyser™ as described in detail in Chapter 3. (Hansatech Instruments Ltd). $F_v$ which is the variable fluorescence, calculated as $F_m$ - $F_0$ was determined before and after the UV exposures. $F_v$ / $F_m$ was also measured and showed similar significant changes to UV-B exposure. Control gametes and zoospores exhibited an optimal quantum yield ($F_v$ / $F_m$) > 0.66. However, $F_v$ is presented here because it was affected more rapidly and to a greater extent, thus it was found to be a more sensitive indicator of UV-B exposure. As $F_v$ is dependent on the chlorophyll concentration, aliquots for each UV-B treatment were taken from one stock solution with an evenly distributed unicell suspension. Prior to making measurements, the thalli or the solution containing the unicells were dark-adapted for 10 min to ensure an oxidised electron transport chain. Further prolonged dark adaptation (25 min) showed no change in $F_0$ and $F_m$. The fluorescence measurements of the unicells were performed while the cells were kept in suspension. Five replicates, each containing $2.9 \times 10^8$ cells per ml, were used for both zoospores and gametes.

## Growth measurements

Gametes and zoospores were settled separately on glass coverslips (500 cells in 100 µl). After 12 h coverslips with the attached unicells were exposed to UV in open Petri dishes covered with 1 cm artificial seawater. Following the exposures the settled embryonic sporophytes and gametophytes were kept at 15 °C in enriched Instant Ocean (50 mg $l^{-1}$ NaNO$_3$ and 10 mg $l^{-1}$

Na$_2$HPO$_4$·12H$_2$O) which was changed daily. A 12:12 h light:dark regime was used and PAR irradiation level was kept constant as in the experiments (25 µmol m$^{-2}$ s$^{-1}$). The physiological variables in the experiment were related to differences in growth rate by measuring daily the area of both mature thalli and randomly chosen embryonic sporophytes and gametophytes on a Quantimet 570 Automatic Image Analyser™ fitted to a microscope (Kyowa, model SD2-PL) during a 7 day period. The relative growth rate was calculated on log transformed data as the slope of the transformed linear growth curves (please see Chapter 3 for more details). 15 germlings were measured in each of five replicates for the reproductive cells and five replicates were chosen for mature thalli.

## Measurement of germination success

The number of germinating cells (scored as cells with one or more cell divisions) and non-germinating cells were counted in the microscope covering 16 patches in each of the 4 replicates containing approximately 500 germlings.

## Statistical analysis

Changes in chlorophyll fluorescence $F_v$, was tested statistically by performing one-way (single factor) analysis of variance (ANOVA). Overall significant differences were followed by Tukey's HSD test to establish where these differences could be found. A two factor ANOVA (factors: type of unicell, UV-B treatment) on log transformed data was used to test differences in germination success between gametes and zoospores and UV-B exposures. Multiple regressions were performed on log transformed growth data.

# 6.4 RESULTS

After 30 minutes exposure to a UV-B irradiance of 0.5 W m$^{-2}$ (equivalent to ambient irradiance in September in SW England) 12 h old free-swimming zoospores of *E. intestinalis* experienced 30 % reduction in variable fluorescence, $F_v$, of the pre-exposure level compared to a non-significant reduction of 5 % in the mature thalli (Table 6.1). This experiment was performed in still water conditions.

In Figure 6.1a significant decreases in $F_v$ (approximately 20 % compared to pre-exposure values) were found in 12 h old free-swimming zoospores after 10 min exposure to 1.8 and 2.2 W m$^{-2}$. Exposures of 1 hour duration caused further decreases of 22 and 42 % respectively (ANOVA, p<0.01). 12 hour old gametes exhibited even greater reductions in variable fluorescence after both 10 and 60 min exposures to all UV-B irradiances. Significant reductions of 19, 22 and 32 % compared to pre-exposure values occurred after 10 min exposure to 0.5 to 1.8 and 2.2 W m$^{-2}$ respectively (ANOVA, p<0.01). Moreover, following 1 hour exposure to the different UV-B irradiances resulted in further reductions of 40, 43 and 65 % respectively in gametes. These experiments presented in Figure 6.1 were all performed in agitated water. The weighted dose-response relationships of $F_v$ in zoospores and in gametes to UV-B exposure, based on the generalised plant response action spectrum (Caldwell 1971) and the inhibition of phytoplankton (*Phaeodactylum*) photosynthesis (Cullen *et al.* 1992), are shown in Figure 6.1c. Exponential models, fitted to the PAS 300 weighted doses resulted in equations of y = exp (4.5476 - 0.1853 * weighted dose) for zoospores and y = exp (4.4273 - 0.33117 * weighted dose) for gametes with r$^2$ = 0.64 and 0.77, respectively.

**Table 6.1 Reduction in chlorophyll fluorescence in mature thalli and 12 h old zoospores following exposure to UV-B.**

| UV-B Exposure (min) | Mature thalli (%) | Zoospores (%) |
|:---:|:---:|:---:|
| 10 | $3.3 \pm 3.6$ | $19.5 \pm 5.5$ * |
| 30 | $4.7 \pm 1.8$ | $29.5 \pm 4.0$ ** |

Chlorophyll fluorescence was measured as $F_v$ and the percentage reduction was calculated from the pre-exposure level. Mean $\pm$ SE (n = 5), *, $p<0.05$; **, $p<0.01$, indicate significant differences between zoospores and mature thalli within each exposure time, one-way ANOVA. UV-B exposure of $0.5$ $Wm^{-2}$ was performed in still water conditions.
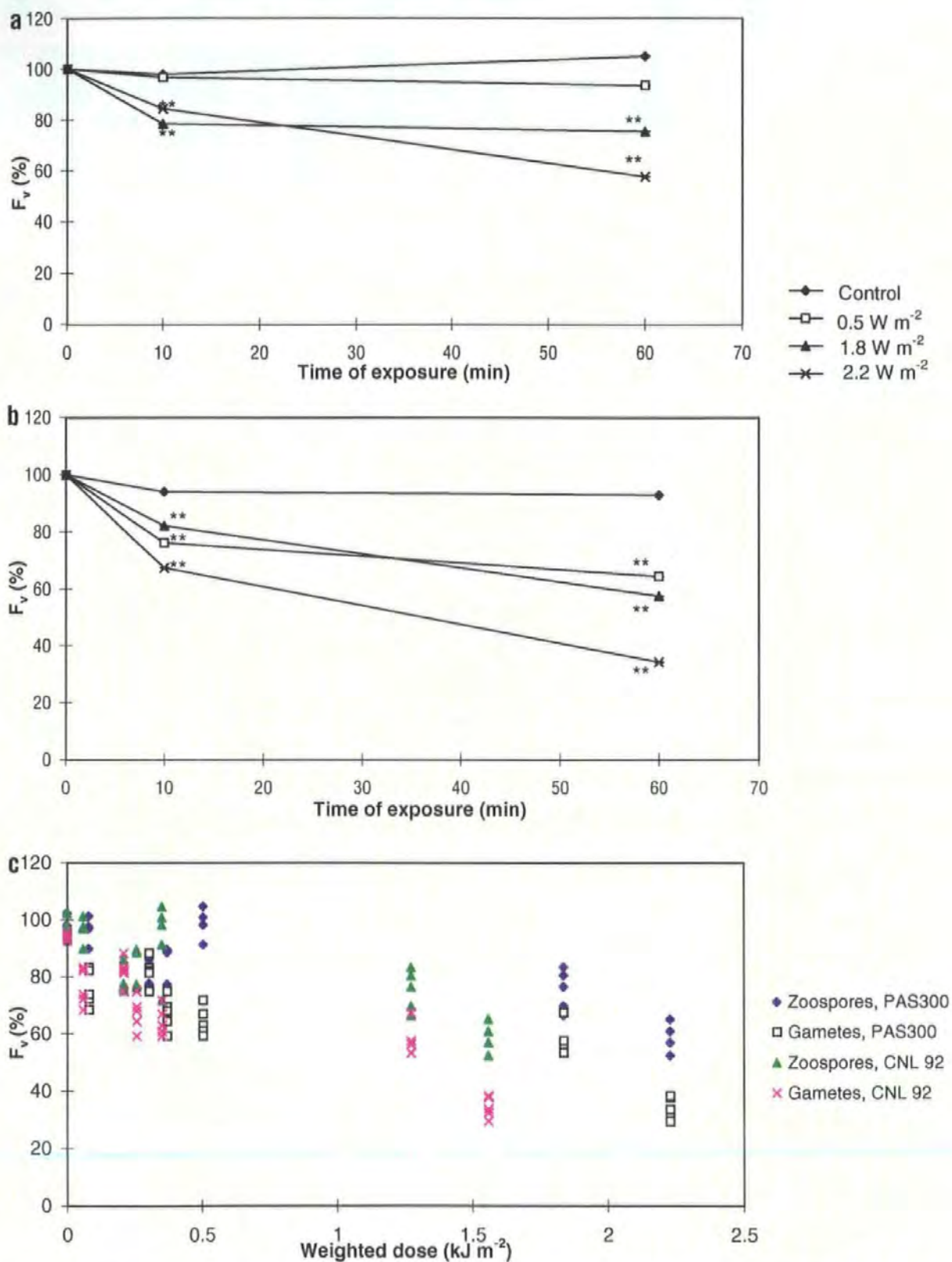
Figure 6.1. Chlorophyll fluorescence $F_v$ (% of pre-exposure value) as a function of exposure time to varying UV-B irradiances in zoospores (a) and gametes (b) of the seaweed species *E. intestinalis* . Mean $\pm$ SE (n = 5), (** indicate significant differences from control levels, one-way ANOVA, p<0.01). (c) $F_v$ (%) as a function of PAS 300 weighted doses (Caldwell 1971) and CNL 92 phytoplankton weighted doses (Cullen *et al.* 1992). Data were derived from Fig 6.1a and b. Each point represents a replicate (n = 5). Each replicate in the three panels contained $2.9 \times 10^8$ cells per ml.

In another series of experiments germination success was measured for zoospores and gametes. The ratio between germinating and non-germinating cells varied significantly between zoospores and gametes 6 days after an initial 1 hour exposure to UV-B (Two-factorial ANOVA, p<0.018), (Figure 6.2). Moreover, an overall significant decrease in germination was also found for all UV-B irradiances (Two-factorial ANOVA, p<0.0001; with no interaction between the two factors: cell type and UV-B treatment, p>0.27). The lack of interaction between cell type and UV-B treatment secures that the two factors are independent (i.e. no auto-correlation). After 1 hr exposure to ambient UV-B irradiance (0.5 W m$^{-2}$) a 50 % decrease in germination was found in fused gametes compared to controls, whereas no effects were detected in settled zoospores. Further exposures to elevated UV-B (1.8 W m$^{-2}$) caused 80 % decreased germination success in gametes compared to 50.5 % in zoospores (two-way factorial ANOVA, p<0.0001).


Similarly, growth rates recorded daily over a 7 day period for both gametophytes (fig. 6.3a) and sporophytes (fig. 6.3b) were inhibited after an initial 1 hour exposure to UV-B performed on day 0. Greater reductions in growth rate occurred with increasing UV-B irradiance for both gametophytes and sporophytes. Slopes of the linear models fitted to log transformed gametophyte data were 0.62 for controls, 0.6, 0.55 and 0.53 for germlings with increasing UV-B irradiance respectively. As indicated on fig. 6.3, slopes of 0.55 and 0.53 were significantly reduced compared to slope for controls (Multiple regression on log transformed data, p<0.01, residual degrees of freedom=135, residual MS$_{error}$=0.05, F-ratio=938.35). Maximum inhibition of 14.5 % compared to controls was measured after exposure to 2.2 W m$^{-2}$. However, the most severe inhibitions were found for sporophytes with reductions up to 16.4 % compared to controls following exposure to 2.2 W m$^{-2}$. Slopes of linear models for sporophytes were 0.63 for control treatment and 0.57, 0.54 and 0.53 with increasing UV-B irradiance. All slopes were significantly reduced compared to controls (Multiple regression on

log transformed data, p<0.01, residual degrees of freedom=135, residual $MS_{error}$ =0.04, F-ratio=1,172.9). $r^2$ values for the fitted linear models, performed on log transformed data, are listed on the figures.
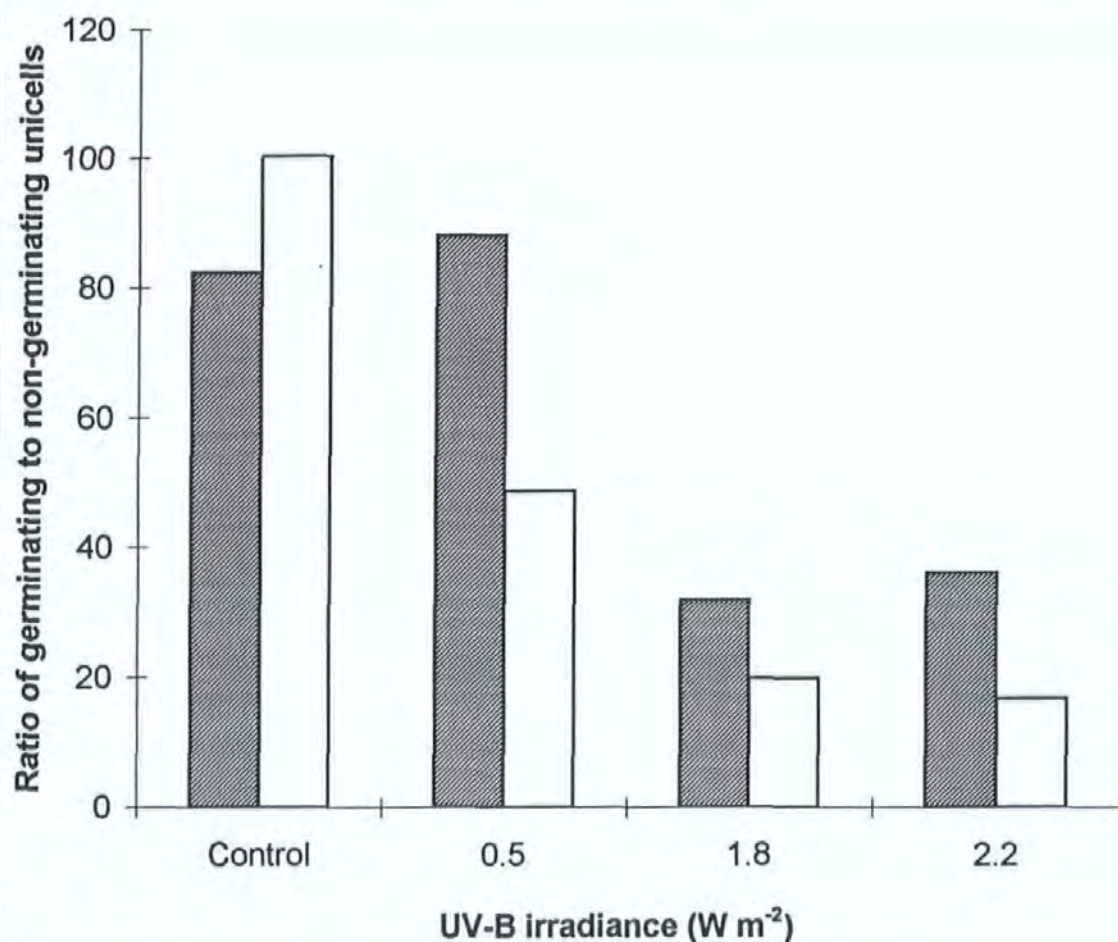
Figure 6.2. Ratio of the number of germinating to non-germinating zoospores and gametes 6 days after 1 h exposure to varying UV-B irradiances. Hatched bars represents zoospores and white bars gametes. A significant overall difference between zoospores and gametes was found (p<0.012), in addition to an overall significant difference in ratio between UV-B irradiances (p<0.0001), two factorial ANOVA (Factors: unicells and UV-B irradiance levels) on log transformed data, with no interaction p>0.27). Overall s.e. = 0.21 of log transformed data.

a



b
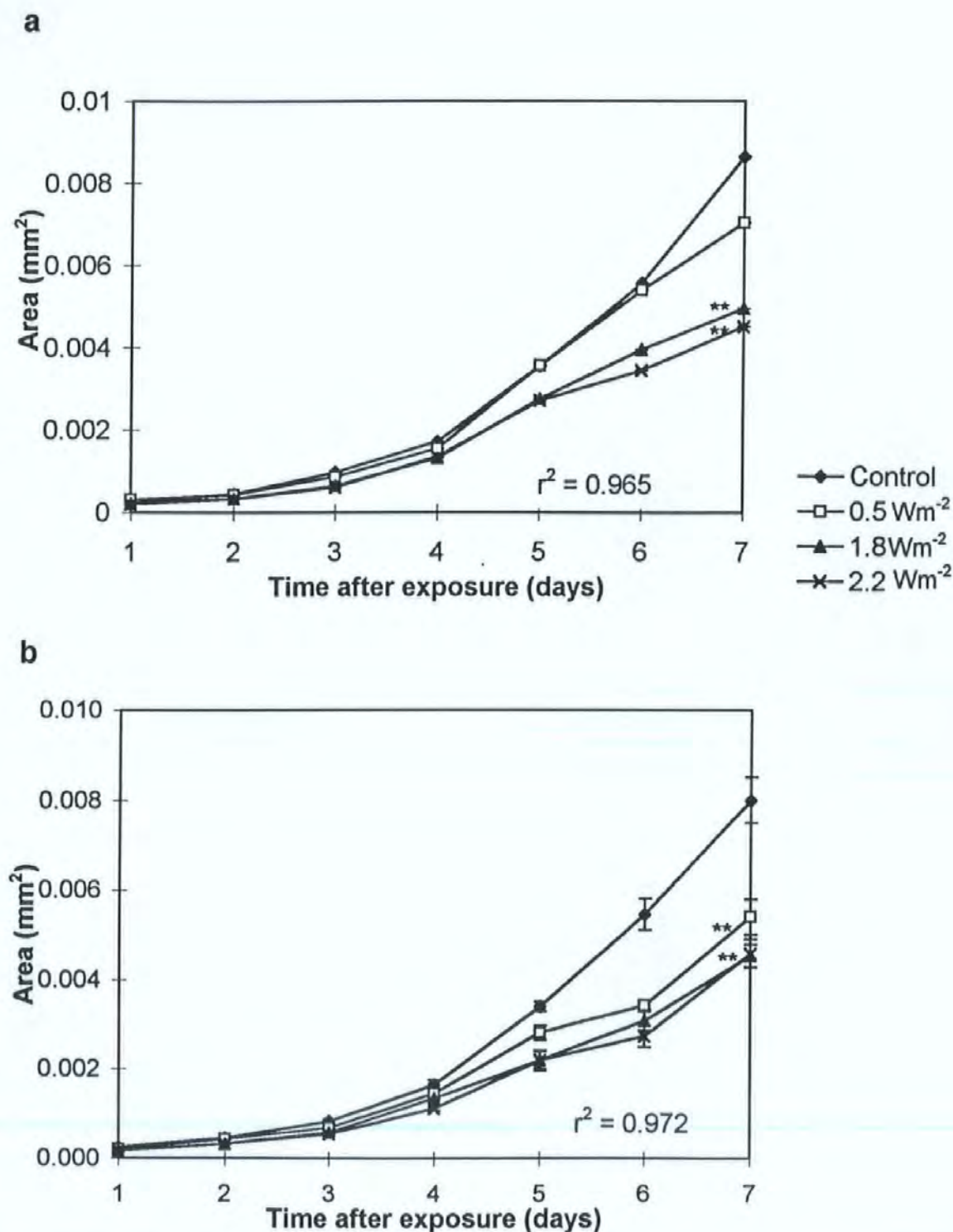


Figure 6.3 Growth of gametophytes (a) and sporophytes (b) measured as increase in area as a function of time after exposure to varying UV irradiances. Multiple regressions were performed on log transformed data, $r^2$ values are shown for the fitted linear models. ** indicate significant differences in the slopes compared to the control, $p<0.01$. 15 germlings were measured per replicate, mean ± SE, n = 5.

to UV radiation (Dring *et al.* 1996b) and to high visible light stress (Hanelt *et al.* 1997, Lüning 1980, Han and Kain 1996, Graham 1996) has been reported.

The greater sensitivity of reproductive cells to UV-B compared to the mature algae may reflect the lack of a secondary cell wall (Lobban and Harrison 1994). Cell wall secretions are first initiated once the spore has attached to the substratum (Lobban and Harrison 1994). Free swimming unicells also exhibit distinctly different photophysiology to the settled stages of macroalgae (Beach *et al.* 1995). For example, photosynthetic performance of settled zoospores of *Ulva fasciata* was more similar to that of mature thalli than to motile zoospores in terms of saturation irradiance ($I_k$) and maximum net photosynthetic rate ($P_{max}$) (Beach *et al.* 1995). Furthermore, the settlement stages of subtidal macroalgae exhibit adaptations (such as a low saturation point for photosynthesis and growth), which enhance survival in low light environments (Kain and Norton 1990, Lüning and Neushul 1978). Low-light-tolerant strategies enable reproductive unicells to utilise light more efficiently and persist in environments into which they have been released and must settle (Beach *et al.* 1995). However, this selective strategy may prove detrimental as UV-B radiation increases with decreasing stratospheric ozone concentrations during spring months.

The rapid decreases in $F_v$ in response to increasing UV-B radiation, in addition to the fact that $F_v$ decreased in a dose-dependent manner to UV-B, indicated that $F_v$ may be suitable as a sensitive biomarker for UV-B exposure. Consistent trends were observed in similar but not identical experiments at intermediate exposure times. Likewise, results of decreased variable fluorescence in chloroplasts and leaves of higher plants following UV exposure have been reported (Björn *et al.* 1986, Renger *et al.* 1986). These results are in accordance with the findings of Larkum and Wood (1993) who concluded that $F_v$ may be used as an indicator of UV-B inhibition. The optimal quantum yield, $F_v / F_m$ ratio, was also measured in this study and showed

similar significant changes to UV-B exposure. However, $F_v$ is presented here because it was affected more rapidly and to a greater extent, thus it was found to be a more sensitive indicator of UV-B exposure. The weighted dose-response relationships of $F_v$ in zoospores and in gametes to UV-B exposure, based on the generalised plant response action spectrum (Caldwell 1971) and the inhibition of photosynthesis in the phytoplankton *Phaeodactylum* (Cullen *et al.* 1992) are shown in fig. 6.1c.

Only one other study has quantitatively measured UV-B effects on early life-stages (3 species of the brown macroalgae *Laminaria*), although most exposures in the study were performed on immature thalli (Dring *et al.* 1996b). Minimal $F_v$ / $F_m$ values were found after 15 min exposure to 0.9 W m$^{-2}$ UV-B in 7 d old gametophytes and in young sporophytes (10 cm length, age unknown) lowest $F_v$ / $F_m$ values occurred after 2 to 4 hours (Dring *et al.* 1996b). UV-radiation combined with PAR in natural sunlight may be even more damaging than UV-radiation alone. 15 to 30 min exposures to direct sunlight were sufficient to kill 1 day old gametophytes of 3 *Laminaria* species (UV irradiance not measured) (Lüning 1980) and young sporophytes of *L. digitata* and *L. hyperborea* (Han and Kain 1996).

Whilst the dose-response experiments in this study, described above, were performed in agitated water, simulating wave action, an interesting comparison could be made to UV-B exposure of zoospores in still water conditions. After only 10 minutes exposure to 0.5 W m$^{-2}$ in still water $F_v$ for the zoospores was reduced by approximately 20 % (Table 6.1) compared to a lack of response in $F_v$ after exposure with agitation (fig. 6.1a). This demonstrates the importance of further investigating the effects of UV attenuation from wave motion before an attempt to estimate possible biological effects of UV-B *in situ* can be made.

Consistent patterns emerged throughout these experiments, with a greater sensitivity in the sexual reproductive part of the life cycle (gametes fusing to form a zygote and developing into the sporophyte) compared with the asexual part of the cycle (zoospores developing into gametophytes). This was observed in free-swimming 12 hour old unicells after only 10 minutes exposure to UV-B radiation. Even at the lowest irradiance level (0.5 W m$^{-2}$) which is comparable to ambient levels in SW England in the month of the experiment (fig. 6.1), gametes experienced a significant decrease in variable fluorescence compared to a lack of response in zoospores. Longer exposure times of 1 hour duration to the highest UV-B irradiance (2.2 W m$^{-2}$) produced a similar response in sensitivity, and resulted in 42 % decrease in F$_v$ in zoospores as opposed to a 65 % reduction in gametes. Likewise, germination of fused gametes was more sensitive to UV-B exposure than germination of zoospores, with gametes being half as likely to germinate successfully following 1 hour exposure to 0.5 W m$^{-2}$ compared to controls, no effects were detected in zoospores. Similarly, greater decreases in relative growth rates were observed in sporophytes during a 7 day period. These decreases in germination success and growth rates clearly showed irreversible damage from UV-B exposure in reproductive unicells of *E. intestinalis*. This pattern of greater sensitivity in the sexual reproductive part of the life cycle could arise from the initial damage to the gametes and could lead to a decreased genotypic variability in the population, although most macroalgae show alternation of asexual and sexual reproduction from one generation to the next (Lobban and Harrison 1994). A shift towards UV-B induced asexual reproduction could have serious implications for algal populations, as this could limit the future potential of greater genetic tolerance to UV and other toxicants through sexual selection. There is an urgent need for more research in this area.

The sensitivity of gametophytes and sporophytes determines whether a species can become established in a given habitat. Recruitment success of macroalgae is naturally extremely low

with survival chances of 1 in 0.5 million and 1 in 9 million for two *Laminaria* species respectively (Chapman 1984). Furthermore, spores of opportunistic (i.e. *E. intestinalis*) and some late successional algal species have slow sinking rates and may remain near the water surface for several days (Hoffmann and Camus 1989). Consequently, further exposure to UV-B (equivalent to 27 and 31 % ozone depletion) resulting in inhibition of photosynthesis and impaired growth rates could have far reaching consequences for primary production in the near shore ecosystems.

Algal species differences in sensitivity to UV exposure have been reported in young gametophytes of macroalgae and in diatoms (Lüning 1980, Karentz *et al.* 1991b). For example, Lüning (1980) found that gametophytes of *L. saccharina* survived longer exposures to winter sunlight than *L. digitata* and *L. hyperborea*. Further, cell size in planktonic diatoms has also been shown to correlate with UV sensitivity. Small cells with larger surface area to volume ratio exhibited higher rates of DNA damage (Karentz 1994). Additionally, Bothwell and co-workers (Bothwell *et al.* 1994) found community composition changes in benthic diatoms, favouring larger cells in the presence of UV. Gametes and zoospores of *E. intestinalis* measure 6 - 8 µm and 8 - 10 µm respectively, in length (Fletcher 1989) and some red algal spore sizes range from 15 - 120 µm (Neushul 1972, Coon *et al.* 1972). Consequently, size of reproductive unicells may also contribute to varying sensitivity to UV radiation among macroalgal species. Any alterations in species diversity within algal communities could alter the transfer of energy through the aquatic food web. This may be the most critical aspect of the ecological consequences of ozone depletion. However, only ca. 8 out of over 5000 existing species of macroalgae have currently been investigated with regard to UV effects on reproduction.

## 6.6 CONCLUSIONS

It was shown that reproductive cells were up to 6 fold more sensitive than adult plants to UV-B exposure, measured as decreases in variable fluorescence and differences in growth rates. Irreversible damage to photosystem II occurred in both gametes and zoospores resulting in decreased growth rates in the young sporophytes and gametophytes. The greatest inhibitions were found in young sporophytes. Moreover, lower germination success in gametes compared to zoospores was detected after only 1 h exposure to UV-B. The implications of a shift towards UV-B induced asexual reproduction in algal populations are unknown. Effects of UV-B exposure in phytoplankton and macroalgae have as yet been investigated almost exclusively in mature or several day old immature algae. However, these results demonstrate a great need to investigate UV-B effects in reproductive unicells of several species, and they underline the importance of conducting such experiments to avoid under-estimation of the ecological significance of elevated UV-B radiation. Moreover, there is an urgent need for research investigating the interactive effects of UV-B and chemical stressors in macroalgae since coastal regions often act as sinks for industrial and agricultural compounds.

# CHAPTER 7

# INTERACTIVE EFFECTS OF UV-B RADIATION AND THE ANTIFOULING COMPOUND IRGAROL 1051 IN THE MARINE MACROALGA *ENTEROMORPHA INTESTINALIS.*

## 7.1 ABSTRACT

The interactive relationship between the s-triazine herbicide Irgarol 1051 and two UV-B irradiances were evaluated in the macroalga *Enteromorpha intestinalis.* The antifouling compound Irgarol 1051 was chosen because very little is known about its associated biological effects, although it is now used regularly on boats and ships. A three-factorial experimental design (UV-B, Irgarol 1051 and time) was used with 2 levels of UV-B irradiance (equivalent to an ambient daily dose in May and 20 % ozone reduction) and an Irgarol 1051 concentration of 1 $\mu g\ l^{-1}$.

Inhibitions in optimal quantum yield of approximately 20 % were found after exposure to UV-B or Irgarol 1051 (applied singly). When the two stressors were applied simultaneously, however, an additive effect resulting in further reductions of up to 19.6 % compared to a single treatment of Irgarol 1051 occurred. A greater reduction in $F_v\ /\ F_m$ of 6.3 % in thalli exposed both to Irgarol 1051 and the highest UV-B irradiance compared to the added effect of the two stressors occurred, indicating a potential synergistic effect. A trend of decreasing values with increasing UV-B exposure, followed by further decreases after exposure to both Irgarol 1051 and UV-B radiation was found for both $F_v\ /\ F_m$ and $F_v$. These decreases in chlorophyll fluorescence were accompanied by reductions in growth rate, amounting to 38.5 % after simultaneous exposure to both stressors.

# 7.2 INTRODUCTION

Intertidal algae must be able to cope with potential changes in salinity, nutrient availability, hydration and temperature as well as changes in visible light and UV due to the continuous tidal changes. The current knowledge regarding macroalgal tolerance to natural stressors has been thoroughly reviewed by Lobban and Harrison (1994), Davidson and Pearson (1996) and Franklin and Forster (1997). Although research into the interactive effects of these natural stressors is well established (Lobban and Harrison 1994 and references therein) almost nothing is known about the interactions between these stressors and UV radiation (Behrenfeld *et al.* 1993). Recently the importance of investigating effects of PAR and UV-A in combination with UV-B has been realised, since it was demonstrated by several workers that both antagonistic or synergistic effects can occur (Rundel 1983, Caldwell *et al.* 1986, Forster and Lüning 1996, Franklin and Forster 1997). Studies in interactions, however, between UV-B exposure and xenobiotic compounds such as trace metals, or organic compounds have very rarely been attempted (Dubé and Bornman 1992) with the exception of Polycyclic Aromatic Hydrocarbons (PAHs) (Gala and Giesy 1992).

Over 1.8 million organic compounds are produced by man at a growing rate of 250 000 new formulations per year. 300-500 of these reach commercial production with a current global production of 100-200 million tons per year (Zhou *et al.* 1996). It has been estimated that up to 30 % of this production reaches the environment, and can cause adverse affects for marine ecosystems (Stumm and Morgan 1981, Malins *et al.* 1988, Pridmore *et al.* 1992, Zhou *et al.* 1996). These compounds may cause effects either on their own or in combination. For example it is well-known that PAHs can be activated photochemically by UV-A and/or B irradiation causing synergistic effects. The consequences are that these compounds become

more reactive and hazardous than the additive effect of the two single stressors (Huang *et al.* 1993, Ren *et al.* 1994).

The addition of herbicides (in particular the s-triazine Irgarol 1051) to antifouling paints has only recently generated attention within scientific circles and in the media. Moreover, no previous work has investigated interactive effects between these herbicides and UV-B exposure. Irgarol 1051 which is highly stable in the marine environment (a half-life of 800 days in sea water) (Ciba-Geigy 1995a) is currently added to over 80 products registered for use as antifouling paints (HMSO 1994) (please see Section 1.2 for more details). Aqueous concentrations exceeding 500 ng l$^{-1}$ have been detected in several Mediterranean locations as well as in areas such as Sussex, Hampshire and Hull in Great Britain (Readman *et al.* 1993, Gough *et al.* 1994, Zhou *et al.* 1996, Tolosa *et al.* 1996). Concentrations as high as 1.7 µg l$^{-1}$ have been reported in marinas in the Côte D'Azur (Readman *et al.* 1993).

With reference to the stressors mentioned above, it is important to consider elevated UV-B radiation as an additional environmental stressor in the marine environment. Thus, combination effects of multiple stressors must be taken into account if we are to accurately assess the effect of springtime ozone depletion in the marine ecosystems. As a first step in assessing the effects of simultaneous exposure of a s-triazine herbicide and UV-B, the aims of this study were two-fold:

a) to investigate the interactive relationship between UV-B radiation and Irgarol 1051 on *in vivo* chlorophyll fluorescence in *Enteromorpha intestinalis*.

b) to relate this physiological parameter to consequent biological effects measured as percentage increase in growth rate.

## 7.3 MATERIALS & METHODS

## Sample collection and preparation

Mature thalli from the intertidal macroalga *Enteromorpha intestinalis* (L.) (Chlorophyta) were collected at low tide (1.4 m depth from mean HWM) from Wembury beach, Devon, in January 1998 (sea water temperature and salinity: 11.6 °C, 35 ‰). The thalli were harvested 12 hours prior to initiating the experiments and were kept in artificial seawater (Instant Ocean, 33 ‰) at constant temperature (15 °C) and in low PAR conditions (fluorescent tubes, Phillips TLD 32W/83 HF™, 59 µmol $m^{-2}s^{-1}$) in growth cabinets. The thalli were maintained in a 12:12h light:dark regime at 15 °C.

## UV-B and Irgarol 1051 treatments and experimental design

A three-factorial design was used to investigate the interactive effects of UV and Irgarol 1051 (the three factors were: UV-B, Irgarol 1051 and time).

The experimental set-up with 6 treatments was as follows:

| Control (PAR, UV-A) | UV-B ambient (+PAR, UV-A) | UV-B elevated (+PAR, UV-A) |
|---|---|---|
| + | + | + |
| 1) Control + ethanol | 3) Control + ethanol | 5) Control + ethanol |
| 2) Irgarol + ethanol | 4) Irgarol + ethanol | 6) Irgarol + ethanol |

After the initial acclimation period the thalli were cut to a length of 15 mm and placed in 20 ml glass containers with either nutrient enriched ($NO_3^-$, 50 mg $l^{-1}$ and $PO_4^{3-}$, 10 mg $l^{-1}$) Instant Ocean™ or Irgarol 1051 solution. Chlorophyll fluorescence was measured prior to the experimental period. Five replicates within each treatment were used for chlorophyll fluorescence measurements. After a ca. 20 % decrease in $F_v / F_m$ in the Irgarol 1051 treated samples (equivalent to a 13 h period in treatment solution) thalli from all treatments were transferred to open Petri dishes, covered with 15 mm of the original treatment solution and exposed to UV.

The chosen UV-B exposure regimes were: unweighted irradiances of 2.4 and 3.3 W $m^{-2}$. These levels were chosen on the basis of previously performed dose-response experiments (Cordi *et al.* 1997). A three hour exposure period was used which is equivalent to doses of 25.7 and 35.7 kJ $m^{-2}$ unweighted UV-B and 7.3 and 10.1 kJ $m^{-2}$ weighted UV-B. These weighted doses correspond to an ambient daily dose in May and 20 % ozone depletion respectively. The ozone depletion level was calculated on the 15.07.97. The weighted doses and the ozone depletion levels were calculated according to the generalised plant response action spectrum (Caldwell 1971) during clear sky conditions at Plymouth. As this study was carried out on thalli collected in January the levels used were comparatively high. The objective of this study was to investigate the interactive relationship between UV-B and Irgarol 1051 in short-term experiments, therefore relative high levels of both UV-B and Irgarol 1051 were chosen to achieve an initial decrease in optimal quantum yield of 20 %. UV-A was kept low, constant and independent of the UV-B exposure levels at an unweighted irradiance of 3.2 W $m^{-2}$. This irradiance is within the same order of magnitude as maximum theoretical levels calculated for 50° latitude (Cornwall) in December (personal comm. with Dr. Driscoll, NRPB; Driscoll *et al.* 1992). During control exposures UV tubes were covered

with Mylar 125 D, and during UV exposures UV tubes were filtered with 35 μm cellulose diacetate foil. PAR irradiance was kept constant and similar to the acclimation period.

## Algal conditions following UV exposure

After the 3 h UV exposure period the algae were transferred to Petri dishes labelled for individual recognition. They were kept in constant temperature cabinets (15 °C) in low PAR conditions of 59 μmol m$^{-2}$ s$^{-1}$ in a 12:12 h light:dark regime. The enriched treatment solutions were changed every 12 h.

## Growth measurements

The physiological chlorophyll fluorescence parameters and the Irgarol 1051 analyses were related to differences in thallus growth by measuring increases in the thallus area. The growth measurements were performed on thalli kept in separate, labelled Petri dishes containing the same treatment solutions used throughout the experimental period. Increases in thallus area were measured daily over a 6 day period on 12 replicates with an Quantimet Image Analyser as described in Chapter 3.

## Preparation of Irgarol 1051 solution and extraction

Test solutions of Irgarol 1051 (>97% purity, Ciba-Geigy, Basel Switzerland) were prepared from a stock solution of 2.5 mg l$^{-1}$ as described by Scarlett *et al.* 1997. A concentration of 1 μg l$^{-1}$ (4 nM) (4x10$^{-5}$ % ethanol) was used in the experiments, this is below maximum concentrations measured in the environment (Readman *et al.* 1993). This concentration was selected from exposure-response experiments performed by Scarlett *et al.* (1997). Control

solutions containing equal amounts of $4 \times 10^{-5}$ % ethanol and Instant Ocean only were also prepared.

**Extraction of Irgarol 1051 in water samples**

Irgarol 1051 extractions from tissue and water samples (in Petri dishes and glass containers) were performed on 3 replicates both at 0 hr and 24 h to determine the extent of Irgarol 1051 loss. As a minimum of 200 ml and 1 g tissue was necessary for the water and the tissue analyses respectively, it was necessary to use a greater volume of water or quantity of tissue for the analyses compared to that used in the experiments. However, care was taken to use an identical water / tissue ratio to that used in the experiments.

Treatment solutions were transferred from exposure vessels into volumetrics to a final volume of 250 cm$^3$ by adding deionised water. Samples were shaken for 2 min with 5 cm$^3$ aliquots of hexane (X3) to extract the Irgarol 1051. Exposure vessels and volumetrics were each rinsed with 5 cm$^3$. Ametryn internal standards were added to the extracts, which were then transferred to -15 °C to facilitate separation of the solvent from the water.

Water samples (200 ml) were stored at 4 °C for no longer than 4 days prior to extraction. Extraction and analysis were carried out as described by Zhou *et al.* (1996). Samples were analysed using GC-MS in selected ion monitoring (SIM) mode. The selected Irgarol 1051 ions were 182, 238, with 253 amu used for quantification, and for the ametryn the ions were 184, 227 with 212 for quantification. Analyses were performed with a Hewlett Packard GC 5890 Series I, equipped with an automated on-column injector and a HP5MS capillary column (30 m X 0.25 mm X 0.25 µm) with an uncoated pre-column (1 m X 0.25 mm) coupled to a Hewlett Packard mass selective detector 5972 (electron impact, quadrupole). The carrier gas was helium; GC conditions were as described by Zhou *et al.* (1996). The

calibration curve for Irgarol 1051 with GC-MS was linear over the range 0 - 10 ng $\mu l^{-1}$ (r = 0.9998) and the limit of detection for the water samples was 5 ng $l^{-1}$.

**Extraction of Irgarol 1051 in algal tissue**

Thalli (1 g wet weight) were cut and mixed with 10 g anhydrous sodium sulphate, placed in cellulose extraction thimbles (20 mm X 50 mm) and internal standard spikes (ametryn) were added. The algal tissue was solvent extracted with 50 $cm^3$ of 1:1 acetone-dichloromethane (DCM) for 20 hours using 25 mm bore soxhlets. The extracts were concentrated under nitrogen; 2 $cm^3$ of isohexane was added and the extract evaporated under nitrogen again. This process was repeated three times to ensure that all the acetone was removed.

The samples were purified using alumina column chromatography eluted with solvents of increasing polarity. Chromatographic glass columns were filled with 3 g of alumina (5 % de-activated with Milli-Q water™) and topped with 1 cm of anhydrous sodium sulphate. The sample extracts were loaded onto the columns in 2 $cm^3$ of isohexane and eluted with 20 $cm^3$ of isohexane followed by 20 $cm^3$ of isohexane-DCM (1:1). The last 20 $cm^3$ were collected and concentrated to 0.3 $cm^3$ under nitrogen. The purified extracts were analysed by GC-MS as described above. The limit of detection for the tissue samples was 1 ng $l^{-1}$. Water and tissue extractions were carried out by Alan Scarlett.

## Statistical analyses

For both chlorophyll fluorescence and the growth data sequential sampling of each thallus was performed. A three factor ANOVA (with or without Irgarol 1051, three levels of UV-B irradiance (0, 1UV-B, 2UV-B) and two time intervals) was used for chlorophyll fluorescence data to investigate the interactive relationship between UV-B and Irgarol 1051. The two time

intervals used were after the 3 h UV exposure and after 31 h. Values measured at these two time periods were subtracted from the pre-exposure value and then analysed. Growth data were analysed by multiple regression analysis on log transformed data calculated as percentage increase in area compared to the first day.

## 7.4 RESULTS

## Chlorophyll fluorescence measurements

An overall significant decrease in $F_v / F_m$ was observed following a 3 hour exposure to two levels of UV-B (2.4 and 3.3 W $m^{-2}$, 3 factorial ANOVA, p< 0.00001, presented in fig. 7.1a). In Figure 7.1a Furthermore, an overall significant decrease in $F_v / F_m$ was also found after 13 h of exposure to Irgarol 1051 and throughout the experimental period (3 factorial ANOVA, p< 0.00001). A significant interaction between UV-B and Irgarol 1051 was found (p<0.02) indicating that the effect of each level of UV-B was dependent of the presence of Irgarol 1051 (and vice versa); thus the effect of Irgarol 1051 differed between the two levels of UV-B. No overall significant time effect was observed between the 3 hr exposure and the 31 hours (p> 0.5). ANOVA tables are presented in Table 7.1.

After the 3 h exposure to the two levels of UV-B (equivalent to an ambient July dose and 20 % ozone depletion) a decrease in the optimal quantum yield of 10.8 and 17.1 % respectively compared to controls, occurred in thalli which were only exposed to UV-B as depicted in Figure 7.1a. In thalli treated only with Irgarol 1051 an initial average decrease of 20 % was found after 13 h. Thalli exposed to both Irgarol 1051 and the two UV-B irradiances experienced a further decrease of 13.0 and 19.6 % respectively compared to thalli treated only with Irgarol 1051, amounting to a total decrease of 40 and 46.6 %.
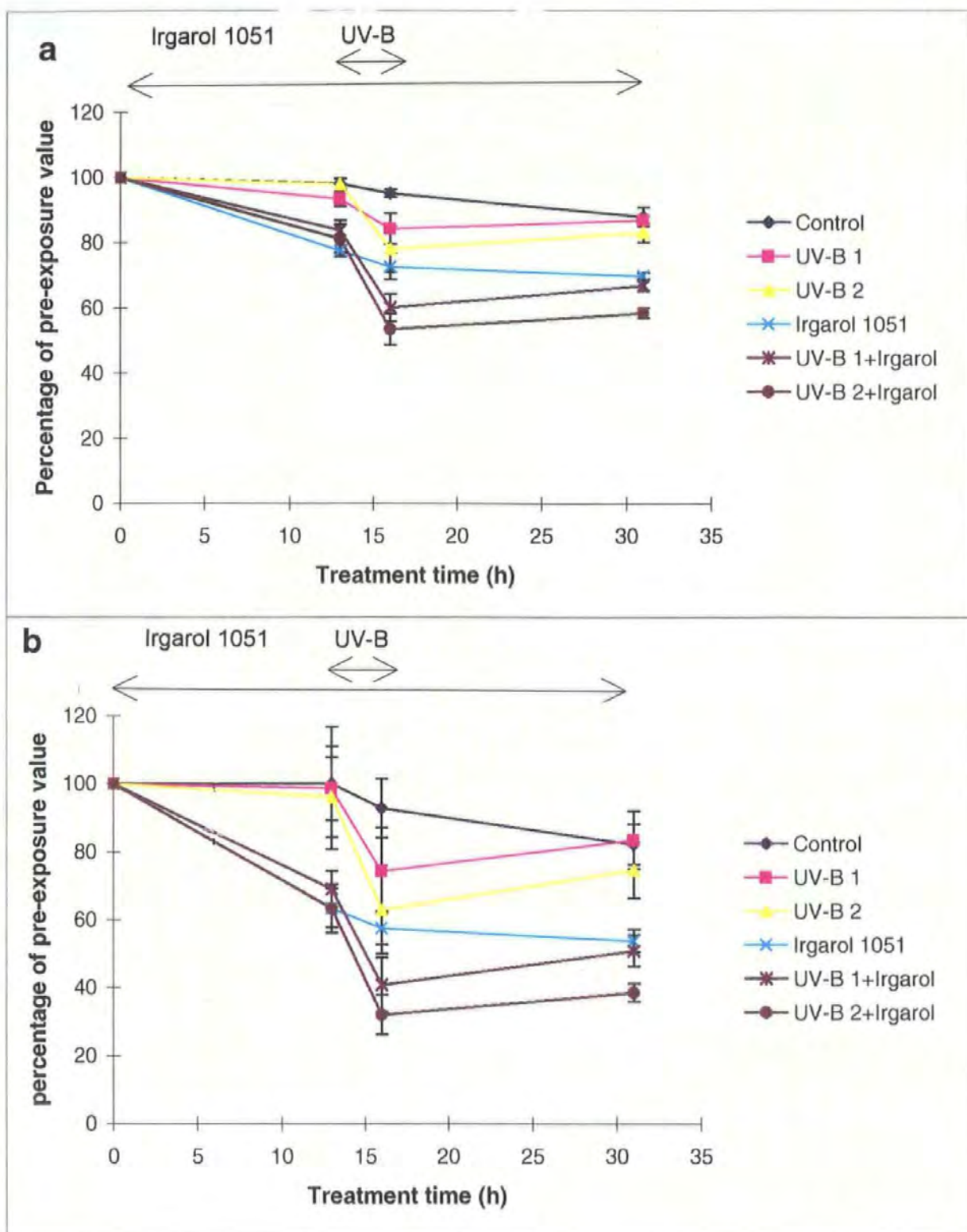
Figure 7.1a, b. Chlorophyll fluorescence as a function of treatment time to UV-B irradiance and/or Irgarol 1051. a) $F_v / F_m$ ratio calculated as percentage of pre-exposure value. b) variable fluorescence, $F_v$, calculated as percentage of pre-exposure value. A 3 hour exposure to unweighted UV-B irradiance of 2.4 W m$^{-2}$ (UV-B 1) or 3.3 W m$^{-2}$ (UV-B 2) and/ or an Irgarol 1051 concentration of 1 µg l$^{-1}$ was used. Means ± SE, n = 5.

Table 7.1. ANOVA tables for the three factor ANOVA analyses (with or without Irgarol 1051, three levels of UV-B irradiance (0, 1UV-B, 2UV-B) and two time intervals). The two time intervals used were after the 3 h UV exposure (refered to as 13 h on figures) and after 31 h. Values were subtracted from the pre-exposure value and then analysed.

## ANOVA table for $F_v$ / $F_m$ data

| Source | Sums of Squares | Df | Mean Square | F-ratio | P-value |
|---|---|---|---|---|---|
| Main effects | | | | | |
| A: irgarol | 0.354 | 1 | | 80.24 | 0.000 |
| B: time | 0.0018 | 1 | | 0.40 | 0.531 |
| C: UV | 0.229 | 2 | | 25.92 | 0.000 |
| Interactions | | | | | |
| AB | 0.006 | 1 | 0.006 | 1.35 | 0.251 |
| AC | 0.038 | 2 | 0.019 | 4.25 | 0.019 |
| BC | 0.095 | 2 | 0.048 | 10.77 | 0.000 |
| Residual | 0.225 | 51 | 0.0044 | | |
| Total, corrected | 0.956 | 60 | | | |

## ANOVA table for $F_v$ data

| Source | Sums of Squares | Df | Mean Square | F-ratio | |
|---|---|---|---|---|---|
| Main effects | | | | | |
| A: irgarol | 1.35E6 | 1 | 1.351E6 | 16.7 | 0.000 |
| B: time | 8335.39 | 1 | 8335.39 | 0.10 | 0.749 |
| C: UV | 1.026E6 | 2 | 513073.0 | 6.34 | 0.0035 |
| Interactions | | | | | |
| AB | 64577.9 | 1 | 64577.9 | 0.80 | 0.376 |
| AC | 222042.0 | 2 | 111021.0 | 1.37 | 0.263 |
| BC | 479101.0 | 2 | 239550.0 | 2.96 | 0.061 |
| Residual | 4.125E6 | 51 | 80878.6 | | |
| Total, corrected | 7.266E6 | 60 | | | |

## ANOVA table for Chlorophyll fluorescence complementary area

| Source | Sums of Squares | Df | Mean Square | F-ratio | P-value |
|---|---|---|---|---|---|
| Main effects | | | | | |
| A: irgarol | 7.52E8 | 1 | 7.521E8 | 25.9 | 0.000 |
| B: time | 1.41E8 | 1 | 1.408E8 | 4.85 | 0.032 |
| C: UV | 1.678E8 | 2 | 8.388E7 | 2.89 | 0.065 |
| Interactions | | | | | |
| AB | 929815.0 | 1 | 929815.0 | 0.03 | 0.859 |
| AC | 5.912E7 | 2 | 2.956E7 | 1.02 | 0.369 |
| BC | 4.983E7 | 2 | 2.491E7 | 0.86 | 0.430 |
| Residual | 1.481E9 | 51 | 2.904E7 | | |
| Total (corrected) | 2.659E9 | 60 | | | |

After 31 h, thalli which were only exposed to UV-B were not significantly different to control thalli. The $F_v / F_m$ ratio in thalli exposed to Irgarol 1051 and the highest UV-B irradiance were still reduced by 29.6 % after 31 h compared to controls. This decrease was equivalent to a further 12 % reduction compared to thalli treated with Irgarol 1051; amounting to 6.3 % more than the inhibition found from an added effect (29.6% compared to 23.3 %).

A similar trend found for $F_v / F_m$, of decreasing levels with increasing UV-B exposure and further decreases after simultaneous exposure to both stressors, was also measured for variable fluorescence, as depicted in Fig 7.1b. An overall significant decrease in $F_v$ was observed following a 3 hour exposure to UV-B and after 31 hours (3 factorial ANOVA, p< 0.004). Reductions of 18.5 % and 29.8 % compared to controls were found after exposure to the two UV-B irradiances respectively. Similarly, an overall significant decrease in $F_v$ occurred after exposure to Irgarol 1051 (3 factorial ANOVA, p< 0.0002). Maximal reductions of 51.9 % and 60.7 % compared to controls were measured after 16 h following simultaneous exposure to Irgarol 1051 and the two UV-B irradiances respectively. No interaction between UV-B and Irgarol 1051 was found (p> 0.2) and no overall time effect was observed between the 3 hr exposure and after 31 hours (p> 0.7).

Likewise, a highly significant decrease in the area calculated from the fluorescence induction curve was detected in thalli treated only with Irgarol 1051 as shown in Figure 7.2 (3 factorial ANOVA, p< 0.00001). A reduction of up to 52 % compared to controls was measured in thalli exposed to Irgarol 1051 for 31 hours. A significant time effect was found after 16 and 31 hours treatment (p< 0.03). However, no significant decrease in the area occurred after exposure to the two UV-B irradiances (p> 0.06).

## Growth measurements

Highly significant decreases in growth rates were observed in thalli from all treatment groups (fig. 7.3) (multiple regression, p< 0.01). Thalli exposed to 3.3 W m$^{-2}$ were initially (day 1) significantly larger than thalli from the other treatments (One-way ANOVA performed on intercepts (raw data), p< 0.05, F-ratio=3.45, degrees of freedom=66, $MS_{error}$=115.8). However, this difference was not reflected in any particular pattern in growth rates between the different treatment groups. Thus, it can be assumed that this initial difference in size did not cause any bias in the measurements of growth rates. Decreases in growth rates of 15.4 % and 23.1 % compared to controls (growth rates of 0.11 and 0.1 compared to 0.13 for controls) occurred in thalli exposed to UV-B irradiances of 2.4 and 3.3 W m$^{-2}$ respectively. Thalli exposed only to Irgarol 1051 suffered reductions of 30.8 % compared to controls (calculated from a growth rate of 0.09). Moreover, thalli exposed to both Irgarol 1051 and the two UV-B irradiances experienced decreases of 35.4 % and 38.5 % (growth rates of 0.084 and 0.08) respectively compared to control thalli.

## Irgarol 1051 extraction analyses

Table 7.2 lists the concentrations of Irgarol 1051 measured in the water samples and in the macroalgal tissue respectively. Approximately 30 % and 45 % of the Irgarol 1051 in the water samples was taken up by tissue contained in glass ware or in Petri dishes respectively after 24 hours.
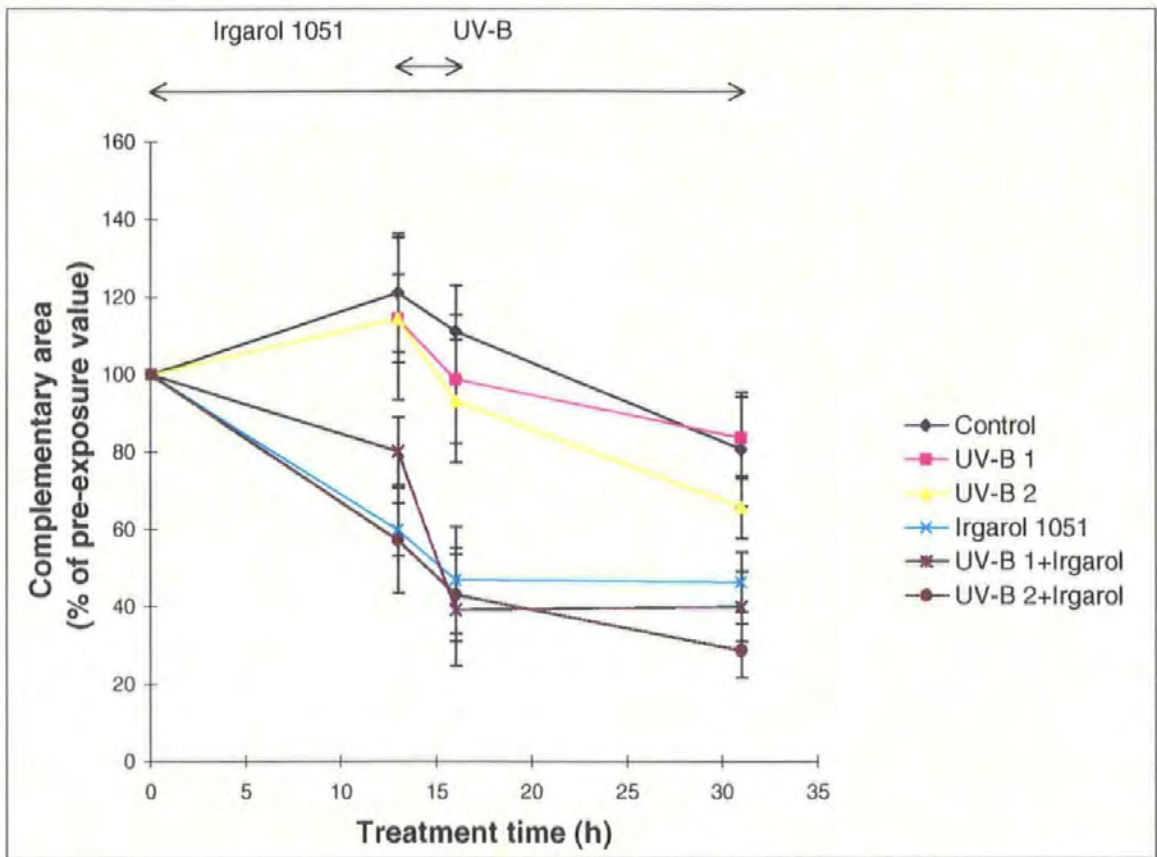
Figure 7.2. Chlorophyll fluorescence complementary area, calculated from the fluorescence induction curve, as a function of treatment time to UV-B irradiance and/or Irgarol 1051. Arrows indicate the UV-B and Irgarol 1051 treatment periods. A 3 hour exposure to unweighted UV-B irradiance of 2.4 $W\ m^{-2}$ (UV-B 1) or 3.3 $W\ m^{-2}$ (UV-B 2) and/ or an Irgarol 1051 concentration of 1 $\mu g\ l^{-1}$ was used. Means ± SE, n = 5.
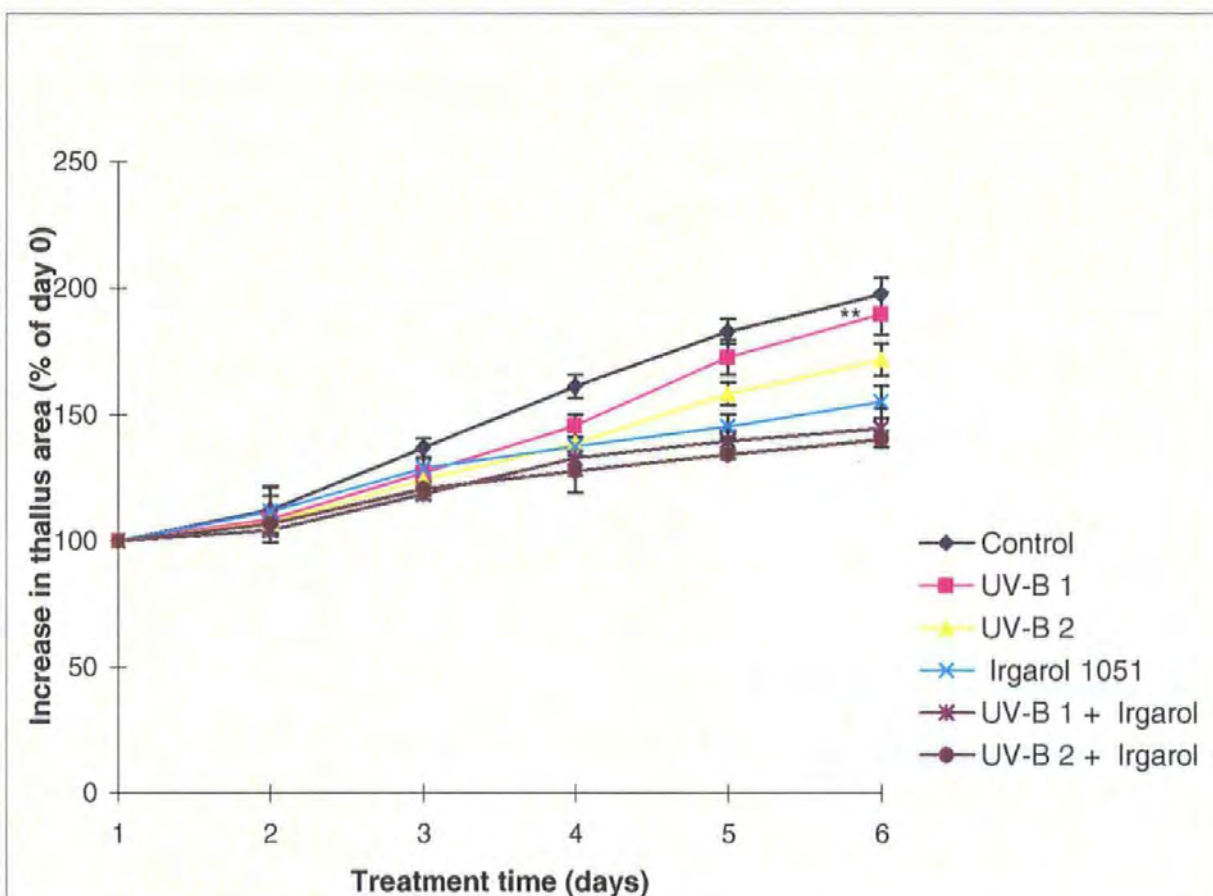
Figure 7.3. Increase in thallus area as a function of treatment time to UV-B irradiance and/or Irgarol 1051. UV-B irradiance of 2.4 (UV-B 1) or 3.3 W m$^{-2}$ (UV-B 2) and/ or an Irgarol 1051 concentration of 1 μg l$^{-1}$ was used. A 3 h exposure to either of the two UV-B irradiances was applied on day 0 in the UV-B treatments. Means ± SE, n = 12, ** indicate significant different slopes of log transformed data of all treatments compared to the slope of the controls, p<0.01.

Table 7.2. Analyses of Irgarol 1051 concentrations in water samples and in tissue samples of

*E. intestinalis*. Means ± SE, n=3, although for control tissue n=5.

| Water samples | | |
|---|---|---|
| Sample | Container used | Concentration (ng $l^{-1}$) |
| Pre-exposure solution | glass | 1091.2 ± 15.0 |
| Tissue 24 h in solution | glass | 773.9 ± 25.6 |
| No tissue, solution left for 24 h | Petri dishes | 601.6 ± 21.8 |
| Tissue samples | | |
| Control tissue | Petri dishes | Below detection limit |
| Tissue 24 h in solution | Petri dishes | 201 ± 6.5 |
| Tissue 48 h in solution | Petri dishes | 327 ± 31.7 * |

The detection limit in tissue samples was 1 ng $l^{-1}$.

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## 7.5 DISCUSSION

### Chlorophyll fluorescence

UV-B radiation and Irgarol 1051 singly and in combination reduces chlorophyll fluorescence following short-term exposures. Moreover, when the two stressors were applied simultaneously, an additive effect on the optimal quantum yield was found, resulting in further reductions of up to 19.6 % compared to a treatment of Irgarol 1051 applied alone. In addition, a greater decrease in $F_v / F_m$ was measured after 31 h in thalli exposed both to Irgarol 1051 and the highest UV-B irradiance compared to the added effect of the two stressors. This greater reduction amounting to 6.3 %, in combination with a significant interaction between the two stressors, may indicate a potential synergistic effect. Furthermore, there was no overall difference in $F_v / F_m$ between 16 h and 31 h, indicating a lack of recovery within this time period (this was also confirmed by the measured lack of a significant time effect). The concurrent exposure of UV-B and Irgarol 1051 also resulted in additive effects in variable fluorescence, $F_v$. Decreases of up to 60.7 % compared to controls were observed in thalli exposed to Irgarol 1051 and the highest UV-B irradiance. A maximum of 45 % of the Irgarol 1051 contained in the water samples was taken up by thalli after 24 hours (Table 7.2) and the treatment solution in the experiments was replaced every 12 h. Thus, it can be assumed that Irgarol 1051 uptake by thalli was not limited during the experimental period.

The triazine type herbicides are well-known inhibitors of electron transfer between $Q_A$ and $Q_B$, the primary and secondary plastoquinone electron acceptors of photosystem II (PS II) (Vermaas *et al.* 1984). These herbicides influence the variable fluorescence yield, as the

proportion of energy trapped by the PS II antenna which is not able to transfer beyond $Q_A$, is re-emitted as variable fluorescence (Samson and Popovic 1988). Similarly, UV-B radiation affects several target sites in photosynthesis. UV-B also impairs the D1 protein associated with PS II, resulting in a decrease of the noncyclic photosynthetic electron transport (Renger *et al.* 1989, Tevini *et al.* 1989, Melis *et al.* 1992, Franklin and Forster 1997). Therefore, as both stressors cause damage to the same target sites, interactive effects from simultaneous exposure can be expected, resulting in further decreases in $F_v$ / $F_m$ and $F_v$. The reductions in chlorophyll fluorescence measured in this study confirm the above findings and show that both the optimal quantum yield and the variable fluorescence are sensitive indicators of combination effects between these two stressors.

The complementary area calculated from the fluorescence induction curve was also very sensitive to treatment of Irgarol 1051, but was not affected by exposure to UV-B. The area is proportional to the PS II electron acceptor pool, which determines the photochemical activity of PS II (Lavorel *et al.* 1986, Samson and Popovic 1988). Melis and Screiber (1979) reported that the area above the induction curve was a direct indicator of PS II photochemistry, and Malkin *et al.* (1981) found a good correlation between this parameter and photosynthetic capacity estimated as $CO_2$ fixation. Samson and Popovic (1988) concluded that this parameter is a reliable and sensitive indicator of herbicide toxicity. However, as no response occurred following UV-B irradiation, it is not a suitable indicator of combination effects between s-triazines and UV-B.

Scarlett *et al.* (1997) measured greater reductions in optimal quantum yield (up to 10 %) after exposure to Irgarol 1051 compared to reductions reported here. These workers also exposed thalli of *E. intestinalis* to 1 µg l^{-1} (4 nM) Irgarol 1051 for 25 h. The difference in sensitivity between the two studies may be caused by a difference in the concentration of Irgarol 1051 at

the collection sites. The average concentration of Irgarol 1051 measured at Sutton Marina, the collection site chosen by these workers, was 84.3 ng l$^{-1}$ compared to a level below detection (1 ng l$^{-1}$) in water samples collected from Wembury (Scarlett *et al.* 1997). Hence, chronic contamination *in situ* may result in greater toxic effects compared to those found in this study. The average levels of Irgarol 1051 detected within marinas so far, e.g. 500 ng l$^{-1}$ at Port Solent (Gough *et al.* 1994) and 700 ng l$^{-1}$ on the Humber, are below the concentration used here (Zhou *et al.* 1996). However, if the current low usage of Irgarol 1051 were to increase, it is possible that estuarine concentrations may also approach or even exceed this level (Scarlett *et al.* 1997). Moreover, concentrations as high as 1.7 µg l$^{-1}$, measured at Côte d'Azur (Readman *et al.* 1993), could on the basis of the data presented here and the data of Scarlett *et al.* (1997), cause up to an extra 20 % decrease in PS II efficiency, amounting to a total decrease of approximately 70 %.

## Growth measurements

Reduced growth rates measured in thalli from all treatment groups indicate non-reversible damage following exposure to UV-B and Irgarol 1051. Increasing UV-B irradiance led to further reductions in growth rates, an effect which was even more pronounced with the addition of Irgarol 1051. Reductions of up to 38.5 % occurred after simultaneous exposure to both stressors. Physiological effects associated with exposure of algae to photosynthetic inhibitors (among several families of herbicides) have previously been reported (Gramlich and Frans 1964, Arvik *et al.* 1973, Hiranpradit and Foy 1992). Hiranpradit and Foy (1992) measured inhibitions in growth rate in several fresh water algal species following treatments of 4 s-triazines (applied singly). Concentrations as low as 0.23 µM of all four herbicides inhibited growth of *Chlamydomonas pyrenoidosa*, *C. reinhardtii* Danegeard, and

*Scenedesmus quadricauda* (Turp.). Gramlich and Frans (1964) found inhibitions in growth rate in *C. pyrenoidosa* following exposure to atrazine at a concentration of 1.0 μM.

Investigations into the effects of herbicide mixtures have been undertaken in aquatic macrophytes (Forsyth *et al.* 1997). Moreover, interactive effects in terrestrial plants following the addition of two herbicides have been measured (Devlin 1973, Bowes 1976, Lym and Messersmith 1985). Lym and Messersmith (1985) reported increased mortality of leafy spurge (*Euphorbia esula* L.) following exposure to the two herbicides, picloram and 2,4-D in Tordon® 202C compared to treatment of picloram alone.

Few workers have investigated the biological effects of Irgarol 1051 contamination. Dahl and Blanck (1996) found inhibited photosynthetic activity and algal biomass in periphyton communities, in short-term (hour) exposures, at Irgarol 1051 concentrations of 1-4 nM (0.25-1 μg $l^{-1}$). Long-term experiments (weeks) produced effects at lower concentrations of 0.25-1 nM (0.063-0.25 μg $l^{-1}$). Furthermore, Irgarol 1051 was 70 times more toxic to microalgal communities than atrazine, and it was concluded that Irgarol 1051 is likely to damage microalgal communities in contaminated coastal waters (Dahl and Blanck 1996). Shifts in community structure have also been observed for phytoplankton (Gustavson and Wängberg 1995). This study demonstrates for the first time the importance of considering possible combination effects between UV-B exposure and s-triazine herbicides. These results are relevant to the real-world situation where organisms are often exposed simultaneously to a variety of stressors, in contrast to many laboratory exposures that aim to elucidate the effects of individual stressors. If the combination effects found here also exist for other species of algae, then clearly the ecological significance of elevated UV-B exposure may be seriously under-estimated in coastal waters contaminated with Irgarol 1051.

# 7.6 CONCLUSIONS

UV-B radiation and Irgarol 1051 applied simultaneously resulted in additive effects on the optimal quantum yield and the variable fluorescence in *E. intestinalis*. A trend found for *in vivo* chlorophyll fluorescence, of greater reductions with increasing UV-B irradiance, followed by further decreases after simultaneous exposure of both stressors, was also reflected in decreases in growth rate.

The results presented here provide useful preliminary insights into the interactive effects of UV-B and Irgarol 1051. They heighten the concern that increased UV-B may result in adverse effects on macroalgal populations, as coastal regions often act as sinks for industrial and agricultural compounds. The fate and effects of Irgarol 1051 require substantial further evaluation. The objective of this study was to investigate the interactive relationship between these two stressors, hence relative high levels of both UV-B and Irgarol 1051 were used to achieve an initial decrease in optimal quantum yield of 20 %. However, the chosen Irgarol 1051 concentration of 1 $\mu$g l$^{-1}$ was within the highest concentrations reported in marinas elsewhere in Europe. More research is needed to investigate the effects of chronic exposures to lower levels of UV-B and Irgarol 1051. Additionally, there is an urgent need to investigate the effects of these stressors in non-target macroalgae. Also, it is important to consider the contamination of the coastal waters with other herbicides (Bester and Hühnerfuss 1993, Gough *et al.* 1994) which are similar to Irgarol 1051 in structure and mode of action, and which will contribute to combined effects (Altenburger *et al.* 1993).

# CHAPTER 8

# EFFECTS OF SIMULTANEOUS EXPOSURE TO UV-B AND IRGAROL 1051 IN TWO NON-TARGET MACROALGAE.

## 8.1 ABSTRACT

The non-target macroalgae *Palmaria palmata* and *Porphyra umbilicalis* (Rhodophyta) were studied to assess possible interactive effects between UV-B radiation and Irgarol 1051. Reductions in chlorophyll optimal quantum yield and growth rates were determined in a 3 factorial design (Irgarol 1051, UV-B and time). Levels of unweighted UV-B ($2.1$ W m$^{-2}$) and Irgarol 1051 ($5$ µg l$^{-1}$) were established in initial dose-response experiments. It was concluded from these experiments that these two non-target algae are relatively tolerant to Irgarol 1051 when applied alone. In the factorial experiment initial reductions in $F_v$ / $F_m$ were found after a single exposure to UV-B irradiation (14.5 % compared to controls in both species) and after 25 hours treatment with Irgarol 1051 (21.6 % in *P. palmata* and 24.7 % in *P. umbilicalis*). However, simultaneous exposure to both stressors caused further decreases in $F_v$ / $F_m$, amounting to 31.3 % in *P. palmata* and 38.4 % in *P. umbilicalis* compared to control thalli. A 90 % recovery of $F_v$ / $F_m$ ratio was observed for *P. umbilicalis*. In *P. palmata*, a lack of recovery in $F_v$ / $F_m$ was found after 25 h exposure to Irgarol 1051 and 50 min exposure to UV-B radiation. Thalli exposed continuously to Irgarol 1051 and 50 min to UV-B were unable to grow. Although non-target algae may not be particularly sensitive to short-term exposures to environmentally relevant concentrations of Irgarol 1051, interactive effects between UV-B and Irgarol 1051 may cause unexpected additive effects on optimal quantum yield and growth rates. In view of springtime changes in the ozone layer and the related

increases in UV-B, interactions between UV-B and chemical stressors should be studied across a wider range of herbicide types and algal species.

## 8.2 INTRODUCTION

The input and number of hydrophobic organic micropollutants (HOMs) such as pesticides, antifouling agents and polynuclear aromatic hydrocarbons (PAHs) released from industry and agriculture have increased dramatically in certain regions over the past decades (North Sea Report). These chemicals enter the aquatic environment via atmospheric transport, spray drift, groundwater leaching, soil run-off and sewage inputs (Zhou *et al.* 1996) and can cause detrimental effects on the marine biota (Reijnders 1986, Malins *et al.* 1988, Pridmore *et al.* 1992). The recent discovery of the ozone hole and the related increases in UV-B will therefore impose an additional environmental stress, apart from the present pollution stress from industrial waste and agricultural fertilisers.

Several surveys have recently documented the existence of Irgarol 1051 in marinas, estuaries and rivers at varying concentrations (Readman *et al.* 1993, Gough *et al.* 1994, Zhou *et al.* 1996, Tolosa *et al.* 1996). The most important question raised from these observations is whether Irgarol 1051, either alone or in combination with other herbicides and elevated UV-B, can adversely affect indigenous algal populations. Dahl and Blank (1996) demonstrated that Irgarol 1051 can produce detrimental effects on the biomass, net photosynthesis and community structure of microalgal populations in coastal water microcosms. Similar results were found for marine phytoplankton in mesocosms treated with environmentally realistic levels of the agrochemical triazine herbicide atrazine (Bester *et al.* 1995). Moreover, effects of elevated UV-B on macroalgal populations have been assessed by several workers (Larkum and Wood 1993, Grobe and Murphy 1994, Dring *et al.* 1996a,b, Cordi *et al.* 1997, Cordi *et*

*al.* 1998a,b). While this work has made a very valuable contribution to our understanding of the isolated effects of triazines and UV-B radiation in the marine environment, the results outlined in Chapter 7, on the fouling alga *E. intestinalis*, are the only ones avaliable on the interactive effects of these two stressors. The experiment described in this chapter may be considered as the first step in assessing the interactive effects of UV-B and Irgarol 1051 in non-target algae.

The aims of this study were to:

a) determine the concentrations at which Irgarol 1051 affects the Photosystem II efficiency in the two non-target macroalgae *Palmaria palmata* and *Porphyra umbilicalis* (Rhodophyta).

b) establish dose-response curves for varying doses of UV-B in both species.

c) investigate combination effects of Irgarol 1051 and UV-B on optimal quantum yield and to relate these changes to adverse effects on growth rate.

## 8.3 MATERIALS & METHODS

The study consisted of two parts, a series of dose-response experiments with UV-B radiation and Irgarol 1051 and a 3 factorial experiment exposing the algae simultaneously to both stressors.

### Sample collection and preparation

Mature thalli of *Porphyra umbilicalis* (L.) (Rhodophyta) and *Palmaria palmata* (L.) (Rhodophyta) were collected at low tide from Wembury beach in May and June 1998 (sea water temperature and salinity: 16.0 °C, 35 ‰). *P. umbilicalis* and *P. palmata* were collected at approximately 1.7 m and 5.2 m depth from the average high water mark respectively. The

thalli were harvested 12 hours prior to initiating the experiments and kept in artificial sea water (Instant Ocean, 33 ‰) at constant temperature (15 °C) and in low PAR conditions (fluorescent tubes, Phillips TLD 32W/83 HF™, 33 $\mu$mol m$^{-2}$s$^{-1}$) in growth cabinets. This PAR irradiance level was kept constant throughout the different experiments. The thalli were maintained in a 12:12h light:dark regime at 15 °C. After the acclimation period branches of *P. palmata* plants were cut at the bases to an approximate length of 15 mm and thalli of from *P. umbilicalis* were cut into discs of 18 mm diameter. The thalli were placed in nutrient enriched ($NO_3^-$, 50 mg l$^{-1}$ and $PO_4^{3-}$, 10 mg l$^{-1}$) Instant Ocean for the experiments.

## Exposure-response experiments

Test solutions of Irgarol 1051 (>97% purity, Ciba Geigy, Basel Switzerland) were prepared from a stock solution of 2500 $\mu$g l$^{-1}$ as described by Scarlett *et al.* (1997). Concentrations of: 1, 2.5, 5, 10, 25, 50, 200, and 500 $\mu$g l$^{-1}$ (0.01% ethanol) were prepared from the stock solution. Both test solutions and the stock solution were allowed to mix thoroughly by magnetic stirrer. Control solutions containing equal amounts of 0.01 % ethanol and Instant Ocean only were also prepared. The thalli were placed in individually marked Petri dishes with either nutrient enriched Instant Ocean or Irgarol 1051 solution. The enriched treatment solutions were changed every 12 hours. Petri dishes were used in this study based on findings from the water sample analyses in the previous chapter that they are as suitable as glass containers for Irgarol 1051 experiments (Please see Table 7.1 in the previous chapter). Care was taken to use a similar water / tissue ratio as in the previous chapter. *In vivo* chlorophyll fluorescence was measured repeatedly on 6 replicates over a 72 h time period.

Dose-response experiments with varying UV-B doses were also performed on both species. An unweighted UV-B irradiance of 2.1 ± 0.2 W m$^{-2}$, equivalent to 0.58 W m$^{-2}$ weighted UV-

B irradiance was used with increasing exposure times. The weighted UV-B irradiance and doses were calculated according to the generalised plant response action spectrum (Caldwell 1971). UV-A was kept low, constant and independent of the UV-B doses at an unweighted irradiance of 9.6 W m$^{-2}$. This irradiance is similar to the maximum theoretical levels calculated for 50° latitude (Cornwall) in January (personal comm. with Dr. Driscoll, NRPB; Driscoll *et al.* 1992). For control exposures, UV tubes were covered with Mylar 125 D and for UV-B exposures UV tubes were filtered with 35 μm cellulose diacetate foil (no UV-C). The thalli were transferred to open Petri dishes, covered with 15 mm of enriched sea water and exposed to UV. Five replicates within each treatment were used for chlorophyll fluorescence measurements.

## UV and Irgarol 1051 factorial experiment

A three-factorial design was used to investigate the interactive relationship between UV-B and Irgarol 1051 over time (the three factors were: UV, Irgarol 1051 and time).

The experimental set-up with 4 treatments was as follows:

| Control (No UV-B + PAR, UV-A) | UV-B ambient + PAR, UV-A |
|---|---|
| + | + |
| 1) Control + ethanol | 3) Control + ethanol |
| 2) Irgarol + ethanol | 4) Irgarol + ethanol |

In the factorial study, a reduction in optimal quantum yield of approximately 25 % was desired for both species following an isolated treatment with either Irgarol 1051 or UV-B.

Therefore, from the dose-response experiments, an Irgarol 1051 concentration of 5 $\mu gl^{-1}$ was chosen for both species. Test solutions of Irgarol 1051 and control solutions were prepared as described above. After 25 hours in the treatment solutions, thalli from all treatments were transferred to open Petri dishes, covered with 15 mm of the original treatment solution and exposed to UV. An unweighted UV-B irradiance of 2.1 W $m^{-2}$, similar to the dose-response experiments, was used. Exposure periods of 50 min and 6 hours were chosen for *P. palmata* and *P. umbilicalis* respectively, to achieve ca. 25 % reduction in $F_v$ / $F_m$. These exposure periods were equivalent to doses of 6.2 and 44.7 kJ $m^{-2}$ unweighted UV-B and 1.8 and 12.6 kJ $m^{-2}$ weighted UV-B. The weighted doses correspond to less than ambient daily dose in April and 30 % ozone depletion respectively (calculated for 15.07.97). The weighted doses and the ozone depletion levels were calculated according to the generalised plant response action spectrum (Caldwell 1971) during clear sky conditions at Plymouth. The ozone depletion level was calculated on the 15.07.97. UV-A and PAR were kept constant and similar to the dose-response experiments. Nine replicates within each treatment were used for chlorophyll fluorescence measurements.

## Algal conditions following UV exposure

After the UV exposure period, the algae were transferred to labelled Petri dishes containing enriched Instant Ocean and the water was changed daily. They were kept in constant temperature cabinets (15 °C) in low PAR conditions of 59 $\mu mol$ $m^{-2} s^{-1}$ in a 12:12 h light:dark regime. The thalli were maintained in these conditions for a further 72 h to investigate recovery patterns.

## Growth measurements

Following the UV exposure separate thalli used only for growth measurements were transferred to 2 l tanks which contained the same treatment solutions throughout the experimental period. The solutions were changed every 2 days. To allow individual identification the thalli were tied to small free-standing glass holders placed on the bottom of the tanks. Thalli of *P. palmata* were tied by the base and thalli of *P. umbilicalis* were tied by making a small hole through the thallus. The measurements were performed on separate thalli, and increases in thallus area were measured daily over a 7 day period with an Quantimet Image Analyser as described in Chapter 3. Nine replicates were chosen for both species.

## Statistical analyses

For both chlorophyll fluorescence and growth data, sequential sampling of each thallus was performed. To investigate the interactive relationship between UV-B and Irgarol 1051, multi factor ANOVAs were applied to the chlorophyll fluorescence data. A Two factor ANOVA (with or without Irgarol 1051; with or without UV-B radiation) was applied to chlorophyll fluorescence data collected after the simultaneous exposure to both UV-B and Irgarol 1051. Further, data collected during the recovery period was analysed using a three factor ANOVA (with or without Irgarol 1051; with or without UV-B radiation; and 3 time intervals). All values were subtracted from the pre-exposure value and then analysed. Growth data were analysed by multiple regression analysis calculated as percentage increase in area compared to the first day.

## 8.4 RESULTS

### Dose-response experiments

The unweighted and weighted UV-B dose-response curves for *P. palmata* appeared to follow square root-X models as depicted in fig. 8.1a. The following calculations are based on the measured data (not the estimated models). An unweighted dose of 3.9 kJ m$^{-2}$ (corresponding to a weighted dose of 1.1 kJ m$^{-2}$, equivalent to approximately ambient daily dose in April) resulted in 27 % reduction in optimal quantum yield compared to control thalli. A 50 % reduction in $F_v$ / $F_m$ compared to control exposures was observed after exposure to an unweighted UV-B dose of 12.0 kJ m$^{-2}$ (equivalent to 3.38 kJ m$^{-2}$ weighted UV-B and an ambient daily dose in May). For *P. umbilicalis* both unweighted and weighted dose-response curves appeared to follow exponential models; the equations are included on Figure 8.1b. An unweighted dose of 44.7 kJ m$^{-2}$ (equivalent to weighted dose of 12.6 kJ m$^{-2}$, equivalent to a 30 % ozone depletion in July) resulted in a 20.1 % reduction in $F_v$ / $F_m$ compared to the control exposure. A 50 % reduction was found after an unweighted dose of 95 kJ m$^{-2}$.

The effect of Irgarol 1051 on $F_v$ / $F_m$ ratio was observed for *P. palmata* after only 3 hours exposure to 25 µg l$^{-1}$ (fig. 8.2a). Incubation for longer periods of time resulted in marked decreases in $F_v$ / $F_m$ ratio with lower Irgarol 0151 concentrations. Apparent equilibration was found after 25 hours for concentrations below 25 µg l$^{-1}$ and after 72 hours for concentrations above 25 µg l$^{-1}$. A 20 % reduction in $F_v$ / $F_m$ was observed after 48 hours treatment with an Irgarol 1051 concentration as low as 1 µg l$^{-1}$, and 30 % reduction was measured after 25 hours treatment with 5 µg l$^{-1}$. An apparent EC 50 (72 h), for this species, was found after exposure to 25 µg l$^{-1}$. Decreases in optimal quantum yield of approximately 20 % was measured for *P. umbilicalis* after 25 h treatment to a concentration of 2.5 µg l$^{-1}$ and 30 % decrease was noted with a concentration of 5 µg l$^{-1}$ (fig. 8.2b). $F_v$ / $F_m$ ratio appeared to reach
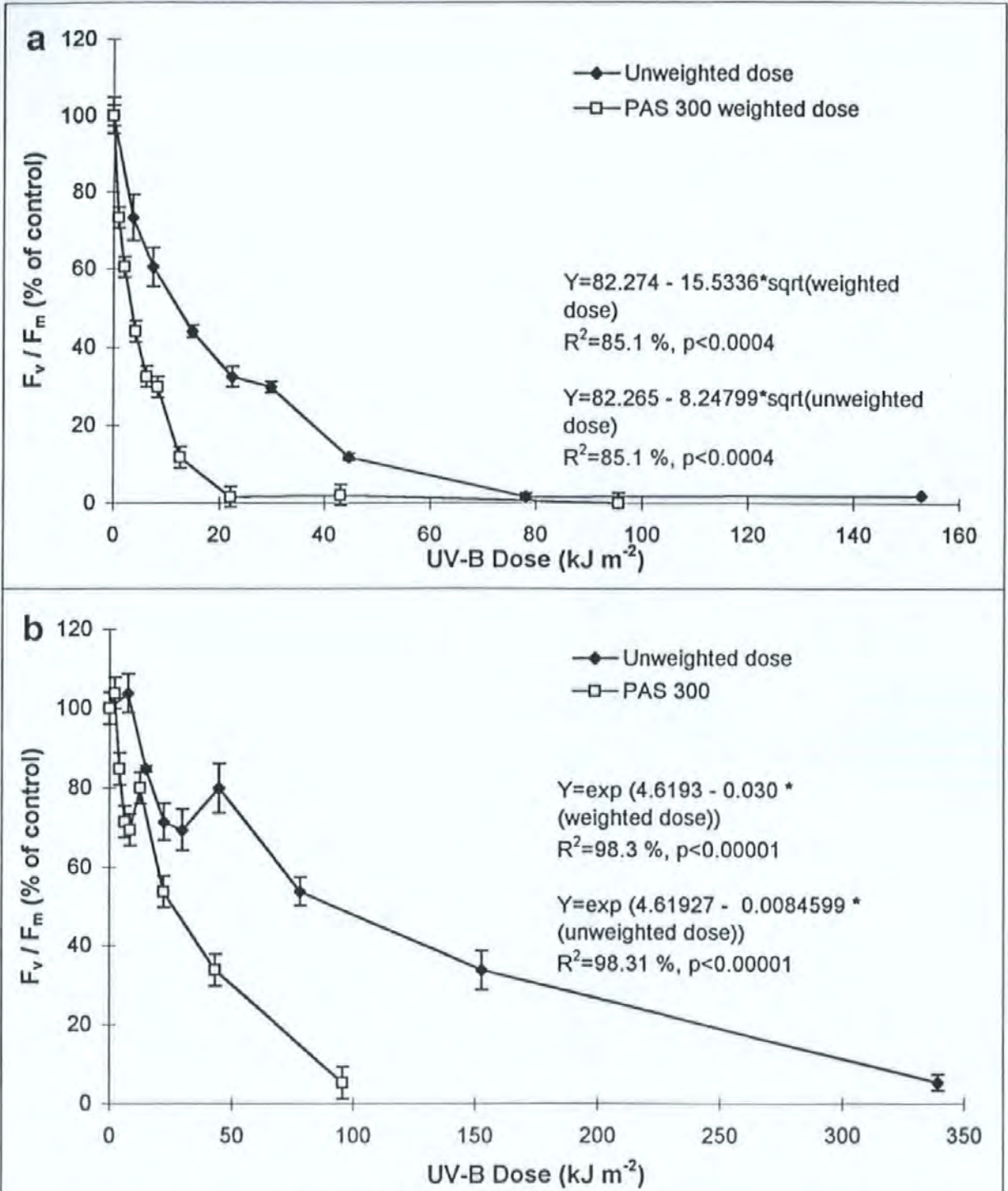
Figure 8.1. Weighted and unweighted dose-response curves for a) *Palmaria palmata* and b) *Porphyra umbilicalis* showing the effect of an unweighted UV-B irradiance of 2.1 W m$^{-2}$ on $F_v / F_m$ ratio in thalli exposed for varying lengths of time. The weighted curves were calculated according to the generalised plant response action spectrum (Caldwell 1971). The equations for best fitted models are included. Means ± SE, n = 5.
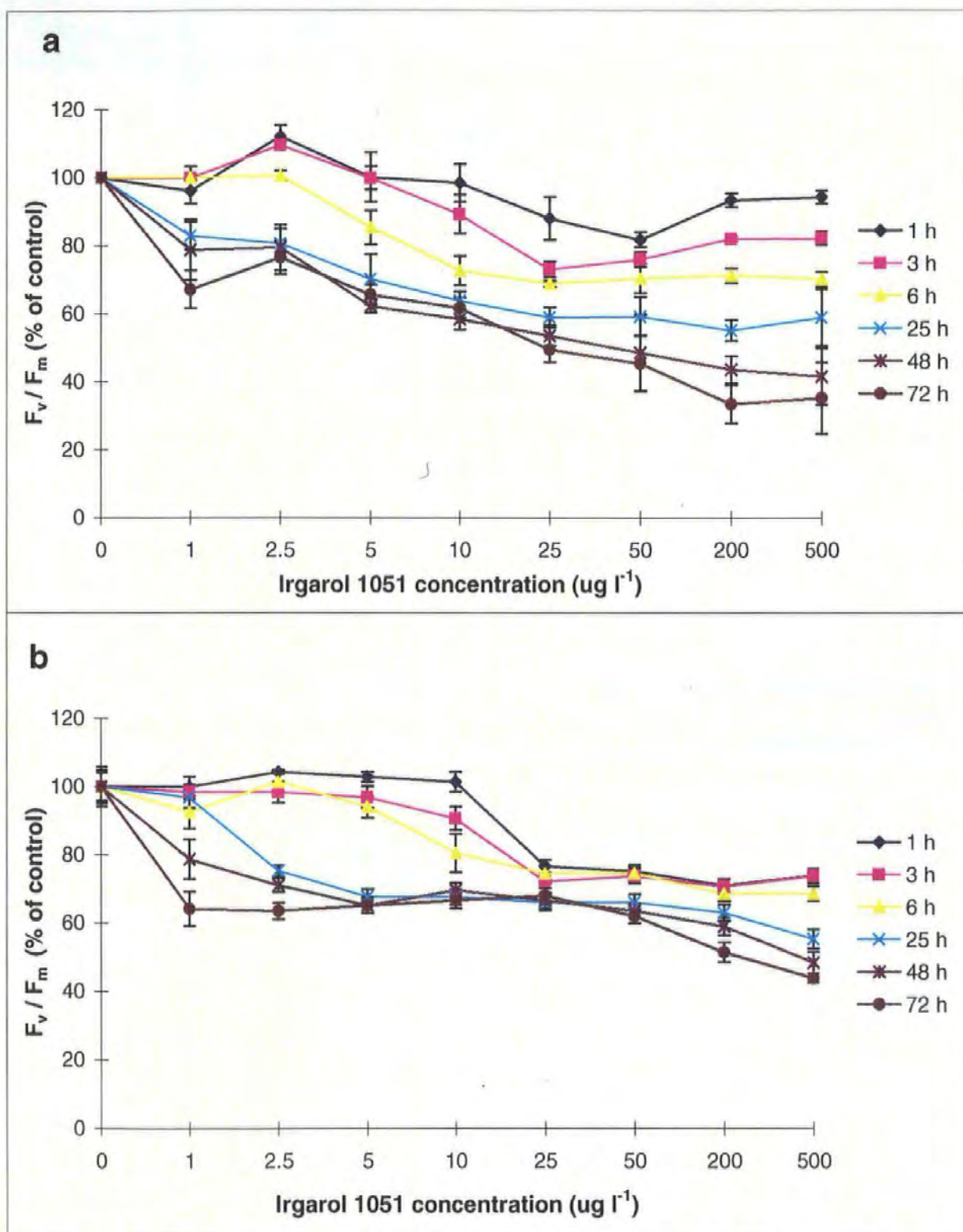
Figure 8.2. Dose-response curves for a) *Palmaria palmata* and b) *Porphyra umbilicalis* showing the effect of varying concentrations of Irgarol 1051 on $F_v / F_m$ ratio in mature thalli incubated in Irgarol 1051 solutions at different time periods. Means $\pm$ SE, n = 6.

a maximum decrease of approximately 40 % after 25 hours incubation with concentrations ranging from 5 to 50 μg l$^{-1}$. An apparent EC 50 (72 h) for *P. umbilicalis* was found after exposure to 250 μg l$^{-1}$.

## UV and Irgarol 1051 factorial experiment

### Analyses performed on *P. palmata*

A 2 factorial ANOVA performed on the data obtained from the simultaneous exposure to both stressors revealed a highly significant overall effect of both UV-B and Irgarol 1051 (2 factorial ANOVA, $p<0.00001$, with no interaction between the two factors ($p>0.056$) (fig. 8.3). Please see Table 8.1 for ANOVA tables. Furthermore, during the recovery period, overall significant effects of Irgarol 1051 and UV-B were noted (3 factorial ANOVA, $p<0.008$). No significant time effects ($p>0.5$) and no significant interactions were measured ($p>0.06$). A decrease in $F_v / F_m$ ratio of 14.5 % compared to controls was observed after the 50 min UV-B exposure to 1.8 kJ m$^{-2}$ (equivalent to ambient daily dose in April) in *P. palmata* (fig. 8.3). These thalli did not exhibit significantly different $F_v / F_m$ ratio to control thalli after 48 and 72 hours recovery. In thalli treated only with Irgarol 1051, an average decrease of 21.6 % was found after 25 h. After a 72 h recovery period a decrease of 15.9 % was still noted in these thalli. Exposure to both Irgarol 1051 and UV-B radiation resulted in a further decrease of 6.5 % compared to thalli treated only with Irgarol 1051, amounting to a decrease of 31.3 % compared to controls and a total decrease of 53.5 % compared to the pre-treatment value. Moreover, these thalli exposed simultaneously to Irgarol 1051 and UV-B did not show recovery during the following 72 hours; a reduction of 21.6 % compared to controls was measured. This decrease was equivalent to the inhibition calculated from an added effect with a total decrease of 36.7 % compared to the pre-exposure level.
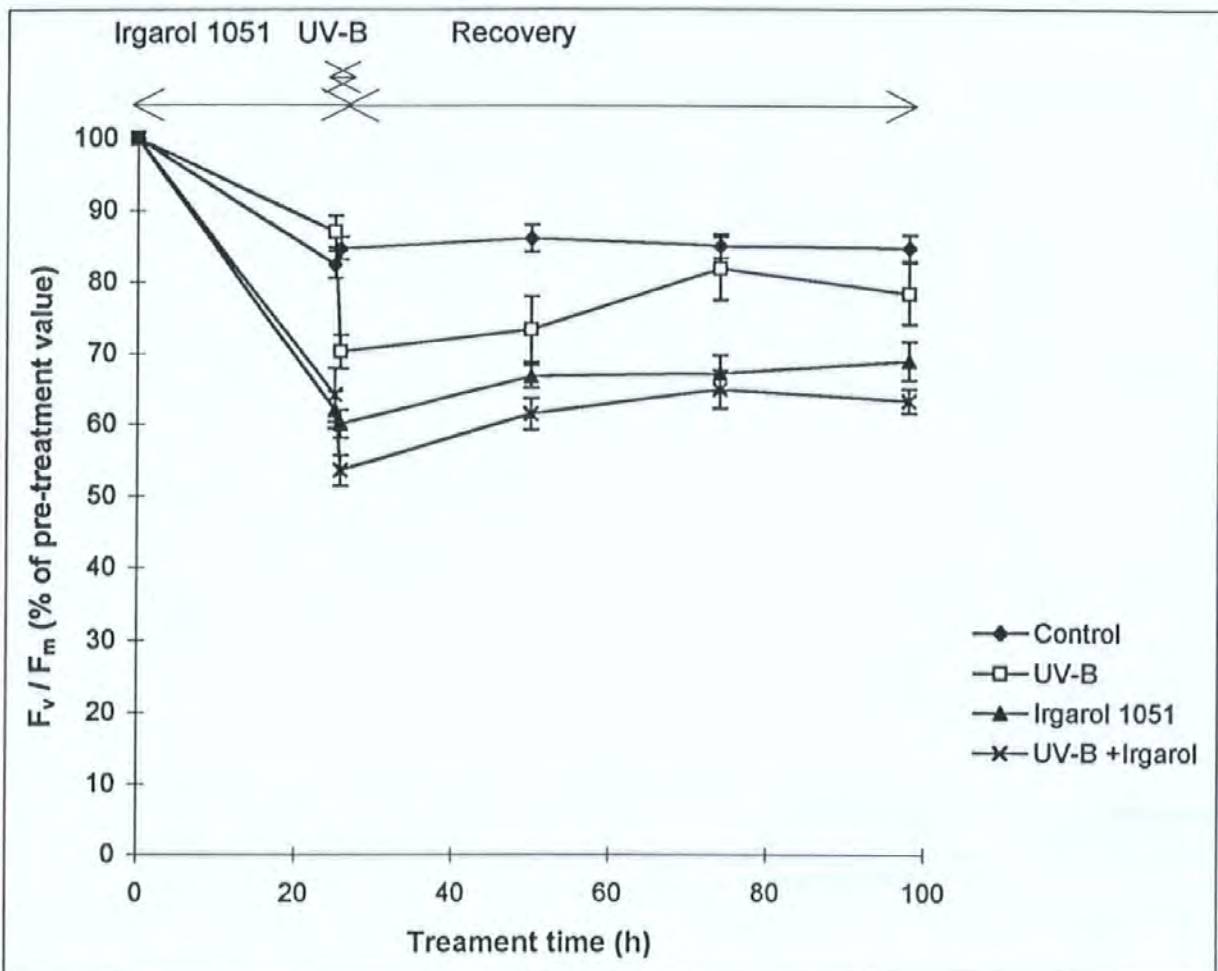
Figure 8.3. $F_v / F_m$ ratio in *Palmaria palmata* as a function of treatment time to UV-B exposure and/or Irgarol 1051. Arrows indicate the period of UV-B exposure, Irgarol 1051 treatment and recovery period. A 50 minute exposure to an unweighted UV-B irradiance of 2.1 W m$^{-2}$ was used and an Irgarol 1051 concentration of 5 µg l$^{-1}$ was applied. Means ± SE, n = 9.

Table 8.1. ANOVA tables for UV and Irgarol 1051 factorial experiments with *P. palmata* and *P. umbilicalis*. Two factorial ANOVAs were performed on data from the simultaneous exposure and three factorial ANOVAs were performed on data from the recovery period. Values were subtracted from the pre-exposure value and then analysed.

### Two factor ANOVA table for *P. palmata*.

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| **MAIN EFFECTS** | | | | | |
| A: irg | 0.190678 | 1 | 0.190678 | 87.90 | 0.0000 |
| B: uv | 0.0481071 | 1 | 0.0481071 | 22.18 | 0.0000 |
| **INTERACTIONS** | | | | | |
| AB | 0.008464 | 1 | 0.008464 | 3.90 | 0.0569 |
| RESIDUAL | 0.069418 | 32 | 0.00216931 | | |
| TOTAL (CORRECTED) | 0.316667 | 35 | | | |

### Three factor ANOVA table for *P. palmata*.

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| **MAIN EFFECTS** | | | | | |
| A: irg | 0.415003 | 1 | 0.415003 | 114.21 | 0.0000 |
| B: time | 0.00377625 | 2 | 0.00188812 | 0.52 | 0.5964 |
| C: uv | 0.0264078 | 1 | 0.0264078 | 7.27 | 0.0083 |
| **INTERACTIONS** | | | | | |
| AB | 0.00284715 | 2 | 0.00142357 | 0.39 | 0.6769 |
| AC | 0.0127792 | 1 | 0.0127792 | 3.52 | 0.0637 |
| BC | 0.0140277 | 2 | 0.00701383 | 1.93 | 0.1506 |
| RESIDUAL | 0.356086 | 98 | 0.00363353 | | |
| TOTAL (CORRECTED) | 0.830927 | 107 | | | |

### Two factor ANOVA table for *P. umbilicalis*.

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| **MAIN EFFECTS** | | | | | |
| A: uv | 0.0783067 | 1 | 0.0783067 | 37.85 | 0.0000 |
| B: irg | 0.210834 | 1 | 0.210834 | 101.90 | 0.0000 |
| **INTERACTIONS** | | | | | |
| AB | 0.00000136111 | 1 | 0.00000136111 | 0.00 | 0.9797 |
| RESIDUAL | 0.0662067 | 32 | 0.00206896 | | |
| TOTAL (CORRECTED) | 0.355349 | 35 | | | |

### Three factor ANOVA table for *P. umbilicalis*.

| Source | Sum of Squares | Df | Mean Square | F-Ratio | P-Value |
|---|---|---|---|---|---|
| **MAIN EFFECTS** | | | | | |
| A: irg | 0.344537 | 1 | 0.344537 | 80.69 | 0.0000 |
| B: time | 0.0330436 | 2 | 0.0165218 | 3.87 | 0.0241 |
| C: uv | 0.005547 | 1 | 0.005547 | 1.30 | 0.2571 |
| **INTERACTIONS** | | | | | |
| AB | 0.0231032 | 2 | 0.0115516 | 2.71 | 0.0718 |
| AC | 0.057963 | 1 | 0.057963 | 13.58 | 0.0004 |
| BC | 0.0410029 | 2 | 0.0205014 | 4.80 | 0.0102 |
| RESIDUAL | 0.418433 | 98 | 0.00426972 | | |
| TOTAL (CORRECTED) | 0.92363 | 107 | | | |

**Analyses performed on *P. umbilicalis***

For *P. umbilicalis*, chlorophyll fluorescence data from the simoultaneous exposure, revealed highly significant UV-B and Irgarol 1051 effects (2 factor ANOVA, p<0.00001) with no interaction between the two factors (p> 0.97), (fig. 8.4). Furthermore, during the recovery period (55, 79 and 103 h) an overall significant decrease in $F_v$ / $F_m$ following treatment to Irgarol 1051 was found (3 factor ANOVA, p<0.0004, Table 8.1 for ANOVA tables), but not to UV-B (p>0.2). Significant interactions were found between Irgarol 1051 and UV-B and between time and UV-B (p< 0.01). A decrease in the optimal quantum yield of 11.8 % was measured compared to the control exposure after the 6 h UV-B exposure (31.8 % compared to pre-exposure value) (fig. 8.4). Thalli treated only with Irgarol 1051 experienced an average decrease of 28.7 % after 25 h. In addition, a simultaneous exposure to both UV-B and Irgarol 1051 resulted in a further decrease of 30.4 % in $F_v$ / $F_m$ compared to control treatment. These reductions in $F_v$ / $F_m$ were all similar to decreases found for *P. palmata*. However, a 90 % recovery compared to control thalli was measured during the following 72 h in all treatment groups in *P. umbilicalis*.

## Growth measurements

Significant decreases in the slopes of growth curves were observed in *P. palmata* from all treatment groups (fig. 8.5a) (multiple regression, p< 0.05). Thalli exposed for 50 minutes to an unweighted UV-B irradiance of 2.1 W m$^{-2}$ experienced decreases of 43.7 % compared to controls (a slope of 2.1 in treated thalli compared to 3.8 in control thalli). Thalli exposed continuously to Irgarol 1051 suffered reductions of up to 81.9 % (calculated from a slope of 0.69). Moreover, a 50 min exposure to UV-B and a continuous exposure to Irgarol 1051 caused thallus shrinking, resulting in a negative slope of the growth curve. *P. umbilicalis*

suffered a decrease in growth rate (26.3 %) after 6 hours exposure to UV-B (a slope of 6.3 compared to 8.6 in control thalli) (fig. 8.5b). During a continuous treatment to Irgarol 1051, a 53.7 % decrease in growth rate occurred compared to controls (a slope of 3.99). However, a simultaneous exposure to both stressors did not result in further significant reductions in the slope of the growth curve (slope of 3.6).
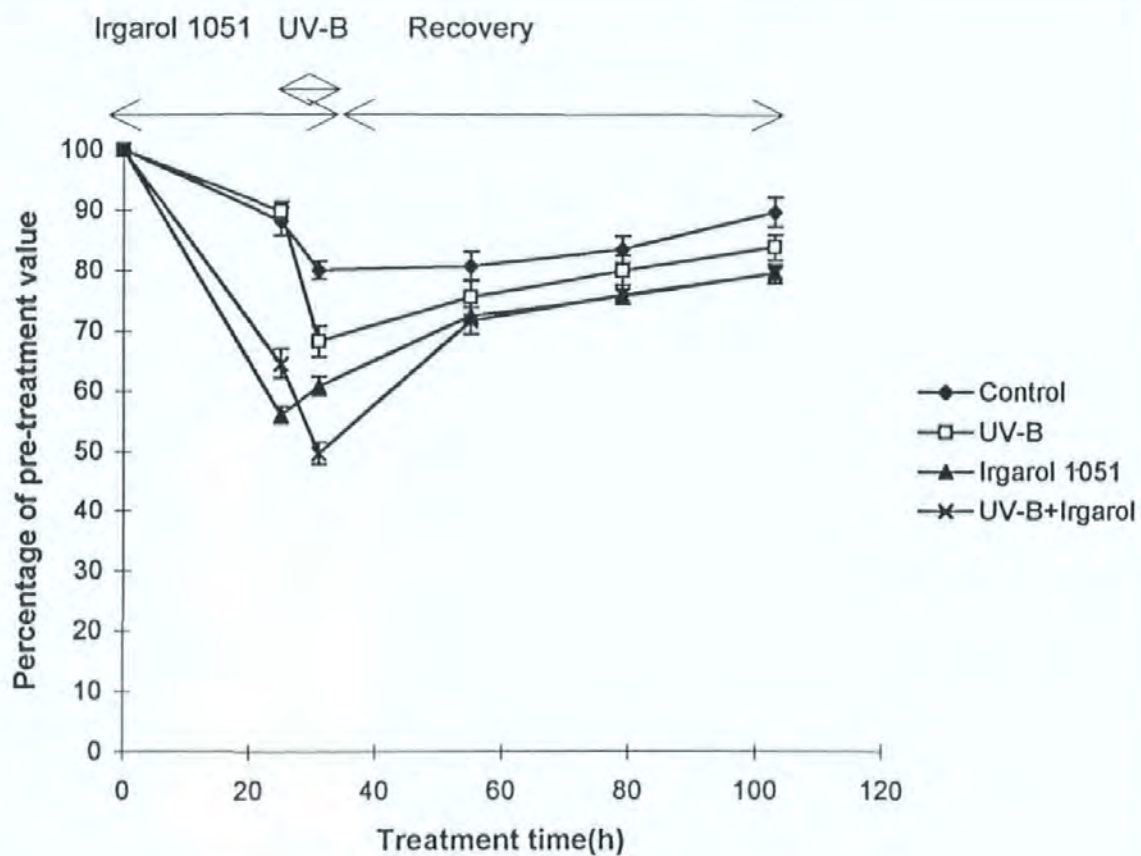
Figure 8.4. $F_v / F_m$ ratio in *Porphyra umbilicalis* as a function of treatment time to UV-B exposure and/or Irgarol 1051. Arrows indicate the period of UV-B exposure, Irgarol 1051 treatment and recovery period. An unweighted UV-B irradiance of 2.1 W m$^{-2}$ and an Irgarol 1051 concentration of 5 μg l$^{-1}$ were applied. Means ± SE, n = 9
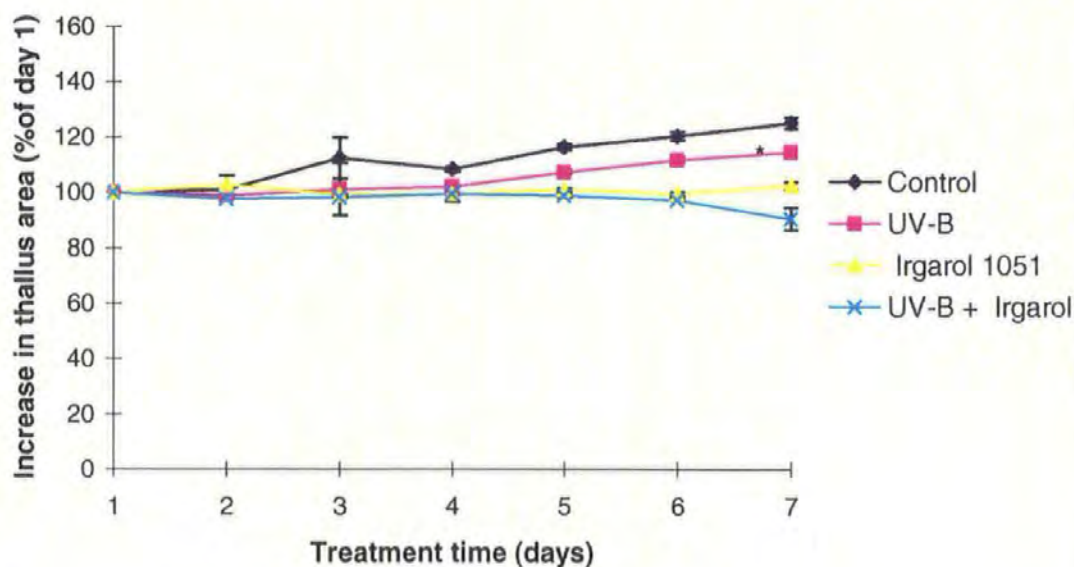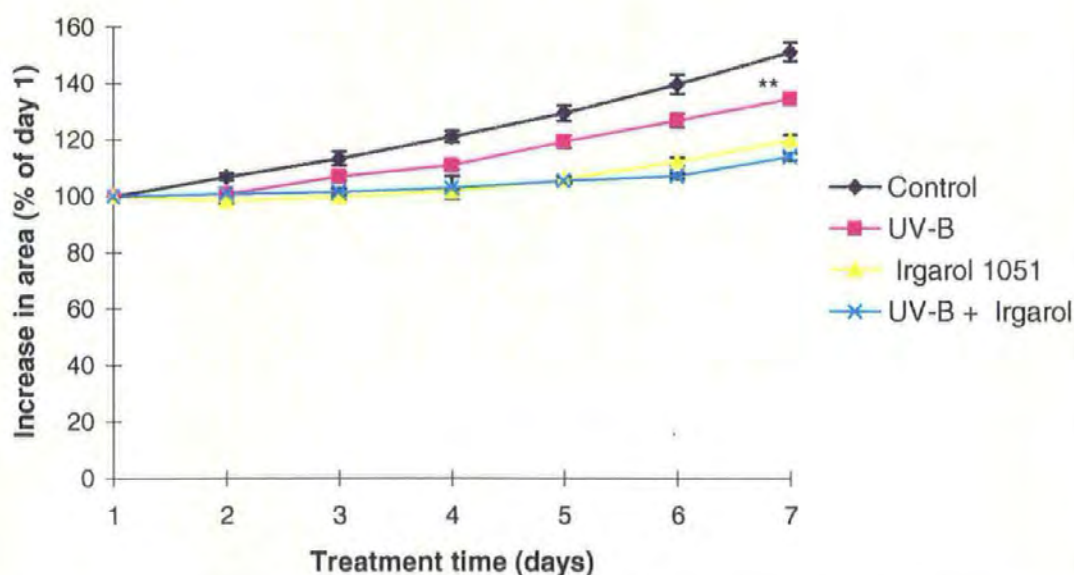
Figure 8.5. Increase in thallus area in a) *Palmaria palmata* and b) *Porphyra umbilicalis* as a function of treatment time to UV-B and/or Irgarol 1051 exposure. For both species a UV-B irradiance of 2.1 W m$^{-2}$ was applied on day 0 in these treatment groups (50 min exposure for *P. palmata* and a 6 hour exposure for *P. umbilicalis* respectively). Irgarol 1051 (5 µg l$^{-1}$) was applied throughout the treatment period. Means ± SE, n = 9. *, ** indicate significant different slopes in all treatment groups compared to non-treated controls. Data was calculated as % increase in area and differences in slopes and intercepts were tested in multiple regression analysis, p<0.05, p<0.01.

# 8.5 DISCUSSION

## Dose-response experiments

Dose-response relationships between varying UV-B doses and optimal quantum yield were established for *P. palmata* and *P. umbilicalis*. Marked differences in sensitivity to UV-B exposure were found between these two species, clearly demonstrated by the difference in ED 50 values (Effective Dose resulting in 50 % deceased $F_v$ / $F_m$ ratio). At an ambient dose of UV-B, equivalent to a daily weighted dose in April (1.1 kJ m$^{-2}$), 27 % decrease in $F_v$ / $F_m$ was found in *P. palmata*. Moreover, at a weighted dose equivalent to 20% ozone depletion in May (5.8 kJ m$^{-2}$, the month of the experiment) a 63 % reduction, compared to controls, was observed in this subtidal species. On the contrary, the intertidal *P. umbilicalis* was very tolerant to short-term exposures of elevated UV-B doses, which confirms the high degree of adaptability of this species to UV irradiation (Dring *et al.* 1996a). This difference in sensitivity between *P. palmata* and *P. umbilicalis* was also observed in an outdoor experiment (please see Chapter 4.1), and may be related to habitat-dependent differences in UV exposure (Larkum and Wood 1993). Habitat-dependent tolerances in photosynthesis and survival to UV-B exposure have been reported by several workers (Herbert 1990, Dring *et al.* 1996a).

The exposure-response curves with varying concentrations of Irgarol 1051 revealed toxicity to thalli of both *P. palmata* and *P. umbilicalis* at a concentration of 1 μg l$^{-1}$. Reductions up to 20 % in $F_v$ / $F_m$ were observed in both species after 48 hours exposure. This was a much lower concentration than the Minimum Inhibition Concentration of 10 μg l$^{-1}$ quoted by the manufactures (Ciba-Geigy 1995b), based on the growth of green algae. Differences in sensitivity between the two macroalgal species were demonstrated in the apparent EC 50 (72 h) values of 25 μg l$^{-1}$ found for *P. palmata* compared to 250 μg l$^{-1}$ for *P. umbilicalis*. Both

171

non-target species exhibited a much greater tolerance to short-term exposure to Irgarol 1051 compared to *E. intestinalis*, a major fouling alga (Scarlett *et al.* 1997 and Chapter 7). A plateau in the dose-response curve at a 40 % decrease in $F_v$ / $F_m$ occurred for *P. umbilicalis* between concentrations of 5 to 50 μg $l^{-1}$. A possible explanation may be a lack of available Irgarol 1051 in the treatment water, thus equilibration was not reached. However, although no direct measurements of Irgarol 1051 were made in this study, a similar water to thallus ratio was used compared to the previous study. In Chapter 7 it was concluded that only 45 % of Irgarol 1051 was taken up after 24 hours (Table 7.1). As the treatment water was changed every 12 h it can be assumed that the uptake of Irgarol 1051 by thalli was not limited during the experimental period. The results demonstrate comparatively high tolerance to short-term Irgarol 1051 exposure, when applied alone. However, there is a need to confirm this apparent tolerance to Irgarol 1051 in chronic exposure experiments.

## UV-B and Irgarol 1051 factorial study

It was demonstrated that UV-B radiation and Irgarol 1051 singly and in combination can reduce chlorophyll fluorescence following short-term exposures in *P. palmata* and *P. umbilicalis*. This is in accordance with the results from the factorial experiment with *E. intestinalis* in the previous study. Moreover, when these two stressors were applied simultaneously an additive effect on the optimal quantum yield was found, resulting in further reductions of 6.5 % for *P. palmata* and 11.2 % for *P. umbilicalis*, compared to a treatment of Irgarol 1051 applied alone. This finding of an additive effect was supported by the lack of interaction between the two stressors after this treatment. No recovery was found for *P. palmata* 3 days after any of the treatments, as indicated by the lack of time effect ($p > 0.59$). However, for *P. umbilicalis* a 90 % recovery compared to controls was found (indicated by the significant time effect, $p < 0.002$). Thus, the greater tolerance noted for *P. umbilicalis* in

the dose-response experiments was also measured when both UV-B and Irgarol 1051 were applied together.

Additive effects of both stressors, demonstrated here at a very low dose of UV-B in *P. palmata* (equivalent to ambient levels in April) could have consequences *in situ*. Hence, multiple stressor effects must be investigated if we are to accurately assess possible consequences of elevated UV-B in the marine environment. In contrast, *P. umbilicalis* exhibited tolerance to high levels of Irgarol 1051 and UV-B, in addition to near recovery in $F_v / F_m$ after 3 days. The underlying mechanisms for this greater tolerance to both stressors in this species has yet to be established. However, the generally high tolerance experienced by species in the intertidal zone to the wide fluctuations in environmental conditions (Beibl 1962) may well extend to tolerance of toxic compounds.

Reduction in chlorophyll fluorescence is a general response to several stressors such as PAR, UV-A and UV-B (Cullen *et al.* 1992, Cordi *et al.* 1997) and to other stressors such as herbicides (Conrad *et al.* 1993, Scarlett *et al.* 1997 and as described in the previous chapter). Thus, this parameter is potentially a very powerful tool in assessing possible interactive effects of stressors which acts on PS II, as has been demonstrated here.

The reduced growth rates measured in thalli of *P. palmata* from all treatment groups indicate non-reversible damage following exposure to UV-B and continuous exposure to Irgarol 1051. A trend of greater decrease in growth rates after exposure to both Irgarol 1051 and UV-B radiation was found for both species. However, in *P. umbilicalis* a reduction in growth rate occurred after UV-B exposure, despite a recovery in optimal quantum yield after 3 days. Similar results indicating a lack of correlation between growth and $F_v / F_m$ have been reported for several species of *Laminaria* and *E. intestinalis* in both mature thalli and young

173

sporophytes (Dring *et al.* 1996b, Cordi *et al.* 1997). This difference could either mean that the physiological processes resulting in reduced growth are independent of photosynthesis (Dring *et al.* 1996b), or could be related to a lack of correlation between $F_v$ / $F_m$ and photosynthesis during short exposures. Indeed a lack of correlation between $F_v$ / $F_m$ and the rate of photosynthetic $O_2$ production has been observed in the Arctic *Palmaria palmata* (Hanelt and Nultsch 1994). This incomplete correlation underlines the importance of including growth measurements in the evaluation of long-term effects of UV-B exposure in *P. umbilicalis*.

An Irgarol 1051 concentration of 5 µg $l^{-1}$ used in this study, has not as yet been recorded in the environment. A survey investigating the environmental concentrations of Irgarol 1051 along the English Channel coast reported local variations in the concentration by a factor of >500 (Gough *et al.* 1994). The highest concentrations were detected in marinas and the lowest concentrations in open waters and rivers. Concentrations between 0.19 - 0.5 µg $l^{-1}$ were measured in estuaries and marinas in Britain (Gough *et al.* 1994) and 1.7 µg $l^{-1}$ was measured in marinas in the Côte D'Azur (Readman *et al.* 1993). Considerable seasonal variations with the highest concentrations in the summertime and lower concentrations in the autumn were also noted. As the objective of this study was to investigate the interactive relationship between UV-B and Irgarol 1051 in two macroalgae, an initial decrease in optimal quantum yield of approximately 25 % following exposure to one stressor alone, was chosen for both species. Thus, relative high levels of Irgarol 1051 and UV-B (only for *P. umbilicalis*) were used to achieve this. However, chronic exposure to levels of Irgarol 1051 as low as 0.063-0.25 µg $l^{-1}$ caused adverse effects in algae communities (Dahl and Blanck 1996). Furthermore, investigations into the reproductive effects caused by heavy metals and organic chemicals (Steele and Thursby 1983, Thursby *et al.* 1985) and UV-B exposure (Chapter 6), have shown that reproduction is a very sensitive endpoint. In addition, Scarlett and co-workers (1997) reported that growth of *E. intestinalis* zoospores was inhibited at an Irgarol

1051 concentration as low as 100 ng l$^{-1}$ when applied singly. Thus, increased sensitivity of earlier life-stages could potentially result in greater detrimental effects on these algal species, than has been suggested by the results presented here.

Both species and phyla specific differences in algal sensitivity to chemicals have previously been demonstrated (Blanck *et al.* 1984, Garten 1990, Sirois 1990, Peterson *et al.* 1994 and Kent and Currie 1995, Wängberg and Blanck 1988), although incorporation of these factors into testing schemes still remains uncommon (Ruzycki *et al.* 1998). The interactive effects between UV-B and the s-triazine herbicide Irgarol 1051 have been established here in *P. palmata* and *P. umbilicalis*. It was shown that simultaneous exposure to both stressors produced greater effects in *P. palmata* compared to a single exposure to either UV-B or Irgarol 1051. This supports the conclusion made by Dubé and Bornman (1992), that if only one stress condition is investigated, one may be underestimating the impact of several stressors.

## 8.6 CONCLUSIONS

From these initial studies, it is apparent that the simultaneous exposure to both Irgarol 1051 and UV-B produced greater reductions in $F_v / F_m$ in the non-target macroalgae *P.umbilicalis* and *P. palmata* compared to single applications of UV-B or Irgarol 1051. Reductions in optimal quantum yield were associated with detrimental effects in growth rates in *P. palmata*, which followed the same pattern of greater reductions when both stressors were applied. Subsequently, the assessment of multiple environmental effects on algae is necessary in obtaining a more meaningful and realistic view of current changes in the marine environment. Moreover, there is a need to investigate the interactive effects of chronic exposures to low levels of Irgarol 1051 and elevated UV-B both in mature thalli and in the reproductive

unicells. Macroalgae form an intrical part of intertidal ecosystems, which support unique and diverse life forms. Therefore, combined effects of enhanced UV-B and xenobiotic compounds, on macroalgae, must be considered in any analysis of the global effects of ozone depletion.

# CHAPTER 9

# DISCUSSION AND CONCLUSIONS

The amount of additional UV-B radiation resulting from even the severest ozone depletion levels reported is minor when it is considered against the background of total ambient solar UV radiation. However, it must be emphasised that the springtime increase in UV-B is a relative temporal shift, not an increase in maximum UV irradiance (Karentz 1991, Stolarski *et al.* 1991). Therefore, the biological consequences of increased UV-B do not rest entirely upon the ability of algae to cope with UV exposure, but on their ability to cope with elevated levels of UV-B in early spring. The seasonal gradient of increasing UV-B levels no longer exists with the occurrence of the ozone hole. Thus, elevated UV-B is encountered during early spring, immediately after minimum irradiation levels during the winter months. Consequently, lowered tolerance in algae, due to long-term adaptation to low winter levels, coincides with abnormally high springtime UV-B irradiances. In addition, UV radiation of shorter wavelengths, which undergoes the greatest changes during ozone depletion also exerts the greatest biological effects (Caldwell *et al.* 1986). Although UV-B has been reported repeatedly to be biologically harmful to both terrestrial and aquatic life (reviews by Häder *et al.* 1995, Franklin and Forster 1997, Häder and Figueroa 1997, Lowe *et al.* 1998), the potential ecological consequences of elevated UV-B are rather more complex to comprehend.

The following sections in this chapter will be related to the overall aims stated in the Introduction and to which extent these aims were met. The key criteria for selection of suitable biomarkers listed in Section 9.1 are followed by the main conclusions from the different chapters related to the four overall aims of this thesis. Section 9.23 discusses the reproducibility of the results and Section 9.24 highlights some challenges related to laboratory

versus field experiments. The possible ecological implications of increased UV-B radiation are mentioned in Section 9.5 and are compared to existing natural stressors and to pollutants in the coastal marine environment. Finally, in Section 9.6, are mentioned some suggestions for future work and the broader implications of UV-B exposure.

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The overall aims of this thesis, as also mentioned in the Introduction, were to:

1) investigate the suitability of several molecular and physiological parameters as biomarkers of UV-B exposure and/or effect in marine macroalgae, and to relate these physiological parameters to consequent biological effects measured as growth rate.

2) estimate the relative sensitivity of the reproductive unicells of *E. intestinalis* to UV-B exposure.

3) examine interactive effects of UV-B radiation and the antifouling compound, Irgarol 1051, using selected biomarkers on both target and non-target macroalgal species.

4) assess differences in susceptibility to UV-B radiation of several macroalgal species from habitats which differ with regard to solar UV and PAR irradiation levels.

## 9.1 Key criteria for selection of biomarkers

Peakall (1992) listed in his book some challenges and obstacles which needs to be addressed in an environmental monitoring programme based on biomarkers with regard to contaminants. The main points from Peakall (1992), which are also applicable to increases in UV-B radiation are mentioned below:

I) The quantitative and qualitative relationship between chemical exposure / UV-B

radiation, biomarker response and adverse effects must be established.

II) Responses due to chemical / UV-B exposure must be distinguished from natural

sources of variability (ecological and physiological variables, species-specific

differences, and individual variability) if biomarkers are to be useful in evaluating

contamination/radiation.

III)The validity of extrapolating from biomarker responses measured in individual

organisms to some higher-level effect at a population or community level must be

established.


I) This first criterion will be considered in two parts: a) the relationship between chemical

exposure / increases in UV-B and the biomarker response and b) the relationship between

biomarker response and adverse effects.


a) The relationship between the stressor and the biomarker response is determined by the

availability of biomarkers which respond to the stressor and also the degree of specificity. In

the Introduction (Section 1.3) four classes of biomarkers were identified:

* biomarkers of exposure (range from general to specific indicators),

* biomarkers of effect (may not provide information of the nature of stress),

* exposure / effect biomarkers (specific and relates the exposure to an effect),

* latent effect biomarkers.


The sensitivity of the biomarker response is equally important for the consideration of this

first relationship. To be useful, biomarkers should be related in a dose-response dependent

manner to UV-B over an environmentally relevant range. This dose-response model must be

based on controlled laboratory studies. In field studies, organisms are exposed to multiple natural and xenobiotic stressors, which can alter the simple dose-response relationship. In the field, it is therefore more realistic to consider a family of dose-response relationships, based on the combination of several stressors.

b) The relationship between biomarker response and adverse effects are related to the link between sublethal responses and lethal responses. For biomarkers to be useful as early warning signals it is desirable that the responses, indicative of sublethal effects are detectable sufficiently in advance of non-compensatory, irreversible events. Furthermore, the biomarker response should be detected at lower doses than are associated with the related mortality (Depledge 1994). However, responses to very low doses may, on the other hand, also pose practical problems, as such responses may be too sensitive indicators of potential problems. The relationship between exposure and adverse effects must be thoroughly investigated for the biomarker approach to have practical use for recognising or predicting ecologically significant events.

II) A very difficult factor to deal with in making ecological predictions based on the biomarker concept is to account for natural sources of variability (ecological and physiological variables, species-specific differences, and individual variability). Inherent differences in morphology and biochemical / physiological status of exposed organisms such as size, age, genotype, environmental conditioning are all important sources of inter-individual differences in response, which needs to be investigated (Depledge 1994).

III) The degree of extrapolation between biomarker responses and related damage on the individual, population or community level vary greatly from biomarker to biomarker (Peakall 1992). To link the chosen molecular and physiological parameters to related damage on the

individual and population level, changes in growth rate and reproduction were also measured as examples of Darwinian fitness parameters.

## 9.2 Aim No. 1: Investigation of molecular and physiological responses as biomarkers of UV-B exposure.

In the following section the suitability of the chosen molecular and physiological parameters as biomarkers of UV-B exposure will be evaluated and related to consequent biological effects. The potential problems related to their use will be highlighted and the findings from the chapters will be placed in the wider contexts of existing work.

### 9.2.1 Molecular responses to UV-B exposure

The molecular basis for many of the changes observed following UV-B exposure has not yet been well defined (Caldwell *et al.* 1995). Responses may result from direct DNA damage, which is an important chromophore (a compound which absorbs radiation and mediates a photochemical reaction) of UV radiation (Zölzer and Kiefer 1989). The formation of photoproducts results in structural changes in DNA molecules which causes incorrect replication of the genetic code. UV-B radiation can accelerate mutation rates and lead to heritable DNA lesions (Atienzar *et al.* 1998 and references therein). In Chapter 5.1 the Random Amplification Polymorphic DNA assay was used to detect DNA damage in *P. palmata* following exposure to varying UV-B irradiances. This technique was used in conjunction with several physiological responses, such as chlorophyll fluorescence, *in vivo* thallus absorptance and growth.

Changes in RAPD patterns were observed as a response to UV-B exposure. Variations in band intensities and the pattern of appearing and disappearing bands could be related to increasing UV-B exposure. Thus, these results suggested that the RAPD technique has potential as a tool for assessing UV-induced toxicity in marine algal populations. This technique appears to be sensitive to relatively low UV-B doses. However, as the formation of photoproducts is a general genotoxic response which can not easily be related to individual or population damage, this parameter may show potential as a general, non-specific biomarker of UV exposure rather than effect. The results further illustrated that utilising several biological responses, from different levels of biological organisation, offer greater possibilities for detecting UV-B induced effects, than do single responses. The biomarker approach proved especially useful when these molecular and physiological responses were linked to changes in Darwinian fitness, measured as decreases in growth rate. To further evaluate the suitability of the RAPD technique as a biomarker of UV exposure, it is necessary to establish the range of inherent natural variability in this response in healthy macroalgal plants.

Induction of HSP 70, on the other hand, which was also investigated as a possible molecular parameter of UV exposure did not show such promising results. A lack of induction of HSP 70 was found in both *Palmaria palmata* and *Enteromorpha intestinalis* after a three hour exposure to relatively high UV-B irradiances. Thus, although this method was both reliable and reproducible it was concluded that this parameter was unsuitable as an indicator of UV-B exposure in these two macroalgae.

## 9.2.2 Physiological responses to UV-B exposure

**Chlorophyll fluorescence**

Predictive dose-dependent relationships between $F_v / F_m$ and UV-B exposure were measured in both *P. palmata* and *E. intestinalis* in Chapter 3. These findings indicated that decreases in $F_v / F_m$ and their recovery may show potential as sensitive biomarkers of UV exposure. Several workers have found similar correlations between optimal quantum yield and UV-B exposure (Björn *et al.* 1986, Renger *et al.* 1986). Larkum and Wood (1993) concluded that $F_v$ may be used as an indicator of UV-B inhibition. Moreover, Björkman (1987) and Demming and Björkman (1987) reported that a decrease in $F_v / F_m$ was linearly related to a decrease in the optimal quantum yield of photosynthesis. In this study, decreased $F_v / F_m$ with no recovery, and reduced growth rates clearly showed irreversible damage to PSII in *P. palmata*. As chlorophyll fluorescence is known to respond to a wide variety of stressors, both natural (Henley *et al.* 1991, Herrmann *et al.* 1995) and xenobiotic (Conrad *et al.* 1993, Sgardelis *et al.* 1994), its potential as a biomarker of exposure is general and non-specific.

Photoinhibition which is a reversible process that only inactivates PS II for limited time periods, is also associated with decreases in optimal quantum yield (Franklin and Forster 1997). Photoinhibition can therefore be regarded as a compensatory response, a protective mechanism belonging to the compensatory zone (please see Section 1.3 for further details). Photoinhibition of photosynthesis by PAR is now an area of active research (Franklin and Forster 1997). As high PAR and UV are closely linked in nature, it is important to be able to distinguish between the two effects and be able to determine whether the response to PAR stress affects the response to elevated UV-B, as has been observed in some higher plant studies (Franklin and Forster 1997). Existing information based on macroalgal studies is contradictory and incomplete (Franklin and Forster 1997). Thus, much more research is needed to establish the degree of extrapolation between changes in chlorophyll fluorescence and related damage on the individual level.

Recent research has reported that chlorophyll fluorescence also undergoes diurnal variations in some macroalgal species (Henley et al. 1991). Thus, is therefore important to establish the range of variability in optimal quantum yield in healthy plants. The establishment of such "normal" reference values must be an integral part of the biomarker approach (Depledge 1994).

## Pigment changes

*In vivo* pigment absorptance was investigated for its suitability as a non-destructive, general biomarker of exposure in Chapter 3. It has the potential to reveal *in vivo* changes in the absorptance spectra of pigments following UV exposure, to characterise sites of damage to pigments and to establish the time course of damage and repair (Smith and Alberte 1994). *In vivo* absorptance of chlorophyll *a*, phycoerythrin and/or carotenoids, as well as phycoerythrobilin and phycocyanin decreased in a dose-response dependent manner to UV exposure and was associated with a decrease in growth rate in *P. palmata*. It was found that *in vivo* spectrophotometric analyses provide reliable, non-invasive means to characterise subtle responses of macroalgae to UV-B exposure. However, changes in the *in vivo* absorptance spectra may not be directly related to pigment concentration changes. Changes in the *in vivo* spectra may also arise from changes in the package effect, caused for example by chloroplast movements, thylakoid organisation and changes in mean cell size etc. (Forster pers. comm). Thus, care must to be taken with the interpretation of the *in vivo* spectra.

With high performance liquid chromatography (HPLC), on the other hand, it is possible to positively identify and quantify chlorophyll and carotenoid pigments. HPLC measurements obtained in several experiments (Chapter 4) supported the *in vivo* spectral data. Other workers have reported similar changes in pigment concentrations in marine diatoms and

cyanobacteria after UV exposure (Döhler 1987, Zündorf and Häder 1991). Thus, these results indicated that changes in *in vivo* thallus absorptance and HPLC analyses could provide early quantitative warning of the detrimental effects of UV-B in marine macroalgae. However, research into the natural sources of variability and the link between these responses and adverse effects is needed for these parameters to have practical use as general biomarkers of exposure.

Pigment synthesis in macroalgae is dependent on light quality (Dring 1981, Ramus 1983) and light quantity (Rüdiger and López-Figueroa 1992, Algarra *et al.* 1991, Talarico 1996). It has been established that the photosynthetic pigments respond to variations of irradiance, photoperiod and spectral variations (Rüdiger and López-Figueroa 1992). Recently, it was demonstrated that phycobili-protein content in red algae and the ratio between phycourobilin / phycoerythrobilin are involved in the physiological acclimation to changes in the light field, and thus may also protect against UV radiation (Yu *et al.* 1981). In experiments described in Chapter 3, the decrease in *in vivo* chlorophyll absorptance was slower than that of the phycobili-proteins and caroteniods, thus indicating a protective role of the accessory pigments. Chloroplast movements may also be induced by excess light and, consequently can reduce photo-destruction of photosynthetic pigments (Hanelt and Nultsch 1991).

A physiological acclimation mechanism protecting against damaging UV irradiance, occur via zeaxanthin, a xanthophyll formed under excess light. The photoprotective role of the xanthophyll cycle has been investigated in *Ulva* spp. (Franklin 1994) and in some brown algae (Uhrmacher *et al.* 1995, Franklin *et al.* 1996), but has not as yet been reported in red algae.This response may show potential as a biomarker as a linear correlation between zeaxanthin content and the degree of photoinhibition was reported in the brown alga *Dictyota dichotoma* (Uhrmacher *et al.* 1995).

Finally, UV absorbing properties of both carotenoids and Mycosporine-like amino acids may also show potential as biomarkers of UV exposure. MAAs, in particular which absorb between 310 nm and 360 nm, may prove useful as specific biomarkers of UV exposure. MAA's have been reported in green, red and brown algae from tropical, temperate and polar regions (Karentz 1994) and the concentration of these compounds has been correlated with depth zonation and UV exposure. Further work is necessary to determine both the wavelength- and dose dependence of induction (Franklin and Forster 1997) and the natural variability of these compounds.

## Membrane damage

Cellular disruption causing membrane damage is partly the mode of action of several aquatic herbicides, ozone, heavy metals and possibly UV. In fact, previous research has shown that the integrity of membranes may also be affected by UV-B irradiance due to decreases in lipid content and impairment of membrane transport systems (Murphy 1983). Determining the leakage of ions in plant or algal cells is a common method of assessing the extent of cell damage resulting from both natural and anthropogenic stressors (Koch *et al.* 1995). Measurements of ion leakage, in Chapter 3, confirmed that exposure to UV-B radiation can induce membrane damage in *P. palmata* and *E. intestinalis*. However, as the extent of damage could not be related in a predictive dose-dependent manner to increasing UV-B irradiance this parameter was judged unsuitable as a biomarker of UV exposure.

## Changes in growth rate

Delays or reductions in growth rate are sensitive measures of adverse effects of UV-B irradiation (Chapter 3,7 and 8). Grobe and Murphy (1994) found an inverse linear relationship between the growth rate of *Ulva expansa* and the duration of UV-B exposure. Furthermore, enhanced growth rates were measured in *Ecklonia* sporophytes in the absence of solar UV radiation (Wood 1987). Apart from these reductions in growth rate subtle changes in allocation of growth to different tissues have been reported for terrestrial plants (Teramura 1983) and may also be possible for algae although there is no reported evidence as yet. Such morphological changes could result in alterations in competition for light and nutrients, and ultimately lead to changes in algal zonation patterns. Reductions in growth rate has the potential of linking biomarkers of exposure to possible effects on the individual and population level. A correlation between the molecular / physiological parameters and changes in growth rate must be established for this to be true. In Chapter 8, however, a recovery in optimal quantum yield was found in *P. umbilicalis* 3 days after UV-B exposure despite a measured reduction in growth rate. Similar results indicating a lack of correlation between growth and $F_v$ / $F_m$ have been reported for several species of *Laminaria* and *E. intestinalis* in both mature thalli and young sporophytes (Dring *et al.* 1996b, Cordi *et al.* 1997, Chapter 3). This difference could either mean that physiological processes resulting in reduced growth are independent of photosynthesis (Dring *et al.* 1996b), or could be related to a lack of correlation between $F_v$ / $F_m$ and photosynthesis during short exposures. Indeed a lack of correlation between $F_v$ / $F_m$ and the rate of photosynthetic $O_2$ production has been observed in *Palmaria palmata* (Arctic) (Hanelt and Nultsch 1994). This incomplete correlation underlines the importance of including "endpoint effects" such as impairment of growth when assessing effects of UV exposure.

## 9.2.3 Reproducibility of results throughout the thesis

Dose-response experiments for both *P. palmata* and *E. intestinalis* which were investigated in several chapters showed some lack of consistency. For example, thalli of *P. palmata* used in dose-response experiments conducted in February and June (Chapter 3), were less sensitive than similar dose-response experiments conducted in May and June (outlined in Chapter 8). On average, thalli exposed to UV-B in Chapter 8 exhibited a 20 % greater reduction in optimal quantum yield compared to thalli exposed in Chapter 3. One of the possible reasons for this variation in sensitivity to UV-B may be related to the collection of thalli. In chapter 3 tips of well-grown, mature thalli were used for the exposures, whereas in Chapter 8 it is possible that younger tips were used. Such differences in sensitivity related to the age and maturity of thalli need to be investigated in greater detail. Similarly, comparisons of dose-response experiments for *E. intestinalis* performed in Chapter 3 and 7 also showed variations of up to 34 % between experiments.

Another reason for the lack of consistency between experiments may be related to time of collection. Seasonal variability in susceptibility to UV-B radiation has been demonstrated by several workers (Häder and Figueroa 1997 and references therein). When conducting UV experiments it is therefore very important to consider that algae collected in the winter months will have a lowered tolerance to UV-B compared to algae collected in spring or summer months. Finally, microhabitat differences may also play an important role in determining the relative sensitivity of algae to UV exposure. The relationships between time and place of sample collection and variations in sensitivity to UV need to be investigated in greater detail.

## 9.2.4 Laboratory verses field-based experiments

While UV-B induced effects throughout this thesis were investigated in laboratory based experiments under controlled conditions with low PAR and UV-A irradiances, different spectral regions of light, such as UV-A and blue light appear to play important photoprotective roles in both plants and algae (Caldwell *et al.* 1989, Caldwell *et al.* 1995). It has been reported that terrestrial plants exposed to solar UV in field studies exhibited reduced sensitivity compared to plants exposed to artificial UV radiation in laboratory experiments (Warner and Caldwell 1983, Mirecki and Teramura 1984). Several workers have also found that the greatest effects of UV on photosynthetic pigments and other responses occurred when terrestrial plants were grown under low PAR irradiance (Mirecki and Teramura 1984, Cen and Bornman 1990). It appears that algae in general differ somewhat from higher plant species in that negative UV-B effects on photosynthesis have been observed under conditions where natural ratios of UVR to PAR are maintained (Post and Larkum 1993). The underlying reasons for this and why the combination of UVR and PAR appear to be more stressful than either one alone are not clear. Reports such as those described above raise a fundamental question for experimental design: how well do laboratory-based experiments, with relatively low PAR and UV-A irradiance, model UV-induced effects on algal photosynthesis, growth and survival in the field?

As described in Section 9.1 the first step in establishing dose-response models must be based on controlled laboratory studies. Once these have been established; field studies, with natural ratios of PAR, UV-A and UV-B (and possibly other natural and xenobiotic stressors) would seem a natural progressive step.

## 9.3 Aim No. 2: Evaluation of the relative sensitivity of the reproductive unicells of *E. intestinalis* to UV-B exposure.

Many macroalgae produce free-swimming reproductive cells which disperse away from the parent population to colonise new sites. These unicells lack the UV protective secondary cell wall (Lobban and Harrison 1994) and possess low-light adaptive strategies (Beach *et al.* 1995), which consequently may make them more susceptible to elevated UV-B. Indeed, in Chapter 6 it was established that the reproductive unicells of *E. intestinalis* suffered greater reductions in both variable fluorescence and growth rates than did mature thalli exposed to the same conditions. Inhibitions of germination success and decreased growth rates were recorded in unicells exposed for 1 hour to elevated UV-B irradiance ($1.8$ W m$^{-2}$ unweighted UV-B irradiance, equivalent to 27 % ozone depletion, Caldwell 1971). Moreover, a greater sensitivity to UV-B exposure was measured in the sexual reproductive part of the life cycle, compared to the asexual part. The only other report which has quantitatively investigated reproductive effects in macroalgae, found supporting evidence of decreased sensitivity to UV radiation with increasing age (Dring *et al.* 1996b). Thus, it was concluded in Chapter 6 that the ecological significance of increases in UV-B radiation may be seriously under-estimated if effects on early lifestages of algae are not considered.

## 9.4 Aim No. 3: Examination of interactive effects of UV-B radiation and the antifouling compound, Irgarol 1051, using selected biomarkers on both target and non-target macroalgal species.

A major consideration (which generally goes unmentioned in photobiology) is that the coastal marine environment is one of the major recipients of anthropogenic pollution. In addition to

trace metals and metalloids, the input of pesticides, antifouling agents and PAHs released from industry and agriculture is increasing (Zhou *et al*. 1996). Furthermore, alterations in salinity, nutrient availability, desiccation, temperature as well as changes in PAR and UV, are examples of natural stress factors which intertidal algae may be subjected to due to the tidal changes (Lobban and Harrison 1994) (fig. 9.1). Thus, elevated UV-B must be considered as an additional stress factor and combination effects between these stressors must be investigated. The results in Chapter 7 and 8 showed that simultaneous exposure to UV-B radiation and Irgarol 1051 resulted in further decreases in optimal quantum yield compared to a single treatment of Irgarol 1051, indicating an additive effect, in both the antifouling alga *E. intestinalis* and the two non-target species *P. palmata* and *P. umbilicalis*. Decreases in chlorophyll fluorescence were accompanied by greater reductions in growth rate in all three species following exposure to both stressors. The findings in these two chapters provide useful preliminary insights into the interactive effects of UV-B radiation and Irgarol 1051. They also strengthen the concern that the ecological implications of increased UV-B radiation may be seriously under-estimated, if combined effects between UV-B and other stressors are not considered.

Contamination of
Trace metals (i.e. Cd, Cu, Pb)
and metalloids (i.e. As, Se)

Ozone depletion related
increases in UV-B
irradiance

Eutrophication and
effluent waste

**Natural stressors:**

Wave motion

Salinity induced stress

Temperature stress

PAR and UV-A irradiation
changes

Desiccation

**Organic pollutants:**

PAHs

Pesticides

Antifouling agents

Macroalgae in the coastal marine environment

Figure 9.1. Examples of natural stressors and xenobiotic compounds which macroalgae in the coastal marine environment are likely to encounter.

## 9.5 Ecological implications of elevated UV-B radiation

This section which deals with the ecological implications of UV-B has been divided into to three sub-sections. The first relates to the fourth aim of this thesis and the main findings are placed in the wider contexts of existing work. The second section deals with how natural stressors in the coastal environment may influence the ecological implications of UV-B radiation and finally possible long-term effects of elevated UV-B will be discussed.

### 9.5.1 Aim No 4: Assessment of species-specific differences in susceptibility to UV-B radiation

The ability to cope with enhanced UV radiation varies widely among different algal species, as observed in Chapter 3 and 4, and confirmed by several workers (Hanelt 1992, Larkum and Wood 1993, Dring *et al.* 1996b, Häder *et al.* 1996a, Bischof *et al.* 1998). This has been related to their vertical distribution along the shore or in the water column (Franklin and Forster 1997, Bischof *et al.* 1998). Intertidal algae generally exhibit a high degree of UV-B tolerance as they are fully exposed to natural sunlight during low tide (Larkum and Wood 1993, Franklin and Forster 1997, Cordi *et al.* 1997, 1998a). Sagert and co-workers (1997), for instance, recorded greater depression of fluorescence yield and slower recovery in thalli of *Chondrus crispus* with increasing vertical depth on the shore. Larkum and Wood (1993) measured the sensitivity to artificial UV radiation of several species of phytoplankton, macroalgae and seagrasses. These workers found that the intertidal *E. intestinalis* and *Porphyra* sp. showed minimal reductions in chlorophyll fluorescence, compared to the subtidal algae *Ecklonia radiata* and *Kallymenia cribosa.*

Increased tolerance to UV-B radiation in intertidal species can be inferred from smaller initial reductions in photosynthetic efficiency, faster recovery, and in some cases little or no reduction in $P_{max}$ (Franklin and Forster 1997). Thus, intertidal algae may be expected to exhibit a high potential for physiological acclimation and genetic adaptation to increases in incident UV-B radiation. UV-B attenuation in coastal waters, on the other hand, with their higher concentrations of suspended particulate matter, ensures that UV-B can not penetrate much below 6 m (Smith and Baker 1979). Hence, deep subtidal algal species are likely to be protected against enhanced UV-B radiation. In contrast, the shallow subtidal species, which become partially exposed to UV during low tide, are likely to be the most vulnerable to shifts in UV-B radiation. Consequently, they are also most liable to suffer detrimental effects. Such subtidal seaweed species depend on the effectiveness of their compensatory mechanisms to offset UV-B induced damage (Examples are listed in Fig. 9.2. This figure is also discussed in greater detail below). Future studies will show whether enhanced UV-B radiation will, over time, lead to shifts in vertical zonation patterns and whether species composition will change in this particularly sensitive group.

## 9.5.2 UV-B radiation and other factors which effects community structure in the coastal marine environment

Numerous factors are influencing and shaping the community structure in the coastal marine environment. Changes in visible light and UV, desiccation, temperature and salinity due to the continuous tidal changes, as well as predation and nutrient availability are all important factors. The current knowledge regarding macroalgal tolerance to natural stressors has been thoroughly reviewed by Lobban and Harrison (1994), Davidson and Pearson (1996) and Franklin and Forster (1997). Although research into the interactive effects of these natural stressors is well established (Lobban and Harrison 1994 and references therein) almost

nothing is known about the interactions between these stressors and UV radiation (Behrenfeld *et al.* 1993). In the following paragraphs interactions between UV-B radiation and some of the above mentioned factors, as well as possible implications for the community structure will be dealt with in more detail.

## PAR, UV-A and UV-B

Recently the importance of investigating effects of PAR and UV-A in combination with UV-B has been realised, since it was demonstrated by several workers that both antagonistic or synergistic effects can occur (Rundel 1983, Caldwell *et al.* 1986, Forster and Lüning 1996, Franklin and Forster 1997). Light availability is an important factor which affects the canopy structure of macroalgae. It has been reported that shading by overlying thalli, which are themselves light adapted or have high levels of UV absorbing pigments, may protect underlying, more sensitive species or thallus regions (Franklin and Forster 1997). It is possible that elevated UV-B may bleach the upper levels of the canopy sufficiently to result in increased light levels reaching the more sensitive, shade adapted leaves underneath. This may ultimately also affect invertebrates and fish which live underneath or among the canopy. Given the potential for interactions of PAR, UV and other intertidal stressors, transplantation and acclimation studies in the field are still required to clarify the role of high natural irradiance in determining changes in community structure.

## Desiccation and temperature

Desiccation and temperature are two natural stressors which may also affect the degree of tolerance to elevated UV-B radiation. It has been reported that lengthy periods of desiccation can reduce photosynthetic rates in emerged macroalgae (Dring and Brown 1981). Desiccation

may provide a degree of protection which is not available to algae experiencing the same level of irradiance in the hydrated state (Franklin and Forster 1997). Preliminary results in algae suggest that desiccation, temperature and other natural stressors trigger protective mechanisms which may also be involved in protection against UV damage. Desiccation and temperature stress can cause intracellular active oxygen species which in turn can trigger enzymatic or biochemical defence systems. In microalgae, enzymes such as superoxide dismutase which are associated with radical scavenging may also have UV protective properties (Lesser 1996). Intertidal algal species may therefore have an advantage over subtidal algae in combating increased UV-B radiation *in situ*. Further biochemical research is needed to elucidate mechanisms of UV protection through desiccation and temperature.

**Predation**

Predation pressure is an equally important factor determining community structure in the coastal marine environment. Research in higher plants has found that UV protecting compounds such as flavonoids may also act as anti-predatory compounds. Moreover, it has been reported repeatedly that concentrations of flavonoids and related phenolic compounds increased when terrestrial plants were exposed to UV-B (Caldwell *et al.* 1995). Apart from UV-B protection afforded by increases in these compounds, there are many other ecological implications associated with changes in these and related compounds. Caldwell and co-workers (1995) mention that flavonoids are important herbivore and pathogen "repellents". Although, not yet investigated it is possible that changes in MAA's and other UV protective compounds in algae could have similar effects on the susceptibility to predators.

Herbivory in many systems is determined by the nutritional value of host tissue, in terms of water, carbohydrate or nitrogen contents (Paul *et al.* 1997). Thus, changes in the C:N ratio or

the palatability of the macroalgae may be compensated for altered consumption rates. Such interactions between predation pressure and UV-B radiation could ultimately affect higher trophic levels.

## 9.6 Future work and broader implications

This study has attempted to develop molecular and physiological techniques for use as early warning signals to UV exposure in macroalgae. Of equal importance, has been the investigation of the sensitivity of macroalgae exposed to UV-B, both in conjunction with an antifouling agent and on its own.

There is a need for further investigations into UV-B-induced effects in macroalgae. In addition to establishing biological weighing functions for macroalgae, which has already been mentioned by several workers (Franklin and Forster 1997), future research activities should include:

- further research into the potential biomarkers investigated in this thesis and the establishment of other, possibly UV specific, biomarkers of exposure and/or effects.

- investigations into the susceptibility of early life stages, in particular the reproductive unicells of several macroalgal species to UV-B exposure.

- considerations of coastal water contamination with other herbicides which are similar to Irgarol 1051 in structure and mode of action.

- assessments of the interactive relationships between other xenobiotic compounds and UV-B radiation in several macroalgal species.

It is hoped that the results obtained and conclusions drawn may enable a more informed assessment of both the particular effects of elevated UV-B in macroalgae and its wider implications for the coastal marine environment.

There is certainly an urgent need to improve our understanding of possible long-term effects of elevated UV-B on aquatic ecosystems (Häder *et al.* 1995). Thus, while this thesis has reported studies focused on the physiological and biochemical responses of macroalgae to UV-B, it is appropriate to end with some assessment of the broader ecological implications of such responses, even though such extrapolation remains speculative given current understanding.

Although there is overwhelming evidence that increased UV-B is harmful to aquatic ecosystems, quantitative estimates of the wider implications are incomplete at this stage (Häder *et al.* 1995). An overview of UV-induced responses at different levels of biological organisation (explained in detail in the previous sections) and their potential long-term implications, as described in detail below, are outlined in Figure 9.2.

Tolerance to xenobiotic compounds or enhanced UV-B levels may be a result of both molecular and physiological acclimation, and genetic adaptation (Weis and Weis 1989). Genetic adaptation, contrary to molecular and physiological acclimation, will result in UV induced resistance which is transferred to the next generation. Molecular and physiological acclimation mechanisms in algae (such as DNA repair or induction of protective pigments etc.), in conjunction with genetic adaptation will, therefore, play a critical role in counter-balancing UV-B induced detrimental effects. Examples of UV-induced acclimation and genetic adaptation are listed below.

# Ozone depletion related increases in UV-B radiation

**Effects at the molecular level**

DNA and RNA damage, Increases in mutation rate

Impaired enzyme activity

**Effects at the physiological level**

Photosynthetic damage, Impairment of membrane integrity, Destruction of pigments,

*In vivo* absorptance changes

**Effects on mature plants**

Impaired growth and morphology

**Effects on early life stages**

Impaired germination and growth

Altered ratio between sexual and asexual reproduction

**Molecular and physiological acclimation:**

DNA repair

Photoinhibition, Induction of protective pigments

**Genetic adaptation,** resulting in increased UV-resistence in algal populations and communities

**Exposure to other stressors (natural and xenobiotic)**

Compensatory responses outweigh UV- induced changes, with no overt alterations in population abundance.

Molecular and physiological acclimation is insufficient to maintain status quo.

• Changes in genetic composition of algal populations

• Reduced population fitness

• Species composition changes

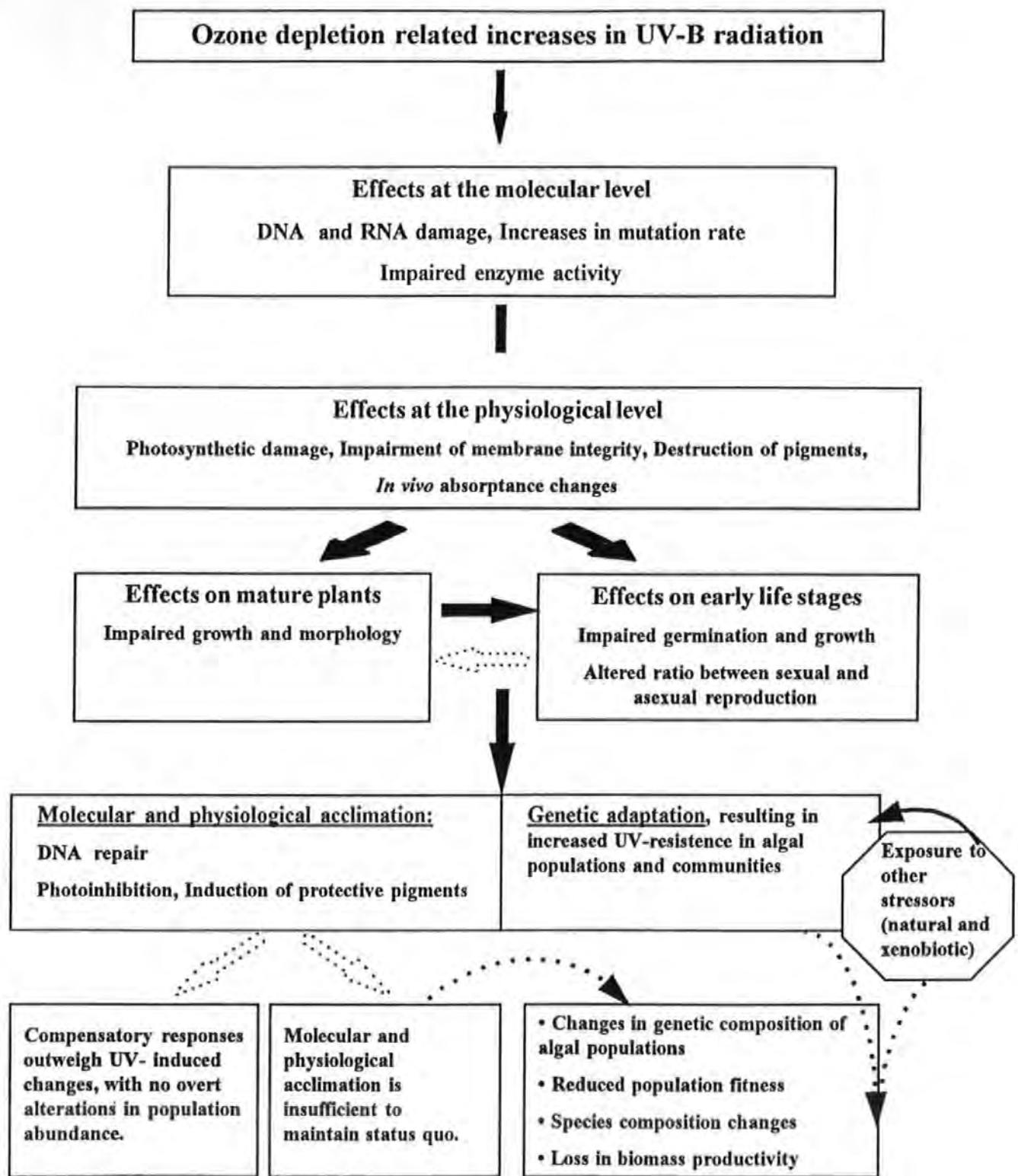• Loss in biomass productivity

Figure 9.2. Responses to ozone related increases in UV-B radiation at different levels of biological organisation, reported in the literature, in marine algae and their potential implications for the coastal marine environment. Black arrows indicate experimentally established links and the dashed arrows indicate interactions for which there are as yet no experimental evidence.

Elevated UV-B may exert selective pressure on algal populations resulting in UV resistant individuals (fig. 9.2). These individuals will subsequently produce more UV-resistant progeny. However, a consequence of such selection pressures may also be genetic composition changes in algal populations (Depledge 1994). Such UV induced changes in genetic composition can in turn lead to lowered tolerance to other natural and/or xenobiotic stressors. For example, Baird *et al.* (1990) who investigated the responses of different genotypes of *Daphnia magna* to cadmium and 3,4 dichloroaniline (DCA), illustrated that a genotype exhibiting resistance to cadmium was ill adapted to tolerate 3,4 dichloroaniline exposure. Thus, the genetic diversity within a population may be eroded as exposure to several stressors occurs. Such genetic diversity reductions may be associated with reduced population fitness, species composition changes and loss in biomass productivity (illustrated by the lower right hand arrow in fig. 9.2).

Compensatory responses such as DNA repair and physiological acclimation may sufficiently outweigh UV induced molecular and physiological damage. However, if these acclimation mechanisms are insufficient, far reaching detrimental effects may also ensue (please see fig. 9.2, lower left hand arrows). For example, a major loss in primary biomass productivity may have significant consequences for the intricate food web in aquatic ecosystems and affect food productivity. Whilst the effects of a 5 % loss in microalgae due to elevated UV-B, have been estimated in terms of subsequent biomass production on fish stocks (Smith and Baker 1989, Weiler and Penhale 1994, Häder *et al.* 1995), no such calculation is available for near-shore primary productivity of macroalgae. The implications of changes in species diversity within algal communities may be far reaching. If the species composition of the primary producers is altered, the quality and quantity of food for secondary consumers may change, with unknown consequences for ecosystem function (Karentz 1991). The impact on the food web would be

significant if populations or species that may tolerate or benefit from elevated levels of UV-B are of lower nutritional value for the next trophic level (Worrest 1982).

Although there is clear evidence that increased UV-B exposure is harmful to marine macroalgae, estimates of the long-term implications on aquatic ecosystems, described in the previous discussion, remain speculative. Work on this level of organisation clearly represents a significant challenge for future research.

# APPENDIX

## PUBLICATIONS AND ABSTRACTS FROM CONFERENCES

Two papers are included in the appendix:

Evaluation of chlorophyll fluorescence, *in vivo* spectrophotometric pigment absorption and ion leakage as biomarkers of UV-B exposure in marine macroalgae. *Mar. Biol.* (1997) **130**, 41-49.

Algal gloom. Ultraviolet radiation could interfere with the base of the marine food chain. An account of the results presented at the Final Year Wellcome Trust Students Meeting by F. Pearce in the *New Scientist*, 8 August (2146), p. 24 (1998).

Finally, abstracts from conferences are included.

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# Evaluation of chlorophyll fluorescence, in vivo spectrophotometric pigment absorption and ion leakage as biomarkers of UV-B exposure in marine macroalgae

**Abstract** The photosynthetic fluorescence ratio $F_v:F_m$, in vivo absorption spectra and ion leakage were evaluated as biomarkers of ambient and elevated UV-B (280 to 320 nm) exposure of the intertidal alga *Enteromorpha intestinalis* (Chlorophyta) and the sublittoral alga *Palmaria palmata* (Rhodophyta). Measurements of thallus growth were also used to assess adverse biological effects. Ambient and elevated UV-B significantly inhibited photosynthesis in both species. It was shown that the $F_v:F_m$ ratio is a sensitive, non-specific general biomarker of UV-B exposure in both species. Moreover, the in vivo absorption of what was tentatively identified as chlorophylls *a* and *b* as well as phycoerythrin and/or carotenoids, phycoerythrobilin and phycocyanin decreased in a dose-response dependent manner and was associated with a decrease in growth rate in *P. palmata*. The intertidal alga *E. intestinalis* showed a greater degree of tolerance to UV-B exposure. These results indicate that changes in the $F_v:F_m$ ratio together with reductions in in vivo pigment absorption could provide an early quantitative warning of the detrimental effects of UV-B in marine macroalgae.

## Introduction

Stratospheric ozone depletion, resulting from the release of anthropogenic chlorofluorocarbons (CFCs), is asso-

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ciated with increased UV-B irradiation (280 to 320 nm) of the earth's surface (Frederick 1993; Kerr and McElroy 1993). Even with strict international controls on the use of CFCs, ozone depletion is predicted to continue well into the next century (Madronich et al. 1995), affecting both Antarctica (Farman et al. 1985) and the northern hemisphere (Stone 1993). In this context, investigations into the effects of enhanced UV-B radiation on phytoplankton and macroalgae is of great importance, since these vital primary producers form the basis of aquatic food webs (Houghton and Woodwell 1989). Macroalgae in particular are major primary producers in near-shore and intertidal ecosystems. Rates of productivity are comparable with those measured in the most productive terrestrial ecosystems (Mann 1972; Duggins et al. 1989).

The biomarker approach has been used extensively by ecotoxicologists for detecting exposure to and effects of environmental contamination during the past decade (Depledge 1989; McCarthy and Shugart 1990; Fossi and Leonzio 1994). One of the most useful definitions of an ecotoxicological biomarker was formulated by the National Academy of Science, USA (National Research Council 1989) and later modified by Depledge (1994): "A biochemical, cellular, physiological, or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms (either individuals or populations) that provides evidence of exposure to and/or effects of one or more chemical pollutants (and/or radiations).".

Biomarkers raise the possibility of determining where an organism lies on the health-status curve (Depledge 1989). Thus, they potentially provide an early warning of pollutant or UV-B-induced reversible departures from homeostasis, or signal the triggering of compensatory biochemical and physiological responses. The simultaneous use of more than one biomarker enhances the detection of effects, since different biomarker responses are induced at different stages of the plant or organism's health-status curve (Depledge 1994). This

clinical approach is comparable to that used in human medicine (Peakall 1992). Biomarkers can be identified that indicate exposure to stress (general non-specific biomarkers), specific categories of stress (natural or related to pollutant exposure), and specific types of stressors (such as acetylcholinesterase inhibition following exposure to organophosphate pesticides). The biomarker approach has proven especially useful when these responses can be linked to changes in Darwinian fitness parameters (Depledge 1989).

The emphasis to date in photobiology has been to establish a correlation between ozone depletion and elevated UV-B. Action spectra and weighting functions have also been derived (Caldwell 1971; Cullen et al. 1992). However, finding biochemical, cellular or physiological responses which can be related in a dose-dependent manner to UV-B exposure, and therefore act as early warning signals to changes in UV-B levels, has rarely been attempted (Campos et al. 1991; Cullen and Lesser 1991). If these early warning signals can be identified, they might be used to signal a change in biological fitness that could have consequences for entire populations. The aim of this study was to: (a) investigate the suitability of chlorophyll fluorescence, changes in in vivo pigment absorption and ion leakage as biomarkers of UV-B exposure in marine macroalgae; (b) to relate these physiological parameters to consequent biological effects measured as growth rate.

## Materials and methods

### Algal collections

The intertidal alga *Enteromorpha intestinalis* (Chlorophyta) and the sublittoral alga *Palmaria palmata* (Rhodophyta) were collected at low tide from Wembury on the southwest coast of Devon, England (Latitude 50°) in February (sea-water temperature = 5 °C) and in June (sea-water temperature = 10 °C). The thalli were harvested 24 h before the experiments and kept in filtered sea water at constant temperature (15 °C) and in low light conditions (25 µmol $m^{-2} s^{-1}$, fluorescent tubes, Phillips TLD 32W/83 HF) in growth cabinets. *P. palmata* was kept in a 12 h light:12 h dark regime, and nitrate ($NO_3^-$) and phosphate ($PO_4^{3-}$) (5:1) were added to the sea water. *E. intestinalis* was kept under constant light (intensity as above); no nutrients were added to avoid sporulation.

### UV treatments and experimental design

Thalli of both species, cut to a length of ~3 cm and covered to a depth of 1.5 cm with filtered sea water, were exposed in open petri dishes. Photosynthetically active radiation (PAR) and temperature conditions were identical to those in the acclimatisation period. PAR was measured with a PAR meter (Sky Instruments). UV-B was supplied by two UV-B tubes (Philips TL 20W/12 RS) and UV-A by two UV-A tubes (Philips 1609 15 W). UV-A was kept constant at 130 µW $cm^{-2}$, which is approximately four times less than that calculated at Latitude 50° (Cornwall) in December (Driscoll et al. 1992 and personal communication). This was maintained independent of the UV-B intensity. UV-A radiation was included in the exposures in view of the finding of Middleton and Teramura (1993) that it modifies the effectiveness of UV-B radiation for several plant characteristics. For instance, UV-A has been found to take part in photorepair and photoprotection (Beggs et al. 1985).

The total irradiance of both UV-B and UV-A tubes was measured using an optical radiometer (MicroPulse Technology) with a MP-229 UV-B sensor and a MP-236 UV-A sensor, respectively. These were calibrated by the manufacturer in the same year. Using the output spectrum of the UV-B tube, a compensation for changes in the spectral response of the Micropulse UV-B sensor was undertaken in 0.5 nm steps by using accurate values for the spectral response of the sensor supplied by Micropulse Technology. For UV-B treatments, the following intensities were measured: 17 µW $cm^{-2}$ (approximate UV-B intensity in Cornwall in February calculated by the National Radiological Protection Board UK; Driscoll et al. 1992 and personal communication), 137 µW $cm^{-2}$ (approximate UV-B intensity in Cornwall in June calculated by the National Radiological Protection Board UK; Driscoll et al. 1992, personal communication). Values of 260, 359, 855, 896 or 1270 µW $cm^{-2}$ were chosen as elevated UV-B intensities. The different UV-B intensities were obtained by adjusting the height of the UV-B tubes above the thalli. UV light was filtered with 35 µm cellulose diacetate foil (AC Converters Ltd.) which showed 0% transmission below 286 nm (UV-C). For the control thalli, UV tubes were covered with Mylar 125 D (Du Pont Ltd) which showed 0% transmission below 320 nm (i.e. filtering out UV-B). Transmission of the filters was measured before and after the experiments with a ATI Unicam UV/Vis spectrometer.

Using the data of Rundel (1983) based on the action spectrum for inhibition of photosynthesis in isolated chloroplasts (Jones and Kok 1966), the increase in biologically effective radiation due to 50% ozone depletion is 4.0× that of ambient; this is approximately of the same magnitude (3.5×) as that estimated for human erythema for 50% ozone depletion (Urbach 1995). Thus, the elevated UV-B exposures of 260, 359, 855, 896 and 1270 µW $cm^{-2}$ used in the present study are thought to represent increases in flux expected for 31, 42 and 100% (855, 896, 1270 µW) ozone depletion, respectively, in the summer months. The relatively high values of UV-B intensity were chosen to investigate the entire range of the dose-response curves.

### Measurements of chlorophyll-fluorescence induction

In vivo chlorophyll fluorescence was measured with a "plant efficiency analyser" (Hansatech Instruments). The ratio $F_v:F_m$ was determined before, immediately following, and 24 and 48 h after UV exposure ($F_m$ = maximal fluorescence; $F_0$ = initial fluorescence; variable fluorescence, $F_v = F_m - F_0$). Before measurements, the thalli were dark-adapted for 10 min to ensure an oxidised electron-transport chain. Prolonged dark adaptation (25 min) showed no change in $F_0$ and $F_m$. The fluorescence was initiated by 1 s red-light pulses with a peak wavelength of 650 nm and an intensity of 3000 µmol $m^{-2} s^{-1}$. Illumination was provided by an array of six high-intensity light-emitting diodes (LEDs, Hansatech Instruments) which were focused onto the thallus surface to provide even illumination.

### In vivo spectrophotometric measurements

Algal pigment absorption spectra were measured in vivo at room temperature to avoid hypsochromic shifts. The samples were placed in a Labsphere (RSA-UC 40), a diffuse reflectance and transmittance accessory, inside the UV/Vis spectrometer (Unicam). Thallus transmission and reflectance spectra were recorded for both species before exposure (0 h), immediately following, and 24 and 48 h after an initial 3 h exposure. The tissue samples were placed in the sphere and a baseline measurement was performed for each thallus to avoid variations in substitution error. A light trap was placed behind the sample to avoid scattering. All spectra were gathered with a 4 nm bandpass from 215 to 830 nm. To obtain the percentage absorption spectra, which is a percentage equivalent of absorptance, the following equation was used: % absorption = 100 − (% transmission + % reflectance).

### Ion-leakage measurements

Ion leakage was measured using a modified version of the method of Axelsson and Axelsson (1987). One gram tissue of *Enteromorpha intestinalis* and 0.4 g of *Palmaria palmata* were used for each replicate, which was split into duplicates to determine method variation; an average variation of ±10% was found. Ion leakage was measured as electrical conductivity with a Corning 220 conductivity meter at room temperature. The tissue was rinsed very quickly in 50 ml ultra-pure water and transferred to 25 ml ultra-pure water (Sample 1). After 2 min, the tissue was drained quickly and transferred to a second beaker containing 25 ml ultra-pure water and boiled for 5 min (Sample 2). The conductivity of both samples was measured at room temperature and the "health index" was calculated on the basis of the ion loss as: Sample 2/(Sample 1 + Sample 2), expressed as percentage.

The minimum value obtained from dead thalli by boiling the tissue in instant ocean for 5 min and then leaving it to equilibrate in instant ocean for 30 min was 20% (Schild et al. 1995).

### Growth measurements

The physiological variables were related to differences in thallus growth by measuring the increase in thallus area on a Quantimet 570 Automatic Image Analyser during a 2 wk period. The process of image capture, image processing and measurements was programmed using QBASIC, the command language of the Quantimet. Distance and light intensity were calibrated prior to measurements.

### Statistical analysis

Statistical analyses were conducted using StatGraphics 6.1. For $F_v:F_m$ data, the spectrophotometric measurements and growth data, sequential sampling of each thallus at all time periods was performed so that each thallus could act as its own control. Interindividual variability was thus taken into account in the statistical analysis. A multivariate analysis of variance for repeated measurements was applied. If the above tests revealed significant differences between groups and overall significant time–treatment interaction, Bonferroni tests were conducted to determine where significant differences occured (Milliken and Johnson 1984). A random design was chosen to analyse ion-leakage data, and statistically tested by performing one-way (single factor) analysis of variance (ANOVA). Tukey's HSD test was used to reveal statistical differences. Growth data was analysed by multiple-regression analysis. Slopes of the regression lines were determined on log-transformed data.

## Results

### Measurements of chlorophyll fluorescence

Fig. 1a and b show significant decreases in the $F_v:F_m$ ratio for all UV-B intensities at almost all exposure times for both *Enteromorpha intestinalis* and *Palmaria palmata*. After 3 h exposure to 260 µW cm$^{-2}$ (equivalent to ≃31% ozone depletion), a decrease of 50% compared to pre-exposure values was found for *E. intestinalis* and 75% for *P. palmata*. At 1270 µW cm$^{-2}$, after 1.5 h exposure, a 75% decrease in $F_v:F_m$ was found for *E. intestinalis* and 80% for *P. palmata*. These results (presented in Fig. 1c and d as dose-response curves) show an exponentially decreasing relationship between UV-B dose and the $F_v:F_m$ ratio for both species. One curve was fitted for each species and one equation was

calculated as a mean of all the points. The comparatively low $F_v:F_m$ values measured for *P. palmata* at 3 and 4.4 J cm$^{-2}$h$^{-1}$ using an UV-B intensity of 260 µW cm$^{-2}$ are here considered as variation, since no measurements were made at this dose using an UV-B intensity of 1270 µW cm$^{-2}$.

A similar experiment performed in June showed recovery to control $F_v:F_m$ ratio for *Enteromorpha intestinalis* at all intensities after an initial 3 h exposure to UV-B (Fig. 2a). However, for *Palmaria palmata* (Fig. 2b) an 18% recovery to 25% of the pre-exposure value was only found at the lowest intensity after 48 h.

### Spectrophotometric measurements

The absorption spectrum for *Enteromorpha intestinalis* (Fig. 3a) demonstrates the presence of chlorophylls *a* and *b* as evidenced in absorption at ≃440 and 680 nm, and at 470 and 657 nm, respectively. The broad shoulder at ≃490 nm is assigned to β-β carotene, lutein and xanthophylls in Chlorophyta (Britton 1984). However, no decrease in absorption at any intensity or time interval occurred. In contrast, a highly significant decrease in absorption was found for *Palmaria palmata* 48 and 72 h after 3 h exposure to 260 µW cm$^{-2}$ (Fig. 3b), ($p < 0.001$, multiple ANOVA). Moreover, a profound decrease in absorption was observed immediately after 3 h exposure to 896 µW cm$^{-2}$ (Fig. 3c), ($p < 0.001$, multiple ANOVA).

Fig. 4 shows that for *Palmaria palmata* 48 h after exposure, the greatest decreases in absorption occurred at 498 nm (characteristic of the phycourobilin chromophore of rhodophycean phycoerythrin and/or carotenoids) and at 568 nm (characteristic of the phycoerythrobilin chromophore of phycoerythrin; Smith and Alberte 1994). The absorption maxima at 436 and 678 nm are attributable to the presence of chlorophyll *a* (Smith and Alberte 1994), and the band at 626 nm probably arises from phycocyanin (Kursar and Alberte 1983). The peak at 336 nm may arise from mycosporine-like amino acids such as porphyra-334 (peak at 334 nm), shinorine (peak at 334 nm), and/or glycine:valine (peak at 335 nm) (Karentz et al. 1991).

### Ion-leakage measurements

A general decrease in the health index compared to the control value was found for *Palmaria palmata* 24 h after exposure to 855 µW cm$^{-2}$, and 48 h after exposure to 359 and 855 µW cm$^{-2}$ (Table 1, $p < 0.001$, ANOVA). No significant decreases in the health index were found for *Enteromorpha intestinalis*.

### Growth measurements

A highly significant decrease in growth rate measured as percentage area increase of thallus was found for
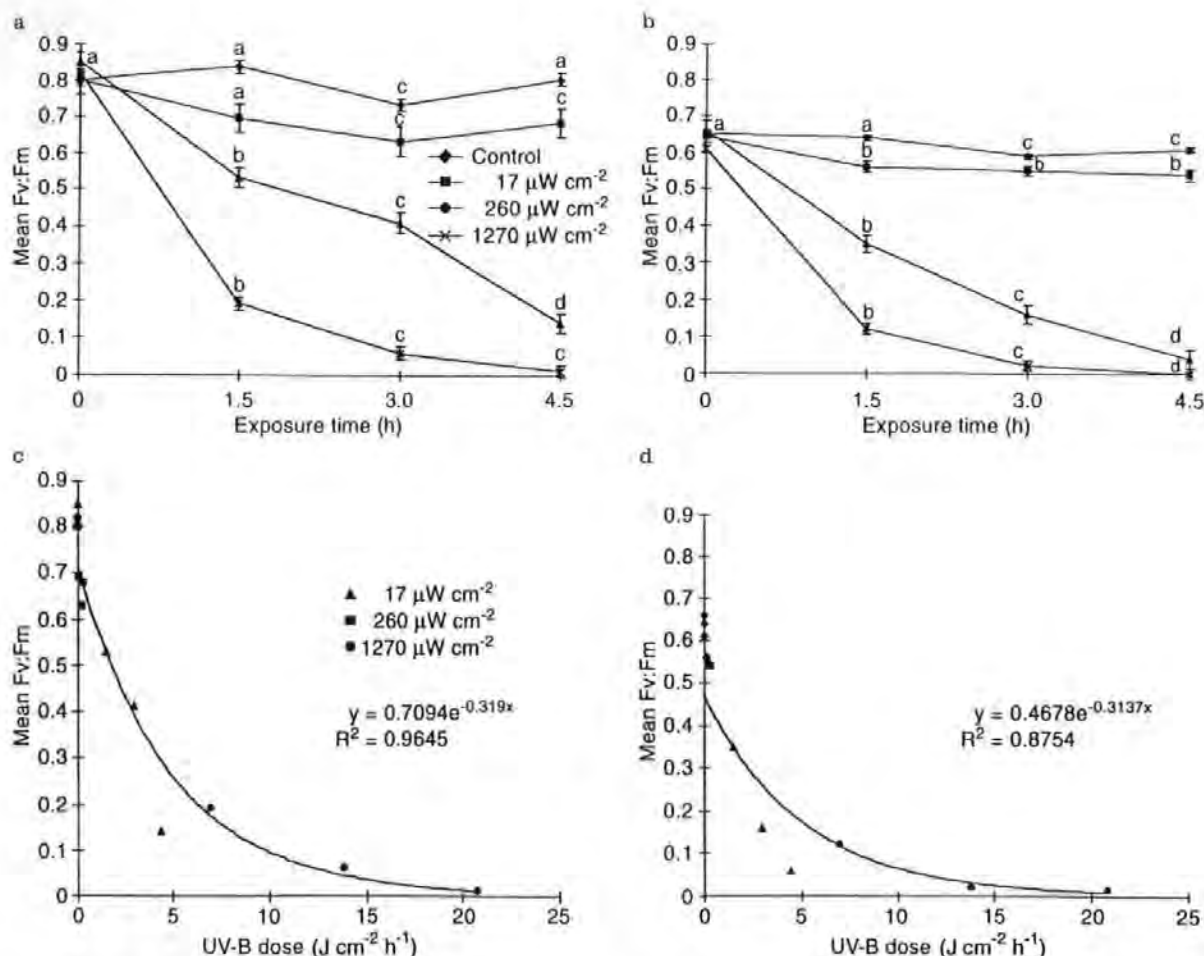
*Enteromorpha intestinalis* at 896 µW cm$^{-2}$ and for *Palmaria palmata* at 137 µW cm$^{-2}$ compared to controls (Fig. 5), ($p < 0.001$, multiple regression). Growth rate for the exponential equation for *E. intestinalis* control group was $\alpha = 0.018$ as opposed to $\alpha = 0.008$ for the treated group ($p < 0.001$). Growth data for *P. palmata* was analysed statistically until Day 11, as the growth rate approached zero after this day. This plateau was thought to be due to the artificial growth conditions in the laboratory. Growth rate for the exponential equation for *P. palmata* control was $\alpha = 0.009$ as opposed to $\alpha = 0.005$ for the treated group ($p < 0.001$).

**Fig. 1** *Enteromorpha intestinalis* (a, c) and *Palmaria palmata* (b, d). a, b Chlorophyll fluorescence-induction parameter $F_v:F_m$ as a function of duration of exposure to different UV-B (280 to 320 nm) intensities; data are means ± SE ($n = 5$); means with different letters within same treatment differ significantly from each other (ANOVA; $p < 0.05$). c, d dose-response curves

## Discussion

It has been shown in this study on *Enteromorpha intestinalis* and *Palmaria palmata* that the $F_v:F_m$ ratio is a sensitive biomarker which responds in a dose-response-dependent manner to exposure to UV-B (Fig. 1). In addition to visible light, solar UV radiation is a strong environmental stress factor which modifies the photosynthetic activity of both plants and algae. In several species of marine benthic algae and phytoplankton, UV-B exposure affects several targets in photosynthesis. UV-B impairs the D1 protein associated with Photosystem II, which results in a decrease in the noncyclic photosynthetic electron transport (Renger et al. 1989). Furthermore, the water-splitting site of Photosystem II, and the reaction centre of Photosystem II can be damaged (Bhattacharjee and David 1987; Bhattacharjee et al. 1987). Herrmann et al. (1995) found decreased $F_v:F_m$ levels both in macroalgae exposed to solar PAR alone and to algae exposed to Solar UV-B and PAR, and was therefore unable to isolate the cause for the decreased chlorophyll fluorescence. Moreover, Helbling et al. (1992) concluded that UV-A radiation accounted for ($> 50\%$ of the photoinhibition caused by total solar UV. However, in the present study, the linear relationship between time of exposure and $F_v:F_m$ values in the control group (low PAR and low UV-A) indicates that the decreases in the treated groups were due to UV-B exposure alone.

Björkman (1987) and Demming and Björkman (1987) reported that a decrease in $F_v:F_m$ was linearly related to a decrease in the optimal quantum yield of photosynthesis. In the present study, decreased $F_v:F_m$
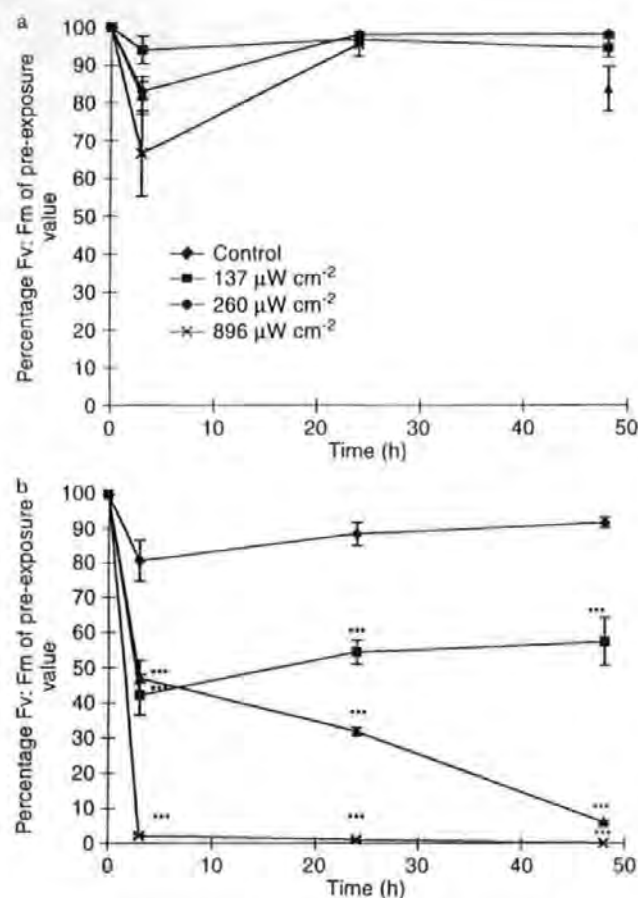
Fig. 2 *Enteromorpha intestinalis* (a) and *Palmaria palmata* (b). $F_v{:}F_m$ as a function of time after exposure to different UV-B intensities; exposure time for all UV-B intensities was 3 h; data are means $\pm$ SE ($n = 5$) [*** significant differences between UV-B-treated thalli and control thalli at $p < 0.001$; overall significant time-treatment interaction ($p < 0.001$) was found for both species]

biotic (Conrad et al. 1993; Sgardelis et al. 1994), chlorophyll fluorescence can be characterised as a very sensitive general biomarker. In conjunction with other biomarkers, some possibly UV-specific, chlorophyll fluorescence may offer a valuable early warning of when solar UV induces early, reversible departures from homeostasis or from the compensatory zone. "Endpoint effects" such as impairment of growth should be used with this biomarker of exposure, since $F_v{:}F_m$ (and $F_v$, data not presented) for *Enteromorpha intestinalis* showed full recovery 24 h after exposure, although a depressed growth rate of 53.9% was observed 12 d after exposure to 896 $\mu$W cm$^{-2}$ (Fig. 5a). Similar results were found in another experiment exposing *E. intestinalis* to 260 $\mu$W cm$^{-2}$ (unpublished data of present authors).

Ion leakage

The decreased health index for *Palmaria palmata* after UV-B exposure (Table 1), indicates damage to the plasmalemma. However, this damage was not found to be a particularly sensitive parameter of UV-B exposure. Moreover, no decrease in the health index was found for *Enteromorpha intestinalis*. Although research into membrane damage caused by UV-B has been limited, it is known that the integrity of the membranes is affected by UV-B (Murphy 1983; Koch et al. 1995), and is due to a decrease in lipid content and damage to membrane-transport systems. Although the cellular and physiological mechanisms responsible for membrane damage are uncertain, the results in this study are important for the identification of the mechanisms of UV-B exposure, especially in view of the lack of bibliographic information on the effects of UV-B on membranes.

and also $F_v$ (data not presented) with no recovery clearly shows irreversible damage to Photosystem II for *Palmaria palmata*. Similar results of decreased $F_v$ values in chloroplasts and leaves of higher plants have been reported (Björn et al. 1986; Renger et al. 1986). Trocine et al. (1981) found that gross photosynthesis is sensitive to UV-B and Larkum and Wood (1993) concluded that $F_v$ may be used as an indicator of UV-B inhibition. The present study confirms these results. Moreover, the finding of decreased $F_v{:}F_m$ after 1.5 h exposure to 17 $\mu$W cm$^{-2}$ (calculated average February intensity) in *P. palmata* (Fig. 1) clearly indicates the sensitivity of this parameter to UV-B. This study shows for the first time a predictive dose-dependent relationship between $F_v{:}F_m$ and UV-B exposure, indicating that the $F_v{:}F_m$ ratio can provide an early quantitative warning of UV-B effects in macroalgae. The greater the response of $F_v{:}F_m$, the further the macroalgae measured here have progressed along the health-status curve.

However, since chlorophyll fluorescence is known to respond to a wide variety of stressors, both natural (Henley et al. 1991; Herrmann et al. 1995) and xeno-

Pigment measurements

*Palmaria palmata* exhibited a steady decline in what has been tentatively identified as chlorophylls *a* and *b* absorption maxima, as well as phycoerythrin and/or carotenoid, phycoerythrobilin and phycocyanin absorptions with increasing UV-B (Figs. 3 and 4). The loss of chlorophyll absorption was discovered to be slower than that of phycoerythrin and/or carotenoids and phycoerythrobilin, indicating that the accessory pigments were impaired first. Döhler (1987) and Zündorf and Häder (1991) reported similar results with pigment changes in marine diatoms and cyanobacteria after UV exposures.

The present study revealed a dose-response-dependent decrease in chlorophyll, phycoerythrin and/or carotenoid and phycoerythrobilin absorption with UV-B intensity, indicating that these pigments may be suitable as general biomarkers of exposure.

The presence of UV-B screening compounds in algae has been well documented since the early work of Sivalingam et al. (1974), and mycosporine-like amino acids have been identified as major screening compounds

**Fig. 3** *Enteromorpha intestinalis* (a) and *Palmaria palmata* (b, c). Average in vivo absorption spectra before and after initial 3 h exposure to 260 μW cm$^{-2}$ (a, b) and before and after initial 3 h exposure to 896 μW cm$^{-2}$ (c) (*, **, *** significant differences between absorption maxima before and after exposure at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $n = 5$)
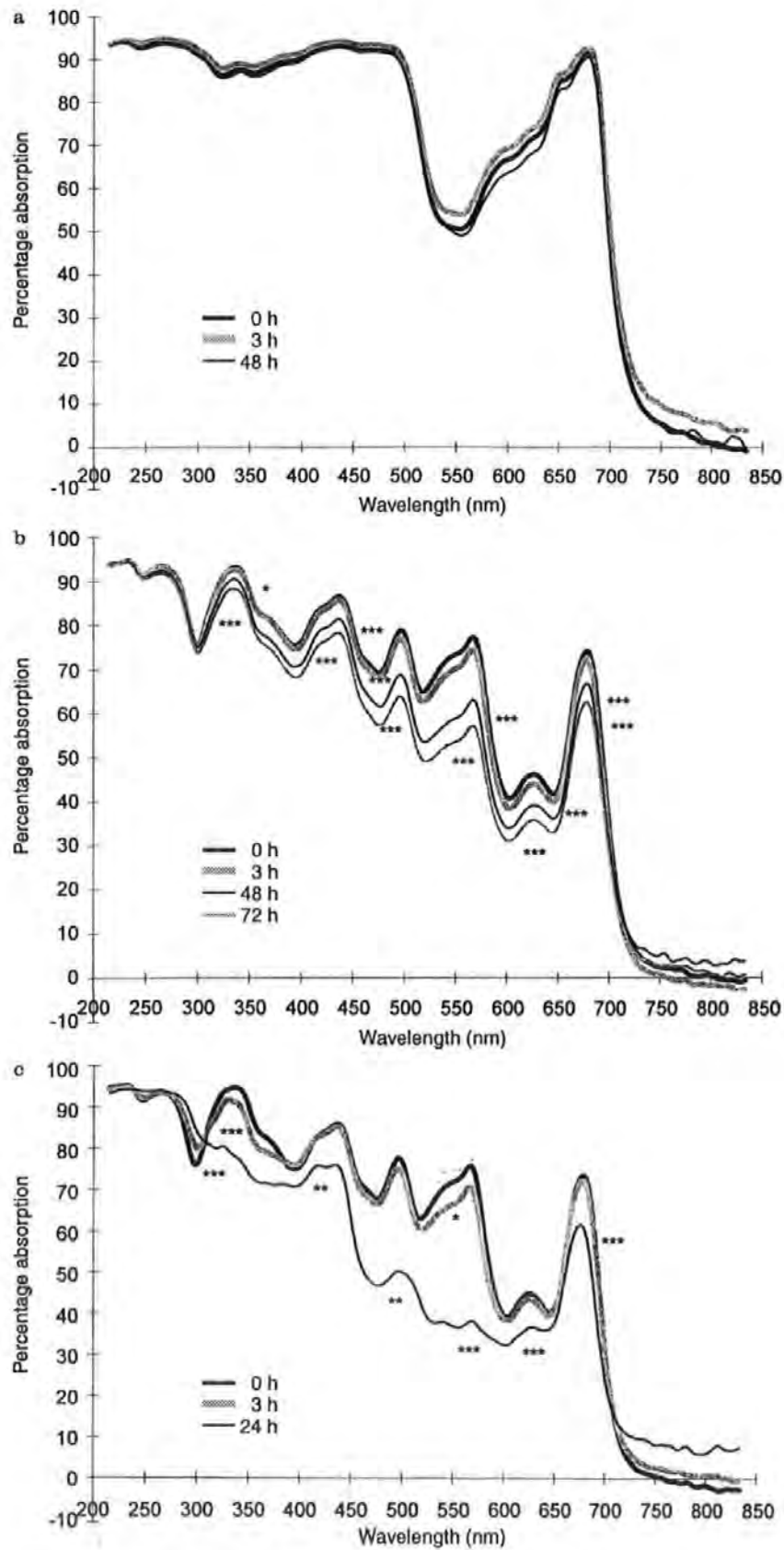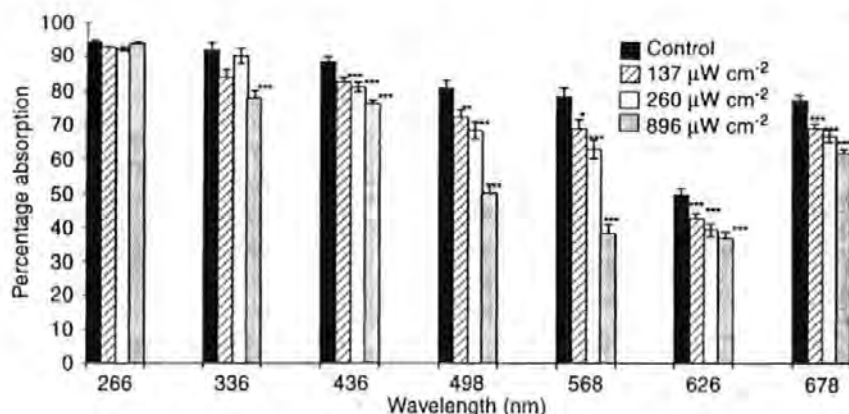
Fig. 4 *Palmaria palmata*. Percentage absorption of thalli as a function of different absorption maxima 48 h after initial 3 h exposure to different UV-B intensities; data are means ± SE ($n = 5$) (*, **, *** significant differences between UV-B treated thalli and control thalli at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively)



in algae, corals and a number of other animals (Dunlap and Chalker 1986; Karentz et al. 1991). These shielding compounds are effective from 310 to 335 nm. Karentz et al. have shown the existence of several of these compounds such as porphyra-334, shinorine, and glycine:valine, in *Palmaria decipiens*. The peak at 336 nm found in the present study indicates the existence of such compounds in *P. palmata*.

Several studies have recorded evidence of induced levels of mycosporine-like amino acids with decreasing depth in the water column (Sivalingam et al. 1974; Dunlap et al. 1986; Post and Larkum 1993). It is interesting to note the window found in pigment absorption between 290 and 315 nm in *Palmaria palmata* ($\simeq 75\%$ absorption) as opposed to *Enteromorpha intestinalis* (95% absorption in this region). This confirms the above findings and can be interpreted in terms of increased screening compounds in intertidal algae compared with sublittoral algae. *E. intestinalis* showed no UV-B-related pigment changes compared to *P. palmata*, and the lack of UV-B effects on photosynthetic efficiency confirms that algae from high-illumination habitats develop pro-

tective mechanisms against the detrimental effects of UV-B. These results, together with the findings of other workers of a site-dependent induction in these compounds, suggest a dose-dependent induction to UV radiation. However, further research is necessary to establish such a relationship and to investigate the suitability of these compounds as specific biomarkers of UV exposure.



Table 1 *Enteromorpha intestinalis* and *Palmaria palmata*. Ion leakage calculated as health index for thalli 24 and 48 h after initial 3 h exposure to different UV-B intensities [** significant differences between UV-B treatments and control group ($p < 0.001$; $n = 5$)]

| UV-B intensity ($\mu W\ cm^{-2}$) | Health index (%)[a]: | | |
|---|---|---|---|
| | Prior to exposure | 24 h after exposure | 48 h after exposure |
| *Enteromorpha intestinalis* | | | |
| Control | 51.9 | 50.4 | 47.2 |
| 260 | – | 55.9 | 53.6 |
| 359 | – | 57.0 | 54.6 |
| 855 | – | 56.5 | 52.3 |
| Dead thalli (*E. intestinalis*) | 20.0 | | |
| *Palmaria palmata* | | | |
| Control | 86.9 | 84.8 | 86.1 |
| 260 | – | 80.7 | 83.0 |
| 359 | – | 80.8 | 73.5 (**) |
| 855 | – | 46.2 (**) | 53.2 (**) |

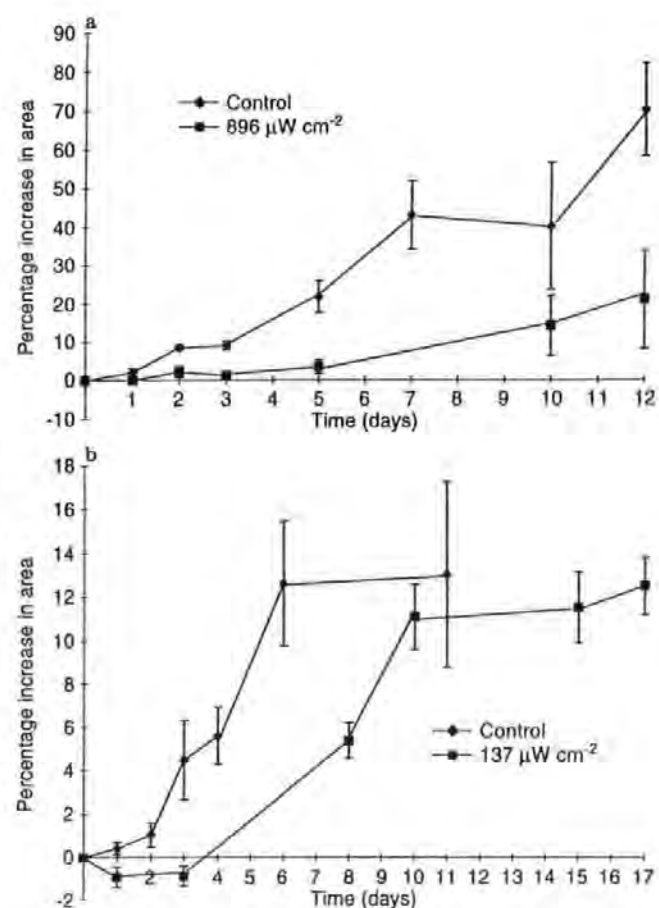[a] See "Materials and methods – Ion-leakage measurements" for calculation of health index

Fig. 5 *Enteromorpha intestinalis* (**a**) and *Palmaria palmata* (**b**). Growth of mature thalli calculated as percentage area increase from Day 0 measured over 12 and 17 d periods after initial 3 h exposure to 896 or 137 $\mu W\ cm^{-2}$, respectively. Data are means ± SE ($n = 5$)

The inhibition in growth found for *Palmaria palmata* in this study confirms the detrimental effects of UV-B even at ambient levels. Similar results of inhibited growth were found by Grobe and Murphy (1994) in the intertidal alga *Ulva expansa* at ambient and elevated UV-B levels, and an inverse linear relationship between growth rate and duration of UV-B irradiance was demonstrated.

## Conclusions

It has been shown that the $F_v:F_m$ ratio is a sensitive, non-specific general biomarker of UV-B exposure in *Enteromorpha intestinalis* and *Palmaria palmata*. Additionally, absorptions identified as being due to chlorophylls *a* and *b*, as well as phycoerythrin and/or carotenoids, phycoerythrobilin and phycocyanin, measured by in vivo spectrophotometry, decreased in a dose-response-dependent manner. Reduction in these physiological parameters was associated with detrimental manifestations such as membrane damage and reduced growth rate in the sublittoral algae *P. palmata*.

To establish biomarkers of exposure which can be used as early warning signals and which are related to biological effects, our future research will seek to determine whether the physiological parameters used in this study follow the same predictive pattern after long-term exposure to solar UV-B.

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# Algal gloom

## Ultraviolet radiation could be interfering with the base of the marine food chain

THE impact of ozone holes on plankton in the Arctic and Southern oceans may be much greater than anyone thought.

Scientists at the University of Plymouth have found that the reproductive cells of algae are several times as sensitive to ultraviolet radiation as mature cells. This means that ozone holes may seriously stunt the growth of marine plankton, the base of the ocean's food chain.
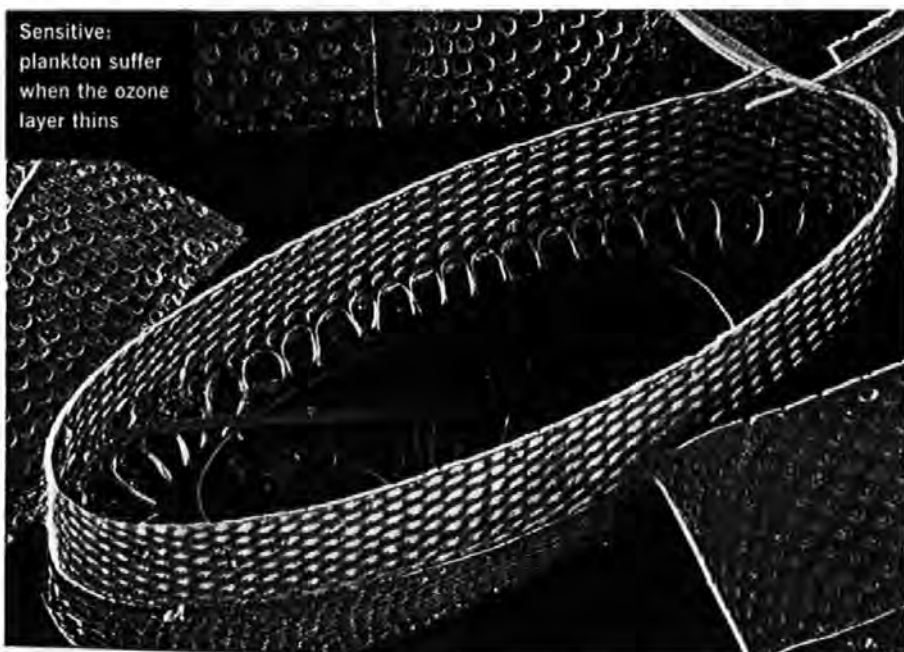
A team led by Britt Cordi of the university's Environmental Research Centre has been studying the sensitivity of the reproductive cells of the species *Entero-morpha intestinalis* to UV-B, the ultraviolet radiation that streams through the ozone holes over the north and south polar regions. In the laboratory they found that asexual spores were six times as sensitive to UV-B as mature algae, measured by the slowdown in their rate of photosynthesis after each increase in exposure.



Sensitive: plankton suffer when the ozone layer thins

Free-swimming gametes, the sexual means of reproduction, were more susceptible still. After one hour's exposure to the equivalent of an ozone hole with 30 per cent of the ozone layer gone, the rate of photosynthesis fell by 65 per cent. This, says Cordi, reduced the growth rate of the organisms by up to 17 per cent and halved their chances of successful germination. The findings were presented at the 1998 final year student meeting of the Wellcome Trust in London last month.

Cordi concludes that because past studies have ignored the early life of the algae, "the ecological significance of elevated UV-B exposure in the marine environment may be seriously underestimated". For several months each southern spring, two thirds of the ozone shield protecting Antarctica and the surrounding ocean from ultraviolet radiation is destroyed. Up to half of the shield over the Arctic region is also lost for a short time. These changes are caused by emissions of chemicals such as CFCs.

Plankton are the ocean's "meadows". They are the main food of krill, the crustaceans on which marine mammals feed. Past monitoring of plankton in the Southern Ocean has measured plankton loss of between 6 and 12 per cent when ozone levels are low. But the new findings suggest that the ecological impact could be greatest at particular times in the spring, during so-called "blooms" when plankton reproduction is at its most intense. **Fred Pearce**

# ABSTRACTS FROM CONFERENCES

The effects of UV-B radiation of *in vivo* thallus absorptance and chlorophyll fluorescence in intertidal marine macroalgae - a field survey and experiment. B. Cordi, P. Hyde, M. E. Donkin, D. N. Price and M. H. Depledge. Copenhagen, Denmark 1997.

Effects of UV-B radiation on the reproductive unicells of the marine macroalga species *Enteromorpha intestinalis*. B. Cordi, J. Peloquin, M. E. Donkin, D. Price and M. H. Depledge. Bordeaux, France 1998.

Damaging effects of UV-B radiation on macroalgae: implications for marine conservation. B. Cordi, M. E. Donkin, D. N. Price and M. H. Depledge. Sydney, Australia 1998.

Development of a genotoxicity assay in aquatic organisms using arbitrarily-primed polymerase chain reaction. F. Atienzar, B. Cordi, A. Evenden, A. Jha, and M. Depledge. Toulouse, France 1997.

DNA fingerprints obtained by AP-PCR to detect DNA damage. F. Atienzar, M. Conradi, B. Cordi, A. Evenden, A. Jha, and M. Depledge. York, UK 1998.

Application of the arbitrarily-primed polymerase chain reaction (AP-PCR) method to detect DNA damage. F. Atienzar, M. Conradi, B. Cordi, A. Evenden, A. Jha, and M. Depledge. Bordeaux, France 1998.

# The effects of UV-B radiation of *in vivo* thallus absorptance and chlorophyll fluorescence in intertidal marine macroalgae - a field survey and experiment.

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A field survey and an outdoor experiment were conducted to assess the use of chlorophyll fluorescence and *in vivo* absorptance spectra as biomarkers of short-term experimental and long-term solar UV radiation exposure in macroalgae. The intertidal macroalga *Porphyra umbilicalis* and the sublittoral macroalga *Palmaria palmata* (Rhodophyta) were collected along a transect on the beach and in the water column respectively. Significant decreases in the absorptance spectra at 497 and 569 nm (consistent with the presence of R-phycoerythrin and/or carotenoids and phycoerythrobilin respectively) occurred in *P. palmata* after exposure to elevated UV-B (2.7 W m$^{-2}$). Furthermore, depth dependent decreases in thallus absorptance at specific wavelengths were found after all exposures, indicating that *in vivo* thallus absorptance may be a useful general indicator of UV exposure in conjunction with other biomarkers. Similarly, a dose-related and site dependent decrease in $F_v / F_m$ was detected in *P. palmata*. A 60 % decrease in thallus absorptance and lack of recovery in chlorophyll fluorescence $F_v / F_m$ ratio indicated irreversible damage to accessory pigments, chlorophyll *a* and photosystem II (PSII) after exposure to elevated UV-B. Moreover, a depth-dependent increase in thallus absorptance between 290 and 325 nm was observed in *P. palmata* in response to short-term experimental elevated UV-B exposure and between 290 and 380 nm in response to long-term solar UV-B. In contrast, the intertidal alga *P. umbilicalis* exhibited a greater degree of tolerance of UV-B exposure.

# Effects of UV-B radiation on the reproductive unicells of the marine macroalgal species *Enteromorpha intestinalis.*

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In March, 1996, it was reported that the Arctic ozone hole was the "deepest ever seen in the Northern Hemisphere" with a maximum decrease of 33 %. This underlines the importance of evaluating potentially damaging effects of UV-B radiation on biota. Although impacts on primary producers are likely to have profound ecological consequences in the marine environment almost nothing is known about UV effects on early life-stages of algae. In laboratory experiments we investigated the relative sensitivity of the reproductive unicells of the macroalga species *Enteromorpha intestinalis* to different UV-B irradiances. Statistically significant decreases in photosynthesis (ca. 20% reductions measured as variable chlorophyll fluorescence (Fv)) occurred in 24 h old gametes after 10 minutes exposure to 0.8 W m$^{-2}$ UV-B irradiation (equivalent to the calculated irradiation level in April in South England (50° N latitude)). Statistically significant decreases in photosynthesis in 24 h old zoospores occurred after 10 min exposure to 2.5 and 3.0 W m$^{-2}$ UV-B radiation. After 60 minutes exposure to these irradiances, photosynthesis fell to ca. 66% of the pre-exposure level in gametes and 42% in zoospores. These decreases in photosynthesis resulted in impaired growth rates of up to 17 %. Moreover, after 30 min exposure to 0.8 W m$^{-2}$ UV-B irradiation photosynthesis in zoospores had decreased by 6.3 times compared to mature plants (29.5 % compared to 4.7 %). The results indicate that the ecological significance of elevated UV-B exposure in the marine environment may be seriously under-estimated if effects on the earlier lifestages of algae are not considered.

# DAMAGING EFFECTS OF UV-B RADIATION ON MACROALGAE: IMPLICATIONS FOR MARINE CONSERVATION.

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Conservation management often focuses on addressing local or regional problems. However, ozone depletion and resulting increases in UV-B exposure are perhaps the greatest long-term threats to ecosystems world-wide. In March, 1996, the Arctic ozone hole was the "deepest ever seen in the Northern Hemisphere". Ecological consequences of the associated increase in UV-B exposure are unknown. In the present study the relative sensitivities of mature thalli (*Palmaria palmata, Porphyra umbilicalis, Enteromorpha intestinalis*) and reproductive unicells (*E. intestinalis*) of macroalgae exposed to different UV-B irradiances have been determined by measuring changes in photosynthesis, pigmentation and growth. Mature thalli exhibited habitat dependent sensitivity. Sublittoral *P. palmata* was twice as sensitive as the two intertidal species. Decreased photosynthesis (ca. 20 %) occurred in 24 h old gametes after 10 minutes exposure to 1.8 and 2.2 $Wm^{-2}$ UV-B irradiance (equivalent to 15 % and 30 % ozone depletion in South England, $P<0.05$). After 60 minutes at 2.2 $W\,m^{-2}$ photosynthesis fell to ca. 66% of the pre-exposure level in gametes and 42% in zoospores. These decreases were associated with a 17% reduction in growth rate. The evidence presented indicates that marine primary production may be vulnerable to elevated UV-B exposure. Consequences for biodiversity and ecosystem processes must be considered in future marine conservation management strategies.

# DEVELOPMENT OF A GENOTOXICITY ASSAY IN AQUATIC ORGANISMS USING ARBITRARILY-PRIMED POLYMERASE CHAIN REACTION

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DNA fingerprinting by arbitrarily-primed polymerase chain reaction (AP-PCR) has been used to differentiate between species, strains and individuals. In this study, we have applied this methodology to asses whether DNA fingerprints could provide a new biomarker assay for genotoxic effects of environmental agents.

There is a growing concern over increasing levels of ultraviolet (UV) radiation and its possible short and long term consequences to aquatic organisms. In a preliminary experiment, *Enteromorpha intestinalis*, a marine macroalgae, was exposed to UV-B under *in vivo* conditions. After DNA extraction, AP-PCR reactions were performed; the *Taq* DNA polymerase amplified the DNA regions from 150 up to 500 base pairs with low efficiency compared to the controls where it amplified bands from 150 up to 1800 base pairs with high efficiency. In an other experiment, under *in vitro* conditions, DNA from *Daphnia magna* (water fleas) was exposed for different periods to UV-C. A dose-dependent decrease for the appearance of bands was observed.

The studies suggested that following UV exposure, DNA amplification could not take place due to presence of thymine dimers, which posed a physical barrier for the movement of the enzyme. It therefore appears that the AP-PCR method could be used as a biomarker to detect the presence of genotoxins in the environment. Studies are underway to examine the usefulness of this method in *Daphnia magna* exposed to different genotoxic agents under *in vivo* conditions.

# DNA fingerprints obtained by AP-PCR to detect DNA damage.

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Although DNA fingerprints obtained by the arbitrarily primed polymerase chain reaction (AP-PCR) technique were used originally to identify or distinguish between individuals, strains and species, we have applied this method to detect DNA damage. The AP-PCR method does detect mutations and we believe that this technique could also reveal the presence of DNA adducts, DNA breakage and possibly other DNA alterations.

The marine macroalga, *Palmaria palmata*, was exposed *in vivo* to 5 different doses of ultraviolet (UV) radiation (1.3 W / m$^2$ UV-A, 0.9, 1.6, 5.6, and 8.0 W / m$^2$ UV-B) for 3 hours. DNA fingerprints were affected with respect to controls. The principal changes were variation in intensity of bands as well as the presence of extra bands. The major event occurring for the 3 first UV doses (1.3 W / m$^2$ UV-A, 0.9, and 1.6 W / m$^2$ UV-B) was an increase of intensity of the bands compared to control with a frequency of 78, 53 and 41 % respectively. For the highest UV-B irradiances (5.6 and 8 W / m$^2$ ), the major modification was a decrease in band intensity compared to control with a frequency of 62.5 and 81 % respectively. The occurrence of extra bands seen in the 3 lowest irradiances of UV, was absent in the higher UV irradiances. However, decrease in band intensity was only observed in the 3 highest UV irradiances. In an other *in vivo* study, *Daphnia Magna* were exposed to benzo(a)pyrene (0.0125-0.2 ppm) for 14 days. DNA fingerprints obtained from exposed organisms also changed when compared to controls. It is believed that, in both studies, the modifications in the patterns are due to the generation of mutations (appearance of band), and to the interaction of *Taq* DNA polymerase with benzo(a)pyrene and UV-induced DNA damage (variation in intensities). In conclusion, the AP-PCR method appears to be a powerful and novel tool for the detection of a wide range of DNA damage at molecular level.

# Application of the arbitrarily-primed polymerase chain reaction (AP-PCR) method to detect DNA damage.

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DNA fingerprints obtained by the arbitrarily-primed polymerase chain reaction (AP- PCR) technique is being used to identify or distinguish between individuals, strains and species. In the present study, we have applied this method to detect genotoxins-induced DNA damage in aquatic organisms.

In a set of experiments, a marine macroalga, *Palmaria palmata,* was exposed to ultraviolet (UV) radiation (0.9-8 W / m$^2$ for 3 hours while the water flea *Daphnia magna* was exposed to benzo(a)pyrene (0.0125-0.2 ppm) for 14 days. DNA from both the organisms was extracted and the AP-PCR method was performed to obtain DNA fingerprints. The principal changes observed in the fingerprints were a variation in intensities of bands and a loss or gain of bands compared to the controls. The qualitative and quantitative changes lead us to conclude that these modifications result from the interaction of *Taq* DNA polymerase with induced DNA damage during the amplification process as well as the quantitative variation of available primers. Our study therefore suggests that the AP-PCR method could be a sensitive tool for the detection of a wide range of DNA damage at molecular level.

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